AMMONIA TRANSPORTATION VIA RHESUS GLYCOPROTEINS IN LONHORNED SCULPIN (*Myoxocephalus octodecemspinosus)* AND SPINY DOGFISH (*Squalus acanthias*).

A Thesis
By
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Abstract

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IN LONHORNED SCULPIN (*Myoxocephalus octodecemspinosus)*
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(August 2016)

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The maintenance of homeostatic equilibrium is one of the main challenges facing marine fishes. Ammonia is a toxic byproduct of protein digestion and therefore must be regulated and excreted. Since the discovery of Rhesus glycoproteins roles in ammonia excretion in 2007, many species of fish have been studied (Nakada et al. 2007b). Rhesus glycoprotein orthologs, Rhag, Rhbg, Rhcg1, and Rhcg2, have been localized to the gills of *Takifugu rubripes,* the puffer fish, while Rhp2 has been localized to the kidney of the marine elasmobranch *Triakis scyllium*, the banded houndshark. This study looked at the marine elasmobranch *Squalus acanthias*, spiny dogfish, and the marine teleost *Myoxocephalus octodecemspinous*, longhorned sculpin. We hypothesize that both the spiny dogfish and the longhorned sculpin utilize Rhesus glycoprotein orthologs in the excretion and movement of ammonia and its byproducts. To date we have cloned a partial fragment of Rhp2 (1040bp) from the spiny dogfish and partial fragments of Rhag (677bp), Rhbg (661bp), and Rhcg1 (610bp), from the longhorned sculpin. The open reading frame from Rhcg2 (1097bp) was also isolated and showed high similarity to other teleost Rhcg2. In this study, immunohistochemistry was used to localize Rhesus glycoprotein orthologs and various ion transporters to the kidney and gills of the spiny

dogfish, and the gills of the longhorned sculpin. Western blot and tissue distribution confirmed expression of Rhesus glycoproteins to the epithelial tissues of the longhorned sculpin.

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Dedication

To my family and friends, whose unconditional love and support made all of this possible.

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Introduction

Homeostasis is key to the survival of all vertebrates and refers to the overall balance between the animal's intracellular space and its external environment. (Withers, 1998). Since the evolution of jawed fishes approximately 443 million years ago, fishes have faced unique challenges associated maintaining internal osmotic and ionic homeostatis (Giles et al. 2015). The fundamental challenge to all fishes with the exclusion of hagfish, is maintaining ionic balance and osmotic homeostasis in environments that pose differences in salinity. Most fishes are restricted to a single environmental niche (stenohaline), but can inhabit aqueous environments that range from the dilute environment of freshwater (0 mOsmol $kg^{-1}l$) to the full salinity of marine environments (1000 mOsmol kg^{-1}) (Edwards and Marshall 2013). Fishes have developed three strategies to maintain homeostasis: osmoconformity, where the fish maintain a similar extracellular fluid (ECF) concentration to that of the environment in which they inhabit. Hagfish are the only marine osmoconformers and there is no evidence of an osmoconforming freshwater fish (Currie and Edwards 2010; Whittamore 2012; Edwards et al. 2015). Hypo-osmoregulation where fishes maintain their ECF slightly lower than that of their marine environment. Finally hyper-osmoregulation, where fishes maintain their ECF slightly higher than that of their dilute freshwater environment (Currie and Edwards 2010).

Elasmobranchs

Marine elasmobranchs are osmconformers and ionoregulators, which is atypical in the marine environment (Whittamore, 2012). These organisms are also ureotelic meaning they retain excess nitrogen and convert the nitrogenous waste product ammonia

into the less toxic form urea (Wood et al 1995; Wilkie 2002). The physiological parameters of marine elasmobranchs mean that they maintain an internal osmolality slightly higher, yet similar to, the osmolality to their external environment (Yancey and Somero 1979; Wood et al. 1995; Anderson et al. 2012; Nawata et al. 2015a) (Fig 1 B). This is achieved by the retention of the regulatory osmolyte urea and the metabolite Trimethylamine Oxide (TMAO) (Beyenbach 2004; Edwards and Marshall, 2013; Nawata et al. 2015a).

Elasmobranchs retain ammonia through various mechanisms, including the gills, that are specialized to preferentially conserve ammonia instead of excrete it, the nephrons in the kidneys are adapted to remove ammonia from the blood stream, and the repurposing of dietary ammonia into urea (CH_4ON_2) (Fig 1 B) (Wood et al. 1995; Wood et al. 2002; Nawata et al. 2015b). The retention of urea aids in the osmotic homeostasis between the intercellular space and the surrounding waters (Wood et al.1995; Anderson et al. 2012; Nawata et al. 2015b). The abundance of urea in the marine elasmobranchs results in the animal being hyperosmotic to their saltwater environment, thus allowing them to conserve water loss and avoid excess salt intake (Li et al. 2013; Hyodo et al. 2014; Nawata et al. 2015a). However, in order to ameliorate the deleterious effect of urea on proteins, urea must be balanced in a 1:2 ratio with the nonelectrolyte TMAO (Seibel and Walsh, 2002; Treberg et al. 2006). TMAO is utilized as an osmolyte in osmoregulation as well as to balance the ability of urea to degrade macromolecules, including proteins (Wood et al. 1995; Kajimura et al. 2006; Walsh et al. 2006).

Elasmobrachs and the Ornithine-Urea cycle

Marine elasmobranchs utilize the Ornithine-Urea Cycle (OUC) to create the osmolyte urea. The OUC is an energetically costly multi-step process that occurs mainly in the kidneys and the mitochondria of liver cells (Kajimura et al. 2006). Urea is produced from ammonia (NH_3) created during protein digestion (Schmidt-Nielson, 1964). Ammonia is sequestered from filtrate in the kidneys and transported through the blood stream into the liver (Wood et al. 1995; Anderson et al. 2012; Nawata et al. 2015a). The unique nephrons of the shark kidney facilitate almost 90% ammonia retention from filtrate in the kidneys (Hyodo et al. 2014). Once in the liver, it is phosphorylated into the carbomoyal phosphate by a rate limiting enzyme carbomoyal phosphate synthetase (Kajimura et al. 2006) (Fig 2). The OUC produces multiple intermediates before the end product urea is created, including citrulline, L-argenine and argininosuccinate (Fig 2). The production of urea is both adenosine triphosphate (ATP)-dependent and metabolically costly, utilizing 5mol of ATP per 1mol of urea produced (Anderson 2001).

This mechanism is not unique to elasmobranchs; some teleost fishes have retained the ability to convert ammonia into less toxic forms such as urea or uric acid (Yancey and Somero 1979; Wood et al. 1995; Wilkie 2002; Nawata et al 2015a). In the special case of the Gulf toadfish (*Opsanus beta)*, this species uses urea as a mechanism to cloak itself against predation (Barimo and Walsh 2006). Due to the energetic cost of producing urea most teleost fishes utilize a more simplistic mechanism to deal with nitrogenous waste.

Teleost Fishes

In contrast to marine elasmobranchs that osmoregulate using urea and TMAO, marine teleost fishes maintain internal homeostasis through ionoregulation and osmoregulation (Evans et al. 2005). The ionic gradient between teleost fishes and their marine ecosystems results in a constant loss of water, coupled with a constant influx of ions (Evans et al. 2005). Due to these physiological conditions, teleost fishes must actively excrete various ions including nitrogenous wastes as well as consume seawater to combat osmotic water loss (Hwang et al. 2011) (Fig 1 A).

Most marine teleost fishes are ammoniotelic, in that their nitrogenous waste is in the form of ammonia, which can be excreted directly across the gill epithelium into the surrounding water (Evans et al. 2005; Nawata et al. 2010; Zhang et al. 2015). The direct excretion of ammonia is restricted to aquatic organisms, as it requires copious amounts of water in which it can disseminate (Evans 2008). The few exceptions to this generalization are the fish species that can enter terrestrial environments for short periods of time; including the mangrove killifish, which can volatilize their waste, as well as the mudskipper, and climbing perch, which utilize active ammonium transport in times of high environmental ammonia (Hung et al. 2007; Clifford et al. 2015).

Embryonic teleost fishes, have demonstrated both ureotelic and ammoniotelic stages throughout their development (LeMoine and Walsh 2013). Immediately after fertilization embryos excrete ~85% of the nitrogenous waste as urea, but 2-3 days post fertilization the embryos shed their chorion and ~75% of their waste is excreted as ammonia (Braun et al. 2009a). Zerbrafish embryos have functioning ornithine-urea cycles, but after hatching it is down regulated and the organism switches to ammoniotelia

and excretes ammonia directly into the environment (Braun et al. 2009a). During embryonic development fish lack functioning gills for excretion of ammonia, so they must detoxify the ammonia by converting it to the less toxic urea (LeMoine and Walsh 2013). This pattern of functioning ornithine-urea cycles during embryonic stages may also be prevalent in other species as it prevents the toxic accumulation of ammonia during development (LeMoine and Walsh 2013).

Ammonia

Total ammonia, both the gaseous form NH_3 and the ionic form NH_4^+, are formed through the degradation of amino acids during protein metabolism (Wood et al. 1995; Houlihan et al. 1995; Braun et al 2009). Ammonia is formed after amino acids are released during protein digestion in the intestine through the removal of carbon during protein catabolism (Evans et al. 2005; Ip and Chew 2010; Wright and Wood 2012). The deamination of the α-amino group on the amino acid results in a carbon byproduct that is used as a source of dietary carbon and glucose production (Ip and Chew 2010). Ammonia is formed mainly in the kidneys, with accessory formation in the muscles, and intestines of vertebrata (Evans 2008; Weiner and Verlander, 2014).

In both marine teleost fishes and elasmobranchs, nitrogenous wastes are a concern for not only osmotic homeostasis, but also the overall health of the fishes. Ammonia presents an issue for all vertebrate organisms as it is a toxic nitrogenous byproduct and therefore fishes must develop mechanisms to excrete it (Treberg et al 2006; Ip and Chew 2010; Nawata et al 2015a). High ammonia levels can have extremly deleterious effects on the body including decreased growth rates (Weiner and Verlander 2014; Sinha et al.

2015), hemolytic anemia (Hung et al. 2008), hepatic encephalopathy in mammals (Lemberg and Fernández 2009), and eventually can lead to death (Wilkie 2002). Acute ammonia toxicity affects fishes through disruptions to the central nervous system (Randall and Tsui, 2002; Albrecht 2007). The increased levels of ammonia lead to the depolarization of the neurons in the brain, which leads to a cascade of chain reactions resulting in cell death and convulsions (Randall and Tsui 2002). This ammonia toxicity can be triggered during exposure to high environmental ammonia, stress events, or swimming, although if exposed to high environmental ammonia, fishes will stop feeding before they produce toxic internal ammonia levels (Randall and Tsui 2002). Marine fishes must have a method to excrete ammonia so that they can avoid the adverse effects associated with high internal ammonia concentrations.

Ion Transporting Tissues

Marine fishes utilize specialized organs in the regulation of ion and osmotic homeostasis. These organs include the gill, kidney, and intestines. As the majority of waste and ion movement occurs at the gills, with some secondary movement occurring at the intestines and kidneys, the following sections will review each of these organs specifically (Schmidt-Nielsen 1964).

Gill

The gills of fishes have long been studied as an excretory organ; not only do they possess an exceptionally large surface area, but also a very high blood flow (Evans and Cameron 1986) (Fig 3). These anatomical considerations, combined with small diffusion

distances from the blood to the surrounding waters, make them an ideal organ for both gas and ion exchange (Evans et al. 2005). These same gill characteristics are ideal for the excretion of ions, specifically ammonia, into the environment with little energy requirements.

The gill is composed of a few major organizational sections. The main structural support for the teleost gill is the bony gill arch, while in elasmobranchs the support comes from the interbranchial septum (Edwards 2000). The next organization level includes both the filaments, which are the main branches that extend from the gill arch, and the lamellae, which extend off from the filament (Edwards 2000; Evans et al. 2005). Each of these sections plays a major role in the gill's ability to be an excretory organ (Fig 3).

The gill arches are the main structural support for the gill; they have blood vessels that run through them to the gill filaments, facilitating blood flow and transportation of ions and waste (Evans et al. 2005). The filaments and lamellae are open to the waters and are used in waste removal and ion exchange (Wilson and Laurent 2002). Water is taken in through the mouth and moves across the gills, both bringing oxygen and ions to the fish, as well as washing away any free ions or gases (Fig 3).

The filament is the main site of ion exchange across the epithelium, due to the vascularization of the filament (Edwards 2000). The filament and lamellae are composed of specific types of cells. On the filament there are two major cell types, mitochondrion rich cells involved in ion transport, and pavement cells, which are theorized to play a role in gas exchange (Wilson and Laurent, 2002; Evans et al. 2005). The mitochondrion rich cells, MRC, have also been called ionocytes, or chloride cells due to the ion transporters localized in their cells (Hill et al. 2008). Ionocytes have an abundance of mitochondria

that produce the ATP necessary to activate ion secretion pathways. The MRC's also have a specialized apical crypt where specific ion transporters and proteins are localized (Claiborne et al. 2002; Guffey et al. 2015). Approximately 90% of the epithelial surface areas of the gills are comprised of pavement cells (Evans et al. 2005)

In the lamellae, as you move distally from the filament, there are very few ionocytes (Evans et al. 2005). The lamellae is composed of a single cell layer, mainly pavement cells and pillar cells, which facilitate gas exchange due to their thin walled squamous cell structure (Wilson and Laurent, 2002; Nakada et al. 2007b). The lamellae are the main site of gas exchange in the fish gill; they are highly vascularized and are the main way for gases to be transferred from the blood stream to the surrounding waters, and vice versa (Evans et al 2005).

The branchial epithelium is also comprised of three more cell types, accessory cells, mucous cells, and neuroepithelial cells (Edwards 2000) (Fig 4). Accessory cells closely associate with MRC's and are normally observed in marine teleost fishes (Laurent and Dunel, 1980). Accessory cells form leaky junctions with MRC's that allow ions to leak across the short junction formed between the two cell types (Laurent and Dunel, 1980; Evans et al. 2005). Mucous cells are localized to the edge of the filament and excrete mucous onto the apical edge of the gill (Laurent and Dunel, 1980; Edwards 2000). Neuroepethelial cells are localized throughout the filament and lamellae on the efferent aspect (i.e., facing the flow of water) of the gill (Zachar and Jonz 2012). Neuroepethelial cells are theorized to be involved in O_2 sensing as well as function in neural stimulus transmission (Zachar and Jonz 2012).

Kidney

The kidneys of marine teleosts and elasmobranchs are secondary sites of ion exchange and ammonia movement (Edwards and Marshall 2013). Vertebrate kidneys are comprised of thousands, up to millions, of nephrons (Schmidt-Nielsen 1964) (Fig 5). The nephron can be broken down in two major groups by location in the kidney, the sections present in the cortex, and the sections present in the medulla (Schmidt-Nielsen, 1997) (Fig 5). The outer layer, the cortex, contains the Malpighian body, the Bowman's capsule, the proximal convoluted tubule, and the distal convoluted tubule (Hill et al. 2008). The inner layer of the medulla contains both Henle's loop, and the collecting duct (Schmidt-Nielsen, 1997). Teleost and elasmobranch kidney morphology differs based on the presence, or reduced presence, of glomeruli (Edwards and Marshall, 2013). Teleost fishes may have fully functioning glomeruli, but this is only common to euryhaline species; sternomarine species normally lack the glomuerli, or it has lost its function in filtration (Beyenbach, 2004; Evans and Claiborne, 2006; Edwards and Marshall, 2013). Independent of the type of kidney fishes have, all kidneys are responsible for filtration of the divalent ions, Mg^{2+}, Ca^{2+}, and SO_4^{2-} as well as the electrolytes and osmolytes Na and Cl. (Beyenbach, 2004).

Intestine

In addition to the gill and the kidney, the intestine is also a site of osmotic and ionic regulation. In marine teleost fishes, the intestine is responsible for the uptake of Na^+, Cl^-, and H_2O (Evans 2008; Whittamore 2011). Marine teleost fishes drink the surrounding water to combat osmotic water loss. The gastrointestinal tract and intestines

absorb H_2O from the saltwater and also act to remove Na^+ and Cl^- (Evans et al. 2005; Evans 2008; Edwards and Marshall, 2013). The posterior end of the intestine secretes HCO_3^- causing the precipitation of the divalent ions Mg^{2+} and Ca^{2+} (Grosell 2006; Edwards and Marshall, 2013).

Mechanisms of Ammonia Excretion

Historical Theories

Ammonia excretion has been studied in fishes for the last 80 years, although there was little consensus regarding the specific mechanisms involved (Evans 1982; Mallery 1983). The original hypothetical models (Fig 7), linked the excretion of ammonia to the uptake of sodium through Na^+/NH_4^+ exchanger on the apical membrane of the branchial epithelium (Claiborne and Evans 1988; Evans et al. 2005). Since that time numerous studies have been conducted, some of which support the existence of a Na^+/NH_4^+ exchange system (Evans 2008; Evans 2010). Studies conducted in freshwater fishes support a model of simple diffusion across the lipid bilayer of NH_3 down its partial pressure gradient, maintained by a boundary layer acidification at the gill (Wilkie 2002; Braun et al 2009). There are also a number of groups that suggest that the diffusion of NH_3 is linked to Na^+/H^+ exchange or an H^+ pump/Na^+ channel mechanism or alternately that there is a mechanism of excretion by which ammonia partially moves by diffusion and partially by electroneutral exchange (Catches et al. 2006; Havrid et al. 2013; Rubino et al. 2014) (Fig 6).

The ion transporters Na^+/H^+ exchanger and H^+-ATPase play a pivotal role in the historical model of ammonia excretion (Evans et al. 2005). The Na^+/H^+ exchangers,

NHE2 and NHE3, are located in the apical side of the MRC's in the gills and move Na^+ into the MRC and exchange H^+ ions to the outside water (Evans et al 2005; Tsui et al. 2008; Li et al 2013; Brix et al. 2015). H^+-ATPase, is a proton pump localized in the MRC and pavement cell of the gills (Edwards et al. 2005; Roa et al. 2014). H^+-ATPase utilizes ATP to move H^+ out of the cells and into the surrounding waters, which causes a slight acidification boundary layer immediate surrounding the gills (Tresguerres et al. 2006; Wright and Wood, 2012). This acidification layer is hypothesized to aid in the movement of ammonia out of the membrane in a freshwater fish model (Wright and Wood, 2012).

More recently a potentially new model to facilitate ammonia excretion has been described and is associated with Rhesus glycoproteins (Rh) (Bakouh et al 2006; Nakada et al 2007a; Nakada et al. 2007b).

Rhesus Proteins

Rhesus proteins belong to a super family of ammonia transport proteins including the ammonia transport protein (Amt) in bacteria, Achaea and plants, methylammonia permease (Mep) in yeast, and Rhesus proteins in animals (Andrade and Einsle 2007). All of the ammonia transport proteins are hydrophobic membrane proteins, have 11-12 transmembrane domains, and function in either uptake or excretion of ammonia (Anstee and Tanner 1993; Andrade and Einsle 2007).

The discovery of Rh proteins came from investigations into the human Rh D, which is responsible for the charge in human blood types. The Rh factor associated with Rh proteins was discovered in the early 1940's due to investigations in fetal maternal blood mismatch, which caused stillbirths or miscarriages (Landsteiner and Weiner, 1941;

Huang and Ye 2010). The Rh proteins in human erythrocytes fall into two categories: 1) the Rh polypeptides, which are non-glycosylated and associate with Rh D; and 2) the glycosylated RhAG, Rh-associated glycoprotein (Huang and Liu 2001; Huang and Peng 2005). The Rh proteins are grouped by molecular weight; non-glycosylated proteins are 30kDa and all named Rh30 proteins, while the glycosylated proteins are 50kDa and are called Rh50 proteins (Nawata et al. 2007; Suzuki et al. 2014). The human Rh proteins are Rh30, RhAG, RhBG, and RhCG, while all non-human protein orthologs are titled Rhag, Rhbg, and Rhcg (Liu et al. 2001; Caner et al. 2015).

In 2007, the first study in fish to identify orthologs of Rh glycoproteins was conducted in *Takifugu rubripes* and localized four orthologs, Rhag, Rhbg, Rhcg1, and Rhcg2 to the gill epithelium (Nakada et al. 2007b). Rh glycoproteins were localized to the pillar, pavement, and mitochondria rich cells of the gill (Fig 9).To date, Rh glycoproteins have been identified in several species of teleost fish (Nakada et al. 2007a; Nakada et al. 2007b; Nawata et al. 2007; Nawata et al. 2010; Wood et al. 2013; Wright et al. 2014; Lawrence et al. 2015) and shark species (Nakada et al. 2010; Nawata et al. 2015a) as well as in the hagfishes (Edwards et al. 2015, Clifford et al. 2015). Rhag is localized to the apical edge of the red blood cells on the lamellae of the gill and is theorized to facilitate the movement of ammonia from the blood stream to the pillar cells (Wright and Wood 2009). Rhbg is located basolaterally on the brachial epithelium pavement cells (Ip and Chew 2010). Rhcg1 is localized to two areas, the apical edge of the pavement cells lining the lamellae, as well as in the apical crypt of the mitochondrial rich cells on the filament of the gill (Nakada et al. 2007b). Rhcg2 is predominately localized on the apical edge of the pavement cells along the lamellae (Weihrauch et al.

2009). All four orthologs of Rh glycoproteins are theorized to facilitate the movement of ammonia (Nakada et al. 2007b).

In addition to the Rh glycoproteins Rhag, and Rhbg, a novel Rh protein ortholog Rhp2, has been identified in the *Triakis scyllium*, which is hypothesized to be the primitive form of Rhcg (Nakada et al. 2010; Nawata et al. 2015a; Nawata et al. 2015b). Rhp2 localizes to the renal tubules of the kidney sinus zone and is theorized to facilitate the sequestration of ammonia from the urine to the blood stream (Nakada et al. 2010). This ammonia reuptake is associated with the OUC in sharks and leads to the production of the regulatory osmolyte urea (Nawata et al. 2015b). The discovery of Rh glycoproteins has helped to create a new hypothesis of ammonia transportation and excretion in both teleosts and chondrictheys (Wright et al. 2014).

While Rh glycoproteins are in the current model of ammonia excretion, it is also important to note the use of the NHE3 ion exchanger in the facilitation of ammonia. NHE exchangers can also preferentially bind NH_4^+ and move it across the membrane into the surrounding waters to maintain acid base regulation (Edwards et al. 2005; Hyndman and Evans 2008; Evans 2010). The Na^+/H^+ exchange proceeds through an electroneutral interchange of one Na^+ for one H^+ (Edwards et al. 2001). The abundance of environmental Na^+ in the marine ecosystem allows for the movement of NH_4^+ from inside the cell to the outside with little no energy input.

Purpose of Study

Many questions about the colocalization of ion transporters and the orthologs of the Rh glycoproteins, as well as the identification of the Rh glycoproteins in the marine teleosts, are still unanswered; this work attempts to fill the voids currently present in this area. To my knowledge, this is the first study to investigate the role of Rh glycoproteins in a stenohaline marine teleost. My project objectives were twofold, 1) use molecular techniques to identify orthologs of the Rhesus glycoproteins in the longhorned sculpin; 2) use protein expression and immunohistochemistry to examine the tissue localization of ammonia transporters, including Rh glycoproteins, in longhorned sculpin and spiny dogfish.

Methods

Animals

The teleost the longhorned sculpin (*Myoxocephalus octodecemspinosus)* and the elasmobranch the spiny dogfish (*Squalus acanthias*) were used to identify and localize orthologs of the Rh proteins. Longhorned sculpin (*M. octodecemspinous)* tissues were obtained from Animal Technician Michele Bailey, Mount Dessert Island Biological Laboratory, Sailsbury Cove, Maine.

Spiny dogfish (*S. acanthias)* gill total RNA was donated to our lab as a generous gift from Greg Goss, University of Alberta, Canada.

Molecular Biology

RNA Isolation

Total RNA isolation was performed using TRI reagent (Sigma-Aldrich,St. Louis, MO) according to previously published methods (Hyndman and Evans 2008). RNA pellets were resuspended in RNase-free Hyclone water (Fisher Scientific, Wilmington, DE) and the concentration of the total RNA measured with a Nanodrop ND-1000 spectrophotometer (Fisher Scientific, Wilmington, DE).

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

cDNA was generated using reverse transcription (SuperScript II; Invitrogen, Carlsbad, CA). Initial Rh sequence amplification was performed using degenerate primers designed against other vertebrate cDNA Rh gene sequences (NCBI database). (Table 1) and polymerase chain reaction (PCR) (3mmol l^{-1} MgCl, 200μmol l^{-1} dNTP mix,

10mmol l^{-1} primer & 1.25units plat taq) in 50µl volume incubated using an MJ Mini thermocycler (Bio-Rad, Hercules, CA). The thermal cycler program was 2 minutes at 92°C, 30 seconds at 92°C, a gradient from 50-68°C depending upon the primers used, 45 seconds at 72°C, repeat 40 cycles from 30 seconds at 92°C, and an extension time of 10 minutes at 72° C (Table 1).

Molecular Cloning and Sequencing

PCR products were cloned into either PROMEGA pGEM® T-easy vector, PROMEGA pGEM® T vector (Promega, Madison, WI), or TOPO PCR 4 vector (Fisher Scientific, Wilmington, DE). Clones containing the correct inserts were identified by restriction digest (Promega, Madison, WI), or PCR screening and then sequenced. Sequencing was conducted at the Mount Dessert Island Biological Laboratory.

Following the amplification of initial sequence, homologous oligo primers were designed and Rapid Amplification of cDNA Ends (RACE) for the 3' and subsequent 5' ends was performed using a SMARTer RACE cDNA amplification kit (Clonetech, Mountain View, CA). The resulting sequenced sections were then assembled using Mac Vector (Mac Vector, Cary, NC).

Tissue Distribution

Tissue specific expression of the four Rhesus genes was conducted using ortholog specific primer pairs and RT-PCR in the gill, kidney, intestine, and the skin of *M. octodecemspinos.* RT-PCR was done according to published protocols (Nawata et al.

2007b). The resulting PCR product was isolated on a 1.5% agarose gel stained with ethidium bromide (Fig 10).

Phylogenetic Tree

The phylogenetic tree was constructed using a ClustalW alignment of Rh glycoprotein sequences; the resulting alignment was analyzed using neighbor joining method and calculation of absolute difference and boot strap confidences estimation (1000 replications) in MacVector (Mac Vector, Cary, NC). Green algae, *Chlamydomonas reinhardtii,* Rhp1 was used as an outgroup (accession number XM_001695412).

Antibodies

Rhesus glycoproteins

Polyclonal antibodies were generated in rabbits against the C-terminus of *Takifugu rubripes* Rhag, Rhbg, Rhcg1, and Rhcg2. These antibodies were used to determine Rh protein expression and colocalization in spiny dogfish gills and kidneys as well as longhorned sculpin gills. The antibody was a gift from Dr Shigehisa Hirose, Department of Biological Sciences, Tokyo Institute of Technology, Yokohama, Japan. These antibodies have been used in previous studies to localize Rh proteins in teleost gills (Nakada et al. 2007b).

H^+-ATPase

A polyclonal antibody raised in a rabbit against spiny dogfish HAT was generated based on the Spiny dogfish HAT sequence (AREEVPGRRGFPGY) (Claiborne et al.

2008). A polyclonal antibody raised against the B subunit of Sculpin H^+-ATPase (AP 7181/7182) was used to determine protein localization and expression in the longhorned sculpin (Catches et al. 2006).

NHE 3

The polyclonal antibody in a rabbit made against the synthetic peptide NHE3 A99 was produced by Biosource international (A99; TDSSHDSGNGDTDHES derived from genbank: EU909191). The antibody was utilized to determine NHE3 localization and expression in the longhorned sculpin.

Na^+/K^+-ATPase

A mouse monoclonal (α5) antibody was raised against the alpha subunit of Na^+/K^+-ATPase by Dr Douglas Fambrough and was obtained from the Developmental Studies Hybridoma Bank (DSHB, John Hopkins Univ., Balitmore, MD USA). This antibody is widely used to determine localization of teleost Na^+/K^+-ATPase in brachial epithelium (Chloe et al. 2002; Edwards et al. 2002; Catches et al. 2006).

$Na^+/K^+/2Cl^-$ Cotransporter

The monoclonal antibody against the human NKCC (T4) was used to localize NKCC in the longhorned sculpin gill. The T4 antibody was acquired from the Developmental Studies Hybridoma Bank (DSHB, John Hopkins Univ., Balitmore, MD USA). The NKCC T4 antibody has been used to recognize both isoforms of NKCC in multiple tissues, including gills (Wilson and Laurent 2002; Horng and Lin 2008).

Western Blotting

Total protein was isolated from longhorned sculpin gill, kidney, and intestines. Tissues were placed into chilled homogenization buffer (250mM Sucrose, 1mM EDTA, 30mM Tris, 100µg/ml PMSF, and 5mg/ml protease inhibitor cocktail) and homogenized. The homogenate was centrifuged at 14,000g for 10 minutes at 4°C to separate the protein from the debris. The protein supernatant was decanted and a BCA (bicinchroninic acid) protein assay was conducted to determine protein concentrations (Thermo Scientific, Rockford, IL). 50µg of total protein was loaded into Ready Mini Gel-TGX-10% acrylamide gels (Bio-Rad, Hercules, CA) and separated by SDS-PAGE (sodium dodecyl sulfate, polyacrylamide gel electrophoresis). Separated proteins were then transferred onto nitrocellulose membranes using a Trans-Blot® Turbo™ (Bio-Rad, Hercules, CA). The membranes were blocked for 24 hr rocking at 4°C in 5% blotto (5% non-fat dry milk powder in 0.1M Tris-buffered Saline with 0.2% Tween-20 (TBST)). Membranes were then incubated in primary antibody 1:500 Rhag, Rhbg, Rhcg1, or Rhcg2 in 5% blotto rocking overnight at room temperature. Membranes were washed three times in 0.1M Tris-buffered Saline (TBS) for 15 minutes, then incubated in the secondary conjugate antibody (1:10000) horseradish peroxidase (HRP)-conjugate goat anti-rabbit and Precision Protein StrepTactin HRP-conjugate (Bio-Rad) in TBS for 1 hour. Following three TBS washes, the membranes were then developed using a chemioluminescence Clarity western system (Bio-Rad, Hercules, CA). Blots were visualized using Bio-Rad Chemi-doc system.

Immunohistochemistry

Immunohistochemistry (IHC) enabled the localization of the transport proteins of interest in tissue sections of gill and kidney (Sanatacana et al. 2014). IHC was done following the published protocols (Edwards et al. 2005).

Fixed tissue samples were processed for paraffin embedding. Gill and kidney tissues were sectioned to 7 μm using a Leica 3 microtome and mounted on Fisherbrand ® Superfrost®/Plus Microscope Slides (Thermo Fischer Scientific Rockford, IL) and incubated overnight. Slides were incubated in a variety of antibodies, including hagfish specific hRhcg antibody (1:500), teleost Rhc1 antibody (1:500), teleost Rha antibody (1:250), teleost Rhb antibody (1:250), teleost Rhc2 antibody (1:250) and detected using goat anti-rabbit or anti-mouse Alexa Fluor® 568, and goat anti-rabbit or anti-mouse Alexa Fluor® 488 (Molecular probes). In addition to localization of all known Rh proteins, NKA, NHE 3, NKCC, and HAT were also localized in *M. octodecemspinos* tissues. Colocalization studies were used to determine locality of Rhesus proteins and the specific ion transporters. Sections were visualized using a Zeiss LSM 510 confocal microscope (Zeiss).

Results

Gene Sequencing and Annotation

Rhag

A partial sequence of Rhag was isolated utilizing *Oncorhynchus mykiss* Rhag primers. BLASTX showed the 677 base pair Rhag fragment has 85% identity to the *Clupea harengus,* Atlantic herring, predicted ammonium transporter Rh type A (XP_012693009.1; gi|831320363|) (Fig 11).

Rhbg

The partial sequence of Rhbg was isolated using PCR and *Oncorhynchus mykiss* Rhbg primers. The 661 base pair fragment has 89 % identity to the *Larimichthys crocea*, large yellow croaker, predicted ammonium transporter Rh type B isoform X1 and X2 (XP_010746197.1; gi|734635239|), as well as 92% identity to *Takifugu rubripes*, Japanese pufferfish, ammonium transporter Rh type B (XP_011617147.1; gi|768957319|) (Fig 12).

Rhcg1

The partial Rhcg1 sequence 610 base pair fragment was isolated using PCR and *Oncorhynchus mykiss* Rhcg primers. The 610 bp fragment has 91% identity to the *Cynoglossus semilaevis*, tongue sole, ammonium transporter Rh type C 1 (XP_008310757.1 ; gi|657750682|) (Fig 13).

Rhcg2

The complete Rhcg2 ortholog was identified and sequenced. The 1097 bp open reading frame has an 89% identity with the *Cynoglossus semilaevis*, tongue sole, predicted ammonium transporter Rh type C2 (XP_008308471.1; gi|657746415|) (Fig 14).

Rhp2

The partial Rhp2 from the spiny dogfish was isolated using banded hound shark primers (Nakada et al. 2009). The 1040 bp fragment had 91% identity with the *Triakis scyllium,* banded hound shark, Rhesus glycoprotein P2 (BAI49727.1; gi|269913342|) and a 96% identity to the *Squalus acanthias*, spiny dogfish Rhp2 (AJF44129.1|; gi|751250147|) (Fig 15).

Tissue Distribution

A 500 bp fragment from each orthologs was amplified to assess expression in the epithelial tissues of *M. octodecemspinosus* (Fig 10). All four orthologs, Rhag, Rhbg, Rhcg1, and Rhcg2, were identified using PCR in the gill, skin, kidney, and intestine of *M. octodecemspinosus* utilizing *Oncorhynchus mykiss* Rh primers (Nawata et al. 2007) (Fig 10). PCR analysis showed qualitative expression of Rhag, Rhbg, Rhcg1, and Rhcg2 in the gills. Rh glycoproteins, Rhbg, Rhcg1, and Rhcg2 were additionally found in the skin, kidney, and intestines.

Western Blot

Use of *Takifufu rubripes* Rhag, Rhbg, Rhcg1, and Rhcg2 antibodies demonstrated a single immunoreactive band (approximately 47kDa) in the longhorned sculpin tissues. These results are similar to the results reported in *Takifugu rubripes* tissues by Nawata et al. 2010 (Fig 16).

Relationship of Rh Glycoproteins

Based upon the multiple alignments of Rh glycoprotein amino acid sequences obtained from the longhorned sculpin, four other teleost fish species, the African clawed fog Rhcg, hagfish Rhcg, banded houndshark Rhp2, and green algae Rhp2; a relationship analysis based was conducted based on neighbor joining (Fig 16). The phylogenetic tree shows longhorned sculpin Rhcg2 being most closely related to other teleost Rhcg2 mRNA, with the exception of the zebrafish Rh glycoproteins.

Immunohistochemistry

Negative controls were conducted simultaneously with experimental slides for the secondary antibodies, goat anti-rabbit or anti-mouse Alexa Fluor® 568, and goat anti-rabbit or anti-mouse Alexa Fluor® 488 utilized in the study (Fig 28).

Co-Localization of Rhag in M. octodecemspinous

Rhag was localized to the apical and basolateral edges of the pillar cells, which surrounded the interlamellar space along the lamella (Figs 18 & 20). NKA was localized exclusively to the ionocytes on the filament (Fig 18). NKCC was localized strongly to the ionocytes in the filament (Fig 20).

Co-Localization of Rhbg in M. octodecemspinous

Rhbg was localized to the basolateral edge of the pavement cells on the lamella (Figs 18 & 20). NKA was localized exclusively to the MRC's on the filament (Fig 18). NKCC was localized strongly in the MRC's of the filament (Fig 20).

Co-Localization of Rhcg1 in M. octodecemspinous

Rhcg1was localized to both the apical crypt of the MRC's in the filament and the edges of the pavement cells of the gill lamaella (Figs 19, 20, 21). The Rhcg1 immunoreactivity along the edges of the pavement cells is much less intense and more diffuse compared to the Rhcg2 expression along the same gill lamella (Figs 19, 20, 21). Rhcg1 was colocalized in the MRC's expressing both NKA and the NKCC, with Rhcg1 immunoreativity seen exclusively in the apical crypt of the MRC (Figs 19 & 20)

Co-Localization of Rhcg2 in M. octodecemspinous

Rhcg2 was localized to the apical edge of the pavement cells lining the gill lamella while NKA localized exclusively to the MRC's (Fig 19). When Rhcg2 colocalized with NKCC, NKCC was found exclusively in the MRC's, while Rhcg2 was only found along the apical edge of the lamellae, with no inmmunoreactivity on the filament (Fig 20).

HAT in S. acanthias and M. octodecemspinous

In the gills of both the spiny dogfish and the longhorned sculpin, HAT was expressed exclusively in the MRC's (Figs 24 & 21). H^+-ATPase expression was more distinct in the spiny dogfish gill, with more diffuse expression occurring in the longhorned sculpin gill. In the kidney, HAT was localized to the renal tubules of the spiny dogfish (Fig 23).

NHEs in S. acanthias and M. octodecemspinous

Kidney

NHE 2 expression was demonstrated along the apical edge of the kidney tubules of the spiny dogfish (Fig 23). NHE 2 closely associated with the apical expression of Rhcg along the edge of the kidney tubules.

Gill

NHE 3 expression in the longhorned sculpin gill was shown in ammonia treated tissues. NHE 3 expression was localized to the ionocytes along with diffuse expression of NKA (Fig 22). NHE 3 expression was specifically found in the apical crypt of the MRC's and along the brush boarders of the filament. When NHE 3 was colocalized with NKA, NKA immunoreactivity was constant in the MRC's along the filament, while NHE 3 localized specifically to the apical crypt of the MRC's (Fig 22).

Co-localization of Rh glycoproteins and NKA in S. acanthias

The Rh glycoproteins, Rhag and Rhcg2, were each colocalized with the ion transporter NKA in the gill of *S. acanthias* (Fig 26). Rhag expression was localized to the edge of the pillar cells in the gill lamellae of *S. acanthias*. (Fig 26). Rhcg2 expression was localized to the apical edge of the lamellae on the gills of *S. acanthias* (Fig 26). When NKA was co-localized with either Rhag or Rhcg2, NKA was expressed solely along the edges of the ionocytes on the filament (Fig 26). Rhbg expression could not be localized in *S. acanthias.*

Discussion

Previous studies, on both marine elasmobranchs and marine teleost fishes, have demonstrated their ability to excrete nitrogenous waste (Claiborne and Evans 1988; Wood et al. 1995; Nakada et al. 2010; Sinha et al. 2015). However, my research is the first in a marine teleost to suggest that Rh glycoproteins may play a predominate role in ammonia excretion. This study isolated and identified partial fragments of the Rh glycoproteins, Rhag, Rhbg, and Rhcg1, as well as the complete open reading frame of an Rhcg2 ortholog in epithelial tissues of *M. octodecemspinous.* In addition, I cloned a partial fragment of the Rhp2 from the elasmobranch *S. acanthias.* This Rhp2 fragment demonstrated a 96% identity to the complete Rhp2 sequence identified by other researchers (Nawata et al. 2015b).

Elasmobranch Rh glycoproteins and Ion Transporters

The examination of Rh expression in the kidney and gill of *S. acanthias* provides important information associated with the unique physiological adaptations of the spiny dogfish, which has the interesting homeostasis profile of osmoconformity and ionoregulation (Whittamore 2012). Results from the dogfish shark showed expression of Rh glycoproteins in both the kidney and the gill. Immunohistochemistry demonstrated Rhcg-like immunoreactivity in epithelial cells of the gill and kidney, while Rhag, Rhcg1, and Rhcg2 were isolated solely from epithelial cells of the gill. These findings suggest Rh glycoproteins play a potential role in the reuptake of ammonia from the kidney filtrate, as well as a possible route for the excretion of excess ammonia across the gills. The role of Rhp2 in the elasmobranch kidney is currently hypothesized to be solely responsible for

the retention of ammonia for the creation of the osmolyte urea (Nawata et al. 2015b). This hypothesis was supported by a previous study that localized Rhp2 to the sinus zone of the kidney, but in the same study no Rhp2 immunoreactivity was demonstrated in the shark gill (Nakada et al. 2010).

The findings of my study support the idea that Rh glycoproteins present in the gill may be important in maintaining osmotic and ionic homeostasis. The direct excretion of ammonia via Rh glycoproteins has been suggested in teleost fish gills, but to date have not been fully investigated in the elasmobranch gill (Nakada et al. 2007b). In my study, IHC localization of Rhag, Rhcg, and Rhcg2 to the gill suggests a similar pattern of transcellular ammonia efflux capability as seen in teleost fishes (Nawata et al. 2007; Nakada et al. 2007a; Nakada et al. 2007b; Tsui et al. 2008). The excess ammonia could be transported by way of the blood stream to the branchial arch, once in the lamellae Rhag present on the pillar cells facilitate the movement of NH3 out of the blood stream, then across the basolaterally located Rhbg into the pavement cells and finally excreted via an apically located Rhcg present on the apical edge of the pavement cells (Figs 9 & 27) (Nakada et al. 2007b). Future work in *S. acanthias* should focus on the utilization of Rh glycoproteins in both the kidney and gill under varying conditions such as elevated salinity and increased internal ammonia loads.

Teleost Rh Glycoproteins and Ion Transporters

Since Claiborne and Evans investigated the preferential movement of ammonia in the *M. octodecemspinous*, the question has been how a marine fish excretes ammonia waste as NH_3 (Claiborne and Evans 1988). Marine teleosts lack the partial pressure

gradient present in freshwater ecosystems to utilize simple diffusion as a mechanism for NH_3 excretion. Moreover, unlike elasmobranchs, these organisms cannot convert ammonia waste to less toxic urea (Claiborne and Evans 1988; Wilkie 2002; Catches et al. 2006; Braun et al. 2009b; Rubino et al. 2014). Since the discovery of Rh glycoproteins as a method of ammonia excretion in 2007, in teleost fishes Rhag, Rhbg, Rhcg1, and Rhcg2 are hypothesized to be integral in NH_3 excretion across the gills of all teleost fishes (Ip and Chew 2010). In my study, all four orthologs were confirmed in *M. octodecemspinous* using both tissue distribution and western blot analysis (Figs 10 &16). Tissue distribution showed Rh Rhag cDNA expressed in gill tissue, while Rhbg, Rhcg1, and Rhcg2 were found in the gill, kidney, skin, and intestines (Fig 10). However, protein expression analysis determined that Rhag, Rhbg, and Rhcg2 were only present in the gill and kidney, while Rhcg1 was present in the gill, kidney, and intestine. The presence of all four Rh glycoprotein orthologs in the gill suggests a conservation and importance of Rh proteins in the function of the gill. These patterns of Rh glycoprotein distribution are also identified using IHCs.

M. octodecemspinous demonstrated strong expression of Rhag along the apical edge of the pavement cells, Rhbg along the basolateral edge of the pillar cells, Rhcg1 and Rhcg2 along the apical edge of the lamellae. Rhcg1, however, localized in both the apical crypt of the ionocytes in addition to the edge of the lamellae. Rh glycoprotein orthologs were double labeled with various ion transporters to both confirm the presence of the ion transporters, as well as serve as markers for ionocytes along the filament. Previous studies have sequenced and investigated the roles of NHE, NKA, and HAT in the *M. octodecemspinous*, though this was before the discovery of the Rh glycoprotein and its

function in homeostasis (Catches et al. 2006). In my study, NHE3, HAT, NKA, and NKCC were all expressed solely in the ionocytes of the gill. Interestingly, NHE 3 expression increased in ammonia treated *M. octodecemspinous* gills compared to the control gill tissues. NHE 3 can be utilized as an NH_4^+ exchanger in times of high ammonia exposure due to the abundance of intercellular ammonia. Due to previous knowledge of the function of the ion transporters as well as current images of their locality in the gill, my results support that these transporters may play a role in ionic homeostasis, but are not the main method of ammonia excretion.

Molecular Identification of Rh glycoproteins

Though the aim of the study was to investigate potential methods of ammonia excretion in *S. acanthias* and *M. octodecemspinous*, Rh glycoprotein cDNA sequence data provided additional support for the presence of Rh gylycoproteins in both organisms. The resulting partial Rhag, Rhbg, and Rhcg1 from *M. octodecemspinous* demonstrated a high similarity to other teleost Rh glycoproteins (Figs 11-13). Additionally, the fully sequenced Rhcg2 from the *M. octodecemspinous* had a high similarity (89%) to *Cynoglossus semilaevis,* tongue sole (XP_008308471.1; gi|657746415|). When the Rhcg2 from *M. octodecemspinous* was compared to other known Rh glycoproteins, the Rhcg2 shows a distinct relationship out with other Rhcg2 orthologs in teleost species (Fig 17). This suggests a high level of conservation of these genes across species. In the elasmobranch *S. acanthias,* the isolated fragment of Rhp2 showed a high identity to the known *Triakis scyllium* Rhp2 (91%) as well as the recently published *S. acanthias* Rhp2

(96%) (Nawata et al. 2015b). All of the isolated Rh glycoprotein orthologs had most similarity in common with in their phylogenetic group, which suggests a high rate of conservation across species for all Rh glycoproteins.

These patterns of inheritance and conservation, seen in both the *M. octodecemspinous* Rh glycoproteins and the *S. acanthias* Rhp2, coincide with previous work on Rh glycoprotein evolution (Nakada et al. 2010). Rhp1 and Rhp2 are currently hypothesized to be the primitive forms of Rhcg, though many researchers are currently investigating the phylogenetic relationship of these ancestral Rh proteins (Liu et al. 2000; Huang and Peng 2005; Nakada et al. 2010; Huang and Peng 2010). The recent finding that Rhcg is present in the Atlantic hagfish, *Myxine glutinosa* and that its expression is increased following ammonia load has created a debate over the evolution of Rhcg (Edwards et al. 2015). This is compounded by findings in elasmobranchs *S. acanthias* and *T. scyllium* (Nakada et al. 2010; Nawata et al. 2015a; Nawata et al. 2015b),that possess an Rhp2 isoform but appear to lack Rhcg cDNA. *M. glutinosa,* being the more ancient of the extant vertebrates,, may also possess an Rhp2, but to date this has not been investigated (Edwards et al. 2015).

Conclusion

The aim of this study was to examine potential routes of ammonia excretion in both *S. acanthias* and *M. octodecemspinous*. Through the use of molecular techniques and immunohistochemistry, I have demonstrated Rh glycoprotein and ion transporter expression in various tissues and propose a new hypothetical model of ammonia excretion in *M. octodecemspinous*. Previous work conducted by Claiborne and Evans in 1988, showed NH_3 excretion as the main method of ammonia excretion in *M.*

octodecemspinous, though at the time of their study, no method of NH_3 excretion had been discovered in marine teleosts (Claiborne and Evans, 1988). This is the first study focusing on *M. octodecemspinous* to isolate a potential method of ammonia excretion since the discovery of Rh glycoproteins role in homeostasis. Through this study I was able to generate a new hypothesized model for ammonia excretion and ion movement in the saltwater teleost *M. octodecemspinous* (Fig 27).

Future Work

Future studies in the *M. octodecemspinous* should focus on both isolating the complete Rh glycoproteins as well as measuring the fluctuations in Rh glycoprotein expression under varying conditions to test the proposed model of ammonia excretion. Currently Rh glycoproteins are theorized to move both ammonia gas and CO_2, since they are of similar molecular weight, though studies have found conflicting evidence of CO_2 movement (Endeward et al. 2008; Nawata and Wood 2008; Wright and Wood, 2012). The CO_2 studies have previously utilized freshwater fishes, such as rainbow trout and zebrafish, or the ureotelic Magadi tilapia, though to my knowledge no studies have been conducted on a marine fish (Nawata and Wood, 2008; Nakada et al. 2010; Wright and Wood, 2012). The key to understanding the role of Rh glycoproteins in CO_2 transport may lie in the marine fish gills. This would be especially significant to understand the preferential movement of ammonia and CO_2 with the global issue of ocean acidification as an increasingly significant threat to marine life.

Tables

Table 1. List of primers used to isolate fragments of Rh glycoprotein orthologs.

Gene	Sequence	Remark
Rhp2	5'-CCGGTACCCCATGTTCCARGAYGTNCA	Degenerate PCR (S) (Nakada et al. 2010)
Rhp2	5'-GGAGTTGAAGGAGGGCCARWANATCCA	Degenerate PCR (AS) (Nakada et al. 2010)
TRhagF1	5'-CAACGCAGACTTCAGCAC	Rainbow Trout Rhag (Natawa et al. 2007)
TRhagR1	5'-CCACAGGTGTCCTGGATG	Rainbow Trout Rhag (Natawa et al. 2007)
TRhbgF1	5'-CATCCTCATCATCCTCTTTGGC	Rainbow Trout Rhbg (Natawa et al. 2007)
TRhbgR1	5'-CAGAACATCCACAGGTAGACG	Rainbow Trout Rhbg (Natawa et al. 2007)
JBRhb479R	5'-CAACACCCCGATGTGGATCTTG	Sculpin Rhbg
JBRhb1053F	5'-ATCATCTCCGTCTTGGGCTTCA	Sculpin Rhbg
JBRhb1361R	5'-GGGGAGCTTCAAGATGAAACCG	Sculpin Rhbg
JBRhbg1340F	5'-CGGTTTCATCTTGAAGCTCCCC	Sculpin Rhbg
JBRhb1492R	5'-CGCGTCTAATTGAGCTTCTCGG	Sculpin Rhbg
ZFRhcg1F	5'-AAGCATATGAACGGGTCCGTCTACC	Zebra Fish Rhcg1 (Nakada et al. 2007)
ZFRhcg1R	5'-AGCCCGACAAACGTTCCTCCGCCGA	Zebra Fish Rhcg1 (Nakada et al. 2007)
FRhcg1F	5'-TGGAGTCTTCATCCGCTACGACGAA	Takifugu Rhcg1 (Nakada et al. 2007)
FRhcg1R	5'-GGCTTGAAATGGCCACAGTGGTGAG	Takifugu Rhcg1 (Nakada et al. 2007)

RhcgDeg1F	5’-TTYCARGAYGTICAYGTIATG	Degenerative Rhcg1
Rhcg1DegR	5’-CARGAYACITGYGGNATHCA	Degenerative Rhcg1
TRhcg1F1	5’-CATCCTCAGCCTCATACATGC	Rainbow Trout Rhcg1 (Natawa et al. 2007)
TRhcg1R1	5’-GTTTCTGTCCAGCAGGCGTC	Rainbow Trout Rhcg1 (Natawa et al. 2007)
Sculp115F	5’-GTGGGCTTCAACTTCCTGATCG	Sculpin Rhcg
Sculp711R	5’-AGCATTCTGGATGTGCACCATG	Sculpin Rhcg
Sculp472F	5’-CAAAGCAGACGCCTCAATGGAT	Sculpin Rhcg
Sculp1122R	5’-CCCCAGATAGGCAACCTCAGAA	Sculpin Rhcg
TRhcg2F1	5’-GCACACTGTTCCTGTGGATG	Rainbow Trout Rhcg2 (Natawa et al. 2010)
TRhcg2R1	5’-CAGCAGGATCTCCCCAGA	Rainbow Trout Rhcg2 (Natawa et al. 2010)

Figures

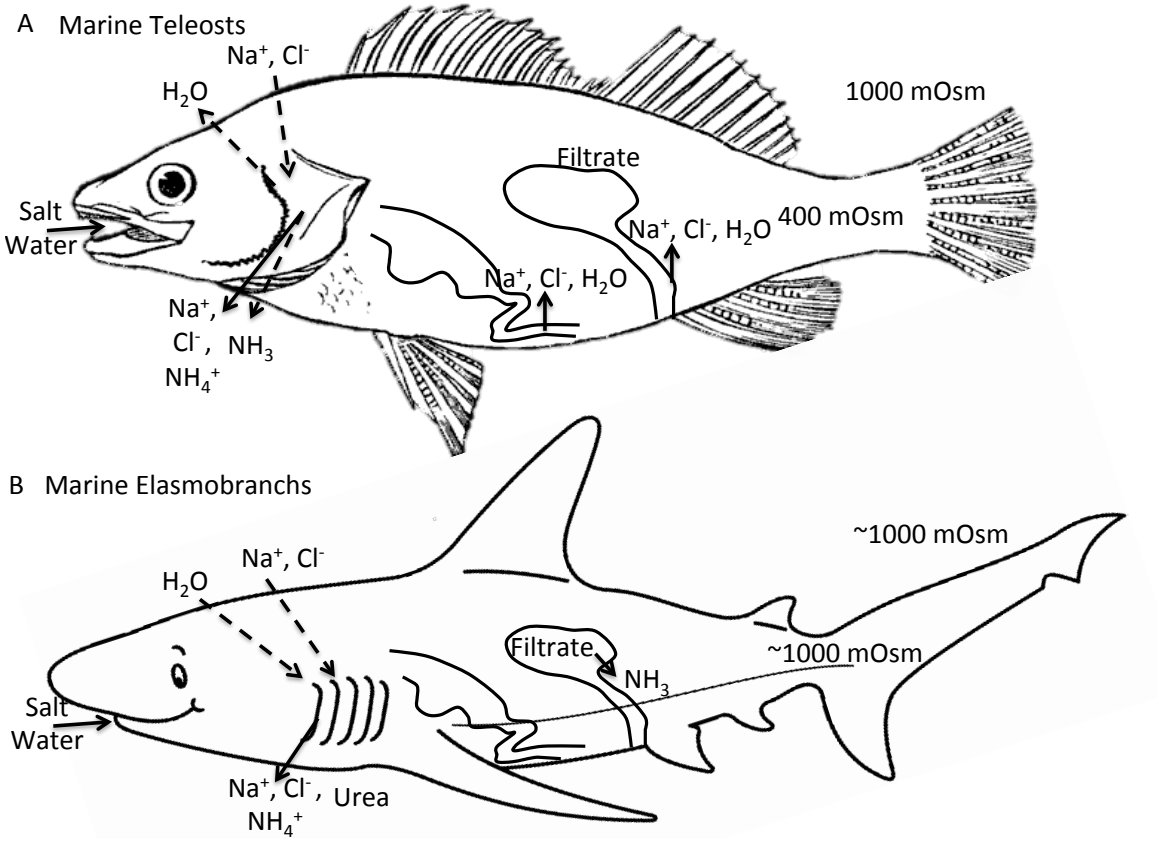

Fig 1. Schematic of the movement of water, ions, and nitrogenous waste in saltwater teleost and elasmobranchs. A. The saltwater teleost fishes exist in a hyperosmotic environment and to combat the osmotic water loss, must constantly drink water. The excess ions ingested from the environment are absorbed in the intestine, transported via the bloodstream, and excreted along the gill. Ammonia is generated from the degradation of protein, transported via the bloodstream, and excreted at the gill (Adapted from Evans 2008). B. The saltwater elasmobranchs exists in a slightly hyposmotic environment due to the retention of the nitrogenous waste product urea. Urea is generated after ammonia is absorbed from the filtrate in the kidneys, transported via the bloodstream, and processed into urea in the OUC in the liver. Urea is then moved along the body to combat osmotic water loss and excess urea is excreted along the gills.

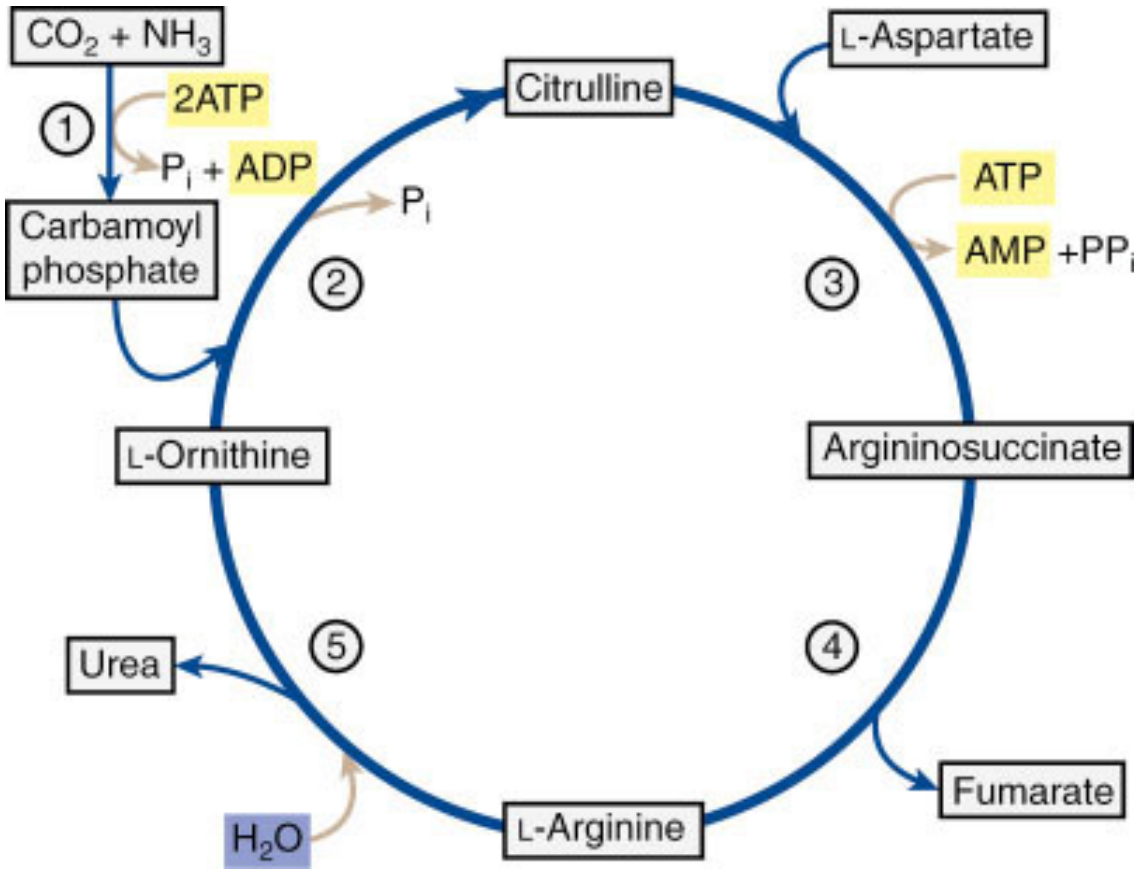

Fig 2. Detailed diagram of the Ornithine Urea Cycle. 1. The introduction of ammonia and carbondioxide to the cycle. 2. The phosphorylated carbamoyl phosphate transitions to the intermediate citrulline. 3. L-aspartate and ATP convert citrulline to argininosuccinate. 4. Fumarate transitions to L-Arginine. 5. The hydrolysis of L-Argenine produces the byproduct Urea and L-Ornithine, which allows the cycle to continue if carbamoyl phosphate is present. (Medical Dictionary, 2011).

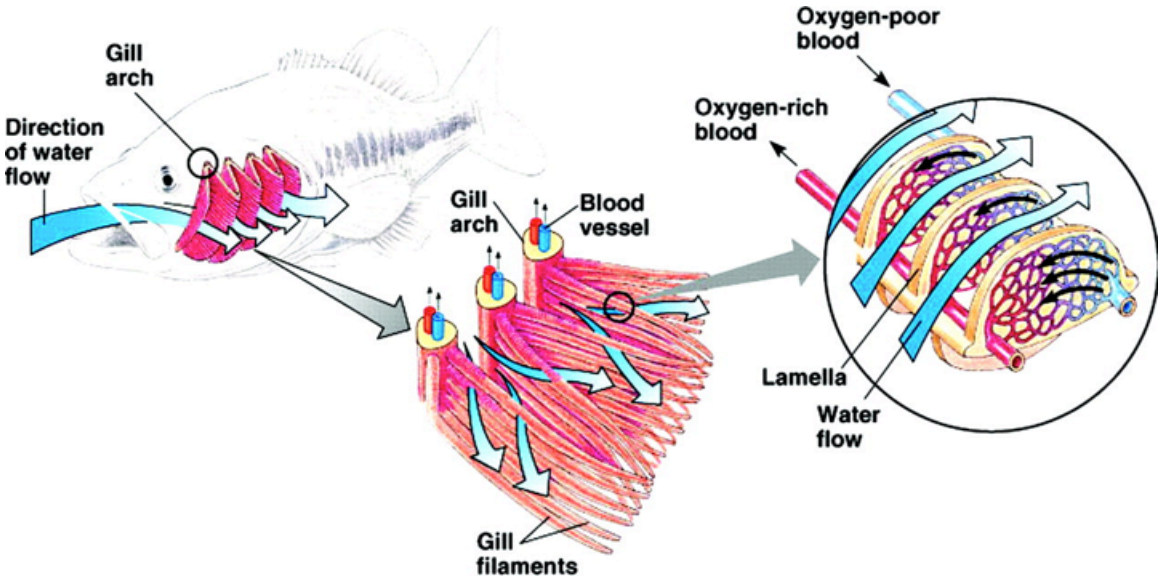

Fig 3. Detailed anatomy of a teleost fish gill. The water flows in through the fishes mouth and moves along the gill filaments and exits through the gill operculum. Water flow brings in oxygen to the fish as well as aids in waste removal across the gill. The gill is composed of the gill arch, which is innervated with the blood supply, gill filament, and the lamellae (Campbell and Reece 2002).

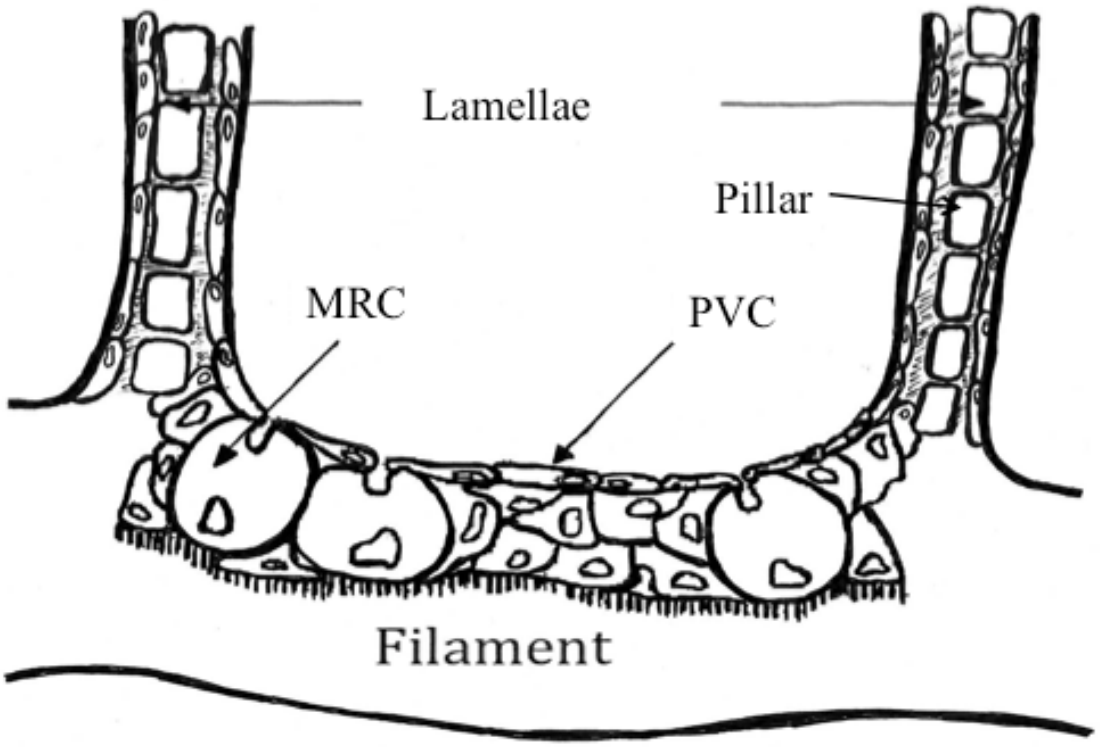

Fig 4. Gill cell types present in a fish gill. Mitochondion Rich Cells, MRC, are prevalent along the main stem of the filament, while Pavement Cells, PVC, line the apical edge of the filament, and pillar cells are in the center of the lamellae (Adapted from Edwards 2000).

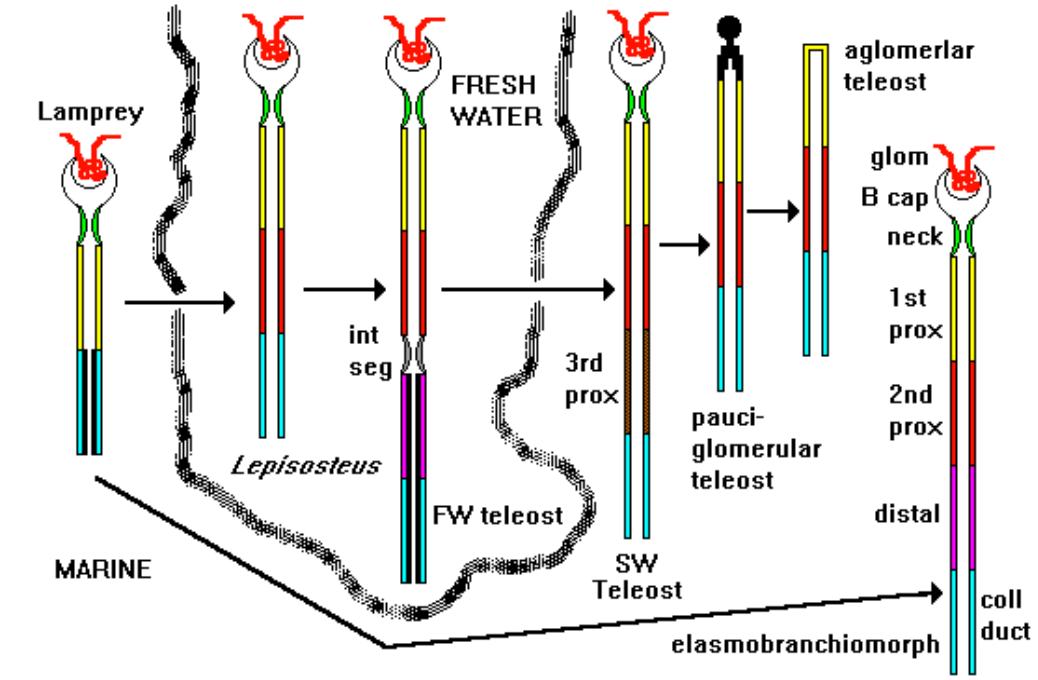

Fig 5. Diagram of kidney nephrons structure in multiple groups of fishes, with phylogenetic groups listed across the bottom. The structural shift from having a glomerous, to being aglomeral teleost, impacts the physiology of the kidney and the osmoregulation of the fish. (http://www.snre.umich.edu/~pwebb/NRE422-BIO440/lec11.html)

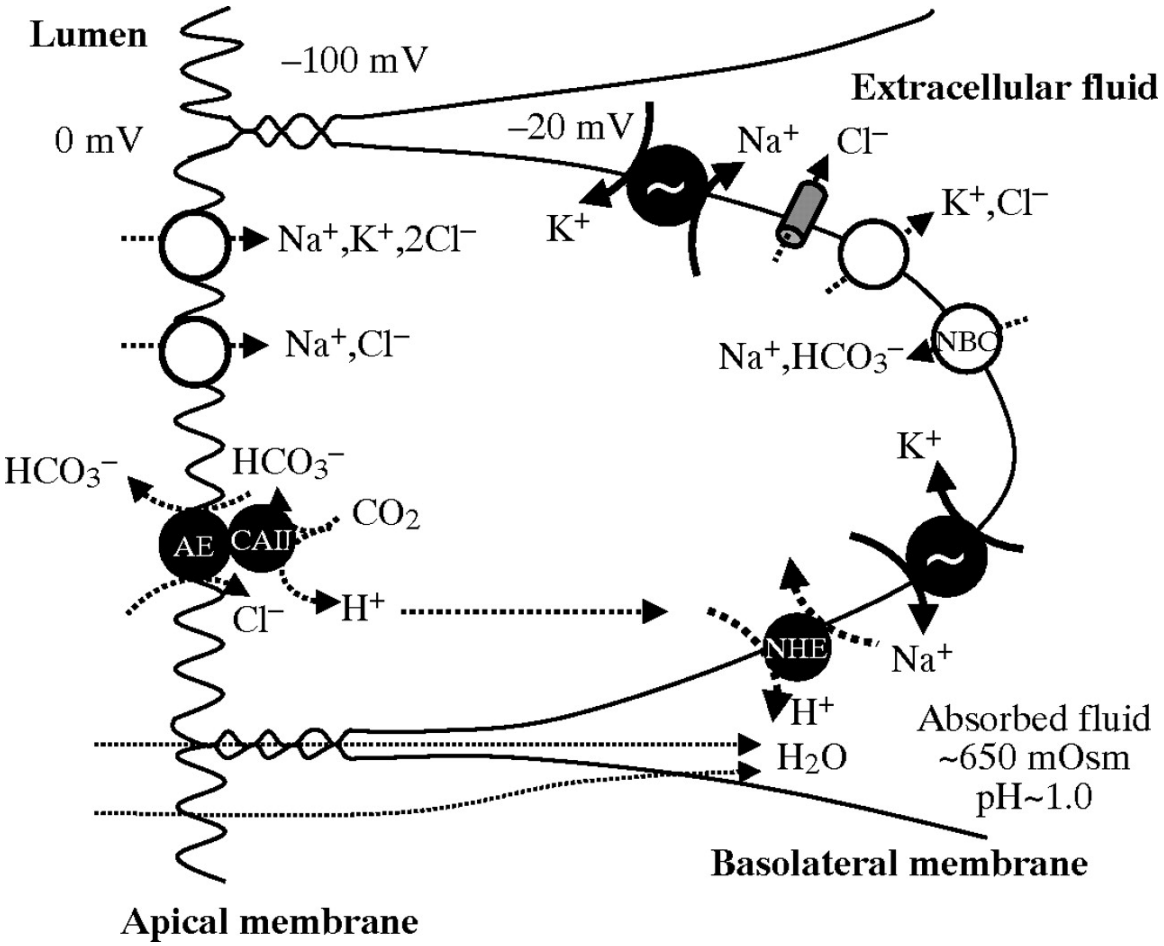

Fig 6. Diagram of the transport processes present in the intestines of marine fish. Both the anion exchanger, AE, and Carbonic anhydrase, CAII, are present on the lumen of the intestines and are responsible for the movement of carbonic acid, carbon dioxie, and chloride ions. The ion tranporters NKA, NHE, and NBC move potassium, chloride, sodium, and carbonic acid across the apical epithelium (Grossel 200)

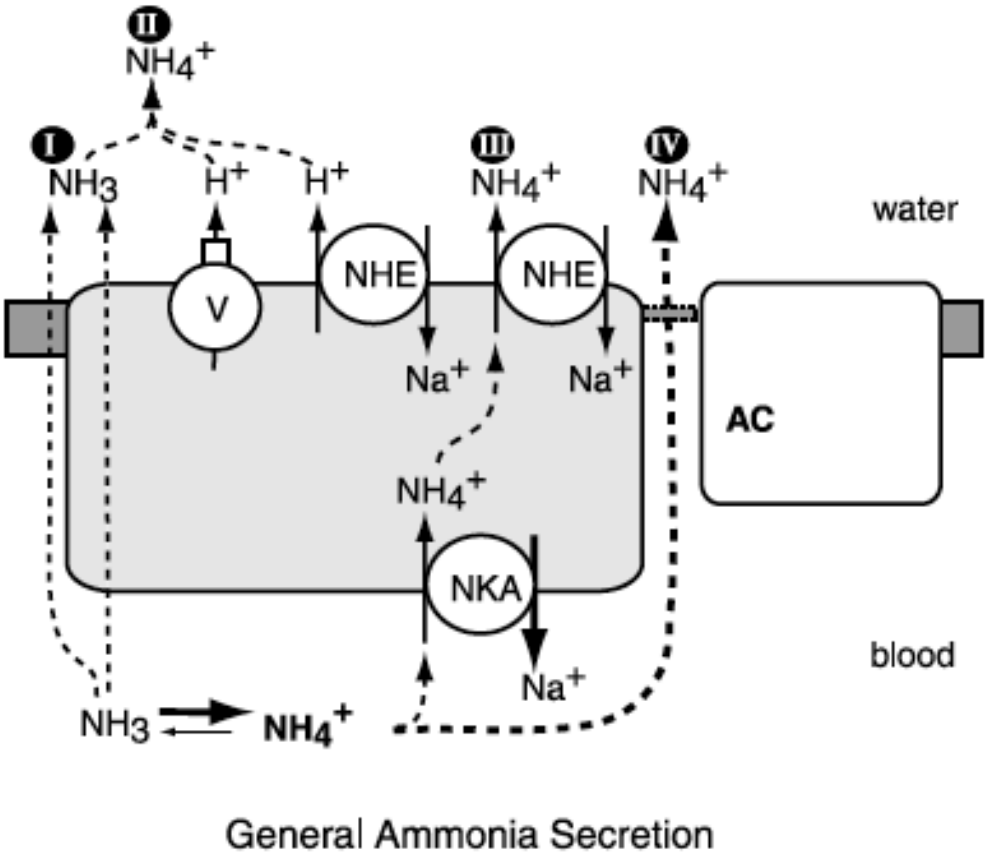

Fig 7. The composite model of ion movement in the gills and red blood cells of agnathans, elasmobranchs, and teleosts. The roman numerals denote the decreasing order of frequency of the pathways. Pathway 1 shows simple diffusion of ammonia across the gill membrane. Pathway 2 shows ammonia movement facilitated by the V-ATPase and NHE. Pathway 3 shows the utlilization of NHE to move ammonia based on charge. Pathway 4 shows direct excretion due to leaky junctions between chloride cells and accessory cells in marine telesots (Evans et al 2005).

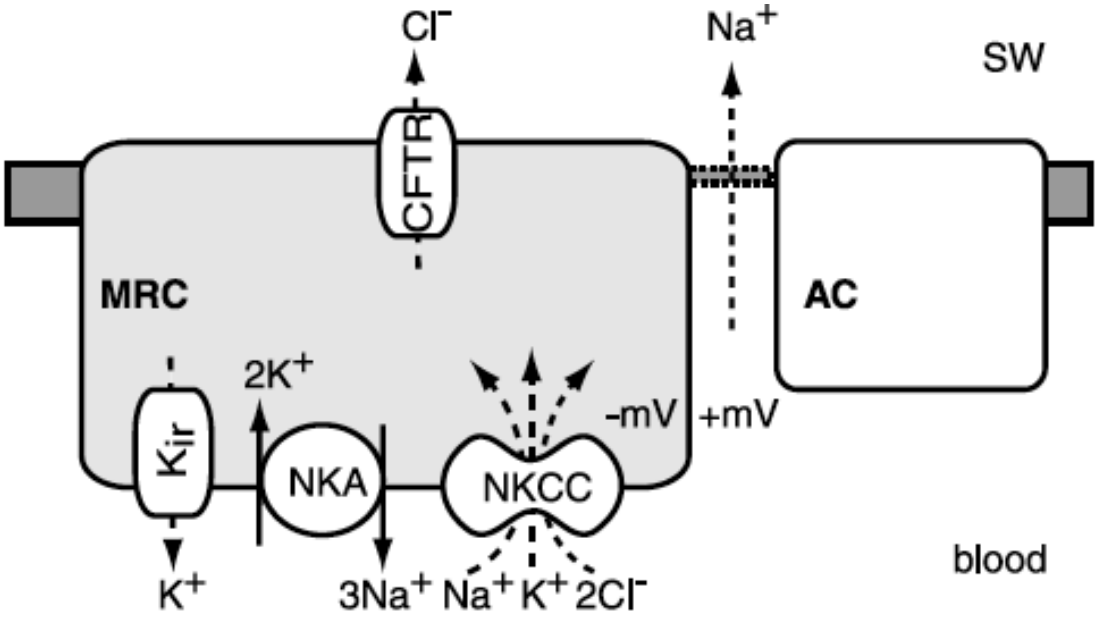

Fig 7. The current model of NaCl movement via ion transporters in the marine teleost gill. Ion transporters Kir, Na+/K+ ATPase, and Na+/ K+/ Cl- cotransporter are localized to the basolateral edge of the MRC and facilitate the movement of ions into and out of the cell. The cystic fibrosis transmembrane protein is localize on the apical edge of the MRC and facilitates the movement of chloride into the surrounding waters. Sodium is shown to leak paracellularly across the junction between the MRC and the accessory cell (Evans et al 2005).

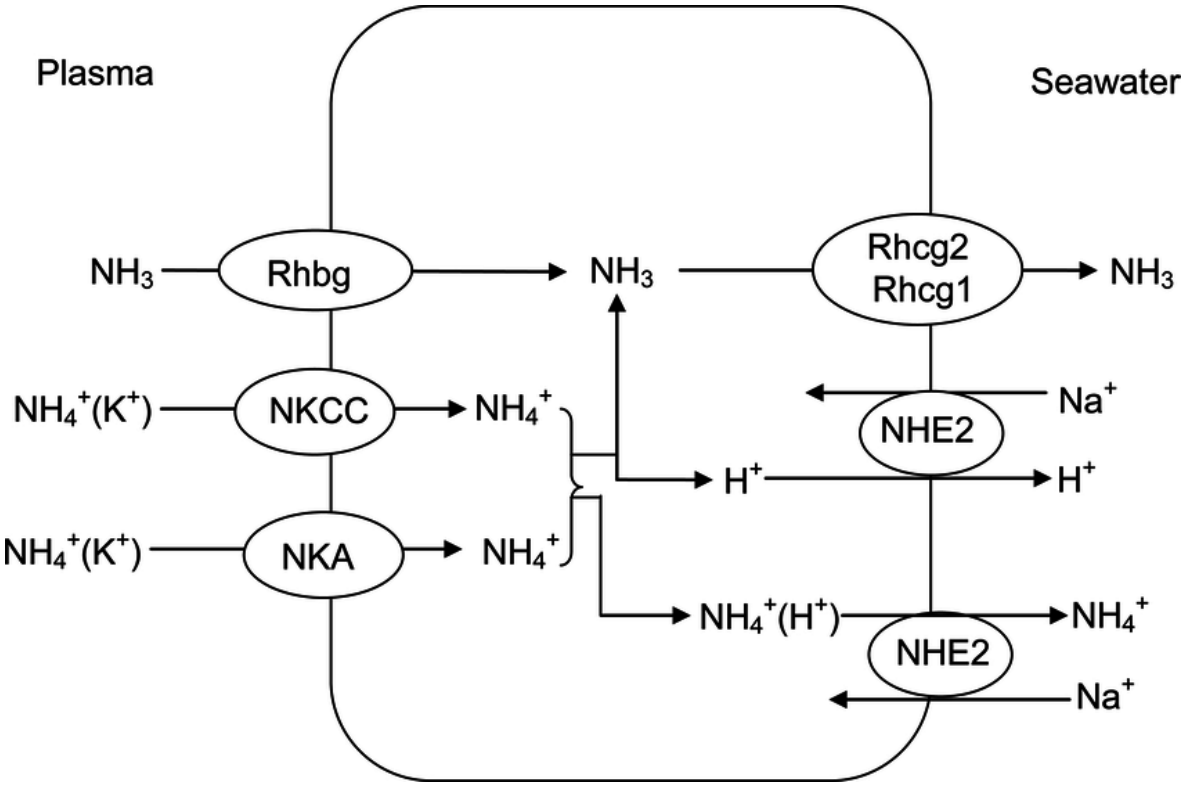

Fig 8. Current hypothesized model of Rh glycoproteins, Rhbg, Rhcg1, and Rhcg2, and ion transporters, NKCC, NKA, and NHE 2 present in the saltwater fish gill. Rhbg, NKCC, and NKA are localized on the basolateral edge of the gill cell, while Rhcg1, Rhcg2, NHE2, and NHEe are localized to the apical edge. These ion transporters and Rh glycoproteins facilitate the movement of ammonia, sodium, potassium, and hydrogen (Ip and Chew, 2010).

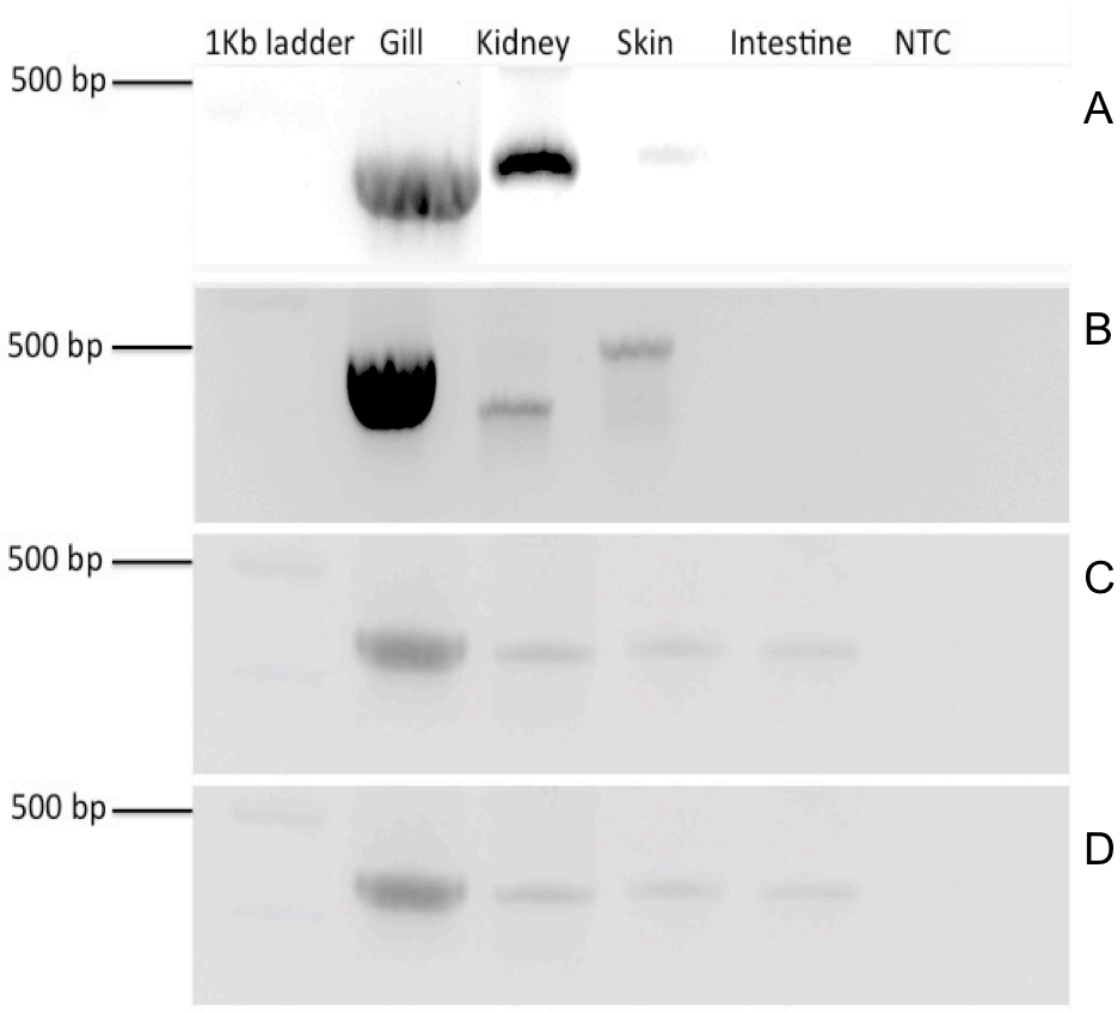

Fig 10. Tissue distribution of Rh proteins isolated through PCR A, Rhag, B Rhbg, C, Rhcg1, and D, Rhcg2 on 1% agarose gels in longhorned sculpin tissues. NTC is a no template control.

5’- GAATTCACTAGTGATTCCACAGGTGTCCTGGATGCCCAGTT

TGGATGCCAGGATGGGAGTCAGGAACTTGAAGCCCAGGGTCGAC

ACGATGCCAGCCACTAATCCAATCAGCATCGCCCCAAATGGCCC

GATGTCCATGTCGGCACATGTTCCCACAGCAACGCCACCGGCCA

AGGTGGCATTCTGAATGTGCACCATGTCCAGTTTTCCTTTGTGCTC

CACAGGCTGGAGATGGCGTAGGCAGAGAGCACGCAGGCAGCCA

GGGAGAGGTAGGTGTTGATCACCGCTGTGAGCTGAGGTAAGCCG

GCATCAGCGATGGCCGAGTTAAAACTGGGCCAGAACATCCACAG

AAAGACGGTTCCAATCATGGCAAACAGATCAGAGTGGTAAACAG

AGCCATCGTTATCATGTCCGTTTTTCAAACTCGGTCGGTAAAGTAC

TCGAGCCACAGCCAGCCCAAAGTAGGCTCCATACGCATGGATGA

TCATGGATGCACCCACGTCACTAGCTCCGAGGATATTGACCACTA

GATGTTCATTGATGGAGAAGATGGTAATCTCCAGTATGGTCATGA

TGAGGAGCTGCACAGGGCTGGTTTTACCCAGAACAGCTCCGAAG

GAGATCAGGACTGTAGCTGTGCTGAAGTCTGCGTTGAATCGAATT

CCCGCGGCC -3’

Fig 11. Longhorned Sculpin Rhag neucleotide sequence (677 bps) with 85% identity to the *Clupea harengus,* Atlantic herring, predicted ammonium transporter Rh type A (XP_012693009.1; gi|831320363|)

5'- GGCCGCGGGAATTCGATTCATCCTCATCATCCTCTTTGGCGT

CCTGGTGCAGTACGACCACGAGACGGACGCCAAGGAATGGCACA

ACCAGACCCACTCTGACTATGAGAACGACTTCTACTTCCGCTACCC

AAGTTTCCAGGACGTGCACGTGATGATCTTCATCGGTTTCGGCTTC

CTCATGACCTTCCTGCAGCGCTACGGCTTCAGCAGCGTGGGCTTCA

ACTTCCTGATCGCAGCCTTCTCCATTCAGTGGGCAACGCTCATGCA

GGGCTTCTTCCACGGCATGCATGGAGGCAAGATCCACATCGGGGT

GGAGAGCATGATCAATGCTGATTTCTGCACCGGCGCTGTGCTCATC

TCGTTCGGAGCCGTTTTGGGTAAAACCAGCCCCGTCCAGCTGCTGG

TCATGGCGATATTCGAGGTCACGCTGTTTGCTGTCAATGAGTTCGT

CCTGCTGTCCGCTCTTGGGGCTAAAGATGCAGGAGGCTCCATGAC

CATCCACACCTTTGGAGCCTACTTCGGCCTCATGGTGACCCGAGTC

CTGTACCGGCCCAACCTGAACAAGAGCAAACACAGGAACAGCTCG

GTGTACCATTCTGACCTGTTTGCTATGATCGGCACCGTCTACCTGTG

GATGTTCTGAATCACTAGTGAATTC- 3'

Fig 12. Longhorned Sculpin Rhbg neucleotide (661 bps) with 89 % identity to the *Larimichthys crocea*, large yellow croaker, predicted ammonium transporter Rh type B isoform X1 and X2 (XP_010746197.1; gi|734635239|), and 92% identity to Takifugu rubripes, Japanese pufferfish, ammonium transporter Rh type B (XP_011617147.1; gi|768957319|)

5'- TTGCACACTGTTCCTGTGGATGTTCTGGCCCAGCTTCAA

CTCGGCCGTCACAGACCACGGGGACGGGCAGCACCGAGCA

GCCCTGAACACCTACCTGGCTTTGGCCTCGACTGTGCTCACT

ACTGTGGCGCTCTCCAGCCTCTTCCAGAAGCACGGAAAACT

AGACATGGTCCACATCCAGAACGCCACTCTTGCTGGAGGTG

TTGCTGTAGGAACTGCAGCAGAGTTCATGCTGATGCCCTACG

GGTCTCTGATCGTAGGATTCTGCTGTGGCATCATCTCCACGC

TGGGATATGTCTACCTCACGCCTTTCATGGAGAAGCACCTGA

AGATCCAGGACACGTGTGGAATCCATAACCTGCATGCCATG

CCCGGCGTCATAGGTGGCATCGTGGGAGCCATTACTGCCGC

GTCTGCAACAGAGTCTGTTTATGGTATTGAGGGGCTCAGGAA

CACCTTTGACTTTGAGGGTGATTTCAAAGACATGTTACCCAC

ACGCCAGGGTGGTCTCCAGGCTGCGGGCCTTTGTGTGGCCA

TCTGCTTCGGTGTGGGTGGAGGTATCCTTGTCGGTTGTATTTT

AAGATTACCTATCTGGGGAGATCCTGCTGAA - 3'

Fig 13. Longhorned Sculpin Rhcg neucleotide and protein sequence (610 bps) with 91% identitity to *Cynoglossus semilaevis*, tongue sole Rhcg1.

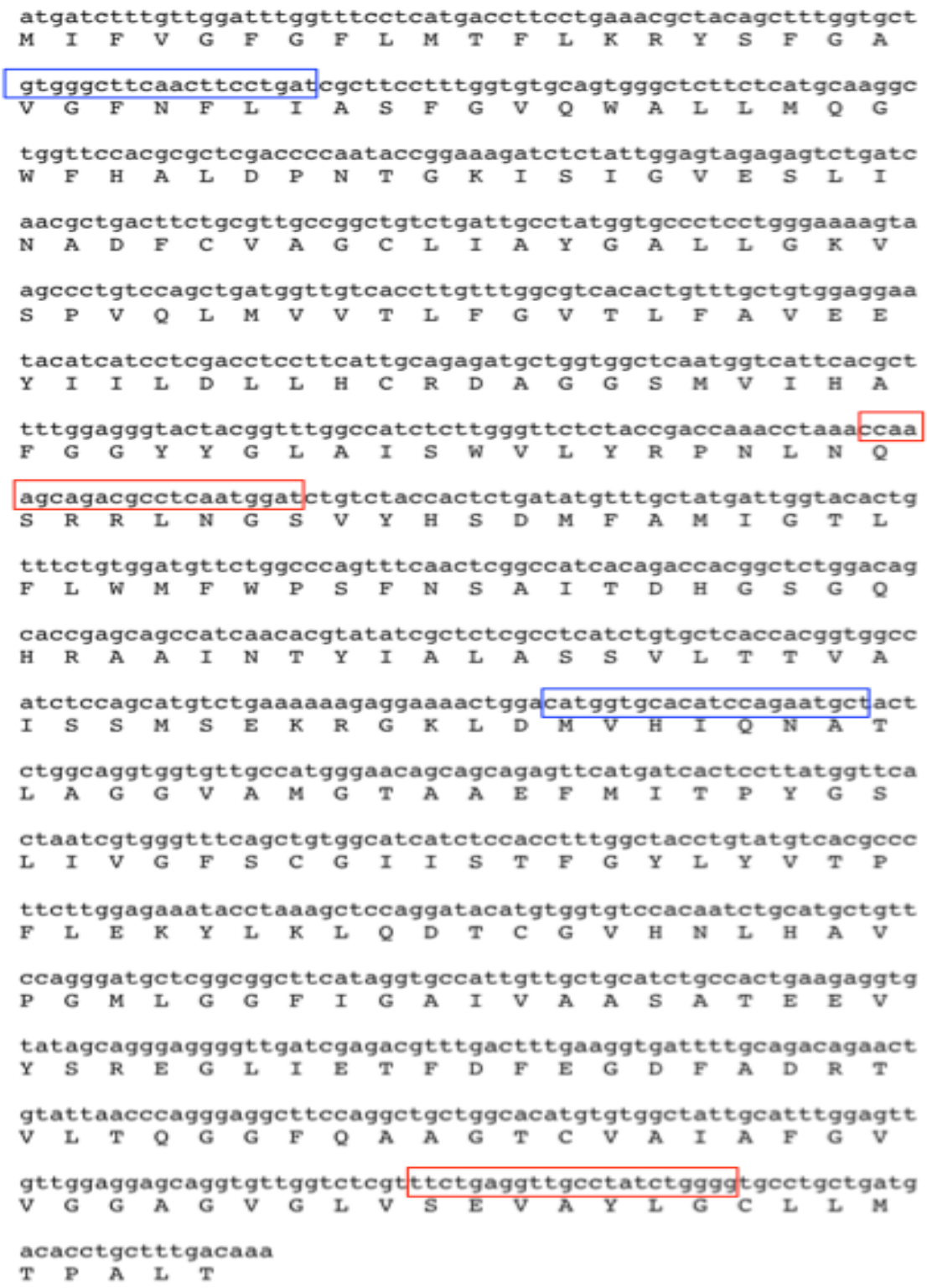

Fig 14. Longhorned Sculpin Rhcg2 nucleotide (1097 bps) and amino acid (365) sequence with 89% identity to *Cynoglossus semilaevis*, tongue sole (XP_008308471.1; gi|657746415|) Sculpin Primers Sculp115F/Sculp711R Shown in Blue, Sculp472F/ Sculp1122 Shown in Red

5'- GNNNNCCCAAATGCAATC-AAGGCG-
TTGGGAGCTCTCCCATATGGTCGACCTGCAGGCGGCCGCACTAGTGA
TTGGGTCGTCATTGAAACAATACTCGTCCTTAGGCTGAGCCAGAAAG
GGTAGTTTTAATATGAAGCCAGTGATGGTCCCTCCTAGGACAGCGAT
GCCCAAACAGACTCCGATAGCTGCCGCCTGGTACTGTGCCTGATCCCA
AGCGCTGCGGCCACCTCCAGCCTTCAGCTGCGGCAGCAATCGGACCAA
TTCGACCAGCTTCCTGTCCCCTTCCTTGGGGACCCTCTCTGGGAAGGT
GTCATACAAGCCCTGGCCATATCTCTCGTCGGCTGTTAACAGGATAG
TGGCAATGCCGGCAATCGCCCCGATAAAGCCGGGGATCCCGTGCAAG
TTGTTGATGCCGCACACGTCTTGGATTTTGAGTCTCTTGGAATCCCG
CGGCCATGGCGGCCGGGAGCATGCGACGTCGGGCCCAATTCGCCCTAT
AGTGAGTCGTATTACAATTCACTGGCCGTCGTTTTACAACGTCGTGA
CTGGGAAAACCCTGGCGTTACCCAACTTAATCGCCTTGCAGCACATCC
CCCTTTCGCCAGCTGGCGTAATAGCGAAGAGGCCCGCACCGATCGCCC
TTCCCAACAGTTGCGCAGCCTGAATGGCGAATGGACGCGCCCTGTAG
CGGCGCATTAAGCGCGGCGGGTGTGGTGGTTACGCGCAGCGTGACCG
CTACACTTGCCAGCGCCCTAGCGCCCGCTCCTTTCGCTTTCTTCCCTT
CCTTTCTCGCCACGTTCGCCGGCTTTCCCCGTCAAGCTCTAAATCGGG
GGGCTCCCTTTAGGG-TTCCGATTTAGTGCTTTACGG-
CACCTCGACCCC--AAAAAACTTGATT--AGGGTGATGGGTTCACGT-
AGTGGG-CCATCGCCCT--
GATAGACGGGTTTTTCGCCCTTTGACGTT- 3'

Fig 15. Spiny dogfish Rhp2 nucleotide sequence (1040 bp) with identity to 91% identity with the *Triakis scyllium,* banded hound shark, rhesus glycoprotein P2 (BAI49727.1; gi|269913342|).

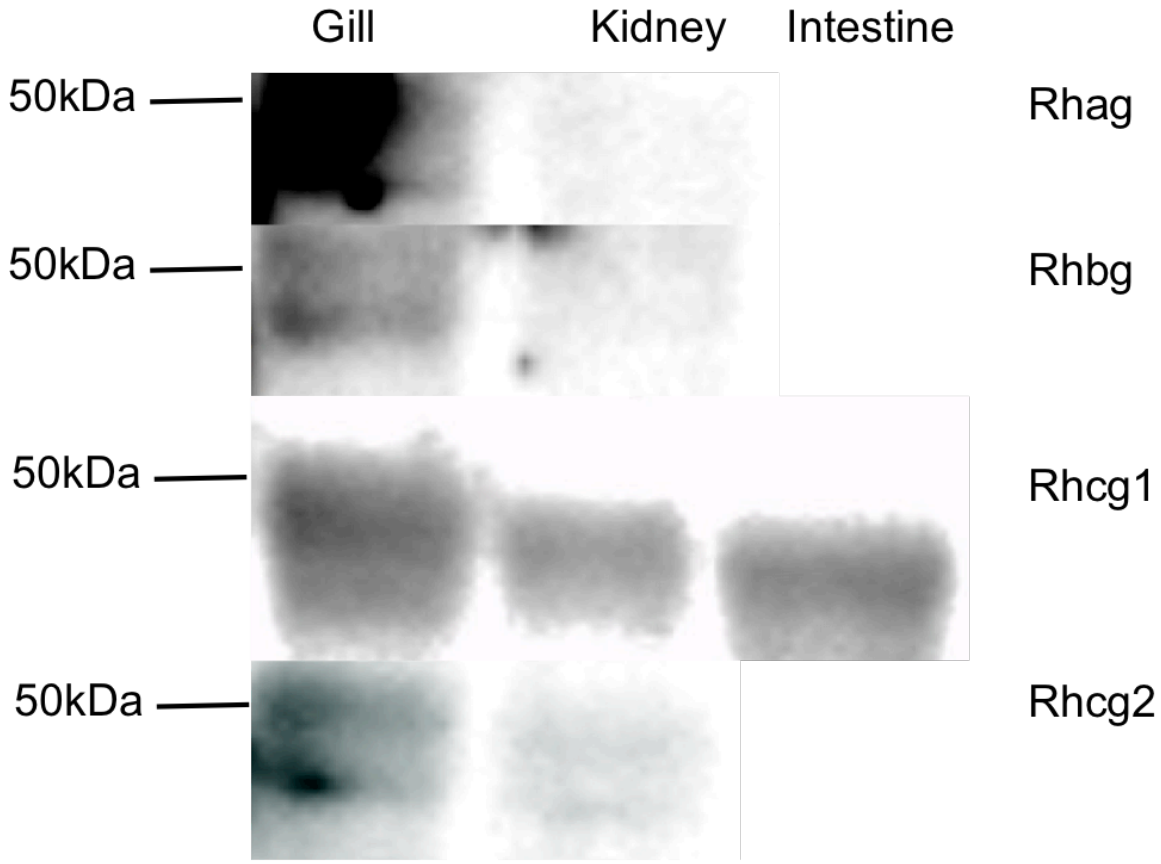

Fig 16. Western Blot analysis of Rhesus glycoproteins in epithelial tissues of longhorned sculpin, A, Rhag, B Rhbg, C, Rhcg, and D, Rhcg2.

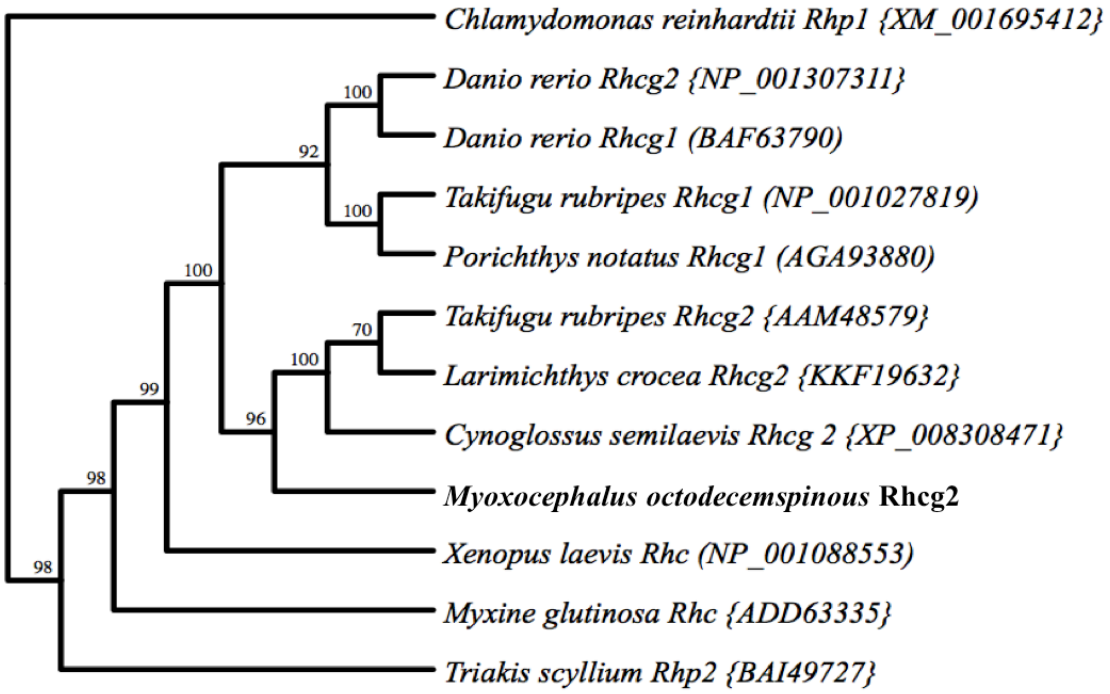

Fig 17. Phylogenetic tree constructed utilizing the neighbor-joining method (bootstrapped with 10,000 replications) of various Rh proteins along with the isolated *M. octodecemspinous* Rhcg2. The tree is rooted using the green algae *Chlamydomonas reinhardtii* Rhp1 Accession number (XM_001695412). Bootstrap confidences and absolute number of differences are shown on the branches, while accession numbers are present in parenthesis on the right.

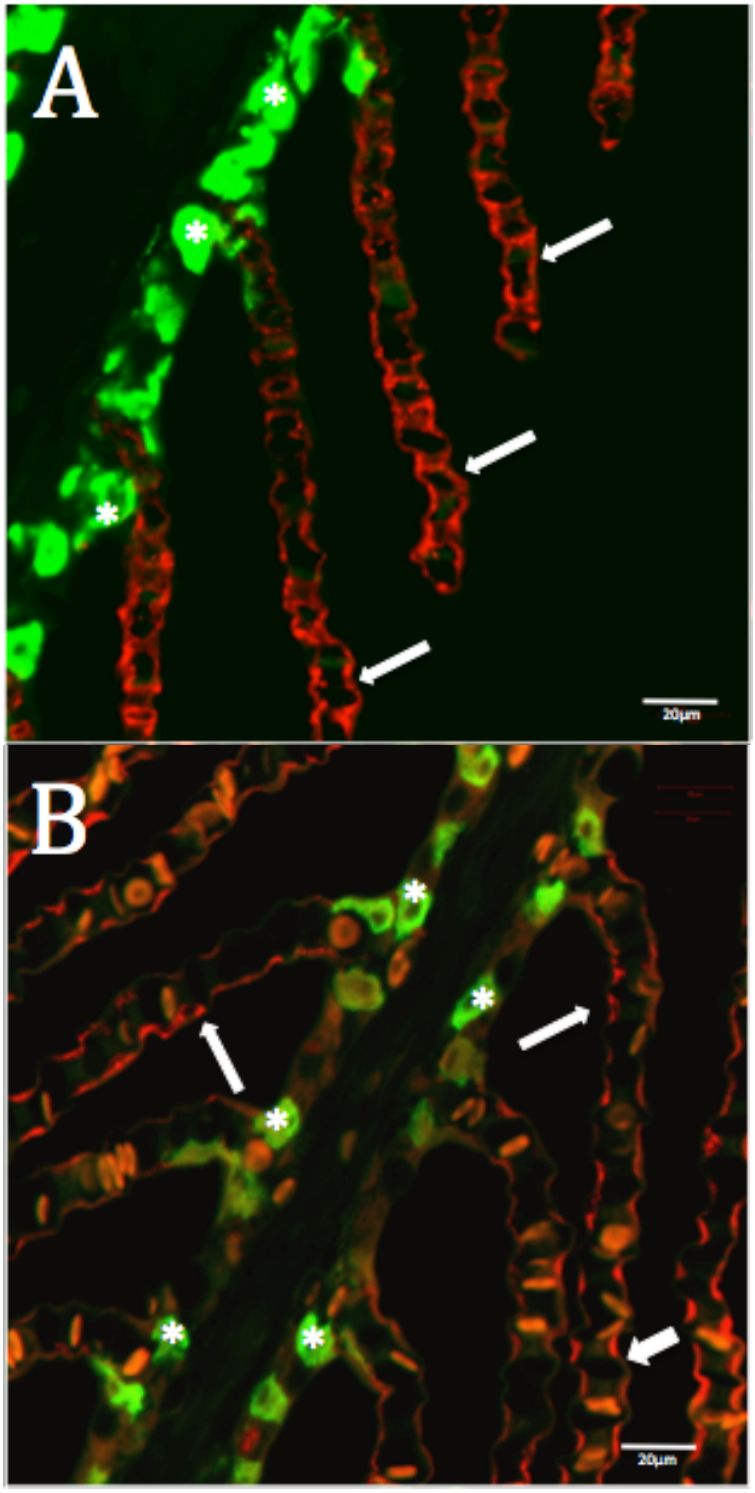

Fig 18. Representative confocal image demonstrating colocalization of NKA, in green, and Rhag(A) and Rhbg(B) in *M. octodecemspinos (*long horned sculpin) gill epithelium. Arrows denote areas of specific Rh glycoprotein expression while asterisks denote iontransporter expression in ionocytes. White Bar = 20µm.

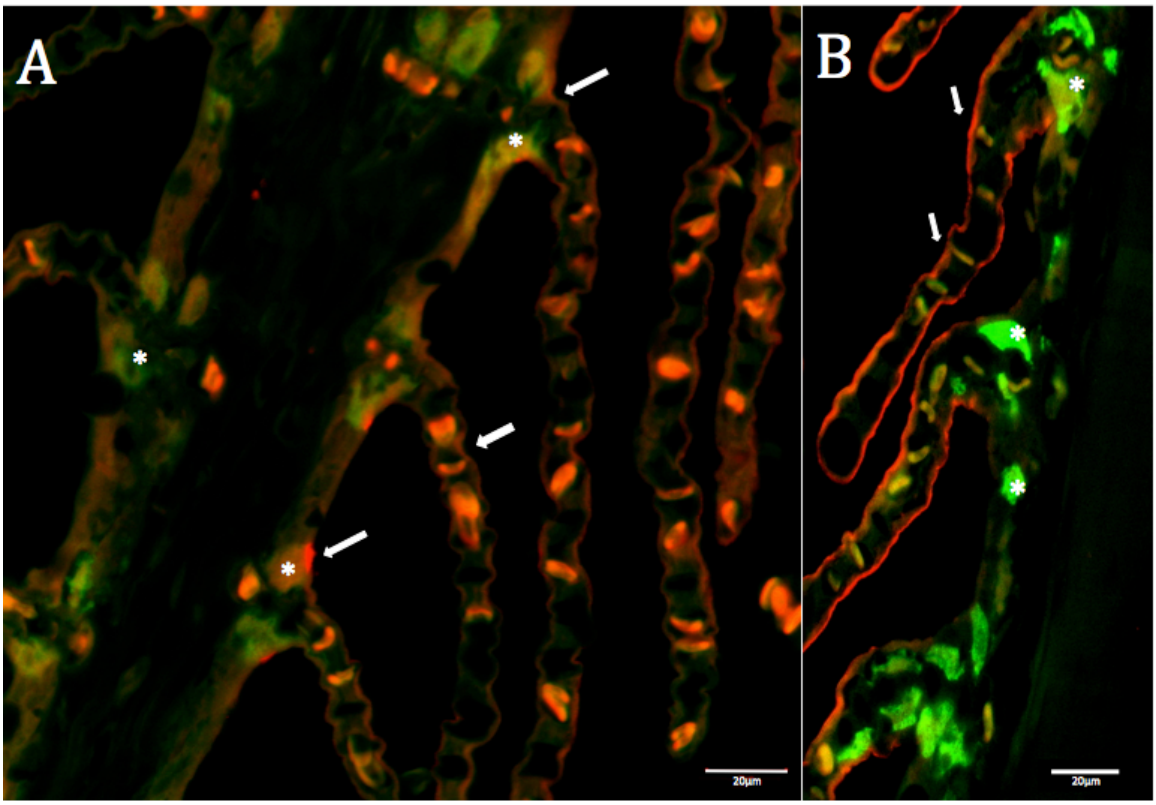

Fig 19. Representative confocal image demonstrating colocalization of NKA, in green, and takifugu Rhcg1(A) and takifugu Rhcg2(B) in *M. octodecemspinos*n gill epithelium. Arrows denote areas of specific Rh glycoprotein expression while asterisks denote iontransporter expression in ionocytes. White Bar = 20µm.

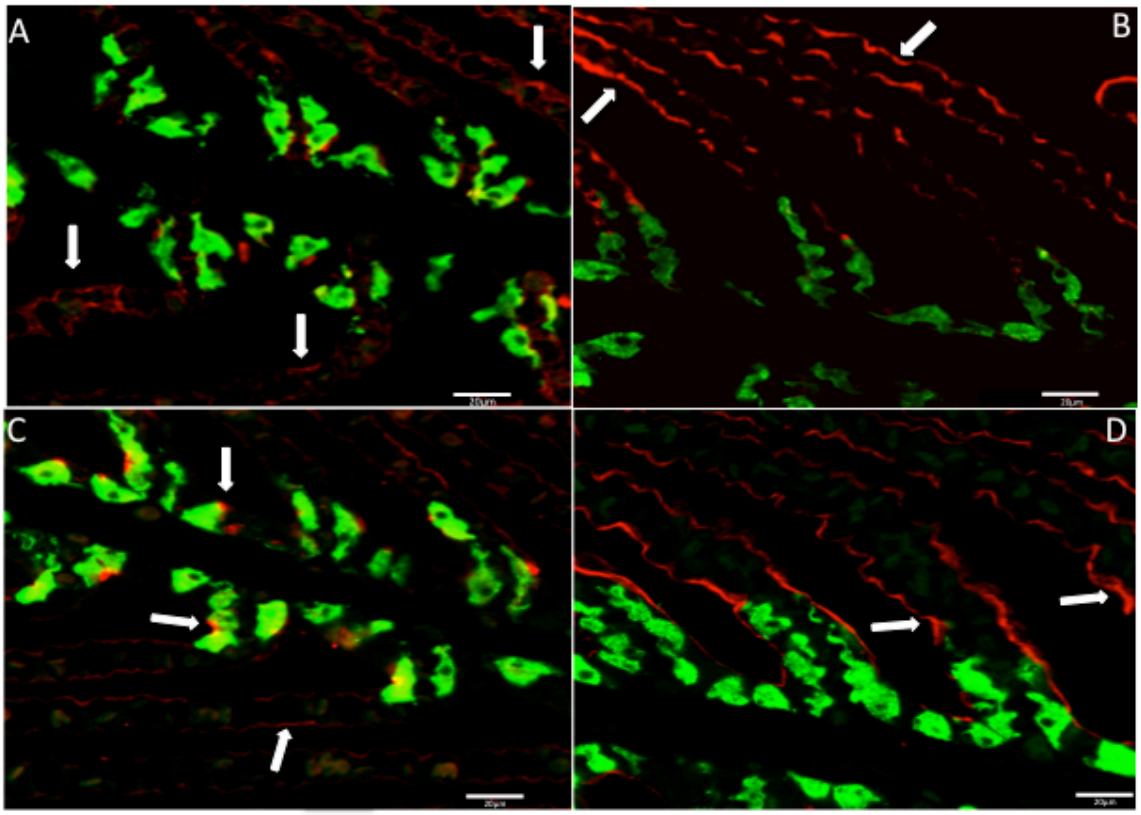

Fig 20. Representative confocal image demonstrating colocalization NKCC, in green, and takifugu Rhag (A), Rhbg (B), Rhcg1(C), Rhcg2(D) in *M. octodecemspinos* gill epithelium. Arrows denote areas of specific Rh glycoprotein expression while asterisks denote iontransporter expression in ionocytes. White Bar = 20μm.

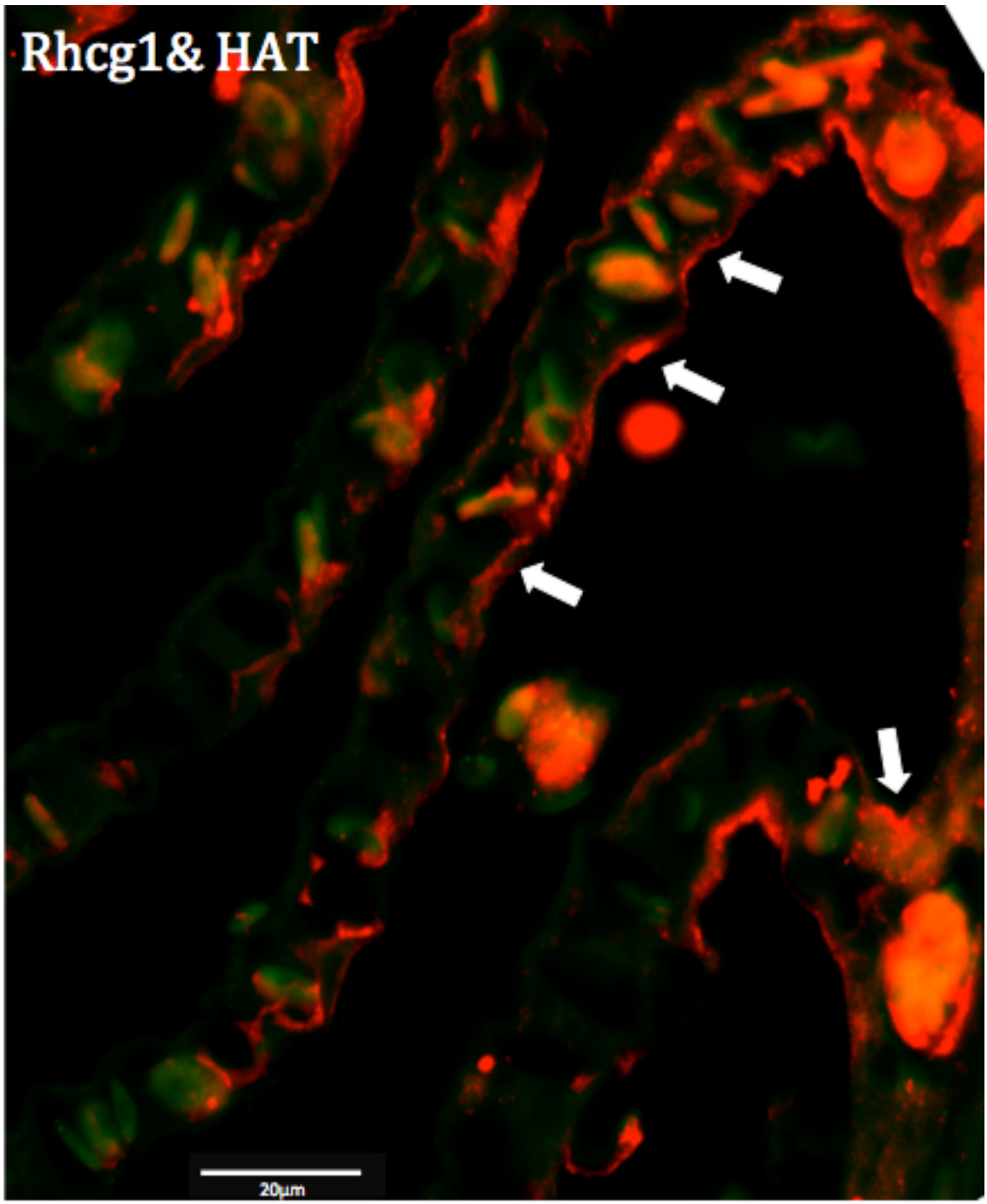

Fig 21. Laser Scanning confocal imagery of *M. octodecemspinos* gills colocalization of takifugu Rhcg1 (1:250) in red with sculpin HAT B (1:250) in green. Arrows indicate areas of Rhcg1 immunoreactivity. White bar = 20µm.

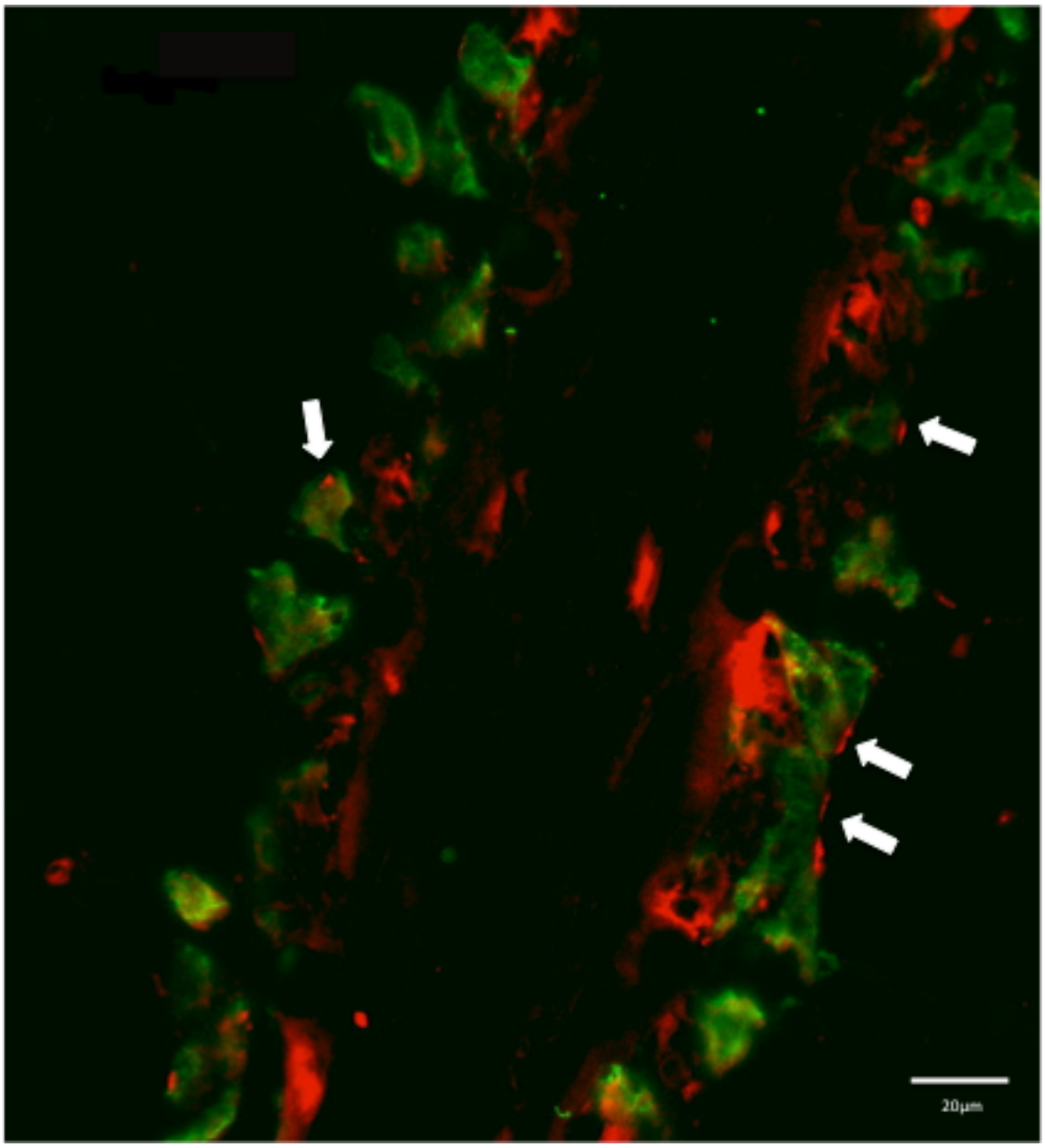

Fig 22. Laser Scanning confocal imagery of ammonia treaed *M. octodecemspinos* gills. A gill stained as a negative control for Alexa Fluor® 568, B gill stained as a negative control for Alexa Fluor® 488, C colocalization of sculpin NHE 3 (1:250) in red with NKA (1:250) in green. Arrows indicate areas of NHE 3 immunoreactivity. White bar = 20µm.

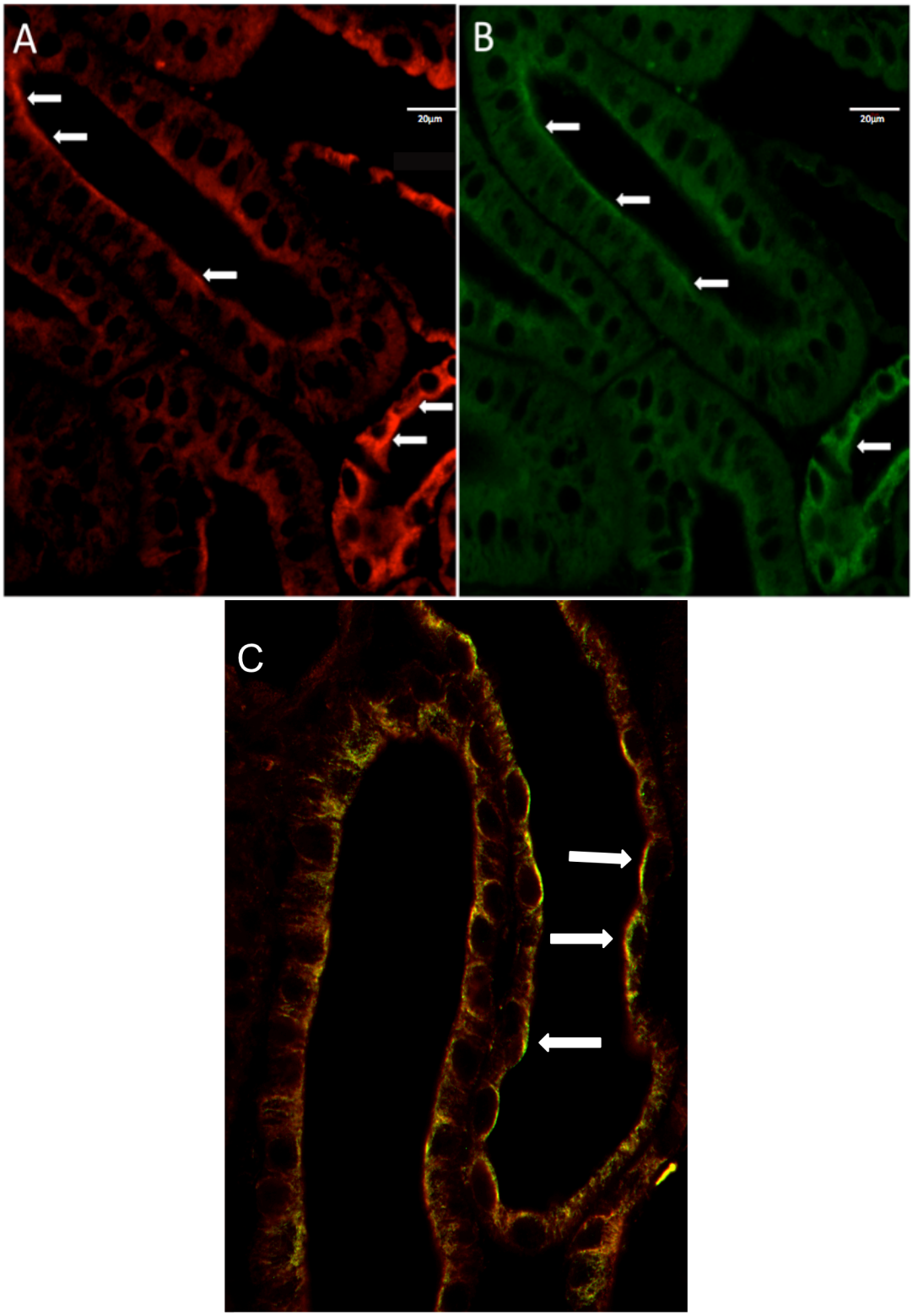

Fig 23. Representative confocal image demonstrating hagfish Rhcg, in red, and either dogfish HAT, B, or NHE 2, C in green in the renal tubules of *S. acanthias* kidney. Arrows denote areas of immunoreactivity of Rhcg, A, HAT, B, or Rhcg and NHE 2 C. White Bar = 20µm.

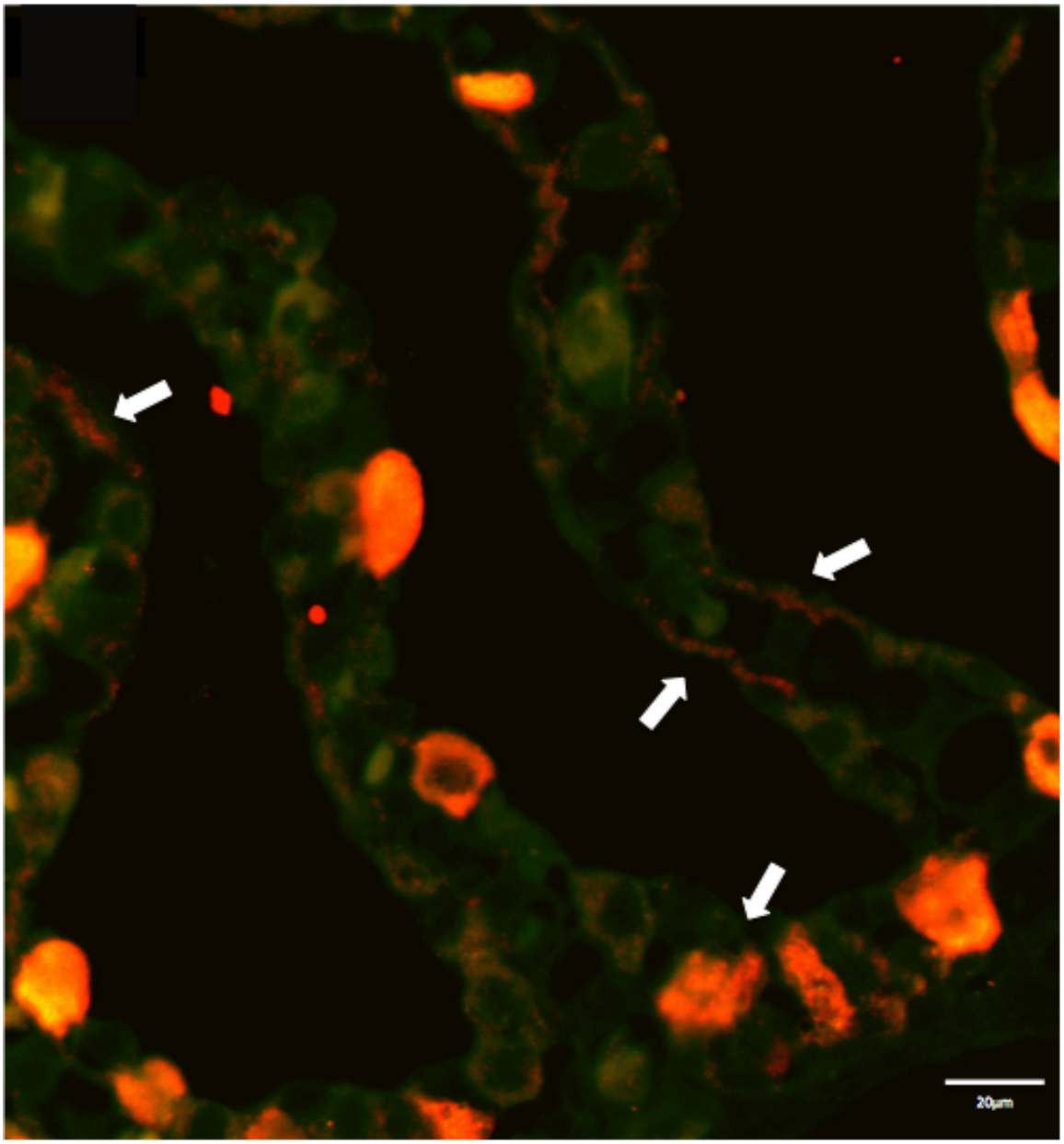

Fig 24. Laser scanning confocal imagery of fluorescently stained *S. acanthias* gills. colocalized takifugu Rhcg1 in red (1:250) and dogfish HAT in green (1:250). Arrows indicate regions of Rhcg1 immunoreactivity. White Bar = 20µm.

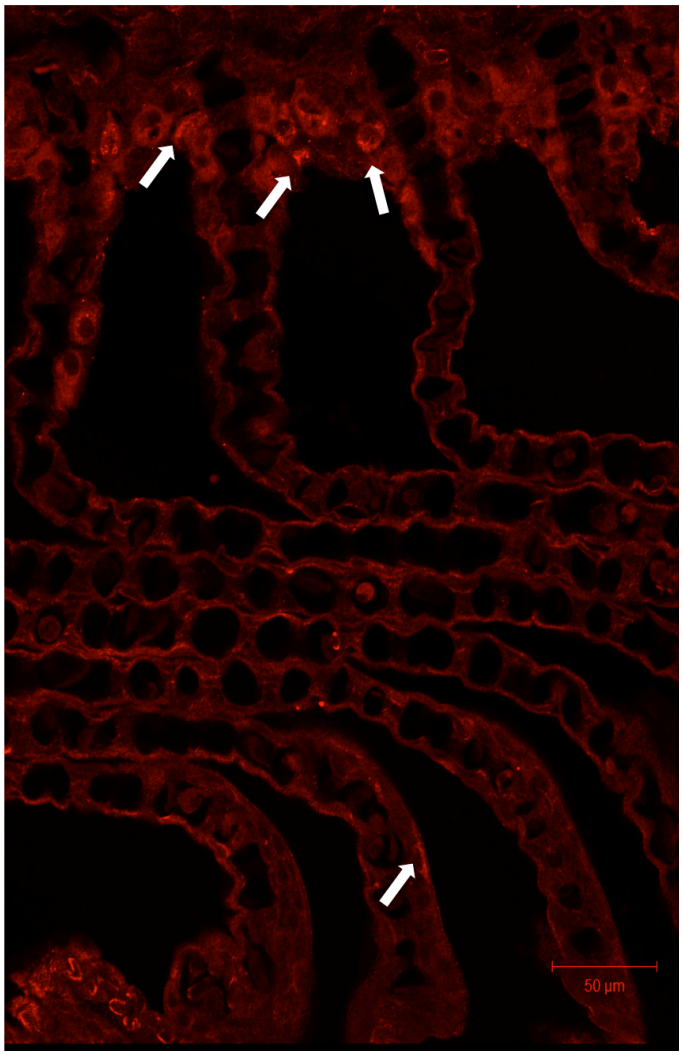

Fig 25. Laser scanning confocal microscopy images of fluorescently labeled hagfish Rhcg, in red, in *S. acanthias* gill epithelium (Edwards, unpublished data 2014). Red bar = 50µm.

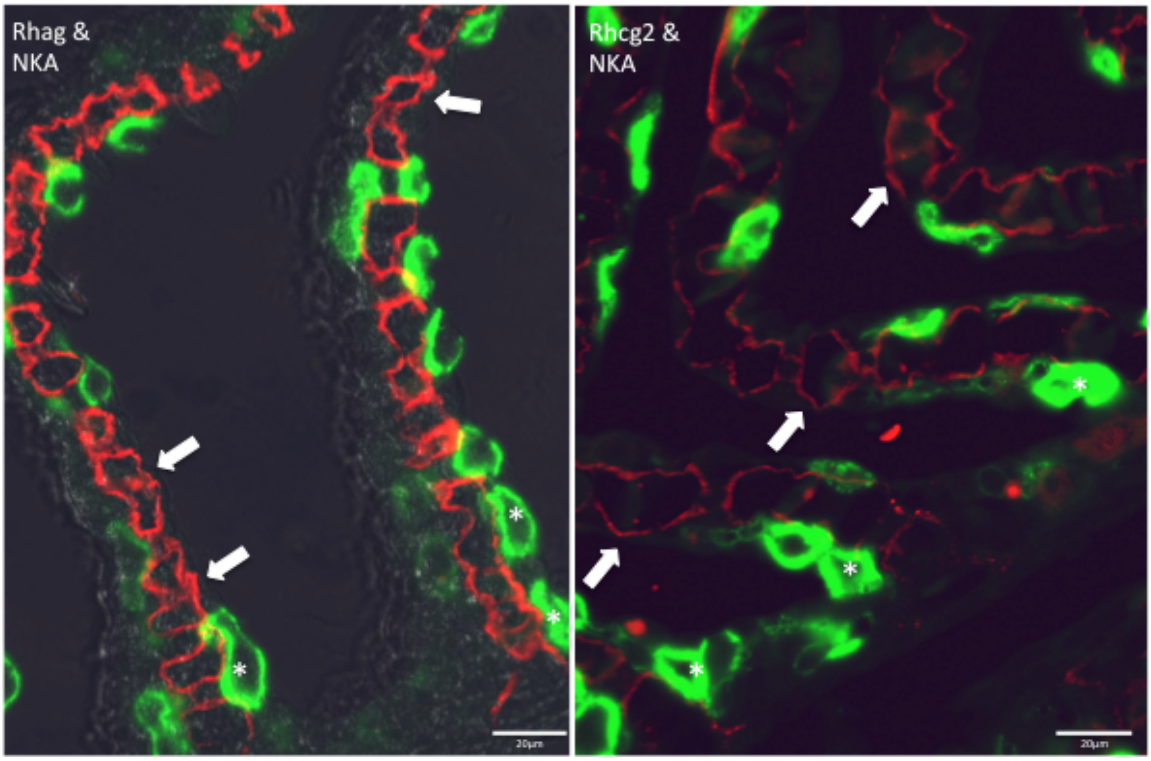

Fig 26. Laser scanning confocal microscopy of fluorescently labeled *S. acanthias* gills A Rhag in red, and NKA in green, B, Rhcg2 in red and NKA in green. Arrows denote areas of immunoreactive Rh expression, while asterisks denote NKA expression in ionocytes. White Bar = 20µm.

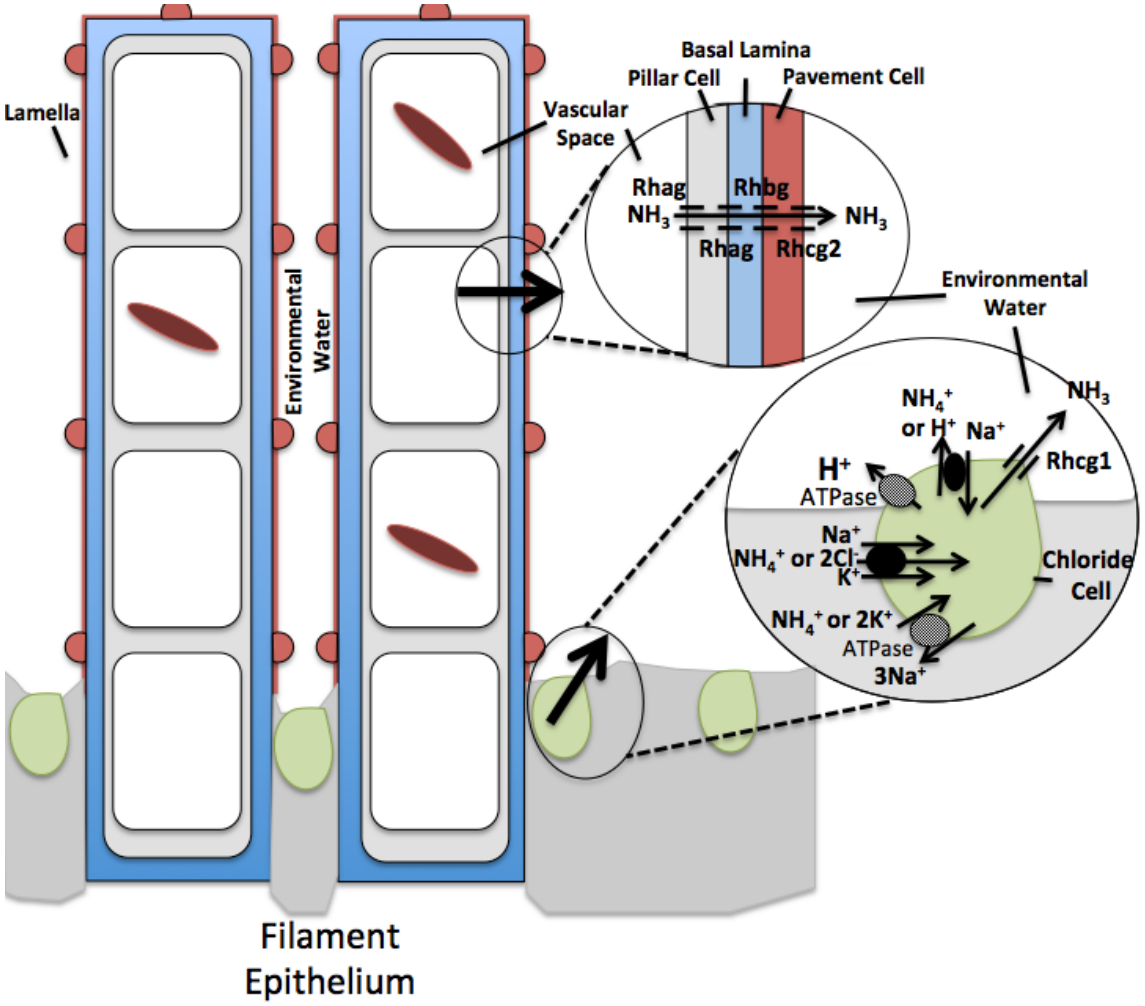

Fig 27. The hypothesized model of ammonia excretion via Rh proteins and ion transporters, NHE3, NKCC, and NKA, in the gill epithelium of *M. octodecemspinous* (Adapted from Weihrauch et al. 2009 and Nakada et al. 2007).

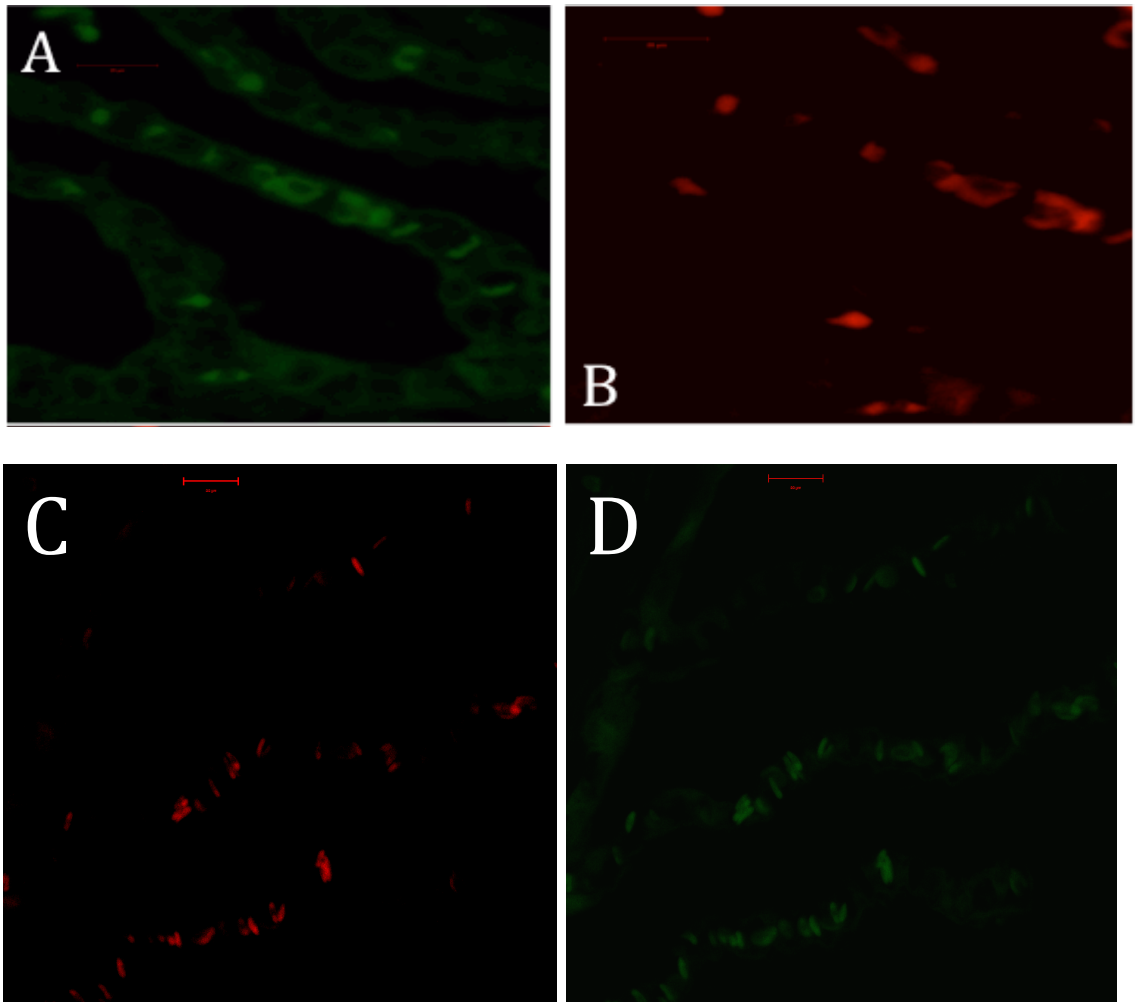

Fig 28. Laser scanning confocal imagery of negative control slides. A, negative control with alexa flora 488 in *S. acanthias* gill, B negative control with alexa flora 568 in *S. acanthias* gill, C negative control with alexa flora 488 in *M octodecemsinous* gill, D negative control with alexa flora 568 in *M octodecemsinous* gill.

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Vita

Laura Victoria Ellis was born in California to parents Gary and Judy. Due to her father's career as a search and rescue pilot in the United States Coast Guard, Laura and her family moved often until his retirement to the mountain of North Carolina. Laura grew up dancing, reading, and being involved in anything scientific. She fell in love with science at a young age and still finds it to be her greatest passion. Her dancing career led her to recently becoming a champion Irish Step dancer in the Drake School of Irish Dance. Her love of dance has let to many travels with her mother along the eastern seaboard for dance competitions.

While in high school in Swannanoa, she came across University of North Carolina Asheville's Biology program and began her journey in science. After graduating with a Bachelors Degree in Ecology and Evolutionary Biology from UNC Asheville, She continued her exploration of zoology and physiology at Appalachian State University under Dr. Susan Edwards. While at Appalachian, Laura found her true place in the scientific field of physiology. To balance out the demands of the lab, Laura maintains her championship status in dance, while also teaching biology to non-majors. Dr. Edwards was instrumental in pushing Laura towards her goals of a Ph.D. in Biology and she can never thank her enough for her help, mentoring, being a fabulous role model, and her part in molding Laura into the scientist she has become.

In Fall 2016 Laura will begin her journey in her Ph.D. in Cellular and Molecular Biology at The University of Arkansas. She could never have imagined how far her journey in science has taken her, and she can't thank each person enough who helped her along her path to get here.