DIFFERENTIAL EXPRESSION OF RH GLYCOPROTEINS IN THE SEA LAMPREY, (*Petromyzon marinus*)

A Thesis
by
SALVATORE DONALD BLAIR

Submitted to the Graduate School
Appalachian State University
In partial fulfillment of the requirements for the degree of
MASTER OF SCIENCE

May 2011
Department of Biology

DIFFERENTIAL EXPRESSION OF RH GLYCOPROTEINS IN THE SEA LAMPREY, (*Petromyzon marinus*)

A Thesis
by
SALVATORE DONALD BLAIR
May 2011

APPROVED BY:

_____
Susan L. Edwards, Ph.D.
Chairperson, Thesis Committee

_____
Ted Zerucha, Ph.D.
Member, Thesis Committee

_____
James B. Claiborne, Ph.D.
Member, Thesis Committee

_____
Steven W. Seagle, Ph.D.
Chairperson, Department of Biology

_____
Edelma D. Huntley, Ph.D.
Dean, Research and Graduate Studies

Abstract

DIFFERENTIAL EXPRESSION OF RH GLYCOPROTEINS IN THE SEA LAMPREY, (*Petromyzon marinus*)

(May 2011)

Salvatore Donald Blair, B.S., Appalachian State University

M.S., Appalachian State University

Chairperson: Susan L. Edwards, Ph.D.

Ammonia is a by-product of protein metabolism. It is a nitrogenous waste that if not excreted, its accumulation is toxic. The exact mechanism by which ammonia is excreted has been a point of controversy for many years. Rh glycoproteins have been recently linked to ammonia excretion in a broad range of organisms, including the fishes. It has been shown that Rh isoforms; Rhag, Rhbg, Rhcg1, and Rhcg2 have been localized in the gills of the teleost, *Takifugu rubripes* and mediate the transport of an ammonia analogue (methylammonium) in vitro. Very little is known about the ability of the more primitive fishes, in particular the agnathans to regulate nitrogenous wastes. In this study we have used immunohistochemistry and western blot analysis to localize several members of the Rh glycoprotein family to various epithelial tissues of the lamprey *Petromyzon marinus*.

Dedication

To my loving parents, Michael and Antoinette Blair

Acknowledgments

Throughout my time here at Appalachian State University and especially in the last two years put towards attaining a Masters Degree there have been numerous people that have supported me. I am forever grateful for the supervision, mentorship, and friendship provided by Dr. Sue Edwards who first introduced me into the path I have chosen to pursue. Without the positive attitude and unwavering encouragement by Sue, none of this would have been possible. I wish to thank my committee members: Dr. Ted Zerucha and Dr. JB Claiborne for their advice and assistance throughout this process. My appreciation also extends to the many exceptional faculty members in the Department of Biology who have made my experience here truly enjoyable by providing superior guidance surpassing the duties of the everyday "Professor": Dr. Gary Walker, Dr. Michael Windelspecht, Betsy Harris, Dr. Terry Carroll, and especially Dr. Jeff Butts for always having an open door, offering considerate advice, and providing a truly treasured friendship that I am honored to hold. My appreciation for microscopy assistance goes out to Dr. Guichuan Hou. I would also like to thank the many people assisting in lab, Ricky de Triquet for lab assistance, comic relief, and overall good times in and out of the country. To Caroline Cochrane and Brian Mikeworth, my many thanks for providing a helpful hand and conversation during long hours in the lab. I owe retention of my sanity to my friends and teammates on the ASU Ice Hockey team. I also wish to express my appreciation towards the Department of Biology and the Graduate School at ASU for their endless support. Funding is a major part of making this experience a reality

and I am very grateful for the support by the National Science Foundation- Research Experience Undergraduate Fellowship, Office of Student Research- Research and travel grants, a Graduate Teaching Assistantship, Edgar Green Scholarship, and the Graduate Research Associate Mentoring Program. Above all however, is my priceless gratitude towards my parents, family, and friends for their unconditional love and support throughout this experience.

Table of Contents

Abstract……iv

Dedication……...……...……v

Acknowledgements……..…….....vi

List of Tables……....ix

List of Figures……....x

Introduction……1

Methods……18

Results……..…25

Discussion……30

Tables…….....40

Figures…….....41

References……67

Biographical Sketch…….....73

List of Tables

Table 1. Primers used for cDNA clone sequencing……..51

List of Figures

Figure 1 Model describing Rh glycoprotein localization in the gill epithelium of the pufferfish *Takifugu rubripes*……...…….52

Figure 2 Model illustrating how the Rh proteins facilitate ammonia excretion…….……53

Figure 3 Western blot control…….……....54

Figure 4 Western blot anadromous lamprey……...…….……...…55

Figure 5 Western blot combined Great Lakes landlocked lamprey……56

Figure 6 Hematoxylin and eosin stained cross section of gill and skin from the lamprey….57

Figure 7 Immunohistochemistry of transformant skin sections……..58

Figure 8 Immunohistochemistry of Rhag in the skin of landlocked and anadromous transformants……59

Figure 9 Immunohistochemistry of Rhbg in the skin of landlocked and anadromous transformants……...…….60

Figure 10 Immunohistochemistry of Rhcg1 in the skin of landlocked and anadromous transformants…….……...….61

Figure 11 Immunohistochemistry of Rhcg2 in the skin of landlocked and anadromous transformants…….……...62

Figure 12 Immunohistochemistry of transformant gill sections…….……63

Figure 13 Double labeling immunohistochemistry of Rhag and Na^+/K^+-ATPase (NKA).....65

Figure 14 Immunohistochemistry of Rhcg1 in transformant gill sections……...…..66

Figure 15. Immunohistochemistry of Rh glycoprotein expression in gills of GL landlocked ammocoetes……..67

Figure 16 Immunohistochemistry of Rhag localized in skin of GL landlocked ammocoete…….……..68

Figure 17 Immunohistochemistry of GL landlocked ammocoete skin sections……..……69

Figure 18 Immunohistochemistry of Rhag and Rhbg in GL landlocked parasite gill sections…….70

Figure 19 Immunohistochemistry of Rh expression in the skin of GL landlocked parasites……71

Figure 20 Lamprey Rhbg nucleotide sequence (663 bp)……72

Figure 21 Lamprey Rhcg nucleotide sequence (1010 bp)……...……..73

Figure 22 Physiological results of *in vivo* nitrogen waste excretion in GL landlocked lamprey……74

Figure 23 Western blot quantification analysis of GL landlocked gill……..75

Figure 24 Western blot quantification analysis of GL landlocked gill…….……76

Figure 25 Overall Rh expression comparison between GL landlocked transformant skin...77

Figure 26. Model of hypothesized ammonia excretion across the skin epithelium……...78

Introduction

It has recently been established that members of the Rhesus (Rh) glycoprotein family are capable of transporting ammonia in a wide range of organisms (Weihrauch et al., 2009; Wright and Wood, 2009). Our current knowledge relating to the Rhesus glycoproteins can be traced back to the late 1930s, in which an unlikely discovery in the field of hematology would launch a new direction of research for the years to come (Levine and Stetson, 1939). Most recently researchers have examined the evolutionary lineage of Rh glycoproteins. Several studies identified the basic structure, function, specific cellular and tissue locations, and most significantly Rh glycoproteins role in organismal ammonia regulation.

Ammonia

Ammonia (total ammonia NH_3 and NH_4^+) is one of the three significant forms of nitrogenous wastes which are excreted by animals, the others being urea and uric acid (Wright and Wood, 2009). All three forms are products resulting from the metabolic breakdown of proteins, during which excess amino acids not utilized for protein production are catabolized into ammonia. This ammonia can then be either directly excreted or converted into a less toxic form. The alternate to ammonia excretion is via conversion of ammonia to urea or uric acid is an energetically costly metabolic process however some animals must invest for this ammonia conversion (Wright, 1995). Mammals and birds are

highly sensitive to ammonia, therefore must excrete the end product of the protein catabolism as urea and uric acid respectively. Unlike terrestrial vertebrates, fish are much more tolerant to ammonia having the ability to withstand higher internal plasma ammonia levels (Wright, 1995). Furthermore, fish have retained the ability to excrete the majority of nitrogenous wastes directly as ammonia (Wright, 1995).

Primary production of ammonia in fish takes place in the liver, with limited production occurring in the kidney, muscle, and intestine (Mommsen and Walsh, 1992). The majority of teleost fish including most actinopterygians and agnathans excrete up to 90% of their nitrogenous wastes as ammonia (Braun et al., 2009a; Evans et al., 2005). In fact the majority of fish are ammonotelic, in that they excrete nitrogenous wastes predominantly as ammonia where as some fish are ureotelic such as the elasmobranches that excrete nitrogenous wastes predominantly as urea. This was supported in the study that demonstrated that the spiny dogfish excreted greater than 90% of the nitrogen wastes as urea (Wood et al., 1995). An example of a strictly ureotelic fish is the Lake Magadi tilapia, in which ammonia excretion is totally absent. These animals excrete their nitrogenous waste as urea. Urea excretion is facilitated by the presence of the ornithine-urea cycle (OUC), which converts ammonia into the less toxic form urea. The Lake Magadi tilapia have been shown to have significant levels of OUC enzymes in the liver (Wood et al., 1989).

Ammonia production resulting from amino acid metabolism, can result in accumulation of ammonia and if not excreted may become toxic. When internal ammonia levels become toxic due to an inability to excrete the excess, this results in disruptive effects to the central nervous system (Binstock and Lecar, 1969), decreases oxidative metabolism (Arillo et al., 1981), or impair oxygen delivery(Arillo et al., 1981) and in severe cases results

in death (Wilkie, 1997; Wright, 1995). Many of these effects are likely due to the ability of NH_4^+ ions to replace K^+ ions in epithelial transporters resulting in disruption of electrochemical gradients (Binstock and Lecar, 1969; Evans et al., 2005). The threat of toxicity and its adverse effects suggest that mechanisms must be in place in order for fish to ensure ammonia is unable to accumulate.

The site of ammonia excretion in fish is primarily across the gill epithelial cells, along with other gas and ion exchanges (Evans et al., 2005). The exact mechanism of ammonia transport and excretion has yet to be agreed upon, however several hypotheses and models have been described (Evans et al., 2005). Models of ammonia transport include: 1) NH_3 diffusion through or between epithelia cells down a concentration gradient, 2) NH_3 trapping involving proton excretion that resulted in NH_4^+ ions outside of the cell, 3) transport of ammonia via a NHE (sodium hydrogen exchanger) and/or Na^+-K^+-ATPase, and 4) NH_4^+ diffusion through leaky junctions between mitochondrial rich cells (MRCs) and accessory cells (Evans et al., 2005). These are still possible routes of excretion, however Rh glycoprotein association with ammonia excretion is gaining strong support among the ion-regulation community (Wright and Wood, 2009).

Rhesus ammonia connection

Rhesus history

An initial study by Levine and Stetson in 1939, noted that the blood of a woman who had delivered a stillborn baby displayed abnormal agglutination of red blood cells. Further studies led to the naming of this feature, "Rh factor," established through the injection of rhesus monkey blood into rabbits and guinea pigs which produce a similar result to that seen

in the 1939 investigation (Landsteiner and Wiener, 1941). Since these initial investigations we now know that the immune blood transfusion complications between mother and fetus are due to the presence or absence of Rh D. Rh D is the major antigenic protein of this blood group, thus we have the common ABO blood typing with O negative and O positive designations representative of absence of Rh D or presence of Rh D respectively (Avent, 2001). The Rh blood group antigen structure is composed of two human erythrocyte protein forms: 1) Rh polypeptides, a non-glycosylated form to which the Rh D along with the Rh CcEe proteins belong; 2) a glycosylated form including RhAG (Rh-associated glycoprotein) (Avent, 2001; Eyers et al., 1994). These forms have a molecular mass of 30kDa and 50kDa respectively, and resulted in the naming of the non-glycosylated Rh30 family and the glycosylated Rh50 family (Ridgwell et al., 1992). Rh protein families are hydrophobic membrane proteins, each spanning up to 12 membrane domains (Anstee and Tanner, 1993). The genes coding for the human Rh polypeptides and RhAG are located on chromosomes 1 and 6 respectively and have been deemed *RHCDE* and *RHAG* (Cherifzahar et al., 1991; Liu et al., 2000; Ridgwell et al., 1992). Liu et al. (2000) further isolated the genes *RhBG* and *RhCG* in humans and *Rhbg* and *Rhcg* in mice, identifying them to code for two proteins found to be homologous to erythroid and non-erythroid Rh glycoprotein (Liu et al., 2000; Liu et al., 2001). The differentiation in the upper and lowercase designations for the various Rh glycoproteins/genes relates to whether they were from humans (RhBG) or from other organisms such as a mouse/fish (Rhbg) (Wright and Wood, 2009). Marini et al. (1994) found these two proteins to be the first evidence of a non-erythroid Rh glycoprotein and demonstrated a homology to ammonia transport proteins (MEP1 and AMT1).

Rhesus and ammonia transport

The first suggestion that Rh glycoproteins may possibly play a role in ammonia transport was by Marini et al. (1994), three years later they characterized specific NH_4^+ transporters and their corresponding genes in both yeast (Mep1 protein) and (Amt1 protein) in a small flowering plant, *Arabidopsis thaliana.* In a short study, Marini et al. (1997) performed a sequence alignment using the earlier characterized proteins (Mep1 and Amt1). They determined that there was a significant similarity between the sequences of human Rh50 and those of the NH_4^+ transporter family. In addition, a smaller conserved region of the amino acid sequence showed a high similarity to that of the human Rh30 proteins as well (Marini et al., 1997). This sequence similarity between the two groups prompted the question of, are members of the Rh glycoprotein family facilitating ammonia transport in animals or do they share an evolutionary ancestor to the NH_4^+ transporters and now have a similar but unique function (Marini et al., 1997).

Interesting evolutionary data regarding the *Rh30* and *Rh50* genes comes from a study providing evidence for two gene duplication events affecting the primate *Rh* gene family. Significantly, the first duplication event occurring 250-346 million years ago caused the evolutionary split between *Rh30* and *Rh50* genes, and subsequently promoted a 2.6 times slower rate of evolution by the *Rh50* genes in comparison to the *Rh30* genes, which also underwent the loss of glycosylation. These results also importantly showed the higher conservation in function among the *Rh50* genes in comparison to the *Rh30* group, and noted their similarity to the *Mep1* and *Amt1* genes looked at by Marini two years earlier (Matassi et al., 1999). A second evolutionary study conducted by Huang and Peng (2005), proved critical in shaping the future path of Rh protein research. This study provided a phylogenetic

relationship between Rh and Amt in addition to showing Rh family member groupings. Initially, bioinformatics results support that Rh proteins function as mainly CO_2 channels in most organisms, and that any function relating to ammonia is a residual possession relating to ancestral Amt proteins. Notably, with a few exceptions they also confirmed the absence of *Rh* genes and the overall prominence of Amt within bacteria. Rh genes were shown to be absent in plants while Amt remained, likely because Rh proteins only excelled in high CO_2 conditions and Amt in plants remained to aid in nitrogen uptake. The toxicity of the nitrogenous wastes produced from amino acid metabolism may explain the loss of Amt genes in vertebrates, and the retaining of the Rh genes. Furthermore, they show vertebrates possess four groups sharing a commonality (*Rh30, RhAG, RhBG*, and *RhCG*), and the presence of an out-group referred to as *Rhp2* (p for primitive) as a non-mammalian cluster. They also note the presence of more *Rh* genes in fish than in mammals resulting from fish possessing two copies of *RhCG*. This data supported the known *Rh* gene data up to that point and led the way for further investigative expression studies (Huang and Peng, 2005).

Rhesus expression

The presence of Rh glycoproteins and their similarity to ammonia transporters resulted in a number of studies to analyze expression patterns of the Rh genes/proteins in many organisms. Marini et al. went on to examine the expression of *RhAG* and *RhGK* (*RhCG*) in yeast cells and found that both homologues were capable of both ammonium uptake and export (Marini et al., 2000). In 2006, a study examined the same expression, this time in HeLa cells and *Xenopus* oocytes and came to similar conclusions. Under flux controlled experiments, the researchers observed that both *RhAG* and *RHCG* were not only

expressed in the cells but they promoted the transmembrane transport of ammonia (Bakouh et al., 2006).

Mammalian Rh expression

With the data linking Rh proteins to other ammonia transport proteins there was an influx of studies examining their specific function in relation to ammonia excretion. Early studies focused on the erythroid glycoprotein RhAG expression, which was observed in many primitive life forms. Initially it was theorized that RhAG possessed ammonia transport properties, however, it was discovered that although RhAG may act to trap ammonia in the blood stream, it did not play any obvious role in direct excretion (Liu and Huang, 1999). Studies showed that RhAG was not expressed in organs associated with ammonia production or excretion, but rather expressed in tissues associated with hematopoietic functions (Liu and Huang, 1999; Liu et al., 2001). The same group identified and characterized the non-erythroid Rh glycoproteins; RhCG/Rhcg and RhBG/Rhbg. The association between Rh glycoproteins and ammonia transport gained great support (Liu et al., 2000; Liu et al., 2001). The study showed RhCG/Rhcg expressed in non-erythroid tissues, the kidneys and testes of human and mouse (Liu et al., 2000). A follow up study, characterized and localized RhBG/Rhbg to the kidney, liver, and skin of human and mouse (Liu et al., 2001). Furthermore, they elucidated the expression of Rhbg and Rhcg to different specific areas of the kidney, (Rhcg to the apical membrane and Rhbg to the basolateral membrane), suggesting that each protein may possess a specific role in the same organ. Notably this role was based on ammonia transport given the similarity to other ammonia transporters and the location in organs dealing with nitrogenous waste excretion (Liu et al., 2000; Liu et al., 2001). Weiner (2006) furthered this finding by examining the expression of *Rhbg* and *Rhcg*

in rats and found comparable results to that of the mice studies. This study localized Rhbg basolaterally in distal nephron region of the renal tissue as well as finding that *Rhcg* was expressed apically. However, there was also significant *Rhcg* basolateral expression, this was in contrast to the findings in the previous mouse study. In addition, they examined *Rh* expression following chronic acidosis in rats and observed a significant three fold increase in apical *Rhcg* expression (Weiner, 2006). This finding led to the conclusion that renal *Rhcg* expression was regulated by chronic metabolic acidosis. Additional expression of *Rhbg* and *Rhcg* in the liver and gastrointestinal tract, suggested that these isoforms play a role in enterohepatic ammonia metabolism (Weiner, 2006).

Aquatic Rh expression

Strong evidence existed for simple NH_3 diffusion as the main route for ammonia excretion in an aquatic environment (Evans et al., 2005), Rh glycoproteins were soon being analyzed as mechanisms for nitrogen regulation among aquatic organisms. In 2004, a study on crabs concluded with the sequencing of a Rhesus like protein from the gill of the green shore crab, *Carcinus maenas*. Named RhCM, it showed a strong amino acid similarity to the human ammonia transporter RhGK. It was hypothesized that RhCM would either be co-localized with a H^+-ATPase pump on the intercellular vesicle membranes, allowing for the acidification of the membrane boundary layer in turn promoting ammonia excretion (Wilson et al., 1994), or have a basolateral location and act as an "overflow valve" in the presence of high ammonia conditions (Weihrauch et al., 2004).

Original fish Rh expression

Following bioinformatical studies determining the presence of *Rh* genes in fish (Huang and Peng, 2005), there was a marked increase in research focusing on Rh glycoproteins and their function in fish species began in 2007. The cornerstone study was by Nakada et al. (2007) using the pufferfish (*Takifugu rubripes*) as their model organism. They demonstrated that ammonia secretion from fish gills was dependent on a set of Rh glycoproteins. This study not only provided expression data at the cellular level, it also provided physiological data demonstrating clear Rh protein function. Cloning the Fugu Rh glycoproteins, Nakada et al. demonstrated the localized expression of the four Rh glycoproteins in the fish gill epithelium through *in-situ* and immunohistochemical analysis. Three of the four proteins were localized to the lamellae of the gill while the other resided in the inter-lamellar junction (Figure 1). Rhag was localized in the apical and basolateral membranes of the pillar cells in the lamellae. Rhbg was localized to the basolateral membrane of the pavement cells which line the lamellae. Rhcg2 was localized to the apical membrane of the pavement cells, while Rhcg1 was localized to the apical membrane of the MRCs in the inter-lamellar space. *Xenopus* oocytes were injected with *Rh* cRNA and were placed in environments with radiolabeled methylammonium. Results showed a significantly greater level of ammonia uptake by the oocytes with pufferfish proteins compared to that of the controls. Upon noting the physiological data showing the uptake by these proteins when injected into the *Xenopus* oocytes, they concluded that the three proteins expressed in the lamellae likely function in ammonia excretion from the vascular space across the plasma membrane into the external environment. The localization of the Rhcg1 protein to the apical side of the MRC was suggested to be with a functioning Na^+-K^+-ATPase pump in further

ammonia excretion. It was concluded that a protein driven pathway would be more efficient than the previous methods theorizing simple NH_3 diffusion. The authors also stated that finding of orthologous fragments of these Rh glycoproteins in other fish data bases suggests that similar ammonia excretion patterns are likely to be seen in other ammonotelic teleost (Nakada et al., 2007b).

Further fish Rh expression studies

Rh expression in euryhaline and marine fishes

The Nakada et al. (2007) study prompted further studies in a wide variety of fish both saltwater and freshwater. *Rhbg*, *Rhcg1*, and *Rhcg2* mRNAs were expressed under control conditions in the gill of the euryhaline killifish, *Kryptolebias*, with *Rhbg* also being expressed in the skin (Hung et al., 2007). This study examined the effect of high environmental ammonia (HEA) on *Rh* gene expression. Results showed a wider range of expression of *Rhbg* in many tissues (liver, kidney, gut, gonad, and muscle), while *Rhcg1* expression was also seen in the skin. HEA results in complications or inhibition to the normal gradient flow of ammonia from the fish to the external environment, in severe cases can stop or reverse the ammonia gradient (Claiborne and Evans, 1988). Following high environmental ammonia exposure, there was little to no elevation of body tissue ammonia levels, suggesting that killifish have an ability to prevent excess ammonia accumulation. It was suggested that the lack of accumulation in the tissues was facilitated by excretion, although the mode of excretion had yet to be determined (Hung et al., 2007). A study on a primitive vertebrate, the pacific hagfish, yielded the presence of Rhag, Rhbg, and Rhcg1, and localized Rhbg to the lining of the blood vessels along the lamellae and filament, and Rhcg1 on the apical side of

the Na^+-K^+-ATPase cells, results similar to seen in pufferfish in the study by Nakada et al. (2007). There was no evidence of Rhag localization in the hagfish tissues (Braun and Perry, 2010). Two *Rhp2* genes have been bioinformatically detected in teleosts genomes by Huang and Peng (2005); attempts at detecting the corresponding mRNAs have failed in all tissues examined to date. However, in the shark, *Triakis scyllium*, *Rhp2* mRNA was detected solely in the kidney, and immunohistochemistry localization analysis determined that *Rhp2* was localized to the basolateral membrane of epithelial cells in the proximal segment of the 2nd loop of the renal tubule. Furthermore it was observed that Rhp2 is regulated by environmental salinity, with increasing expression associated with higher salinities. The study suggested that Rhp2 might play a role in ammonia reabsorption in order for urea synthesis (Nakada et al., 2010). Another study examining Rh expression during high environmental conditions in pufferfish determined that there was a significant up-regulation of *Rhcg1* and a down-regulation of *Rhag* and *Rhbg*. The apically located Rhcg1 on the MRCs likely dealt with the excretion of ammonia while the down-regulation of the other two Rh proteins suggests their involvement with *influx* of ammonia which would be redundant in those conditions (Nawata et al., 2010).

Rh expression in freshwater fish

In contrast to the exclusive patterns of Rh expression shown by Nakada et al. (2007), an examination of the freshwater rainbow trout, *Oncorhynchus mykiss*, showed that under control conditions all three mRNAs (*Rhbg, Rhcg1,* and *Rhcg2*) were expressed in both the pavement cells as well as on MRCs. However, under high environmental ammonia conditions, expression patterns were altered with an increase in Rhbg and Rhcg2 expression

seen in pavement cells of the gill and expression of Rhcg2 in the skin also, while Rhcg1 expression visualized only in the MRCs (Nawata et al., 2007).

Another function of Rh glycoproteins is a suggested route for CO_2. To investigate the role of *Rh* gene expression in the transport of CO_2, trout were exposed to hypercapnic conditions (increased environmental CO_2) and *Rh* mRNA expression quantified. This study concluded that although variable changes in mRNA expression occurred, the increases in Rh gene expression were not attributed directly to the hypercapnic conditions, but rather were due to elevated plasma ammonia levels resulting from the treatment. This suggests that Rhbg and Rhcg are likely not co-transporters of both ammonia and CO_2 in fish; however, a noticeable increase in Rhag expression in hypercapnia conditions may point to the possibility that Rhag in the erythrocytes could respond to both ammonia and CO_2 (Nawata and Wood, 2008).

The Asian weatherloach is a fish highly tolerant of ammonia, localization studies have demonstrated co-localization of Rhcg1 and H^+-ATPase on the apical gill epithelia in the same mitochondria rich cell and pavement cell populations, and noted an increase in expression of *Rhcg1* and *Rhcg2* mRNA expression in the skin following aerial exposure (Moreira-Silva et al., 2010). Sashaw et al. (2010) recently reported rainbow trout embryo *Rhcg2* mRNA expression being upregulated due to alkaline water while *Rh* expression did not change in high environmental ammonia conditions. These results were in contrast to previous data for adult rainbow trout by Nawata et al. (2007), showing upregulation of *Rh* mRNA resulting from subjection to high environmental ammonia. This study also proposed that the Rh isoform Rhcg2 may be more active in rainbow trout than the other Rh proteins, suggesting species specific Rh protein expression (Sashaw et al., 2010).

Looking at potential up-regulation events, it was observed that following feeding rainbow trout demonstrated a significant increase in *Rhcg2* mRNA expression levels in the gill, suggesting that an increase in internal ammonia production due to influx of protein may trigger up-regulation or Rh proteins to compensate for the ammonia load. The study also suggested that ammonia excretion via Rh proteins is coupled with Na^+ uptake via a sodium hydrogen exchange pump (NHE) (Zimmer et al., 2010).

Developmental Rh expression

Developmental studies to date have focused primarily on examining Rh expression in the Zebrafish, *Danio rerio*. In adult freshwater zebrafish Rhcg1 has been localized not only to the gill epithelial cells but also to the epithelial cells of the distal tubule (absent in pufferfish and most seawater fish) in the kidney. In the larval zebrafish, Rhcg1 was localized to the apical side of MRCs on the yolk sac membrane, suggesting the potential role that Rhcg1 could play in ammonia excretion at that stage of development (Nakada et al., 2007a). Rh expression in the skin of zebrafish embryos described the apical localization of Rhcg1 in association with H^+-pump rich cells (HRCs) located on the skin and yolk sac (Shih et al., 2008). Hypothetically this localization would enable the facilitated diffusion of NH_3 through these protein channels (Shih et al., 2008). Developmental Rh expression was again looked at in the zebrafish larvae however, unlike before in the trout embryonic study by Nawata et al. (2008), there was no consistent specific localization of Rh observed in the zebrafish embryos. The study proposed that the scattered locations of Rhbg and Rhcg1 in the embryos suggested an absence of a direct cooperative pathway for ammonia flux early in development. However, they had the ability to work independently to excrete ammonia. They suggested that Rhag expression did not play a direct role in external ammonia excretion but rather

functions in additional aspects such as maintaining structural integrity of red blood cells in zebrafish embryos (Braun et al., 2009a). The same group also looked at adult zebrafish in comparison to the embryos and larvae with regards to Rh expression. Results showed that *Rhag*, *Rhbg*, and *Rhcg1* mRNAs were predominantly expressed in the adult gills with variable expression in other organs such as the brain. Localization of Rhag, Rhbg, and Rhcg1 in the zebrafish gill, mirrored the results of Rh expression in the pufferfish, (Nakada et al., 2007) and also observed that high environmental ammonia increased mRNA levels of *Rh* in the embryos with no effect on the adult gill, while phloretin increased mRNA expression of *Rh* in both the embryos and adult gill (Braun et al., 2009b). A developmental study looking at Rhesus glycoproteins in rainbow trout embryonic stages, noted a significant increase of mRNA levels of *Rhcg2* in the later stages of development ~60 days post fertilization, although all three *Rh* genes were expressed starting at day 14 including *Rhbg, Rhcg1* and *Rhcg2* (Hung et al., 2008). This study demonstrated similar *Rh* expression patterns to the studies on zebrafish, however, while zebrafish expression includes many Rh members, studies show Rhcg2 to be the much more active than the other isoforms in the rainbow trout. This proposition was suggested by Sashaw et al. (2010).

Model of freshwater ammonia excretion

The currently accepted model for ammonia excretion via the gills of freshwater fish in relation to Rh glycoproteins was recently updated in a review by Wright and Wood (2009) (Figure 2). Rhag located on erythrocytes facilitates the NH_3 efflux bringing the ammonia from the gill; however, the model shows that ammonia can also be brought to the gills by plasma. Rhbg, located on the basolateral side of the gill epithelial cell allows for passage of NH_3 into the intercellular space from the blood. NH_3 then moves into the exterior water

environment, passing through the apically located Rhcg1 or Rhcg2 protein. This diffusion is facilitated by the acidification of the gill water boundary layer. This acidification is brought about by the efflux of protons either provided through an H^+-ATPase or an isoform of NHE. This "ammonia pump" maintains the overall blood-to-water partial pressure of NH_3 gradient as well as coupled to Na^+ uptake (Wright and Wood, 2009).

It is tempting to accept any number of proposed hypotheses on the mode of ammonia excretion in fish including the current freshwater model above. However, in reviewing the literature there are many inconsistencies and exceptions that are species specific (ex. Nakada et al., 2007; Nawata, Hung et al., 2007), life stage specific (Sashaw et al., 2010), or condition specific (Nawata and Wood, 2008) which influence the expression of the Rh glycoproteins and their role in ammonia excretion. It seems that the specific cellular and tissue expression in many of the animals examined varies and adapts depending on what circumstances are defined at the time of analysis. This idea may support the evolutionary long term conservation of the Rh genes (Huang and Peng, 2005) through their ability to be expressed, up-regulated, or down-regulated when the particular conditions trigger them to function. Overall, researchers have come a long way from identifying the "Rh factor" in human blood to realizing the important role that these Rh glycoproteins play in ammonia regulation not only fish but a wide range of organisms.

Sea lamprey

Sea lamprey, *Petromyzon marinus*, are thought to have evolved approximately 450 million years ago, making it one of the earliest known vertebrates (Doolittle, 1983). This

primitive fish along with its close relative the hagfish are the only living representatives of the agnathans, or jawless vertebrates (Heimberg et al., 2010). Two separate populations of the sea lamprey are found in North America: the anadromous form, which spends a portion of its life cycle in both freshwater and seawater and the invasive landlocked Great Lakes population, that spends its entire life cycle in freshwater. Both populations undergo four major divisions in the life cycle: ammocoete, transformant, parasite, and upstream migrant. This unique life cycle of the sea lamprey begins with the hatching of eggs that have been laid in freshwater rivers and streams. These eggs develop into an ammocoete larval stage, characterized as a filter feeding form, which burrows into the river bottom substrate for up to 3-8 years. The larval form undergoes metamorphosis into a freely swimming juvenile lamprey or transformant. The metamorphosis of the anadromous sea lamprey prepares the animals to migrate downstream to the sea. Upon reaching the seawater the lamprey grow into adulthood becoming parasitic and feed on the blood and fluid of other fishes. After a brief stage of feeding in the sea, adult lampreys cease feeding, return to the freshwater rivers and migrate upstream to spawn and die (Beamish and Potter, 1975). This unique life style not only belongs to the anadromous population, but a similar lifecycle is present in the invasive land-locked Great Lakes population. Instead of migrating to the sea, the transformants find their way to the large freshwater lakes where they grow into adulthood and parasitize on numerous freshwater fish including salmon and lake trout (Potter and Beamish, 1977). The lamprey then return to the rivers to complete the lifecycle, spawn and die (Potter and Beamish, 1977). A fundamental change associated with the life cycle is the alteration in feeding mechanisms: a transition from filter feeding drifting nutrients in the freshwater rivers, to gorging on the blood and fluids of other fish as adult parasites. It is

hypothesized that due to the different feeding styles throughout the life cycle, lamprey must cope with fluctuating ammonia levels. The two main feeding stages of the lamprey are the ammocoete filter feeding stage and the parasitic stage. Upstream migrants do not feed and the internal organs degenerate and the lamprey die soon after spawning (Sower et al., 1985). Due to lack of the functioning feeding mechanism (oral disk), juvenile lampreys cannot feed until full transformation is complete allowing them to attach to their prey. Upon parasitic feeding, lampreys have to deal with their own internal ammonia production but also must manage the nitrogenous waste products gained through the ingestion of blood and body fluids of their prey. During this parasitic feeding stage plasma ammonia levels have been shown to be significantly elevated (Wilkie et al., 2004).

Purpose of Study

The overall goal of this research was to examine the expression of Rh glycoproteins throughout the life cycle of the sea lamprey, *Petromyzon marinus*. Our hypothesis was that Rh expression would be increased during feeding stages in the lamprey lifecycle such as its parasitic phase. We also hypothesized that an observed difference in Rh expression would be evident due to increasing salinities incurred by the native anadromous population compared to the Great Lakes landlocked population. The specific objectives of this study was to 1) observe Rh glycoprotein expression in epithelial tissues through Western Blot (WB) and Immunohistochemistry (IHC) analysis; 2) clone partial lamprey Rh gene sequences.

Methods

Sea lamprey and tissue collection

It should be noted that animal collection is seasonally dependent and unreliable at times.

Anadromous sea lamprey

Two stages of sea lamprey life cycle (*Petromyzon marinus*) from the anadromous population were collected. The upstream migrant lampreys were caught by a local fisherman via dip netting on the Columbia River in Maine, USA. Fish were transported to Mount Desert Island Biological Laboratory where they were housed in dechlorinated running freshwater tanks.

Transformant anadromous sea lampreys were ordered from Acme Lamprey Company, Harrison ME. Transformant lampreys were housed at Appalachian State University in dechlorinated freshwater aquariums on a normal day/night cycle. After a month acclimation period, water was brought up to 10% salinity. Following a weeklong acclimation period, upstream migrant and transformant fish were sacrificed and gill and skin samples were take for Western Blot (WB) and Immunohistochemistry (IHC) analysis. WB samples were snap frozen in liquid nitrogen and stored at -80°C. IHC samples were placed in 4% paraformaldehyde and stored at 4°C overnight.

Great lakes landlocked lamprey

Three stages of the sea lamprey from the Great Lakes landlocked population were collected. Lampreys were provided by Dr. Nick Johnson, Hammond Bay Biological Station, Millersburg, MI. Ammocoete larval stage and transformants were collected via electrofishing from local rivers feeding into Lake Huron. Parasitic lampreys were collected from local commercial fisherman removing them from salmon and lake trout hosts. All lampreys were housed in running Lake Huron water tanks and parasitic lampreys were given lake trout as hosts to feed on. Dissection of lamprey gill and skin tissue from all 3 life cycle stages was conducted at Hammond Bay Biological Station. WB samples were snap frozen in liquid nitrogen and stored at -80°C. IHC samples were placed in 4% paraformaldehyde and stored 4°C overnight.

Antibodies

Heterologous fish antibodies raised against amino acid sequence fragments encoding a part of the COOH terminus of Fugu Rhag (amino acid residues 386–441), Rhbg (407–458), Rhcg1 (426–485), and Rhcg2 (420–481) a gracious gift courtesy of Hirose Lab, were used for all Rh glycoproteins being studied (Nakada et al. 2007). These primary antibodies were utilized in both the western blot analysis and immunohistochemistry staining.

Western blot

Snap frozen gill and tissue samples were homogenized in ice-cold buffer solution; homogenization buffer contained sucrose, tris base, 100mM EDTA, phenylmethylsulfonyl fluoride (PMSF) and protease inhibitor cocktail and had a final pH of 7.8. Homogenized samples were transferred to 1.5ml tubes and centrifuged at 14,000 RPM at 4°C for 10 min.

Supernatants were transferred to clean 1.5ml tubes for protein concentration analysis using a Pierce® BCA Protein Assay Kit (Thermo Fisher Scientific, Rockford, IL). Protein from zebrafish gill and skin was used for positive controls (Figure 3). 25 µg of lamprey gill and skin protein and 25 µg of zebrafish gill protein was loaded into NuSep 4-20% LongLife gels for electrophoresis at 50V for 30 min and 90V until completion (~1 hour). Separated proteins were blotted on to Bio-Rad Immun-Blot PVDF membranes for 75 minutes at 90V. Membranes were incubated overnight at 4°C in blotto (5% carnation non-fat powdered milk and 100ml TBS with 0.2% triton) to block non-specific binding sites. Membranes were then incubated in primary anti-body diluted in blotto (1:1000 Rhag, Rhbg, Rhcg1, and Rhcg2) at 4°C on a rocker table overnight. Membranes were then washed 3 times in TBST with 0.2% triton (0.1M Tris buffered saline, pH of 7.4.) After washing, membranes were incubated in secondary antibody diluted in TBST (1:3000 goat-α-rabbit-AP or 1:15,000 goat-α-rabbit-HRP + StrepTactin-HRP Conjugate) rocking at room temperature (~23°C) for one hour. Excess secondary antibody was removed by three consecutive washes in TBST, each for seven minutes, with a final wash in TBS containing no triton to remove residual detergent. Following washes, membranes were covered with either Immun-Star™ AP Substrate or Immun-Star™ HRP Luminol/Enhancer and Peroxide Buffer for 5 min at room temperature to undergo the chemiluminescent reaction. Antibody binding was detected by exposing Hyperfilm™ ECL (high performance chemiluminescent film- Amersham Biosciences, Piscataway, NJ) to the membranes. Relative quantification of protein expression was performed by scanning in images of film and analysis was performed by Quantity One® software (Bio-Rad, Hercules, CA) and statistical analysis using Microsoft Excel 2007.

Immunohistochemistry

Anadromous lamprey

Tissues from both upstream migrants and transformants were fixed overnight in 4% paraformaldehyde (PFA), then dehydrated through a series of graded ethanol, cleared with xylene and embedded in paraffin wax using a Shandon Citadel 1000 (Thermo Scientific, Rockford, IL) and oriented for cross sectioning in wax blocks. Microtome (Leica Biocut 2030) sectioning of gill and skin tissues was conducted; 7μm thick paraffin wax sections were fixed onto Fisherbrand® Superfrost®/Plus Microscope Slides (Thermo Fischer Scientific Rockford, IL) and stored at room temperature. Slides were de-waxed through a series of Protocol® Safeclear washes and rehydrated through a series of graded ethanol to DI water with a final 5 min wash in phosphate buffered saline (PBS) solution. Non-specific binding sites were blocked by incubating in 5% Normal Goat Serum in PBS for 20 min. Tissue sections were incubated overnight at room temperature in a humidified chamber in primary antibody (Rhag, Rhbg, Rhcg1, Rhcg2: 1:500; NKA (α5) 1:500) diluted in blocking solution. The following day, excess primary antibody was removed by washing in PBS. Tissue sections were incubated in secondary antibody (1: 2000 Alexa Fluor® 568 goat α rabbit IgG; 1:2000 Alexa Fluor® 488 donkey α rabbit IgG; 1:2000 Alexa Fluor® 488 goat α mouse IgG) diluted in washing solution at room temperature for 1 hour. Slides then underwent 3 more washes in PBS to remove excess secondary antibody. Slides were mounted onto cover slips with ProLong® Gold antifade reagent (Invitrogen™, Eugene, OR). Slides were visualized using scanning laser confocal microscopy (Zeiss LSM 5, Carl Zeiss, USA).

Great lakes landlocked lamprey

Gill and skin tissues from the Great Lakes landlocked population were fixed in 4% PFA overnight. Tissues were then washed in several changes of PBS and with a final overnight infiltration bath in 30% sucrose in PBS. Tissues were then frozen in Tissue-Tek® Optimal Cutting Temperature (OCT) using liquid nitrogen and sliced at 7um thick with a cryostat (Leica CM 11000). Tissue sections were dried onto VWR VistaVision™ HistoBond® Microscope slides and stored at room temperature for IHC analysis as previously described, with the following modifications: an initial 10 min wash in PBS with 0.1% triton, followed by 3 x 5 min washes in PBS to clear residual OCT compound. The 5% NGS was diluted in the primary washing solution (PBS w/0.1% triton). The remaining steps were the same as in the previous IHC protocol.

RNA isolation and cDNA synthesis

Total RNA from the gills of sea lamprey was isolated via homogenization in Tri Reagent® (Tri Reagent, Molecular Research Center, Inc. Cincinnati, Ohio), following protocol utilizing a bromochloropropane (Tri Reagent, Molecular Research Center, Inc. Cincinnati, Ohio) extraction, isopropanol precipitation, with 75% ethanol washing of precipitated RNA. Final RNA was solubilized in molecular grade treated water and checked for purity and concentration using Nanodrop spectroscopy at 260/280 nm. First strand cDNA was synthesized from 5ug total RNA using Superscript III reverse transcriptase enzyme with cDNA synthesis primed with an Oligo-dT primer (Invitrogen, Carlsbad, CA).

Polymerase chain reaction

Degenerate primers (Table 1) based on conserved *Rh* sequences were used to amplify target sequence of approximately 600 bp. The polymerase chain reaction (PCR) was performed using a hot start method (Platinum *Taq*, Invitrogen Carlsbad, CA) in a 50ul volume using a PCR express thermocycler (Hybaid, Franklin, MA). PCR reagent concentration for all reactions was 3 mmol I^{-1} MgCl, 200 umol I^{-1} dNTP mix, 10mmol $I^{-1}$for each primer and 1.25 units *Taq*. The thermal cycling parameters included initial incubation at 94°C (2 min) allowing activation of *Taq* polymerase followed by 35 cycles of 94°C (30 sec) 54°C (45sec) 72°C (60 sec) with a final extension time of 72°C for 5 min. The PCR products were analyzed by gel electrophoresis (1% agarose in 0.5 × TBE buffer, 45 mM Tris borate, 1 mM EDTA) and visualized under UV light following ethidium bromide staining.

Cloning and sequencing

Using TOPO TA Cloning® Kit for Sequencing the resulting 600 bp product was ligated into the pCR2.1 plasmid vector (Invitrogen, Carlsbad, CA) and transformed into One Shot® TOP10 Chemically Competent *E. coli* cells and plated onto LB agar plates containing 50 µg/ml ampicillin. Positive clones were selected and grown up in LB broth (5 µg/ml ampicillin). Plasmid DNA was isolated by alkaline lysis (Manual FastPlasmid™ Mini Kit, 5PRIME) and analyzed by restriction digest using *Eco*R1 and agarose gel electrophoresis. Clones containing an insert were then sequenced on an Applied Biosystems 3100 model DNA Sequencer (Mount Desert Island Biological Laboratory DNA sequencing facility).

Sequence Analysis

Related sequences were detected via searching the GenBank database using the standard BLAST algorithms at the National Center for Biotechnology (NCBI). Consensus sequence was constructed using Assembly Align (Oxford Molecular Group). Sequence alignments were carried out using CLUSTAL W. Rh sequences were retrieved from the protein, genomic, and nucleotide databases of GenBank and Ensemble by BLAST searches. Further sequence analysis was performed by Kaben G. Nanlohy, (Weiming Li Lab, Department of Fisheries and Wildlife, Michigan State University).

Hematoxylin and eosin staining

In order to provide a histological reference for comparison by designating specific cellular structures in the gills and skin, hematoxylin and eosin (H and E) staining was performed. Gill and tissue sections were adhered to slides as prescribed above and taken through the H and E staining sequence, using Permount® (Fisher Scientific) as a mounting medium. Images were acquired using an Olympus IX81 Inverted Light Microscope.

Results

Western blot

Heterologous *anti-fugu Rh* antibodies were used to visualize protein expression in the lamprey. Western blots were performed using quantified protein from the gill and skin of both anadromous and landlocked lamprey at various stages of the life cycle, detecting Rh glycoprotein expression. Immunoreactivity of the Rh glycoprotein antibodies was confirmed with an immunoreactive band at approximately 50kDa, (Figure 3).

Anadromous lamprey

Rh protein expression in the anadromous population of sea lamprey both transformants and upstream migrants, demonstrate a clear immunoreactive proteins for Rhag, Rhcg1, and Rhcg2 at the predicted size of ~ 50kDa (Figure 4). Total Rh protein expression significantly increased ($P<.05$) in transformants acclimated to 10% salinity when compared to skin samples from freshwater transformants (Figure 25). Rhbg expression was only evident in skin samples from the 10% salinity transformant (Figure 4). Density measurements are inversely proportional to the amount of expression, thus the shorter columns represent higher expression, while the taller columns represent lower expression (Figure 25).

GL landlocked lamprey

Rh expression in the Great lakes landlocked population was present throughout all life cycle stages (ammocoetes, transformants, and parasites) in gill and skin with a few exceptions. Rhbg expression was absent in the gills and skin of the ammocoetes as well as the gills of the parasite. Also, Rhcg2 expression was not detected in the gills of the parasites. Rhag, Rhbg, Rhcg1, and Rhcg2 expression was detected in the rest of the samples. Visibly stronger expression of Rh isoforms was observed in the skin than in the gills of each of the three lamprey life cycle stages, with the greatest expression associated with Rhcg2 (Figure 5).

Quantification of Rh protein expression

Quantification of the GL landlocked lamprey western blots enabled comparisons between expression of the different Rh isoforms; between the skin and gill, and between the different life stages. While the mean expression of many appeared to be different, statistical analysis yielded very few significant differences likely due to the small sample size (n=4). Rhag expression was significantly increased in GL landlocked ammocoete skin samples in comparison to parasite (P=0.05) but no significant difference was observed in comparison to skin samples from transformants. While there were no differences between transformant and ammocoete Rhbg expression, there was in fact a significant increase in skin Rhbg expression in parasitic animals in comparison to ammocoetes (P=0.05) (Figure 23 & Figure 24). The calculated density is inversely proportional to amount of expression, thus shorter bars represent higher expression and taller bars represent lower expression.

Histology

Hematoxylin and eosin staining of transformant skin and gill sections as well as adult upstream migrant gill sections were successfully performed for cellular localization referencing. Specific morphological characteristics in the skin and gill epithelium are labeled for visual comparison with immunohistochemistry results (Figure 6).

Immunohistochemistry

Negative controls were conducted in the absence of primary antibody in conjunction with experimental samples (Figure 7 and Figure 12)

Transformant skin comparisons

Comparisons between anadromous and landlocked transformants demonstrated obvious differential localization in skin sections (Figures 8-11). Rhag was localized to large club or mucous cells in the mid region of the skin epithelium of the anadromous transformant, while absent in the GL landlocked transformant (Figure 8). Rhbg was localized to cells on the basal layer of the skin epithelium in the anadromous transformants, compared to a wider range of Rhbg expression that extended from the basal layer to the dermal layer of the epithelium of the GL landlocked transformants (Figure 9). Anadromous transformant Rhcg1 expression was localized to cells on the apical surface of the skin, and absent in the skin of the GL landlocked transformants (Figure 10). Rhc2 expression was seen throughout the skin epithelium of the anadromous transformant, however strong Rhc2 expression was confined to the epidermal cells on the apical side of the epithelium of the GL landlocked transformants (Figure 11).

Transformant gill comparisons

Overall transformant gill Rh expression was much less defined than observed in the skin. In the GL landlocked transformants, Rhag expression was specifically localized to the apical crypts of Na^+/K^+-ATPase (NKA) positive mitochondrial rich cells (MRCs) (Figure 13). Rhag expression was absent in the anadromous transformant gill. Weak Rhcg1 expression was observed in the MRCs of the gills of both the anadromous and the GL landlocked transformants (Figure 14).

Anadromous upstream migrants

Rh expression was absent in the anadromous upstream migrants.

GL landlocked ammocoetes- gill

The gills of the GL landlocked ammocoetes were characterized by Rhag, Rhcg1, and Rhcg2 expression ranging from the large MRC-like cells in the interlamellar space to outlining the apical cells on the lamellae (Figure 15).

GL landlocked ammocoetes- skin

Rhag (Figure 16) expression in the skin was localized to the apical layer of the epithelium. Similar expression was seen in Rhcg1, apically located (Figure 17C). Rhbg was localized to the club/mucous cells, with Rhcg2 expression present in both the club/mucous cells as well as the apical layer (Figure 17B, D).

GL landlocked parasites

The gills of GL parasites were characterized by Rhag expression on the apical surface of the MRCs, while Rhbg was localized to the pavement cells lining the lamellae (Figure 18A,B). There was no evidence of Rhag expression in the skin. However, the other three isoforms were expressed throughout the skin epithelium with Rhcg1 seen more apically orientated (Figure 19).

Gene sequencing

To date we have partial lamprey cDNA sequences for two Rh glycoproteins in the sea lamprey: Rhbg and Rhcg (Figure 20 and 21). They are 663 and 1010 base pairs long, respectively. BLASTX results showed the lamprey Rhbg to be similar to (66% identity) *Takifugu rubripes* Rhbg mRNA for Rh type B glycoprotein (AB218980.1; gb|AY116074.1|). Lamprey Rhcg was shown to be most similar to (99% identity) that of Atlantic hagfish, *Myxine glutinosa* Rh type C glycoprotein (GU733440.1; ADD63335.1), with a lower identity (64% identity) seen with the teleost pufferfish, *Takifugu rubripes* Rh type C2 (NP_001027934.1; GI:74136151).

Discussion

This is the first study to demonstrate the presence of Rh isoforms Rhag, Rhbg, Rhcg1, and Rhcg2 in the epithelial tissues of the sea lamprey, *Petromyzon marinus*. In this study we set out to answer two main questions: 1) Is Rh glycoprotein expression altered during the different stages in the life cycle of the sea lamprey? 2) Is Rh expression altered in animals that are strictly freshwater dwelling in comparison to their anadromous counterparts? Our working hypothesis was that Rh expression would at its highest during the feeding phase (parasite) of the lamprey life cycle due to the additional nitrogenous waste that accompanies a blood meal. Our hypothesis was based and supported by previous physiological studies conducted by our collaborator Dr. Mike Wilkie (unpublished) in which ammonia excretion was examined in the different stages of the GL landlocked sea lamprey (Figure 22).

In testing these hypotheses, various life stages from two populations of the sea lamprey were collected. Transformants and upstream migrants from the anadromous population were acquired. Ammocoetes, transformants, and parasites from the Great lakes (GL) landlocked population were also obtained. Ideally, animals representing all four stages from both populations would have been used, however due to an unreliable animal supply, experiments were limited to the accessible lamprey.

The scope of this study did not include the physiological analysis of the Rh glycoproteins ability to mediate ammonia transport in the sea lamprey; it aimed to localize to

the Rh isoforms in epithelial tissues known to be associated with ammonia excretion in higher fishes (Nakada et al., 2007; Wright and Wood, 2009).

Life stage Rh expression

The examination of Rh expression in the different life stages provides some valuable information associated with not only the various morphological changes that occur but also the associated feeding behaviors at each developmental phase. The ammocoete, which feeds by filtering nutrients out of the water column (Beamish and Potter, 1975), showed expression of Rh glycoproteins in both the gills (pharyngeal basket) and the skin. Protein expression results indicate the absence of Rhbg expression in both the gills and skin, IHC confirms this lack of Rhbg expression in the gills. However, there was evidence of Rhbg expression in the club cells of the skin, no detection was evident in western blot (WB) analysis. This finding whilst contraindicative may be due to the indiscriminant nature of the Rhbg immunoreactivity that occurred in a small number of club cells resulting in a low overall protein concentration of Rhbg. The presence of the remaining 3 isoforms (Rhag, Rhcg1, Rhcg2) in both skin and gill, suggests that active ammonia excretion is capable via combination of early gill excretion and excretion across the skin epithelium. Rhag in the western blot and not in the IHC of the skin was again puzzling, but it is possible that the skin WB protein samples may have been contaminated with red blood cells, where Rhag has been shown to be expressed (Nawata and Wood, 2008). Our data did not demonstrate any significant differences between Rh expression in the gill and skin of ammocoetes. This is also consistent with the physiological data that showed little to no difference in the ammonia excretion rates between the gill and skin of the GL ammocoetes (Figure 22) (Wilkie, unpublished).

The transformant is in a transitioning phase in which it prepares to make the change from being a filter feeding organism to a parasite. It undergoes the development of the oral disk and as a result develops parasitic capabilities enabling feeding on the blood and fluids of other fish (Beamish and Potter, 1975). High amounts of expression by all four isoforms of the Rh glycoproteins were observed in both gills and skin, stressing the importance of both branchial and extrabranchial ammonia excretion during this life stage. According to the protein expression results, GL transformant gill and skin express Rhag, Rhbg, Rhcg1, and Rhcg2. IHC results indicated Rhbg and Rhcg2 were confined to the skin, with Rhag and Rhc1 expression localized within the gills. This localization pattern is suggestive that expression of Rh glycoproteins can be altered depending on the physiological needs of the animal at a given time. A similar finding was observed by Nawata et al. (2010), in which exposure to high environmental ammonia resulted in an upregulation of Rh isoform (Rhcg1), while the expression of others (Rhag and Rhbg) were simultaneously downregulated in the gill epithelium. The differential localization of these isoforms is also consistent with the physiological evidence for GL transformants in which the extrabranchial excretion of ammonia is decreased in comparison to that of the ammocoete (Figure 22).

The feeding strategy of the parasitic lamprey is to feed on the blood and body fluids of other fishes. The ingestion and breakdown of proteins within the blood meal has been shown to result in a significant increase of ammonia excretion (Wilkie et al., 2004). Protein expression studies examining Rh expression in the GL landlocked parasite following feeding showed an increasing trend but no significant differences between Rh glycoprotein isoform expression in the gills and skin. There was a significant increase (P=0.05) of Rhbg expression in the skin of the parasite in comparison to the skin of the ammocoete. However,

the presence of the Rh isoforms in both branchial and extrabranchial sites suggest that the skin of landlocked parasites may play a role in ammonia excretion in addition to excretion occurring across the gill epithelium. This addition of extrabranchial support to ammonia excretion is most likely associated with the inability of the gills to accommodate a large increase in parasite plasma ammonia concentration following blood meal consumption.

Anadromous upstream migrants

Rh expression in the upstream migrants was totally absent. The upstream migrant stage in this study was represented by the anadromous population only. This stage of the lamprey life cycle is characterized by non-feeding, spawning, and death (Beamish and Potter 1975). Upon nearing the end of their migratory journey, with all energy spent on reaching their destination and reproduction, the internal organs are atrophied and the blood cells are lysing (personal observation); all of which are consistent with this stage of the life cycle being preprogrammed for death.

Anadromous and GL landlocked transformant

The most fascinating aspect of this study was an interesting comparison of Rh protein expression discovered between skin samples from the anadromous GL landlocked transformants. Anadromous transformants demonstrated strong skin expression of Rhag localized to club/mucous cell, Rhbg along the basal layer of the dermis, Rhcg1 along the apical aspect of the epidermis, and a ubiquitous distribution of Rhcg2 throughout the skin. However, the distribution of the Rh immunoreactivity was quite different in the GL landlocked transformants. Rhag was absent, Rhbg was expressed in both the basal and dermal layers, Rhcg1 was absent, and Rhcg2 was strongly expressed along the apical aspect

of the epidermis. The fact that the same stage of life cycle from differing populations demonstrates different localization patterns of Rh expression is intriguing. This suggests two possible scenarios: 1) The greater number of Rh isoforms expressed in the anadromous transformants may be due to variable environments which this population encounters through its migration from freshwater to 100% salinity and back to freshwater. The pattern of Rh protein expression seen in the lamprey skin is similar to the expression pattern of RhBG and RhCG in the skin of mangrove killifish (Hung et al., 2007). In the killifish it was proposed that the RhBG and RhCG facilitate the diffusion of ammonia (NH_3) across the gill and skin, following which it would be subsequently trapped by protons to form NH_4 (Hung et al., 2007). The acidification of the mucous layer of the gill has been long suggested to increase the diffusion of ammonia across the branchial epithelium (Wright and Wood, 2009). It has also been shown that the skin of larval zebrafish characterized by cells rich in H^+-ATPase (HRCs), would also assist in the creation of a gradient allowing for greater diffusional excretion of ammonia (Shih et al. 2008). It is possible that in a shift from freshwater, where H^+ excretion is predominantly via H^+-ATPase, to seawater different Rh isoforms may be able to work better in-conjunction with Na^+/H^+ exchangers (NHE) than others. The idea of H^+ excretion via H^+-ATPase or NHE enhancing ammonia excretion is supported by many studies such as Zimmer, Nawata et al. (2010) along with, Wright and Wood (2009) stating that ammonia excretion is enhanced by the acidification of the gill water boundary layer by an efflux of H^+. In this case, it appears that the GL landlocked transformants which spend their entire life in freshwater have adapted to requiring a limited number of Rh isoforms to meet the demands for ammonia excretion. This data whilst interesting requires further

extensive studies in order to demonstrate the relationship between Rh glycoproteins and other ion-regulatory exchangers.

2) The pattern of localization of Rh isoforms in the anadromous transformant appears to be the formation of a direct cooperative pathway for ammonia flux. Nakada et al. (2007) initially suggested a direct transcellular ammonia efflux pathway across the lamellae of the gill. Our results suggest that there appears to be a similar pathway of excretion across the lamprey skin epithelium. In our study, IHC results suggest the outward movement of ammonia through the skin via connecting Rh protein channels starting with Rhbg on the basal layer of the epithelium, moving through Rhcg2 pathways into the large mucous/club cells expressing Rhag where it may be stored, and finally excreted into the external water via apically located Rhcg1 (Figure 26). Future studies examining this pathway in sea lamprey via radiolabeled methylammonium as demonstrated in Nakada et al. (2007) would be highly beneficial.

Extrabranchial Rh expression

The findings of this study support the hypothesis of extrabranchial ammonia excretion. Localization of Rh expression in the skin strongly suggests that ammonia excretion in lamprey may not solely be occurring across the branchial epithelium. Physiological ammonia flux data in GL lamprey has determined that significant amounts of ammonia were being excreted across extrabranchial and branchial locations in both ammocoetes and transformants life stage (Wilkie, unpublished) (Figure 22). The localization patterns of Rh expression we have seen in the skin of the GL landlocked sea lamprey in this

study is supported by these physiological findings and further work is planned to correlate these findings in both anadromous and landlocked populations.

GL landlocked quantified Rh expression

Quantification of protein expression was conducted with the aim to identify significant differences in Rh isoform expression between the different life stages. Due to the low number of animals, statistical significance was difficult to achieve. Rh expression levels visually appeared to differ in many instances. However there were only two comparisons that produced significant differences ($P<0.05$) in Rh expression between the skin of ammocoetes and the skin of parasites. Rhag was expressed at significantly higher levels in the ammocoetes than in the parasites, while Rhbg was expressed at higher levels in the parasites than in the ammocoetes, bringing about the suggestion that the location of ammonia excretion may alter depending on life stage. Due to the feeding strategy and underdeveloped gills of ammocoetes, it makes sense that the skin would play a large role in ammonia excretion as supported by Wilkie's data (Figure 20). The fact that adult lampreys including the parasitic and upstream migrant stage have fully functioning gills with large surface area as seen in the H & E stained gill sections, suggests that the gills play a larger role in ammonia excretion at that life stage (Figure 8).

This study also aimed to test if adaption to salinity altered Rh expression by acclimating a group of anadromous transformants from freshwater to 10% salinity seawater. Animal fatalities incurred during this time period due to phenol exposure limited our results to a single animal, resulting in an inability to draw a firm conclusion. Preliminary western

blot results comparing the skin protein of the anadromous transformant in 10% salinity to a transformant in freshwater demonstrates a curious relationship. The fact that Rhag, Rhbg, Rhcg1, and Rhcg2 were represented with more intense expression in the skin of the 10% salinity transformant compared to that of the anadromous transformant in freshwater and quantification of the overall Rh expression yielded significant increases ($P<0.05$) in expression in the 10% salinity transformant skin, prompting the suggestion that adjustment of environmental salinity may increase overall Rh expression (Figure 25). One possible explanation is that increasing salinity facilitates an increase of H^+ excretion. Choe and Evans (2003) observed that Atlantic stingrays produce a greater net acid excretion rate in seawater acclimated animals in comparison to those in freshwater. The increased acid excretion, we hypothesize, may result in an increased ammonia excretion driven by the acidification of the gill boundary layer as stated previously. As this assumption is based on a single observation it requires further investigation to determine the relationship between salinity and ammonia excretion via Rh glycoproteins.

Molecular identification of Rhbg and Rhcg in the gills of sea lamprey

Finally while the aim of this project was to determine Rh protein expression, the lamprey Rh cDNA sequence data provides additional Rh mRNA evidence to support protein expression in this primitive vertebrate. The resulting partial Rhbg and Rhcg cDNA sequences show a relatively high identity to other known ammonia transporters in both Atlantic hagfish (GU733440.1; ADD63335.1) and the pufferfish (AB218980.1; gb|AY116074.1|; NP_001027934.1; GI:74136151). The molecular evidence demonstrated an extremely high Rhcg sequence similarity (99% identity) between the lamprey and hagfish. This is a reasonable finding, which is mostly due to these primitive agnathans being the

closest relatives to each other. Evolutionary distance also supports the lesser degree of similarity (64% identity) seen between the primitive lamprey and the teleost pufferfish. Until full length sequence information is acquired either by annotation of the lamprey genome or further polymerase chain reaction (PCR) our knowledge of the presence of Rh genes in the lamprey is limited.

Pitfalls

There are other considerations that must be addressed given the variable expression seen in the various lamprey life stages, populations, tissues, and cells. From the data available it is highly possible that the expression of Rh glycoproteins by these animals is highly variable and directly related to the external and internal environment at time of tissue collection. Electrochemical gradient imbalance could come into play with the small bodied GL landlocked ammocoetes, which were gathered via electrofishing. It has been shown that electrofishing causes significant increases in cortisol levels in effected animals (Mesa and Schreck, 1989). Although *in vivo* experiments have not directly related cortisol with increased Rh expression, cortisol does regulate the expression of many ionregulatory genes and Tsui et al. (2009) found that *Rhbg* and *Rhcg* mRNA levels were increased due to combined cortisol and high environmental ammonia stimulations levels have been correlated with increased Rh expression (Wright and Wood, 2009). The use of MS-222 (Tricane Methanesulfonate) as an anesthetic or in lethal doses prior to decapitation of animals for tissue culture may inadvertently cause a false increase/decrease in Rh glycoprotein expression. It has been observed that within minutes, ammonia excretion can be increased due to high internal accumulation (personal observation of hagfish) and MS-222, which has an NH_2 side group, is rapidly metabolized and excreted via the gills (Maren et al., 1968).

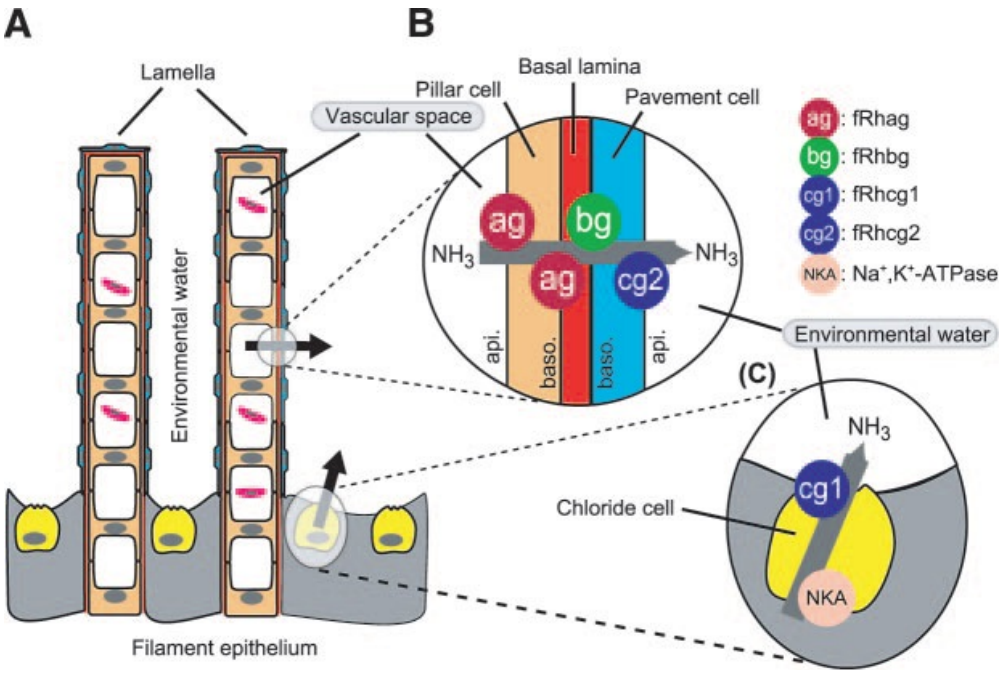
Parasitic lampreys were removed from lake trout but the length of attachment and ammonia concentration in the blood meal is unknown. Heterologous antibodies were used in both WB and IHC analysis, and with the development of monoclonal antibodies specific to lamprey we could produce much more defined immunolabeling results. With these considerations in mind, this study still provides strong evidence for the presence of Rh glycoproteins in the sea lamprey.

Conclusion

The excretion of nitrogenous waste products is critical to the survival of all vertebrates. Ammonia is a highly toxic nitrogenous waste product and as a result must be excreted or converted to less toxic products such as urea. Multiple strategies are employed by different groups of aquatic invertebrates and vertebrates to excrete ammonia across the gills, skin and/or via renal routes. Based on the overarching hypothesis that the extant agnathan fishes represent the evolutionary transition between invertebrate and vertebrate organisms, these phylogentically ancient organisms can provide critical knowledge toward determining how and when key physiological processes evolved in the vertebrate lineage. The sea lampreys serve as an excellent model to study various modes of ammonia excretion due to their complex life cycle. This study has confirmed the localization of the Rh isoforms in both gill and skin in the sea lamprey and preliminary western blot results have suggested that differing expression not only is associated with life stage, but also a single observation suggesting higher Rh expression due to salinity. As the ancestors of the sea lamprey were likely the first vertebrates to successfully invade environs less saline than seawater, further work aims to shed light on the role that environmental salinity played in the evolution of Rh-mediated ammonia excretion in vertebrates.

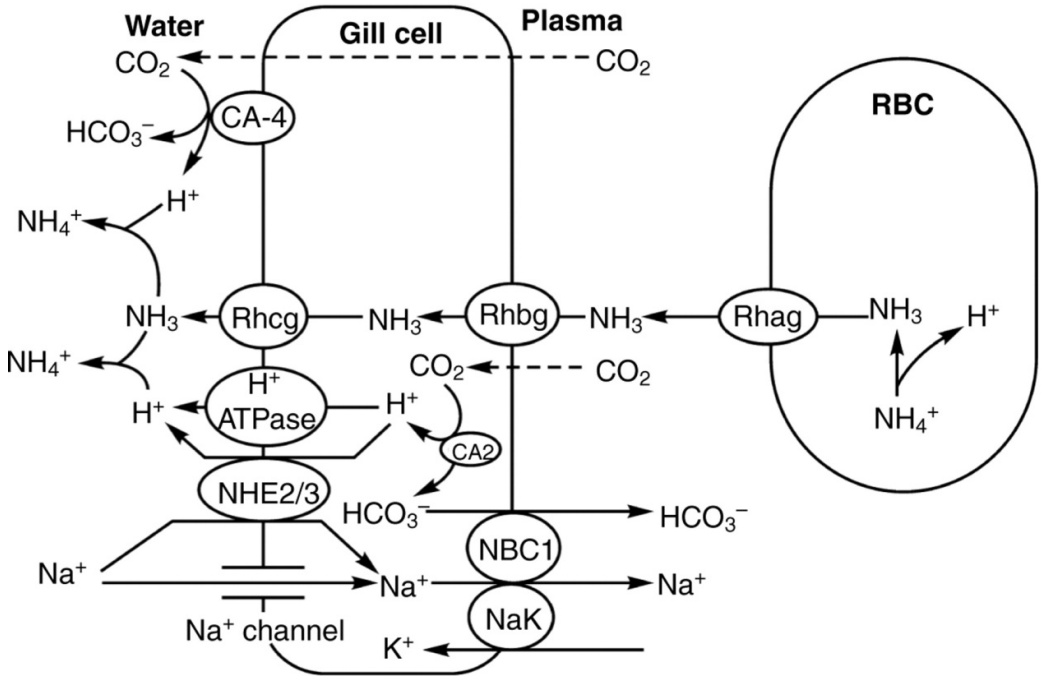
Table 1. List of primers used to perform Rh gene PCR amplification.

Primers	Tm	Sequence	Remarks
RhCgF1	54.8°C	5′ GAARRYYTSATYAACCGNBAYTT 3′	Heterologous
RhCgR1	58.5°C	5′ TGNAYNCCACANGTRTCMTGGAK 3′	Heterologous
HagRhBF8	58.3°C	5′ TGGTCTTGCTGTCTCCAGAGTAATG 3′	Homologous
HagRhRB7	60.7°C	5′ AATGGCTCCAGAAAGTCCTCCCAG 3′	Homologous
HagRhCF5	56.0°C	5′ TGGTGGATTTTTTGGTCTCGC 3′	Homologous
HagRhCB5	58.0°C	5′ CAATGATACCTCCGCAGAAGCC 3′	Homologous



(Nakada, Westhoff et al., 2007)

Figure 1. Model describing Rh glycoprotein localization in the gill epithelium of the pufferfish *Takifugu rubripes*. Rhag was localized to the apical and basolateral sides of the pillar cells of the lamellae. Rhbg was localized to the basal side of the pavement cells lining the lamellae. Rhcg1 was co-localized on chloride cells with sodium potassium ATPase (NKA) activity. Rhcg2 was localized to the apical side of the pavement cells (Nakada et al., 2007b).



(Wright and Wood, 2009)

Figure 2. Model illustrating how the Rh proteins facilitate ammonia excretion. NH_3 transported from blood cells into plasma via Rhag located on RBC membrane. Basolaterally located Rhbg facilitates movement of NH_3 from plasma into epithelial gill cell. Excretion of NH_3 from gill cell to external environment mediated by apically located Rhcg protein isoforms (Wright and Wood, 2009) .

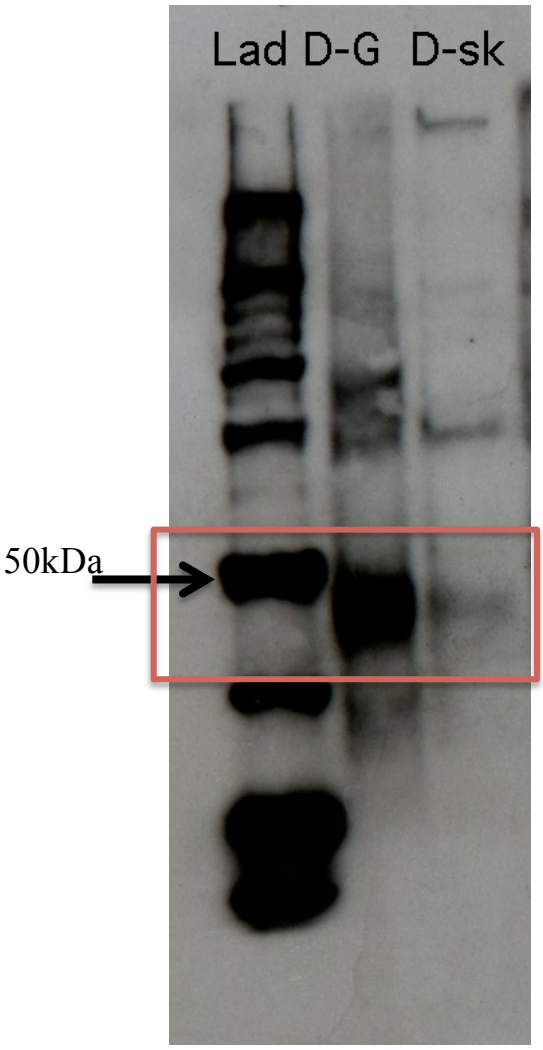


Figure 3. Western blot control. Quantified protein from zebrafish, *Danio rerio*, gill and skin tissue was used. Immunolabeling with Rh glycoprotein antibodies acted as positive control for analyzing the lamprey protein, as Rh glycoproteins have successfully been identified in the zebrafish (Nakada, Hoshijima et al. 2007). Lane 1 is our standard, lane 2 (D-G) is gill protein, and lane 3 (D-sk) is skin protein. Rhcg1 is detected with dark bands ~50 kDa, shown in red box.

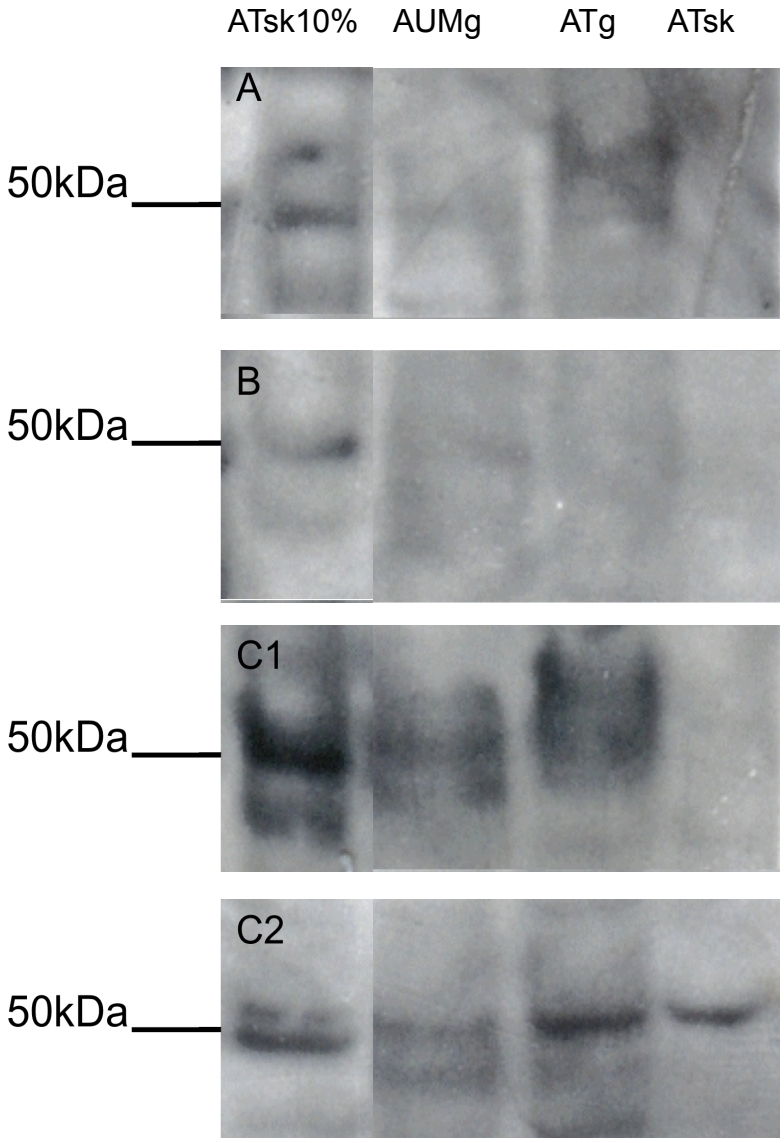


Figure 4. Western blot anadromous lamprey. Quantified proteins results compared Rhag (A), Rhbg (B), Rhcg1 (C1), and Rhcg2 (C2) (~50 kDa) in the anadromous transformant skin (MTsk10%) in 10% salinity, anadromous upstream migrant gill (AUMg), anadromous transformant gill (ATg), and anadromous transformant skin (ATsk). Rhag expression is detected in all tissues with very light band in the MTsk. Rhbg only strongly expressed in the skin of the transformant lamprey acclimated to 10% salinity. Rhcg1 and Rhcg2 expression was detected in all samples. Darker bands signify higher expression of Rh glycoproteins, while lighter bands signify less expression.

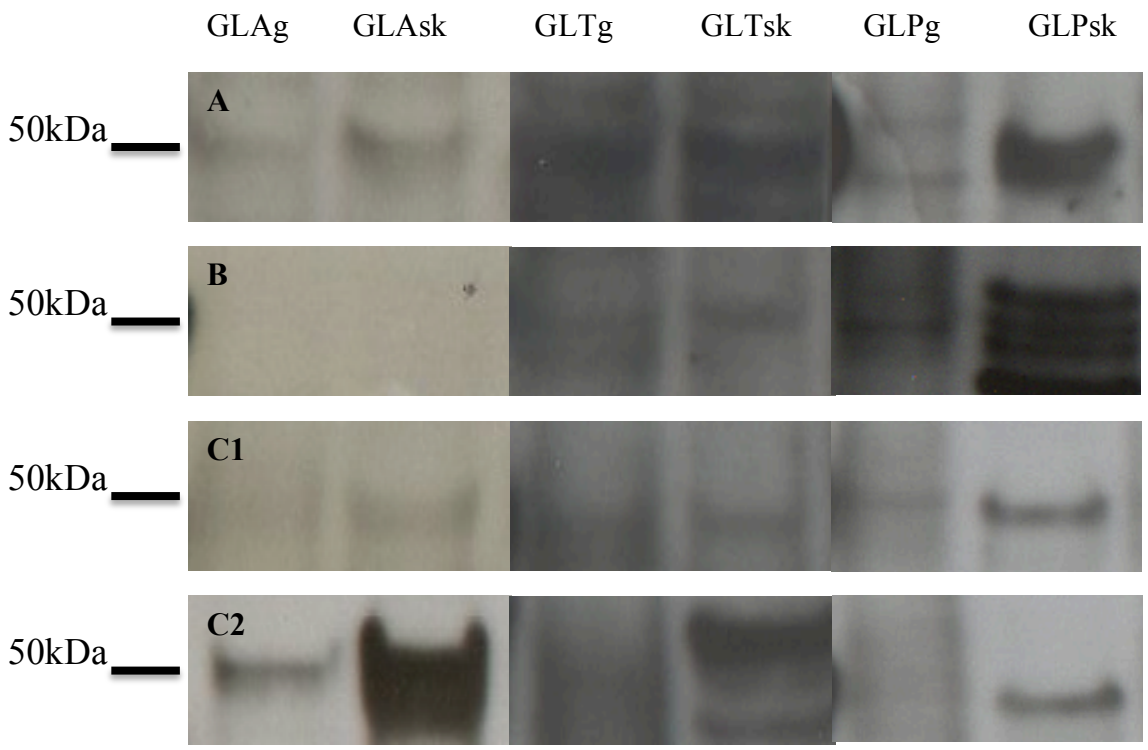


Figure 5. Western blot combined Great Lakes landlocked lamprey. Columns or lanes designate quantified protein from Great lakes ammocoete gill (GLAg), Great lakes ammocoete skin (GLAsk), Great lakes transformant gill (GLTg), Great lakes transformant skin (GLTsk), Great lakes parasite gill (GLPg), and Great lakes parasite skin (GLPsk). Rows of Rhag (A), Rhbg (B), Rhcg1 (C1), and Rhcg2 (C2) detection is shown by the presence or absence of a band (~50kDa) from top to bottom, respectively. Rh expression was observed in all samples with the exception of absence of Rhbg in the gill and skin of the ammocoetes. Rhc2 was absent in the parasite gill. Darker bands signify higher expression of the Rh glycoproteins, while lighter bands signify less expression.

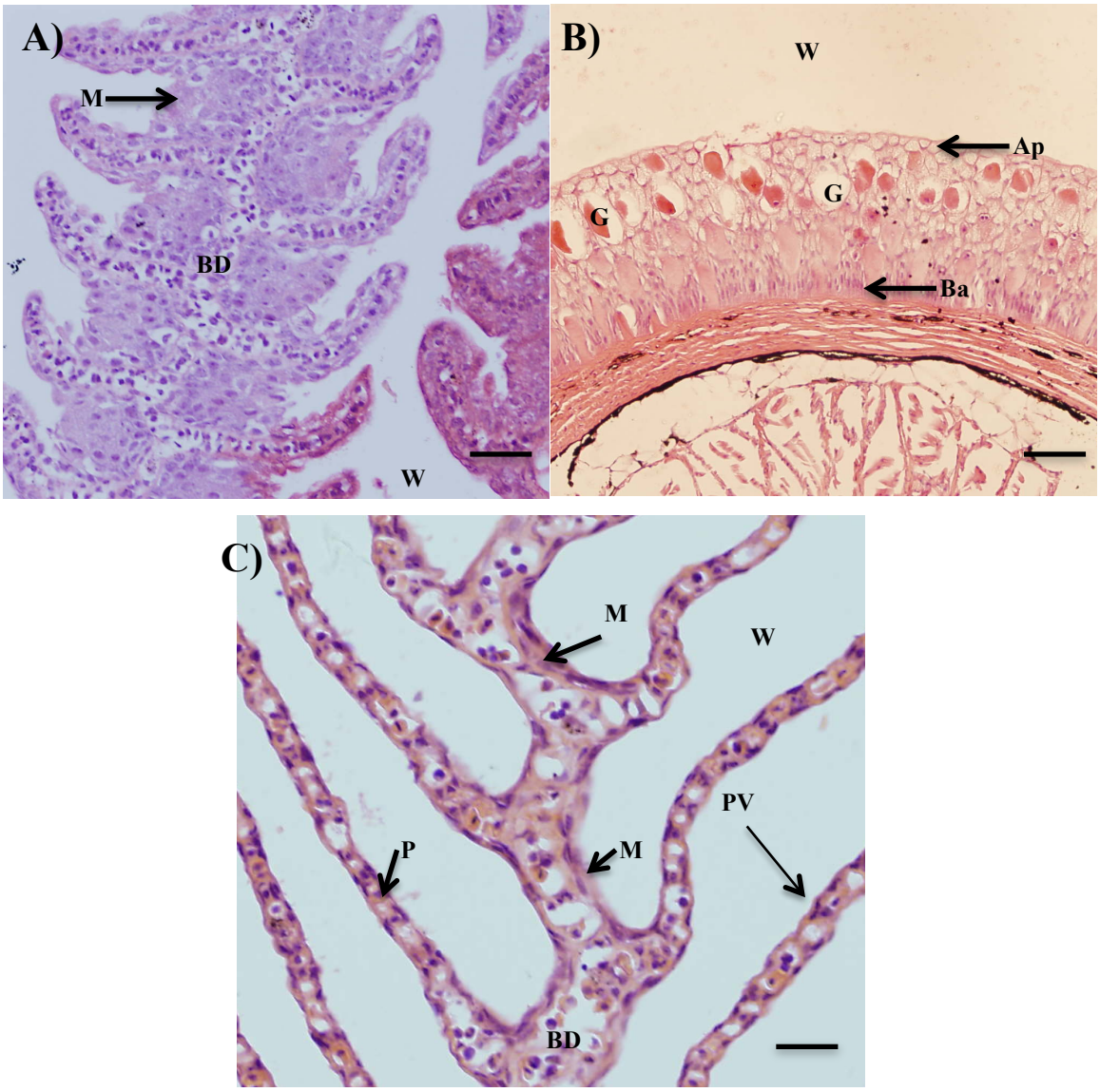


Figure 6. Hematoxylin and eosin stained cross section of gill and skin from the lamprey. A) Transformant gill section showing locations of blood (BD) in the filament of the gill and surrounding external water (W). In between the gill filaments in the interlamellar space lie the mitochondria rich cells (M). B) Transformant skin section showing the apical (Ap) epithelium facing the water (W) and the deeper basal (Ba) region of the skin. Goblet cells (G) also known as mucous or club cells are dispersed within the mid region of the skin epithelia. C) Upstream migrant gill section showing the blood (BD) filled filament with the elongated branching lamellae. The lamellae are composed of central stacking pillar cells (P) with apically located pavement cells (PV) lining the lamellae. The interlamellar space is also composed of mitochondria rich cells (M). This is also representative of the parasite gill section, differing only by larger enhanced mitochondria rich cells. Black bar ≈ 50 μm.

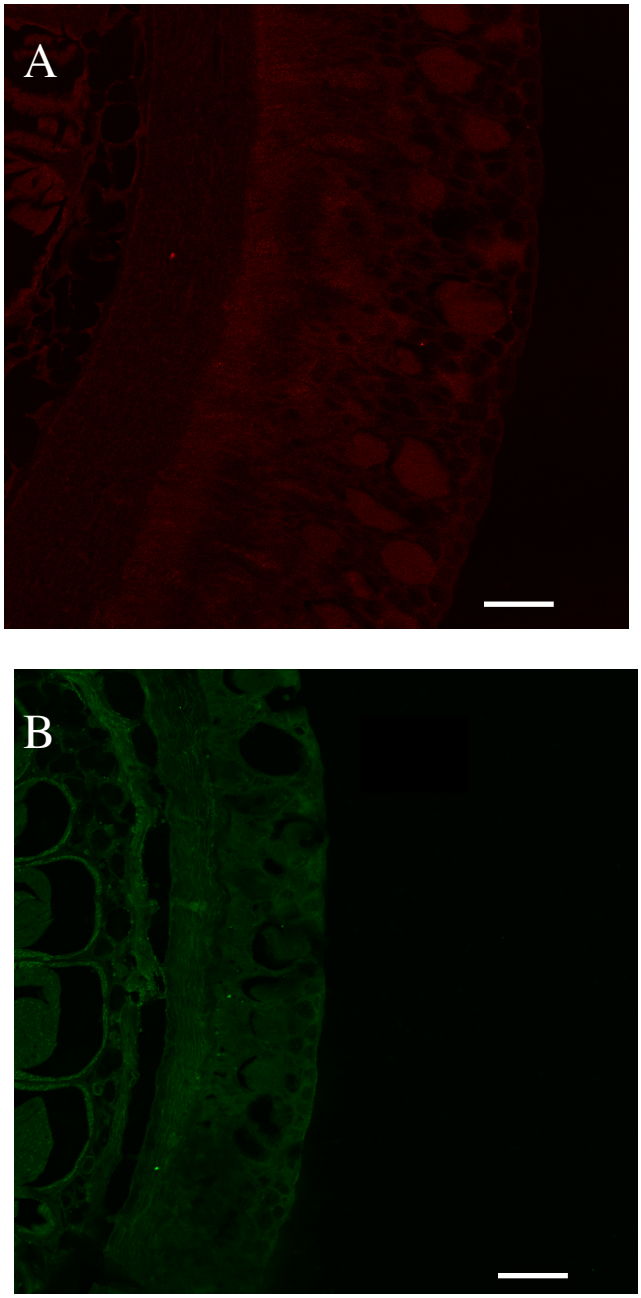


Figure 7. Immunohistochemistry of transformant skin sections. A) Anadromous transformant skin section, stained as negative control (anti-RB 568 nm) no primary antibody. B) Great lakes landlocked transformant skin negative control (anti-RB 488nm) with no primary antibody. White bar = 50 μm.

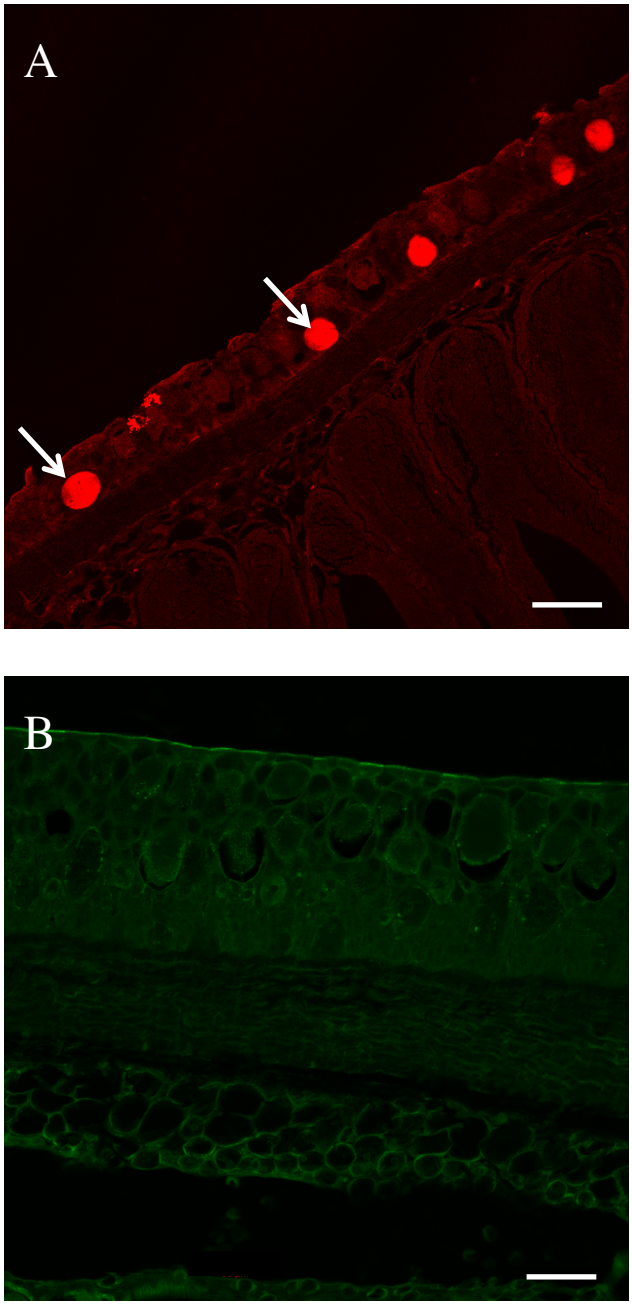


Figure 8. Immunohistochemistry of Rhag in the skin of landlocked and anadromous transformants. A) Rhag expression localized to large club cells or mucous cells of the skin epithelium of the anadromous transformant. B) Rhag expression is absent in the skin of Great Lakes landlocked transformant. White bar = 50 μm.

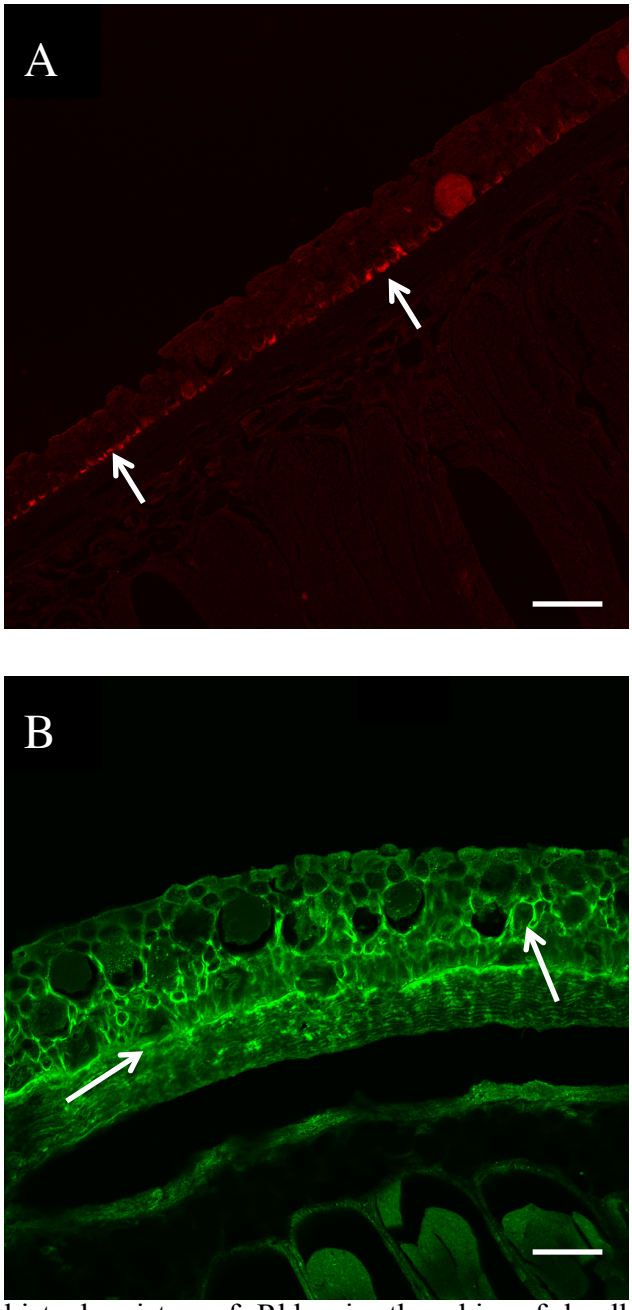


Figure 9. Immunohistochemistry of Rhbg in the skin of landlocked and anadromous transformants. A) Rhbg expression localized to the basal layer of the skin epithelium of the anadromous transformant. B) Rhbg expression is ubiquitous from the basal layer and mid region and tapers off towards the apical cells in the skin of Great Lakes landlocked transformant. White bar = 50 μm.

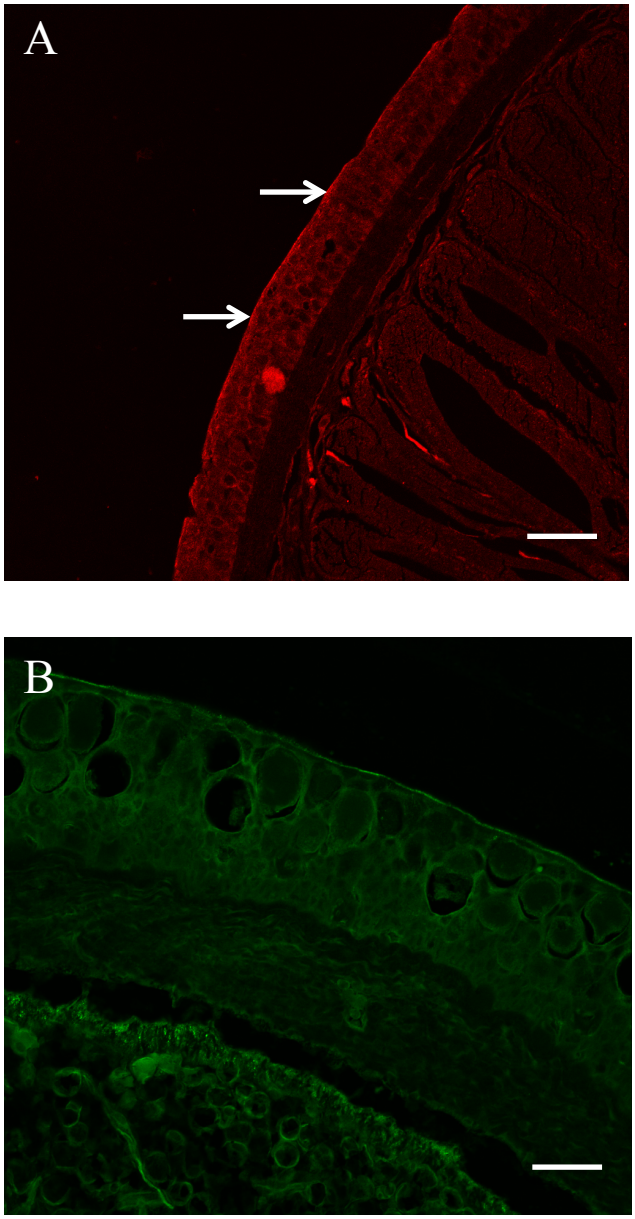


Figure 10. Immunohistochemistry of Rhcg1 in the skin of landlocked and anadromous transformants. A) Rhcg1 expression localized to the apical layer of the skin epithelium of the anadromous transformant. B) Rhcg1 expression is minimal to absent in the skin of Great Lakes landlocked transformant. White bar = 50 μm.

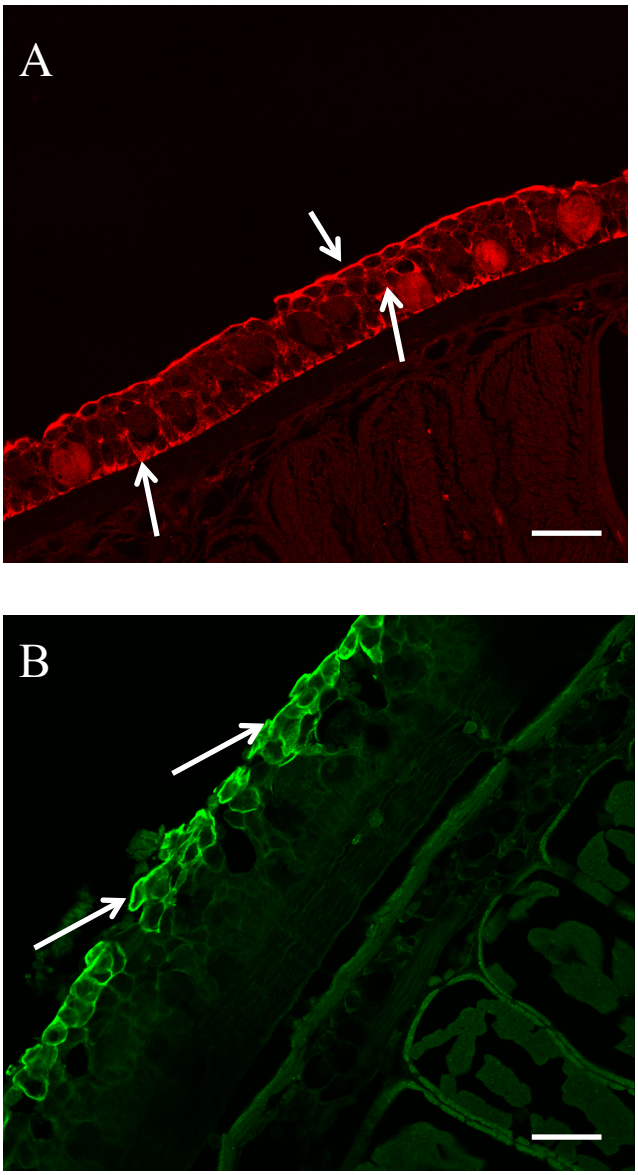


Figure 11. Immunohistochemistry of Rhcg2 in the skin of landlocked and anadromous transformants. A) Rhcg2 expression is ubiquitous throughout the skin epithelium of the anadromous transformant. B) Rhcg2 expression is located to cells on the apical side of the skin of Great Lakes landlocked transformant. White bar = 50 μm.

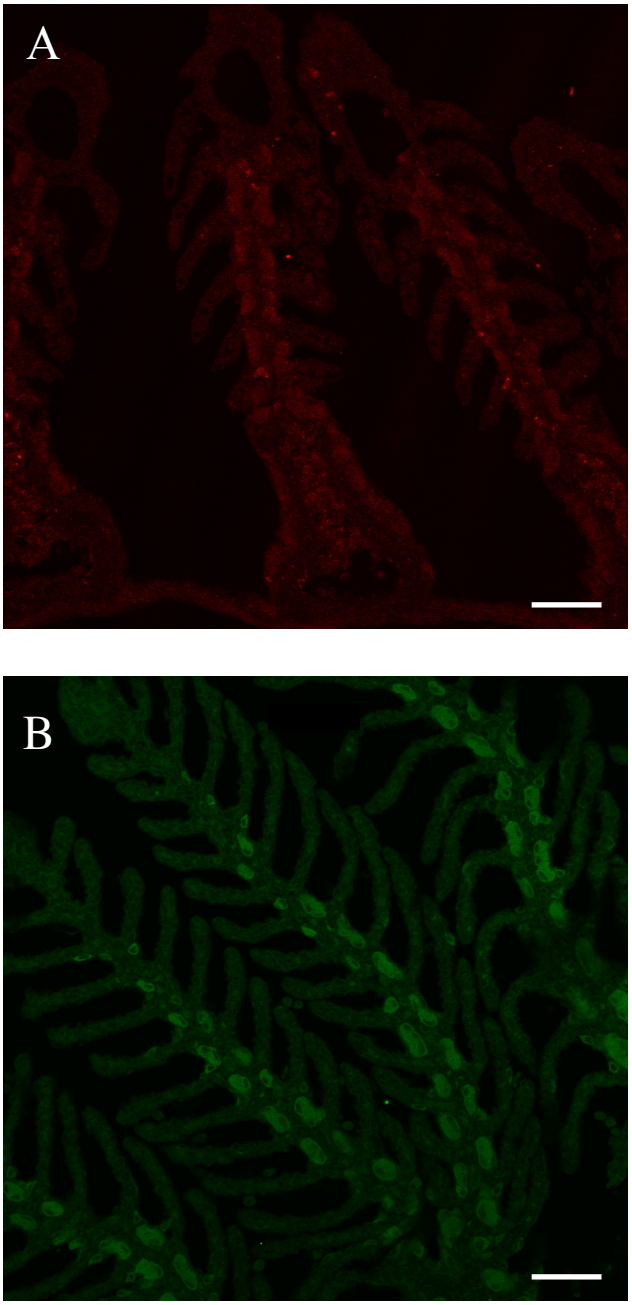


Figure 12. Immunohistochemistry of transformant gill sections. A) Anadromous transformant gill section stained as negative control (anti-RB 568 nm) no primary antibody. B) Great lakes landlocked transformant gill negative control (anti-RB 488nm) with no primary antibody. White bars = 50 μm.

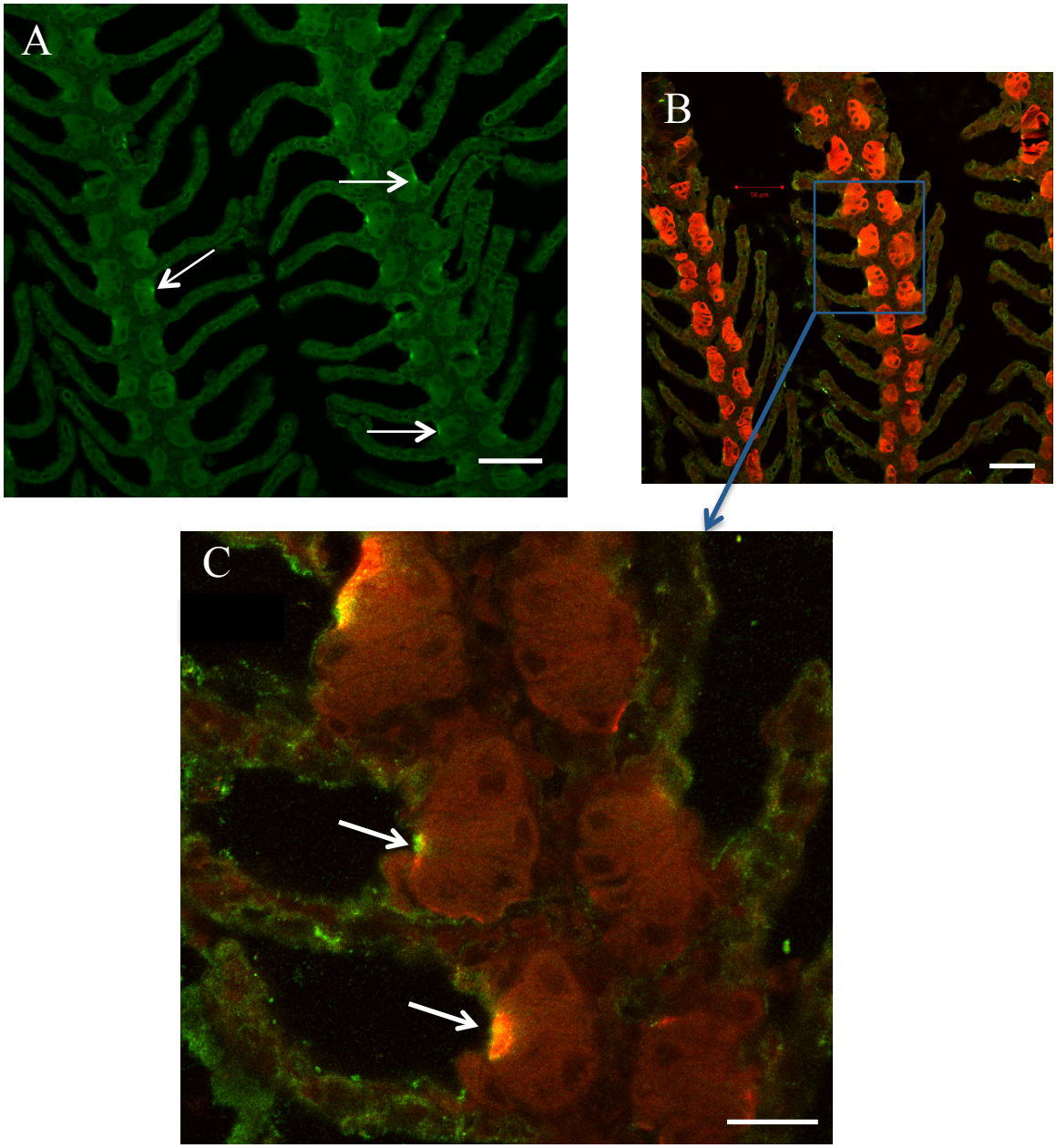


Figure 13. Double labeling immunohistochemistry of Rhag and Na^+/K^+-ATPase (NKA) in the gills of Great Lakes landlocked transformants. A) Rhag expression shown with single immunolocalization in the interlamellar space. B) Co-localization of Rhag (green) on the apical side of mitochondrial rich cells (MRCs) in the interlamellar space, rich in NKA (red) expression. White bar = 50 μm. C) Higher magnification of Rhag expression showing localization specifically to the apical crypt of hypothesized in the gills of Great Lakes landlocked transformant. White bar = 20 μm.

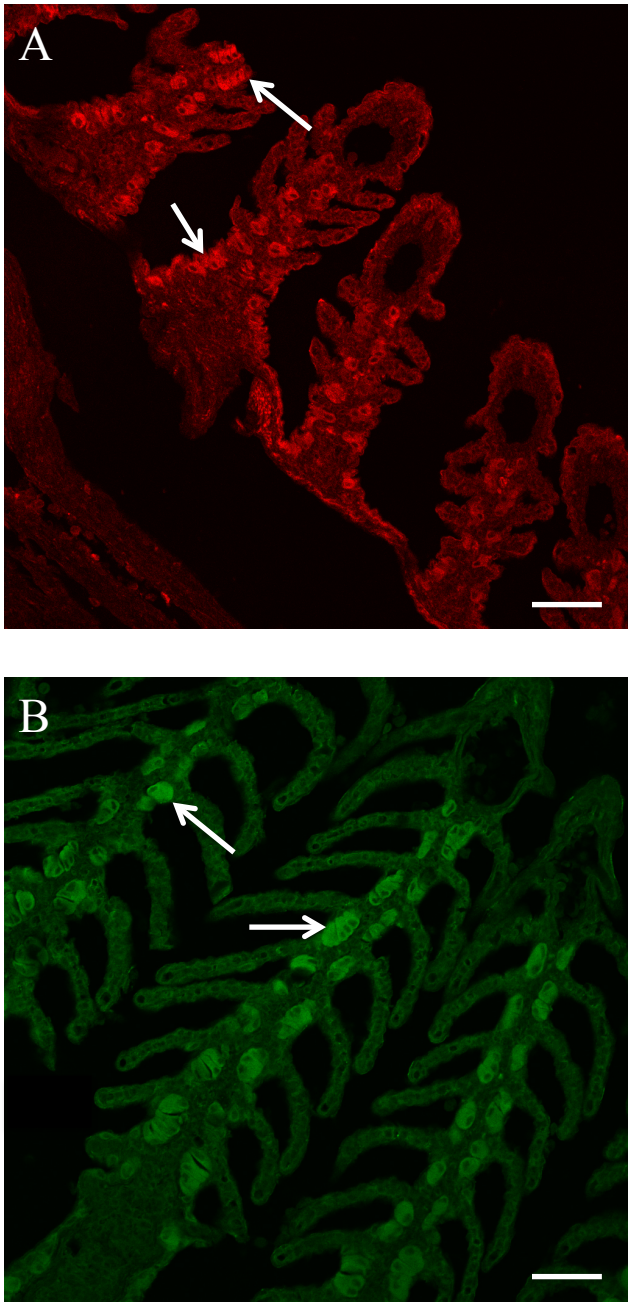


Figure 14. Immunohistochemistry of Rhcg1 in transformant gill sections. A) Rhcg1 expression is seen outlining the apical lamellae as well as concentrated in the hypothesized mitochondrial rich cells (MRCs) of the anadromous transformants B) Rhcg1 expression is minimal but compared to negative control, is localized to the hypothesized MRCs of the Great lakes landlocked transformants C) and D) Negative controls for comparison of Anadromous transformant gill and Great lakes landlocked transformant gill, respectively. All white bars = 50 μm.

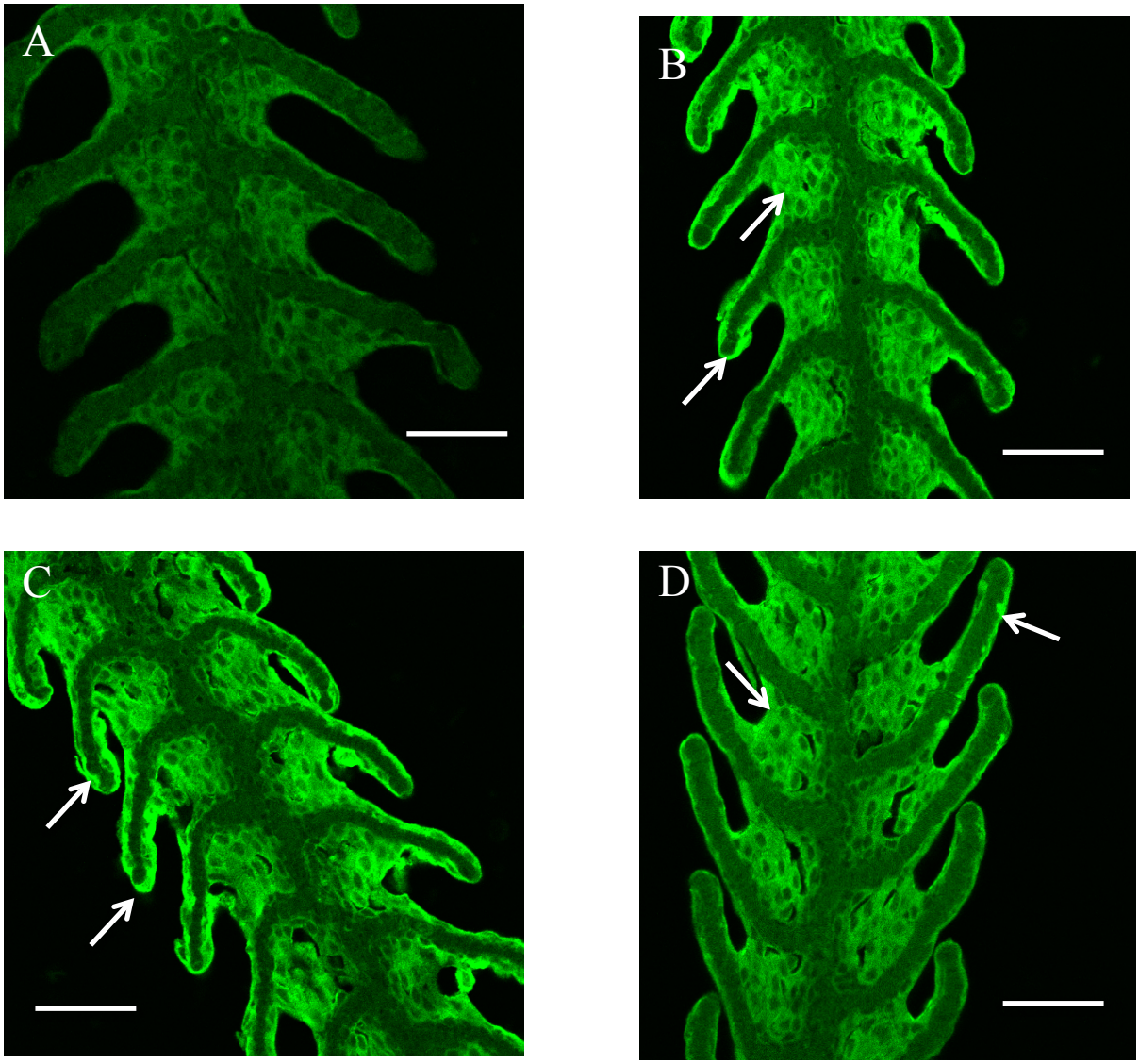


Figure 15. Immunohistochemistry of Rh glycoprotein expression in gills of GL landlocked ammocoetes. A) Section stained as negative control showing no expression for comparison. B) Rhag is localized to the mitochondria rich cells (MRCs) as well as outlining the apical sides of the lamellae. C) Rhcg1 localized to the apical lamellae lining with little expression in the MRCs. D) Rhcg2 localized to the MRCs with little expression in outlining the lamellae. White bar = 50 μm.

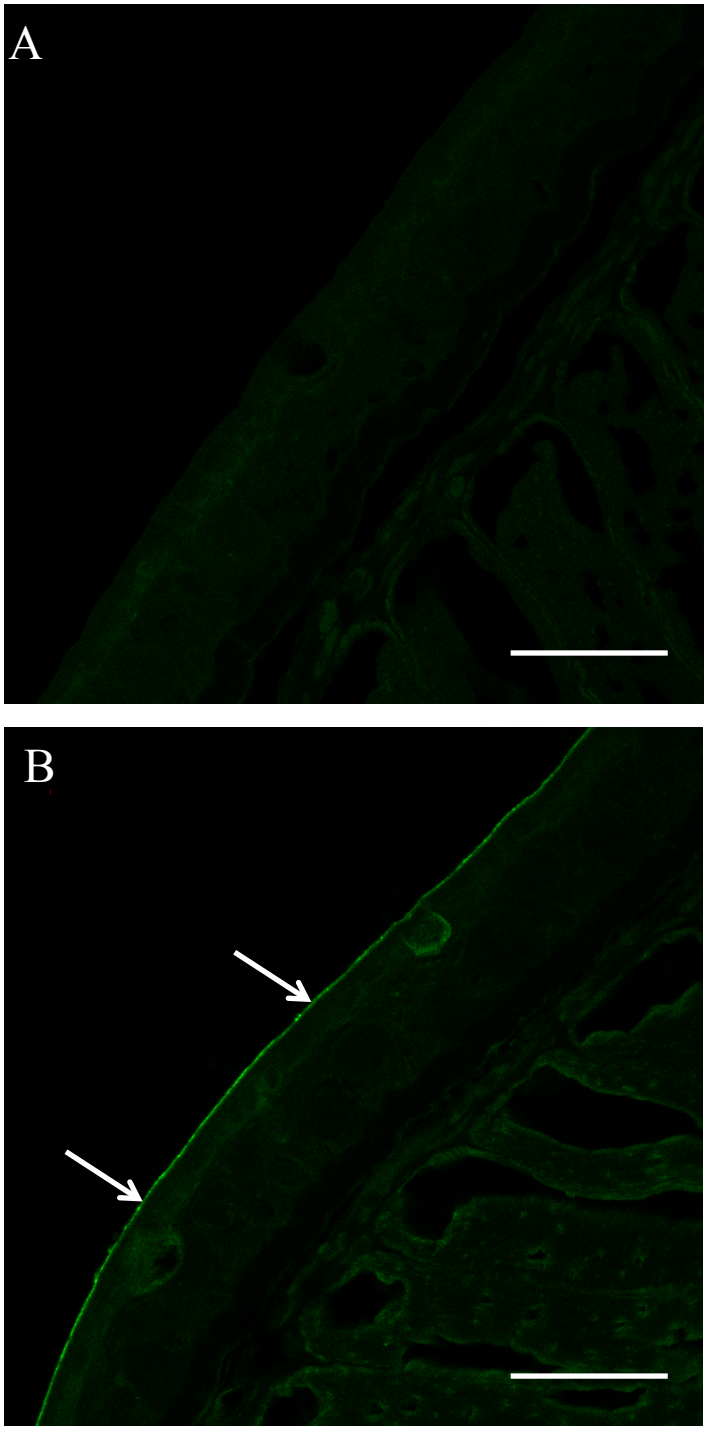


Figure 16. Immunohistochemistry of Rhag localized in skin of GL landlocked ammocoete. A) Negative control stained skin section for comparison. B) Rhag is localized to the apical layer of cells lining the skin epithelium. White bar = 50 μm.

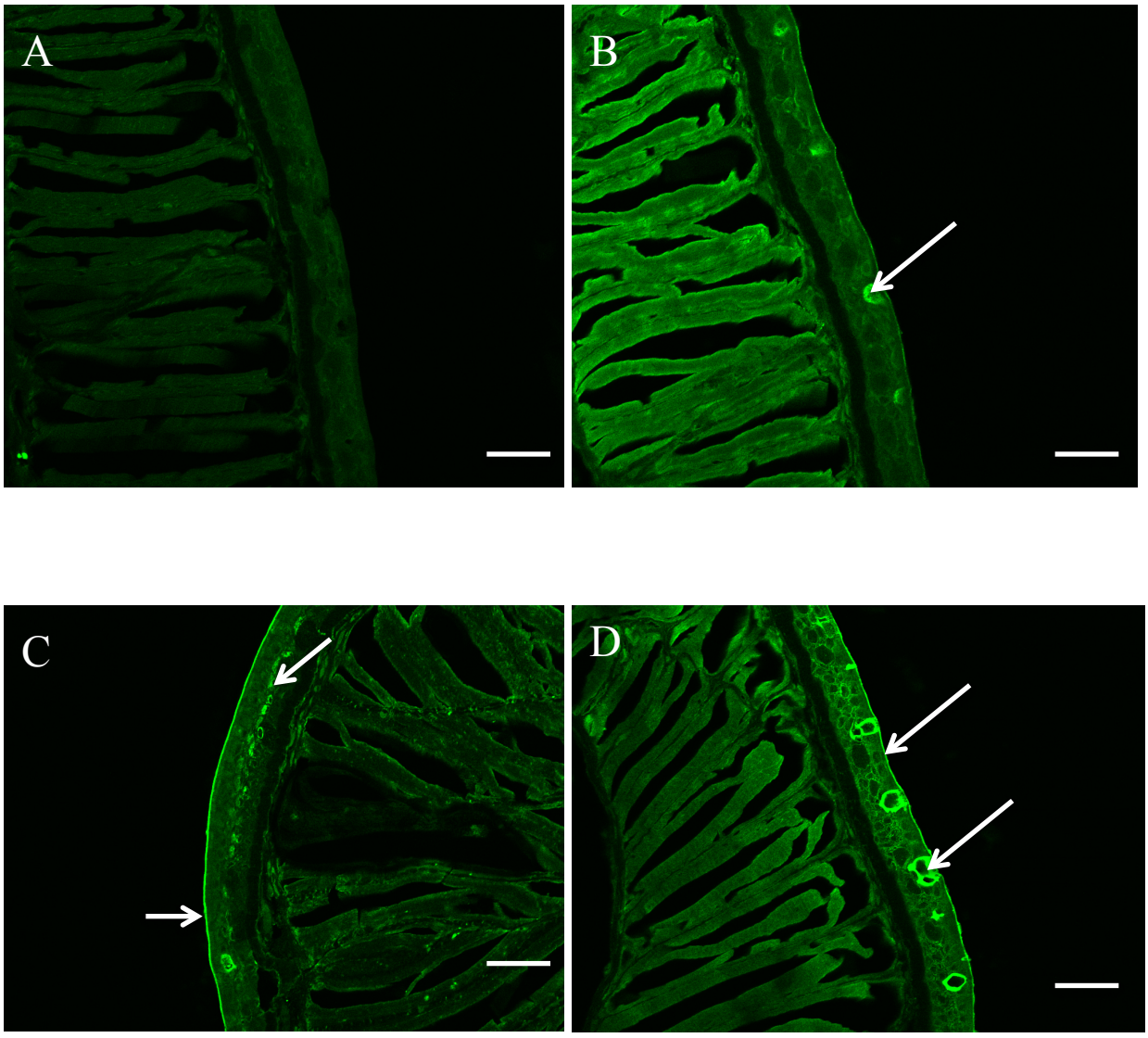


Figure 17. Immunohistochemistry of GL landlocked ammocoete skin sections. A) Negative control showing no staining. B) Rhbg localized to the club cells. C) Rhcg1 localized to apical cells lining skin epithelium as well as to a basal layer of cells. D) Rhcg2 localized to both the club cells and to the apical lining of cells on the outside of the skin epithelium. White bar = 50 μm.

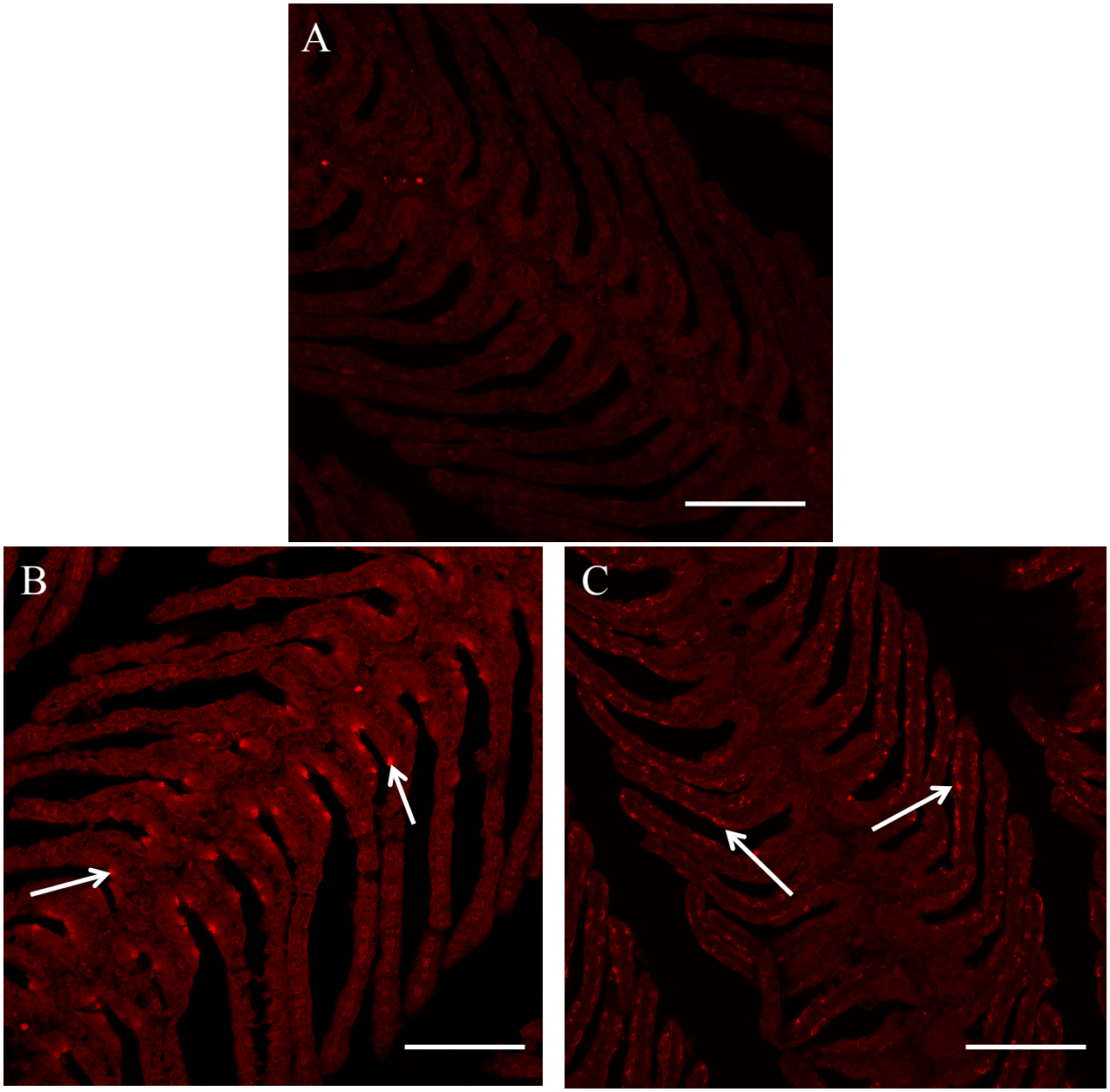


Figure 18. Immunohistochemistry of Rhag and Rhbg in GL landlocked parasite gill sections. A) Negative control showing absence of expression. B) Rhag expression on the apical sides of mitochondrial rich cells in the interlamellar space. C) Rhbg localized to the pavement cells lining the lamellae. White bars = 100 μm.

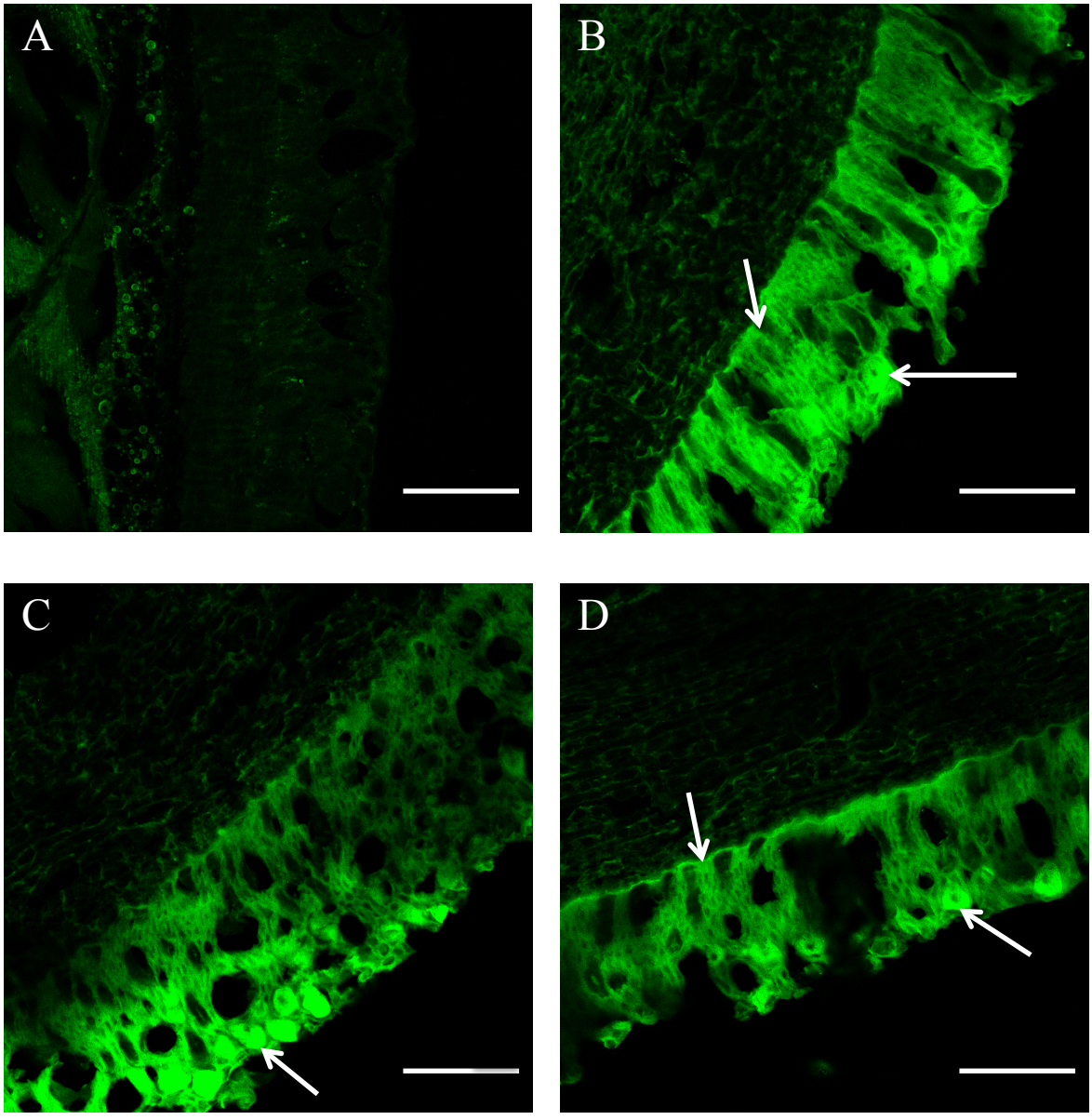


Figure 19. Immunohistochemistry of Rh expression in the skin of GL landlocked parasites. A) Negative control showing absence of Rh expression. B) Rhbg, (C) Rhcg1, and (D) Rhcg2 localized to the skin epithelium showing ubiquitous staining from basal through mid region extending to apically facing cells. White bar = 100 μm.

5′-**GGGGTACAAGTTTTTGATCGCCACCTTCGGCATCCAGT**
GGGCCACCCTCATGCAGGGCTGGTTCTGGCACCTGGGTCC
GGACGGCAAGATCCTAGTCGGGGTCATGAACATGATGAA
CAACGACTTTTTCTGGGGCTCTGTGCTCATCATCTTTGGCG
CCCTGCTGGGCAAGACCACCCCGGTGCAGCTTCTCTTCATG
GCCCTGCCGATCATCAATCTGTACTCGGTCAACAAGTACA
TCGTCCTGCACCTCCTCATGTGCAAGAATGACGGCGGCTC
GATGACGATCCACACGTTCGGCGCGTACTTCTTACTCCCCG
TGTCGCGCGTGCTCTACCGCCCGGGACTCAAGGACGGGCA
CCCCAAGAACGGCTCCGTCTACCACTCGGACGTCTTCTCGA
TGATGAGGACCATTTTCCTCTTCCTCTTCTGGCCGACCTTC
AACTCCTCCATCTCGGCGGCGGGTAACGACCAGCACAGGA
CCTCCATCAAGACCTACTACTTGCTCACGGCCTCCGTGGTG
GTCACCTACGCCATATCCAGCCTCACCGAGGAGCACGGCA
AACTCGACATGGTCCACATCCTCAACACGACGCTGGTGGG
AGGGGTTGCGATGGGGACGGCCGGCGAGATGATGATGAT
GACGTACTGTTCGCTCATCGT-3′

Figure 20. Lamprey Rhbg nucleotide sequence (663 bp). BLASTX results yield lamprey Rhbg to be similar to (66% identity) *Takifugu rubripes* Rhbg mRNA for Rh type B glycoprotein (AB218980.1; gb|AY116074.1|).

5′-CAAATCAGTATAGGGCGATTGATTTAGCGGCCGCGAATTCGCC
CTTTGGTGGATTTTTTGGTCTCGCGGTTTCTCGAGTCCTATACCGTC
CAGGCCTCAAGGAGCCCCACCGAAAGGCATCTTCAGTCTATCACT
CTGATTTATTTGCTATGATTGGTACCCTGTTCCTATGGATTTACTGG
CCGAGCTTTAATTCTGCAATTTCAGAAAAAGGTGTAAATCAGACTC
GAGCAGTAATCAATACATACTACACGCTGGCCTCGTGTACTGTAAC
TACGTGTATCCTATCAAGCTTGGTTGACAAGAGCGGCCGAATCAAC
ATGGTCCATTTGCAGAATTCGACGTTGGCCGGAGCAGTTGCGGTTG
GAACGGCAGCAGAAATGATGCTCACTCCGTACGGGTCCCTTATCGT
GGGGTTAATTCTGGGAACGCTCAGCACACTTGGATACACCTTCATC
ACGCCTGCCCTGGAAAAATACCTCCATGTTCAAGACACCTGTGGCA
TTCATAACCTGCATGCCCTTCCTGGCTTCTGCGGAGGTATCATTGA
AGGGCGAATTCGTTTAAACCTGCAGGACTAGTCCCTTTAGTGAGGG
TTAATTCTGAGCTTGGCGTAATCATGGTCATAGCTGTTTCCTGTGTG
AAATTGTTATCCGCTCACAATTCCACACAACATACGAGCCGGAAGC
ATAAAGTGTAAAGCCTGGGGTGCCTAATGAGTGAGCTAACTCACAT
TAATTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTC
GTGCCAGCTGCATTAATGAATCGGCCAACGCGCGGGGAGAGGCGG
TTTGCGTATTGGGCGCTCTTCCGCTTCCTCGCTCACTGACTCGCTGC
GCTCGGTCGTTCGGCTGCGGCGAGCGGTATCAGCTCACTCAAAGGC
GGTAATACGGTTATCCACAGAATCACGGGATAACGCAGAAAGAAC
ATGTGAGCAAAAACGCCCAGCAAAAGGCCAGGAACCCGTAAAAAA
G-3′

Figure 21. Lamprey Rhcg nucleotide sequence (1010 bp). Lamprey Rhcg to be similar to (99% identity) that of Atlantic hagfish, *Myxine glutinosa* Rh type C glycoprotein (GU733440.1; ADD63335.1), as well as similarity to (64% identity) that of the pufferfish, *Takifugu rubripes* Rh type C2 (NP_001027934.1; GI:74136151).

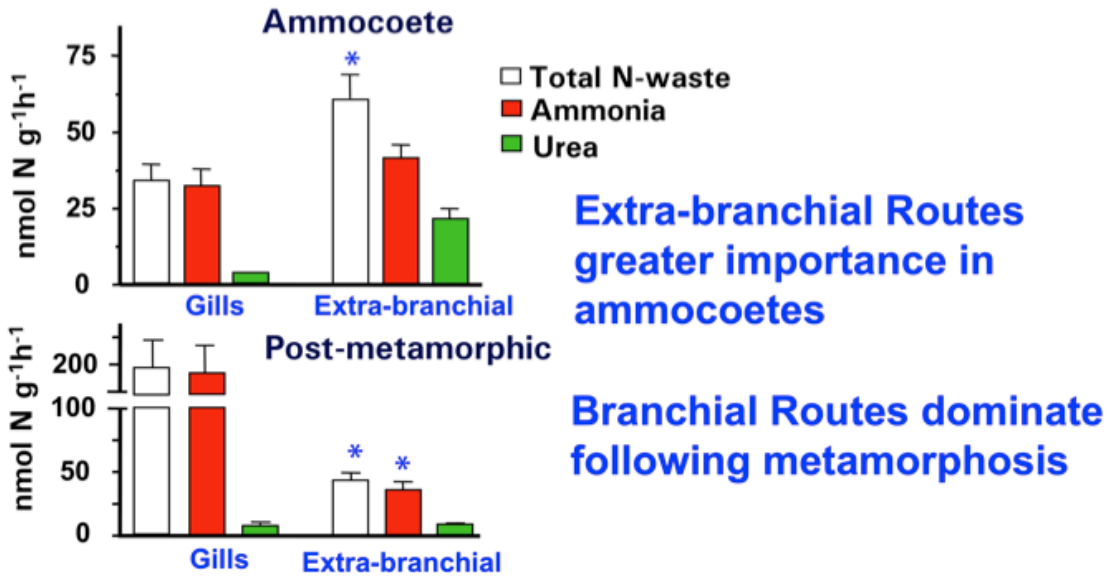


Figure 22. Physiological Results of *In Vivo* Nitrogen Waste Excretion in Great Lakes FW Lamprey. Total ammonia N-waste excretion is occurring through mechanism routes other than gill, in ammocoete and transformant. Ammocoete demonstrate significant extra-branchial routes although both in all life stages (Wilkie, unpublished).

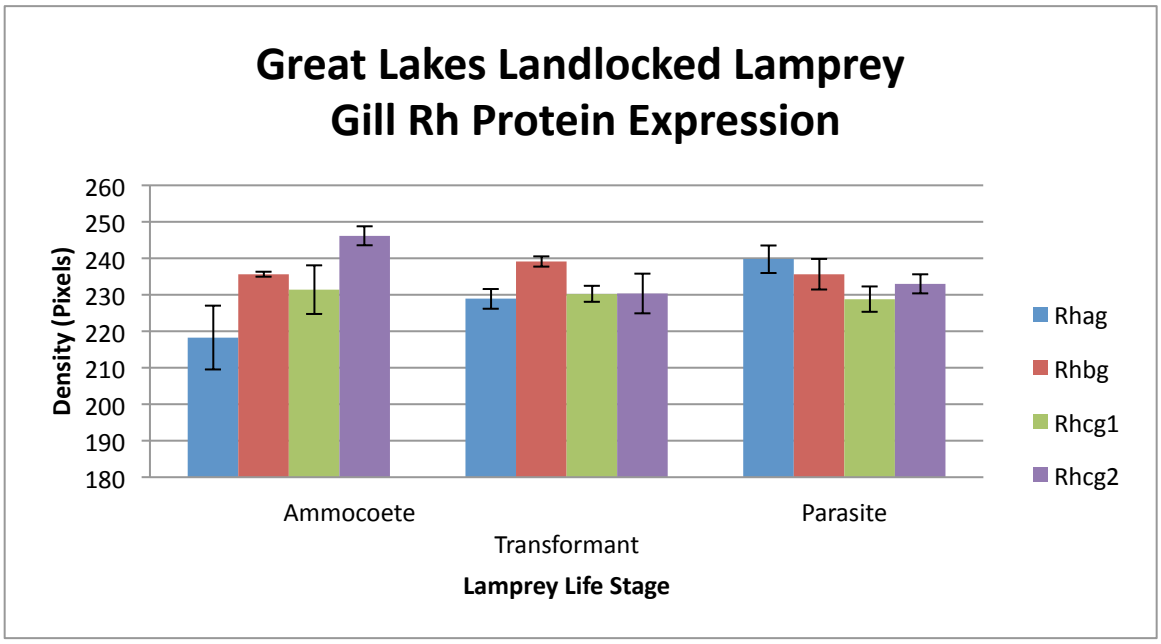


Figure 23. Western blot quantification analysis of GL landlocked gill Rh protein expression in Ammocoetes, Transformants, and Parasites. No significant differences were calculated. The calculated density is inversely proportional to amount of expression, thus shorter bars represent higher expression and taller bars represent lower expression.

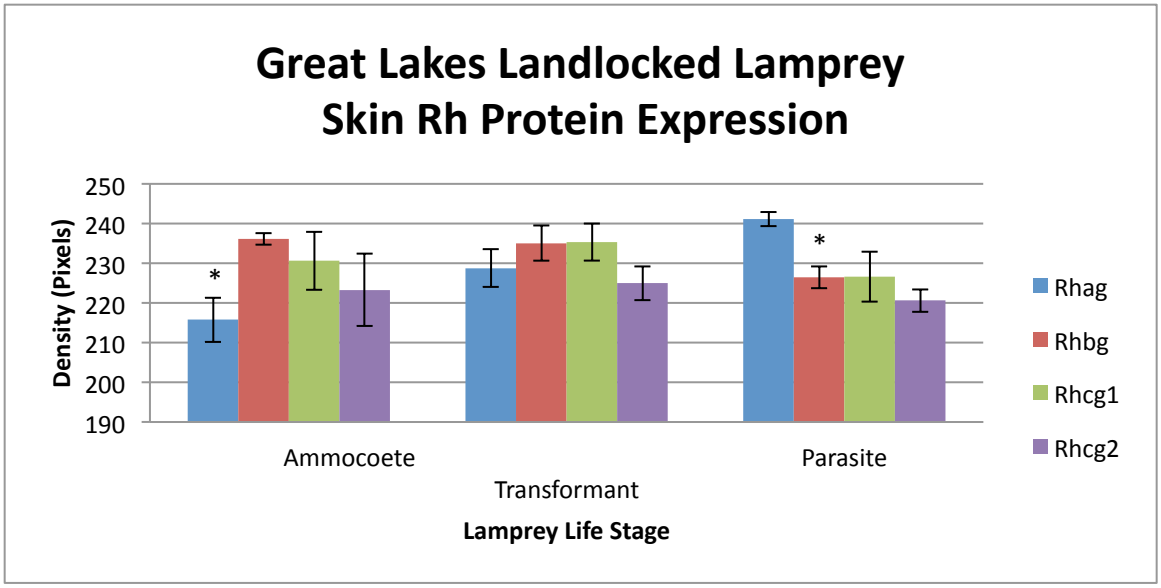


Figure 24. Western blot quantification analysis of GL landlocked gill Rh protein expression in Ammocoetes, Transformants, and Parasites. Rhag in the skin of GL landlocked ammocoetes were significantly higher ($P< 0.05$) than Rhag in the skin of GL landlocked parasites. Rhbg in the skin of GL parasites was significantly higher ($P< 0.05$) than Rhbg in the skin of GL landlocked ammocoetes. The calculated density is inversely proportional to amount of expression, thus shorter bars represent higher expression and taller bars represent lower expression.

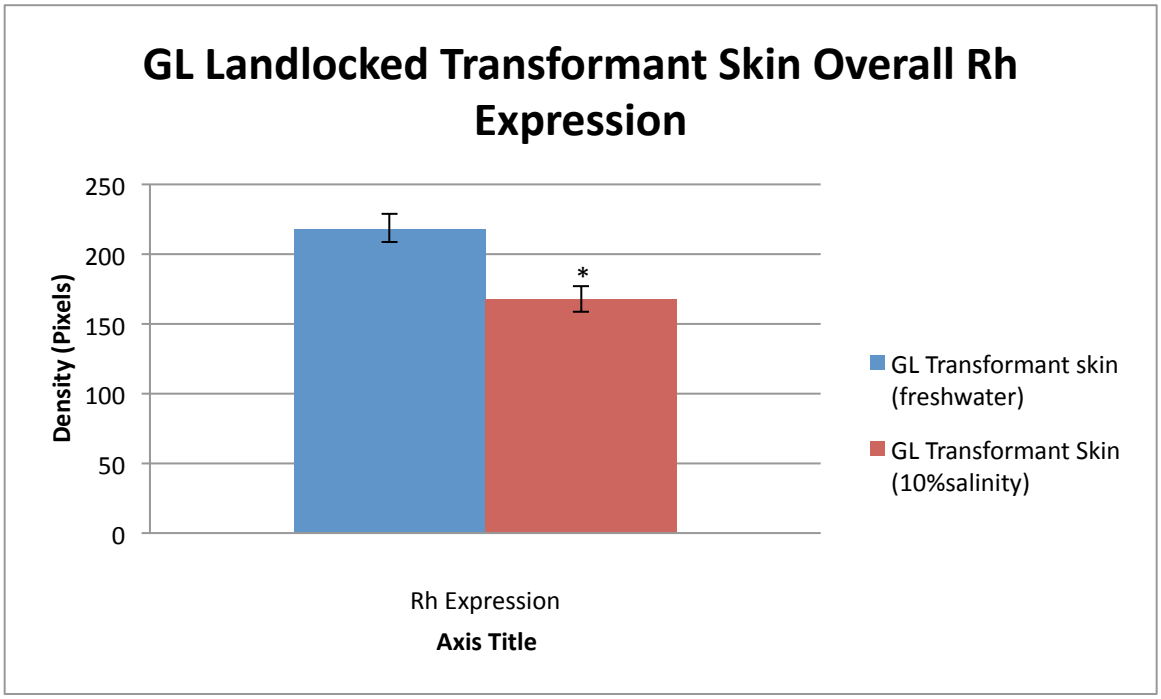


Figure 25. Overall Rh expression comparison between GL landlocked transformant skin. Overall Rh protein expression was significantly increased in skin of transformant at 10% salinity ($P<0.05$) in comparison to freshwater transformant skin. The calculated density is inversely proportional to amount of expression, thus shorter bars represent higher expression and taller bars represent lower expression.

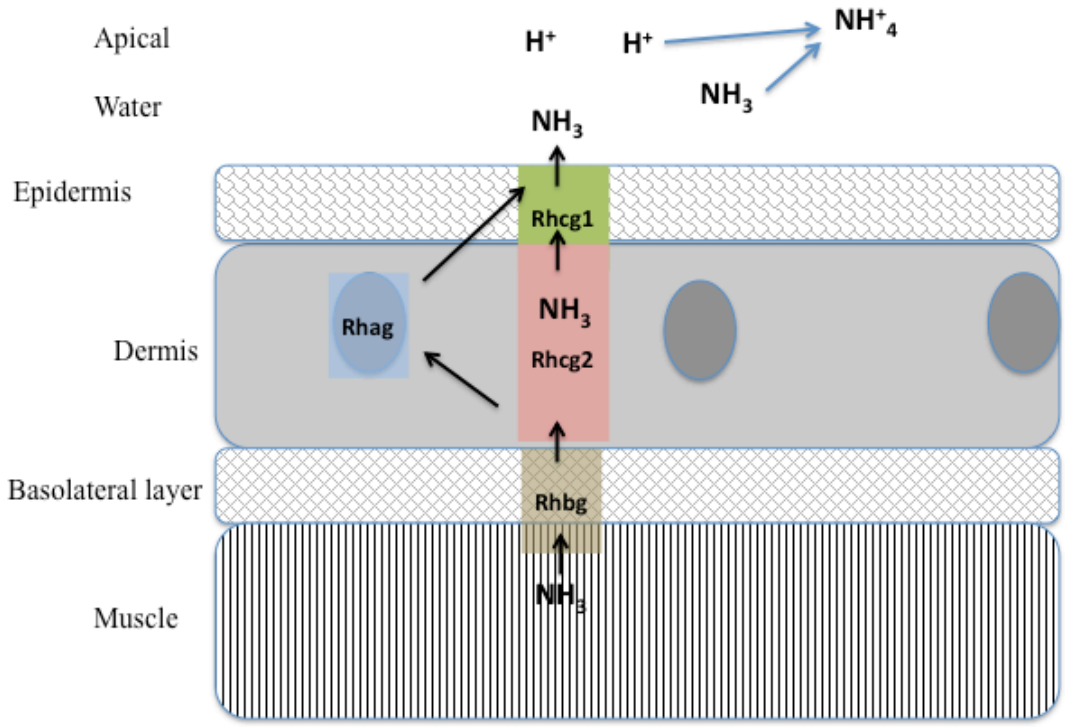


Figure 26. Model of hypothesized ammonia excretion across the skin epithelium. The anadromous transformant skin expressed all 4 Rh isoforms. Rhag was localized to the large mucous or club cells, Rhbg was localized to the basolateral dermal layer, Rhcg1 to the epidermal cells of the apical surface, and Rhcg2 expressed throughout the dermal layer. The locations of these Rh glycoproteins may provide a direct cooperative pathway across the skin epithelium enabling ammonia excretion into the environment.

References

Anstee, D.J., and Tanner, M.J. (1993). Biochemical aspects of the blood group Rh (rhesus) antigens. Baillieres Clin Haematol *6*, 401-422.

Arillo, A., Margiocco, C., Melodia, F., Mensi, P., and Schenone, G. (1981). Ammonia toxicity mechanism in fish: studies on rainbow trout (*Salmo gairdneri* Rich). Ecotoxicol Environ Saf *5*, 316-328.

Avent, N.D. (2001). A new chapter in Rh research: Rh proteins are ammonium transporters. Trends Mol Med *7*, 94-96.

Bakouh, N., Benjelloun, F., Cherif-Zahar, B., and Planelles, G. (2006). The challenge of understanding ammonium homeostasis and the role of the Rh glycoproteins. Transfus Clin Biol *13*, 139-146.

Beamish, F., and Potter, I. (1975). The biology of the anadromous Sea lamprey (Petromyzon mannus) in New Brunswick. Journal of Zoology *177*, 57-72.

Binstock, L., and Lecar, H. (1969). Ammonium ion currents in the squid giant axon. J Gen Physiol *53*, 342-361.

Braun, M.H., and Perry, S.F. (2010). Ammonia and urea excretion in the Pacific hagfish *Eptatretus stoutii*: Evidence for the involvement of Rh and UT proteins. Comparative Biochemistry and Physiology a-Molecular & Integrative Physiology *157*, 405-415.

Braun, M.H., Steele, S.L., Ekker, M., and Perry, S.F. (2009a). Nitrogen excretion in developing zebrafish (Danio rerio): a role for Rh proteins and urea transporters. Am J Physiol Renal Physiol *296*, F994-F1005.

Braun, M.H., Steele, S.L., and Perry, S.F. (2009b). The responses of zebrafish (*Danio rerio*) to high external ammonia and urea transporter inhibition: nitrogen excretion and expression of rhesus glycoproteins and urea transporter proteins. Journal of Experimental Biology *212*, 3846-3856.

Cherifzahar, B., Mattei, M.G., Levankim, C., Bailly, P., Cartron, J.P., and Colin, Y. (1991). Localization of the Human Rh Blood-Group Gene Structure to Chromosome Region 1p34.3-1p36.1 by Insitu Hybridization. Human Genetics *86*, 398-400.

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

Claiborne, J.B., and Evans, D.H. (1988). Ammonia and Acid-Base-Balance during High Ammonia Exposure in a Marine Teleost (*Myoxocephalus-Octodecimspinosus*). Journal of Experimental Biology *140*, 89-105.

Doolittle, R.F. (1983). The Structure and Evolution of Vertebrate Fibrinogen. Annals of the New York Academy of Sciences *408*, 13-27.

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

Eyers, S.A., Ridgwell, K., Mawby, W.J., and Tanner, M.J. (1994). Topology and organization of human Rh (rhesus) blood group-related polypeptides. J Biol Chem *269*, 6417-6423.

Heimberg, A.M., Cowper-Sal-lari, R., Semon, M., Donoghue, P.C., and Peterson, K.J. (2010). microRNAs reveal the interrelationships of hagfish, lampreys, and gnathostomes and the nature of the ancestral vertebrate. Proc Natl Acad Sci U S A *107*, 19379-19383.

Huang, C.H., and Peng, J. (2005). Evolutionary conservation and diversification of Rh family genes and proteins. Proc Natl Acad Sci U S A *102*, 15512-15517.

Hung, C.C., Nawata, C.M., Wood, C.M., and Wright, P.A. (2008). Rhesus glycoprotein and urea transporter genes are expressed in early stages of development of rainbow trout (*Oncorhynchus mykiss*). J Exp Zool A Ecol Genet Physiol *309*, 262-268.

Hung, C.Y., Tsui, K.N., Wilson, J.M., Nawata, C.M., Wood, C.M., and Wright, P.A. (2007). Rhesus glycoprotein gene expression in the mangrove killifish Kryptolebias marmoratus exposed to elevated environmental ammonia levels and air. J Exp Biol *210*, 2419-2429.

Landsteiner, K., and Wiener, A.S. (1941). Studies on an agglutinogen (Rh) in human blood reacting with anti-rhesus sera and with human isoantibodies. J Exp Med *74*, 309-320.

Levine, P., and Stetson, R.E. (1939). An unusual case of intra-group agglutination. Journal of the American Medical Association *113*, 126-127.

Liu, Z., Chen, Y., Mo, R., Hui, C.C., Cheng, J.F., Mohandas, N., and Huang, C.H. (2000). Characterization of human RhCG and mouse Rhcg as novel nonerythroid Rh glycoprotein homologues predominantly expressed in kidney and testis. Journal of Biological Chemistry *275*, 25641-25651.

Liu, Z., and Huang, C.H. (1999). The mouse Rhl1 and Rhag genes: Sequence, organization, expression, and chromosomal mapping. Biochemical Genetics *37*, 119-138.

Liu, Z., Peng, J.B., Mo, R., Hui, C.C., and Huang, C.H. (2001). Rh type B glycoprotein is a new member of the Rh superfamily and a putative ammonia transporter in mammals. Journal of Biological Chemistry *276*, 1424-1433.

Maren, T.H., Embry, R., and Broder, L.E. (1968). The excretion of drugs across the gill of the dogfish, Squalus acanthias. Comp Biochem Physiol *26*, 853-864.

Marini, A.M., Matassi, G., Raynal, V., Andre, B., Cartron, J.P., and Cherif-Zahar, B. (2000). The human Rhesus-associated RhAG protein and a kidney homologue promote ammonium transport in yeast. Nat Genet *26*, 341-344.

Marini, A.M., Urrestarazu, A., Beauwens, R., and Andre, B. (1997). The Rh (rhesus) blood group polypeptides are related to NH_4^+ transporters. Trends Biochem Sci *22*, 460-461.

Marini, A.M., Vissers, S., Urrestarazu, A., and Andre, B. (1994). Cloning and expression of the MEP1 gene encoding an ammonium transporter in Saccharomyces cerevisiae. Embo Journal *13*, 3456-3463.

Matassi, G., Cherif-Zahar, B., Pesole, G., Raynal, V., and Cartron, J.P. (1999). The members of the RH gene family (RH50 and RH30) followed different evolutionary pathways. Journal of Molecular Evolution *48*, 151-159.

Mesa, M.G., and Schreck, C.B. (1989). Electrofishing mark recapture and depletion methodologies evoke behavioral and physiological-changes in cutthroat trout. Transactions of the American Fisheries Society *118*, 644-658.

Mommsen, T.P., and Walsh, P.J. (1992). Biochemical and Environmental Perspectives on Nitrogen-Metabolism in Fishes. Experientia *48*, 583-593.

Moreira-Silva, J., Tsui, T.K., Coimbra, J., Vijayan, M.M., Ip, Y.K., and Wilson, J.M. (2010). Branchial ammonia excretion in the Asian weatherloach *Misgurnus anguillicaudatus*. Comp Biochem Physiol C Toxicol Pharmacol *151*, 40-50.

Nakada, T., Hoshijima, K., Esaki, M., Nagayoshi, S., Kawakami, K., and Hirose, S. (2007a). Localization of ammonia transporter Rhcg1 in mitochondrion-rich cells of yolk sac, gill, and kidney of zebrafish and its ionic strength-dependent expression. Am J Physiol Regul Integr Comp Physiol *293*, R1743-1753.

Nakada, T., Westhoff, C.M., Kato, A., and Hirose, S. (2007b). Ammonia secretion from fish gill depends on a set of Rh glycoproteins. FASEB J *21*, 1067-1074.

Nakada, T., Westhoff, C.M., Yamaguchi, Y., Hyodo, S., Li, X., Muro, T., Kato, A., Nakamura, N., and Hirose, S. (2010). Rhesus glycoprotein p2 (Rhp2) is a novel member of the Rh family of ammonia transporters highly expressed in shark kidney. J Biol Chem *285*, 2653-2664.

Nawata, C.M., Hirose, S., Nakada, T., Wood, C.M., and Kato, A. (2010). Rh glycoprotein expression is modulated in pufferfish (*Takifugu rubripes*) during high environmental ammonia exposure. Journal of Experimental Biology *213*, 3150-3160.

Nawata, C.M., Hung, C.C.Y., Tsui, T.K.N., Wilson, J.M., Wright, P.A., and Wood, C.M. (2007). Ammonia excretion in rainbow trout (*Oncorhynchus mykiss*): evidence for Rh glycoprotein and H^+-ATPase involvement. Physiological Genomics *31*, 463-474.

Nawata, C.M., and Wood, C.M. (2008). The effects of CO_2 and external buffering on ammonia excretion and Rhesus glycoprotein mRNA expression in rainbow trout. J Exp Biol *211*, 3226-3236.

Potter, I.C., and Beamish, F.W.H. (1977). Freshwater biology of adult anadromous sea lampreys Petromyzon-marinus. Journal of Zoology *181*, 113-130.

Ridgwell, K., Spurr, N.K., Laguda, B., MacGeoch, C., Avent, N.D., and Tanner, M.J. (1992). Isolation of cDNA clones for a 50 kDa glycoprotein of the human erythrocyte membrane associated with Rh (rhesus) blood-group antigen expression. Biochem J *287 (Pt 1)*, 223-228.

Sashaw, J., Nawata, M., Thompson, S., Wood, C.M., and Wright, P.A. (2010). Rhesus glycoprotein and urea transporter genes in rainbow trout embryos are upregulated in response to alkaline water (pH 9.7) but not elevated water ammonia. Aquat Toxicol *96*, 308-313.

Shih, T.H., Horng, J.L., Hwang, P.P., and Lin, L.Y. (2008). Ammonia excretion by the skin of zebrafish (*Danio rerio*) larvae. Am J Physiol Cell Physiol *295*, C1625-1632.

Sower, S.A., Plisetskaya, E., and Gorbman, A. (1985). Changes in plasma steroid and thyroid hormones and insulin during final maturation and spawning of the sea lamprey, Petromyzon marinus. Gen Comp Endocrinol *58*, 259-269.

Tsui, T.K., Hung, C.Y., Nawata, C.M., Wilson, J.M., Wright, P.A., and Wood, C.M. (2009). Ammonia transport in cultured gill epithelium of freshwater rainbow trout: the importance of Rhesus glycoproteins and the presence of an apical Na^+/NH_4^+ exchange complex. J Exp Biol *212*, 878-892.

Weihrauch, D., Morris, S., and Towle, D.W. (2004). Ammonia excretion in aquatic and terrestrial crabs. J Exp Biol *207*, 4491-4504.

Weihrauch, D., Wilkie, M.P., and Walsh, P.J. (2009). Ammonia and urea transporters in gills of fish and aquatic crustaceans. J Exp Biol *212*, 1716-1730.

Weiner, I.D. (2006). Expression of the non-erythroid Rh glycoproteins in mammalian tissues. Transfus Clin Biol *13*, 159-163.

Wilkie, M.P. (1997). Mechanisms of ammonia excretion across fish gills. Comparative Biochemistry and Physiology a-Physiology *118*, 39-50.

Wilkie, M.P., Turnbull, S., Bird, J., Wang, Y.S., Claude, J.F., and Youson, J.H. (2004). Lamprey parasitism of sharks and teleosts: high capacity urea excretion in an extant vertebrate relic. Comp Biochem Physiol A Mol Integr Physiol *138*, 485-492.

Wilson, R., Wright, P., Munger, S., and Wood, C. (1994). Ammonia excretion in freshwater rainbow trout (*Onchorynchus mykiss*) and the importance of gill boundary layer acidification: lack of evidence for Na^+/NH_4^+ exchange. J Exp Biol *191*, 37-58.

Wood, C., Auml, Rt, P., and Wright, P. (1995). Ammonia and urea metabolism in relation to gill function and acid-base balance in a marine elasmobranch, the spiny dogfish (Squalus acanthias). J Exp Biol *198*, 1545-1558.

Wood, C.M., Perry, S.F., Wright, P.A., Bergman, H.L., and Randall, D.J. (1989). Ammonia and urea dynamics in the Lake Magadi tilapia, a ureotelic teleost fish adapted to an extremely alkaline environment. Respir Physiol *77*, 1-20.

Wright, P. (1995). Nitrogen excretion: three end products, many physiological roles. J Exp Biol *198*, 273-281.

Wright, P.A., and Wood, C.M. (2009). A new paradigm for ammonia excretion in aquatic animals: role of Rhesus (Rh) glycoproteins. J Exp Biol *212*, 2303-2312.

Zimmer, A.M., Nawata, C.M., and Wood, C.M. (2010). Physiological and molecular analysis of the interactive effects of feeding and high environmental ammonia on branchial ammonia excretion and Na^+ uptake in freshwater rainbow trout. Journal of Comparative Physiology B-Biochemical Systemic and Environmental Physiology *180*, 1191-1204.

Biographical Sketch

Salvatore Blair was born in Lansing, MI to parents Michael and Antoinette in July of 1987. Tired of the long cold Michigan winters and following job opportunities, North Carolina became the new home to the Blair family. Growing up much of his childhood was spent in the outdoors and fishing became his most true passion. At the age of 4, soccer and ice hockey became lifelong sporting activities and the majority of weekends were spent with his parents traveling to games in different cities across the East coast of the US.

He attended high school at The Fayetteville Academy and upon first setting eyes on the campus of Appalachian State University and surrounding mountains he knew that this was his perfect college atmosphere. Sal spent his weekends playing ice hockey on the ASU Ice Hockey club team; where in his last year on the team would win the conference championship. Any time there was no hockey to be played and work was complete, you could likely find him standing in one of the many mountain streams fly-fishing for the elusive trout.

He received a Bachelor of Science degree in biology following the pre-medical tract with aspirations of attending veterinary school. However after spending a summer at Mount Desert Island Biological Laboratory working with Sue Edwards, he decided to stay in the fish physiology world and complete a Master of Science degree at Appalachian State University. In the fall of 2011 he will attend the University of Alberta in Edmonton, Canada and enter the Doctoral program of Physiology, Cell and Developmental Biology.