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Rh Glycoprotein as an Ammonia Transport Molecule

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RH GLYCOPROTEIN AS AN AMMONIA TRANSPORT MOLECULE

by

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(Under the Direction of James Claiborne)

ABSTRACT

Fish use their gills to excrete ammonia in order to eliminate nitrogenous waste. We hypothesize that this mechanism is accomplished by one or more transport proteins in the Rh glycoprotein (RhxG) family. Longhorn sculpin (*Myoxocephalus octodecemspinosus*) cDNA was amplified using polymerase chain reaction (PCR) and then the PCR products were visualized on an agarose gel. The cDNA from the gel bands was then sequenced and the gene sequence fragments were assembled and completed by rapid amplification of the cDNA ends (RACE). By this process we have obtained large portions of the gene sequences of the four known paralogues located in the sculpin gill (RhA, RhB, RhC1, and RhC2). Also, *in vivo* ammonia-loading experiments were done to determine the effect of increased internal ammonia on protein and mRNA expression. Treatment groups were exposed to a single ammonium bicarbonate, distilled water, or ammonium chloride (5 mM kg^{-1}) infusion; then gill tissue was collected 4 hr post-infusion and analyzed using quantitative PCR to test changes in mRNA levels and dot blots for changes in RhxG protein levels. Preliminary QPCR data showed a trend of increase in response to ammonia loading. A second infusion test, with a chronic (8 hr) double load of ammonium bicarbonate, was completed

with QPCR and dot blot analysis done on the gill tissue. Ambient water samples were also collected to determine *in vivo* ammonia efflux. In conclusion, from this data we have found protein expression changes in response to increased internal ammonia.

INDEX WORDS: Ammonia excretion, Rh Glycoprotein

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DEDICATION

In the beginning was the Word, and the Word was with God, and the Word was God. John 1:1

Soli Deo Gloria

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Introduction

Nitrogenous waste products are a result of the breakdown of the amino acids in the proteins we digest for nutrition, and are universal to all higher vertebrates. Animals excrete this waste in the form of three main nitrogen products: ammonia, urea, and uric acid. These three products have a direct relationship between water conservation and energy requirements, meaning the more energy spent the more water conserved. Ammonia, the least water conserving form and therefore the least energy requiring, is highly toxic. Due to its toxicity it must either be excreted or converted into the less toxic variants, such as urea or uric acid. Uric acid, which is mainly excreted by birds, reptiles, and many terrestrial invertebrates, requires the least amount of water loss along with the highest energy cost and does not appear to be excreted in significant quantities by fishes (Wood, 1993; Wright, 1995). Humans and other mammals convert their nitrogenous wastes primarily to urea (with minimal amounts of uric acid and ammonia) to deter water loss and use their kidneys (urinary/renal system) to deal with the excretion. Urea, like uric acid, is also much less toxic than ammonia, although it is more expensive to produce requiring at least two additional molecules of ATP (Mommsen and Walsh, 1991). To understand ammonia excretion in more detail, a species that is a known ammonotelic with a higher tolerance to elevated ammonia levels needs to be examined. Teleostean fish follow these stipulations quite well and are known to use their gills as the

dominant site of gas exchange, osmoregulation, acid-base regulation, as well as for the excretion of nitrogenous wastes making the fish gill an excellent model to further understand ammonia excretion. Most teleostean fish are known to be ammonotelic, though some situations do dictate urea synthesis over ammonia such as high pH environments, stress, confinement, or air exposure (Evans et al., 2005). Also, ammonia is thought to make up >80% of nitrogenous waste excretion among fish, outside of ureotelic elasmobranches and unique teleosts such as the gulf toadfish and the Lake Magadi tilapia (Wilkie, 1997; Wood, 1993; Wright, 1995). Therefore, it is important to look at the fish gill to understand how the excretion of ammonia is handled.

The process of ammonia excretion has been described by either simple diffusion or by the use of a transport protein in four different pathways, as shown in Figure 1. The bulk of evidence generated over the last 25 years points to the simple diffusion of ammonia (NH_3) down the favorable blood-to-water gradient across the membrane in freshwater teleosts (Wilkie, 2002). This is supported by the fact that a moderate decrease in external water pH, which would decrease the concentration of NH_3 in external water by protonation to ammonium, increasing the rate of total ammonia excretion, and increases in external water pH decreased the rate of total ammonia excretion. Also, ammonium injections caused an initial increase in total ammonia excretion, in excess of net acid excretion, suggesting that at least some of infused ammonium disassociated and crossed gills as NH_3. The infusion, in addition, caused a metabolic acidosis,

which presumably reflects residual H^+ left in blood after disassociation (Evans et al., 2005; Wilkie, 2002). However, at physiological pH the majority of ammonia exists as ammonium (NH_4^+); and due to its positive charge, it cannot penetrate the lipid phase of cell membranes (Knepper et al., 1989). Thus, it is unlikely that appreciable passive NH_4^+ diffusion takes place under typical conditions. While NH_3 is about 10-1000 times more permeable in gill epithelia than NH_4^+ (Wood, 1993), NH_3 lipid solubility is only moderate in comparison to other neutral molecules such as carbon dioxide (Knepper et al., 1989). Thus, it seems unlikely that simple diffusion can be responsible for the entirety of ammonia excretion.

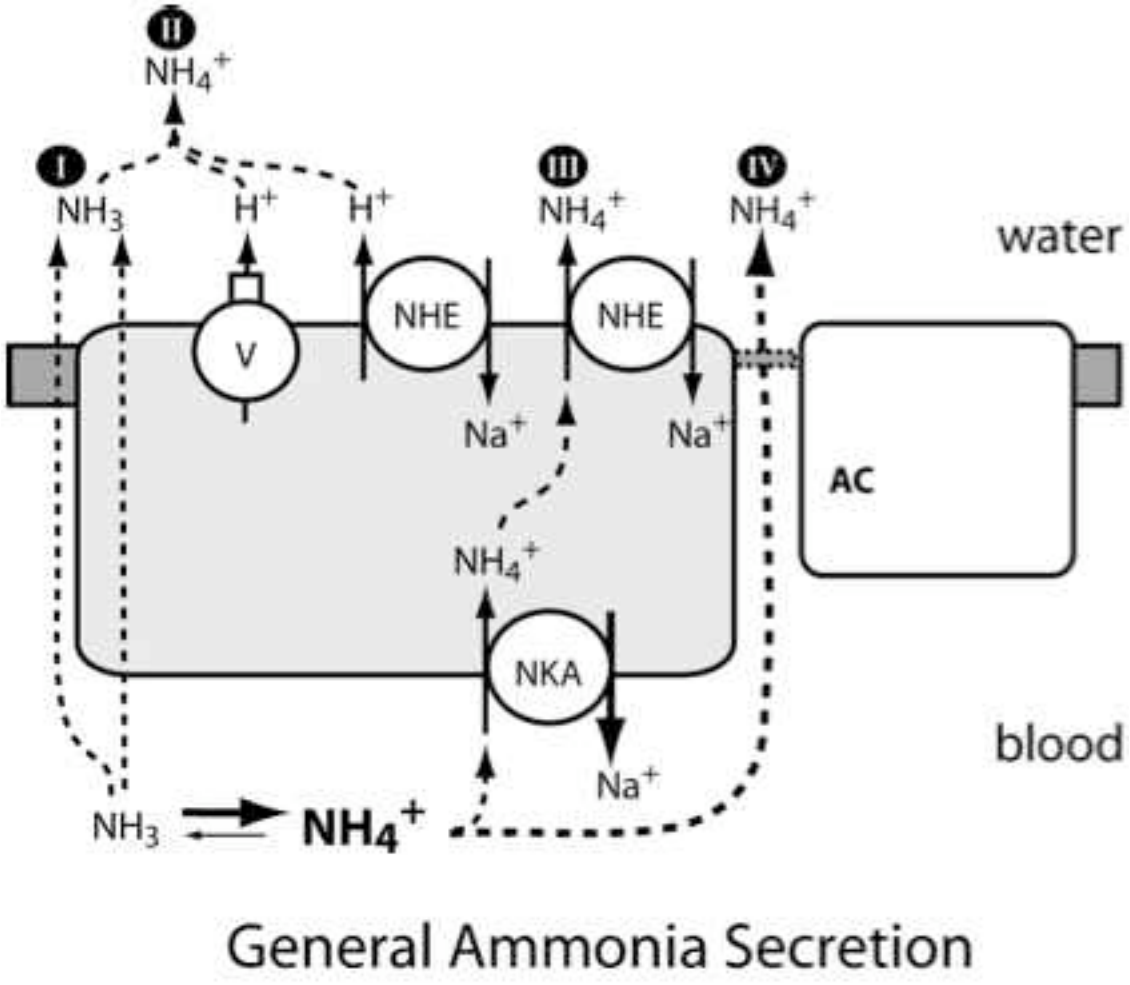


Figure 1: Previously proposed pathways of ammonia excretion. 1) Simple diffusion through cellular membrane. 2) Ammonia trapping, using simple diffusion and proton excretion by V-ATPase and NHE to allow NH_4^+ to pass. 3) Non-specific Ion transport, the utilization of transport molecules for ions of similar size and charge as shown using NKA and NHE. 4) Leaky paracellular junction as found between the chloride cell and the accessory cell in seawater teleosts. Taken from Evans (2005).

Another route for ammonia excretion across the membrane is the mediation by transport systems that are not specific to ammonia. The presence of electroneutral Na^+/NH_4^+ exchange in fresh water fish gills was proposed by August Krogh (1939) 70 years ago. In this model, Na^+ uptake across the apical

side of the gill is tied to NH_4^+ extrusion, where NH_4^+ replaces H^+ on an electroneutral Na^+/H^+ antiport. This would require the electroneutral Na^+/H^+ exchange (NHE) to be energized by inwardly directed Na^+ gradients (Grinstein and Wieczorek, 1994).

There is some discussion on whether the occurrence of this mechanism exists or if it is mistaken for ammonia trapping, in which the same protein channels, NHEs and V-ATPases, are pumping out protons as per their normal function and these protons are joining with the ammonia molecules that are freely diffusing across the membrane as in the first mechanism pathway. The Na^+/NH_4^+ exchange system was hypothesized from observations that amiloride (NHE inhibitor) addition and Na^+ removal from external water decreased net ammonia excretion rates as well as the observation that ammonium salts injections increased Na^+ absorption in freshwater teleosts (Wilkie, 1997). However, when HEPES was added to external water (effectively binding all loose protons and therefore inhibiting ammonia trapping) total ammonia excretion was uninhibited while Na^+ uptake suffered a 90% inhibition (Evans et al., 2005; Wilkie, 2002).

Members of the NHE family are thought to play an important role in transepithelial ion movements, intracellular pH homeostasis, and cell volume regulation (Claiborne, 2002). Like mammalian kidney nephrons, fish gills have apical NHEs for systemic acid-base and Na^+ regulation and basolateral NHEs for cell pH and volume regulation (Choe et al., 2002). Choe (2005) later suggested

that this model would also be feasible in fresh water as well as salt water as an ammonia excretion system.

The final pathway previously suggested for ammonia transport across the gills is that of ammonium diffusion. Albeit this form of ammonia excretion is considered minimal in all fishes except seawater teleost and lampreys which are known to have shallow tight junctions between the mitochondrion rich cells (MRCs) that would allow the small charged molecule to pass (Evans et al., 2005). This pathway was proposed after sculpin exposed to high external ammonia concentrations did not develop a metabolic acidosis; suggesting that ammonia loading occurred not in the basic NH_3 form but in NH_4, the acid-base neutral form (Claiborne and Evans, 1988).

Recent studies have focused on Rhesus proteins as possible ammonia excretion transport molecules. It has been established that there are at least four Rh proteins in mammals, RhDg and RhAg in red blood cells and RhBg and RhCg in other tissues, along with copies very similar to RhDg and RhAg also found in RBCs RhCEg and RhPgs that are rarely looked at (Huang and Peng, 2005). These proteins have been associated with the microbial ammonium transporter (Amt) proteins (Marini et al., 1997). Many research groups think that Amt proteins concentrate the NH_4^+ ion against a gradient and that they function as active transporters (Ludewig et al., 2007). Several groups think that human and mouse Rh proteins also transport ammonium and are Amt functional equivalents in mammals (Marini et al., 2000). Both views have been challenged in that

regard by statements that Amt proteins are gas channels for NH_3 and possibly for CO_2 (Soupene et al., 2004).

Marini (1997) first suggested that glycosated Rh proteins played a role in ammonium transport. This idea was formed when it was pointed out that higher organisms depended on non-specific transporting systems for ammonia excretion while lower organisms, such as bacteria, yeasts, and plants, had specific ammonia transporters (Amts). Recent functional studies from several laboratories have indicated that human Rh orthologues play a main role as ammonium transporters, also placing them in polarized distribution in the gut, kidney, and liver (Planelles, 2007).

Weiner's review (Weiner and Hamm, 2007) examines closely the literature on ammonia flux in the human renal system. With essentially none of the urinary ammonia being filtered by the glomerulus, it must be excreted into the urine or the renal vein by the proximal tubule, thick ascending limb of the loop of Henle, and the collecting duct (Weiner and Hamm, 2007). They also included studies of proposed functions of specific Rh proteins. RhAg, found in erythrocytes, was shown to mediate efflux of the ammonia analog Methylammonia (MA) suggesting that RhAg acts as a mammalian blood ammonia transporter (Weiner and Hamm, 2007). Weiner (2003) had earlier used RhBg and RhCg antibodies to detect the proteins in renal systems of the rat and mouse. They showed immunoreactivity of RhCg to be apical and located in the connecting segment, initial collecting tubule, cortical collecting duct, outer medullary collecting duct, and the inner

medullary collecting duct; with RhBg found basalaterally in the connecting segment, initial collecting tubule, outer medullary collecting duct, innermedullary collecting duct, and also low levels found in the distal convoluted tubule (Weiner and Verlander, 2003). He also suggested the possibility of the Rh proteins acting as a CO_2 transporter.

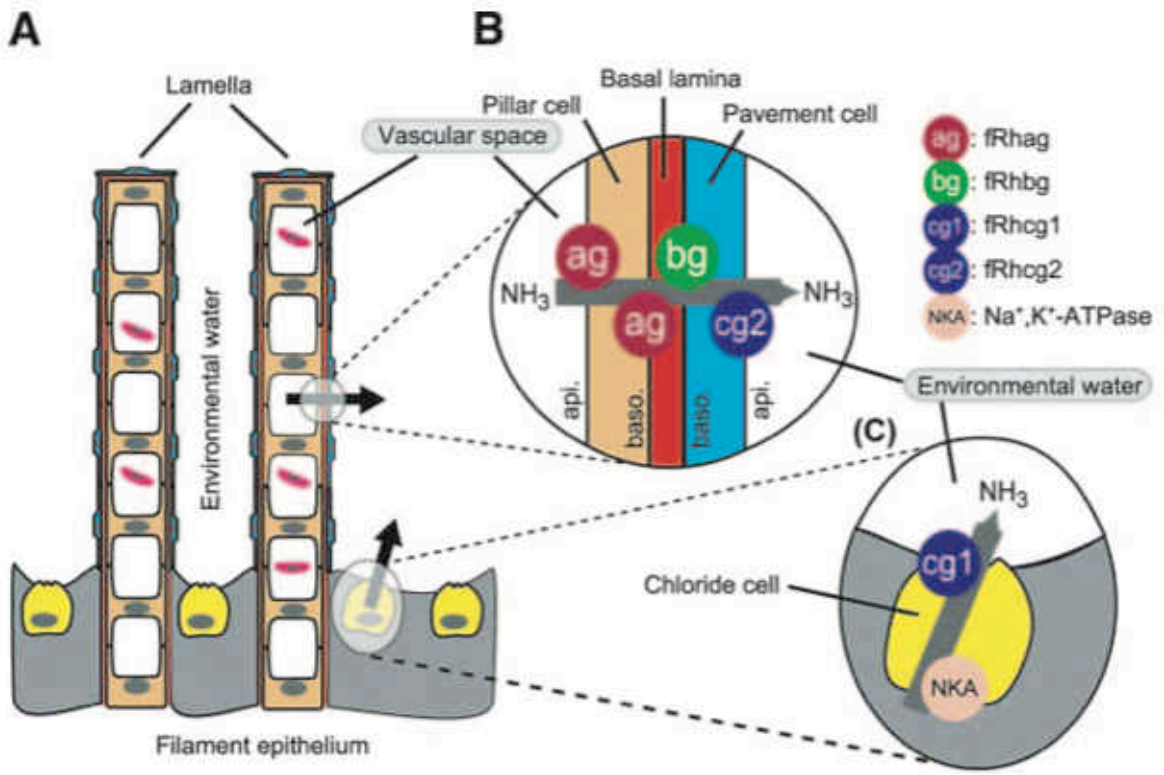
The classical thought that gases can pass through membranes by simple diffusion has been questioned due to the increasing number of exceptions (Endeward et al., 2008). Endeward specifically looks at RhAg in the red blood cell (RBC) as a CO_2 transporter. While comparing RBCs Rh_{null} and AQP-1_{null} (aquaporin) to test basal CO_2 permeability of the red cell membrane; they found the normal inhibitory affects of 4,4`-diisothiocyanato-stilbene-2,2`-disulfonate (DIDS) to be greatly lessened in the null sets suggesting their role in CO_2 transport (Endeward et al., 2008). In another study on Rh1 in green algae a filial strain was grown with the absence of Rh1. In normal conditions (air, and acetate) it grew similarly to its parental strain, however, in CO_2 heavy conditions its growth was restricted. Also, these $Rh1_{null}$ lines, when grown on acetate, poorly express three proteins involved in the carbon concentrating mechanism, whereas the parental strains have no difficulty. The failure to produce these proteins indicates limited internal CO_2, thus, pointing to the conclusion that Rh1, and possibly even more Rh proteins, are involved with CO_2 transport (Soupene et al., 2004).

The dynamics of ammonia excretion in fish cannot be explained without a cursory understanding of the structure of the fish gill. Being the center of

osmoregulation, acid-base regulation, and respiration, surface area is key. Along the structural gill arches are the rows of gill filaments from which sprout the lamellae. The lamellar epithelium is made up of pavement cells (PVTs), although they cover a great deal of the gill's surface area they are generally considered to play passive roles (Laurent and Dunel, 1980). Located in between the lamellae on the gill filament are the mitochondrion rich cells (MRCs) also known as the chloride cells. In contrast to the PVTs these cells are considered to be the primary sites of active physiological processes in the gills. They receive their name from being filled with mitochondria as well as to their NaCl secretory function ("chloride cells") and are the site of expression for the active transport enzyme Na^+-K^+-ATPase that indirectly drives NaCl secretion (Philpott, 1980). A further, more in depth review can be found in the Evans et al. review (Evans et al., 2005).

While working with the Rh glycoprotein orthologues in pufferfish (*Takifugu rubripes*), Nakada et al. (2007) showed results that suggested the four Rh glycoproteins found in the gill (fRhag, fRhbg, fRhcg1, and fRhcg2) are all likely to be involved in the elimination of ammonia. Their results also showed no cross-reactivity between the orthologues, thereby confirming the specificity of the transport. They used immunolocalization of the four orthologues to determine that RhAg is found on both sides of the pillar cells, RhBg is found on the basolateral side of the pavement cells, RhC1g is found on the apical side of the MRCs, and RhC2g is found on the apical side of the pavement cells.

From this they suggested a mechanism, as shown in Figure 2, for both the facilitated and active diffusion of ammonia across the gill epithelium. Facilitated diffusion would occur by ammonia passing through both sides of the pillar cell utilizing RhAg cell and then through the basolateral RhBg and apical RhC2g of the pavement cell. In cases where the gradient was not sufficient for this, active transport would occur by NH_4^+ occupying the K^+ spot in the basolateral Na^+ / K^+ ATPase antiport (NKA) of the MRC creating enough of a gradient inside the cell for it to passively diffuse across through the apical RhC1g (Nakada et al., 2007). Similar immunolocalization results have been found in the longhorn sculpin (Claiborne et al., 2008).



AMMONIA TRANSPORTERS IN THE FISH GILL

Figure 2: Model of ammonia excretion in fish. A) Cross section of gill. B) Protein channel mediated excretion of ammonia through gill lamellae epithelium. Combined utilization of RhAg on both the basolateral and apical sides of the pillar cell and RhBg on the basolateral with RhC2g on the apical sides of the pavement cell are shown as partial pathway of ammonia excretion. C) Ammonia excretion through RhC1g on the apical side of the MRC, using the NKA on the basolateral side for import of the ammonia (ammonium ion replaces the K^+). Taken from Nakada (2007).

This information leads us to a question. Does ammonia transport utilize the previously described pathways of simple diffusion and non-specific ion channels, or does it follow a pathway utilizing the Rh glycoproteins as channels to cross the gill epithelium membrane? Nakada's proposed method needs to be tested against a new species to ground his claims, and in this study we will

examine the presence of the Rh orthologues in the marine longhorn sculpin and measure the mRNA and protein level reaction to ammonia loading. From this data we will determine whether our results suggest the proposed pathway of ammonia transport across gill epithelium by Rh glycoprotein ammonia channels or one of the previously proposed pathways of ammonia excretion. We will determine the DNA sequence of sculpin Rh orthologues from the gill, and use that information to run further functional and physiological tests on sculpin exposed to ammonia stress while measuring mRNA and protein production.

Methodology

Animal Holding Conditions

Longhorn sculpin were collected by commercial fisherman from Frenchman Bay, ME and maintained in aquariums with running 100% seawater at (12-15°C) until needed. The sculpin were restricted from eating for one week before being used for experimentation. Individual fish were removed from the primary holding tank with some being immediately weighed and sacrificed while others were anesthetized with MS-222 (1:10,000 dilution, 7-10 minutes), weighed, and then cannulated. Sacrificing of fish involved a brain/spinal pithing method of quickly cutting the spinal cord and pithing the brain area of the fish. Gill filaments were surgically removed and soaked in a PBS wash to eliminate the blood. Then the tissue was either stored in RNAlater media (Ambion) at -20°C according to manufactures protocol for later RNA isolation, or flash frozen in liquid nitrogen and stored at -80°C for protein work.

Cannulation was performed by surgically inserting polyethylene E-50 size tubing (filled with teleost Ringer solution) into the sculpin peritoneal cavity through a 18 gauge needle and using a blocked 23 gauge needle to plug the tube as described previously (Claiborne et al., 1997). The cannula was held in place by stitching the tube into the skin of the fish. Cannulated sculpin were placed into individual plexiglass containers (2.4L ± 5%) with aerated 100% running seawater at (12-15°C) and acclimated for at least 20 hours before injections. Sculpin were

injected via the implanted cannula with 5mM/kg of NH_4Cl, NH_4HCO_3, or the equivalent amount of distilled water, according to calculated conversion by weight.

RNA Isolation

Isolation of total RNA from gill homogenates of long-horned sculpin was performed using the TRI reagent (Molecular Research Center, Inc.) method of extraction. As per standard protocol extracted tissue was homogenized in 15 ml round-bottom Falcon tubes in 1ml TRI Reagent per 50-100mg tissue. The homogenate was stored at room temperature for 5-15 minutes then transferred in 1 ml aliquots to 1.5 ml microcentrifuge tubes. Bromochloropropane (BCP; 10.1 ml per 1.0 ml TRI reagent used) was then added. The mixture was incubated at room temperature for 2-15 minutes and then centrifuged at 12,000 g for 10 minutes at 4°C. The procedure will separate the mixture into three layers: RNA (top, aqueous phase), DNA (interphase), and protein (lower, organic phase). The aqueous phase, containing RNA, was transferred to a new 1.5 ml tube. The RNA was then precipitated with 0.5 ml isopropanol for each ml TRI reagent used in the initial homogenization.

The sample was incubated at room temperature for 8 minutes and was centrifuged at 12,000 g for 8 minutes at 4°C. The supernatant was removed from resulting RNA pellet. The RNA pellet was then washed with 75% ethanol and centrifuged at 7,500 g for 5 minutes at 4°C. The resulting RNA was quantified and checked for purity using UV spectrophotometry at 260/280 nm and the

integrity verified by agarose gel electrophoresis (1% agarose gel using a MOPS/formaldehyde buffer system) and ethidium bromide staining.

Reverse Transcription cDNA Synthesis

Reverse transcription was performed on sculpin total RNA using the Superscript III Kit (Invitrogen) according to manufacturers' protocol. Superscript III Reverse transcriptase utilizes the avian myeloblastosis virus RNase H- reverse transcriptase and is designed to have higher thermal stability, and produce high yield and more full-length cDNA transcripts. Sculpin total RNA was heated for 5 minutes at 70°C and put on ice to remove secondary structure. The reverse transcription reaction cycle was one cycle of: 60 minutes at 50°C, 15 minutes at 70°C, In order to check for genomic DNA amplification a negative and positive superscript III reaction was performed. An amount of 0.5 μg/μl total sculpin RNA was used in the reverse transcription reaction.

Polymerase Chain Reaction

To do the Polymerase chain reaction (PCR) procedure on the sculpin cDNA obtained from the reverse transcription a FastStart *Taq* polymerase kit (Roche) and a Hybaid thermo cycler machine was used in a 50-μl (1x) reaction.

An alternative protocol was followed using the Phusion High-Fidelity PCR kit from Finnzymes. This protocol can be done using two different methods; a standard method (as described by manufacturer) and a wide range step down method. (Frey et al., 2007)

The wide range step down method was designed to create optimal conditions for any sequence to amplify so 4 different procedures are used simultaneously, A, B, C, and D. For A and B 5x HF (high fidelity) PCR buffer was used with high concentration of primer (5μl) for A and low concentration of primer (.5μl) for B. For C and D 5x GC (for GC rich sequences) PCR buffer was used with high concentration (5μl) for C and low concentration (.5μl) for D. This spread of buffer and concentration creates optimal conditions for any cDNA to amplify. For step down, the thermal cycler runs standard until it reaches the annealing temperature at which point it steps down (65°-55°C) decreasing in .5° increments so that it hits every possible annealing temperature.

Cloning

Cloning was done using a Zero Blunt TOPO PCR Cloning Kit (Invitrogen) and was completed using manufacturers protocol. LB-Agar was used to make the agarose plates and Kanamycin was the antibiotic used to kill the bacteria untransformed with plasmid. Colonies were selected and PCR was run with a standard Phusion protocol to determine if cDNA was present in the colonies.

Then the plasmid isolation was done using Roche's High Pure Plasmid kit following the manufacturers protocol. High Pure filter tubes and centrifugation achieved the elution of plasmid DNA. The eluted plasmid was then transferred to sterile .5 ml tubes and stored in the refrigerator (4°C) until sequencing.

Rapid Amplification of cDNA Ends (RACE)

To do RACE a step down Phusion PCR protocol was used with 3` Oligo dT primer for the downstream and 5' Gene Specific primer for the upstream.

For some of the sequences a modified rolling circle procedure, similar to that found in Polidoros et al (2006), was used where the cDNA was fashioned into a circle by CircLigase™ (Epicentre Biotechnologies) that ligates intramolecularly sticking the 5' and 3' ends together using phosphoralated Oligo DT (dideoxythimine) primers, shown in Figure 3.

It was then amplified using nested inverse primers, designed using Oligo Primer Analysis Software against the known coding sequence, and standard Phusion PCR technique. The product's resulting linear single stranded cDNA, with the unknown 5' and 3' ends (along with the untranslated portion) next to each other, was then cloned and sent for sequencing. The returning sequence was then used to design more inverse primers until both the 3' and 5' ends were found.

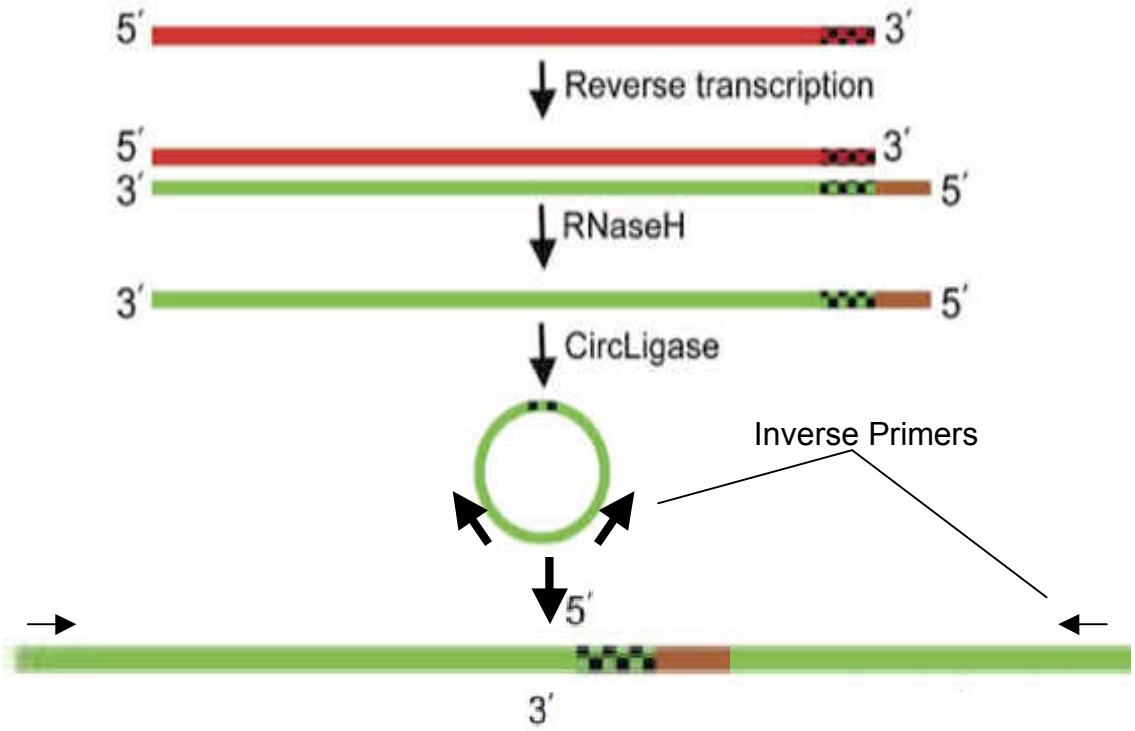


Figure 3: Circularization of RNA for inverse primer sequencing. Image shows the process of taking RNA to a circlized cDNA state, using the CircLigase© ssDNA Ligase enzyme, from which the inverse primers can be used to sequence the 5' and 3' ends. Adapted from Polidoros (2006)

Primer Design

All primers were designed using Oligo Primer Analysis Software (Molecular Biology Insights, Inc). For standard PCR reactions used to gain smaller segments of the coding sequence this was done by using primers designed previously for the stickleback or by using the already sequenced stickleback genome as a framework for the sculpin. Primers were design as pairs with preference to low 3' ΔG, high 5'ΔG (suggested parameter given by primer design software), absence of a hairpin loop formation, high annealing temperature (65°-75°C was the optimal range), and a nucleotide length of 20-24

bases. This was also the manner in which inverse primers were designed. See Table 2 for a list of primers used.

For quantitative RT-PCR primers were designed with the same preferences as standard PCR primers along with a few extras. The optimal quantitative primers primed for a product between 50bp and 150bp in length and one or both of the primers would lie across an exon-intron boundary so that no genomic contamination would be primed. Integrated DNA Technologies (IDT) manufactured all primers.

DNA Sequencing

The sequencing process for each of the four genes involved first designing standard PCR primers for the sculpin to gain small segments of the entirety of the sequence. Depending on the clarity of the PCR product there were different methods of preparation for sequencing. If a clear single band occurred, the product could be shipped directly for sequencing, after an ExoSap-IT (USB) method preparation. This procedure involved taking 5 μl of the PCR reaction, done according to manufacturer protocol, and 2 μl of ExoSap-IT reagent and incubating them at 37° C for 15 minutes and 80° C for 15 minutes. 0.8 μl of each primer (forward and reverse) at 10μM, and 21.2 μl of sterile water was added to 2μl of the ExoSap reaction to yield a total volume of 24 μl that was to be shipped for sequencing.

In cases where multiple, but clear bands occurred a gel purification procedure was done. This procedure used a NuSieve GTG (Cambrex) agarose

gel to run the PCR product Gel Green (Biotium), it was then viewed over a Dark Reader (Clare Chemical Research) and the individual bands cut out. The gel was then liquefied using β-Agarase I (New England BioLabs) and prepared for shipment.

In cases where multiple products overlapped or smearing occurred cloning was used to get one specific desired product out of multiple amplified products. Once these fragments of the gene were amplified and separated they were sent to Mount Desert Island Biological Laboratory (MDIBL) Sequencing Facility for nucleotide sequencing.

Ammonia Loading

The ammonia loading method began with a control period for each fish. This consisted of an 8-hour period in which no infusions occurred and the external ammonia levels were measured to determine ammonia efflux under normal conditions.

Two methods were used for ammonia loading, acute and chronic. With the acute ammonia load the fish was infused once with 5mM/kg of NH_4CL, NH_4HCO_3, or distilled water (equivalent volume). After 4 hours the fish was sacrificed (N=6 per treatment). With the chronic ammonia load the fish was infused 4 times with either 5mM/kg of NH_4HCO_3 or distilled water (equivalent volume) every 2 hours, over a total 8 hour period at which point the fish was sacrificed (N=3 per treatment). During every load the water system was closed so to maintain the excreted ammonia levels for measurement purposes. The

plexiglass box was flushed with fresh water after the control period and at the 4-hour point during the chronic loading. Table 1 shows a time flow chart of the ammonia loading method.

Table 1: Time flow chart of ammonia loading method.

Hour 0	**Hour 8**	**Hour 10**
Control period begins Water sample taken	Control period ends Water sample taken Box flushed with fresh seawater 1st infusion Water sample taken	2nd infusion Water sample taken
Hour 12	**Hour 14**	**Hour 16**
Water sample taken Box flushed with fresh seawater 3rd infusion Water sample taken	4th infusion Water sample taken	Water sample taken Fish sacrificed

Ammonia Assay

During the chronic ammonia loading water samples (100μl) were taken to determine ammonia flux and stored frozen at -20°C. A control period was measured for each fish by sampling water before and after an 8-hour period in which the fish was resting in a closed circuit (meaning that seawater was not running) environment. The water was then sampled at each infusion point, as well as the sacrifice point. The water samples were kept at -20°C. The assay used was a modified Solorzano phenol-hypochlorite method (Solorzano, 1969).

The procedure was the same but the materials were proportionally reduced to run on a 220μl reaction scale. The assay was read by a Tecan Sunrise microplate reader using a standard concentration curve to determine total ammonia in water in triplicate.

Protein Expression

Dot blots were used for protein quantification using methods described by Hyndman and Evans (2009). Using a primary antibody against the protein of interest as well as a secondary chemiluminescent antibody against the primary antibody we can calculate the relative concentration of the protein of interest compared to a standard by densitometry.

Gill tissue that had been collected and flash frozen in liquid nitrogen and stored at -80°C was homogenized in 2 mL of ice-cold buffer (250 mM sucrose, 30 mM Tris, 1 mM EDTA, 0.5% of Sigma protease inhibitor cocktail, and 100 μg/mL pheynylmethylsulfonyl fluoride; pH 7.8) and centrifuged at 12,000g for 10min at 4°C and the supernatant decanted. The protein content of the supernatant was measured using Pierce's BCA protein assay kit (Rockford, IL). The supernatant was then heated at 65°C for 15min, then diluted to 2.5μg/μL in 10 mM TBS (25mM/L Tris, 150mM/L NaCl; pH 7.4), and continued heating at 65°C until blotted. A randomly selected control sample, selected from the group of fish being tested, was then diluted (in 10mM TBS) in a seven point series of two-fold dilutions for a concentration curve and the concentrations made known by a

microplate reader. Proteins were blotted in 1μL dots, in triplicate, onto nitrocellulose membrane (Millipore, Billerica, MA), and left to air dry for 15min.

The membrane was then placed in blotto (non-fat milk in TBS; pH 7.4) and incubated for 1hr at room temperature with shaking, and then was placed in primary antibody (rabbit anti protein; RhAg, RhBg, RhC1g, or RhC2g specific at 1/250 concentration) to incubate overnight. Then the membrane was washed three times in fresh 10mM TBS with 1% Tween (TBST) and incubated in 1/1500 alkaline phosphatase-conjugated, goat anti rabbit secondary antibody for 1hr at room temperature. Again the membrane was washed three times in TBST. The membrane was developed utilizing a chemiluminescent signal (Biorad) per manufacturer's protocol and developed on ECL hyperfilm (Amersham, Piscataway, NJ). The film was digitized on a flat bed scanner and densitometry determined by Biorad's Quantity One software. All values were standardized to the dilution curve and made relative to protein content (relative units/mg) protein.

Fish specific polyclonal antibodies (developed against homologous epitopes in the marine puffer fish, *Takifugu rubripes* (Nakada et al., 2007) and used successfully for immunohistochemical localization in the sculpin (Claiborne et al., 2008) were utilized to measure protein expression levels in dot blots. Western blots on the antibodies used confirmed single band products (Claiborne and Edwards unpublished).

Quantitative RT-PCR

For mRNA expression the QPCR method as described in Hyndman and Evans (2009) was completed using an Invitrogen Platinum SYBR Green qPCR SuperMix-UDG kit on the Strategene mx4000 machine at MDIBL, as well as LightCycler FastStart DNA Master SYBR Green I kit on the Roche LightCycler 1.5 machine at GSU, according to manufacturer's protocol.

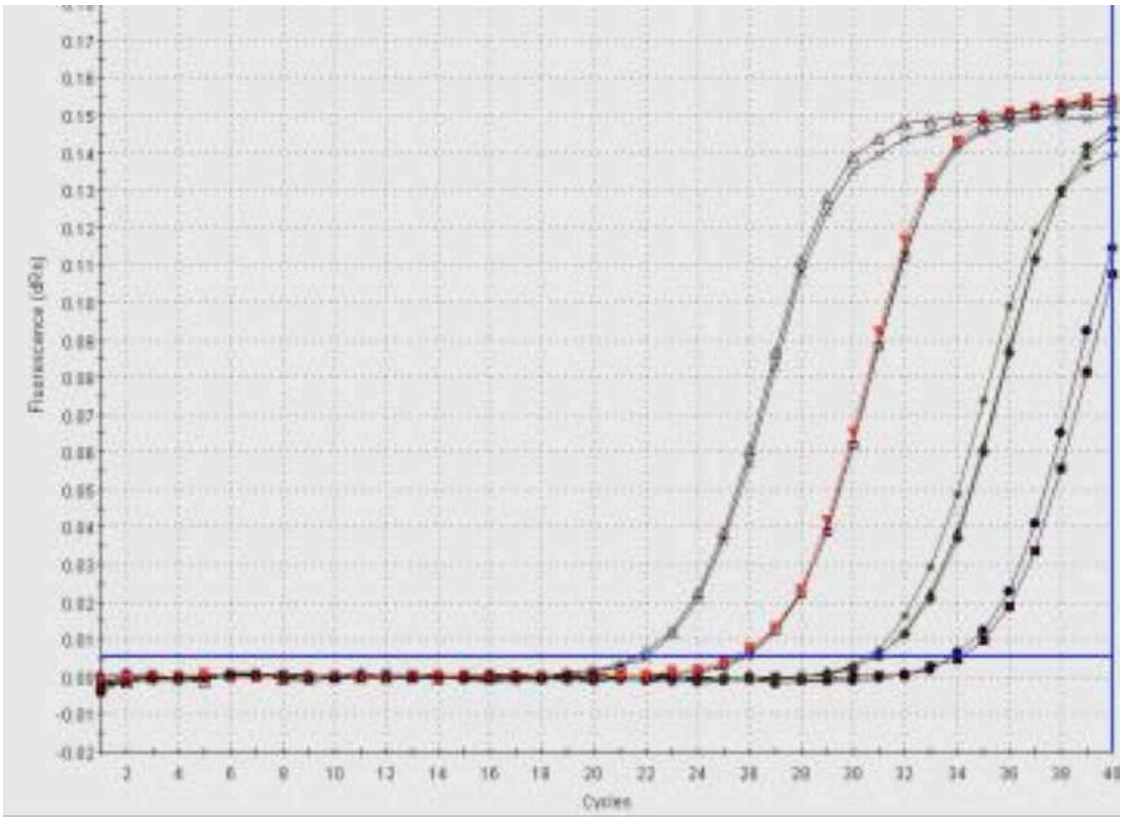


Figure 4: Example of quantitative RT-PCR results. Shown with fluorescence on the Y and number of cycles on the X. The first duplicate has the highest concentration of mRNA because its' luminescence became visible at cycle 23 compared to the second duplicate which becomes visible at cycle 26. More mRNA means higher concentrations of cDNA, and since the cDNA is what the luminescent dye attaches to so that it becomes visible it will become visible at an earlier cycle.

In RT-PCR the PCR works as normal using polymerase to amplify single strand cDNA (copied from high quality mRNA) into double strand DNA. SYBR Green is a florescent molecule that will only bind to double stranded DNA. Therefore, the higher the expression level (mRNA), the higher the cDNA level and the faster the concentration of SYBR increases causing fluorescence. We measure this timed florescence value as crossing threshold (C_T), the point where the bound SYBR becomes more visible than the background light, and is then plotted as a log relationship, example shown in Figure 4. Primers were designed to span exon-intron boundaries to prevent priming of genomic DNA, the products were kept, ideally, between 50 and 150 base length, a 3' ΔG (Gibb's free energy) value below -9 allowed for more specific binding, and annealing temperatures were in the range of 58°C.

To quantify relative mRNA levels a concentration curve of known quantities was used for each gene each run at 1:1, 1:10, 1:100, 1:1000, and 1:10,000 and the equation of this known concentration line was used to quantify our unknown concentration, which was run at a 1:10 dilution to ensure that the C_T did not fall out of the parameters of the concentration curve. All samples were run in triplicate, except those done at GSU, which were done in duplicate due to limited space for samples in the Roche machine. To standardize the results, a putative housekeeping gene, ribosomal protein L8 (NCBI: DQ066926), was initially used as an internal control (Small et al., 2008). This was then found to be effected by ammonia loading so Ubiquitin was then used based on Hibblers's

study on housekeeping genes as internal controls {, 2008 #263} The Pfaffl (Pfaffl, 2001) mathematical process was applied for standardization and in order to equalize the efficiencies of the gene amplification. The final result is a comparison of relative increase/decrease of mRNA expression in the gene of interest standardized to the internal control.

Statistics

All statistical tests were run in Excel (Microsoft). Student's t tests were used to determine statistical significance in most cases. Bonferroni adjustments were made to the α levels to correct for type 1 error in repeated measurements by dividing the set α of 0.05 by N, where N is the number of tests to that point, i.e. (0.05/1, 0.05/2, 0.05/3, etc.).

Results

Sequencing

Sequencing products received back from MDIBL were pieced together into contigs (recreating the DNA sequence) using MacVector with Assembler (MacVector Inc). To date the entirety of the RhBg coding sequence has been established along with the 3' end of RhAg and RhC2g. Continuing efforts are being made to establish the 3' end of RhC1g as well as the remaining 5' ends of RhAg, RhC1g, and RhC2g to go along with the large internal coding sequence fragment. Figures 15-18 in the appendix show the established DNA and translated protein sequences for the Rh orthologues. A protein homology alignment shows significant percent identity in all of the proteins when compared to their paralogues in stickleback: RhAg 50%, RhBg 91%, RhC1g 90%, and RhC2g 91% identical, see Figure 19. The phylogenetic tree resulting from the protein alignment is seen in Figure 20. In Figure 21 the Kyte/Doolittle hydrophilicity charts are shown for each protein showing that like all proteins in the Rh family there are 7 membrane-spanning regions.

In Vivo Ammonia Assay

During the chronic ammonia (NH_4HCO_3) infusion, water samples were taken from the external water environment of the fish so that an ammonia assay determining the ammonia flux of the fish under these ammonia stress loads could be completed. Preliminary data confirmed that the infused ammonia load

remained in the fish and did not leak out of the cannula or sutures (meaning that there was no ammonia in the water due to faulty procedure).

Figure 5, shows the clear difference in slopes between the control period and the experimental period. A definitive change in the slope from pre-infusion control period to the experimental group infusion period and only a minor increase in initial slope from pre-infusion control period to control group infusion period is seen. The slope of the line during the control periods of both treatments was approximately 0.65. During the treatment phase the control group's average slope was 0.98 while the experimental group's average slope was 4.45. With this visual difference in slopes observed, the next thing to look at would be if a statistical difference is shown between the rates.

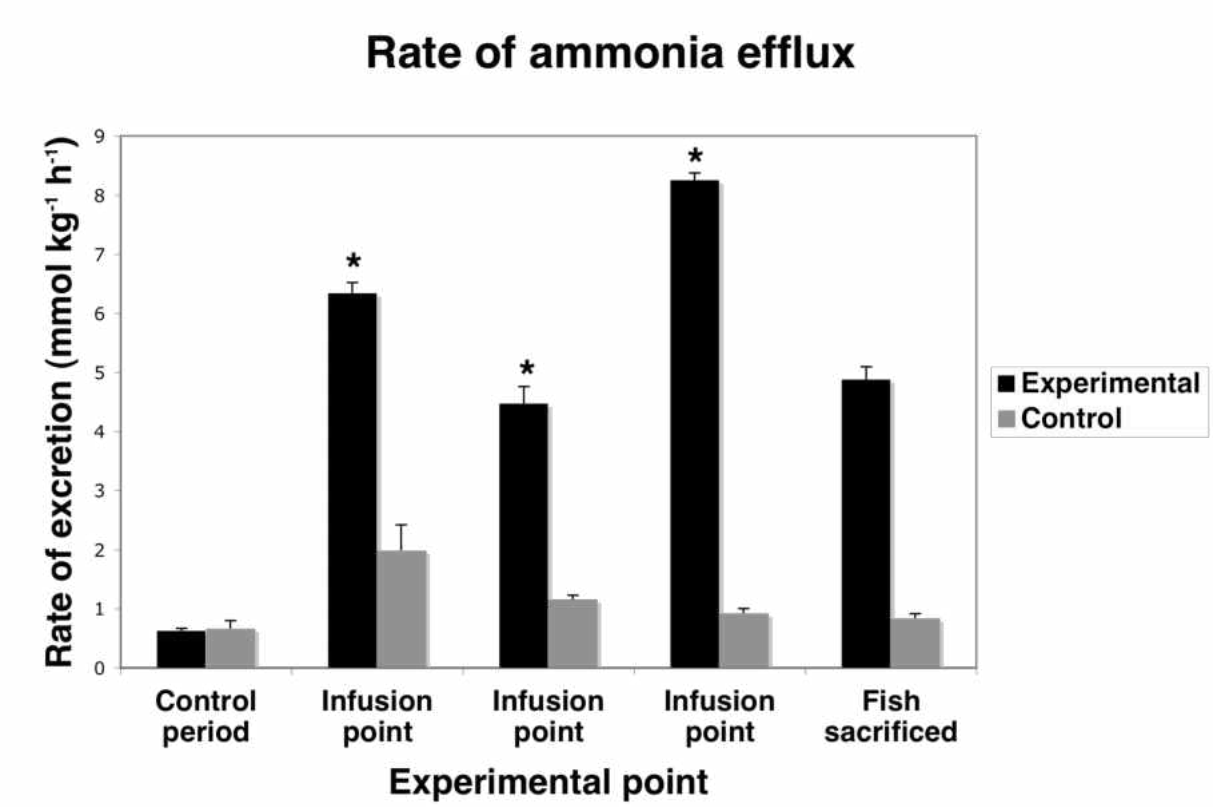


Figure 5: Average total ammonia per fish. Graph showing the average total ammonia per fish in the water. Experimental NH_4HCO_3 treatment and water control, x±SE N=3 for each group.

There is a significant difference of ammonia excretion rates between the control set and the experimental set as seen in Figure 6. In reaction to the imposed ammonia stress the excretion rate increased 10 fold from a pre-infusion control rate average of 0.66 ± 0.044 mmol kg^{-1} h^{-1} to an average of 6.3 ± 0.18 mmol kg^{-1} h^{-1} ($p<0.001$, N=3) which ends up as an approximate 14.8 fold increase in the rate of excretion. It is interesting to note that this maintained level of heightened ammonia excretion that by the end of the 8-hour infusion period the experimental group had actually excreted more (an average of 38.66% more)

that the amount of ammonia infused into the fish (20mM/kg). This increase in rate comes in comparison with the control group average, which increases from

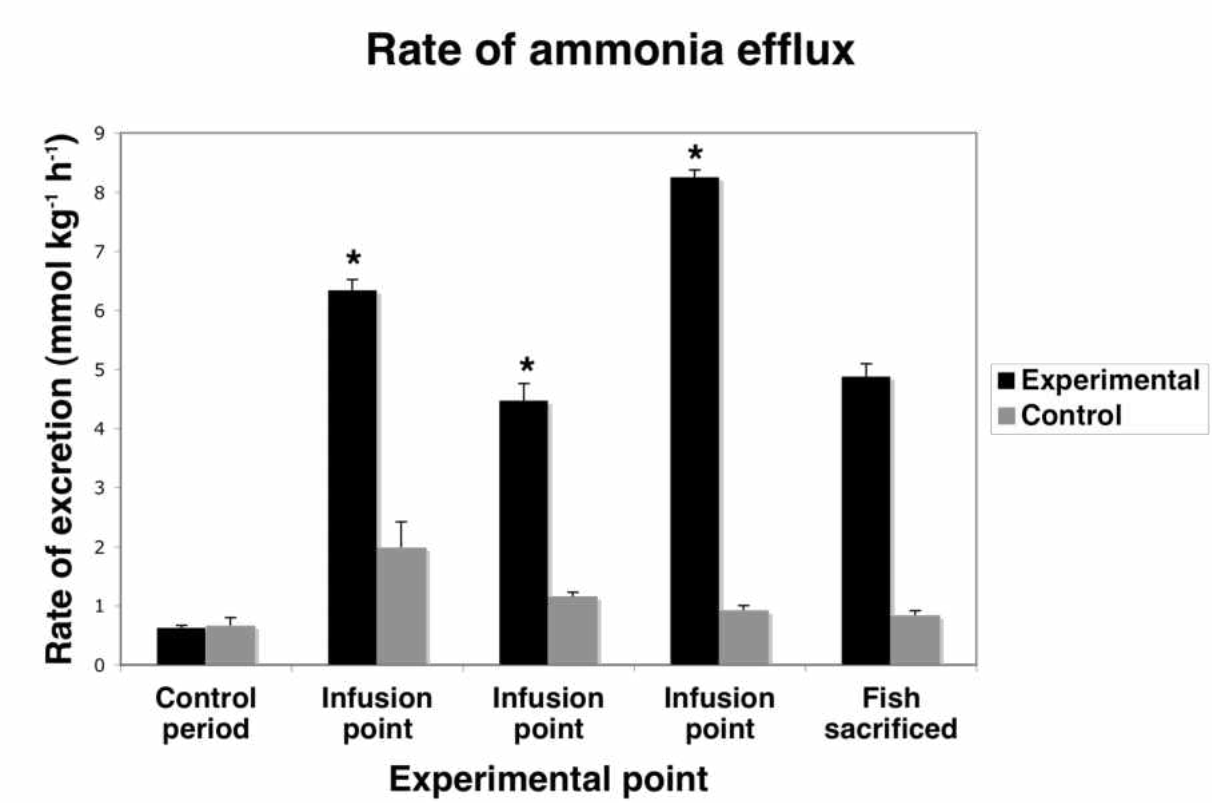


Figure 6: Rate of ammonia efflux. Experimental NH_4HCO_3 treatment and water control, x ± SE N=3 for each group. Control rates compared to pre-infusion control (time 0): P=0.167, p=0.313, 0.604, and p=0.447 respectively for time 2, 4, 6, and 8. Experimental rates compared to pre-infusion control (time 0): p=0.004*, p=0.023*, p=0.0009*, and p=0.013 respectively for time 2, 4, 6, and 8 utilizing Bonferroni adjusted α rates.

its pre-infusion control rate average of 0.66 ± 0.13 mmol kg^{-1} hr^{-1} to its highest rate (at the 4 hour time point immediately following the third infusion) of 2.305 ± 0.15 mmol kg^{-1} h^{-1}, a 3.5 fold increase.

Figure 7 shows that when looking at ammonia output there is a significant difference (p=0.0001) between the pre-infusion period and the infusion period in the experimental group.

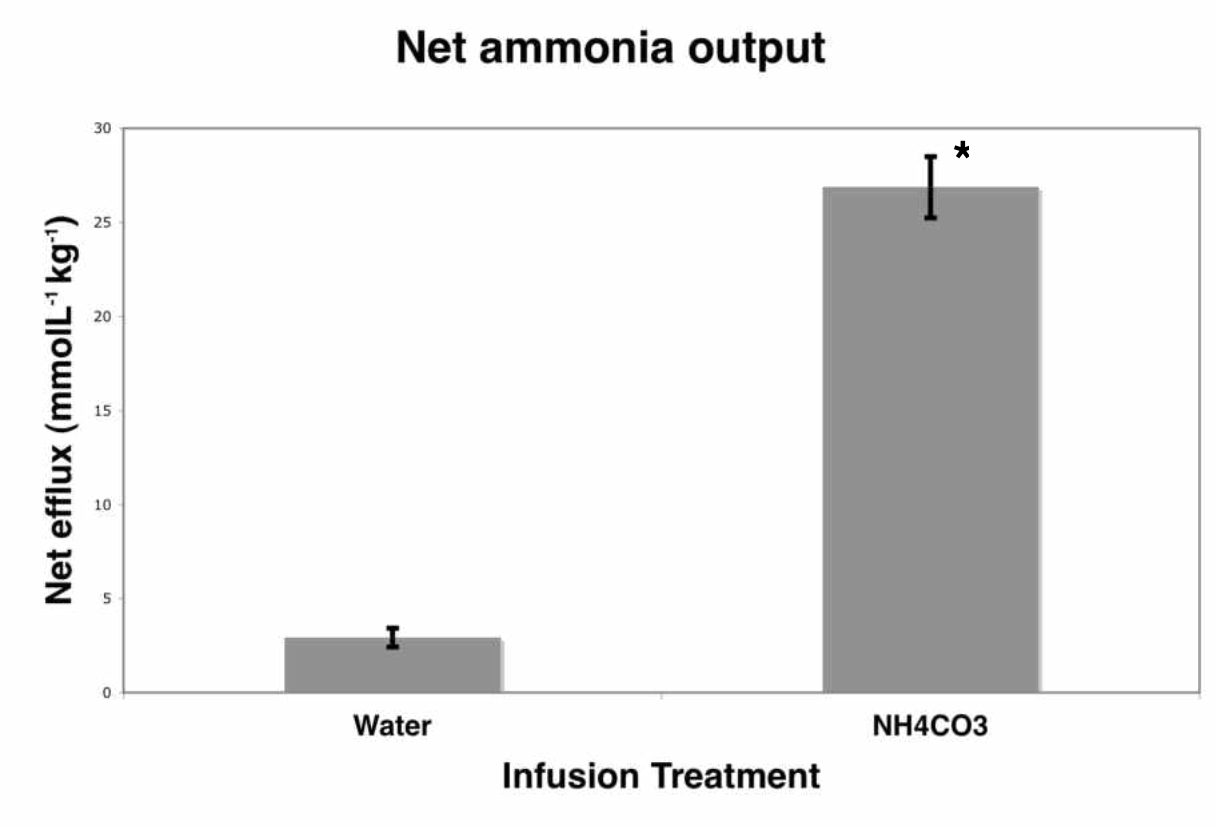


Figure 7: Total ammonia output. Graph showing the total ammonia output in water per fish, as compared to its pre-infusion control period. Experimental NH_4HCO_3 treatment and water control, n = 3 for each group, showing standard errors. Control p=0.3, experimental p=0.014*.

Quantitative RT-PCR: mRNA Expression

Acute:

To look at the results of the quantification of mRNA expression the results will be broken down into the two physiological loads given to the fish: acute and

chronic. When looking at the following results a couple of things need to be noted. Between the two types of ammonia administered, ammonia chloride (NH_4Cl) and ammonia bicarbonate (NH_4HCO_3), NH_4HCO_3 was the only one that actually caused valid changes. The NH_4CL treatment group changes were overshadowed due to high variance from fish to fish as seen in Figure 8.

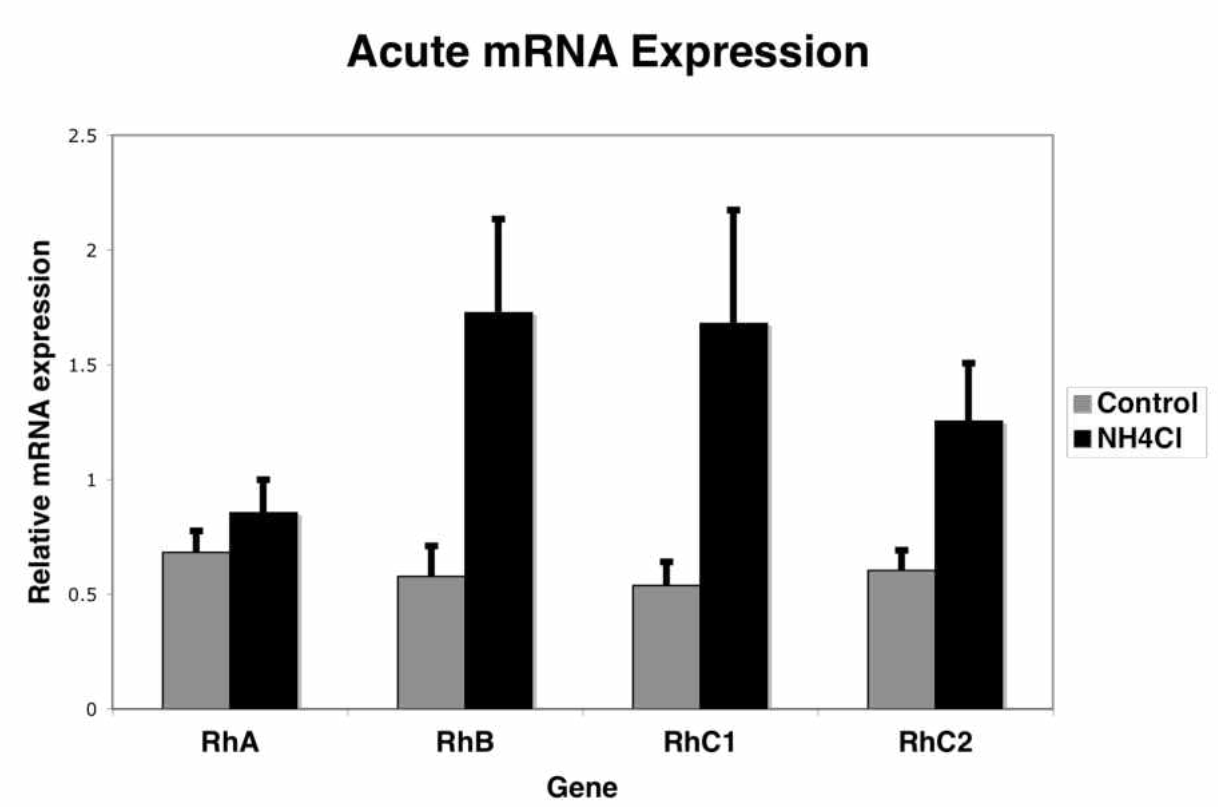


Figure 8: Acute relative NH_4Cl mRNA expression. Graph showing relative expression of sculpin RhAg, RhBg, RhC1g, and RhC2g mRNA compared to Ubc internal control in the gill after acute ammonia stress of NH_4Cl treatments, and water control, x±SE N=6 for each group. RhAg p=0.62, RhBg p=0.2, RhC1g p=0.28, RhC2g p=0.25.

Looking at Figure 9 it is observed that there are no significant changes in relative mRNA level of treated fish compared to control fish. There is an insignificant decrease in relative RhAg mRNA expression when the fish are

exposed to NH_4HCO_3, and there is a slight, still insignificant, increase in expression of RhBg, RhC1g, and RhC2g.

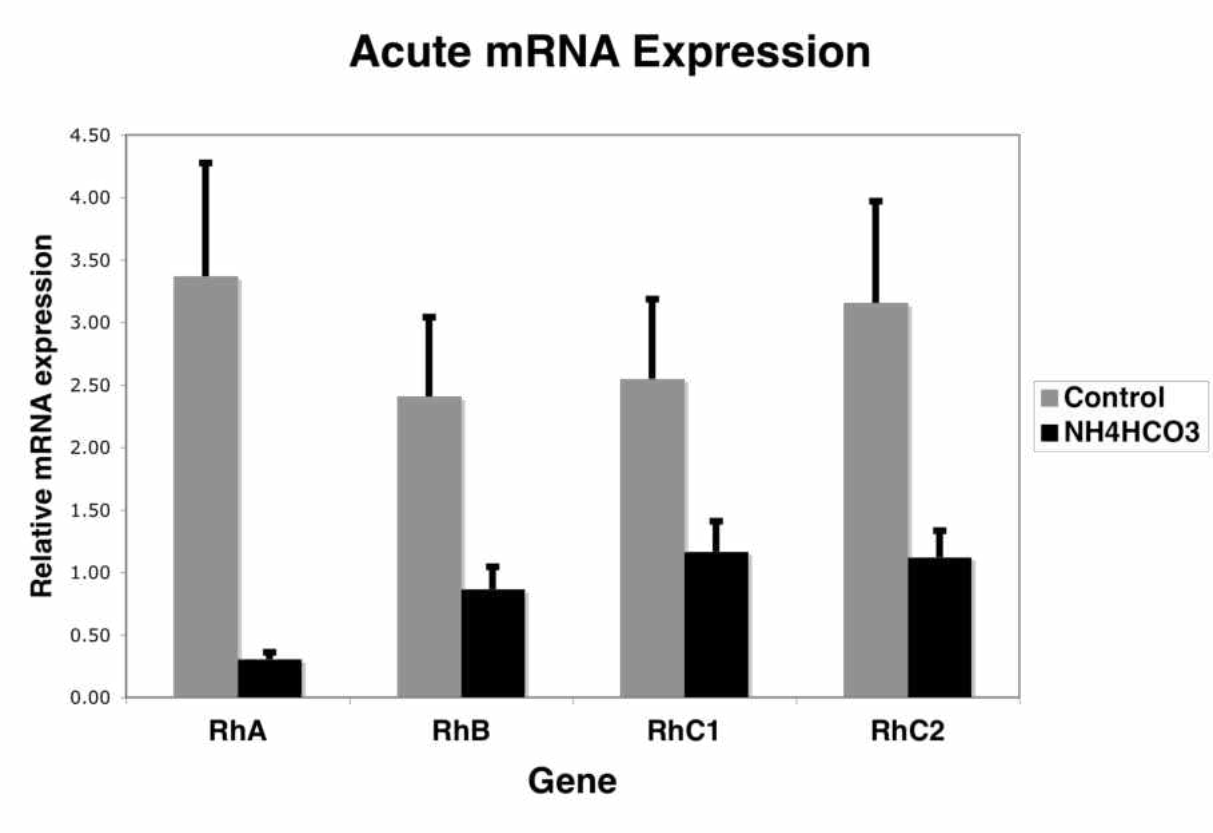


Figure 9: Acute relative NH_4HCO_3 mRNA expression. Graph showing relative expression of sculpin RhAg, RhBg, RhC1g, and RhC2g mRNA compared to Ubc internal control in the gill after acute ammonia stress of NH_4HCO_3 treatments, and water control, x±SE N=6 for each group. RhAg p=0.27, RhBg p=0.47, RhC1g p=0.26, RhC2g p=0.35.

Chronic:

In the chronic ammonia loading series there are similar results to the acute series with no significant changes in mRNA expression of any gene due to ammonia stress. In Figure 9 there is again an insignificant decrease in relative

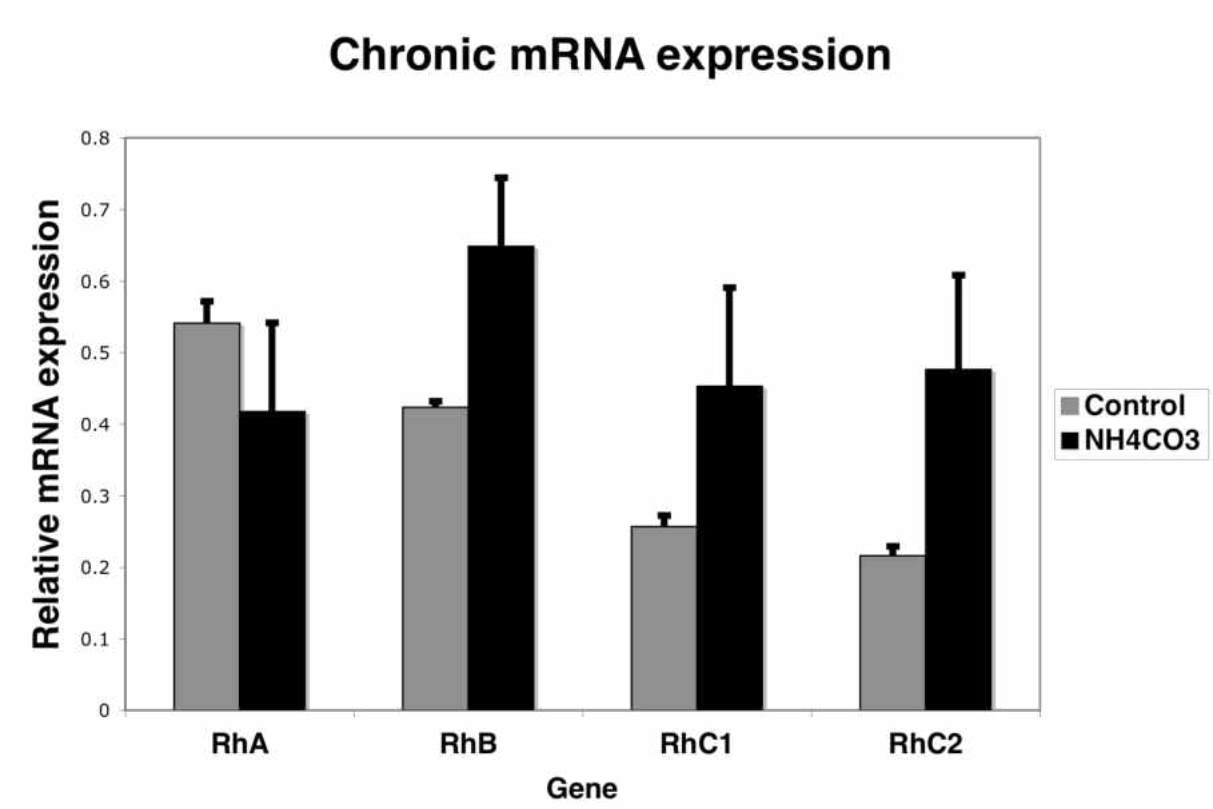
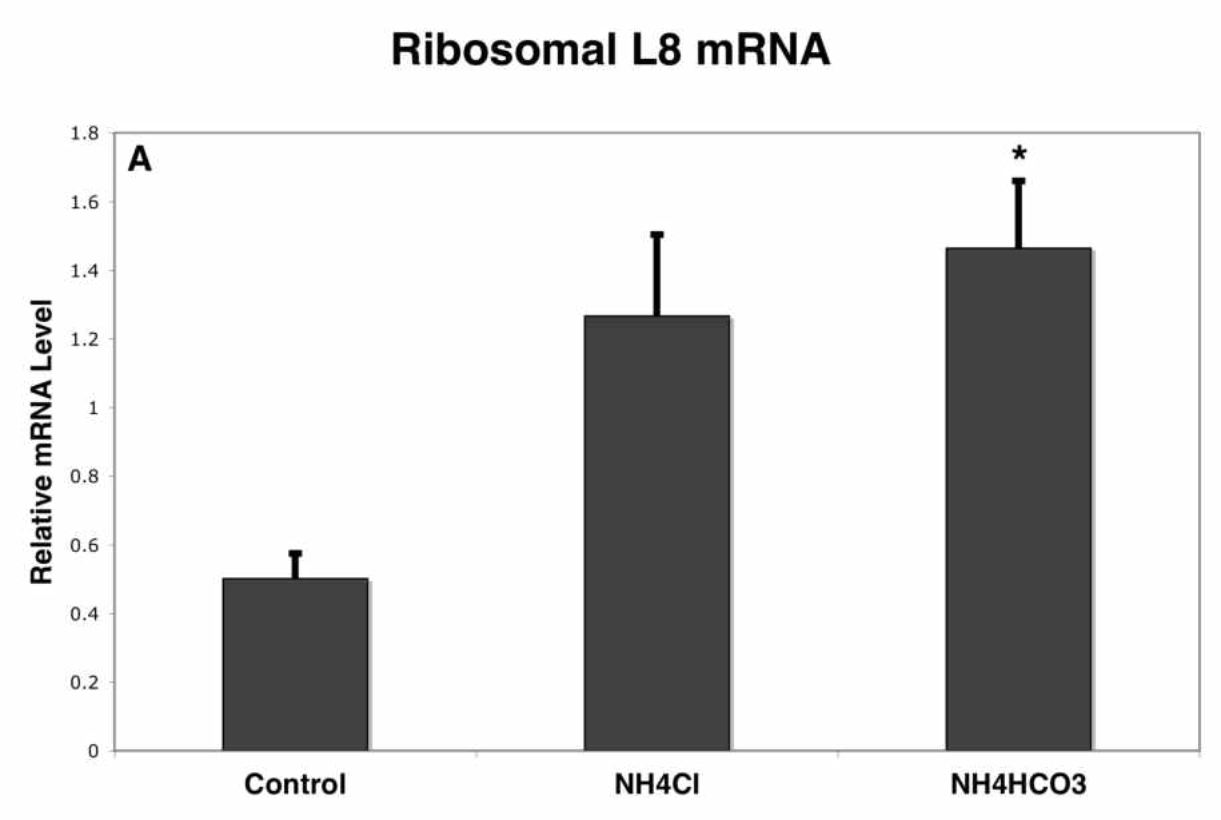


Figure 10: Chronic relative NH_4HCO_3 mRNA expression. Graph showing relative expression of sculpin RhAg, RhBg, RhC1g, and RhC2g mRNA compared to Ubc internal control in gill after chronic ammonia stress of NH_4HCO_3 treatment and water control, x±SE N=3 for each group, RhAg p=0.65, RhBg p=0.3, RhC1g p=0.51, RhC2g p=0.38.

mRNA expression of RhAg compared to control following ammonia stress, as opposed to RhBg, RhC1g, and RhC2g where there is again insignificant increases in relative mRNA expression.

Internal Control:

One thing to note is the variability found in the internal control gene. At first ribosomal protein L8 was used as the internal control gene until it was found to increase in a statistically significant manor when exposed to acute levels of NH_4HCO_3. This is evident when looking at Figure 21 there is a statistically significant change when exposed to an acute load of NH_4HCO_3, relative levels of L8 mRNA increased. Though in the chronic loading (Figure 10) there was no statistically significant change.



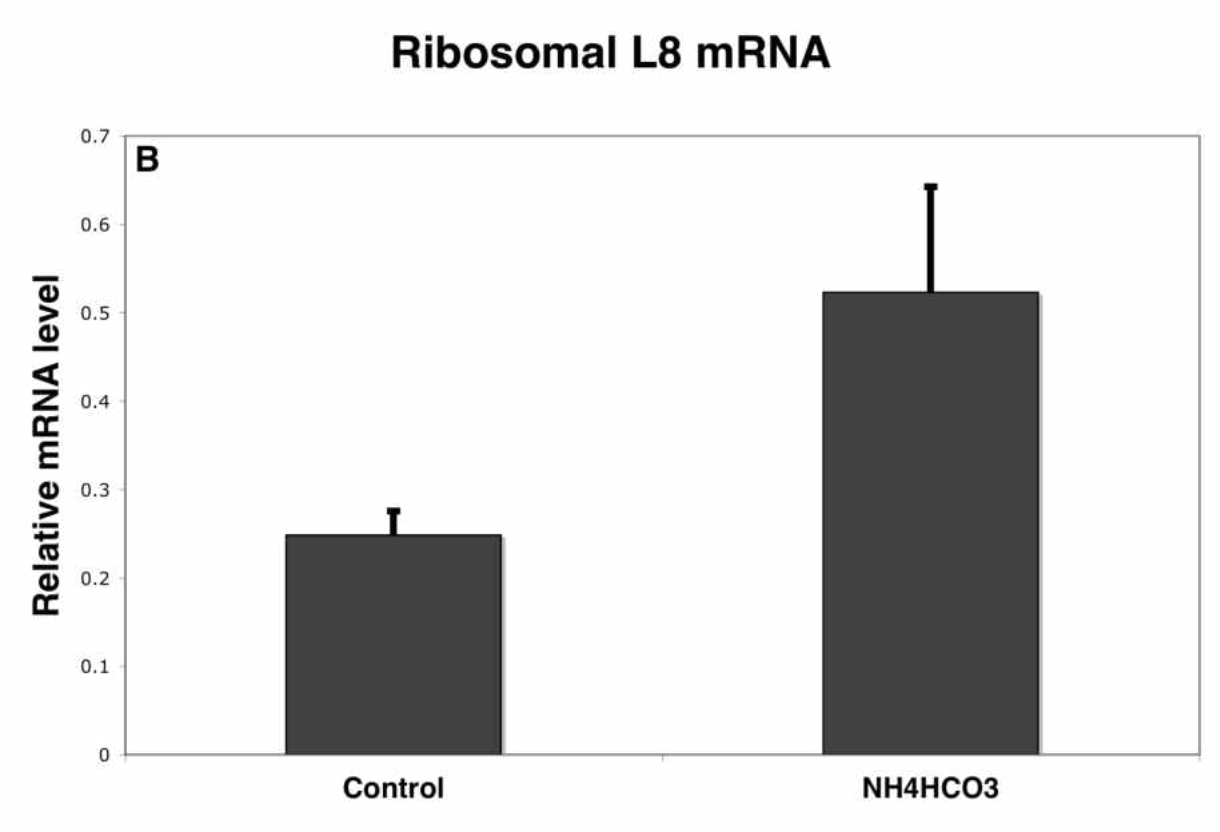


Figure 11: L8 mRNA expression after ammonia stress. A) Graph showing the expression of sculpin L8 mRNA in gill after acute ammonia stress of NH_4Cl, NH_4HCO_3 treatments, and water control, x±SE N=6 for each group. NH_4Cl p=0.75, NH_4HCO_3 p=0.044*. B) Graph showing the expression of sculpin ribosomal L8 mRNA in gill after chronic ammonia stress of NH_4HCO_3 treatment and water control, x±SE N=3 for each group p=0.325.

In light of this, two new internal control stability genes were tested to replace L8. Based on Hibbler's (2008) work, primers for L13A ribosomal binding protein and Ubiquitin were tested for viability in sculpin. Ubiquitin (Ubc) was found to be viable and unaffected by acute or chronic ammonia loads as can be seen in figures 12 and 13 and was, therefore, used as the internal control.

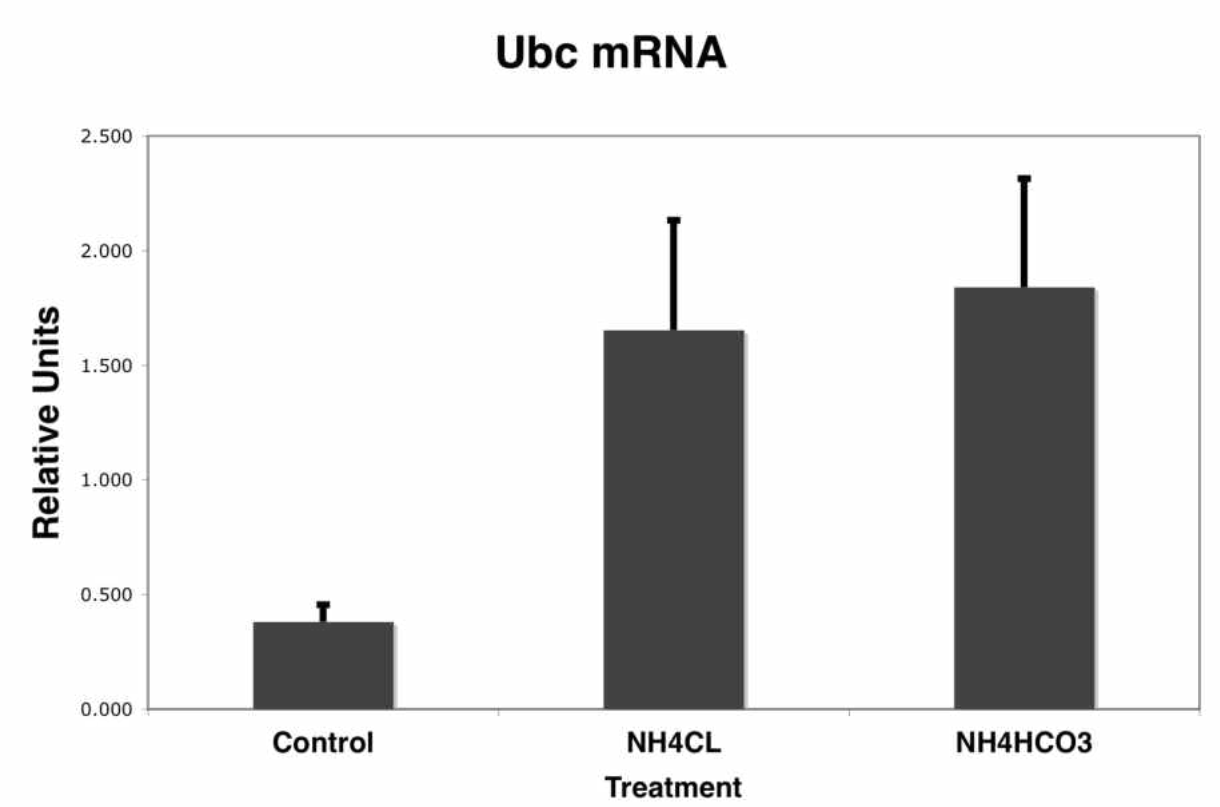


Figure 12: Acute Ubc mRNA expression. Graph showing the expression of sculpin Ubc mRNA in gill after acute ammonia stress of NH_4Cl, NH_4HCO_3 treatments, and water control, x±SE N=6 for each group. NH_4Cl p=0.75, NH_4HCO_3 p=0.044*.

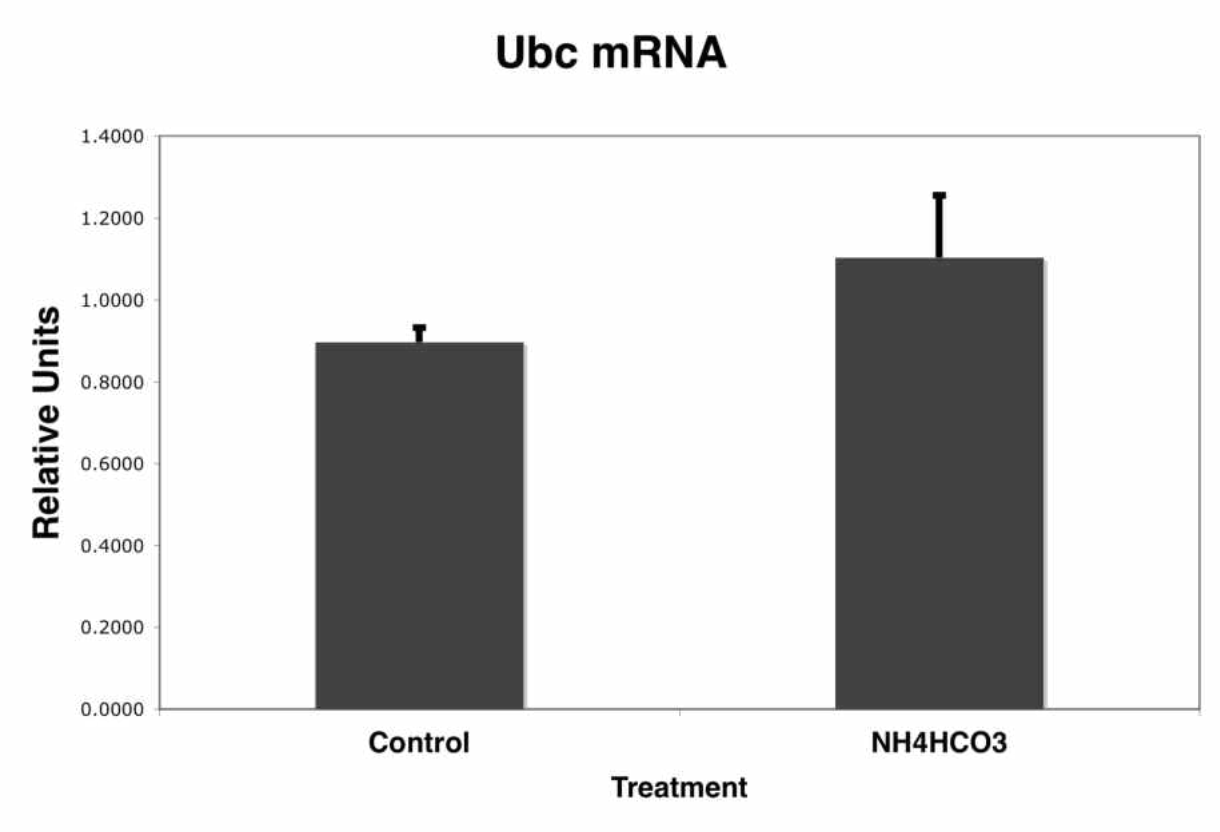


Figure 13: Chronic Ubc mRNA expression. Graph showing the expression of sculpin Ubc mRNA in gill after chronic ammonia stress of NH_4HCO_3 treatment and water control, x±SE N=3 for each group p=0.325.

Dot Blots: Protein Expression

Acute:

The densitometry was done by comparing the brightness of bound chemiluminescent, protein-specific antibodies to a curve of known protein concentrations that were also bound with chemiluminescent antibodies, an example of the this can be seen in Figure 14.

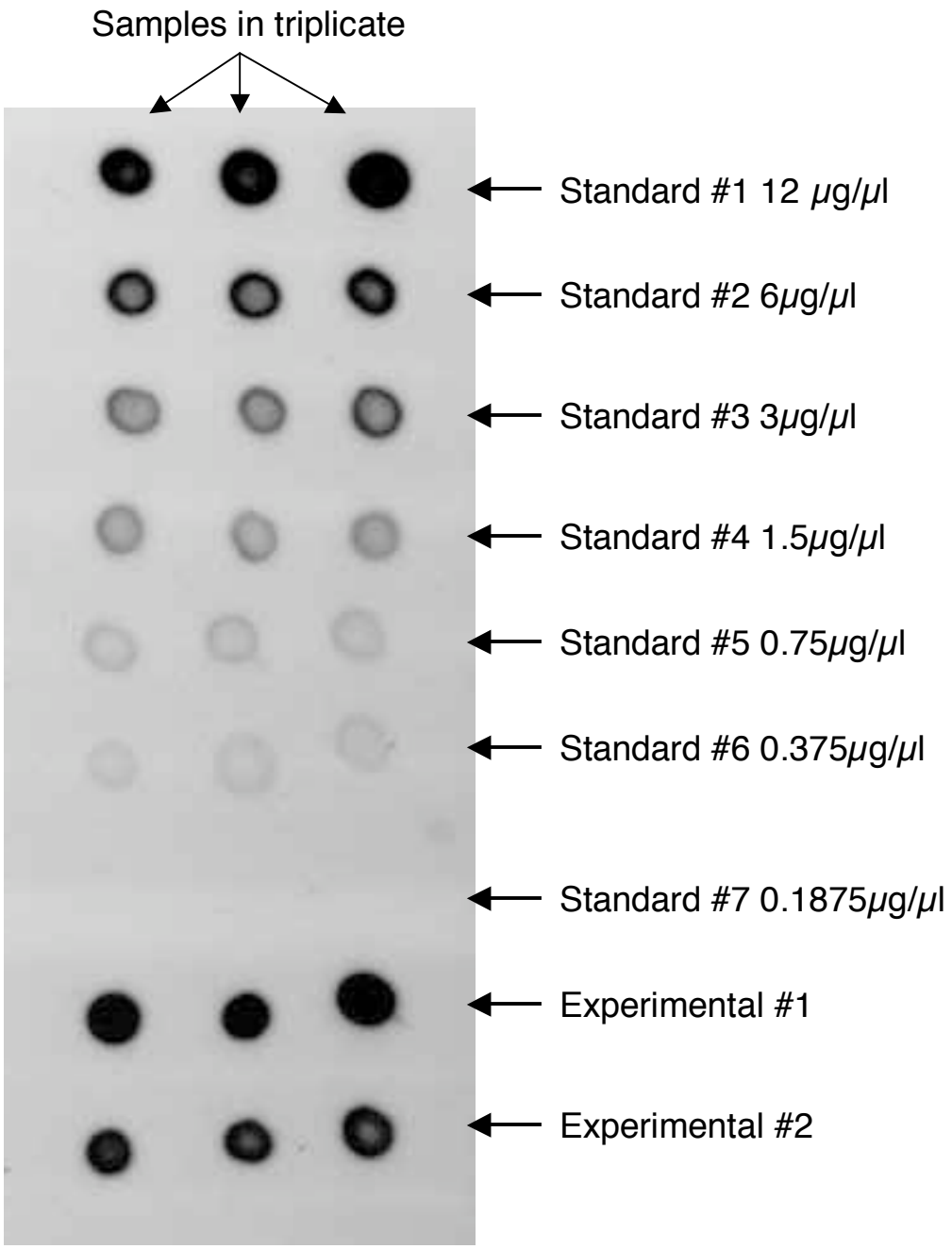


Figure 14: Example image of dot blots. Shows a concentration curve and two experimental samples done in triplicate. The concentration curve starts at the top and goes down with a 1:1 dilution (protein: water) each time for a seven-point concentration curve. At the bottom two experimental points are shown that were compared to the curve to determine relative concentration of protein. The densitometry comparison was done with Bio-rad's Quantity One program.

In Figure 15 no significant changes occur after an acute exposure to NH_4Cl. The first thing apparent is seen in Figure 16 where a significant decrease in relative RhAg protein expression in response to an acute NH_4HCO_3 stress is shown. No significant changes occur in RhBg or RhC2g. RhC1g shows an insignificant increase (approximately 30% increase), however, it should be noted that p=0.06 and without the high fish to fish variability between control and experimental this could possibly be a significant increase.

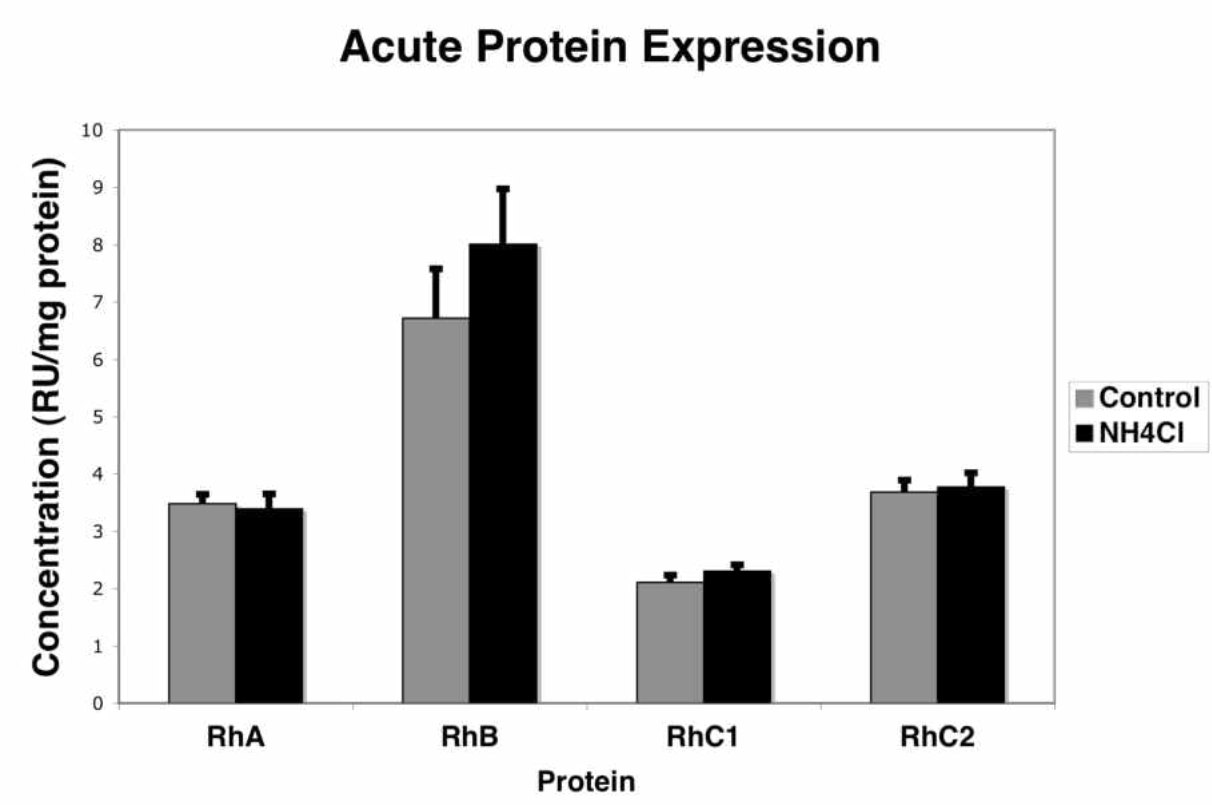


Figure 15: Acute relative NH_4Cl protein expression. Graph showing the relative expression of sculpin RhAg, RhBg, RhC1g, and RhC2g protein in gill after acute ammonia stress of NH_4Cl treatments, and water control, x±SE N=6 for each group. RhAg p=0.88, RhBg p=0.63, RhC1g p=0.57, RhC2g p=0.88.

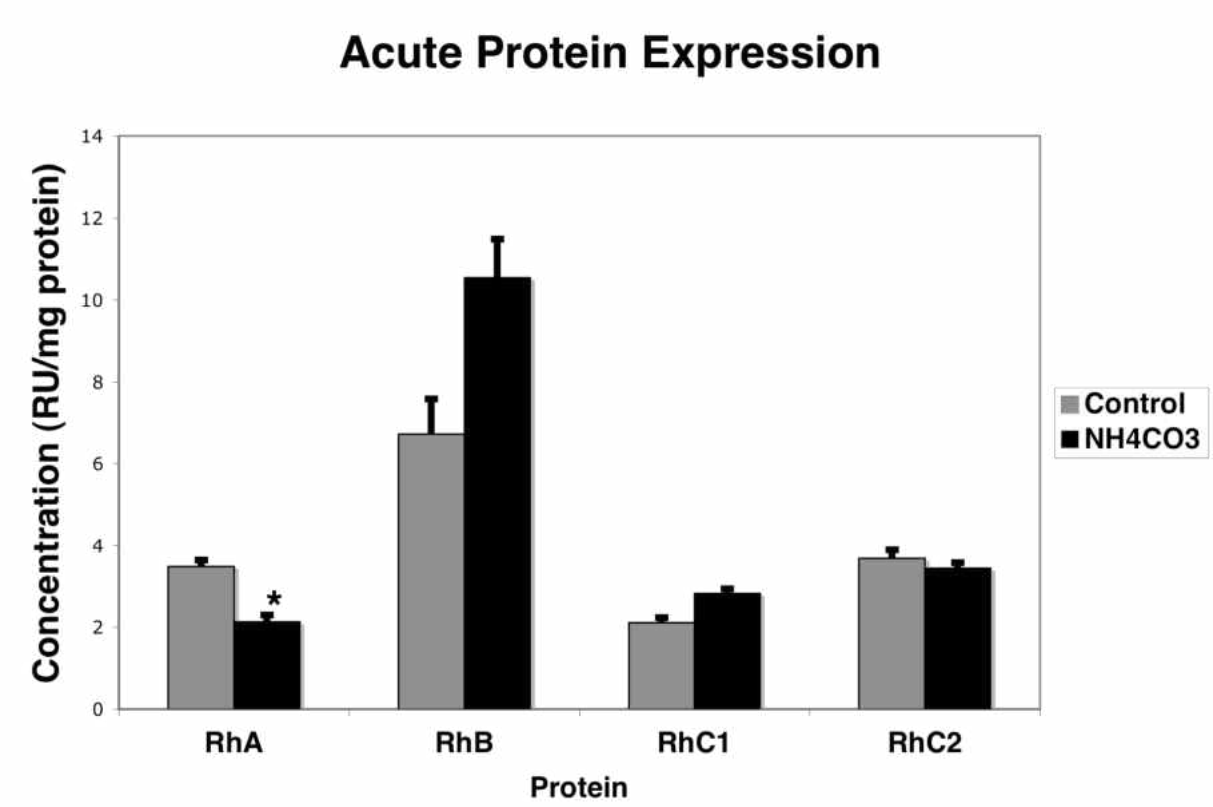


Figure 16: Acute relative NH_4HCO_3 protein expression. Graph showing the relative expression of sculpin RhAg, RhBg, RhC1g protein in the gill after acute ammonia stress of NH_4HCO_3 treatments, and water control, x±SE N=6 for each group. RhAg p=0.015*, RhBg p=0.16, RhC1g p=0.06, and RhC2g p=0.63.

Chronic:

In the chronic series there is an insignificant increase (p=0.06, N=3) of relative RhAg protein levels when exposed to a chronic NH_4HCO_3 stress, see Figure 17. Also, it is interesting to note that the relative protein expression levels for RhAg went from significantly down regulated with an acute ammonia stress load to nearly significantly up regulated with a chronic ammonia stress load. Figure 17 also shows no significant changes in RhBg or RhC2g protein

expression, however, it does show a significant increase (approximately 7x) in relative RhC1g protein level expression due go a chronic NH_4HCO_3 stress.

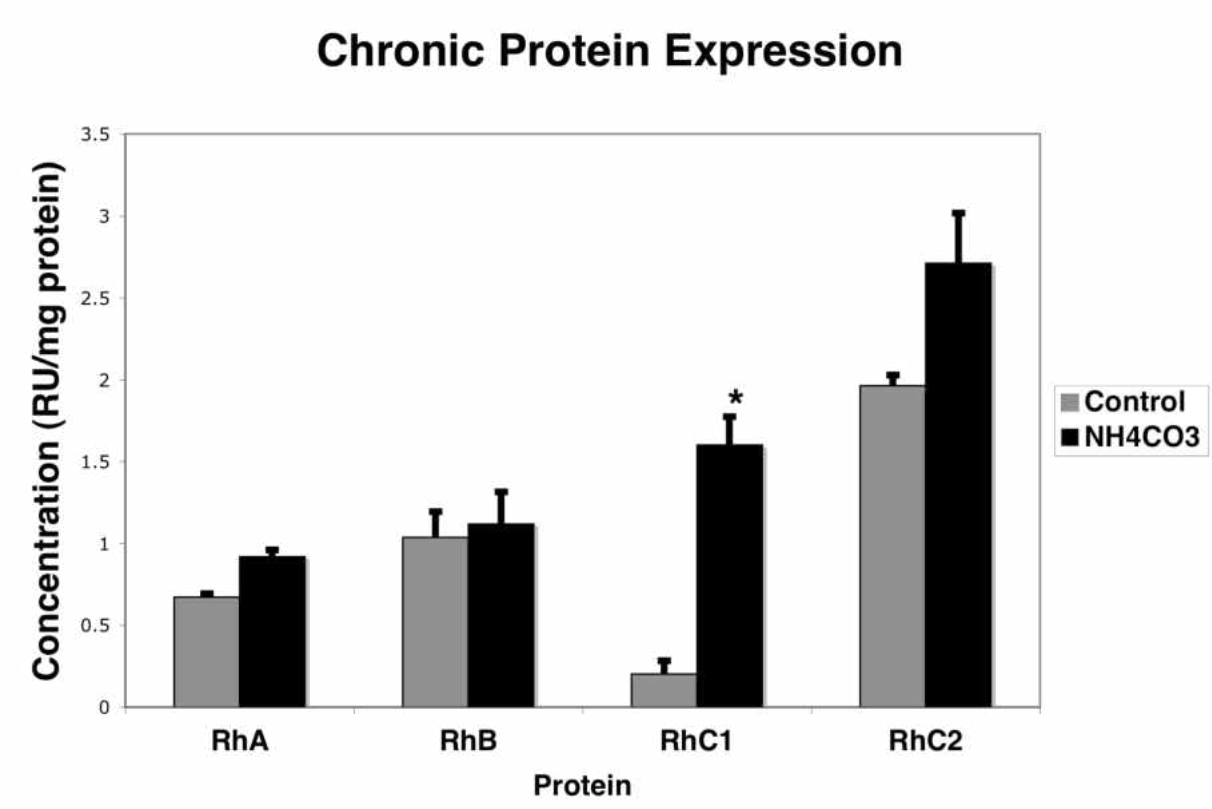


Figure 17: Chronic relative NH_4HCO_3 protein expression. Graph showing the expression of sculpin RhAg, RhBg, RhC1g, and RhC2g protein in gill after chronic ammonia stress of NH_4HCO_3 treatment and water control, x±SE N=3 for each group. RhAg p=0.06, RhBg p=0.88, p=0.02*, RhC2g p=0.29.

Table 2: Summary of Results

	mRNA	
Gene	**Acute**	**Chronic**
RhAg	Trend of decrease ⇓	Trend of decrease ⇓
RhBg	Trend of increase ⇑	Trend of increase ⇑
RhC1g	Trend of increase ⇑	Trend of increase ⇑
RhC2g	Trend of increase ⇑	Trend of increase ⇑
	Protein	
Protein	**Acute**	**Chronic**
RhAg	Significant decrease ⇓ **	Trend of increase ⇑
RhBg	Trend of increase ⇑	Trend of increase ⇑
RhC1g	Trend of increase ⇑	Significant increase ⇑ **
RhC2g	Trend of increase ⇑	Trend of increase ⇑

Discussion

The question at hand is how ammonia is excreted from the gills of the longhorn sculpin, either by one of the previous methods (such as simple diffusion or non-specific ion transport) or through the newer model employing Rh glycoproteins. In this study the presence of the Rh orthologues in the sculpin gill has been examined, and functional and physiological tests have been completed to determine the response of the Rh orthologues mRNA and protein levels to ammonia loading.

In vivo ammonia assays showed that ammonia loads injected into the fish were excreted into the external environment. These ammonia excretion rates follow extrapolations of previous experiments using acute loading stress (Claiborne and Evans, 1988). Figure 6 showed that there was no statistical difference between the pre-infusion period and the infusion period in the control group, however, there is a significant difference ($p=0.0001$) between the pre-infusion period and the infusion period in the experimental group. This would be expected that when you infuse excess ammonia into the fish that the fish would excrete it in some manner and not store it due to its toxicity.

Four orthologue gene sequences (RhAg, RhBg, RhC1g, and RhC2) that have been previously linked to ammonia excretion (Nakada et al., 2007) were found in the sculpin gill. They were found to be significantly homologous with the Rh paralogues found in stickleback with RhBg, RhC1g, and RhC2g being 90% identical, and RhAg being 50% identical (with an e value of -89) (Figure 24). A

phylogenetic tree amongst a large group of fishes and humans placed all of the genes along with their corresponding paralogues (Figure 23) and hydrophilicity figures (Figure 25) showed plausibly that they shared the common 7 membrane spanning regions common among the Rh family.

To determine if these four proteins were responsible for the ammonia excretion, quantitative RT-PCR methods and dot blot methods were utilized to measure mRNA and protein expression, respectively. No significant changes in RhAg, RhBg, RhC1g, or RhC2g were observed following NH_4Cl infusion at any point in the experiment. This is most likely due to the variation from fish to fish experienced throughout the experiment, which was most evident when looking at NH_4Cl results. Mean relative mRNA levels appeared to increase following ammonia loading, but the changes were not significantly different between the experimental and control levels of mRNA in either the acute or chronic ammonia load groups.

Changes did occur in protein expression response to ammonia loading. RhC1g protein level following the acute ammonia load increased (by ~30%) when compared to controls ($p=0.069$). In contrast, the chronic ammonia group exhibited dramatically higher RhC1g expression (~7x higher; $p=0.021$) over the control fish, thus suggesting that it may play a role in ammonia excretion.

RhAg protein levels also responded significantly to ammonia loading. There was a significant down regulation ($p=0.015$) of protein expression when exposed to acute NH_4HCO_3 and a nearly significant up regulation ($p=0.06$, but

without the fish to fish variability caused by using separate fish for control this value could be significant) when exposed to chronic levels. This is a very interesting change with no clear explanation of what is happening. It is plausible that initial response to ammonia stress is that of removing this pathway to force another, such as the RhC1g pathway, for reasons such as additional control of the excretion process over shorter periods of time. Following the continued stress it is feasible that the RhAg pathway was then allowed back “on” to compensate for the continual ammonia stress to the fish.

The variation in qPCR relative mRNA levels measured between animals was high, so this may have masked changes due to the ammonia loads. The large increase in RhgC1 protein expression following the acute load (with little parallel increase observed in mRNA) may also imply that the regulation of this system is accomplished by post-transcription changes such as alterations in translation rates, membrane shuttling, and/or adjustments to protein channel half-life (Cavet et al., 1999) this way a decrease in the breakdown of pathways can still allow an increase in expression without a change in mRNA. We postulate that apical RhgC1 in gill MRCs (Claiborne et al., 2008) is up regulated to allow the rapid excretion of ammonia across the gills.

When comparing our results to the model set forth by Nakada et al. (2007) in we see that it is a plausible model, Figure 18 shows both the lamellar epithelium pathway as well as the chloride cell pathway along with the other previously proposed methods discussed earlier. Clearly RhC1g could be utilized

in a key role with its localization in the chloride cell using NKA to pump the ammonia into the cell. Also with no change in the RhBg or the RhC2g protein expression it seems valid to expect these proteins to be players in a more general, passive approach to excreting ammonia levels that would be produced under normal physiological conditions. With this explanation RhAg could then be easily hypothesized to be used as a door or switch to direct ammonia excretion to the NKA-RhC1 pathway in an acute ammonia stress and back to the RhA-RhB-RhC2 pathway under normal or extreme chronic ammonia stress periods.

Also to be considered is the possibility that the RhXgs are playing some role in CO_2 transport. This is something that is being looked into more intensely today by different research groups, and should be considered in future research.

In summary, we postulate that apical RhgC1 in gill MRCs is up regulated to allow the rapid excretion of ammonia across the gills in accordance with the model set forth by Nakada et al. (2007) as well as RhAg is being utilized as a type of secondary pathway in its' ammonia excretion route of RhA-RhB-RhC2 in the lamellar epithelial cells. Also our research implies that the regulation of this system is accomplished by post-transcription changes such as alterations in translation rates, membrane shuttling, and/or adjustments to protein channel half-life this way a decrease in the breakdown of pathways can still allow an increase in expression without a change in mRNA.

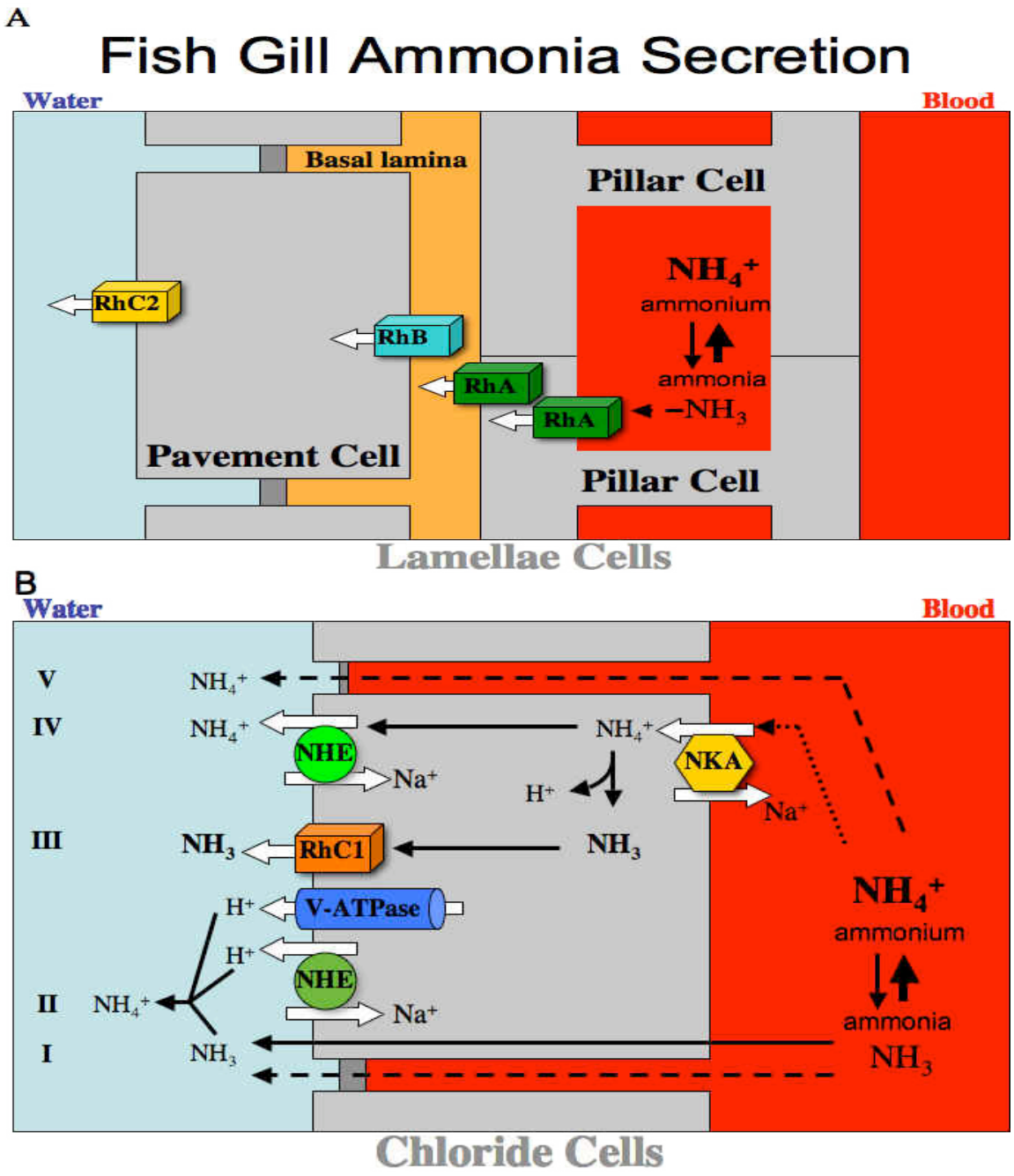


Figure 18: Method of ammonia excretion. Image showing the method of ammonia excretion in the gill of the marine teleost longhorn sculpin. A) Lamellae epithelium cells showing an ammonia excretion pathway from blood through RhAg to exit the pillar cell into the basal lamina and then through the basolateral side of the pavement cell via RhBg and out the apical side of the pavement cell using RhC2g. B) Restatement of the possible methods of ammonia excretion in a chloride cell utilizing the pathways shown in Figure old mechanisms as well as that of RhC1g on the apical side of the chloride cell with the basolateral NKA to bring ammonium inside the cell (instead of K^+).

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Appendix

Table 3: List of all Primers used

Reference #	SeqName	Sequence
	GacRhB,SBRhBG	
357	SBRhBG-120U24F2#357	GCTATGGCACAGCAAGAACCACTC
358	SBRhBG-468U25F7#358	GGTCACGCTGTTTTCTGTCAACGAG
359	SBRhBG-490L25B2#359	CGTTGACAGAAAACAGCGTGACCTC
360	SBRhBG-991L24B6#360	TCAGTTTGTTCTCCAGGACGGGTG
	GacRhC2,SBRhCG	
361	SBRhCG-22U21F1#361	TTGAGAGGTTTTTTCTGCCCG
362	SBRhCG-182U21F3#362	ACAACATCACCAGCGACATCG
363	SBRhCG-632U24F9#363	CCATCTCCTGGATTCTCTACCGAC
364	SBRhCG-998U24F19#364	TCTCCACTTTTGGCTACCTGTTTG
365	SBRhCG-735L24B3#365	CAGGAACAGTGTGCCAATCATAGC
366	SBRhCG-1346L23B13#366	TCGTCAAAGCAGTTGTCATCAGC
367	SBRhCG-1220L22B9#367	TGGGTTCCTACCGTTCTGTCTG
368	SBRhCG-1340L20B12#368	AAGCAGTTGTCATCAGCGGG
	GacRhC2 full	
373	SBRhCG--30U20F1#373	GCAGCATCATTCCAAGACCG
374	SBRhCG--29U22F1#374	CAGCATCATTCCAAGACCGTTC
375	SBRhCG-597L20B2#375	CGCCAAACAAGGTGACAACC
376	SBRhCG-609L23B4#376	CAAACAGTGTGACGCCAAACAAG
377	SBRhCG-633U24F2#377	CATCTCCTGGATTCTCTACCGACC
378	SBRhCG-633U24F2a#378	CATCTCTTGGGTTCTCTACCGACC
379	SBRhCG-802U22F4#379	ATCAACACCTACATCGCCCTCG
380	SBRhCG-802U22F4a#380	ATCAACACGTATATCGCTCTCG
381	SBRhCG-1721L23B2#381	CAGTCTTCAACCTCCCATCAAGG

382	SBRhCG-1723L24B3#382	AACAGTCTTCAACCTCCCATCAAG
383	SBRhBG--48U24F1#383	TAAAAGCTCCTCTCTCGTCGCCTC
384	SBRhBG-284L24B9#384	AAGTCGTTCTCGTAGTCGGAGTGG
385	SBRhBG-401L22B11#385	ATCAGGAAGTTGAAGCCCACGC
386	SBRhBG-206U20F3#386	TCATCGGTTTCGGCTTCCTC
387	SBRhBG-457U21F5#387	GCCATTTTTGAGGTCACGCTG
388	SBRhBG-470U23F7#388	TCACGCTGTTTTCTGTCAACGAG
389	SBRhBG-884U23F9#389	GTGAAATGATGCTGACGCCTTAC
390	SBRhBG-886U22F10#390	GAAATGATGCTGACGCCTTACG
391	SBRhBG-906L23B4#391	GTAAGGCGTCAGCATCATTTCAC
392	SBRhBG-989L22B6#392	AGTTTGTTCTCCAGGACGGGTG
393	SBRhBG-991L22B7#393	TCAGTTTGTTCTCCAGGACGGG
394	SBRhBG-1611L20B8#394	TTGTTCCGCCTCCTGAATGC
395	SBRhBG-1613L21B9#395	TCTTGTTCCGCCTCCTGAATG
	GacScpRhB	
406	GacScpRhB-18U19#406	AGGACCCTCCGGCGTGTGT
407	GacScpRhB-518L24#407	CCCAAAACGGCTCCGAACGAGATG
408	GacScpRhB-128U22#408	GGTCACCAACATGCGGCTCAAG
409	GacScpRhB-561U24#409	CTGCTGGTCATGGCGATATTCGAG
410	GacScpRhB-1257L19#410	CCTGGTAGGACGCGGTGAT
411	GacScpRhB-270U24#411	AACGACTTCTACTTCCGCTACCCA
412	GacScpRhB-608L24#412	CCCAGGGCAGTTACCAGGACGAAC
	GacScpRhB	
435	GacRhB-InMoc02-508U20#435	GCGCTGTGCTCATCTCGTTC
436	GacRhB-InMoc02-863L24#436	GCTGCCAGGGAGTAGTAGGTGTTC
437	GacRhB-InMoc02-825U24#437	GTCACGGCTCACGGAGACGACCAG
438	GacRhB-InMoc02-1583L19#438	GGGCGGGGCTAAGGTTCTG
439	GacRhB-InMoc02-830U19#439	GGCTCACGGAGACGACCAG

440	GacRhB-InMoc02-1549L24#440	CTCAGGGTTTTTCTACATCGGACT
	GacRhC2	
441	GacRhC2-82U24A#441	TCAGGGAATTGTGCGACCGTCAAA
442	GacRhC2-622L21A#442	CGGCATCTCTGGCATGTATGA
443	GacRhC2-121U20B#443	GTAAGAGTCTCCCGGCAGTT
444	GacRhC2-673L22B#444	GAGATGGAAAGGCCGTAGTAAG
445	GacRhC2-500U20C#445	CGTGGCGGGCTGCTTGATAG
446	GacRhC2-1537L24C#446	GTGATGAAGACAGACGGCCAATAG
447	GacRhC2-731U22D#447	CCGTCTGCATGGTTCCGTTTAT
448	GacRhC2-1071L22D#448	ATGAGAGGCGAGAGGTAGACAT
	L8RibosomeFishdegen	
449	Fish-L8-F1#449	GGATACATCAAGGGAATCGTGAARGAYATHAT
450	Fish-L8-R1#450	CAGTTTCGCTTGGCCTTGTAYTTRTGRTA
451	Fish-L8-F2#451	GGGAATCGTGAAGGACATCATHCAYGAYCC
452	Fish-L8-R2#542	CCGAAAGGGTGCTCCACNGGRTTCAT
	GacRhA	
453	GacRhA-431U23#A453	GCGGACTTCAGCACAGCTACAGT
454	GacRhA-1353L24#A454	CAGATAATTAAACCTCCGCTTTGT
455	GacRhA-626U24#B455	CTGGCTGTGGCTCGGGTACTTTAC
456	GacRhA-792U24#C456	CGGCAGTGATCAACACCTACCTCT
457	GacRhA-999U24#D457	CTACTGTGGGCTTCAAGTACCTTA
458	GacRhA-1353L20#D458	TAATTAAACCTCCGCTTTGT
	ScpRhC2 Race	
509	ScpRhC1-1285U22-InG-GR3#509	GCT GGC ACA TGT GTG GCT ATT G
510	ScpRhC1-1340U22-InG-GR3#511	TCG TTT CTG AGG TTG CCT ATC T
	ScpRhC1-2077L19-InG-	

	GR3#510	
	Cau L8	
512	Cau-L8f1-1U20#512	AGC ACA GAA AAG GTG CTG CT
513	Cau-L8f1-433L20#513	ACC AGC AAC AAC ACC AAC AA
514	Cau-L8f1-25U24#514	TCC GTC ATA TCG ACT TCG CTG AAC
515	Cau-L8f1-331L22#515	GAG ATG ACT GTG GCG TAG TTT C
	Anchor-dT25	
520	Anchor-dT25-VN#520	TTT TTT TTT TTT TTT TTT TTT TTT TVN
516	Anchor-dT25-VN-5'Phos#516	/5Phos/TTT TTT TTT TTT TTT TTT TTT TTT TVN
	ScpRh A - B Race	
517	ScpRhAfI-GR3-1001U24#517	CCC TGG GCT TCA AGT TCC TGA CTC
518	ScpRhAfI-GR3-1208U24#518	GGG CTG TTA CAG GTT TAA TAA TGA
519	GR3-25L20#519	TAC GTA ACG GCA TGA CAG TG
521	ScpRhBfI-GR3-488U21#521	GGC GCT GTG CTC ATC TCG TTC
522	GR3-25L24#522	ACG CTA CGT AAC GGC ATG ACA GTG
523	ScpRhBfI-GR3-692U19#523	CGG CCC AAC CTG AAC AAG A
	Rh-QPCR	
524	ScpRhA-Q1-963U22-524	GGCGATGCTCATTGGATTAGTG
525	ScpRhA-Q1-1018L22-525	TGGATGCCAGGATGGGAGTCAG
526	ScpRhA-Q-1189U17-526	GGGTTCGCTCTGGTTGG
527	ScpRhA-Q-1220L22-527	AACGGCAACTTCATTATTAAAC
528	ScpRhB-Q1-693U22-528	GGCCCAACCTGAACAAGAGCAA
529	ScpRhB-Q1-821L20-529	GCCGTGCGGTGCTGGTCATC
530	ScpRhB-Q1-570U22-530	CGCTGTTTGCTGTCAATGAGTA
531	ScpRhB-Q1-668L17-531	ACTCGGGTCACCATGAG
532	ScpRhC1-Q1-1450U22-532	ACGTCCCACCAATTCTCCATTA
533	ScpRhC1-Q1-1487L18-533	TCCGCTACGTCTTTGTTC
534	ScpRhC1-Q1-657U21-534	GGCTCCATGGTGATCCACACA

535	ScpRhC1-Q1-764L22-535	CATGGCAAAGACATCTGAGTGA
536	ScpRhC1-Q1-971U19-536	CGCCACTCTTGCTGGAGGT
537	ScpRhC1-Q1-1080L22-537	TGAAAGGCGTGAGGTAGACATA
538	ScpRhC2-Q-1218U22-538	GGGGTTGATCGAGACGTTTGAC
539	ScpRhC2-Q-1344L22-539	AGGCAACCTCAGAACGAGACCA
540	ScpRhC2-Q-1082U19-540	CGCCCTTCTTGGAGAAATA
541	ScpRhC2-Q-1205L22-541	ATCAACCCCTCCCTGCTATACA
	Inv-PCR	
542	ScpRhA-Inv-1784U21-542	CGATCGCCCTTTCCACAGTTC
543	ScpRhA-Inv-829L24-543	GGAGATGGCGTAGGCAGAGAGCAC
544	ScpRhC1-Inv-1031U24-544	CGGGTCTCTGATCGTAGGATTCTG
545	ScpRhC1-Inv-897L22-545	GCGCCACAGTAGTGAGCACAGT
546	ScpRhC2-Inv-1623U24-546	CACCGGTCCTCTGCCACTCTTGTC
547	ScpRhC2-Inv-1527L20-547	TATGAGCGCCTTAATGTTTC
	ScpRhC1-GR3 RACE	
548	ScpRhC1-GR3-720U20-548	CGGCCAAACCTGAACCAGAG
549	ScpRhC1-GR3-2239L24-549	ACGCTACGTAACGGCATGACAGTG
550	ScpRhC1-GR3-n-1070U24-550	CACGCTGGGATATGTCTACCTCAC
551	ScpRhC1-GR3-n-2239L19-551	ACGTAACGGCATGACAGTG
552	ScpRhB-sp-838U17-552	GGCCATGAACACCTACT
553	ScpRhB-sp-1256L22-553	GATGGACCTGAAACCTTTTAAT
	L8-QPCR	
556	ScpL8-Q-365U22-556	CACACCGGACAGTTCATCTACT
557	ScpL8-Q-460L19-557	GGGCTTCTCCTCCAGACAG
558	FheL8-F1-Q-558	CGTTTCAAGAAAAGGACGGAGC
559	FheL8-R1-Q-559	GGAGATGACGGTGGCGTAG
	Rh-QPCR	
560	ScpRhA-InG-Q-662U17-560	AAAACGGACATGATAAC

561	ScpRhA-InG-Q-724L20-561	CAGAACATCCACAGAAAGAC
562	ScpRhA-InG-Q-1028U17-562	TCCTGGCATCCAAACTG
563	ScpRhA-InG-Q-1112L20-563	CAAAGCCACAGCTACGATAC
564	ScpRhA-InG-Q-1101U20-564	TGGATTGGCTGGTATCGTAG
565	ScpRhA-InG-Q-1224L17-565	ACGGCAACTTCATTATT

Sequence: ScpRhA-ct05.mv Range: 1 to 1410

```
        10         20         30         40         50         60         70         80         90        100
CAGCCCTGTGCAGNTCTTCATCATGACCATAATNGAGATTNCCTTNTTTCCATCNATGAACATTTGGTGGCCAATNTCCTCAAAGCTAATGACGTGGGTG
GTCGGGACACGTCNAGAAGTAGTACTGGTATTANCTCTAANGGAANAAAGGTAGNTACTTGTAAACCACCGGTTANAGGAGTTTCGATTACTGCACCCAC
 Q  P  C  A  X  L  H  H  D  H  N  X  D  X  L  X  S  I  X  E  H  L  V  A  N  X  L  K  A  N  D  V  G>

       110        120        130        140        150        160        170        180        190        200
CATCCATGATCATCCATGCGTATGGAGCCTACTTTGGGCTGGCTGTGGCTCGAGTACTTTACCGACCGAGTTTGAAAAACGGACATGATAACGATGGCTC
GTAGGTACTAGTAGGTACGCATACCTCGGATGAAACCCGACCGACACCGAGCTCATGAAATGGCTGGCTCAAACTTTTTGCCTGTACTATTGCTACCGAG
A  S  M  I  I  H  A  Y  G  A  Y  F  G  L  A  V  A  R  V  L  Y  R  P  S  L  K  N  G  H  D  N  D  G  S>

       210        220        230        240        250        260        270        280        290        300
TGTTTACCACTCTGACCTGTTTGCCATGATTGGAACCGTCTTTCTGTGGATGTTCTGGCCCAGTTTTAACTCGGCCATCGCTGAGCCCGGCTTACCTCAG
ACAAATGGTGAGACTGGACAAACGGTACTAACCTTGGCAGAAAGACACCTACAAGACCGGGTCAAAATTGAGCCGGTAGCGACTCGGGCCGAATGGAGTC
  V  Y  H  S  D  L  F  A  M  I  G  T  V  F  L  W  M  F  W  P  S  F  N  S  A  I  A  E  P  G  L  P  Q>
```

```
      310       320       330       340       350       360       370       380       390       400
CTCACAGCGGTGATCAACACCTACCTCTCCCTGGCTGCCTGCGTGCTCTCTGCCTACGCCATCTCCAGCCTGGTGGAGCACAAAGGAAAACTGGACATGG
GAGTGTCGCCACTAGTTGTGGATGGAGAGGGACCGACGGACGCACGAGAGACGGATGCGGTAGAGGTCGGACCACCTCGTGTTTCCTTTTGACCTGTACC
 L  T  A  V  I  N  T  Y  L  S  L  A  A  C  V  L  S  A  Y  A  I  S  S  L  V  E  H  K  G  K  L  D  M>

      410       420       430       440       450       460       470       480       490       500
TGCACATTCAGAATGCCACCTTGGCCGGTGGCGTTGCTGTGGGAACATGTGCCGACATGGACATCGGGCCATTTGGGGCGATGCTCATTGGATTAGTGGC
ACGTGTAAGTCTTACGGTGGAACCGGCCACCGCAACGACACCCTTGTACACGGCTGTACCTGTAGCCCGGTAAACCCCGCTACGAGTAACCTAATCACCG
V  H  I  Q  N  A  T  L  A  G  G  V  A  V  G  T  C  A  D  M  D  I  G  P  F  G  A  M  L  I  G  L  V  A>

      510       520       530       540       550       560       570       580       590       600
TGGCATCGTGTCGACCCTGGGCTTCAAGTTCCTGACTCCCATCCTGGCATCCAAACTGGGCATCCAGGATACCTGCGGCGTCCACAATCTGCACGGCATG
ACCGTAGCACAGCTGGGACCCGAAGTTCAAGGACTGAGGGTAGGACCGTAGGTTTGACCCGTAGGTCCTATGGACGCCGCAGGTGTTAGACGTGCCGTAC
  G  I  V  S  T  L  G  F  K  F  L  T  P  I  L  A  S  K  L  G  I  Q  D  T  C  G  V  H  N  L  H  G  M>

      610       620       630       640       650       660       670       680       690       700
CCTGGCATCCTGGGTGGATTGGCTGGTATCGTAGCTGTGGCTTTGGGGAAAAAAGAGGGAGGTGATGCTGCCATGCAAGCTGCTGCCTTGGCTTCATCCC
GGACCGTAGGACCCACCTAACCGACCATAGCATCGACACCGAAACCCCTTTTTTCTCCCTCCACTACGACGGTACGTTCGACGACGGAACCGAAGTAGGG
 P  G  I  L  G  G  L  A  G  I  V  A  V  A  L  G  K  K  E  G  G  D  A  A  M  Q  A  A  A  L  A  S  S>
```

```
       710        720        730        740        750        760        770        780        790        800
TCGGGTTCGCTCTGGTTGGAGGGGCTGTTACAGGTTTAATAATGAAGTTGCCGTTCTGGGGTCAGCCTCCAGACCAGAACTGCTATGATGACTCTCTATA
AGCCCAAGCGAGACCAACCTCCCCGACAATGTCCAAATTATTACTTCAACGGCAAGACCCCAGTCGGAGGTCTGGTCTTGACGATACTACTGAGAGATAT
L  G  F  A  L  V  G  G  A  V  T  G  L  I  M  K  L  P  F  W  G  Q  P  P  D  Q  N  C  Y  D  D  S  L  Y>

       810        820        830        840        850        860        870        880        890        900
CTGGGAGGTTCCTGAGGAGGAGAACGAGGAGAACGAGGAGAGCTTGGCTCACGCCGATCACTCAAAGAACAAAGCAGAGGTTTAAATATCTGCTCACCGT
GACCCTCCAAGGACTCCTCCTCTTGCTCCTCTTGCTCCTCTCGAACCGAGTGCGGCTAGTGAGTTTCTTGTTTCGTCTCCAAATTTATAGACGAGTGGCA
  W  E  V  P  E  E  E  N  E  E  N  E  E  S  L  A  H  A  D  H  S  K  N  K  A  E  V  *>

       910        920        930        940        950        960        970        980        990       1000
CAATCCAGCTTGAAAACTATGAAGTAAAGGACTATTAACCTGCTCATATCAATTTGTCAATTATATTAATGTCATTTTTTTTTTTTTTTAAATACATGAT
GTTAGGTCGAACTTTTGATACTTCATTTCCTGATAATTGGACGAGTATAGTTAAACAGTTAATATAATTACAGTAAAAAAAAAAAAAAATTTATGTACTA

      1010       1020       1030       1040       1050       1060       1070       1080       1090       1100
TTGCTGAACTAACCCACACACGTCAATTTTATAAACTGTTTTGTGAGCAAAAAAAAAAAAAAAAAAAACACTGTCATGCCGTTACGTAGCGAAGGGCGA
AACGACTTGATTGGGTGTGTGCAGTTAAAATATTTGACAAAACACTCGTTTTTTTTTTTTTTTTTTTTGTGACAGTACGGCAATGCATCGCTTCCCGCT
```

```
      1110       1120       1130       1140       1150       1160       1170       1180       1190       1200
ATTCTGCAGATATCCATCACACTGGCGGCCGCTCGAGCATGCATCTAGAGGGCCCAATTCGCCCTATAGTGAGTCGTATTAAAATTCACTGGCCGTCGTT
TAAGACGTCTATAGGTAGTGTGACCGCCGGCGAGCTCGTACGTAGATCTCCCGGGTTAAGCGGGATATCACTCAGCATAATTTTAAGTGACCGGCAGCAA

      1210       1220       1230       1240       1250       1260       1270       1280       1290       1300
TTACAACGTCGTGACTGGGAAAACCCTGGCGTTACCCAACTTAATCGCCATGCAGCACATCCCCCTTTCGCAGCTGGCGTATAGCGAAAAGCCCGCACGA
AATGTTGCAGCACTGACCCTTTTGGGACCGCAATGGGTTGAATTAGCGGTACGTCGTGTAGGGGGAAAGCGTCGACCGCATATCGCTTTTCGGGCGTGCT

      1310       1320       1330       1340       1350       1360       1370       1380       1390       1400
TCGCCCTTTCCACAGTTCGCAGCCTATCGTACGGCAGTTAAGGTTTACCCTATAAAGAAAAACCGTTACTTTGTTTGTGAGGACAATGAAATAATGACCC
AGCGGGAAAGGTGTCAAGCGTCGGATAGCATGCCGTCAATTCCAAATGGGATATTTCTTTTTGGCAATGAAACAAACACTCCTGTTACTTTATTACTGGG

      1410
CGGGCCGACG
GCCCGGCTGC
```

Figure 19: Sequence results of RhAg showing coding sequence and 3' untranslated sequence along with amino acid sequence.

Sequence: ScpRhB-ct07 Range: 1 to 1317

```
        10         20         30         40         50         60         70         80         90        100
TGTGTGTGTGAGAGAGGCAGAACTTTTTGAGCTGGATCTTTAAAGCCCCTCTCATCCCCTCAGCCTCTCCTCCTCGTCCCCACCGGGCAGCGAAGCCATG
                                                                                                      M>

       110        120        130        140        150        160        170        180        190        200
ACGGACGCGGCGACCAACATGCGGCTGAAGCTGCCGATCGCCTGCTTCATCCTGCAGATCATCCTCATCATCCTCTTCGGCGTCCTGGTGCAGTACGACC
 T  D  A  A  T  N  M  R  L  K  L  P  I  A  C  F  I  L  Q  I  I  L  I  I  L  F  G  V  L  V  Q  Y  D>

       210        220        230        240        250        260        270        280        290        300
RCGAGACGGACGCCAAGGAATGGCACAACCAGACCCACTCTGACTATGAGAACGACTTCTACTTCCGCTACCCAAGTTTCCAGGAYGTGCACGTGATGAT
X  E  T  D  A  K  E  W  H  N  Q  T  H  S  D  Y  E  N  D  F  Y  F  R  Y  P  S  F  Q  D  V  H  V  M  I>

       310        320        330        340        350        360        370        380        390        400
CTTCATCGGTTTCGGCTTCCTCATGACCTTCCTGCAGCGCTACGGCTTCAGCAGCGTGGGCTTCAACTTCCTGATCGCAGCCTTCTCCATTCAGTGGGCC
  F  I  G  F  G  F  L  M  T  F  L  Q  R  Y  G  F  S  S  V  G  F  N  F  L  I  A  A  F  S  I  Q  W  A>
```

```
      410       420       430       440       450       460       470       480       490       500
ACGCTCATGCAGGGCTTCTTCCACGGCATGCATGGAGGCAAGATCCACATCGGGGTGGAGAGYATGATCAATGCTGATTTCTGCACCGGCGCTGTGCTCA
 T  L  M  Q  G  F  F  H  G  M  H  G  G  K  I  H  I  G  V  E  S  M  I  N  A  D  F  C  T  G  A  V  L>

      510       520       530       540       550       560       570       580       590       600
TCTCGTTCGGAGCCGTTTTGGGTAAAACCAGCCCCGTCCAGCTGCTGGTCATGGCGATATTCGAGGTCACGCTGTTTGCTGTCAATGAGTACGTCCTGCT
I  S  F  G  A  V  L  G  K  T  S  P  V  Q  L  L  V  M  A  I  F  E  V  T  L  F  A  V  N  E  Y  V  L  L>

      610       620       630       640       650       660       670       680       690       700
GTCCGCTCTTGGGGCTAAAGATGCAGGAGGCTCCATGACCATCCACACCTTTGGAGCCTACTTCGGCCTCATGGTGACCCGAGTCCTGTACCGGCCCAAC
  S  A  L  G  A  K  D  A  G  G  S  M  T  I  H  T  F  G  A  Y  F  G  L  M  V  T  R  V  L  Y  R  P  N>

      710       720       730       740       750       760       770       780       790       800
CTGAACAAGAGCAAACACAGGAACAGCTCGGTGTACCATTCTGACCTGTTCGCTATGATCGGCACCATCTACCTGTGGATGTTCTGGCCCAGCTTCAACT
 L  N  K  S  K  H  R  N  S  S  V  Y  H  S  D  L  F  A  M  I  G  T  I  Y  L  W  M  F  W  P  S  F  N>

      810       820       830       840       850       860       870       880       890       900
CTGCYGTCACGGCTCACGGAGATGACCAGCACCGCACGGCCATGAACACCTACTACTCCCTGGCAGCCTGCACGCTGTCCACGTACGGCATGTCCGCCAT
S  A  V  T  A  H  G  D  D  Q  H  R  T  A  M  N  T  Y  Y  S  L  A  A  C  T  L  S  T  Y  G  M  S  A  I>
```

```
      910       920       930       940       950       960       970       980       990      1000
CACGGCTCACGACGGCAAGCTGGACATGGTGAGGTCGCTGCTGATGGTCCGGATCGCCAACTTTAGGGATCAAACTGAGGCAAAAGGATTCCTCCCAGAG
  T   A   H   D   G   K   L   D   M   V   R   S   L   L   M   V   R   I   A   N   F   R   D   Q   T   E   A   K   G   F   L   P   E>

     1010      1020      1030      1040      1050      1060      1070      1080      1090      1100
AGGGATTCTCAGTTTGACAGCTTCTAGTTCCCAATAAATCAGGTTAAAACCAATAGAAATCCAACCTATTTTTAGGCACTTTTCCCAAAAATGTCATACC
 R   D   S   Q   F   D   S   F   *>

     1110      1120      1130      1140      1150      1160      1170      1180      1190      1200
CAAACTAAAAAGGGTGTATATCTGCAACCACTAGGGCTATTTGAATAATGTTGGTGTTATAATAAAAGGTACACATGTGAGATTTAGTTCAGATGTGTAA

     1210      1220      1230      1240      1250      1260      1270      1280      1290      1300
ATATCAGTCTATATGTTACTGGTTAAGAGTAAATGAAGATGACACACAGGAGAAAATTAAAAGGTTTCAGGTCCATCCAAAAAAAAAAAAAAACACTGTC

     1310
ATGCCGTTACGTAGCGT
```

Figure 20: Sequence results of RhBg showing entirety of coding sequence along with 3' and 5' untranslated ends, also showing amino acid sequence.

Sequence: ScpRhC1-ct01.nucl Range: 1 to 886

```
        10        20        30        40        50        60        70        80        90       100
TAAAGTCAGTGCANTCCANCTGCTGGTTTTGACCTTGTTTGGGATCACATTGTTTGCTGTTGAGGAATATATTATCCTAGATATCATACATGCCAGAGAT
ATTTCAGTCACGTNAGGTNGACGACCAAAACTGGAACAAACCCTAGTGTAACAAACGACAACTCCTTATATAATAGGATCTATAGTATGTACGGTCTCTA
  K  V  S  A  X  X  L  L  V  L  T  L  F  G  I  T  L  F  A  V  E  E  Y  I  I  L  D  I  I  H  A  R  D>

       110       120       130       140       150       160       170       180       190       200
GCTGGAGGCTCCATGGTGATCCACACATTTGGAGCTTACTATGGTCTCTCCATCTCGTGGATGCTCTATCGGCCAAACCTGAACCAGAGCGATCGCCTGC
CGACCTCCGAGGTACCACTAGGTGTGTAAACCTCGAATGATACCAGAGAGGTAGAGCACCTACGAGATAGCCGGTTTGGACTTGGTCTCGCTAGCGGACG
 A  G  G  S  M  V  I  H  T  F  G  A  Y  Y  G  L  S  I  S  W  M  L  Y  R  P  N  L  N  Q  S  D  R  L>

       210       220       230       240       250       260       270       280       290       300
AGGGCTCCGTCTATCACTCAGATGTCTTTGCCATGATTGGCACCCTCTTCCTGTGNATGTTCTGGCCCAGCTTCAACTCGGCCATCACAGACCATGGGGA
TCCCGAGGCAGATAGTGAGTCTACAGAAACGGTACTAACCGTGGGAGAAGGACACNTACAAGACCGGGTCGAAGTTGAGCCGGTAGTGTCTGGTACCCCT
Q  G  S  V  Y  H  S  D  V  F  A  M  I  G  T  L  F  L  X  M  F  W  P  S  F  N  S  A  I  T  D  H  G  D>
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      310       320       330       340       350       360       370       380       390       400
CGGGCAGCACCGAGCAGCCCTGAACACCTACCTGGCTTTGGCCTCGACTGTGCTCACTACTGTGGCGCTCTCCAGCCTCTTCCAGAAGCACGGAAAACTA
GCCCGTCGTGGCTCGTCGGGACTTGTGGATGGACCGAAACCGGAGCTGACACGAGTGATGACACCGCGAGAGGTCGGAGAAGGTCTTCGTGCCTTTTGAT
  G   Q   H   R   A   A   L   N   T   Y   L   A   L   A   S   T   V   L   T   T   V   A   L   S   S   L   F   Q   K   H   G   K   L>

      410       420       430       440       450       460       470       480       490       500
NACATGGTCCACATCCAGAACGCCACTCTTGCTGGAGGTGTTGCTGTAGGAACTGCAGCAGAGTTCATGCTGATGCCCTACGGGTCTCTGATCGTAGGAT
NTGTACCAGGTGTAGGTCTTGCGGTGAGAACGACCTCCACAACGACATCCTTGACGTCGTCTCAAGTACGACTACGGGATGCCCAGAGACTAGCATCCTA
 X   M   V   H   I   Q   N   A   T   L   A   G   G   V   A   V   G   T   A   A   E   F   M   L   M   P   Y   G   S   L   I   V   G>

      510       520       530       540       550       560       570       580       590       600
TCTGCTGTGGCATCATCTCCACGCTGGGATATGTCTACCTCACGCCTTTCATGGAGAAGCACCTGAAGATCCAGGACACGTGTGGAATCCATAACCTGCA
AGACGACACCGTAGTAGAGGTGCGACCCTATACAGATGGAGTGCGGAAAGTACCTCTTCGTGGACTTCTAGGTCCTGTGCACACCTTAGGTATTGGACGT
F   C   C   G   I   I   S   T   L   G   Y   V   Y   L   T   P   F   M   E   K   H   L   K   I   Q   D   T   C   G   I   H   N   L   H>

      610       620       630       640       650       660       670       680       690       700
TGCCATGCCCGGCGTCATAGGTGGCATCGTGGGAGCCATTACTGCCGCGTCTGCAACAGAGTCTGTTTATGGTATTGAGGGGCTCANGAACACCTTTGAC
ACGGTACGGGCCGCAGTATCCACCGTAGCACCCTCGGTAATGACGGCGCAGACGTTGTCTCAGACAAATACCATAACTCCCCGAGTNCTTGTGGAAACTG
  A   M   P   G   V   I   G   G   I   V   G   A   I   T   A   A   S   A   T   E   S   V   Y   G   I   E   G   L   X   N   T   F   D>
```

```
       710       720       730       740       750       760       770       780       790       800
TTTGANGGTGATTTCAAAGACATGTTACCCACACGCCAGGGTGGTCTCCAGGCTGCGGGCCTTTGTGTGGCCATCTGCTTCGGTGNGGGTGNAGGTATCC
AAACTNCCACTAAAGTTTCTGTACAATGGGTGTGCGGTCCCACCAGAGGTCCGACGCCCGGAAACACACCGGTAGACGAAGCCACNCCCACNTCCATAGG
 F  X  G  D  F  K  D  M  L  P  T  R  Q  G  G  L  Q  A  A  G  L  C  V  A  I  C  F  G  X  G  X  G  I>

       810       820       830       840       850       860       870       880
TTGTCGGGTGNATTTTNAGANTACCTATCTGGGGNAATCCTGCAAANGAAANTNGTTTTGATGANGAACCCTACTGGGAACTCCCT
AACAGCCCACNTAAAANTCTNATGGATAGACCCCNTTAGGACGTTTNCTTTNANCAAAACTACTNCTTGGGATGACCCTTGAGGGA
L  V  G  X  I  X  R  X  P  I  W  G  N  P  A  X  E  X  X  F  D  X  E  P  Y  W  E  L>
```

Figure 21: Sequence results of RhC1g showing majority of coding sequence along with the amino acid sequence.

Sequence: ScpRhC2-ct05f Range: 1 to 1860

```
        10         20         30         40         50         60         70         80         90        100
ACATCGAAAATGACTTCTACTTCAGATATCCAAGCTTCCAGGACGTCCATGTCATGATCTTTGTTGGATTTGGTTTCCTCATGACCTTCCTGAAACGCTA
TGTAGCTTTTACTGAAGATGAAGTCTATAGGTTCGAAGGTCCTGCAGGTACAGTACTAGAAACAACCTAAACCAAAGGAGTACTGGAAGGACTTTGCGAT
   I   E   N   D   F   Y   F   R   Y   P   S   F   Q   D   V   H   V   M   I   F   V   G   F   G   F   L   M   T   F   L   K   R   Y>

       110        120        130        140        150        160        170        180        190        200
CAGCTTTGGTGCTGTGGGCTTCAACTTCCTGATCGCTTCCTTTGGTGTGCAGTGGGCTCTTCTCATGCAAGGCTGGTTCCACGCGCTCGACCCCAATACC
GTCGAAACCACGACACCCGAAGTTGAAGGACTAGCGAAGGAAACCACACGTCACCCGAGAAGAGTACGTTCCGACCAAGGTGCGCGAGCTGGGGTTATGG
  S   F   G   A   V   G   F   N   F   L   I   A   S   F   G   V   Q   W   A   L   L   M   Q   G   W   F   H   A   L   D   P   N   T>

       210        220        230        240        250        260        270        280        290        300
GGAAAGATCTCTATTGGAGTAGAGAGTCTGATCAACGCTGACTTCTGCGTTGCCGGCTGTCTGATTGCCTATGGTGCCCTCCTGGGAAAAGTAAGCCCTG
CCTTTCTAGAGATAACCTCATCTCTCAGACTAGTTGCGACTGAAGACGCAACGGCCGACAGACTAACGGATACCACGGGAGGACCCTTTTCATTCGGGAC
 G   K   I   S   I   G   V   E   S   L   I   N   A   D   F   C   V   A   G   C   L   I   A   Y   G   A   L   L   G   K   V   S   P>
```

```
      310       320       330       340       350       360       370       380       390       400
TCCAGCTGATGGTTGTCACCTTGTTTGGCGTCACACTGTTTGCTGTGGAGGAATACATCATCCTCGACCTCCTTCATTGCAGAGATGCTGGTGGCTCAAT
AGGTCGACTACCAACAGTGGAACAAACCGCAGTGTGACAAACGACACCTCCTTATGTAGTAGGAGCTGGAGGAAGTAACGTCTCTACGACCACCGAGTTA
V  Q  L  M  V  V  T  L  F  G  V  T  L  F  A  V  E  E  Y  I  I  L  D  L  L  H  C  R  D  A  G  G  S  M>

      410       420       430       440       450       460       470       480       490       500
GGTCATTCACGCTTTTGGAGGGTACTACGGTTTGGCCATCTCTTGGGTTCTCTACCGACCAAACCTAAACCAAAGCAGACGCCTCAATGGATCTGTCTAC
CCAGTAAGTGCGAAAACCTCCCATGATGCCAAACCGGTAGAGAACCCAAGAGATGGCTGGTTTGGATTTGGTTTCGTCTGCGGAGTTACCTAGACAGATG
  V  I  H  A  F  G  G  Y  Y  G  L  A  I  S  W  V  L  Y  R  P  N  L  N  Q  S  R  R  L  N  G  S  V  Y>

      510       520       530       540       550       560       570       580       590       600
CACTCTGATATGTTTGCTATGATTGGTACACTGTTTCTGTGGATGTTCTGGCCCAGTTTCAACTCGGCCATCACAGACCACGGCTCTGGACAGCACCGAG
GTGAGACTATACAAACGATACTAACCATGTGACAAAGACACCTACAAGACCGGGTCAAAGTTGAGCCGGTAGTGTCTGGTGCCGAGACCTGTCGTGGCTC
 H  S  D  M  F  A  M  I  G  T  L  F  L  W  M  F  W  P  S  F  N  S  A  I  T  D  H  G  S  G  Q  H  R>

      610       620       630       640       650       660       670       680       690       700
CAGCCATCAACACGTATATCGCTCTCGCCTCATCTGTGCTCACCACGGTGGCCATCTCCAGCATGTCTGAAAAAAGAGGAAAACTGGACATGGTGCACAT
GTCGGTAGTTGTGCATATAGCGAGAGCGGAGTAGACACGAGTGGTGCCACCGGTAGAGGTCGTACAGACTTTTTTCTCCTTTTGACCTGTACCACGTGTA
A  A  I  N  T  Y  I  A  L  A  S  S  V  L  T  T  V  A  I  S  S  M  S  E  K  R  G  K  L  D  M  V  H  I>
```

```
      710       720       730       740       750       760       770       780       790       800
CCAGAATGCTACTCTGGCAGGTGGTGTTGCCATGGGAACAGCAGCAGAGTTCATGATCACTCCTTATGGTTCACTAATCGTGGGTTTCAGCTGTGGCATC
GGTCTTACGATGAGACCGTCCACCACAACGGTACCCTTGTCGTCGTCTCAAGTACTAGTGAGGAATACCAAGTGATTAGCACCCAAAGTCGACACCGTAG
  Q   N   A   T   L   A   G   G   V   A   M   G   T   A   A   E   F   M   I   T   P   Y   G   S   L   I   V   G   F   S   C   G   I>

      810       820       830       840       850       860       870       880       890       900
ATCTCCACCTTTGGCTACCTGTATGTCACGCCCTTCTTGGAGAAATACCTAAAGCTCCAGGATACATGTGGTGTCCACAATCTGCATGCTGTTCCAGGGA
TAGAGGTGGAAACCGATGGACATACAGTGCGGGAAGAACCTCTTTATGGATTTCGAGGTCCTATGTACACCACAGGTGTTAGACGTACGACAAGGTCCCT
 I   S   T   F   G   Y   L   Y   V   T   P   F   L   E   K   Y   L   K   L   Q   D   T   C   G   V   H   N   L   H   A   V   P   G>

      910       920       930       940       950       960       970       980       990      1000
TGCTCGGCGGCTTCATAGGTGCCATTGTTGCTGCATCTGCCACTGAAGAGGTGTATAGCAGGGAGGGGTTGATCGAGACGTTTGACTTTGAAGGTGATTT
ACGAGCCGCCGAAGTATCCACGGTAACAACGACGTAGACGGTGACTTCTCCACATATCGTCCCTCCCCAACTAGCTCTGCAAACTGAAACTTCCACTAAA
M   L   G   G   F   I   G   A   I   V   A   A   S   A   T   E   E   V   Y   S   R   E   G   L   I   E   T   F   D   F   E   G   D   F>

     1010      1020      1030      1040      1050      1060      1070      1080      1090      1100
TGCAGACAGAACTGTATTAACCCAGGGAGGCTTCCAGGCTGCTGGCACATGTGTGGCTATTGCATTTGGAGTTGTTGGAGGAGCAGGTGTTGGTCTCGTT
ACGTCTGTCTTGACATAATTGGGTCCCTCCGAAGGTCCGACGACCGTGTACACACCGATAACGTAAACCTCAACAACCTCCTCGTCCACAACCAGAGCAA
  A   D   R   T   V   L   T   Q   G   G   F   Q   A   A   G   T   C   V   A   I   A   F   G   V   V   G   G   A   G   V   G   L   V>
```

1110 1120 1130 1140 1150 1160 1170 1180 1190 1200

CTGAGGTTGCCTATCTGGGGTGACCCTGCTGATGACAACTGCTTTGATGATGAAGTTTACTGGGAGGTTCCTGAGGATGAGGAGAGCATCCCTCCTGTTT

GACTCCAACGGATAGACCCCACTGGGACGACTACTGTTGACGAAACTACTACTTCAAATGACCCTCCAAGGACTCCTACTCCTCTCGTAGGGAGGACAAA

L R L P I W G D P A D D N C F D D E V Y W E V P E D E E S I P P V>

1210 1220 1230 1240 1250 1260 1270 1280 1290 1300

TGGAGTACAACAACCACATGATACACAAGCACCAAGACATATCCGAGTCAAACTTCTCTGTGGAGCAAAGTTAGAAACATTAAGGCGCTCATATAACTTT

ACCTCATGTTGTTGGTGTACTATGTGTTCGTGGTTCTGTATAGGCTCAGTTTGAAGAGACACCTCGTTTCAATCTTTGTAATTCCGCGAGTATATTGAAA

L E Y N N H M I H K H Q D I S E S N F S V E Q S *>

1310 1320 1330 1340 1350 1360 1370 1380 1390 1400

GAACTTGCTACAGATGTGAAATGACAGGAGCCACCTGAAACAACGTGCAGTTCTACAAAGAAAGGAACACACCGGTCCTCTGCCACTCTTGTCTCGCACC

CTTGAACGATGTCTACACTTTACTGTCCTCGGTGGACTTTGTTGCACGTCAAGATGTTTCTTTCCTTGTGTGGCCAGGAGACGGTGAGAACAGAGCGTGG

1410 1420 1430 1440 1450 1460 1470 1480 1490 1500

TCTTACACCTGACAGATCTGTGAATGATTGGAAGCTTAAGGGTATGATGGTGGTAATCAAATGTCTCATGAATGGAAAAATGATTCACAGTTAAAACTGA

AGAATGTGGACTGTCTAGACACTTACTAACCTTCGAATTCCCATACTACCACCATTAGTTTACAGAGTACTTACCTTTTTACTAAGTGTCAATTTTGACT

```
      1510       1520       1530       1540       1550       1560       1570       1580       1590       1600
TTTTATTTTCGATGAAATCTTTCACTACATTTACTTTCTCTTTCATTTTGGATGAAACTTCATATGATTTGCAGCTGCTTAGTGACTGTAAAGTATCAGG
AAAATAAAAGCTACTTTAGAAAGTGATGTAAATGAAAGAGAAAGTAAAACCTACTTTGAAGTATACTAAACGTCGACGAATCACTGACATTTCATAGTCC

      1610       1620       1630       1640       1650       1660       1670       1680       1690       1700
TTCTGAATCCAAGCAGCACGATGGGAAGTCAAATAACACTTAGTTAATGTTGTGTAAAATGATTATAATGTAAAGAAGAACTGGGAAAAATGTGTACATC
AAGACTTAGGTTCGTCGTGCTACCCTTCAGTTTATTGTGAATCAATTACAACACATTTTACTAATATTACATTTCTTCTTGACCCTTTTTACACATGTAG

      1710       1720       1730       1740       1750       1760       1770       1780       1790       1800
AGTTATTGTTGTCTTTAATAATTATAGACAGTTTTAAGCTTTTATGTCATGTCTTTTAAAGTTGATGAATATTGNCATTCATAATGNTTGCCATTTTANT
TCAATAACAACAGAAATTATTAATATCTGTCAAAATTCGAAAATACAGTACAGAAAATTTCAACTACTTATAACNGTAAGTATTACNAACGGTAAAATNA

      1810       1820       1830       1840       1850       1860
TCTCAATAATGNANACTGNTGACATACGGTATGTAAATGAAGGNGTTTANNAAANAANCA
AGAGTTATTACNTNTGACNACTGTATGCCATACATTTACTTCCNCAAATNNTTTNTTNGT
```

Figure 22: Sequence results of RhC2g showing the coding sequence along with 3' untranslated end, also showing the amino acid sequence.

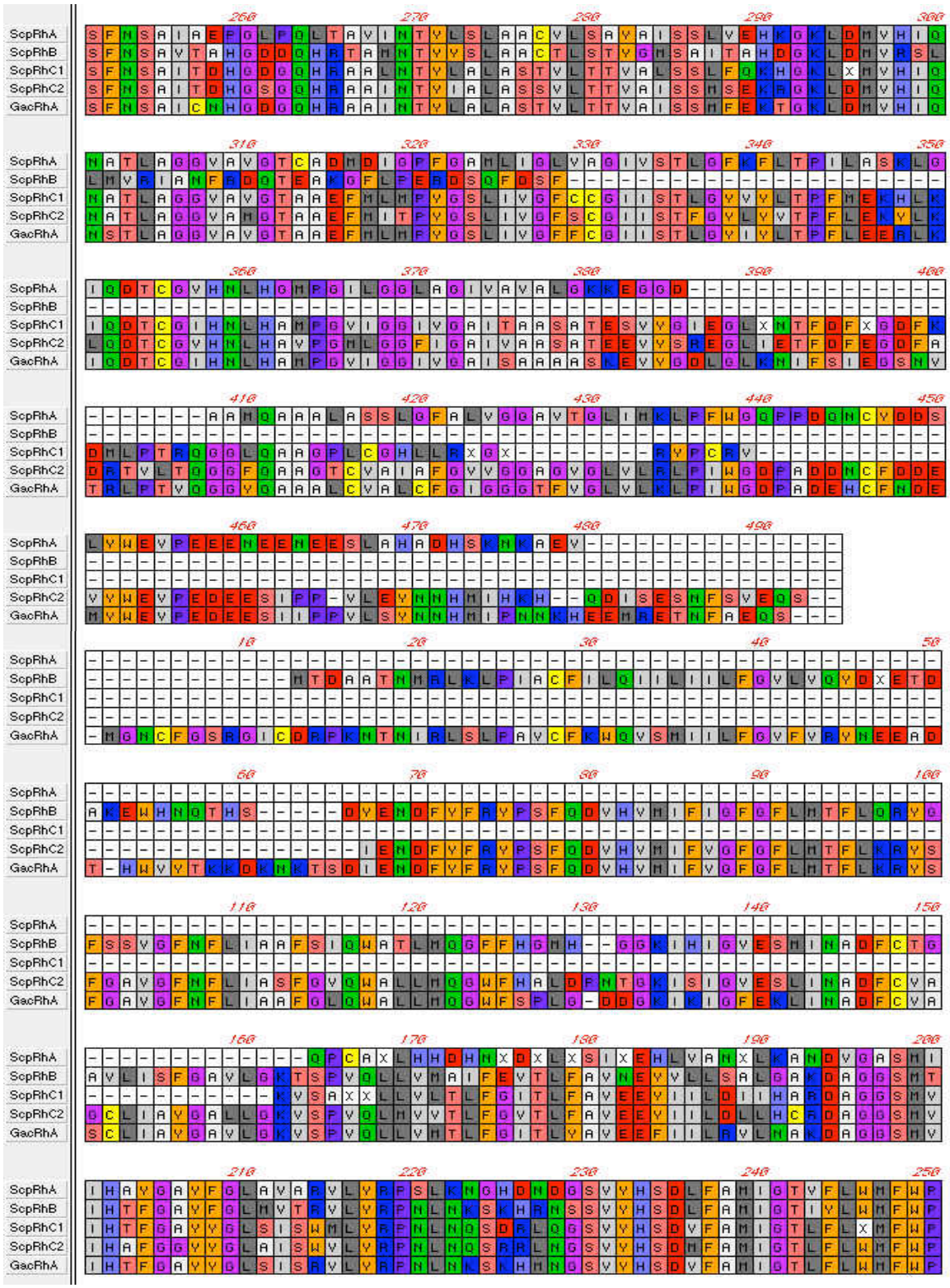

Figure 23: Protein homology of the four Rh orthologues of the sculpin with RhAg from the stickleback as a baseline reference protein.

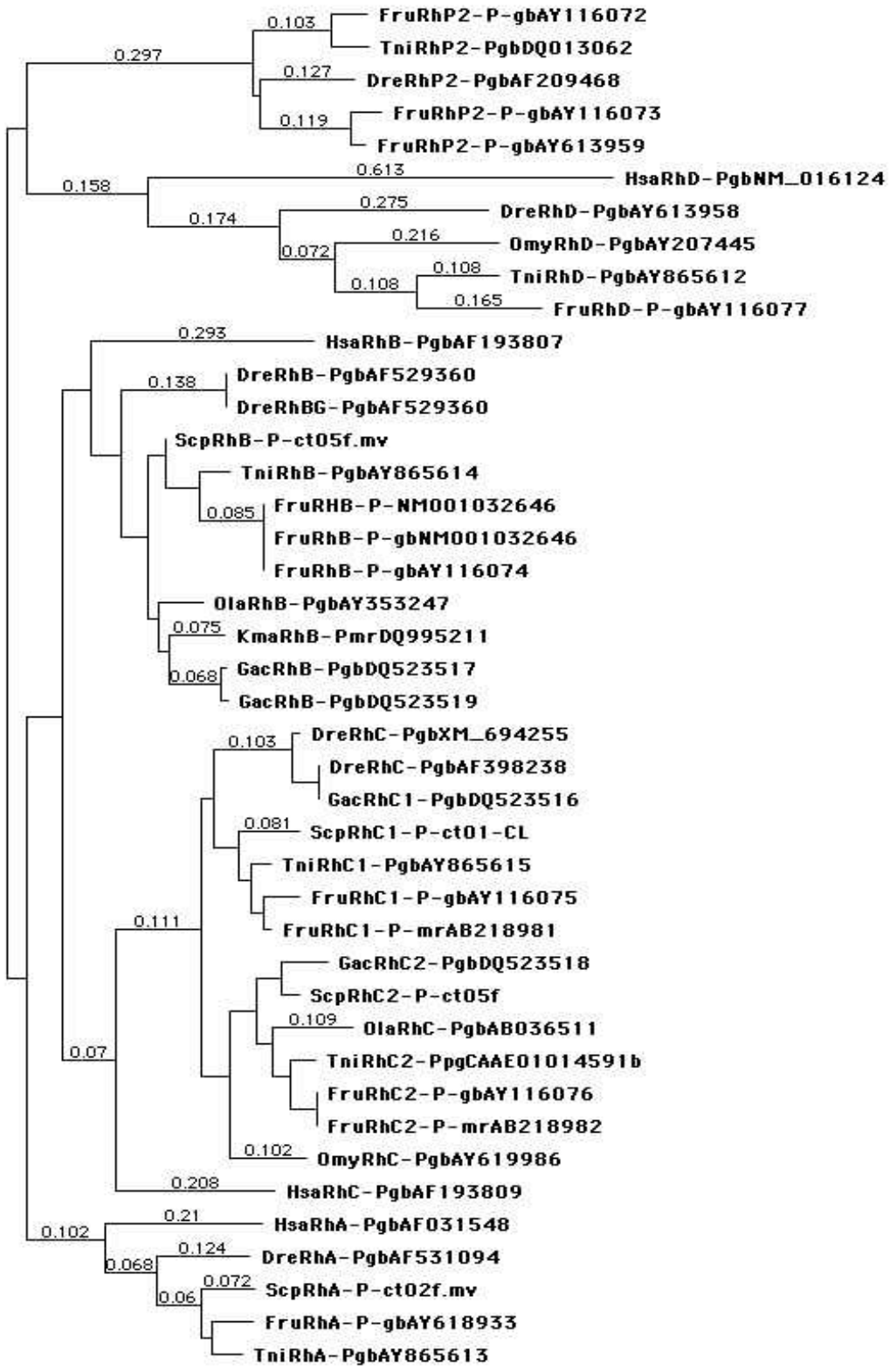

Figure 24: Nearest neighbor joining, Poisson-corrected phylogeny of fish Rh proteins with Human reference point. Genebank accession numbers shown.

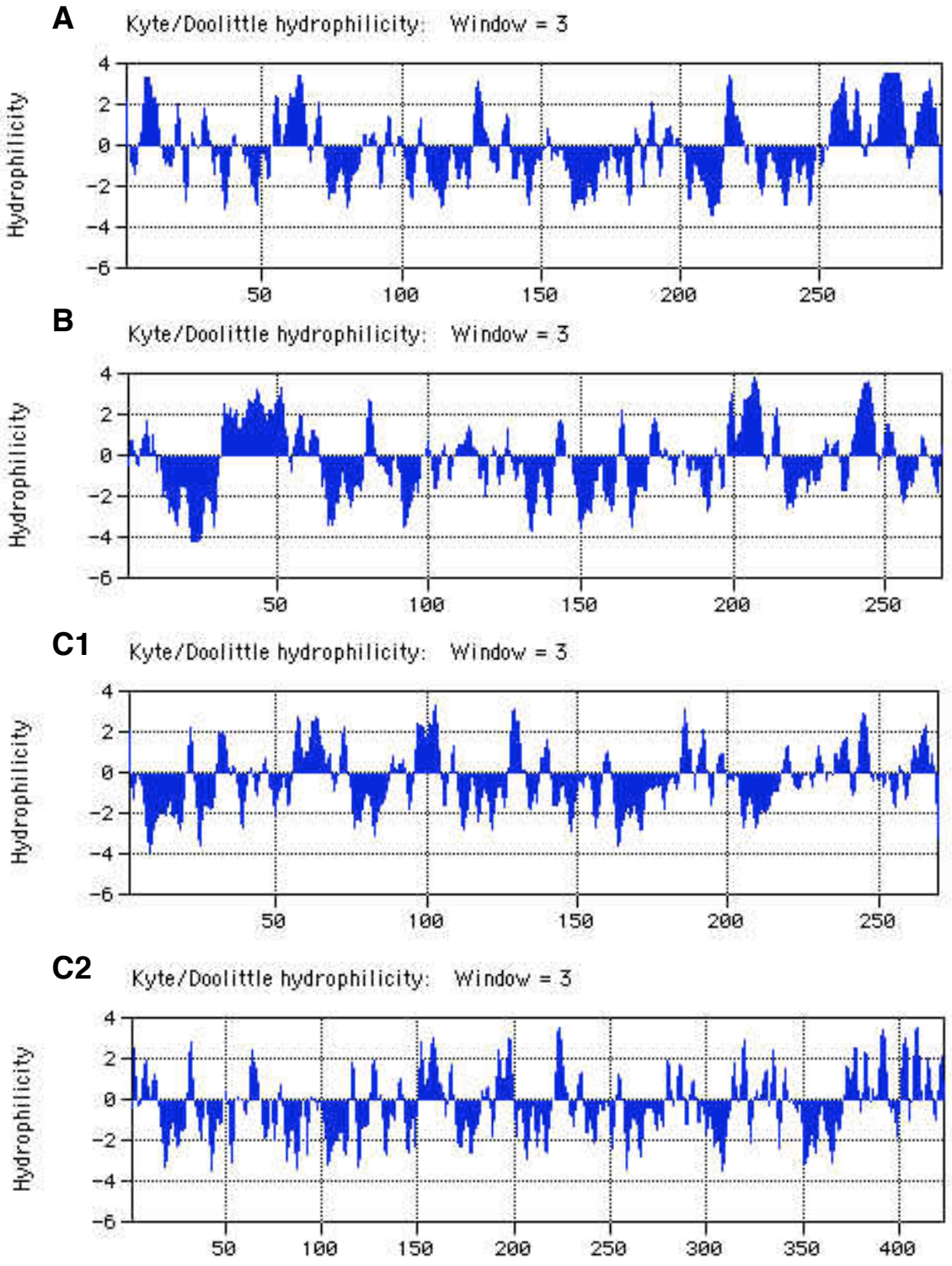

Figure 25: Kyte/Doolittle hydrophilicity diagrams for RhAg, RhBg, RhC1g, and RhC2g showing the hydrophilic regions of the protein sequence and thus the most likely regions for membrane spanning for the protein.