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Cloning and Sequencing of Sodium-Hydrogen Exchanger in the Gills of the Long-Horned Sculpin (*Myoxocephalus octodecimspinosus*)

Danielle Lee Gunning

CLONING AND SEQUENCING OF SODIUM-HYDROGEN EXCHANGER IN THE GILLS OF THE LONG-HORNED SCULPIN (MYOXOCEPHALUS OCTODECIMSPINOSUS)

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CLONING AND SEQUENCING OF SODIUM-HYDROGEN EXCHANGER IN THE

GILLS OF THE LONG-HORNED SCULPIN

(*MYOXOCEPHALUS OCTODECIMSPINOSUS*)

A Thesis

Presented to

the College of Graduate Studies of

Georgia Southern University

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In Partial Fulfillment

of the Requirements for the Degree

Master's of Science

In the Department of Biology

_____

by
Danielle Lee Gunning

December 2000

November 14, 2000

To the Graduate School:

This thesis, entitled " Cloning and Sequencing of Sodium-Hydrogen Exchanger in the Gills of the Long-Horned Sculpin, *Myoxocephalus octodecimspinosus*," written by Danielle Lee Gunning is presented to the College of Graduate Studies of Georgia Southern University. I recommend that it be accepted in partial fulfillment of the requirements for the Master's of Science Degree in Biology.

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Alison Morrison-Shetlar, Supervising Committee Chair

We have reviewed this thesis
and recommend its acceptance:

_____

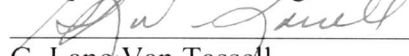JB Claiborne, Committee Member

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Quentin Fang, Committee Member

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Mark Musch, Committee Member

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John Averett, Department Chair

Accepted for the College of Graduate Studies

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G. Lane Van Tassell
Dean, College of Graduate Studies

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Chapter I

Introduction

Acid-Base Homeostasis.-Both the regulation of internal pH and the maintaince of

a proper balance of ions are equally important functions of physiological homeostasis.

Deviation from the normal range can impact proper physiological function. The low

solubility of O_2 in water and the high solubility of CO_2 in the aquatic environment require

fishes to maintain a high ventilation rate compared to air-breathers. Due to this, changes

in the external environment can cause acid-base disturbances in both marine and

freshwater fishes (Claiborne et al. 1997). The uptake and excretion of ions such as

sodium and chloride is a fundamental feature of ionic regulation. Cellular mechanisms,

such as transport proteins are means by which cells can regulate their ionic and osmotic

balance (Shuttleworth 1988).

The gills of fish are the primary site of gas exchange, acid-base regulation and

osmoregulation (Lin and Randall 1995). This is due to the large surface area and thin

epithelial membranes of the secondary lamellae. Diffusion of gases across the gill

membrane is further enhanced by the counter-current flow of the blood to the secondary

lamellae. The gill epithelium consists of five cell types: mucous, neuro-epithelial,

pavement, undifferentiated or stem cells and chloride cells. Epithelial cells are permeable

to respiratory gases such as O_2, CO_2, and NH_3 and play a major role in gas exchange.

Chloride cells (or mitochondria-rich cells) contain most of the ion transport pathways,

which require energy in the form of ATP, needed for osmoregulation. It has been hypothesized by Lin and Randall (1995) that in freshwater fish the primary function of chloride cells is acid-base regulation, while the pavement cells primary function is to maintain Na^+ homeostasis.

Cells require acid-base regulation to maintain a proper physiological pH. Fishes must maintain a blood pH of 7.7-8.0 and an intracellular pH of 7.2-7.5 because many enzymes that control biochemical processes are pH sensitive (Heisler 1993). Low or high pH can alter these enzymes thereby inhibiting their function. The disassociation of acid-base relevant ions, in particular hydrogen (H^+) and bicarbonate (HCO_3^-), in osmoregulation and ion regulation, can impact the acid-base status of fishes. The primary responsibility of acid-base regulation falls on the chloride cells of the gills, although transport across the skin and kidneys may play a minor role (Heisler 1993). The majority (> 80%) of the acid excreted by most fish is through the gills because this organ can respond immediately to acid-base disturbances, whereas the kidneys may take hours or days to respond (Claiborne 1998; McDowall 1997).

The mechanisms of transport for ions that regulate pH by marine fishes have yet to be explained fully; however, more significant evidence has accumulated for the mechanisms for freshwater species. Transport proteins postulated to be involved in acid-base, ion, and osmoregulation, in freshwater and saltwater fish are the sodium-hydrogen exchanger (NHE) and/or the hydrogen-adenosine triphosphate (H^+-ATPase) pump, working in conjunction with the chloride-bicarbonate (Cl^-/HCO_3^-) exchanger. The proton pump, which facilitates the active transfer of H^+ out of the cell and passive uptake of Na^+ via sodium channels, is the predominate mechanism hypothesized to be used by

freshwater fish for acid-base regulation (Perry and Fryer 1997). The sodium/hydrogen exchanger is the transport protein thought to be involved in ion regulation and acid excretion in most marine species (Claiborne et al. 1997).

Freshwater Model.-The hydrogen-adenosine triphosphatase (H^+-ATPase) pump or proton pump functions in biological energy conversion in living cells to maintain cytoplasmic pH, and acidifying the interior of several organelles of eukaryotic cells. There are three classes of H^+-ATPases: P-ATPase, F-ATPase, and V-ATPase. Na^+/K^+ ATPase and gastric H^+- ATPase are in the family of plasma membrane H^+-ATPase or P-ATPase. F-ATPase or mitochondrial H^+-ATPase function in eubacteria, chloroplasts, and mitochondria to synthesize ATP drive by a proton-motive force generated by the electron transport chain. Vacuolar H^+-ATPase (V-Type) is present in archaebacteria and vacuolar systems of eukaryotic cells and acts to generate a proton-motive force at the expense of ATP and cause limited acidification of the internal space of several organelles of the vacuolar system (Nelson 1984)

Freshwater fish are hyperosmotic to their environments; therefore they tend to gain water and lose salts by diffusion across the gills. Ions are also lost in the urine. To prevent this, freshwater fish excrete a large volume of dilute urine and actively transport salts back into their blood (Claiborne et al. 1997). Since the sodium gradient across the apical membrane cannot drive the Na^+/H^+ exchanger in freshwater fish, an alternative mechanism is employed. The freshwater model for acid-base regulation involves the use of a proton pump coupled with a sodium channel (Perry et al 1997). There is some disagreement with the freshwater model. Lin and Randall have hypothesized that the proton pump is used for ion transport and not acid-base regulation (1995). The proton

transport is initiated by the consumption of ATP, which then actively drives

hydrogen ion movement across the membrane and generates a negative potential in the

apical membrane. This then drives sodium influx via the sodium channel. The functional

significance of the H^+-ATPase in fish gill is to generate an electrochemical gradient for

Na^+ uptake from a dilute medium (Figure 1); hence, Na^+ concentration is the predominant

regulator of the H^+-ATPase in fish gills (Lin and Randall 1995). Fish gill epithelium

contains a vacuolar-type ATPase analogous to that of the rat and human kidney and the

vacuoles of plants, fungi, and archaebacteria (Lin and Randall 1995).

However, a study done by Perry and coworkers (1997) disagrees with the findings

of Lin and Randall. Perry and coworkers have shown through immunological studies that

the H^+-ATPase being expressed in the gill tissue is actually a P-type ATPase and not a V-

type. Not only do the two studies disagree with the type of proton pump, but also with

the response to acid-base disturbances. During alkalosis, the primary response is

stimulation of Cl^- uptake via chloride cell associated apical membrane Cl^-/HCO_3^-

exchangers and during acidosis the surface area is decreased (Perry et al 1997).
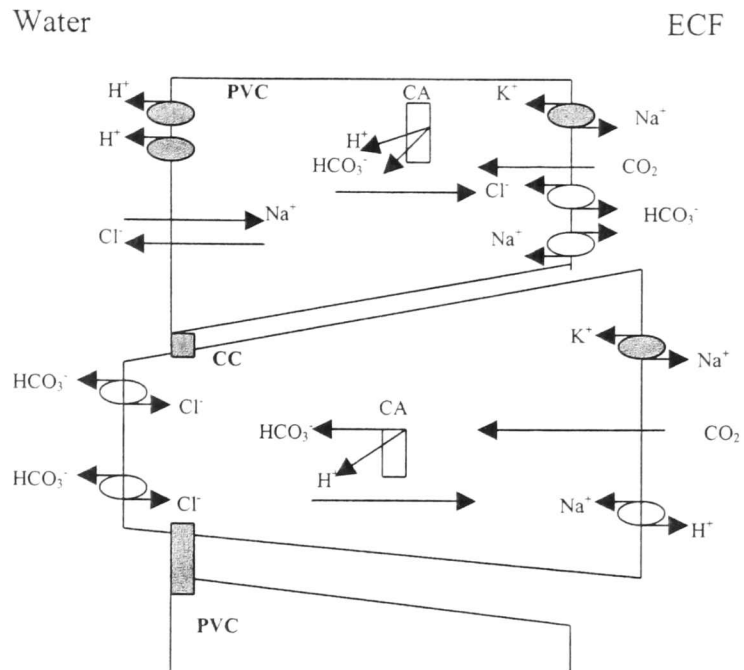
Figure 1: Hypothetical model for acid-base exchanges in the freshwater fish. The proton transport is initiated by an electogenic proton-translocating ATPase that pumps hydrogen ions to one side of the membrane and generates a negative potential on the other side that drives Na+ flux via a Na+ conductive channel. Filled circles indicate ATPases. CA= carbonic anhydrase, PVC= pavement cell, CC = chloride cell, and ECF = extracellular fluid. Modified from Claiborne (1998).

Saltwater Model.-Saltwater fish face a different problem; they are hypoosmotic to their environment. The high salt concentration of the ocean draws water out of the fish, and salts diffuse in across the gills. To prevent dehydration, marine fish drink seawater and actively excrete excess salts. The saltwater model for acid-base regulation involves the use of an electroneutral sodium/hydrogen exchanger (Claiborne et al. 1997).

The sodium/hydrogen exchangers (NHE) are plasma membrane transport proteins that under physiological conditions exchange one extracellular Na^+ ion for one intracellular H^+ ion (Figure 2). NHE's are primarily responsible for the regulation of intracellular pH and may also function in transcellular absorption of Na^+, cell volume regulation, and cell proliferation (Tse et al 1993; Yun et al 1995; Towle et al. 1997).

NHE's can be found in almost every cell type from prokaryotes to eukaryotes (Wakabayashi et al. 1997). The sodium/hydrogen exchanger was initially detected in the intestine, urinary bladder, and gall bladder (Yun et al. 1995) and has been found in mammalian cells including fibroblasts, neurons, hepatocytes, muscle, erythrocytes, lymphocytes, neutrophilils, platelets, macrophages, and epithelial cells from the renal canal. Six mammalian isoforms, NHE1-6, and one trout isoform βNHE, have been identified and cloned. Each isoform differs in its activity and expression levels to a variety of stimuli, including growth factors, tumor promoters, hormones, and chronic extracellular acidification (Grinstein and Rothstein 1986). Depending on the isoform, NHEs also exhibit varying degrees of inhibition to amiloride, a diuretic that competes with Na^+ for its external binding site (Bianchini and Pouysségur 1994) and its analogues as well as exhibit a wide range of membrane localization (Bianchini and Pouyssegur

1994; Noel et al. 1996).   These physiological and biochemical differences aid in

identifying which isoform is being expressed.

All isoforms of the NHE family are structurally similar, they possess a 10-12

membrane spanning hydrophobic N-terminus and a cytoplasmic hydrophilic C-terminus

(Noel et al 1996; Figure 3). The N-terminus is the more conserved region especially the

fifth membrane-spanning domain within the NHE gene family. This suggests that this

part of the protein plays a crucial role in the structure and/or ion transport mechanism.

The NHE isoforms share a 40-60% overall amino acid homology, with only
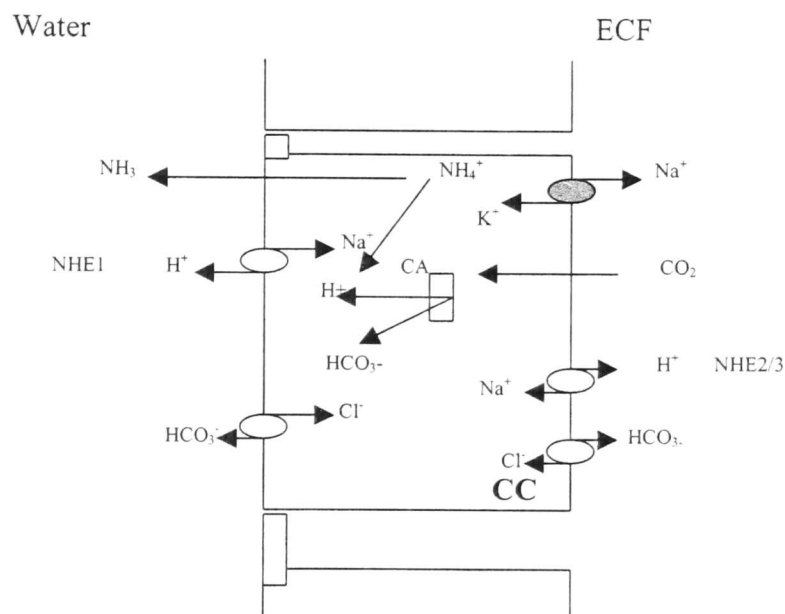
Figure 2: Hypothetical model for acid-base exchanges in the seawater fish. The
sodium/hydrogen exchangers (NHE) are plasma membrane transport proteins that under
physiological conditions exchange one extracellular Na^+ ion for one intracellular H^+ ion,
working in conjunction with the chloride-bicarbonate ($Cl^-/HCO3^-$) exchanger and
carbonic anhydrase. Filled circles represent ATPase. CC = Chloride cell, CA =
Carbonic anhydrase, and ECF = extracellular fluid. Modified from Claiborne (1998).

20-40% homology in the cytoplasmic domain. For that reason, it has been proposed

that the C-terminus is isoform specific and responsible for the regulation and activation

of Na^+/H^+ exchange sites (Noel et al 1996; Yun et al. 1995).
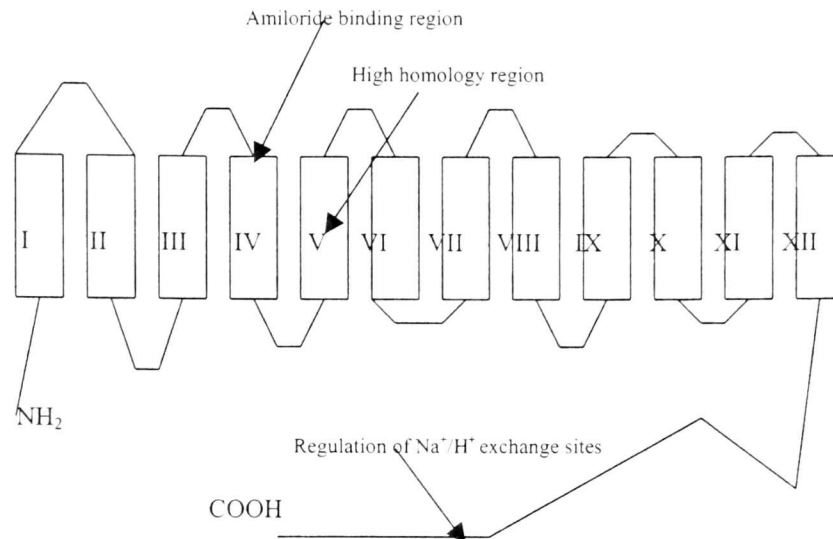
Figure 3: Representative diagram of the hypothetical topology of the NHE family. Picture was modified from Fliegel et. al. (1993).

NHE Isoforms.-NHE1 was the first Na^+/H^+ exchanger isoform cloned from human

genomic DNA and has been the most widely studied NHE isoform. NHE1 is a

glycoprotein that is found in the membrane as a homodimer (Noel et al. 1996), is ~820

amino acids long, and has been mapped to human chromosome 1 and rat chromosome 5.

NHE1 has been cloned from a variety of mammals including human, rabbit, pig, and

Chinese hamster (Yun et al. 1995). Northern analysis and ribonuclease protection assays

have indicated the presence of NHE1 mRNA in nearly all mammalian cells (Tse et al.

1993). Immunohistochemical studies using antibodies against NHE1 have shown it to be

located on the basolateral membrane in virtually all cell types, tissues, and species (Yun

et al 1995; Wakabayshi et al 1997). Activation of NHE1 is usually a result of conditions

such as increase in acid load, cell volume change, or growth factors (Bookstein et al. 1997). It has also been shown that NHE1 is remarkably conserved across multiple species having at least 90% amino acid identity (Tse et al. 1993).

In contrast to the other isoforms, NHE1 has defined physiological roles. Known as a "housekeeper", NHE1 functions in pH homeostasis, volume regulation, and cell proliferation.

NHE2 (~813 amino acids) is abundantly expressed in the epithelial cells of the ascending and descending colon, ileum, jejunum, and stomach and has been cloned from rabbit, rat, and human DNA (Bookstein et al. 1997). NHE2 mRNA has also been found in the adrenal gland, kidney, and uterus (Tse et al. 1993). NHE2 has been mapped to human chromosome 2 and rat chromosome 9 (Bookstein et al 1997). It is believed that NHE2 is found on the apical membrane and is most likely involved in gut Na+ absorption (Bookstein et al. 1997).

Unlike NHE1, the precise physiological roles of NHE2 are unclear. However, when transfected into cells, NHE2 is capable of regulating pH, cell volume, and proliferation similar to NHE1 (Orlowski and Grinstein 1997).

A more specialized NHE isoform, NHE3 (~832 amino acids) is expressed only in the intestinal and renal epithelial cells and has been cloned from rabbit, rat, and human DNA (Tse et al. 1993). NHE3 has been mapped to human chromosomes 5 and 10 (Yun et al. 1995). The tissue distribution of the NHE3 message suggests that it might be the apical NHE exchanger involved in transepithelial Na^+ absorption (Bookstein, DePaoli et al. 1994). The activity of NHE3 in the brush boarder membrane is increased in response

to chronic metabolic acidosis, chronic renal failure, and in hypertension NHE3 appears to be more resistant to amiloride than both NHE1 and NHE2.

NHE4, which is shorter in length than the other cloned isoforms (~717 amino acids), is found in the stomach, kidney medulla, hippocampus, brain, uterus, and skeletal muscle. It is the only isoform that has not been successfully transfected and expressed in heterologous systems (Yun et al 1995; Noel, Roux et al. 1996), . However, NHE4 has been mapped to rat chromosome 9, and human NHE4 has been mapped to chromosome 2. Immunofluorescence labeling of NHE4 in both the rat and rabbit kidney have shown the NHE4 isoform to be located on the basolateral membrane (Peti-Peterdi et al 2000). However, there is disagreement with the localization of NHE4 to the basolateral membrane. Bookstein and coworkers(1997) have shown that NHE4 is an apical transporter. Little is known about this isoform, which is functionally and pharmacologically different from the other isoforms. NHE4 is the only isoform to exhibit no amiloride sensitivity (Yun et al. 1995). It is thought to play a role in cell volume and pH regulation since it is found in the inner medulla collecting tubules of the kidney and epithelial cells of the stomach (Bookstein et al 1994; Bookstein et al. 1997).

NHE5 is an 896 amino acid protein and is closely related to NHE3 in structure and amiloride sensitivity (Baird et al. 1999). It has been mapped to human chromosome 16 and exhibits 59-73% homology to the other cloned isoforms of the NHE family. NHE5 has been detected in the brain, testis, spleen and skeletal muscle. Northern blot analysis showed that NHE5 is widely distributed in the brain; suggesting that it may be a neuron-specific isoform. Due to its similarities with NHE3, and expression in the brain,

it is thought to be involved in the control of intracellular pH in the hippocampal and other neurons (Baird et al. 1999).

The most recent isoform of the NHE family to be discovered is NHE6. Even though it has a wide tissue distribution, NHE6 is most abundant in mitochondria-rich tissues such as brain, skeletal muscle, and heart. This may indicate that NHE6 is important for mitochondrial function and the regulation of intramitochondrial Na^+ and H^+ levels. Although there is no information available to determine a definite physiological role for NHE6, it has been hypothesized that NHE6 may also be involved in aiding the efflux of Ca^{2+} and NH_4^+ from the mitochondrial matrix (Numata et al. 1998).

βNHE (759 amino acids) was first isolated from trout red blood cells (Bianchini and Pouysségur 1994). This isoform has the most homology with NHE1 (Borgese et al. 1994). Due to their high degree of similarity, NHE1 and βNHE are thought to be phylogenetic variants of the same gene (Noel et al. 1996). Like NHE1-3, βNHE is a glycoprotein but differs in that it resides in the membrane in a non-functional state even when intracellular pH drops to 6.3. pH only activates this isoform when it falls below 6 (Malapert et al. 1997). βNHE has been cloned from trout cephalic kidney, fish haematoporetic tissue, and circulating erythrocytes (Borgese et al. 1992).

Objectives.-In the present study the long-horned sculpin, *Myoxocephalus octodecimspinosus*, was used to detect a cDNA for a protein homologous to a mammalian NHE isoform. The specific objectives of this research were to: 1) obtain the complete sequence of an sodium/hydrogen exchanger; 2) establish the structure of the NHE DNA sequence; 3) find out which isoform is being transcribed in the gill tissue, and; 4)

compare known isoforms to the sculpin sequence to determine which regions of the sequence are homologous.

Significance.-In mammals, the lungs and kidneys accomplish acid-base regulation. The gills of fish are comparable to the lungs and kidney of mammals because they are used for respiration and the elimination of toxic waste. However, the cellular mechanism by which fish maintain homeostasis in the gill is not completely understood. At least one of the NHE isoforms is hypothesized to regulate acid-base and ion balance across the gill epithelia (Claiborne et al. 1999). The significance of this research is that it may lead to a better understanding of the cellular mechanisms behind acid-base regulation, and provide insight into the physiology of the mammalian kidney and other organs. Further, NHE research is important because NHE plays a role in many human pathophysiological states, including hypertension, epilepsy, diabetes mellitus, and cardiac ischaemia (Cox et al 1997; Dyke and Lopaschuk 1998; Fischer et al. 1999).

Chapter II

Materials and Methods

Brief Overview.-The purpose of this project is to clone and sequence the full-length message of the NHE isoform expressed in the gill tissue of the long-horned sculpin, *Myoxocephalus octodecimspinosus.* Recently, in our laboratory, two NHE isoforms, a βNHE-like and a NHE2-like, partial product have been cloned and sequenced from a single reverse transcription-polymerase chain reaction (RT-PCR) product obtained from the gill tissue of the long-horned sculpin using NHE degenernate primers. Sculpin specific primers (NHE2F and NHE2R) were designed against the conserved regions of the NHE2 partial sequence (Claiborne et al. 1999). To address the objectives of this research, NHE2F and NHE2R sculpin specific primers were used in multiple RT-PCRs, 5'/3' rapid amplification of cDNA ends (RACE) and multiple PCRs to screen a sculpin gill cDNA library. The partial NHE2 product was used as a species-specific probe against a sculpin gill cDNA library. This research was conducted at Mount Desert Island Biological Laboratory (MDIBL) in Salisbury Cove, Maine and Georgia Southern University (GSU) in Statesboro, Georgia.

Tissue Extraction.- The long-horned sculpin, *Myoxocephalus octodecimspinosus*, were obtained by local fishermen from Fisherman's Bay in Salisbury Cove, Maine and kept in saltwater tanks at The Mount Desert Island Biological Laboratory. The fish were sacrificed by brain/spinal pithing. Total gill arches were removed from both sides of the sculpin with sterile scissors and forceps. Before the arches were weighed, they were trimmed to remove cartilage and/or bone so that only the gill filaments were retained. Once weighed, the tissue was put immediately into Tri Reagent (Sigma)(1 mL for each 50-100 mg of tissue) and maintained on ice.

Acid Loading.-Several sculpin were subjected to acid infusion to determine the expression levels of the NHE in the gill tissue. Acid infusions were done according to the procedure of Claiborne (1997) except fish were not fitted with blood cannulea. The amount of acid given to the fish depended on body weight. Two fish at each time period were sacrificed at 0 hours, 2 hours, and 6 hours and gill tissue was extracted for total RNA isolation.

RNA Isolation.-Before tissue extraction, all instruments used were cleaned and sterilized using ETOH and a Bunson burner. Tissue was homogenized in Tri Reagent until completely suspended. The protocol for Total RNA isolation followed the method provided by Sigma (see Appendix B). Total RNA was resuspended in 30 μL of sterile water and the concentration determined by spectophotometry at 260 nm and 280 nm ((1 OD at 260nm = 40μg RNA) and purity ($A_{260/280}$)) before use in reverse transcription polymerase chain reaction (RT-PCR).

Reverse Transcription.-Polymerase Chain Reaction (RT-PCR)-Total RNA
samples extracted from the gill tissue of the long-horned sculpin were used in a series of
RT-PCR experiments. Two different RT-PCR kits were used along with two different
thermal cyclers. The GeneAmp RNA PCR® kit (Perkin Elmer), SuperScript
Preamplification™ System (GibcoBRL), PCR Core System II (Promega), Perkin Elmer
480 DNA Thermal Cycler and Hybaid PCR Express Thermal Cycler were used for
these experiments. The RT cycle for the GeneAmp RNA PCR® kit and the RT cycle
for the SuperScript Preamplification™ System were set according to the protocol
supplied with each kit. A PCR master mix was prepared and added to the RT mixture.
The concentration and volumes varied depending on the kit being used. A final volume
of 50 μL was used for most reactions (Appendix C).

3'/5' RACE (Rapid Amplification of cDNA Ends).-3'/5' Race (Clontech
SMART™ RACE cDNA Amplification Kit) was used to isolate the complete
sequence of either the 5' end or the 3'end of the sequence of interest. The protocol
was followed according to the directions provided by Clontech. Gene specific primers
(GSP), NHE2 reverse primer for the 3' end and NHE2 forward primer for the 5'
reactions were used (Appendix D). The GSP primers were designed against the
conserved regions of the NHE2 partial sequence (Claiborne et al 1999; Figure 4).

5'-ttcctgracagctacstggcctatctggtggccgagctcttcgccatctcatccatcatggc**CATTGTGACCTGTGCCCTC**acc
atgaagtactacgtcgaagaaaatgtttcccagcgttcctgcacaaccatccgccatgtgatcaagatgcttggctccatctccgagaccctcatcttctt
ctttctgggggtcgtcgccataacaacagaacatgagtggaactggggctacattctgttcacattgctgtttgctttcgtatggagggggtctgggtgtctt
ggtgctgactcagatcattaac**CCTTTCCGCACCATCCCATT**taacttgaaagatcaatttggtctggcntacggtggccttc-3'

Figure 4: Partial NHE2-like sequence obtained from the gill tissue of the long-horned
sculpin. Letters in all capitals are the primers NHE2F and NHE2R.

Quantitative RT-PCR.-Quantitative RT-PCR was used to determine the expression levels of the mRNA. Ambion's QuantumRNA™ Classic 18S Internal Standard kit was used to determine the expression levels of NHE2 RNA in sculpin gill under acidic conditions. The protocol supplied with the kit was used for each application (Appendix E). Ambions's internal standards produce a band of approximately 450 kb. In order to quantify the expression levels of NHE in the gill tissue, sculpin specific primers were chosen on their ability to produce a band twice that size. Sculpin specific primers, NHE2F and SCULP2-R689 were used in each of the reactions. (See Table 1 for description of the primers.) Once relative expression was determined ANOVA and Students T-test were used to determine significance.

Gel Electrophoresis.-All RT-PCR, 5'/3' RACE, PCR, Quantitative PCR and Miniprep products were visualized by gel electrophoresis using a 1% agarose (Fisher) gel stained with ethidium bromide (Sigma). 1xTBE was used as a running buffer (Sambrook et. al., 1989). Ten microliters of each DNA sample was added to 1 μl of 10xDNA loading dye and loaded into separate lanes aligned with a DNA ladder (Promega 1 Kb) to estimate size of the DNA. Samples were run on average for 45 minutes at 80V. Gels were visualized and photographed (FisherBiotech camera, Fisher Scientific) over an ultraviolet light (UV Transilluminator, UPV, Inc.).

Sub-Cloning.-All RT-PCR products and RACE products were sub-cloned to prevent loss of template. Invitrogen's Original TA Cloning® kit with pCR®2.1 vector was used for the procedure. Ligation and transformation steps were followed according to the methodology set by the manufacturers of the kit.

cDNA Library screening.-The NHE2 partial sequence (Claiborne et al. 1999) was used as a species-specific probe to screen a cDNA library previously constructed in our lab. Nested PCR using sculpin specific NHE2 primers reamplified the NHE2 partial sequence.

The partial NHE2-like sequence was used as a species-specific probe to screen the sculpin gill cDNA libraries. The NHE2 probe was radiolabeled with α ^{32}P- dCTP following the Promega protocol except that 5 µl of α ^{32}P- dCTP and 1 µl of each of dATP, dGTP, and dTTP was substituted for the normal dNTP mixture. The PCR was run at an annealing temperature of 57°C for 40 cycles. Excess (free) radioactivity was removed by column chromatography. A column was prepared by filling a 2ml pipette with Sephadex G50 fine (Sigma) in 1 X SSC creating the matrix for size separation. The column was washed with 1 X SSC several times and the probe solution added to the column. The probe was purified using 1X SSC and fractions were collected drop wise. Probe localization and determination was done using a Geiger counter. The fractions containing the probe were pooled and stored at 4°C until the cDNA library was screened.

Agar plates containing 10µg/ml of ampicillin were used to plate out the sculpin gill cDNA library. The cDNA library was plated out to a final concentration that resulted in almost confluent plates. After incubating the agar plates overnight, the colonies were then transferred to autoclaved, circular nylon membranes (Amersham). This transferal was accomplished by placing nylon membranes on top of the plates until they were completely saturated, and marks were made on the membranes and plates to designate the orientation, and then membranes were removed with forceps. These filters were

then a mirror image of the plates. After the lifts, each membrane was soaked

colony side up in a series of solutions for 5 minutes, 1) 5mM EDTA and 1% SDS, 2)

.5mM NaOH and 1.5M NaCl, 3) 1M Tris pH 8.0, and 4) 2 X SSC. During transfer

between solutions, nylon membranes were blotted on filter paper to remove excess

liquid. The solutions caused disruption of the bacterial cells and denaturation of their

plasmid DNA. The membranes were allowed to dry and then placed in an UV cross

linker (Fisher Scientific) for 2 cycles (optimum crosslinking) permanently binding the

DNA to the filters. The cross-linked membranes are then incubated overnight at 42°C

in prehybridizing solution (12ml of 20 X SSC, 4ml of 100% Denharts solution, 2ml of

20 X SDS, 0.2ml of 50μg sperm DNA, and ultra pure water up to 40 mls). Membranes

are removed from the prehybridizing solution and placed in hybridizing solution (20ml

of formamide, 12mls of 20 X SSC, 2ml of 20 X SDS, 0.2mls of 50μg/ml of salmon

sperm DNA, and 4ml of 100X Denhardts) with the radioactive NHE probe and

hybridized for 48 hours at 42°C. Membranes were washed 4X 30 minutes with washing

solution (200 ml of 20 X SSC, 50ml of 10X SDS, up to 2 liters of water) at 68°C with

shaking to remove unbound probe, and membranes were placed between two layers of

Saran wrap and exposed to autoradiography film (Kodak XOMAT) with intensifying

screens (Kodak) for 24 to 48 hours at -70°C. The film was developed and positive

signals were aligned with the agar plate and positive colonies were isolated. Each

positive colony was prepared for plasmid DNA isolation (Birnboim and Doly, 1979)

and sent off to Medical College of Georgia, the University of Georgia, and the Mount

Desert Biological Laboratory for sequencing.

Overnight Cultures.-Colonies were screened for inserts using the blue-white screening method (Sambrook et al. 1989). Colonies containing PCR inserts, white colonies, were identified for overnight cultures. 5-7 mls of LB Broth (Gibco) containing 50 µg/ml ampicillin (Sigma) was poured into 15 ml tubes. Colonies were picked from agar plates using an autoclaved pipette tip and transferred to 5 ml LB culture medium and grown at 37°C with shaking (250 rpm) for plasmid isolation.

Isolation of Plasmid DNA.-Plasmid DNA was isolated using the alkaline lysis method of Birnboim and Doly, (1979). See Appendix E for protocol.

Restriction Digest.-Digestion of DNA with restriction enzymes was performed using EcoR1 (Gibco BRL) to ensure the inserts of the correct size were present in all isolated plasmids. The vectors used contain EcoR1 restriction sites on either site of the insert position; therefore, the restriction enzyme cuts the plamid DNA just before and just after the insert. At least two distinct bands, a high molecular weight band of the vector and a smaller band of the insert, were visible after the product was run on an agarose gel and viewed using a UV light. To perform the digest, 1 µl of the plasmid DNA was mixed with 1.0 µl 10x React 3 Eco R1 buffer, 1 µl EcoR1 enzyme and 7.0 µl sterile water. The reaction was mixed well and incubated in a water bath at 37°C for 1 hour.

Southern Blot.- Plasmid DNA was isolated by alkaline lysis (see Isolation of Plasmid DNA) and cut with the restriction enzyme EcoR1 (see Restriction Digest). The products of the restriction digest were run on a 1% agarose gel stained with ethidium bromide. To facilitate transfer of DNA to membrane, the gel was gently agitated for 15 minutes in 100 ml of a 0.5 M NaOH, 1.0 M NaCl solution in a tray for denaturation,

rinsed in 200 ml of distilled water, and then gently agitated in 100 ml of 1M KOAc for 15 minutes. The DNA was then ready for transfer onto nylon membrane (Amersham). The blotting method of Sanger et. al., (1985) was used to transfer the DNA to the membrane and on completion, the membrane was ready to be probed.

The final step of the Southern blot was prehybridization and hybridization of the radiolabeled sculpin PCR product to the cDNA fragments on the nylon membrane. This hybridization procedure was the same as that used in the cDNA library screening except a Hybritube (Gibco BRL) was used instead of petri dishes. Otherwise, the same hybridization and washing procedure were followed, after which the nylon membrane was exposed to X-ray film and left at -70°C for 6 hours. Finally, the film was developed and examined to determine positive hybridization of the radioactive probe to homologous DNA.

Automated Sequencing. -Automated sequencing was used to sequence all DNA samples. The samples were sequenced at the Medical College of Georgia, University of Georgia, and The Mount Desert Island Biological Laboratory. All samples were prepared according to the standards set by each of the facilities.

DNA Sequence Analysis.-DNA sequencing information obtained was entered into MacVector and DNAStar sequencing analysis software. ClustalW alignment was used to pair all the sequences from the 3' end together and all of the sequences from the 5' end together. Sequences were then translated into amino acid using translation frame shifts to obtain open reading frame. Areas of overlap were then found and the fragments were aligned to generate the full-length sequence.

Protein Structure Analysis.-Hydrophobicity and potential membrane-associated helical regions were predicted using the algorithms of Kyte and Doolittle(Kyte and Doolittle 1982). The predicted secondary structure of the protein was determined using the methods of Chou and Fasman.

Chapter III

Results

Total RNA.-Total RNA was obtained from the sculpin gill tissue for use in RT-PCR, 3'/5' RACE, and quantitative PCR to determine which NHE isoforms are being expressed. Stock RNA samples were diluted so that the final concentration was 1 $\mu g/\mu l$ and tested with the sculpin specific NHE2 primers in RT-PCR experiments to determine the viability of the RNA. All gill RNA samples should have produced 270 base NHE2 band using NHE2F/NHE2R primers. If the NHE2 band was produced, then the RNA extraction was considered to be successful and the quality of the RNA was good (Figure 5). To determine the quantity and purity, spectrophotometric readings were taken at 260 and 280 nm. RNA samples that gave a 260/280 reading of 1.8 - 2.0 were considered to be of good quality. Total RNA of good quality was then used in RT-PCR, 3'/5' RACE and quantitative PCR experiments.
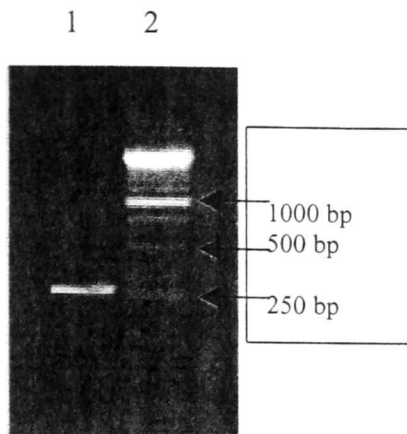
Figure 5: RT-PCR of sculpin RNA using NHE2F and NHE2R primers. The positive band (lane1) is ~270 base pairs in length and verifies that the RNA is of good quality. The RT-PCR product seen here is the NHE2-like partial sequence used as a species-specific probe to screen the sculpin gill cDNA libraries. Products are visualized on a 1% agarose gel stained with ethidium bromide.

cDNA Library Screening.- The sculpin gill cDNA library was screened in an attempt to isolate the full-length cDNA of the NHE isoform being expressed in the gill tissue of the long-horned sculpin. A cDNA library is a representation of all the mRNA expressed in the tissue at the time of extraction. The partial NHE2-like sequence (Claiborne et al. 1999) was used as a species-specific probe to screen the sculpin cDNA libraries. In order to radiolabel the DNA fragment, 32P-dCTP was incorporated in the PCR experiment. The radiolabeled DNA was separated from free radioactivity by column chromatography. A Geiger counter was used to verify that the initial fractions collected from the column contained the radiolabeled PCR product. The counts of the radioactivity as measured by the Geiger counter were low in the first several tubes, rose to a peak corresponding to the radiolabeled fragment, fell again to background levels, and finally rose to a high level corresponding to the free radioactivity. The radioactive probe was then denatured and incorporated into hybridizing solution and used to screen the sculpin gill cDNA libraries. The screening procedure was followed, after which the film

was developed and examined. Several dark spots were visible on most filters. These spots corresponded to radiolabeled probe hybridized to plasmid DNA containing homologous DNA. Plugs of agarose containing these colonies were cut from the original plates and positive colonies were again screened so that a single colony could be selected. An example of first, second, and third screen filters can be seen in Figures 6-8.

After picking positive colonies, plasmid DNA was isolated from each culture and cut with restriction enzyme EcoR1. The products of the restriction were run on a 1% agarose gel and blotted to nylon membrane. The partial NHE2 product used to screen the sculpin gill cDNA libraries was used to probe the membrane. A Southern blot was used for confirmation of positive colonies. Positive bands that bound the radioactive probe containing homologous DNA were selected from the Southern blot and sequenced.

Two different sculpin gill cDNA libraries were screened, a library made from the gills of fish adapted to 20% seawater and a library of fish adapted to 100% seawater. Once the colonies were isolated, they were purified using alkaline method and sent off for sequencing. A total of six partial and full-length cDNA sequences were obtained from the library screening process. From the sculpin gill seawater library, a partial sequence with high homology of a zinc finger protein was found (Figure 24). The full-length sequence of glutathione S- transferase (GST) (Figure 25) and hemoglobin, alpha subunit (Figure 29) was acquired from the sculpin gill 20% seawater library. Along with the full-length sequences, several partial sequences were also found in the 20% sculpin gill seawater library. They are: phenylalanyl-tRNA synthetase beta subunit (Figure 28), nuclear RNA helicase (Figure 26), and a class II aminotransferase (Figure 27). Sequences for each can be found in Appendix F. No positive colonies containing NHE

were detected using those conditions and so higher stringency conditions are

currently being used to acquire the full-length sequence.

Figure 6: Representative autoradiograph of a first screen filter that has been labeled with a radioactive ^{32}P. Positive colonies appear as black dots on the film.
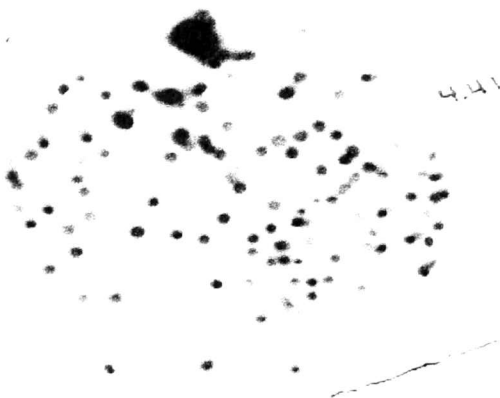
Figure 7: Representative autoradiograph of a second screen filter that has been labeled with a radioactive ^{32}P. Positive colonies appear as black dots on the film. As the screening process progresses, more black colonies appear on the film.
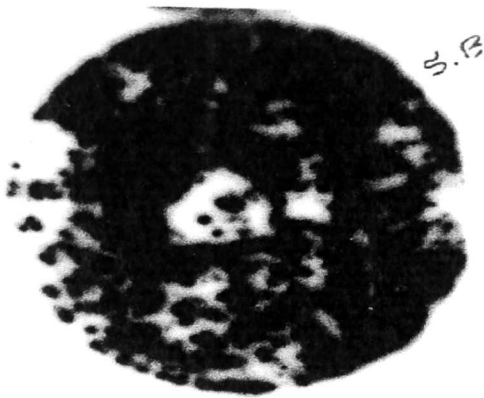
Figure 8: Representative autoradiograph of a third screen filter that has been labeled with a radioactive ^{32}P. Positive colonies appear as black dots on the film. The film is almost completely black from positive colonies. Now a single colony can be isolated and sent off for sequencing.

3'/5' RACE.- Total RNA isolated from seawater long-horned sculpin were used in 3' and 5' RACE reactions to obtain the full-length message of the NHE isoform being expressed in the gill tissue. Single band 3' and 5' RACE products from the sculpin RNA using NHE2F for the 3' reactions and NHE2R for the 5' reactions were acquired. The best RACE products were obtained using an annealing temperature gradient ranging from 57°C to 68°C during the PCR experiment. All other cycling parameters were followed according to the Clontech protocol . The PCR products produced were strong bands of ~2000 base pairs in length for the 3' end and ~1500 base pairs for the 5'end (Figure 9). Both products were cloned and an EcoR1 restriction digest analysis indicated that the PCR products had not been successfully ligated. Since the RACE products could not be ligated, direct PCR sequencing was done in order to obtain sequence information. When the RACE products were sequenced using the sculpin specific primers used to generate the original product, no sequence data was obtained.
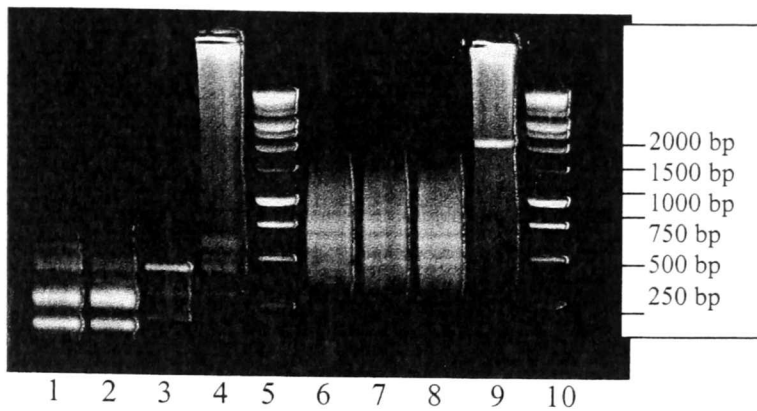
Figure 9: Ethidium bromide stained 1% agarose gel of 3'/5' RACE products. Lanes 1-4 are 5' RACE products at annealing temperatures ranging from 58.7°C to 64°C. Lanes 5 and 10 are a 1 kb ladder, bottom band is 250 bp and they increase by 250 bp increments up to 1000 bp. Lanes 6-9 are 3' RACE products at annealing temperatures ranging from 58.7°C to 64°C.
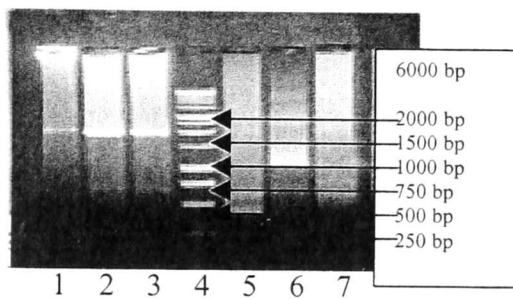
Figure 10: Ethidium bromide stained 1% agarose gel of 3'/5' RACE PCR products using the new sequencing primers. Lane 1 is the 3'RACE using NHE2F, lane 2 is 3' RACE using CRB-seq-F1, and lane 3 is 3' RACE with CRB-seq-F2. Lane 4 is a 1 kb ladder, bottom band is 250 bp and they increase by 250bp increments. Lanes 5-7 are 5' RACE PCR products. Lane 6 shows the expected band using CRB-seq-R1 primer. A list of primers can be found in Table 1.

Primers internal or nested to the known partial NHE2 sequence were designed. The new nested primers, CRB-seq-F1, CRB-seq-F2, and CRB-seq-R1 were tested by reamplifing the RACE products by PCR (Figure 10). The new primers were then used to sequence each of the original RACE products. Automated sequencing of the PCR products was done at Mount Desert Island Biological Laboratory (MDIBL). When the sequence results were entered into BLAST, both the 5' and 3' RACE products matched NHE. Both sequences matched significantly to NHE2 with a 76% homology at the amino acid level when compared to the known rat NHE2 sequence. However, the sequence was not complete. New primers were generated based on the conserved regions on the known rat NHE2 sequence and the new sculpin sequence (Table 1). A total of sixteen primers were generated for sequencing. Using these primers the full-length sequence from the sculpin was found. Direct PCR sequencing using the new primers was done until the complete sequence was found (Figure 11). Multiple PCR products were sequenced multiple times to obtain the consensus sequence.

The full-length sculpin cDNA contains a 2918 nucleotide open reading frame, encoding a protein of 908 amino acids (Figures 11-12). Sequence data was submitted to the into Blast NIH web server database for comparison to other known sequences. Blast results indicated that on the nucleotide and amino acid level, this sculpin NHE product best matches rat NHE2. Amino acid sequences of all published NHE2 isoforms and the sculpin NHE are compared in Figure 13. The 5' region of the sculpin NHE is highly homologous to the published mammalian NHE2 isoforms, whereas the 3' end is significantly different.

1-CAGGGCCAAAAGTTAAGCTCATATAGGGTAACAGGGCAACGCAGAGTACGCGGGGAAA-58
59-AAGCGAATATGCCAGTGTTTACAATGGATTACCCAAGAATACAGATCCCATTTGAGAT-117
118-C**ACTATGTGGGTGCTGCTGG**₁CTTCATTCGCAAAAATTGGTTTCCATGTGTACAACA-175
176-AGATCACCATCTGGGTGCCGGAGACCTGCCTTCTGATCACCATCGGCCTGATCGTAG-234
235-GTGGCATCATGCACTCCGTCCACGAGGAGCCACCTGCTGTGCTCAGCAGCAACGTCT-292
293-TCTTCCTCTACATGCTGCCCCCGATTGTCCTGGACTCGGGCTACTTCATGCCCACCAG-351
352-GCCTTTCTTCGAGAACATTGGCACGGTGCTGTGGTTTGCAGTGGTGGGGACGCTGTG-409
410-GAACAGCATCGGCATCGGCATGTCTCTGTTCGCCATATGCCAGATCGAGGCGTTTGG-467
468-TGTGCAGGACATCAACCTCCAGGAGAACCTGCTGTTTGCCACCATCATCTCGGCCGT-526
527-GGACCCCGTGGCCGTGCTGAGCGTGTTCGAGGACGTGTCTGTGAACGAGCAGCTCTA-584
585-CATCGTGGTGTTTGGAGAATGCCTCTTCAATGATGCCGTCACTGTGGTGTTGTACAAC-642
643-ATGTTCAACTTTGTGGCGGAGATGCCGGT**GGTGGAGCCAGTGGATGTTT**₂GCCTCGG-700
701-GGTGGCCCGGTTCTTCGTGGTCGGGCTCGGCGGGATGGGCTTCGGCATCCTGTTCGGT-759
760-TTCACGGCCG**CCTTCACCACAAGATTCACC**₃TCCAAGGTCCGAGAGATCGAGCCGCT-817
818-TTTCATATTCATGTACAGCTACCTGGCCTATCTGGTGGCCGAGCTCTTCGCCATCTCAT-875
876-CCATCATGGC**CATTGTGACCTGTGCCCTC**₄ACCATGAAGTACTACGTCGAAGAAAAT-933
934-GTTTCCCAGCGTTCCTGCACAACCATCCGCCATGTGATCAAGATGCTTGGCTCCATCT-992
993-C**CGAGACCCTCATCTTCTTCTTTCTTG**₅GGGT**CGTCGCCATAACAACAGAAC**₆ATG-1048
1049-AGTGGAACTGGGGCTACATTCTGTTCACATT**GCTGTTTGCTTTCGTATGGAGG**₇G-1104
1105-GTCTGGGTGTCTTGGTGCTGACTCAGATCATTAAC**CCTTTCCGCACCATCCCATT**₈-1161
1162-TAACTTGAAAGATCAATTTGGTCTGGCCTACGGTGGCCTACGAGGTGCAATTTCAT-1218
1219-TCGCCTTGGTCTTCACCCTTCCTGACACCATCGGACGCAAGCAGCTCTTCATCACTG-1260
1261-C CACCATTTCTATCATCCTCT**TCACTGTCTTTCTCCAGGGC**₉**ATCAGTATCAGAC**-1317
1318-**C**₁₀TTTGATTGAGTTCATCAACGTCCGCAGAACCAACCGCAATCTTGACACCATCAA-1374
1375-CGTAGAGATTCACTGCAGGCTCATGGAGCACACCATGGCTGGCTTAGAGGACCTCT-1431
1432-GTGGGCAGTGGAGCCACTTCTACTGGAAGGACAAGTTTATGAAGTTCAACAATCGA-1489
1490-ATCCTGCGGAAGATCCTGATCCGAGACAACCGGGCC**GAGTCCAGTATCGTGGC**-1546
1547-**G**₁₁CTTTATAAGAAGCTGGAACTGCAGAACGCCATGGAGATCCTGGACACTGTGTCT-1604
1605-GGAGACATGAGCGCTGCTCCGTCCATTGTCTCTCTCTATGAGGAAAAGACTAAACC-1660
1661-TAAGAAGTTCCTGGCTTCGGACCTGAAGGACATGCATGACATCCTGTCCAAGAACA-1716
1717-TGTACAAGATCAGGCAGC**GGACGGTGGCTTACACAAC**₁₂TAAGCATGCCCTG**CCC**-1771
1772-**AATGACAGCCAAAGCA**₁₃AAGAGAT**ATTGATAAGACGCCACGCC**₁₄AGCATCCGCC-1828
1829-GCARCCTCCGCCCGGGCAGCTTTCAGT**CATCGGTGGTCCCAAATCC**₁₅CACAAATA-1885
1886-CTTCTCTCTTCCTGCTGGGAAAGGTCTGGACTCAAAATTCCCCCCTGCTGGGCAGAC-1943
1944-GGATGAGGAAACCATGTCAGAGGTGGCCTACCCCTCCAGATGGTCTCGGCTTAGGC-2001
2002-AGCCCGCACGCTCCTCCTCCAGAGCCATGATGCCTCTCCGCCGGCTGGACACCCTC-2058
2059-ACAGAGGTGCACTCTGTCGACATGGTGGACGAGAGCTCAGGGGAGGGCAGAGGC-2113
2114-AGGTCGGGGCGCGGCACGTCCAGAACACACTCTGCCTCCAGTGATCCCCACATTCC-2170
2171-TGTTCCTCATCGTCGCGTTGACAACGAACATGGCTCAGCTGATAACTTTAGAGACG-2227
2228-GGCATCATGAAGAGCAGCAGCAGCCTTCGTCCCCGCCTCCAGGCTGGGCTGCTGAA-2284
2285-GCCCGAGACCACGCGGCACGAAACCCTCTGCTCAGGCGGCCTCAGTGGAACCCCA-2341
2342-AGATGTAAAAGTGTTCCTGGGCTTTCAAGAGAAGATGGACTGCCACAGACTCCAG-2398
2399-GGTCTCTACAGACGTGTCCATAGACCAAATGAAGGATCACTGGAGGTCCATTAAC-2455
2456-TTCTTATTAGGGGTGAGCCATCCTCAAATGAGCCACAATTGACAGTAATGCCTTGA-2512
2513-AGTCAGAGGTGTTGGCCACACCAGAAGCACAGTGCACTCTAGTGAAGCAACTGAA-2569
2570-TATGAACTGTCACTAGTGACTCATTCTGCAAATTCTATCTGCAGTTATTTCACCCAT-2626
2627-TCACATCAATGTGATTTTCAATACTTTATCTTGGTGTAGGATTTTTAAAGACTATGT-2683
2684-AATTTTAAACTTTTTTTTTTAAGAATAATTTCTTTTTCCTTGGAATAAAAACTTATTT-2740
2741-GACTTTGTTGCCTATTGGGGGGAAAGTGGCCAATTGAGGATCCAAGTTGTTTTTTTTT-2797
2798-TTAGGCCTTTTTTCAAAACTTTTGGGATAACTGGGGGCCTTTTTTTTTTTTTCCTTGTG-2854
2855-GACTAACCCCTTTTTTGGTTTTTTTTGGAAAAAAGTTTTGGGAAAAAAAAAAAAAAA-2911
2912-AAAAAA-2918

Figure 11: Full-length sequence of the obtained sculpin NHE. Letters in bold and underlined represent primer binding sites. Numbers correspond to their sequences found in Table 1.

1-QGQKLSSYRVTGQRRVRGEKAN**M**PVFTMDYPRIQIPFEITMWVLLASFAKIGFHVYNKITI-61
61-LWFAVVGTLWNSIGIGMSLFAICQIEAFGVQDINLQENLLFATIISAVDPVAVLSVFEDV-121
122-SVNEQLYIVVFGECLFNDAVTVVLYNMFNFVAEMPVVEPVDVCLGVARFFVVGLGG-178
179-MGFGILFGFTAAFTTRFTSKVREIEPLFIFMYSYLAYLVAELFAISSIMAIVTCALTMKY-240
241-YVEENVSQRSCTTIRHVIKMLGSISETLIFFFLGVVAITTEHEWNWGYILFTLLFAFVWR-302
303-GLGVLVLTQIINPFRTIPFNLKDQFGLAYGGLRGAISFALVFTLPDTIGRKQLFITATISII-365
366-LFTVFLQGISIRPLIEFINVRRTNRNLDTINVEIHCRLMEHTMAGLEDLCGQWSHFYWKD-426
427-KFMKFNNRILRKILIRDNRAESSIVALYKKLELQNAMEILDTVSGDMSAAPSIVSLYEEK-487
488-TKPKKFLASDLKDMHDILSKNMYKIRQRTVAYTTKHALPNDSQSKEILIRRHASIRRXLR-548
549-PGSFQSSVVPNPTNTSLFLLGKVWTQNSPLLGRRMRKPCQRWPTPPDGLGLGSPHAPPP-609
610-EP*CLSAGWTPSQRCTLSTWWTRAQGRAEAGRGAARPEHTLPPVIPTFLFLIVALTTNM-669
670-AQLITLETGIMKSSSSLRPRLQAGLLKPETTRHETLCSGGLSGTPRCKSVPGLSREDGLP-730
731-QTPGSLQTCP*TK*RITGGPLTSY*G*AILK*ATIDSNALKSEVLATPEAQCTLVKQLNMN-792
793-CH**LILQILSAVISPIHINVIFNTLSWCRIFKDYVILNFFF*E*FLFPWNKNLFDFVAYWG-855
856-ESGQLRIQVVFFLGLFSKLLG*LGAFFFFPCGLTPFLVFFGKKFWEKKKKKK-908

Figure 12: Full-length sequence for Sculpin NHE. Complete sequence is 908 amino acids long. The first M is the start codon and is underlined and in bold and a * represents stop codons. The first stop codon is in bold and underlined.

Amino acid sequences of all published mammalian NHE isoforms and the sculpin NHE are compared in Figure 13, and their hydropathy profiles are presented in Figures15-16. Hydropathy analysis showed that the sculpin NHE2 has a similar theoretical membrane topology as the other plasma membrane Na^+/H^+ exchangers, with multiple membrane spanning domains in the N-terminal half of the protein and an extensive cytoplasmic domain in the C-terminal half. The greatest similarity between these proteins occurs in the N-terminal regions, which contains the transmembrane domains. The C-terminal region, which contains the cytoplasmic domain, shows the least similarity. Secondary structural analysis also showed that the sculpin NHE2 has a similar predicted secondary structure to that of the NHE gene family (Figures 17-18). Phylogenetic analysis of the sculpin NHE with the mammalian NHE gene family shows that the sculpin NHE is most closely related to the mammalian NHE2. The tree shows

that the sculpin NHE is located on the same branch as the mammalian NHE2 and

NHE4 (Figure 14).

```
NHE1        1 MVLRSGICGLSPHRIFPSLLVVALVGLLPVLRSHGLQLSPTASTIRSSE       50

Rat NHE2    1    MGPSGTAHRMRAPLSWLLLLLLSLQVAVPAGALAETLLDAPGARGAS       47
NHE3        1 QLRDSAEGCDEERTRYHGMRVGSWSNAAAMWHPALGPGWKPLLALAVAV       49
Rat NHE4    1        MGPAMLRAFSSWKWLLLLMVLTCLEASSYVNESSSPTGQQTP-       42
Rat NHE5    1                       MLRVALLLLPGLPLAGVGAT       20
NHE6        1 PVRNGREAGGDMARRGWRRAPLRRGVGSSPRARRLMRPLWLLLAVGVFDW       50
BNHE        1     MPAFSCAFPGCRRDLLVIVLVVFVGIG---------LP-----IEASA       34
sculpin     1                                          QGQKLS-        6


NHE1       51 PPRERSIGDVTTAPPEVTPESRPVNHSVTDHGMKPRKAFPVLGIDYTHVR      100
Rat NHE2   48 S--------------N---PPSPASVVAPGTTPFEESRLPVFTLDYPHVQ       80
NHE3       50 T--------------------SLRGVRGIEEEPNSGGSFQIVTFKWHHVQ       79
Rat NHE4   43 --------------------DARFAASSSDPDE--RISVFELDYDYVQ       68
Rat NHE5   21 E-------------------EPTQEPGPLGEPP---GLALFRWQWHEVE       47
NHE6       51 AG------------------ASDGGGGEARAMDEEIVSEKQAEESHRQD       81
BNHE       35 P---------------AYQSHGTEGSHLTNITNTKKAFPVLAVNYEHVR       68
sculpin     7 --------------------SYRVTGQRRVRGEKANMPVFTMDYPRIQ       34


NHE1      101 TPFEISLWILLACLMKIGFHVIPTISSIVPESCLLIVVGLLVGGLIKGVG      150
Rat NHE2   81 IPFEITLWILLASLAKIGFHLYHKLPTIVPESCLLIMVGLLLGGIIFGVD      130
NHE3       80 DPYIIALWILVASLAKIVFHLSHKVTSVVPESALLIVLGLVLGGIVWAAD      129
Rat NHE4   69 IPYEVTLWILLASLAKIGFHLYHRLPHLMPESCLLIIVGALVGSIIFGTH      118
Rat NHE5   48 APYLVALWILVASLAKIVFHLSRKVTSLVPESCLLILLGLALGGIVLAVA       97
NHE6       82 SANLLIFILLLTLTILTIWLFKHRRARFLHETGLAMIYGLLVGLVLRYGI      131
BNHE       69 KPFEIALWILLALLMKLGFHLIPRLSAVVPESCLLIVVGLLVGGLIKVIG      118
sculpin    35 IPFEITMWVLLASFAKIGFHVYNKITIWVPETCLLITIGLIVGGIMHSVH       84


NHE1      151 ET-------------------PPFLQSDVFFLFLLPPIILDAGYFLPLR      180
Rat NHE2  131 EKS-----------------PPAMKTDVFFLYLLPPIVLDAGYFMPTR      161
NHE3      130 HIA-----------------SFTLTPTLFFFYLLPPIVLDAGYFMPNR      160
Rat NHE4  119 HKS-----------------PPVMDSSIYFLYLLPPIVLESGYFMPTR      149
Rat NHE5   98 KKA-----------------EYQLEPGTFFLFLLPPIVLDSGYFMPSR      128
NHE6      132 HVPSDVNNVTLSCEVQSSPTTLLVTFDPEVFFNILLPPIIFYAGYSLKRR      181
BNHE      119 EE-----------------PPVLDSQLFFLCLLPPIILDAGYFLPIR      148
sculpin    85 EEP-----------------PAVLSSNVFFLYMLPPIVLDSGYFMPTR      115
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NHE1       181 QFTENLGTILIFAVVGTLWNAFFLGGLMYAVCLVGGEQ---INNIGLLDN
Rat NHE2   162 PFFENLGTIFWYAVVGTLWNSIGIGLSLFGICQIEAFG---LSDITLLQN 208
NHE3       161 LFFGNLGTILLYAVIGTIWNAATTGLSLYGVFLSGLMG---ELKIGLLDF 207
Rat NHE4   150 PFFENIGSILWWAGLGALINAFGIGLSLYFICQIKAFG---LGDINLLQN 196
Rat NHE5   129 LFFDNLGAILTYAVVGTLWNAFTTGVALWGLQQAGLVAP--RVQAGLLDF 176
NHE6       182 HFFRNLGSILAYAFLGTAISCFVIGSIMYGCVTLMKVTGQLAGDFYFTDC 231
BNHE       149 PFTENVGTILVFAVIGTLWNAFFMGGLLYALCQIESVG---LSGVDLLAC 195
sculpin    116 PFFENIGTVLWFAVVGTLWNSIGIGMSLFAICQIEAFG---VQDINLQEN 162


NHE1       228 LLFGSIISAVDPVAVLAVFEEIHINELLHILVFGESLLNDAVTVVLYHLF
Rat NHE2   209 LLFGSLISAVDPVAVLAVFENIHVNEQLYILVFGESLLNDAVTVVLYNLF 258
NHE3       208 LLFGSLIAAVDPVAVLAVFEEVHVNEVLFIIVFGESLLNDAVTVVLYNVF 257
Rat NHE4   197 LLFGSLISAVDPVAVLAVFEEARVNEQLYMMIFGEALLNDGISVVLYNIL 246
Rat NHE5   177 LLFGSLISAVDPVAVLAVFEEVHVNETLFIIVFGESLLNDAVTAVLYKVC 226
NHE6       232 LLFGAIVSATDPVTVLAIFHELQVDVELYALLFGESVLNDAVAIVLSSSI 281
BNHE       196 LLFGSIVSAVDPVAVLAVFEEIHINELVHILVFGESLLNDAVTVVLYNLF 245
sculpin    163 LLFATIISAVDPVAVLSVFEDVSVNEQLYIVVFGECLFNDAVTVVLYNMF 212


NHE1       278 EEFANYE----HVGIVDIFLGFLSFFVVALGGVLVGVVYGVIAAFTSRFT 323
Rat NHE2   259 KSFCQMK----TIQTVDVFAGIANFFVVGIGGVLIGILLGFIAAFTTRFT 304
NHE3       258 ESFVTLG--GDAVTGVDCVKGIVSFFVVSLGGTLVGVIFAFLLSLVTRFT 305
Rat NHE4   247 IAFTKMHK-FEDIEAVDILAGCARFVIVGCGGVFFGIIFGFISAFITRFT 295
Rat NHE5   227 NSFVEMG--SANVQATDYLKGVASLFVVSLGGAAVGLVFAFLLALTTRFT 274
NHE6       282 VAYQPAGDNSHTFDVTAMFKSIGIFLGIFSGSFAMGAATGVVTALVTKFT 331
BNHE       246 EEFSKVG----TVTVLDVFLGVVCFFVVSLGGVLVGAIYGFLAAFTSRFT 291
sculpin    213 NFVAEMP----VVEPVDVCLGVARFFVVGLGGMGFGILFGFTAAFTTRFT 258


NHE1       324 SH--IRVIEPLFVFLYSYMAYLSAELFHLSGIMALIASGVVMRPYVEANI 371
Rat NHE2   305 HN--IRVIEPLFVFLYSYLSYITAEMFHLSGIMAITACAMTMNKYVEENV 352
NHE3       306 KH--VRIIEPGFVFVISYLSYLTSEMLSLSAILAITFCGICCQKYVKANI 353
Rat NHE4   296 QN--ISAIEPLIVFMFSYLSYLAAETLYLSGILAITACAVTMKKYVEENV 343
Rat NHE5   275 KR--VRIIEPLLVFLLAYAAYLTAEMASLSAILAVTMCGLGCKKYVEANI 322
NHE6       332 KLREFQLLETGLFFLMSWSTFLLAEAWGFTGVVAVLFCGITQAHYTYNNL 381
BNHE       292 SH--TRVIEPLFVFLYSYMAYLSSEMFHLSGIMALIACGVVMRPYVEANI 339
sculpin    259 SK--VREIEPLFIFMYSYLAYLVAELFAISSIMAIVTCALTMKYYVEENV 306


NHE1       372 SHKSHTTIKYFLKMWSSVSETLIFIFLGVSTVAGS-HHWNWTFVISTLLF 420
Rat NHE2   353 SQKSYTTIKYFMKMLSSVSETLIFIFMGVSTVGKN-HEWNWAFVCFTLAF 401
NHE3       354 SEQSATTVRYTMKMLASGAETIIFMFLGISAVDPVIWTWNTAFVLLTLVF 403
Rat NHE4   344 SQTSYTTIKYFMKMLSSVSETLIFIFMGVSTVGKN-HEWNWAFVCFTLAF 392
Rat NHE5   323 SHKSRTAVKYTMKTLASSAETVIFMLLGISAVDSSKWAWDSGLVLGTLFF 372
NHE6       382 STESQHRTKQLFELLNFLAENFIFSYMGLTLFTFQNHVFNPTFVVGAFVA 431
BNHE       340 SHKSYTTIKYFLKMWSSVSETLIFIFLGVSTVAGP-HAWNWTFVITTVIL 388
sculpin    307 SQRSCTTIRHVIKMLGSISETLIFFFLGVVAITTE-HEWNWGYILFTLLF 355
```

```
NHE1      421 CLIARVLGVLGLTWFINKFRIVKLTPKDQFIIAYGGLRGAIAFSLGYLLD
Rat NHE2  402 CLIWRALGVFVLTQVINWFRTIPLTFKDQFIIAYGGLRGAICFALVFLLP 451
NHE3      404 ISVYRAIGVVLQTWILNRYRMVQLETIDQVVMSYGGLRGAVAYALVVLLD 453
Rat NHE4  393 CQIWRAISVFTLFYVSNQFRTFPFSIKDQLIIFYSGVRGAGSFSLAFLLP 442
Rat NHE5  373 ILFFRALGVVLQTWVLNQFRLAPLDKIDQVVMSYGGLRGAVAFALVILLD 422
NHE6      432 IFLGRAANIYPLSLLLNLGRRSKIGSNFQHMMMFAGLRGAMAFALAIRDT 481
BNHE      389 CLVSRVLGVIGLTFIINKFRIVKLTKKDQFIVAYGGLRGAIAFSLGYLLS 438
sculpin   356 AFVWRGLGVLVLTQIINPFRTIPFNLKDQFGLAYGGLRGAISFALVFTLP 405


NHE1      471 KKHFPMCDLFLTAIITVIFFTVFVQGMTIRPLVDLLAVKKKQETKRSINE
Rat NHE2  452 ATVFPRKKLFITAAIVVIFFTVFILGITIRPLVEFLDVKRSNKKQQAVSE 501
NHE3      454 EKKVKEKNLFVSTTLIVVFFTVIFQGLTIKPLVQWLKVKRSEQREPKLNE 503
Rat NHE4  443 LTLFPRKKLFVTATLVVTYFTVFFQGITIGPLVRYLDVRKTNKKE-SINE 491
Rat NHE5  423 RTKVPAKDYFVATTIVVVFFTVIVQGLTIKPLVKWLRVKRSDYHKPTLNQ 472
NHE6      482 ATYARQMMFSTTLLIVFFTVWVFGGGTTAMLSCLHIRVGVDSDQEHLGVP 531
BNHE      439 NSHQ-MRNLFLTAIITVIFFTVFVQGMTIRPLVELLAVKKKKESKPSINE 487
sculpin   406 DTIG-RKQLFITATISIILFTVFLQGISIRPLIEFINVRRTNRNLDTINV 454


NHE1      521 EIHTQFLDHLLTGIEDICGHYGHHHWKDKLNRFNKKYVKKCLIAG---ER 567
Rat NHE2  502 EIHCRFFDHVKTGIEDVCGHWGHNFWRDKFKKFDDKYLRKLLIR----EN 547
NHE3      504 KLHGRAFDHILSAIEDISGQIGHNYLRDKWSNFDRKFLSKVLMRR---SA 550
Rat NHE4  492 ELHIRLMDHLKAGIEDVCGQWSHYQVRDKFKKFDHRYLRKILIR----RN 537
Rat NHE5  473 ELHEHTFDHILAAVEDVVGHHGYHYWRDRWEQFDKKYLSQLLMRR---SA 519
NHE6      532 ENERRTTKAESAWLFRMWYNFDHNYLKPLLTHSGPPLTTTLPACCGPIAR 581
BNHE      488 EIHTEFLDHLLTGVEGVCGHYGHYHWKEKLNRFNKTYVKRWLIAG---EN 534
sculpin   455 EIHCRLMEHTMAGLEDLCGQWSHFYWKDKFMKFNNRILRKILIR----DN 500


NHE1      568 SK-EPQLIAFYHKMEMKQAIELVESGGM--------------------- 594
Rat NHE2  548 QP-KSSIVSLYKKLEIKHAIEMAETGMI--------------------- 574
NHE3      551 QKSRDRILNVFHELNLKDAISYVAEGERRGSLAFIRSPSTDNMVNVDFST 600
Rat NHE4  538 QP-KSSIVSLYKKLEMKQAIEMAETGLL--------------------- 564
Rat NHE5  520 YRIRDQIWDVYYRLNIRDAISFVDQGGHVLSSAGLTLPSMP-------- 560
NHE6      582 CLTSPQAYENQEQLKDDDSDLILNDGDIS------------------- 610
BNHE      535 FK-EPELIAFYRKMELKQAIMMVES---------------------- 558
sculpin   501 RA-ESSIVALYKKLELQNAMEILDTVSG--------------------- 527


NHE1      595 --GKIPSAVS-TVSMQNIHPKSLPSERILPAL---SKDKEEEIRKILRNN 638
Rat NHE2  575 --STVPSFASLNDCREEK-IRKLTP-----------GEMDEIREILSRN 609
NHE3      601 PRPSTVEASVSYFLRENVSAVCLDMQSLEQRRRSIRDTEDMVTHHTLQQY 650
Rat NHE4  565 --SSVASPTPYQSERIQG-IKRLSP-----------EDVESMRDILTRN 599
Rat NHE5  561 SRNSVAETSVTNLLRESGSGACLDLQVIDTVR-SGRDREDAVMHHLLCGG 609
NHE6      611 --LTYGDSTVNTEPATSSAPRRFMGN---------SSEDALDRELAFGDH 649
BNHE      559 --GQLPSVLPSTISMQNIQPRAIPR------V---SKKREEEIRRILRAN 597
sculpin   528 --DMSAAPSIVSLYEEKTKPKKFLA-----------SDLKDMHDILSKN 563
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```
NHE1      639 LQKTR-QRLR---SYNRHTLVADPYEEAWNQMLLRR--------------
Rat NHE2  610 LYQIR-QRTLS---YNRHNLTADTSERQAKEILIRRRHSLRESLRKDNSL  655
NHE3      651 LYKPR-QEYKHL--YSRHELTPNEDEKQDKEIFHRTMRKRLESFKSAKLG  697
Rat NHE4  600 MYQVR-QRTLS---YNKYNLKPQTSEKQAKEILIRRQNTLRESLRKGQSL  645
Rat NHE5  610 LYKPR-RRYKAS--CSRHFISEDAQERQDKEVFQQNMKRRLESFKSTKHN  656
NHE6      650 ELVIRGTRLVLPMDDSEPPLNLLDNTRHGPANAYNYSLSDLNNLHMNLNR  699
BNHE      598 LQNNK-QKMRS-RSYSRHTLFDADEEDNVSEVRLRKT--------KMEME  637
sculpin   564 MYKIR-QRTVA---YTTKHALPNDS--QSKEILIRRHASIRRXLRPGSFQ  607
                      .

NHE1      671 QKARQLEQKINNYLTVPAH-KLDS-PTMSRARIGSDPLAYEPKEDLPVIT
Rat NHE2  656 NRERRASTSTSRYLSLPKN-TKLPEKLQKKNKVSNADGNSSDSDMDGTTV  704
NHE3      698 INQNKKAAKLYKRERAQKR--RNSSIPNGKLPMENLAHNFTIKEKDLELS  745
Rat NHE4  646 PWVKPAGTKNFRYLSFPYS-NPQPARRGARAAESTGNPCCWLLHFLLCRA  694
Rat NHE5  657 ICFTKSKPRPRKTGHKKKDGVANPEATNGKPPRDLGFQDTAAVILTVESE  706
NHE6      700 NLYYLKVPVHVSECVSYINAIYMANKEYKYPVHGRLNTNYIPLSFPGCTL  749
BNHE      638 RRVSVMERRMSHYLTVPAN-RESPRPGVRRVRFESDNQVFSADSFPTVHF  686
sculpin   608 SSVVPNPTNTSLFLLGKVW-TQNSPLLGRRMRKPCQRWPTPPDGLGLGSP  656

NHE1      719 IDPASPQS-PESVDLVNEELKGKVLGLSRDPAKVAEEDEDDD--GGIMMR  765
Rat NHE2  705 LNLQPRA----RRFLPDQFSKKASPAYKMEWKNEVDVGSARA--PPSVTP  748
NHE3      746 EPEEATNYEEISGGIEFLASVTKDVASDSGAGIDNPVFSPDEDLDPSILS  795
Rat NHE4  695 MVEKIWG----PGGQETQPRLLCRNLN                         717
Rat NHE5  707 EEEESESSETEKEDDEGIIFVARATSEVLQEGKVSGSLEVCP--SPRIIP  754
NHE6      750 NTGSVLGSYFHSDVVCSVSFMSQLKCKKNRIFSFLNLPDTNP--EYQFSN  797
BNHE      687 EQPSPPST-PDAVSLEEEEEE-----VPKRPSLKADIE------------  718
sculpin   657 HAPPPEPNCLSAGWTPSQRCTLSTWWTRAQGRAEAGRGAARP--EHTLPP  704

NHE1      766 SKETSSPGTDDVFTPAPSDSPSSQRIQRCLSDPGPHPEPGEGEPFFPKGQ  815
Rat NHE2  749 APRSKEGGTQTPGVLRQPLLSKDQRFGRGREDSLTEDVPPKPPPRLVRRA  798
NHE3      796 RVPPWLSPGETVVPSQRARVQIPNSPSNFRRLTPFRLSNKSVDSFLQADG  845
Rat NHE4  718                                                    717
Rat NHE5  755 PSPTCAEKELPWKSGQGDLAVYVSSETTKIVPVDMQTGWNQSISSLESLA  804
NHE6      798 CELIFLCARGGRVRRECYFLGNLGRRGSFVGKLLLIAAAFVLIVFFPFSL  847
BNHE      719 -------GPRGNASDNHQGELDYQRLARCLSDPGPNKDKEDDDPFMSC    759
sculpin   705 VIPTFLFLIVALTTNMAQLITLETGIMKSSSSLRPRLQAGLLKPETTRHE  754


NHE1      816                                                    815
Rat NHE2  799 SEPGNRKGRLGNEKP                                    813
NHE3      846 PEEQLQPASPESTHMNYRLWHTTYWTLVSSVICCCPARIAPCRACPPTCP  895
Rat NHE4  718                                                    717
Rat NHE5  805 SPPCTQPPTLTRLPPHPLVPEEPQVPLDLSSDPRSSFAFPPSLAKAGRSR  854
NHE6      848 VACCGATSNWHLILPHSGWGVKCGDPFPPLAVKAQIHNLQYTVVQKRRLQ  897
BNHE      760                                                    759
sculpin   755 TLCSGGLSGTPRCKSVPGLSREDGLPQTPGSLQTCPNTKNRITGGPLTSY  804
```

```
NHE1       816                                                                           
815
Rat NHE2   814                                                                        813
NHE3       896 KPRPTNAHFDCHFCLWCSWDRARAPGPTQCPRSVGGCAPGTQNALIPKAS     945
Rat NHE4   718                                                                        717
Rat NHE5   855 SE--SSADIPQQQELQPLMGHKDHTHLSPGPANSHWCIQFNRGGRL        898
NHE6       898 MTSETLCLFFQTKTVEGVTSSRLSQSTGVYSSGHLINIWRGWGGQRARGE    947
BNHE       760                                                                        759
sculpin    805 NG--NAILKNATIDSNALKSEVLATPEAQCTLVKQLNMNCHNNLILQILS    852


NHE1       816                                                                        815
Rat NHE2   814                                                                        813
NHE3       946 --PTPTPSSAKTRFSSGLYRTATARSGARRDAAVIAAWPMAQLRGSPSVA    993
Rat NHE4   718                                                                        717
Rat NHE5   899                                                                        898
NHE6       948 NREWWFQRSLFPNVNIFYTRNVNIRNLIAANFLIMYFICIAGFVVRSFYI    997
BNHE       760                                                                        759
sculpin    853 --AVISPIHINVIFNTLSWCRIFKDYVILNFFFNENFLFPWNKNLFDFVA    900


NHE1       816                                                                        815
Rat NHE2   814                                                                        813
NHE3       994 PPQPASVLRVQRTLGVLMHSLALGRIFNSIWCPQDTTLPPAVFLPWFSSN    1043
Rat NHE4   718                                                                        717
Rat NHE5   899                                                                        898
NHE6       998 DLNCCSLAAKQEQNVKFLNLLCLIIICNSVVNVNSNFMNPRSFHTAPKSV    1047
BNHE       760                                                                        759
sculpin    901 YWGESGQLRIQVVFFLGLFSKLLGNLGAFFFFPCGLTPFLVFFGKKFWEK    950


NHE1       816                                                                        815
Rat NHE2   814                                                                        813
NHE3       1044 GSVVLPPHPYPEASPCNCLPPLFNEEKDPNEACSGLNHCWYKQRSQKLRF   1093
Rat NHE4   718                                                                        717
Rat NHE5   899                                                                        898
NHE6       1048 IREIEYSGALIAAGWLLCVQQVNNSS-------FSWYVLILITNAAMHFF   1090
BNHE       760                                                                        759
sculpin    951 KKKKK                                                                 955


NHE1       816                                                                        815
Rat NHE2   814                                                                        813
NHE3       1094 VEVAPPKSTSFNIGSSFLCVPRIYTPVALSDPFQVSSPDCPITLNSTAEI   1143
Rat NHE4   718                                                                        717
Rat NHE5   899                                                                        898
NHE6       1091 FTVLKEEHRTISHFIFGVNKNILFCNADNTNRCIECLEECFLMLILTCSN   1140
BNHE       760                                                                        759
sculpin    956                                                                        955
```

```
NHE1       816
815
Rat NHE2   814                                                                          813
NHE3       1144 FGSSSSDFPVQPLPAWALPSKTRCPANTIPKLGTLQMARQAGLGARSGPL 1193
Rat NHE4   718                                                                          717
Rat NHE5   899                                                                          898
NHE6       1141 FRRKTNFFGFFFPQVNVALPNILSSNNIDLFTNCSDFPVKNTLLNKTCTY 1190
BNHE       760                                                                          759
sculpin    956                                                                          955


NHE1       816                                                                          815
Rat NHE2   814                                                                          813
NHE3       1194 EAFLGQNYSQLWHLSKEGSTSPRSRGMQESSLNTFCPVPRSSGPPPNNIG 1243
Rat NHE4   718                                                                          717
Rat NHE5   899                                                                          898
NHE6       1191 SPKGIYLCYCKHSLRFNGNSNVVPFRGSFDCWHSHLLCSGSDFPAMQESL 1240
BNHE       760                                                                          759
sculpin    956                                                                          955


NHE1       816                                                                          815
Rat NHE2   814                                                                          813
NHE3       1244 PLEGKRLGPQPLSTSSEVQCSRVTNVLLPTHNKLCLHHREIEMGLKVDLM 1293
Rat NHE4   718                                                                          717
Rat NHE5   899                                                                          898
NHE6       1241 PNRVCFTNCVPLHAVNSFLLMYVNGVNTSVSLTHKVIQVVKGYKNFNNYL 1290
BNHE       760                                                                          759
sculpin    956                                                                          955


NHE1       816                                                                          815
Rat NHE2   814                                                                          813
NHE3       1294 TSWRLLKQSTQVSAQETKYCVSGTRLTVTFRGVTGKSVNLYNCCSNFVDR 1343
Rat NHE4   718                                                                          717
Rat NHE5   899                                                                          898
NHE6       1291 FYSVGLLTSLYVNTSHDKFSNSSISACIRLFTRETDKANVNNICLHTLSR 1340
BNHE       760                                                                          759
sculpin    956                                                                          955


NHE1       816                                                                          815
Rat NHE2   814                                                                          813
NHE3       1344 KRAGRSESKASPPNILHLLQSVAPPPNNACQYAVDCLVHTQPSDAQHLVS 1393
Rat NHE4   718                                                                          717
Rat NHE5   899                                                                          898
NHE6       1341 GTKPAVLTSASGRLSSPLICVHISKQGRKNKLCSDAYETPENTLMKYSLS 1390
BNHE       760                                                                          759
sculpin    956                                                                          955
```

```
NHE1       816                                                                        815
815
Rat NHE2   814                                                                        813
NHE3      1394  TALEAFVPFRIEVQPLTMPGSVISPDYLELPFGVLPRQAVLGYCTRGVLR  1443
Rat NHE4   718                                                                        717
Rat NHE5   899                                                                        898
NHE6      1391  YHHAQVELLLDLFSCCLWNLRVNMANTLHLLKLFFKVSWHLSNLSVFGNV  1440
BNHE       760                                                                        759
sculpin    956                                                                        955
```

```
NHE1       816                                                                        815
Rat NHE2   814                                                                        813
NHE3      1444  GIYQPLLCRQVSQTFLCQDTALTPHPICWTWGPIPTAELEAKCDETVPQQ  1493
Rat NHE4   718                                                                        717
Rat NHE5   899                                                                        898
NHE6      1441  LLKNNTFNLYVFFVFSGVLGQMLNWFLLGKSVIKKVIALQITT         1483
BNHE       760                                                                        759
sculpin    956                                                                        955
```

```
NHE1       816                                                                        815
Rat NHE2   814                                                                        813
NHE3      1494  LSPCSRYPEIPKLAAGGDQSVEPFDTLSMFNLCTGHVLNVCELHHWANPY  1543
Rat NHE4   718                                                                        717
Rat NHE5   899                                                                        898
NHE6      1484                                                                       1483
BNHE       760                                                                        759
sculpin    956                                                                        955
```

```
NHE1       816                                                                        815
Rat NHE2   814                                                                        813
NHE3      1544  GTVLTRKPHSQNPTSCTNMIHTVATFHSNGPSCSCHCCTIECKILNMYFL  1593
Rat NHE4   718                                                                        717
Rat NHE5   899                                                                        898
NHE6      1484                                                                       1483
BNHE       760                                                                        759
sculpin    956                                                                        955
```

```
NHE1       816                                                                        815
Rat NHE2   814                                                                        813
NHE3      1594  RKTIVKVFFLLGENFYLFNTELNPTLWYYKVYKFRKFVFPLLYFLENNFI  1643
Rat NHE4   718                                                                        717
Rat NHE5   899                                                                        898
NHE6      1484                                                                       1483
BNHE       760                                                                        759
sculpin    956                                                                        955
```

```
NHE1        816                                                          815
Rat NHE2    814                                                          813
NHE3       1644 TVFNILCINNLFMKGNLKNSSKPEIYFEWLKLCINVVNIIIVELNFNSQN 1693
Rat NHE4    718                                                          717
Rat NHE5    899                                                          898
NHE6       1484                                                         1483
BNHE        760                                                          759
sculpin     956                                                          955
```

Figure 13: ClustalW alignment of all Published NHE sequences aligned with the sculpin NHE sequence. Homologous regions are in bold. Sequences for each of the published NHE isoforms can be obtained from the BLAST NIH webserver. The IPC numbers for each isoform are: NHE1-P19634, NHE2-P48763, NHE3-AAB50819, NHE4-P26434, NHE5-Q920X2, NHE6-Q59819, and βNHE-Q01345.
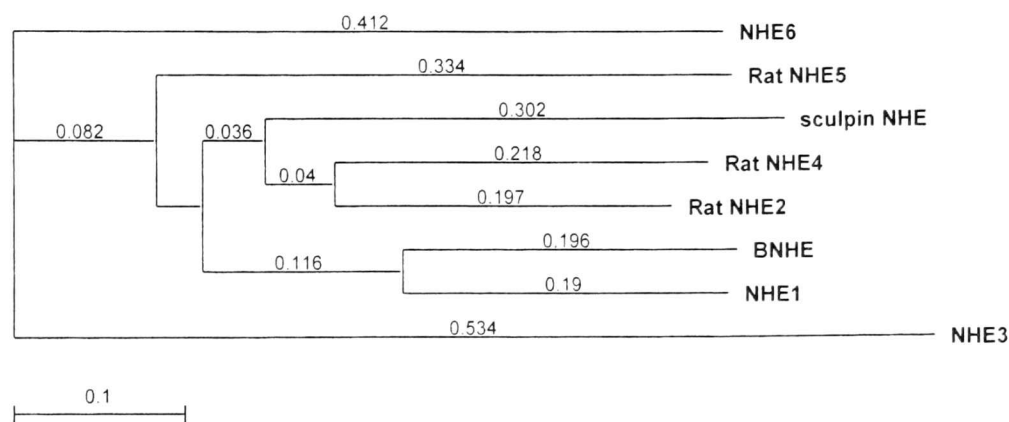

Figure 14. Phylogenetic tree of the mammalian NHE gene family with the sculpin NHE2-like sequence. The tree was plotted using the open reading frames of each sequence. The tree shows that the sculpin NHE is located on the branch closest to NHE2 and NHE4. Numbers represent the differences between two sequences.

Kyte/Doolittle hydrophilicity: Window = 7

Figure 15: Hydrophilicity plot for the rat NHE2 protein was determined according to the algorithm of Kyte and Doolittle using a window of 7 amino acids. Positive values correspond to hydrophilic segments and negative numbers correspond to hydrophobic segments.

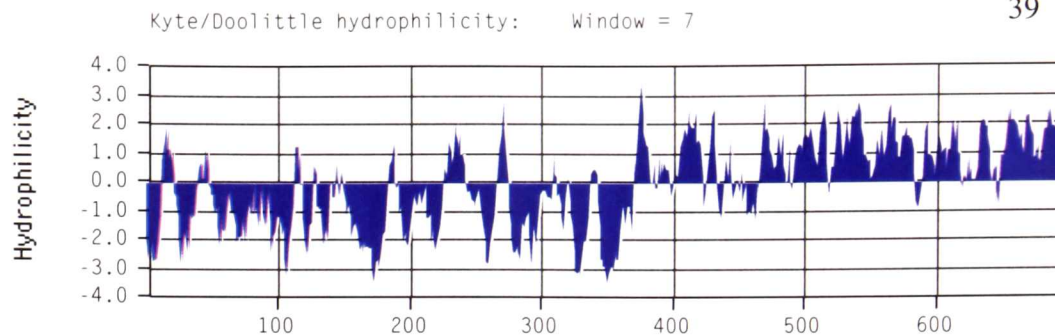Kyte/Doolittle hydrophilicity: Window = 11
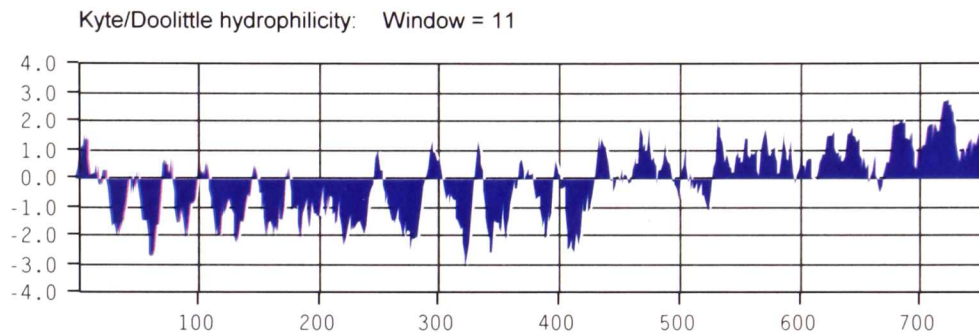
Figure 16: Hydrophilicity plot for sculpin NHE2-like protein was determined according to the algorithm of Kyte and Doolittle using a window of 11amino acids. Positive values correspond to hydrophilic segments and negative numbers correspond to hydrophobic segments.

Figure 17: Predicted secondary structure of sculpin NHE2-like protein using the methods of Chou and Fasman. The secondary structure was plotted using the open reading frame.

Figure 18: Predicted secondary structure of the rat NHE2 protein using the methods of Chou and Fasman. The secondary structure was plotted using the open reading frame.

Sub-Cloning.- Using the new primers generated for sequencing (Table 1), RT-PCR was carried out to produce fragments of the NHE2-like sequence. The 3' and 5' RACE products were not success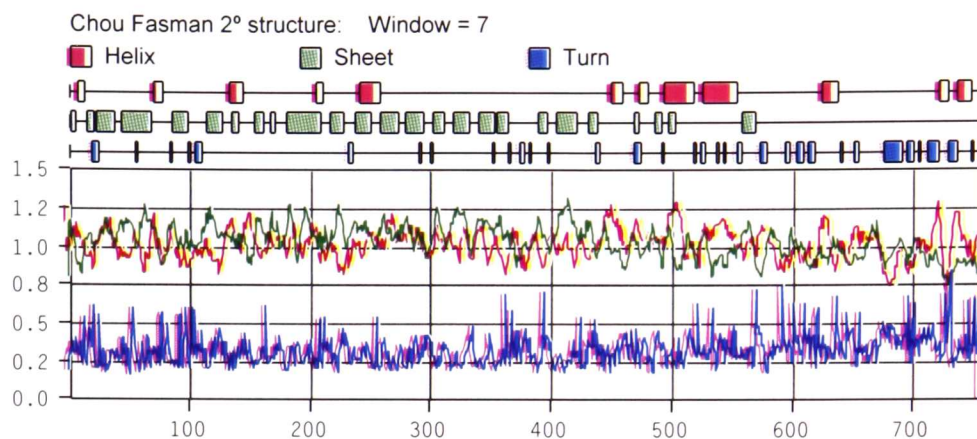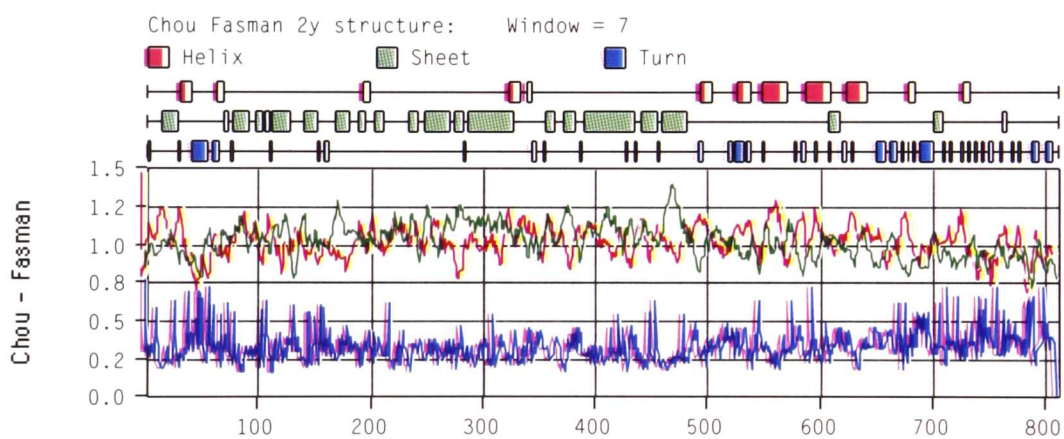fully cloned due to their size, therefore primer combinations were chosen on their ability to produce fragments under 1000 base pairs in length for the entire sequence (Figure 19). All of the primer combinations were successful except F144 and CRB-seq-R1. All positive RT-PCR products were ligated into pCR™2.1 vectors and transformed into OneShot™ Competent Cells. Positive colonies were isolated through blue/white screening, mini-prepped, and restriction digest was preformed (Figure 20). Positive colonies containing an insert of the correct size were sequenced, glycerol stocked and stored at -70°C. Sequences obtained confirmed that the primers amplify the sculpin NHE2-like sequence. As a result 3'/5' RACE is no longer needed to generate the full-length message of the sculpin NHE2-like sequence.
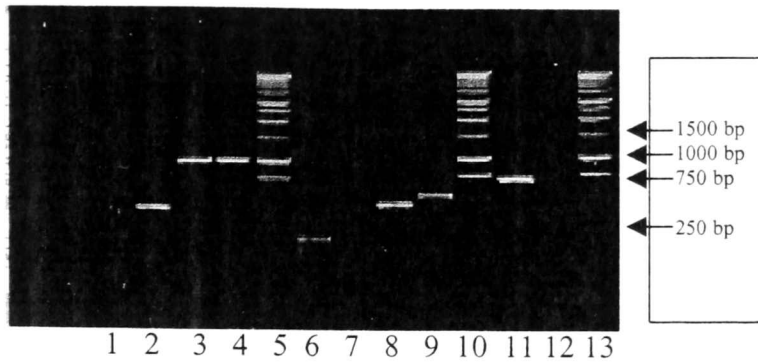
Figure 19: Ethidium bromide stained 1% agarose gel of RT-PCR using total sculpin gill RNA. Each lane represents a different combination of primers from Table 1, except lanes 5, 7,10, and 12. Primer combinations were chosen so that each region of the sculpin NHE could be amplified without using RACE. Lanes 5 and 10 are 1 kb ladders and lane 12 is the negative control. Lane 7 was primer combination F144 and CRB-seq-R1 which did not produce a band.
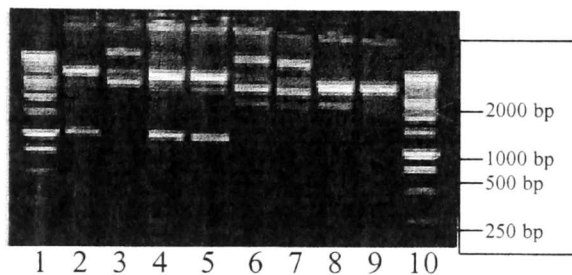
Figure 20: 1% agarose gel stained with ETBr containing 8 different plasmid DNA products with different combinations of the sequencing primer inserts after restriction digest with EcoR1.

Quantitative PCR.- To measure changes in the expression of mRNA in the gill tissue of the long-horned sculpin following acidosis, total RNA was extracted from several sculpin under acidic conditions and analyzed by relative RT-PCR using sculpin specific NHE2 primers. Prior to the quantitative PCR experiments, the linear range for amplification of the mRNA was determined. The linear range of the reaction is defined as the period of the PCR in which the amplification efficiency is at its maximum and

remains constant over a number of cycles (Ambion). Ethidium bromide staining
requires large amounts of product for visualization; therefore, ^{32}P was incorporated into
the amplification process. After the PCR was run, bands were cut out of the gel and run
through a scintillation counter. The linear cycle for the experiments was found to be 27
cycles (Figures 21-22).
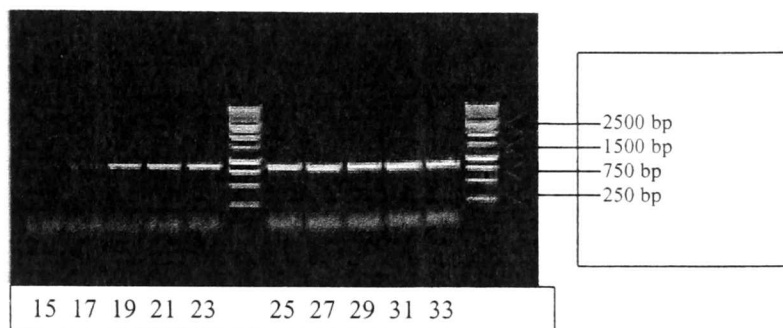
Figure 21: Results from RT-PCR determination of linear range. Tubes were taken out of
the thermocycler after each odd cycle beginning with cycle 15 and ending with cycle 33.
Bands were cut out of the gel and radioactivity determined by a scintillation counter.

Figure 22: Linear range of sculpin RNA spans between cycles 21 and 31. To give a
maximum range on both sides of the linear range, cycle 27 was chosen. The linear range
was determined by measuring the counts per minute (CPM) for each band then taking the
log of the CPM.

Ambion's quantitative PCR kit was chosen because it uses an internal standard to determine relative expression; however, the internal standard competes for available resources with the target mRNA. Given that, it is critical for the internal standard to be amplified from the RT-PCR at a level similar to the target mRNA. The experiment was run using the number of cycles determined from the linear range experiment and I found that the optimal ratio of internal standard and competimers was 2:8.

After determining the optimal parameters, quantitative PCR experiments were run using the RNA from six fish. For each of the experiments, RNA from each fish was run in triplicate. The experiments were performed twice. The relative expression was established by dividing the internal standard by the NHE2 product. Statistical analysis by ANOVA of the relative expression for each fish indicated that the results were not significant ($p > .02$; Figure 23). There does, however, seem to be an increasing trend of expression of NHE2 mRNA from zero hours through the six-hour time period with the peak at two-hours. There is less agreement between the two animals measured at hour six.

**Relative Expression of mRNA for Sculpin
Under Acidic Conditions**
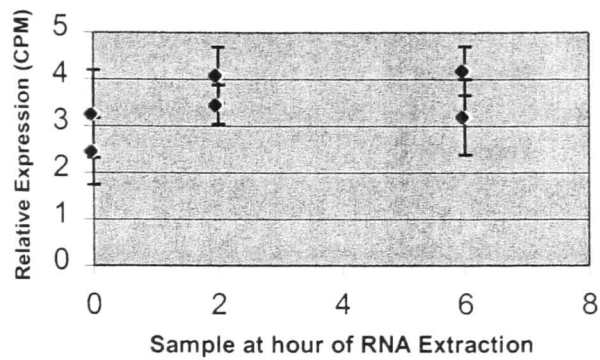
Figure 23: Relative expression for individual fish following acidosis. Standard error bars are shown for each fish (n=3 for quantitative PCR replicates). There is an increasing trend of mRNA produced after acid injection for hours two and six, however, for one of the fish in the 6-hour range, there is a decrease of expression from two hours. Taking the CPM for the NHE2 band and dividing it by the CPM for the internal standard band determined relative expression.

Chapter IV

Discussion

Sequence Analysis.-Sculpin specific primers NHE2F and NHE2R were successful in amplification of 3' and 5' RACE products. The RACE products were sequenced and found to match rat NHE2 (76% homologous) on the amino acid level by BLAST; however, the entire sequence was not complete. Further sequence was obtained from the RACE products using the primer walking method. In this method, a new primer was made each time sequence information was obtained moving further into the sequence (Table 1). The full-length sculpin cDNA contains a 2918 nucleotide open reading frame, which encodes a protein of 908 amino acids (Figures 11-12).

The full-length sequence of the sculpin NHE isoform sequenced is most similar to the NHE2 isoform. Blast comparisons of sculpin NHE sequence to all known sequences reveal the highest homology (76%) to rat NHE2, and are highly significant. βNHE, the only other fish isoform cloned to date, exhibits a 74% homology to human NHE1 (Malapert et al. 1997), and the crab NHE exhibits a 72% homology to the rat NHE isoforms (Towle et al. 1997). Following the precedent set by Malapert (1997) and Towle (1997), the 76% homology of the sculpin NHE sequence is of high enough homology to call it an NHE2-like isoform. In addition, hydropathy analysis showed that the sculpin NHE2 has the same membrane topology as the other plasma membrane Na^+/H^+ exchangers and is predicted to have 10-12 membrane-spanning domains and a long

cytoplasmic C terminus. Six mammalian isoforms and several fish isoforms of the NHE have been cloned from various epithelial cells. Multiple alignment of the sculpin NHE amino acid sequence with examples of the six mammalian isoforms and the βNHE fish isoform revealed a number of regions of homology particularly in the putative membrane-spanning domains (Figure 13). The membrane-spanning segments share a great deal of identity among all the isoforms, this suggests that this region participates in the transport of Na^+ and H^+ across the membrane. In contrast, the hydrophilic C-terminal region exhibits a lower degree of similarity among the isoforms, signifying that this is the region responsible for regulation of the NHE (Orlowski 1997). The substantial difference of the C-terminal region of the sculpin NHE2-like isoform is most likely due to the specific role that NHE plays in the gill epithelia.

When compared individually, each of the transmembrane segments of the sculpin gill NHE isoform is much more homologous to the corresponding region in NHE2 than to the corresponding segments of NHE1, NHE3, NHE5, NHE6, and βNHE. The transmembrane domains of the sculpin NHE and the rat NHE2 exhibit strong homology with 81% identity at the amino acid level. However, in the cytoplasmic domain of the sculpin NHE isoform, the homology falls to 20%. The noticeable difference of the C-terminal region of the sculpin NHE in relation to the mammal isoforms is most likely due to the specific function of NHE within the gill epithelia (Bookstein et al 1997). Between the mammalian isoforms the C-terminal region is significantly different, suggesting that the C-terminal region is responsible for regulation of Na^+/H^+ exchanger and, therefore, identifies which isoform is being expressed (Orlowski and Grinstein 1997).

The translation of the sculpin NHE2-like sequence showed a secondary structure pattern similar to that of the NHE gene family (Figures 17-18). Membrane proteins are generally composed of transmembrane domains and extracellular loops. In a study done by Liu and Deber (1998) on protein construction, they found that β-conformation in transmembrane proteins actually promote the α-helical conformation and that the α-helical conformation correlates with hydrophobicity. In both the rat NHE2 and the sculpin NHE2 secondary structural analysis, (Figures 17-18) the model is made up of α-helices with β-sheets intertwined in the transmembrane domains. Hydrophilicity plots (Figures 15-16) reinforce the α-helical conformation based on hydrophobicity. This supports the α-helical conformation of transmembrane proteins.

Multiple alignments of the sculpin NHE amino acid sequence with the representatives of the NHE gene family was done to create a phylogenetic tree (Figure 14). The diagram illustrates the possible relationship of the sculpin NHE2-like isoform to the other known NHE's. The tree shows that the sculpin NHE is located on the branch closest to the Rat NHE2 and Rat NHE4 isoforms. This reinforces not only the idea that the sculpin NHE is indeed a member of the NHE gene family, but most likely and NHE2-like isoform.

The presence of an NHE2-like isoform fits the saltwater model for acid-base regulation in the gill tissue as described on page 5. The postulated role of the apical isoform in the gills is the excretion of H^+ into the water and Na^+ absorption into epithelial cells. NHE is very sensitive to intracellular pH (Bookstein et al 1997), and therefore is activated by changes in H^+ concentration. NHE2 is also characteristically involved in regulation of net Na^+ transport (Bookstein et al. 1997). Alterations of acid-base relevant

ions in the external media have been shown to cause acid-base disturbances in most fishes, as a result, an apical isoform is needed to compensate for acid-base disturbances. The majority of the acid excreted by most fish is through the gills and not the kidney because the gills respond immediately to acid-base disturbances, whereas the kidneys may take hours or days to respond (Claiborne 1998; McDonald 1998). Although this study has identified a single NHE isoform in the gill of the sculpin, it is unlikely that NHE2 is the only isoform being expressed. A partial sequence of βNHE was detected in the gill tissue of the long-horned sculpin (Blackston 1998), as was NHE1 with antibodies (Choe 1999). Recent evidence suggests that gene duplication might be responsible for the creation of gene families like NHE and that fishes have gone through three entire gene duplications (Meyer and Schart 1999). The 'one-two-four rule' states that vertebrates tend to have more genes often four, belonging to the same gene family (Meyer and Schart 1999). Given that, it is probable that there are more isoforms of the NHE gene family being expressed in the gill epithelia.

Even though the full-length sequence was obtained from the gill tissue of the long-horned sculpin, it could not be sub-cloned. All sub-cloning attempts with the 5' RACE products and the 3' RACE products were unsuccessful. However, when the full-length message was generated by RT-PCR, the fragments could then be sub-cloned. This is most likely due to either the size of the template or the fact that the sculpin NHE2-like message may be toxic to the bacteria. If the full-length message were toxic then it would kill the bacteria. All RT-PCR products were under 1000 base pairs in length, which is significantly smaller than the original RACE products, and could be sub-cloned without complications. Despite the fact that the RT-PCR products could be sub-cloned, it does

not rule out the possibility that the full-length message may be toxic gene. A toxic

gene is described as an insert that prevents the cell from harboring that clone, which will

in turn have detrimental effects on the cell (personal communication from Invitrogen's

technical support). The pCR2.1 vector was designed to allow ligations of PCR products

up to 10 kb in size. All troubleshooting suggestions made by the company were followed

with no successful results. This could also play a role in the library screening process. If

NHE2-like message generated by the sculpin were indeed toxic, then the attempts to

incorporate it into the library would also be unsuccessful. This could be a reason why the

library screening processes has been unsuccessful.

Quantitative PCR.- Recent studies have demonstrated that NHE2 can be activated

in the presence of a pH gradient, decreases in the pH of the cell cause an upregulation of

NHE2 (Bookstein et al. 1997). Sculpin were infused with an acid load of HCl in order to

study the time course of mRNA expression in the gill tissue of the long-horned sculpin.

Quantitative PCR was used to determine the mRNA expression of the sculpin NHE2-like

isoform under acidic conditions. Previous work done by Claiborne, and coworkers

(1997) demonstrated that sculpin under acidic conditions were able to compensate for the

added acid load by over-excreted H^+ into the water. It was hypothesized that activation

of an apical NHE aided in the recovery of the additional acid load (Claiborne et al. 1997).

Towle and coworkers (1997) found that the level of NHE mRNA expression is much

higher in the gill than any of the other tissues of the euryhaline crab, *Carcinus meanas,*

which function in acid-base homeostasis.

The present results may indicate an increase in transcription of NHE2 mRNA in

the gill tissue of the long-horned sculpin under acidic conditions, while not statistically

significant due to the low sample number of sculpin used (Figure 21). PCRs were run in triplicate for each fish, and this procedure was repeated twice. Some variations among the PCR reactions for individual fish are apparent. This could be due to parameters set for the reaction. Between fish variation may possibly be due to fluctuations in P_{O2}, P_{CO2}, temperature, and salinity as these parameters can affect the acid-base balance of many fish (Claiborne et al. 1997). Fish already in acid-base disequilibria may exhibit high baseline levels of NHE2 mRNA. This might account for the variation in expression of NHE among the different fish. For these reasons, quantitative PCR experiments using more fish are needed to determine if the increasing trend of NHE2 expression in the gill tissue is significant.

If NHE2 mRNA expression does indeed increase, this would be consistent with the previous research on NHE2. The apical isoform of the NHE gene family, NHE2 has been shown to be capable of regulating pH (Yun et al. 1995). Increased acid concentration in the sculpin triggered an up regulation in the message level of NHE2 in the gill. This corresponds with the saltwater model for acid-base regulation (Figure 3) and the previous work done on the long-horned sculpin by Claiborne et al (1997). An apical isoform is needed to move the hydrogen ions out and sodium ions in to buffer the excess acid load.

Library Screening.- Multiple NHE isoforms have been identified in mammalian tissues. These isoforms exhibit differences in membrane localization, sensitivity to inhibition by amiloride, ion specificity, activation and size (Orlowski et al. 1992). For this and reasons mentioned earlier in this chapter, it has been suggested that there could be more than one isoform of NHE in the long-horned sculpin. To determine if more than

one isoform of NHE is present in the gill tissue of the long-horned sculpin, cDNA libraries were screened using a partial sculpin NHE2 sequence as a probe. Two different sculpin gill cDNA libraries were screened, a library made from the gills of fish adapted to 20% sea water and a library made from fish adapted to 100% sea water.

A total of six partial and full-length cDNA sequences were obtained from the library screening process (Appendix F). From the sculpin gill seawater library a partial sequence with high homology of a zinc finger protein was found. The full-length sequence of glutathione s- transferase (GST), hemoglobin, alpha subunit, and nonhistone chromosomal protein HMG was acquired from the sculpin gill 20% seawater library. Along with the full-length sequences, several partial sequences were also found in the sculpin gill 20% seawater library. They are: phenylalanyl-tRNA synthetase beta subunit, nuclear RNA helicase, and a class II aminotransferase. It is likely that the cDNA sequences were found either because of the low stringency, or the probe used in the screening process. The experiments were repeated increasing the stringency of the screening process, but no NHE isoforms were detected. Therefore, these experiments should be repeated with the 5' sequence of the sculpin NHE2-like isoform as a probe to determine if other NHE isoforms can be detected. As with the PCR sub-cloning, one reason why no NHE sequences have been detected could be due to a toxic effect of the complete sequence in bacteria.

The greatest similarity between the NHE isoforms occurs in the N-terminal regions, which contains the transmembrane domains. Using the cloned fragments of the sculpin NHE2-like sequence, a new probe was made to screen the libraries. The new probe is ~900 base pairs in length and is located in the transmembrane spanning domains of the

sequence. The new probe was made from sculpin gill RNA using R689 and NHE2F primers in the RT-PCR reactions. Therefore, sculpin NHE sequence may bind to the homologous regions of other NHE isoforms if present in the sculpin gill cDNA library.

Summary.- The major objectives of this study were 1) obtain the complete sequence a the sodium/hydrogen exchanger; 2) establish the structure of the NHE DNA sequence; 3) find out which isoform is being expressed in the gill tissue, and; 4) compare the sculpin to other mammalian isoforms to determine which regions of the sequence are homologous. The amino acid sequence of the sculpin NHE2-like isoform is highly similar to human, rat, and rabbit NHE2, exhibiting 76% amino acid homology. The high degree of similarity indicates that the sculpin cDNA clone is most likely an NHE2-like isoform.

Several pieces of evidence suggest that the predicted protein encoded by the sculpin NHE2-like transcript is likely to be an isoform of the NHE gene family. The sculpin cDNA shares several structural features with the previously characterized NHE2 isoforms. The computer generated analyses of the primary structure of the sculpin NHE revealed a high degree of similarity in their hydrophobic and hydrophilic regions and in their predicted secondary structure that is consistent with the other NHE2 isoforms having similar transmembrane organizations. The highest similarity occurred in the N-terminal region on the 5' end of the sequence. Located within these regions are 12 hydrophobic membrane-spanning domains that show even higher degrees of amino acid identity. Since the transmembrane spanning domains most likely participate in cation transport, the higher degree of similarity of these domains is consistent with the

possibility that these proteins have similar ion specificities (Wakabayashi 1997). In contrast, the C-terminal region is highly hydrophilic and exhibits the lowest degree of similarity. This region, which is most likely on the cytoplasmic side of the membrane, contains the area that is responsible for regulating the transport activity.

The presence of multiple sodium-hydrogen exchangers in particular tissues, such as the kidney, stomach, and intestine may reflect their differential location and specific function. Blackston et al. (1997) demonstrated using degenerate primers in RT-PCR that a βNHE-like isoform might also be present in the gill tissue of the long-horned sculpin. Choe et al. (1998) showed the immunological presence of an NHE1-like isoform in the gill tissue of the sculpin. Wall (Master's thesis 2000) demonstrated the immunological presence of NHE1, NHE2, and NHE3-like isoforms in the gill tissue of the killifish. This implies the existence of a basolateral isoform different to the predicted apical location of the NHE that was cloned and multiple isoforms within the gill epithelia.

The physiological significance of the sculpin NHE2-like isoform is not fully understood. Preliminary expression studies using quantitative PCR have revealed a trend for the up regulation of the message of the NHE2-like isoform. Additional quantitative PCRs are needed to determine if the trend is significant. As a result, we know that the message is being transcribed, but it is unknown if the translated protein functions within the cell. Expression studies using oocytes are needed to determine the physiological role of the NHE2-like isoform within the gill epithelia. In addition to expression studies, sculpin specific antibodies can now be created and used in immunohistochemical studies. This should give a better definition to the physiological significance as to the role that NHE plays in acid-base regulation in fish gill epithelia.

Literature Cited

Baird, N. R., J. Orlowski, et al. (1999). "Molecular cloning, genomic organization and functional expression of Na^+/H^+ exchanger isoform 5 (NHE5) from human brain." J. Biol. Chem. **274**(7): 4377-4382.

Bianchini, L. and J. Pouysségur (1994). "Molecular structure and regulation of vertebrate Na^+/H^+ exchangers." J. Exp. Biol. **196**: 337-345.

Blackston, C. R., W. Hollimon, et al. (1997). "Isoforms of the Na^+/H^+ antiporter (NHE), in gill mRNA of the marine long-horned sculpin (*Myoxocephalus octodecimspinosus*)." Bull.MDIBL **36**: submitted 1997.

Bookstein, C., A. M. DePaoli, et al. (1994). "Na^+/H^+ exchangers, NHE-1 and NHE-3, of rat intestine. Expression and localization." J. Clin. Invest. **93**: 106-113.

Bookstein, C., W. Musch, et al. (1994). "A unique sodium hydrogen exchange isoform (NHE-4) of the inner medulla of the rat kidney is induced by hyperosmolarity." J. Biol. Chem. **269**: 29704-29709.

Bookstein, C., Y. Xie, et al. (1997). "Tissue distribution of Na^+/H^+ exchanger isoforms NHE2 and NHE4 in the rat intestine and kidney." Am. J. Physiol. **273**: C1496-C1505.

Borgese, F., M. Malapert, et al. (1994). "The cytoplasmic domain of the Na^+/H^+ exchangers (NHEs) dictates the nature of the hormonal response: Behavior of a chimeric human NHE1/trout βNHE antiporter." Proc. Natl. Acad. Sci. USA **91**: 5431-5435.

Borgese, F., C. Sardet, et al. (1992). "Cloning and expression of a cAMP-activated Na^+/H^+ exchanger; evidence that the cytoplasmic domain mediates hormone regulation." Proc. Natl. Acad. Sci. USA **89**: 6765-6769.

Choe, K. P. (1998). NHE1 expression in the gills of the long horned sculpin.

Claiborne, J. B. (1998). Acid-Base Regulation. In "The Physiology of Fishes" D. H. Evans, Ed. Boca Raton, CRC Press: 179-200.

Claiborne, J. B., C. R. Blackston, et al. (1999). "A mechanism for branchial acid excretion in marine fish: identification of multiple Na^+/H^- antiporter isoforms (NHE) in gills of two seawater teleosts." J. Exp. Biol. **202**(3): 315-324.

Claiborne, J. B., E. Perry, et al. (1997). "The mechanisms of acid excretion across the gills of marine fish." J. Exp. Zool. **279**: 509-520.

Cox, G. A., C. M. Lutz, et al. (1997). "Sodium/hydrogen exchanger gene defect in slow wave epilepsy mutant mice." Cell **91**: 139-148.

Dyck, J. and D. Lopaschuk (1998). "Glucose metabolism, H^+ production and Na^+/H^+ exchanger mRNA levels in ischemic hearts from diabetic rats." Molecular and Cellular Biochemistry **180**: 85-93.

Fischer, H., A. Seelig, et al. (1999). "New Drugs for the Na^+/H^+ Exchanger. Influence of Na^+ Concentration and Determination of Inhibition Constants with a Microphysiometer." J. Membrane Biology **168**: 39-45.

Fliegel, L. and O. Fröhlich (1993). "The Na^+/H^+ exchanger: an update on structure , regulation and cardiac physiology." Biohem. J. **296**: 273-285.

Grinstein, S. and A. Rothstein (1986). "Mechanisms of regulation of the Na^+/H^+ exchanger." J. Membr. Biol. **90**: 1-12.

Heisler, N. (1993). Acid-base regulation. "The physiology of fishes." D. H. Evans Ed. Boca Raton, CRC Press. **1**: 343-378.

Kyte, J. and R. F. Doolittle (1982). "A simple method for displaying hydropathic character of a protein." J. Molec. Biol. **157**: 105-132.

Lin, H. and D. Randall (1995). Proton pumps in fish gills. Cellular and molecular approaches to fish ionic regulation. C. M. Wood and T. J. Shuttleworth. London, Academic Press. **14**: 229-255.

Lin, H. and D. J. Randall (1993). "H^+ATPase activity in crude homogenates of fish gill tissue-inhibitor sensitivity and evironmental and hormonal regulation." J. Exp. Biol. **180**: 163-174.

Liu, Li-Ping and C. M. Deber (1998). "Guidelines for membrane protein engineering derived from De Novo designed model peptides." Biopolymers **47**: 41-62.

Malapert, M., H. Guizouarn, et al. (1997). "Regulation of Na^+/H^+ antiporter in trout red blood cells." J. Exp. Biol. **200**: 353-360.

McDowall, R. M. (1997). "The evolution of diadromy in fishes (revisited) and its place in phylogenetic analysis." Rev. Fish. Biol. **7**: 443-462.

Meyer, A. and M. Schart (1999). "Gene and genome duplications in vertebrates: the one-to-four (-to-eight in fish) rule and the evolution of novel gene function." Curr. Opin. Cell Biol. **11**: 699-704.

Nelson, J. S. (1984). Fishes of the world. New York, John Wiley and Sons.

Noel, J., D. Roux, et al. (1996). "Differential localization of Na^+/H^+ exchanger isoforms (NHE1 and NHE3) in polarized epithelial cell lines." J. Cell. Science **109**: 929-939.

Numata, M., K. Petrecca, et al. (1998). "Identification of a mitochondrial Na^+/H^+ exchanger." J. Biol. Chem. **273**(12): 6951-6959.

Orlowski, J. and S. Grinstein (1997). "Na^+/H^+ exchangers of mammalian cells." J. Biol. Chem. **272**(36): 22373-22376.

Orlowski, J., R. A. Kandamsamy, et al. (1992). "Molecular cloning of putative members of the Na/H exchanger gene family." J. Biol. Chem. **267**(13): 9331-9339.

Perry, S. F. (1997). "The chloride cell: Structural and function in the gills of freshwater fishes." Annu. Rev. Physiol. **59**: 146-150.

Peti-peterdi, J., R. Chambrey, et al. (2000). "Macula densa Na^+/H^+ exchange activities mediated by apical NHE2 and basolateral NHE4 isoforms." Am. J. Renal Physiol. **278**: F452-F463.

Sambrook, J., E. F. Fritsch, et al. (1989). Molecular cloning: a laboratory manual. Cold Springs Harbor, Cold Springs Harbor Laboratory Press.

Shuttleworth, T. J. (1988). Salt and water balance- Extrarenal mechanisms. Physiology of Elasmobranch fishes. T. J. Shuttleworth. Berlin, Springer-Verlag: 171-199.

Towle, D. W., M. E. Rushton, et al. (1997). "Sodium/proton antiporter in the euryhaline crab *Carcinus maenas*: Molecular cloning, expression and tissue distribution." J. Exp. Biol. **200**: 1003-1014.

Tse, C. M., S. A. Levine, et al. (1993). "Cloning and expression of a rabbit cDNA encoding a serum-activated ethylisopropyl amiloride resistant epithelial exchanger isoform NHE2." J. Biol. Chem. **268**: 11911-11964.

Wakabayashi, s., M. Shigekawa, et al. (1997). "Molecular physiology of vertebrate Na$^+$/H$^+$ exchangers." Physiol. Rev **77**(1): 51-74.

Yun, C. H., C. M. Tse, et al. (1995). "Mammalian Na$^+$/H$^+$ exchanger gene family: structure and function studies." Am. J. Physiol. 269: G1-G11.

Appendices

Appendix A- Table 1: List of primers used, their sequences, and optimum annealing temperatures. Numbers to the position in sequence column correspond to the sequence information in Figure 11.

Primer Name	Nucleotide Sequence	Annealing temperature	Position in sequence
SC-NHE2-F	CATTGTGACCTGTGCCTC	57°C	4
SC-NHE2-R	AATGGGATGGTGCGGAAAGG	57°C	8
CRB-SEQ-F1	CGAACCCTCATCTTCTTCTTTCTG	60-64°C	5
CRB-SEQ-F2	CGTCGCCATAACAACAGAAC	60-62°C	6
CRB-SEQ-R1	CCTCCATACGAAAGCAAACAGC	60-62°C	7
SCULP2-F538	TCACTGTCTTTCTCCAGGGGC	60-62°C	9
SCULP2-F542	CAGGGCATCAGTATCAGACC	60-62°C	10
SCULP2-F616	GAGTCCAGTATCGTGGCG	60-62°C	11
SCULP2-R333	AAACATCCACTGGCTCCACC	60-62°C	2
SCULP2-R365	GGTGAATCTTGTGGTGAAGG	60-62°C	3
SCULP2-F704	ATTGATAAGACGCCACGCC	60-62°C	14
SCULP2-F725	CATCGGTGGTCCCAAATCC	60-62°C	15
SCULP2-R701	TGCTTTGGCTGTCATTGGG	60-62°C	13
SCULP2-R689	GTTGTGTAAGCCACCGTCC	60-62°C	12
SCULP-F144	ACTATGTGGGTGCTGCTGG	60-62°C	1
SCLP2-R128	TATTCGCTTTTTCCCCCGC	60-62°C	

Table 2: List of primers and the size of band in base pairs (BP) produced during PCR for the sculpin NHE2-like sequence. An "XXX" can not be used in a PCR reaction.

Primer	NHE2R	SEQ-R1	R333	R365	R689	R701
F144	1038 BP	1038 BP	567 BP	663 BP	1635 BP	1671 BP
NHE2F	270 BP	210 BP	XXX	XXX	861 BP	903 BP
SEQ-F1	210 BP	90 BP	XXX	XXX	747 BP	783 BP
SEQ-F2	150 BP	84 BP	XXX	XXX	717 BP	777 BP
F538	XXX	XXX	XXX	XXX	453 BP	489 BP
F542	XXX	XXX	XXX	XXX	441 BP	477 BP
F616	XXX	XXX	XXX	XXX	219 BP	255 BP
F704	XXX	XXX	XXX	XXX	XXX	XXX
F725	XXX	XXX	XXX	XXX	XXX	XXX

Appendix B- Protocol for RNA isolation

The correct volume of BCP (200 μL per 100 mg) was added to the TRI reagent
and mixed by inversion for fifteen seconds. The sample was allowed to sit for two to
fifteen minutes until the sample turned pink and centrifuge at 12,000 rpm for fifteen
minutes at four degrees Celsius. After centrifugation, three phases should be present,
transfer the aqueous RNA layer (top layer) to a new tube. To precipitate RNA out of
solution, add isopropanol (.5 mL per 1mL of TRI reagent) to the samples and allow
sample to stand at room temperature for five to ten minutes, then centrifuge at 12,000
rpm for ten minutes at four degrees Celsius. RNA should form a white pellet, remove
supernatant and wash pellet by adding one mL of 75% ETOH per one mL of TRI
reagent, vortex and centrifuge at 7500 rpm for five minutes at four degrees Celsius.
Remove supernatant and allow pellet to dry for five to ten minutes then resuspend in
30 μL of sterile water. A spectrophotometer was used to determine the concentration
(1 OD at 260nm = 40μg RNA) and purity ($A_{260/280}$) of the RNA sample before use in
RT-PCR.

Appendix C-Protocol for RT-PCR

Table 3: Components of the Perkin Elmer Gene Amp® RNA PCR Kit and the volumes used for a single RT-PCR reaction

Reverse-Transcription Master Mix

Component	Volume
25 mM MgCl$_2$	2.0 μl
10X PCR Buffer	1.0 μl
10 mM dGTP	1.0 μl
10 mM dATP	1.0 μl
10 mM dCTP	1.0 μl
10 mM dTTP	1.0 μl
20 U/μl RNase Inhibitor	0.5 μl
50 U/μl MuLV Reverse Transcriptase	0.5 μl
50 μM Oligo dT	0.5 μl
RNA Sample	1.0 μl
Ultra Pure H$_2$0	0.5 μl
Total RT Master Mix volume per reaction	10 μl

Polymerase Chain Reaction Master Mix

25 mM MgCl$_2$	2.0 μl
10X PCR Buffer	4.0 μl
Ultra Pure H$_2$O	31.5 μl
5 U/μl AmpliTaq DNA Polymerase	0.5 μl
Gene Specific Primer Upstream	1.0 μl
Gene Specific Primer Downstream	1.0 μl
Total PCR Master Mix per Reaction	50 μl

RT cycle was set as follows:

    1.) 10 minutes @ 25°C to anneal the oligo dT primers,

    2.) 15 minutes @ 42°C to elongate the first strand cDNA,

    3.) 5 minutes @ 95°C to denature the newly synthesized cDNA strand from the

        mRNA strand, and

    4.) 5 minutes @ 5°C to maintain the reaction in the denatured state.

PCR cycle was set for 40 cycles of:

    1.) 95°C for 1 minute (denaturation),

    2.) 57-64°C for 1 minute (annealing), and

3.) 72°C for 1 minute (elongation)

Appendix D- Protocol for 3'/5' RACE

1. First Strand Synthesis

Combine the following in a .5 ml microcentrifuge tubes:

5' RACE Ready cDNA
   3 µl RNA sample
   1 µl 5' CDS primer
   1 µl Smart II oligo

3' RACE Ready cDNA
   3 µl RNA sample
   1 µl 3' CDS primer
   1 µl Ultra Pure H_2O

Incubate the tubes at 70°C for 2 minutes. Then cool the tubes on ice for 2 minutes. Spin the tubes briefly and then add the following to each reaction tube:

   2 µl 5X First-Strand buffer
   1 µl DTT (20 mM)
   1 µl dNTP Mix (10 mM)
   1 µl MMLV reverse transcriptase (200 units/µl)
   10 µl Total Volume

Incubate the tubes at 42°C for 1.5 hour in an air incubator. Dilute the first strand reaction product with 100 µl Tricine-EDTA buffer. Then heat the tubes at 72°C for 7 minutes. Store samples at -20° until needed.

2. PCR for RACE

Prepare enough PCR master mix for all of the PCR reactions plus one extra to ensure sufficient volume. For each 50 µl reaction, mix the following reagents:

34.5 µl PCR grade water
5 µl   10X Advantage 2 PCR Buffer
1 µl   d NTP Mix (10 mM)
1 µl   50X Advantage 2 Polymerase Mix
41.5 µl Total Volume

Prepare PCR reactions for each of the RACE experiments as follows:

5' RACE	3' RACE
2.5 µl 5' Ready cDNA	2.5 µl 3' Ready cDNA
5 µl UPM	5 µl UPM
1 µl NHE2R	1 µl NHE2F
41.5 µl Master Mix	41.5 µl Master Mix
50 µl Final Volume	50 µl Final Volume

RACE was set for 35 cycles of:

   1). 95°C for 30 seconds

   2). 68°C for 3 minutes

RACE products were run on a 1% agarose gel stained with ethidium bromide for 45 minutes at 80V.

Appendix E- Protocol For Quantitative RT-PCR

1. Determination of Linear Range

The RT-PCR was followed according to the protocol for Perkin Elmer's RT-PCR kit (appendix C) except that 6μl of ^{32}P (α dCTP) was added to the PCR master mix. Gene Specific Primers NHE2F and SCULP2-R689 were used to produce the NHE2 product. PCR cycle was set for 35 cycles of:

1.) 95°C for .5 minute (denaturation),

2.) 62°C for .5 minute (annealing), and

3.) 72°C for .5 minute (elongation).

Tubes were taken out of the thermocycler after each odd cycle beginning with cycle 15. PCR products were run on a 1% agarose gel stained with ethidium bromide for 45 minutes at 80V. Bands were then cut out of the gel and put in 10 ml of scintillation fluid and then ran through the scintillation counter. The log of CPM was taken and the results were graphed to determine the linear range.

2. Determine the Optimal Ratio of 18S Primers: Competimers

Primers: Competimer mixtures were prepared following the methods set by the kit. The PCR cocktail was made exactly as before in the determination of the Linear Range. The cycling parameters were kept the same except that the number of cycles is reduced to the amount determined by the linear range experiment. PCR products were visualized by gel electrophoresis.

3. Relative Quantitative PCR experiment

Once the cycling parameters, linear range, and the optimal 18S primer: competimer ratios have all been established, the quantitative PCR experiments may be run. Using the information gained by the previous experiments, set up the RT-PCR reactions using the conditions identified in the preliminary work.

Appendix F- Protocol for Isolation of Plasmid DNA

To isolate the plasmids from the bacterial colony, 1.5 ml of overnight cultures was poured into 1.5 ml centrifuge tubes and centrifuged for 1-2 minutes at 1400 rpms at 4°C to pellet the bacteria. The supernant was poured out and 100 μl of Solution 1 (1X TE buffer) was added to each tube. The tubes were vortexed until the bacterial pellet went into solution. Then, 150μl of Solution 2 (0.2 N NaOH/1% SDS solution) were added, the tubes were inverted 2-3 times to mix and incubated on ice for 2 minutes. To precipitate the bacterial debris, 200μl of Solution 3 (3M KOAc solution, pH 4.8) were added. Again, the samples were inverted 2-3 times to mix. A white precipitate formed along the side of the tube, and the supernant containing the isolated plasmid DNA was removed carefully into a new tube. The plasmids were precipitated out of solution by adding 600μl of isopropanol to the supernant, vortexed, and incubated on ice for 10 minutes, then centrifuged at 14,000rpm, at 4°C, for 10 minutes. The supernant was poured off; the pellet was allowed to dry, then resuspended in 100μl of sterile water. To remove any residual proteins, 100μl of 1:1 phenol:chloroform was added, vortexed, and centrifuged for 2 minutes. The aqueous phase containing the plasmid DNA was removed to a new tube and precipitated by adding 250μl of 100% EtOH and incubated at -70°C for 1 hour or at -20°C overnight and then the samples were centrifuged for 20 minutes. The supernant was poured off and the pellets were dried. To degrade any remaining RNA, the plasmid DNA was resuspended in 10μl of 20μg/ml Rnase solution and incubated at 37°C for 31-45 minutes. Now the samples are ready for restriction digest.