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Gill Structure & Function in Parasitic and Non-parasitic Lampreys: The Effects of Metamorphosis and Freshwater-Seawater Transfer

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Gill Structure & Function in Parasitic and Non-parasitic Lampreys: The Effects of Metamorphosis and Freshwater-Seawater Transfer

by

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AUTHOR'S DECLARATION

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

I understand that my thesis may be made electronically available to the public.

Abstract

Lamprey *(Petromyzontiformes*) are a phylogenetically ancient group of jawless fishes that begin their lives as filter-feeding larvae (ammocoetes) before undergoing a complex metamorphosis into juvenile lamprey that involves major changes to their internal and external body plan. Some parasitic species, such as the sea lamprey (*Petromyzon marinus*), migrate to sea following metamorphosis, where they use their oral discs and rasping tongue to attach to and ingest vast quantities of blood from fishes. Thus, sea lamprey have to counter the simultaneous challenges of hyposmoregulation in sea water and the generation of large quantities of ammonia due to the catabolism of protein-rich blood. A goal of this study was to characterize how changes in the structure and function of the gills facilitated osmoregulation and nitrogenous waste (N-waste) excretion by sea lamprey following metamorphosis, particularly after acclimation to sea water and the ingestion of blood from teleost fishes. Accordingly, key features of the lamprey gill including the distribution and abundance of Na^+/K^+-ATPase (NKA) and H^+-ATPase (V-ATPase) pumps involved in ion regulation, and ammonia transporting Rhesus glycoproteins and urea transporting proteins, were investigated using through immunohistochemical staining and Western blotting techniques.

In contrast to the sea lamprey, there are other species of lamprey that remain in fresh water following metamorphosis. Many of these species are non-parasitic including the northern brook lamprey (*Ichthyomyzon fossor*), but some such as the closely related silver lamprey (*Ichthymyzon unicuspis*) are parasitic. To learn more about how an exclusively FW existence affected ion transport and ammonia excretion by lampreys, the gills of post-metamorphic (juvenile) northern and silver lamprey were compared to those of larval and juvenile sea

lamprey. As in sea lamprey, the gills of both species were characterized by the presence of Rhesus c-like glycoprotein (Rhcg-like) and urea transport (UT) protein but, the distribution of these proteins more closely resembled those of larval sea lamprey than juvenile sea lamprey. In both the silver and northern brook lamprey, Rhcg-like protein co-localized with V-ATPase, suggesting that H^+ excretion was coupled with Rhcg-like protein mediated diffusion trapping of NH_3. Similarly, UT abundance in both species was comparable to that of the larval sea lamprey. I conclude that in freshwater lampreys, NH_3 extrusion via apical Rhcg-like proteins is coupled to V-ATPase mediated H^+ excretion, which maintains favourable diffusion gradients by trapping NH_3 as NH_4^+. Given that the lampreys and teleosts have evolved along separate lineages for at least 360 million years, I propose that this method of ammonia excretion is an ancient strategy used by aquatic organisms to facilitate ammonia excretion across the gills in fresh water. In contrast, the need for V-ATPase trapping of NH_3 as NH_4^+ is not required in sea water, in which the Rhcg-like proteins were restricted to the basolateral membrane and co-localized with NKA in sea water mitochondrion-rich cells (SW MRCs). These findings suggest that Rhcg-like protein may mediate ammonia excretion by loading the SW MRC with ammonia, with the resulting NH_4^+ pumped out of the cell via substitution for H^+ on an apical Na^+/H^+ exchanger, or via an outwardly directed NH_4^+ electrochemical gradient that favours excretion via paracellular junctions.

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List of Abbreviations

Amm	Ammocoete
aMRC	Ammocoete-type mitochondrion-rich cell
CF	Condition Factor
DAPI	4’,6-diamidino-2-phenylindole
Early Met.	Early Metamorphosis
ENaC	Epithelial Na^+ Channel
FW	Fresh water
IMRC	Intercalated mitochondrion-rich cell
J_{amm}	Ammonia excretion rate
J_{urea}	Urea excretion rate
Juv – Fed	Juvenile sea lamprey allowed to feed on trout
Juv – FW	Juvenile sea lamprey in fresh water
Juve – SW	Juvenile sea lamprey acclimated to 100% seawater (34ppt)
Late Met.	Late metamorphosis
Mid Met.	Mid-metamorphosis
MR	Mitochondrion-rich cell
NB	Northern brook lamprey *(Icthyomyzon fossor*)
NHE	Na^+/H^+-exchanger
NKA	Na^+/K^+-ATPase
N-waste	Nitrogenous Waste
PBS	Phosphate buffered saline
PVDF	Polyvinylidene difluoride
OUC	Ornithine Urea Cycle
Rh	Rhesus
Rhag/Rhag-like	Rhesus a(like) glycoprotein
Rhbg/Rhbg-like	Rhesus b(like) glycoprotein
Rhcg/Rhcg-like	Rhesus c(like) glycoprotein

Rhcg1	Rhesus c1 glycoprotein
Rhcg2	Rhesus c2 glycoprotein
S	Silver lamprey (*Icthymoyzon unicuspis*)
SDS	Sodium dodecyl sulphate
SL - amm	Sea lamprey ammocoete (*Petromyzon marinus*)
SL – post	Post-metamorphic sea lamprey (*Petromyzon marinus*)
SW	Sea water (34ppt)
SW MRC	Seawater-Type Mitochondrion-Rich Cell
TPBS	TWEEN 20 phosphate buffered saline
TTBS	TWEEN 20 Tris-buffered saline
UT	Urea transporter
V-ATPase	Vacuolar-Type H^+-ATPase

Chapter 1

General Introduction

1.1 Lamprey Life Cycle and Metamorphosis

Sea lamprey (*Petromyzon marinus*) spend the first 3 to 7 years of their lives in freshwater rivers and streams as burrow-dwelling larvae that filter-feed on organic detritus (>97.8%), and smaller amounts of algae and bacteria, though proportions vary seasonally (Beamish 1980; Sutton and Bowen 1994; Tetlock et al. 2012; Hansen et al. 2016). In sea lamprey, this larval stage is followed by a seven-stage metamorphosis into a free-swimming, parasitic juvenile stage (Fig. 1.1) (Youson 1980). During this time, sea lamprey in their native range migrate downstream to marine environments to feed on the blood of fishes for 12-20 months (Hansen et al. 2016). One population of sea lamprey, however, is found in the Laurentian Great Lakes basin which they likely entered in the early 1800's and subsequently impacted native fisheries (Gruber et al. 2012; Bravener and McLaughlin 2013; Hansen et al. 2016). Within lakes, sea lamprey will feed upon many teleost species including alewife (*Alosa pseudoharengus*), American shad (*Alosa sapidissima*), white sucker (*Catostomus commersonii*), burbot (*Lota lota*), rainbow trout (*Oncorhynchus mykiss*), lake trout (*Salvelinus namaycush*), and other large-bodied fishes (Farmer 1980). In the marine environment, juvenile parasitic sea lamprey not only feed on a variety of teleosts, but they have also observed feeding on elasmobranchs, including basking sharks (*Cetorhinus maximus*) (Wilkie et al. 2004). Following this parasitic juvenile phase, the much larger adult animals migrate up freshwater rivers and streams where they spawn and die (Fig. 1.1) (Tetlock et al. 2012; Hansen et al. 2016).

Metamorphosis in lampreys is associated with many physiological changes including the development of a lateral line system, changes in blood composition, colouration, and external

and internal body structure (Rall et al. 1961; Youson 1984; Gelman et al. 2008). Most notable are the changes related to diet in parasitic species, including changes in the digestive tract, and the development of a rasping tongue, oral disc, and large eyes. As larvae, lamprey possess a simple, tubular digestive tract that is approximately half of their body length (Sutton and Bowen 1994). During metamorphosis, they develop longitudinal folds within the intestine thus increasing the absorptive surface area of the gut and allowing more efficient nutrient absorption as they begin their parasitic phase (Battle and Hayashida 1965; Youson and Connelly 1978). They also undergo biliary atresia, the loss of the gall bladder, and evidence suggests that the posterior intestine may take up the role of bile synthesis and secretion (Langille and Youson 1983; Yeh et al. 2012).

The dome-shaped oral hood of larval sea lamprey becomes an enlarged oral disc with many teeth and is essential for attachment to host fishes during feeding (Youson and Potter 1979; Youson 1980). The endostyle, which produces mucus to trap the detritus which the larvae feed on, becomes the thyroid gland in the juvenile and adult sea lamprey (Wright and Youson 1980). A rasping tongue develops allowing the sea lamprey to more effectively pierce the skin of the fish and consume blood and tissue (Wright and Youson 1976). The animals also develop the ability to produce and secrete lamphredin from the buccal glands. This secretion has lytic and anticoagulant properties, allowing the animals to more efficiently ingest the blood of their host (Renaud et al. 2009). Together, these changes allow sea lamprey to ingest large amounts of blood of their hosts and grow at very high rates during this parasitic phase. However, this mode of feeding by juvenile sea lampreys comes with its own set of challenges.

While the oral disc augments blood ingestion, it may also impair respiration by preventing the lamprey from unidirectionally irrigating the gill while attached to their hosts or to the substrate. In larval lamprey (ammocoetes), the gills are unidirectionally ventilated, in which water currents generated by a velum are drawn into the oral cavity and directed through the pharynx to the gills. After crossing the gills, the water then exits via triangular-shaped branchiopores. Like other fishes, this facilitates efficient gas exchange as the blood flows in a counter-current direction through the lamellae of the gills, opposite to the flow of water (Randall 1972; Wilson and Laurent 2002). After metamorphosis, juvenile sea lamprey use tidal ventilation by pumping water in and out of their newly-formed gill pouches, with the water entering and leaving via the branchiopores (Randall 1972). The branchiopores also change shape during metamorphosis, switching from triangular to ovular openings (Youson and Potter 1979). There is an increase in both the total number and length of gill filaments during metamorphosis, as well as a decrease in the spacing between lamellae (Lewis and Potter, 1976). While these structural changes of the gill facilitate respiration during the juvenile, parasitic phase, less is known about how other physiological processes mediated by the gill, including nitrogenous waste (N-waste) excretion, ion transport and acid-base regulation change with the animal's stage of development.

In most fish species, the gill serves as the primary site of respiration, osmoregulation, ionoregulation, acid-base regulation and N-waste excretion (Evans et al. 2005). These processes are often interconnected, and a single transporter can be involved in more than one of these processes. For example, respiratory CO_2 excreted via the gills is hydrated within the cytosol of branchial (gill) epithelial cells to HCO_3^- and H^+, via carbonic anhydrase (CA) resulting in H^+

excretion via acid-base regulatory transporters such as an Na^+/H^+ exchanger (NHE) or an active H^+-ATPase (Evans et al. 2005). Bicarbonate on the other hand is transported out of the cell via apical anion exchange processes (e.g. Cl^-/HCO_3^- exchange; Marshall 2002). Because these processes facilitate Na^+ uptake and Cl- uptake in exchange for H^+ and HCO_3^-, respectively, they are also critical for regulating internal ion balance (Evans et al. 2005; Edwards and Marshall 2013). Acidification of the gill boundary layers due to either the hydration of CO_2 and/or V-ATPase-mediated H^+ extrusion, may also augment ammonia excretion via Rh glycoproteins by trapping NH_3 as NH_4^+, which sustains NH_3 diffusion gradients across the gill (Wilkie 2002; Weihrauch et al. 2009; Wright and Wood 2009). The overarching goal of my thesis is to ascertain how changes in gill structure that accompany metamorphosis in lampreys affect the arrangement of the molecular machinery involved in the excretion of N-wastes, and the related iono- and acid-base regulatory processes. A particular focus was to relate changes in patterns of N-waste excretion, ion exchange, and metabolic H^+ excretion to changes in the sea lamprey diet throughout metamorphosis, and to the challenges posed by the FW-SW transition.

1.2 Ammonia Toxicity and the Effects of Feeding on Nitrogenous Waste Excretion

The change from detritus to a blood diet in lamprey requires an increased capacity to break down amino acids due to the large protein content of blood meals (Wilkie et al. 2006). The breakdown of amino acids creates N-wastes in the form of ammonia which must then be removed from the body or detoxified into less harmful forms, such as urea, in order to avoid the adverse effects of internal ammonia accumulation (Randall and Tsui 2002). Excess protein is broken down via proteolysis into amino acids, which are then deaminated to yield a carbon

skeleton, which can be utilized for gluconeogenesis or be catabolized via the citric acid cycle to generate reducing equivalents used in the production of ATP. However, this process also results in the generation of highly toxic ammonia (Figure 1.2) (Ip and Chew 2010; Bucking 2017). This ammonia can also be converted to urea via processes such as the ornithine-urea cycle (OUC), uricolysis, or arginolysis. In sea lamprey, urea is primarily generated by the catabolism of excess purines through the process of uricolysis, and by the breakdown of arginine into ornithine and urea via arginase (Fig. 1.2) (Wilkie 2002; Wilkie et al. 2004).

If internal ammonia rises too much, due to activities such as feeding or exposure to high environmental ammonia or pH, fish may experience acute ammonia toxicity (Person-Le Ruyet et al. 1995; Wicks et al. 1999). There are many consequences of acute ammonia toxicity as it targets the nervous system causing hyperexcitability, the depletion of energy stores, and eventually death (Arillo et al. 1981; Wilkie et al. 2011). In mammals and fish this occurs largely due to the increased activation of N-methyl-D-aspartate (NMDA) receptors, resulting in an uncontrolled influx of Ca^{2+} thus depolarizing neurons in the CNS (Felipo and Butterworth 2002; Wilkie et al. 2011). High environmental ammonia also leads to an increased production of reactive oxygen species leading to oxidative damage and swelling within the brain (Lisser et al. 2017). Other acute effects such as acidosis are also possible (Arillo et al. 1981; Monfort et al. 2002).

Animals use multiple strategies to prevent acute ammonia toxicity, including the active excretion of ammonium ions (NH_4^+), and the conversion of ammonia to less toxic substances such as glutamine and urea (Randall and Tsui 2002; Ip and Chew 2010). In teleosts, N-waste

excretion rates generally increase with both meal size and relative protein content of the meal (Ramnarine et al. 1987; Chakraborty and Chakraborty 1998; Wicks and Randall 2002; Kajimura et al. 2004; Wu and Gatlin III 2014). Typically, this increase results in a rapid increase in the excretion rates of ammonia and urea immediately following the meal, followed by a slow return to baseline excretion rates (Ramnarine et al. 1987; Wood et al. 2001).

Upon completion of metamorphosis, parasitic species of lamprey begin feeding on fishes, consuming large amounts of blood and tissue which is richer in protein than the larval diet of primarily organic detritus (Wilkie et al. 2006). In sea lamprey, the catabolic enzymes responsible for breaking down these amino acids, such as glutaminase, glutamate dehydrogenase, serine dehydratase, alanine aminotransferase and aspartate aminotransferase are upregulated during feeding appropriately (Wilkie et al. 2006; Bucking 2017). Ammonia excretion, the primary form of N-waste, in sea lamprey was observed to increase by as much as 20-fold following feeding in the parasitic life stage (Wilkie et al. 2006). In addition to changes in ammonia excretion, a 15-fold increase in urea excretion was observed in sea lamprey after feeding on trout and increases as high as 450-fold were measured following feeding on basking sharks (Wilkie et al. 2004). Elasmobranchs retain high amounts of urea in their blood, which makes their blood slightly hyperosmotic to sea water allowing them to retain water in their marine environment (Hazon et al. 2003).

1.3 Freshwater Nitrogenous Waste Excretion Mechanisms in Other Fish

A basic understanding of how ammonia and urea are excreted across the gills in different fish species is emerging, but the mechanisms of excretion remain poorly understood in lampreys.

In most teleost species, the gills function as the primary site of N-waste excretion (Sayer and Davenport 1987; Kajimura et al. 2004). For both freshwater and marine fishes, the main N-waste excreted is ammonia, with less toxic urea as the second largest contributor (Boucher-Rodoni and Mangold 1985; Sayer and Davenport 1987; Walsh et al. 2001; Kajimura et al. 2004; Braun et al. 2009a; Bucking 2017). Lower amounts of whole amino acids and proteins may also be excreted (Walsh et al. 2001; Kajimura et al. 2004), though the amounts and relevance of these excretion products is minimal. The relative amounts of urea excreted by fishes can be influenced by life stage and environmental factors. For example, rainbow trout (*Oncorhynchus mykiss*) and Atlantic cod (*Gadus morhua*) use urea as their primary nitrogenous waste product as embryos and larvae but excrete primarily ammonia as juveniles and adults (Wright et al. 1995; Chadwick and Wright 1999; Steele et al. 2001). Rates of N-waste excretion can also be affected by factors such as external pH, ammonia concentrations, and diet as discussed previously (Wilkie and Wood 1996; Wilkie et al. 1999; Wilkie 2002; Braun et al. 2009a,b). One fish, the Lake Magadi tilapia (*Alcolapia grahami*), excretes virtually all of its N-wastes as urea due to its highly alkaline environment in the Great Rift Valley of Africa (Randall et al. 1989; Wood et al. 1989).

Historically, it was thought that ammonia was excreted via simple diffusion across the gills but it is now clear that the process in most teleosts also involves facilitated diffusion through Rhesus (Rh) glycoproteins (Weihrauch et al. 2009; Wright and Wood 2009). Rh glycoproteins were first discovered in the erythrocytes of mammals and their role in ammonia excretion by fishes was only recently characterized. The Rh glycoproteins include Rhag, Rhbg, Rhcg1, and Rhcg2. Morpholino knockdowns of some of the genes coding for these proteins

severely inhibited ammonia excretion in zebrafish (*Danio rerio*), confirming their importance for this process (Braun et al. 2009a). Work done over the last decade on teleosts suggests that Rhag is responsible for the transport of ammonia from within red blood cells to the plasma, Rhbg facilitates ammonia transport from the plasma into the gill cell across the basolateral membrane, and Rhcg-like proteins transporting ammonia from across the apical membrane of the gill cell to the external environment (Fig. 1.3) (Nakada et al. 2007; Weihrauch et al. 2009; Wright and Wood 2009: Nawata et al. 2010; Wright and Wood 2012; Edwards et al. 2015). The excretion of ammonia across the apical membrane is also thought to be coupled with H^+-ATPase proton excretion in FW and Na^+/H^+ exchange in SW. It is thought that this arrangement results in the protonation of un-ionized ammonia (NH_3) into its ionized ammonium (NH_4^+) form, which helps to maintain a favourable NH_3 partial pressure gradient, promoting the facilitated diffusion of NH_3 across the apical surface gill via Rhcg-like protein (Fig. 1.3) (Wright and Wood 2009).

In addition to the excretion of ammonia, various fish species also excrete urea. Some teleosts, such as the Lake Magadi tilapia, utilize the ATP-fueled ornithine-urea cycle (OUC) to convert ammonia to urea as it is an efficient way to continue detoxifying ammonia when ammonia excretion is not possible (Randall et al. 1989; reviewed by Wilkie 2002). It is believed that urea, like ammonia, is excreted using facilitated diffusion via specific transporters on the gill epithelium (Fig. 1.3) (Walsh et al. 2001; McDonald et al. 2012). The mRNA for toadfish urea transporters (UTs), proteins which are very similar to those of the mammalian kidney, have been isolated and used to detect urea transporters (UTs) across multiple fish species (McDonald et al. 2009; McDonald et al. 2012). It has also been reported that UTs are found almost exclusively

within the gills, however their distribution varies greatly between species (Braun et al. 2009b). In some species, UT is localized within mitochondrion-rich cells, while in others such as the gulf toadfish, UT is localized basolaterally on the epithelium (Braun et al. 2009b; Bucking et al. 2013).

1.4 Effects of Salinity on Gill Function

Fishes living in freshwater environments must contend with osmotic gradients favouring the influx of water and the loss of ions across the gills (Fig. 1.1). To cope, freshwater fishes drink little or no water, take up ions (Na^+, Cl^-) from the water via freshwater mitochondrial rich cells, and produce vast quantities of dilute urine (Marshall 2002). Meanwhile, the opposite is true in salt water environments in which seawater-dwelling fishes, including sea lamprey, drink salt water and produce minimal quantities of extremely concentrated urine to reduce water loss (Fig. 1.1) (Logan et al. 1980; Evans et al. 2005; Edwards and Marshall 2013). The ingested water is absorbed into the blood along the esophageal and intestinal tract, and the co-ingested ions including Na^+ and Cl^- are transported via the circulation to the gills where these solutes are excreted by seawater-type mitochondrion-rich cells (SW MRCs) (Marshall 2002).

In freshwater, the primary goal is the uptake of Na^+ and Cl^-ions to counter diffusive ion losses to the water. Typically, this occurs through a combination of an apical V-type H^+-ATPase (V-ATPase), an epithelial sodium channel (ENaC) utilizing the electrical potential generated by H^+ excretion to passively uptake Na^+ across the apical membrane, and a basolateral Na^+/K^+-ATPase (NKA) to pump Na^+ into the plasma (Avella and Bornancin 1989, Lin and Randall 1993). Meanwhile in sea water, V-ATPase involvement is limited, but NKA activity increases to

create a favourable gradient for a Na^+-K^+-Cl^--cotransporter (Marshall, 2002). As in teleosts, the mitochondrion-rich cells of lampreys play an important role in ionoregulation, and distinct changes occur in these cells throughout metamorphosis. Ammocoetes possess an ammocoete-type mitochondrion-rich cell (aMRC) whose function so far remains unclear, though it has been suggested that it may be involved in waste excretion by larval lampreys (Bartels et al. 1998). Following metamorphosis, juvenile and adult lamprey lose the aMRC and develop seawater-type mitochondrion-rich cells (SW MRC), responsible for the excretion of Na^+ and Cl^- into the environment as described above (Peek and Youson 1980; Bartels et al. 1998; Bartels and Potter 2004; Reis-Santos et al. 2008). The relationship between these changes in gill structure and ionoregulatory mechanisms of lampreys have been addressed in relatively few studies however (e.g. Bartels and Potter 2004; Reis-Santos et al. 2008). Even less is known about how these developmental changes in gill structure affect the patterns and mechanisms of ammonia and urea excretion by lampreys following metamorphosis and during the freshwater to seawater transition.

It is well established that metamorphosis results in an upregulation of N-waste production capacity, which is accompanied by marked increases in ammonia and urea excretion. These increases are most pronounced following feeding by parasitic juvenile sea lampreys (Wilkie et al. 2004; 2006). Thus, one goal of the present study was to relate changes in gill structure and function to observed changes in N-waste excretion and ionoregulation at different stages in the sea lamprey life cycle, in response to feeding, and during the FW to SW transition that characterize the parasitic juvenile life stage.

1.5 Evolution of Petromyzontiformes and 'Paired' Lamprey Species

The lamprey lineage has persisted for at least 360 million years, with the parasitic life style evolving within the last 280 million years (Potter and Hilliard 1987; Gess et al. 2006). Although the general body plan of lampreys appears similar to the fossil record the lamprey lineage has been constantly evolving and diversified into 41 different species (Docker et al. 2012). While the sea lamprey has been studied extensively, less is known about the ontogeny of gill function in other species of lampreys, including other parasitic lamprey species such as the Arctic lamprey (*Lethenteron camtschaticum*) and silver lamprey (*Ichthyomyzon unicuspis*) (Docker et al. 2012; Bartels et al. 2012; Makhrov et al. 2013; Yamazaki and Nagai 2013). Of these 41 species, the majority (23) are non-parasitic species, that were derived from a parasitic ancestor (Potter and Gill 2003). Very shortly following metamorphosis, these non-parasitic lampreys, such as the northern brook (*Ichthyomyzon fossor*) and American brook (*Lethenteron appendix*) lamprey, begin their maturation into breeding adults, and then die (Docker et al. 2012).

Some subsets of lamprey species are considered "paired" or satellite species, denoting a group consisting of an ancestral, anadromous parasitic species and one or more derived, exclusively freshwater species which may be parasitic or non-parasitic (Artamonova et al. 2011). The concept of "paired" species is disputed however, as some argue that these are simply different morphs of a single species (Leach 1940; Docker et al. 2012). These paired or satellite species are nearly impossible to distinguish morphologically in the larval stages, and during the early stages of metamorphosis (Leach 1940; Artamonova et al. 2011). These species are also indistinguishable by DNA barcoding, contributing to the debate of whether these lampreys are

different ecotypes of a single species, and that different morphology is a result of differences in gene regulatory mechanisms (Docker et al. 2012; Ren et al. 2016).

The northern brook and silver lamprey provide a valuable opportunity to understand how differences in feeding may affect N-waste excretion mechanisms. However, study of these species is constrained by their limited distribution and population, and as both species are designated as of special concern in Canada (COSEWIC 2007; 2011). Although indistinguishable as ammocoetes, northern brook lamprey and silver lamprey are identifiable as juveniles due to differences in external morphology. The non-parasitic northern brook species typically have smaller eyes and oral discs than their parasitic counterpart as well as less well-developed dentition (Hardisty and Potter 1971; Vladykov and Kott, 1979; Potter et al. 2015). Silver lamprey, true to their name, also appear more silver in colour, while the northern brook lamprey is more brown in pigmentation, though this trait can vary widely within a species (Scott and Crossman 1973).

Little is known about how the gill physiology of these freshwater species differs from anadromous lamprey like the sea lamprey, specifically as it relates to ionoregulation and N-waste excretion. Bartels and Potter (2004) suggested that SW MRCs, are not present in the gill epithelium during freshwater phases of the anadromous sea lamprey and are entirely absent from exclusively freshwater lamprey. Similarly, the absence of a blood-feeding parasitic phase could result in substantial differences in the presence and organization of N-waste excretion mechanisms in the gills of parasitic and non-parasitic lamprey species.

1.6 Hypotheses and Objectives

The overarching goal of my thesis was to relate changes in the abundance and organization of transporters involved in ion regulation, ammonia and urea excretion in the gills of lamprey to their stage of development (larval, metamorphosing, juvenile). A particular focus was to ascertain how the lamprey gill is re-organized as sea lamprey enter the juvenile phase of their life cycle, which is accompanied by marked changes in their diet and habitat.

My first objective was to investigate how ammonia and urea excretion patterns change during the complex life-history of the sea lamprey, and whether or not this resulted in changes in the machinery used to excrete these N-wastes. I predicted that excretion rates would increase markedly upon completion of metamorphosis, and following the ingestion of blood during the juvenile, parasitic phase (Wilkie et al. 1999; 2006). Based on our understanding of the mechanisms of N-waste excretion in teleosts, I predicted that Rhesus (Rh) glycoproteins would be localized within the gill mitochondria rich cells, such as the aMRC in ammocoetes, and within the SW MRCs in juvenile lampreys. I also predicted the Rh glycoproteins and urea transporter (UT) proteins would be upregulated as the animals completed metamorphosis and approached their juvenile blood-feeding life stage (Wilkie et al. 2004; Evans et al. 2005; Braun et al. 2009b; Nawata et al. 2010).

A second objective was to understand how the transition from fresh water (Fig. 1.3) to sea water (Fig. 1.4) influenced mechanisms the organization of the gill and mechanisms of nitrogenous waste excretion. I hypothesized that there would be changes in the distribution and abundance of proteins such as Na^+/K^+-ATPase (NKA) and vacuolar-type H^+-ATPase (V-

ATPase) associated with acclimation to sea water (Evans et al. 2005; Weihrauch et al. 2009; Wright and Wood 2009). Specifically, I expected that NKA expression would increase, and V-ATPase expression would decrease as the sea lamprey completed metamorphosis and prepared for the transition to SW. I also predicted that there would be a re-organization of Rh glycoprotein distribution in the gills due to the need re-structure the gills for a seawater existence.

Finally, my third objective was to determine how parasitism of fishes by juvenile lampreys, and an anadromous life history influenced the evolution of N-waste excretion pathways in sea lampreys. To achieve this goal, I studied the gills of a pair of closely related juvenile lamprey species, the silver and northern brook lampreys. I expected that the parasitic silver lamprey would have greater expression of Rh glycoproteins and UTs within the gill epithelium than the closely related non-parasitic northern brook lamprey due to the marked differences in their diet

To investigate these objectives, I used several experimental and analytical techniques. Both ammonia (J_{amm}) and urea (J_{urea}) excretion rates were measured in sea lamprey of different life stages and acclimated to different salinities. Internal concentrations of ammonia and urea were also determined using colorimetric and enzymatic assays. Immunohistochemical staining was used to elucidate the mechanisms of ammonia and urea excretion through localization of Rh glycoprotein and UT, and their colocalization with the associated transporters NKA and V-ATPase within the gills of sea lamprey throughout their life-cycle, and in the post-metamorphic northern brook and silver lampreys. Finally, western blot analysis of Rh glycoprotein, UT, and

NKA were used to quantify changes in the expression of these proteins as sea lamprey metamorphosed and began their juvenile parasitic phase.

Figures

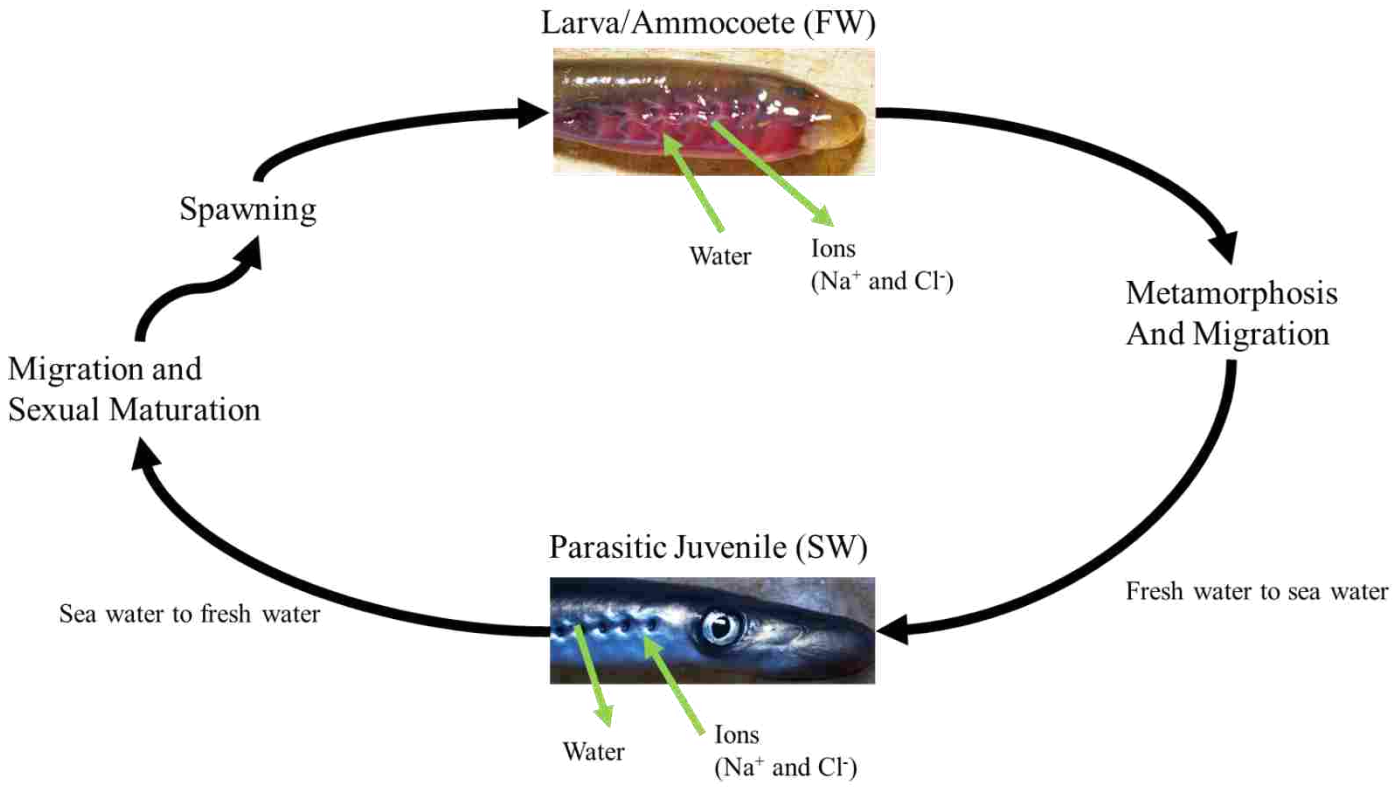

Figure 1.1. Schematic illustrating the sea lamprey (*Petromyzon marinus*) life cycle (black arrows) and the different osmo- and iono-regulatory pressures they face in fresh water (FW) and sea water (SW) environments (green arrows). In FW, ammocoetes must prevent osmotic water influx and ion loss, while in SW, juvenile lamprey must reduce water loss and excrete excess ions.

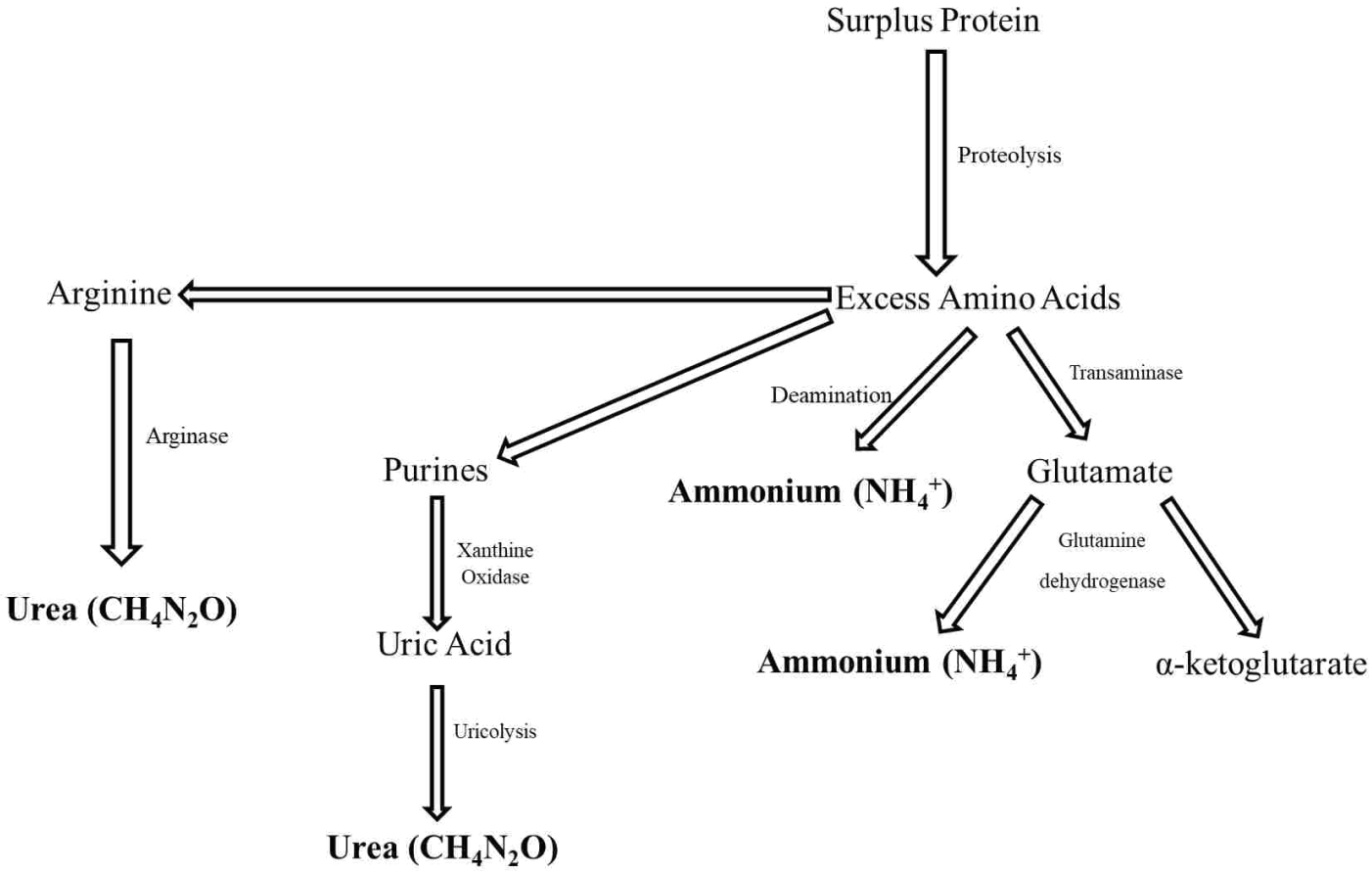

Figure 1.2. The breakdown of protein and amino acids in lamprey, producing the primary nitrogenous waste products of ammonia and urea (bold). The various products shown can be produced via the catabolism of proteins or taken up directly from the host.

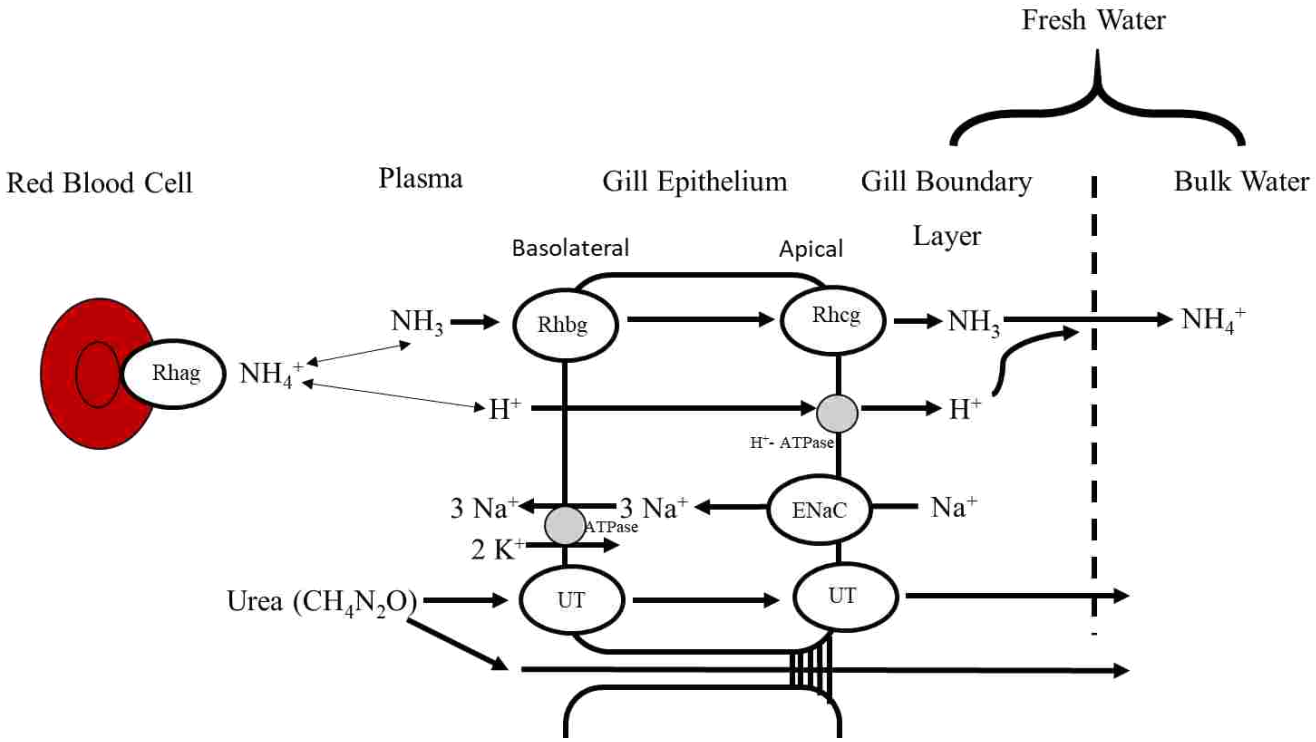

Figure 1.3. Proposed mechanisms of N-waste excretion and ion uptake by fresh water teleost fishes. Rhesus a-like glycoprotein (Rhag) transports NH_4^+ from the red blood cell into the plasma. Rhbg-like protein moves NH_3 across the basolateral membrane of the gill epithelium while Rhcg-like protein facilitates movement across the apical membrane. Meanwhile, H^+ is actively excreted via an H^+-ATPase. This protonation of the boundary layer facilitates the uptake of Na^+ via an epithelial Na^+ channel (ENaC), and Na^+/K^+-ATPase allows the active uptake of Na^+ across the basolateral membrane of the gill epithelium. Urea excretion may be facilitated by urea transporter (UT) across the membrane, or through the paracellular spaces. Figure based on Wright and Wood 2009 and Weihrauch et al. 2009.

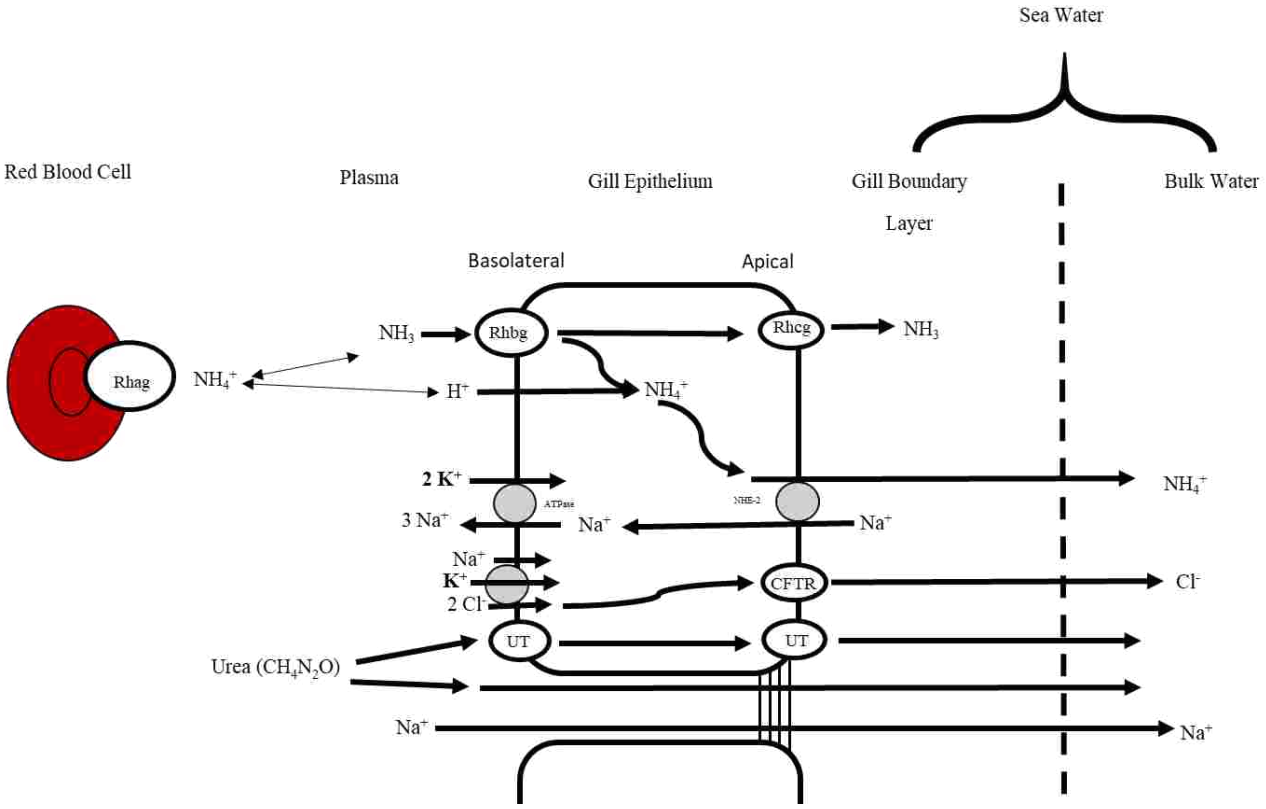

Figure 1.4. Proposed metabolon for N-waste excretion and ion uptake by teleost fishes in sea water. Rhesus a-like glycoprotein (Rhag) transports ammonia from the red blood cell to the plasma, while Rhbg-like protein and Rhcg-like protein facilitate ammonia transport across the basolateral and apical membranes of the gill epithelium respectively. Ammonia may also be protonated within the cell, and substitute for H^+ on an apical Na^+/H^+ exchanger as NH_4^+. The apical passive uptake of Na^+ through this exchanger is associated with a basolateral Na^+/K^+-ATPase for Na^+ uptake. This facilitates a basolateral NKCC, moving Na^+, K^+, and 2 Cl^- into the epithelial cell. Cystic fibrosis transmembrane conductance regulator (CFTR) facilitates the movement of Cl^- across the apical membrane. This creates a favourable electrochemical gradient for Na^+ to move through the paracellular spaces into the bulk water. Urea excretion may be facilitated by urea transporter (UT) across the membrane, or through the paracellular spaces. Figure based on Evans et al. 2005 and Weihrauch et al. 2009.

Chapter 2

Re-organization of the pathways of branchial ammonia and urea excretion during metamorphosis and the freshwater-seawater transition of the anadromous sea lamprey (*Petromyzon marinus*)

Introduction

Sea lamprey (*Petromyzon marinus*) begin their complex life cycle as larvae called ammocoetes, living burrowed in the sediment of streams and rivers, filter feeding on organic detritus (Sutton and Bowen 1994; Beamish and Lowartz 1996). This ammocoete phase persists for 3-7 years before the animals undergo a seven-stage metamorphosis, transforming into free-swimming, parasitic juveniles which migrate into larger water bodies such as the sea and lakes (Youson 1980). During metamorphosis, sea lamprey undergo physiological and anatomical changes to prepare for their parasitic blood-feeding phase. These changes include the development of an oral disc with many teeth used for attachment to host fishes, a rasping tongue to assist in penetrating the tissue, and complex eyes (Youson 1980). As juveniles, sea lamprey parasitize large bodied teleost and even elasmobranch fishes for at least 12-20 months before migrating back to freshwater rivers and streams as adults, before they eventually spawn and die (Farmer 1980; Wilkie et al. 2004; Wilkie 2011; Beamish et al. 2018).

The transition from filter- to blood-feeding results in a marked increase in the protein content of the sea lamprey diet and is accompanied by an increase in their capacity to break down proteins and amino acids, and to excrete nitrogenous wastes (N-wastes) (Wilkie et al. 2004, 2006). The ability to detoxify ammonia, the primary N-waste product, either through excretion or conversion to less harmful compounds such as urea, is particularly important to avoid the many adverse effects of internal ammonia accumulation and toxicity (Randall and Tsui, 2002). An increase in the capacity to deaminate amino acids to excrete ammonia and urea following metamorphosis has been reported previously in sea lamprey (Wilkie et al. 1999; 2004; 2006). Yet, relatively little is known about how changes in gill structure and function facilitate

the changes in J_{amm} and J_{urea} by sea lamprey following metamorphosis, and at the onset of the parasitic phase.

Anadromous populations of sea lamprey must also prepare for the transition from fresh water (FW) to sea water (SW) at the completion of metamorphosis, which is reflected by major differences in the structure and function of the gills as the fish switch from hyper- to hyposmoregulation (Marshall 2002; Bartels and Potter 2004). In the freshwater fish model (Chapter 1, Figure 1.3), the presence of V-type H^+-ATPase (V-ATPase) facilitates Na^+ uptake, through the pumping of H^+ across the apical membrane of gill epithelial cells, generating a transapical electrochemical gradient that promotes Na^+ uptake via apical Na^+ channels, and to facilitate proton trapping of NH_3 excreted via Rhesus c-like glycoproteins (Rhcg-like; Wright and Wood 2009; Edwards and Marshall 2013). A V-ATPase was identified in freshwater acclimated pouched lamprey (*Geotria australis*) (Choe et al. 2004), but neither V-ATPase or Rh glycoproteins have been characterized in sea lamprey gill. As in other fishes (Chapter 1, Figure 1.3) there are also Na^+/K^+-ATPase (NKA) transporters which actively pump $3Na^+$ into the paracellular spaces in exchange for $2K^+$, which are present on the basolateral membrane of juvenile *G. australis* and sea lamprey gills (Choe et al. 2004; Reis-Santos et al. 2008). NKA is likely more involved in the sea water mechanism of N-waste excretion due to its increased importance for ionoregulation, while V-ATPase would have a deceased role as the active excretion of protons is not needed in sea water (Weihrauch et al. 2009. However, this has not been directly studied in lamprey. Even less is known about the role that urea transporters (UT) play in the excretion of urea in lamprey, which contributes significantly to total N-waste

excretion in all phases of the sea lamprey life cycle, particularly after feeding in juvenile sea lampreys (Wilkie et al. 2004; 2006).

The objectives of the present study were to (i) characterize the proteins involved in N-waste excretion (Rhcg-like; UT) and their association with gill-mediated ion (NKA) and acid-base regulation (V-ATPase), (ii) investigate how the reorganization of the gills during metamorphosis and seawater acclimatization affect the distribution and abundance of these transporters, and (iii) deduce models of ammonia and urea excretion for the different stages of the sea lamprey life cycle. This was accomplished by comparing the rates of J_{amm} and J_{urea} and internal plasma ammonia and urea concentrations of ammocoetes and juvenile lamprey to one another and ascertaining how acclimation to SW and a blood diet affects these processes. Immunohistochemical staining and western blotting were used to characterize how the distribution and relative abundance of Rhcg-like proteins, UT, NKA and V-ATPase were altered during metamorphosis, through the FW-SW transition, and during feeding in juvenile lamprey.

Materials and Methods

Experimental Animals and Holding

Lamprey Collection and Housing

Larval sea lamprey were collected in June of 2016 and June of 2017 from tributaries of the Richibucto River, New Brunswick, using pulsed DC backpack electrofishing (Mode LR20, Smith-Root, Vancouver, WA). The animals were transported to the Animal Care Facility at Wilfrid Laurier University in Waterloo, Canada where they were housed in static, 30L aquaria filled with well water (pH ~8.1, alkalinity ~250mg/L $CaCO_3$), and a layer of sand, approximately

7 cm in depth, to provide burrowing substrate. Water was replaced weekly, ranging in temperature from 18-20˚C, and animals were maintained under a 12-hour light-dark cycle. Ammocoetes were fed a slurry of baker's yeast on a weekly basis (1g/animal), but metamorphosing animals were not fed during this non-trophic period of their life cycle (Late June until December). Details on the feeding of parasitic juvenile lamprey are outlined below. All animal holding and experimental protocols were approved by the Wilfrid Laurier Animal Care Committee, and followed Canadian Council of Animal Care guidelines.

A total of 239 larval sea lamprey (ammocoetes) were identified as the most likely candidates to undergo metamorphosis, based on their body length, mass, and condition factor (CF$\geq$1.5, length $\geq$ 120 mm, mass $\geq$ 2.5 g) as outlined by Holmes and Youson (1994). Groups of these lamprey were then placed in separate aquaria (N = 50) as described above. Sub-sets of animals were sampled throughout metamorphosis and blood and gill tissue was collected for measurements of plasma ammonia and urea, along with other tissues (for a parallel, collaborative study). A sub-set (N=39) of these lampreys were housed in 10cm x 10cm x 30cm mesh compartments, each uniquely identified so that animals could be distinguished from one another. These chambers were filled with ~10cm of sand to allow adequate space for burrowing. The animals were weighed every 2 weeks to track changes in body mass of transforming and non-transforming animals between July and December.

Parasitic Lamprey Feeding Protocol

Parasitic juvenile lampreys were fed rainbow trout, following WLU Standard Operating Procedure I-14 (Animal Care Committee, Wilfrid Laurier University, 2014). In all cases, the

fully metamorphosed juvenile lamprey were removed from their aquaria, and placed in separate tanks at the end of November. These animals were held in 30L aquaria and acclimated to 10% seawater (3.4ppt), created using Instant Ocean ® Sea Salt (Instant Ocean ®, St. Blacksburg, Virginia, USA), which we have found prevents the onset of fungal infections in post-metamorphic animals. The animals were held in these aquaria until the beginning of January when feeding studies were started.

Parasitic juvenile sea lamprey (N = 21; mean mass = 2.3 ± 0.12 g) were transferred to a 400L tank containing juvenile rainbow trout (N= 53; mass = 55.9 ± 1.6 g) filled with 10% sea water (SW; T = 13.0 ± 0.4 ˚C), under the 12 h light:dark regime described above. The tank was equipped with charcoal filters and bio-filter media, and water was replaced (50%) weekly. The trout were fed 2% body mass of EWOS pellets (Surrey, B.C., Canada) twice weekly. This setup was monitored a minimum of three times per day. It was not necessary to remove any trout as they experienced no adverse events as a result of the parasitism. Once every two weeks, all lamprey were removed from the tank and weighed to ensure that the lamprey did not exceed a lamprey:trout body mass ratio of 1:10, the accepted criterion established by the WLU ACC in consultation with the university veterinarian.

Experimental Protocols

Changes in Body Mass of Metamorphosing and Non-metamorphosing Larval Sea Lamprey

Biweekly, animals expected to be undergoing metamorphosis (both actual transformers and ammocoetes) were removed from their uniquely identified chambers by gently agitating and lifting the chamber from the sand. The chamber containing each lamprey was then used to

transport the lamprey to an anesthetic bath ($0.1g \cdot L^{-1}$ MS-222 buffered with $0.2g \cdot L^{-1}$ sodium bicarbonate) for up to 5 minutes. Animals were then weighed, photographed and the stage of metamorphosis deduced based on the external characteristics outlined in Youson and Potter (1979). After measurements were completed, chambers were placed back into the sand and the animals were returned to their chambers.

Ammonia and Urea Excretion Measurements

Prior to flux measurements, each of the post-metamorphic, juvenile animals were acutely transferred to 30L aquaria containing 10% SW, at ~13˚C for a minimum of one week. A subset of animals (N=10) were then acclimated to 33%, 66%, and 100% SW over a 9-day period, 3 days per increment until fully acclimated to full strength SW (34ppt). The remaining lamprey that had been held in 10% SW were given the opportunity to feed on rainbow trout as described above for ~2 weeks. Measurements of net ammonia (J_{amm}) and urea (J_{Urea}) excretion rates were measured in ammocoetes (N = 13) and in juvenile sea lamprey immediately following the completion of metamorphosis in fresh water (N = 20), acclimation to 10 % SW (N = 20), 100 % SW (N = 10), or after feeding in 10 % SW (N = 4). Measurements were made using 110 mL darkened flux chambers to which water flow had been cut-off prior to initiating measurements (Wilkie et al. 1999). Each flux chamber was gently aerated to ensure the water was well oxygenated and thoroughly mixed, and positioned in a water bath to maintain temperature at 12-13˚C. Water samples were collected using a sampling port attached to the lid of the chamber from which 5 mL water samples were collected every 4 h for a maximum of 12h. The water samples were saved and stored frozen (-30°C) in 7mL plastic scintillation vials until analyzed for ammonia and

urea excretion. At the conclusion of the experiment, the animals were euthanized with an overdose of anesthetic (1.5g·L^{-1} MS-222; Birceanu et al. 2009), weighed and processed for collection of blood and gill as described below. Other tissues were also collected for use in a parallel collaborative study.

Collection of Blood and Gills from Ammocoetes, Metamorphosing and Juvenile (Post-metamorphic) Sea Lamprey

Sampling occurred six times between the end of June and early December in 2016 and again from Mid-July 2017 until the end of February in 2018. At each sample period, groups of lamprey (N ~ 12) were anaesthetized and then euthanized with respective doses of 0.75 g·L^{-1} and 1.5 g·L^{-1} of MS-222 (Birceanu et al. 2009), buffered with two parts $NaHCO_3$, followed by the immediate collection of blood samples and gill sections. Other tissues, including liver, intestine, tail and brain, were snap-frozen in liquid nitrogen (N_2), then stored at -80˚C and saved for a separate, collaborative study. The gill tissue was collected as whole animal cross sections 0.5 cm thick, from the branchial region, and were fixed for light microscopy in 10% formalin in PBS (137mM NaCl, 2.7 mM KCl, 8 mM, Na_2HPO_4 and 2 mM KH_2PO_4), pH 7.4, at 4˚C in glass 20 ml scintillation vials for approximately 24 hours before being rinsed twice and stored in 70% ethanol at 4˚C. Whole blood was collected in heparinized hematocrit tubes through a whole body incision behind the last branchiopore and spun for 3 minutes at 8000 rpm. Plasma was then transferred into 0.5 ml polypropylene centrifuge tubes and flash frozen in N_2 and stored at -80˚C until analyzed.

Analytical Methods

Water and Blood Sample Analysis

Water ammonia was quantified spectrophotometrically using the sodium salicylate-hypochlorite colorimetric assay, in which ammonia reacts with sodium salicylate and hypochlorite forming a blue indophenol, with an optimal absorbance of 650nm (Verdouw et al. 1978).

Water urea concentrations were measured spectrophotometrically with ferric chloride, thiosemicarbizide, and diacetyl monoxime, in which urea produces a pink chromagen with an optimal absorbance of 525nm (Rahmatullah and Boyde 1980).

Plasma ammonia concentrations were measured using an enzymatic assay (Ammonia Assay Kit AA0100; Sigma-Aldrich, St. Louis, Missouri, USA), in which ammonia is converted to alpha-ketoglutarate, in the presence of glutamate dehydrogenase. The corresponding drop in absorbance, measured at 340 nm, due to the reduction of NADPH to $NADP^+$ is proportional to the ammonia concentration of the sample. Plasma urea samples were diluted 1:1 in 8% PCA, and analyzed spectrophotometrically using the ferric chloride, thiosemicarbizide and diacetyl monoxime assay as described previously for measuring water urea (Rahmatullah and Boyde 1980).

All water and blood spectrophotometric analyses were completed using an Epoch 2 microplate spectrophotometer (BioTek Instruments Inc., Winooski, Vermont, USA). Standard curves required a linearity of $R^2 > 0.95$ for the plate to be accepted.

Immunohistochemical Staining

Fixed gill tissues stored in 70% ethanol, as described above, were dehydrated and embedded in paraffin wax using a Citadel 1000 tissue processor through a 16-hour dehydration and infiltration cycle. This process involved two changes of 70% ethanol, one change of 95% ethanol, three changes of 100% ethanol, three changes of xylene, and two changes in paraffin wax. The prepared tissues were then cut on a microtome into 5μm sections and placed on aminopropyltriethoxysilane (APS) coated slides and stored at room temperature until staining.

Staining protocols were based on those outlined by Wilson et al. (2007). Immediately prior to staining, slides were placed in an oven at 60˚C for 20 minutes to begin the dewaxing process. This was followed by three changes of xylene and two changes of 100% ethanol at five minutes each. A hydrophobic circle was drawn around each section (Super PAP pen, Sigma-Aldrich, St. Louis, Missouri, USA). The slides were rinsed in de-ionized (DI) water and treated with 1% sodium dodecyl sulfate in PBS, pH 7.3 (Brown et al. 1996), for five minutes to unmask antibody epitopes which may have been made unavailable by the fixation process (Shi et al. 2007). The slides were then rinsed again with DI water, PBS with TWEEN 20 (TPBS) for five minutes. A blocking solution (BLØK, MilliporeSigma, Burlington, Massachusetts, USA) was applied to each section and left for 20 minutes.

To detect Rhesus (Rh) glycoproteins, polyclonal rabbit antibodies developed against *Takifugu rubripes* Rhcg1 (Nakada et al., 2007; provided courtesy of Professor S. Hirose, Tokyo Institute of Technology, Japan) were applied to the sections. The investigation of urea transporters (UTs) utilized a polyclonal rabbit antibody developed against UT in *Danio rerio* (Braun et al. 2009a; provided courtesy of Dr. S.F. Perry, U. of Ottawa, Ontario, Canada). The

presence of V-type H^+-ATPase was investigated using a rabbit polyclonal antibody for the B subunit (Wilson et al., 2007). These antibodies were used at a dilution of 1:200 in BLØK that also contained a 1:100 dilution of a mouse monoclonal antibody for Na^+/K^+-ATPase (clone α5) and were incubated overnight at 4˚C. The α5 antibody was deposited to the DSHB by Fambrough, D.M. (Developmental Studies Hybridoma Bank, University of Iowa, USA). The following morning, slides were rinsed in TPBS (5, 10, and 15 min) in Coplin jars. Secondary antibodies of goat anti-rabbit Alexa Fluor 488 and goat anti-mouse Alex Fluor 555 (Thermo Fischer Scientific, Waltham, Massachusetts, USA) were applied at a dilution of 1:500 and incubated at 37˚C for one hour. Slides were rinsed again in TPBS as described above and 4',6-diamidino-2-phenylindole (DAPI) was applied with TPBS for 10 minutes to stain cell nuclei within the sections. Coverslips were mounted with 1:1 glycerol PBS with 0.1% NaN_3 as a preservative. For each slide, a negative control section containing BLØK solution containing no primary antibody was used in place of the primary antibody dilutions described above. All other steps for negative control sections were identical to those described here.

The stained sections were then analyzed for the presence of Rhesus c-like glycoproteins (Rhcg-like), NKA, V-ATPase, and UT based on the detection of fluorescent antibodies bound to the gill sections. Photos were taken using a Leica DM5500 photomicroscope with a Hamamatsu C11440 ORCA-Flash 4.0 digital camera using Leica Application Suite X (LASX) software (Leica Microsystems 2016, Wetzlar, Germany). Figures were assembled using GIMP GNU Image Manipulation Program (2.8.22; 2017).

Western Blotting

Single whole animal cross sections of the branchial region were homogenized in 1 mL of 0.3% sodium deoxycholic acid in SEI buffer solution containing 250mM sucrose, 10mM Na_2EDTA and 50mM imidazole, pH 7.4 using a bead homogenizer at 4000rpm for 2 x 10 s (Bertin Instruments, Precellys® 24 Tissue Homogenizer, Montigny-le-Bretonneux, France). Homogenized samples were then centrifuged at 5000rpm for 60 seconds at 4˚C. A sample (5µL), drawn from the supernatant immediately below the layer of lipid, was used to determine protein concentration using the bicinchoninic acid (BCA) protein assay (G-Biosciences, Overland, Missouri, USA; Smith et al. 1985). Two hundred µL of supernatant were then transferred to a clean 1.5mL centrifuge tube, to which an equal volume of 2x Laemmli's buffer (0.125M Tris-HCl pH 6.8, 20% glycerol, 4% sodium dodecyl sulphate (SDS), 0.01% Bromphenol Blue, 100 mM dithiothreitol) was added. Samples were then heated at 70˚C for 10 min and stored at 4˚C. Following the determination of the total protein concentration, each sample was diluted to 1µg/µL using 1x Laemmli's buffer.

Exactly 20 µg of lamprey tissue protein were loaded into each lane of a 10% polyacrylamide SDS gel with a 4%T stacking gel. These samples were electrophoresed for 15 minutes at 75V, followed by 60 minutes at 100V (Bio-Rad PowerPac™ HC Power Supply, Hercules, CA). Bio-Rad dual-colour molecular weight marker was used in addition to a pooled sample from all lamprey tissues on each gel that was processed to check for consistency between gels. Gels and polyvinylidene difluoride (PVDF) membranes were equilibrated in 10% methanol transfer buffer (48 mM Tris Base, 39 mM Glycine) for 15 and 30 minutes respectively. Protein

transfer from gel to PVDF membrane was conducted using a wet transfer stack at 100V for 1 hour with a TE 22 Mighty Small Transfer Tank (Hoefer Inc., Holliston, MA).

After drying, membranes were wet with methanol, followed by MilliQ water, treated with 1% Ponceau's stain in 5% acetic acid for five minutes, destained in 1% acetic acid and photographed to assess the quality of the transfer. The membranes were then washed for 5, 10, and 15 minutes in Tris-buffered saline (20 mM Tris, 500 mM NaCl) with 0.1% Tween 20 (TTBS) followed by blocking for at least three hours in 5% skim milk powder in TTBS. Membranes were briefly rinsed in TTBS and antibodies were then applied, diluted in 1% BSA in TTBS and 0.05% NaN_3 at the following concentrations: UT – 1:5000 (Braun et al. 2009); Rhcg1 – 1:1000 (Nakada et al., 2007; provided courtesy of Professor S. Hirose, Tokyo Institute of Technology, Japan), NKA – 1:500 (Wilson et al. 2007), V-ATPase (B) – 1:500 (Wilson et al. 2007), and tubulin (12G10, DSHB) – 1:500. Samples were left to incubate overnight at room temperature, then washed three times in TTBS the following morning as specified above. They were then incubated in secondary antibody for 1 hour. Membranes incubated for UT, Rhcg, NKA, or V-ATPase received the goat anti-rabbit IgG HRP conjugated secondary antibody at a concentration of 1:25000, while those incubated for tubulin received the goat anti-mouse IgG HRP conjugated secondary antibody at 1:25000. The membranes were then rinsed again in TTBS as before.

Finally, 2 mL of BioRad Clarity ECL Detection Solution (Hercules, CA) were applied to each membrane for at least five minutes, and detection of chemiluminesence was carried out using an Azure c300 Imaging System (Azure Biosystems, Dublin, CA) under a chemiluminescent increment exposure, the images were then exported for analysis.

Quantification of protein expression was performed using ImageJ software's gel analysis toolset (NIH, Maryland, USA). Values were measured based on the area under the curve, minus the background expression of the gel (SPARQ-ed, University of Queensland, Australia). Size and intensity of bands were analyzed and adjusted based on a pooled sample run on every gel. This pooled sample was created by mixing 20 µL of all samples which was vortexed and processed as described above.

Calculations

Excretion rates of ammonia and urea were calculated using the following equation for each time interval:

$$J_{N\text{-}waste} = \frac{[N_{initial} - N_{final}] \times V}{M \times \Delta T}$$

where N is the concentration of ammonia or urea in $nmol \cdot L^{-1}$, V is the volume of water in litres, M is the mass of the animal in grams, ΔT is the amount of time elapsed in hours and $J_{N\text{-}Waste}$ is the excretion rate of nitrogenous wastes in $nmol \cdot g^{-1} \cdot h^{-1}$, either J_{amm} or J_{urea}.

Statistical Analysis

All statistical analyses were carried out with an alpha level of 0.05. Results for mass throughout metamorphosis are presented as mean ± SEM. A student's t-test was applied to determine whether total mass loss during metamorphosis differed between transforming and non-transforming sea lamprey.

A One-Way ANOVA of excretion rates was used to compare independent groups of ammocoetes, 10% SW acclimated post-metamorphic, 100% SW post-metamorphic, and fed lamprey. This was followed by a Tukey's honestly significant difference (HSD) post-hoc test. Life-stage was considered the independent variable, and excretion rates the dependent variable.

Plasma ammonia and urea were both analyzed using One-Way Analysis of Variance (ANOVA). Life stage was again considered the independent variable with plasma ammonia and urea concentrations as the dependent variables. This was followed by a Games-Howell post-hoc test as this test is robust to differences in sample size and significant differences in variances between groups.

All western blotting data were analyzed using One-Way ANOVAs. Life stage was considered the independent variable with relative protein concentration as the dependent variable. For the analysis of Rhcg-like protein and UT, analysis was followed by a Tukey's Honestly Significant difference test. A Games-Howell post-hoc test followed this analysis for NKA due to unequal sample variances between groups.

Results

Differences in Mass Loss Between Metamorphosing and Non-metamorphosing Sea Lamprey

Of the 39 animals isolated in individual holding chambers, 26 underwent metamorphosis and 13 remained in the larval stage (non-transformers). All animals averaged between 3.0-3.5g throughout the entire metamorphic period and there was no significant difference in initial weight between transforming and non-transforming animals. All animals lost an average of 0.360

± 0.032g or 10.8% of their starting body mass throughout the metamorphic period (data not shown). Transforming lamprey lost significantly more mass on average (~0.1g), than the non-transforming ammocoetes ($p<0.05$).

Effects of Life Stage on J_{amm} and J_{urea} in Sea Lamprey

In ammocoetes, J_{amm} averaged 50 ± 8 nmol $N \cdot g^{-1} \cdot h^{-1}$, which was not significantly different from the rates of juveniles, which averaged 28 ± 9 nmol $N \cdot g^{-1} \cdot h^{-1}$ immediately following metamorphosis in fresh water (Figure 2.1A). The J_{amm} of juveniles tested in 10 % SW or 100 % SW were not significantly different from ammocoetes, averaging approximately 60 nmol $N \cdot g^{-1} \cdot h^{-1}$. Of the 21 juvenile, parasitic animals placed in the feeding tank with trout, only six survived until the end of the feeding period. Of these six, four were directly observed to have been feeding on trout. Amongst the four animals that were observed to have been feeding, J_{amm} was almost 5-fold higher, averaging 291 ± 105 nmol $N \cdot g^{-1} \cdot h^{-1}$ (Fig. 2.1A). J_{amm} of parasitic animals was significantly higher than all other groups ($p<0.05$; see appendix A). Feeding was confirmed by inspecting the gut for evidence of feeding.

In ammocoetes, J_{urea} averaged 24.3 ± 4.7 nmol $g^{-1} \cdot h^{-1}$. After the completion of metamorphosis, J_{urea} was more than 60% lower in juvenile sea lamprey in FW but doubled to approximately 15 nmol $g^{-1} \cdot h^{-1}$ after acclimation 10 % SW and did not change after transfer to 100 % SW (Figure 2.1B). After feeding on trout, however, J_{urea} more than tripled to 44 ± 13 nmol $g^{-1} \cdot h^{-1}$, compared to the rates measured in the non-feeding juveniles in 100 % SW (Figure 2.1B). J_{urea} of parasitic lamprey was significantly higher than all other juvenile groups, but not significantly different than the ammocoetes.

Changes in Plasma Ammonia and Urea Concentrations During and Following Metamorphosis

Internal ammonia levels varied widely among different stages of the sea lamprey, peaking in the post-metamorphic, juvenile individuals at 1252 ±228 $\mu mol \cdot L^{-1}$ and proceeding to decrease following salinity acclimation and feeding (Fig. 2.2A). One-way ANOVA indicated significant differences between groups ($p<0.05$; see appendix A) and the subsequent Games-Howell post-hoc indicated a significant decrease in internal ammonia in stage 2 at 183.8 ± 30.1 $\mu mol \cdot L^{-1}$ ($p<0.05$), and an increase in the post-metamorphic and seawater acclimated juveniles ($p<0.05$).

There was little variation in internal urea levels during the early stages of metamorphosis (Fig. 2.2B), but there was a decrease of 44% between stages 1 and 5, and the seawater acclimated juvenile animals ($p>0.05$). Internal urea increased markedly following feeding in juvenile sea lamprey, however due to a small sample size (n=4) and large variation in this group, the results for the current experiment remain inconclusive ($p>0.05$).

Distribution and Abundance of Rhcg-like Proteins and Associated Transporters in the Gills of Metamorphosing Sea Lamprey

Immunohistochemical analysis was undertaken on the gills of 6-8 animals per life stage (ammocoete, stage 1-7 of metamorphosis, juvenile lampreys in FW, 10 % SW or 100 % SW) to determine if the presence/absence, distribution, and relative staining intensity for Rhcg-like protein (using the Rhcg1 antibody), V-ATPase, NKA, and UT, changed during metamorphosis. DAPI staining was also used to visualize cell nuclei. Examples of typical micrographs at low magnification are illustrated in Figure 2.3.

Rhcg-like protein was present in the pavement cells lining the lamellae and was likely found on ammocoete-type mitochondrion-rich cells (aMRC), based on distribution and location (Bartels et al. 1998), in the interlamellar regions of the gills of sea lamprey ammocoetes (Fig. 2.4A). As metamorphosis progressed, the amount of Rhcg-like proteins appeared to decrease (Fig. 2.4B, C) until the juvenile stages when it was localized within the interlamellar regions of seawater-type mitochondrion-rich cells (SW MRC – green), identifiable based on the characteristic staining for NKA (red; Reis-Santos et al. 2008) (Fig. 2.4C, D, E). NKA was diffusely distributed along the lamellar epithelium of larval lamprey, but staining intensity increased in the early and later stages of metamorphosis (Fig. 2.4B,C). By late metamorphosis, NKA was mainly restricted to the interlamellar regions of the gill epithelium, with the signal strength peaking in the juvenile stages (Fig. 2.4D,E,F). The colocalization of NKA with Rhcg-like protein in the SW MRCs was most pronounced in juvenile sea lamprey that had been feeding on rainbow trout (Figure 2.4F).

Western blotting produced a single thin band at 48kDa, the expected molecular weight of Rhcg-like protein. Blotting for NKA blotting resulted in a single broad band at 111kDa, also at the expected molecular weight. Western blotting revealed no significant difference ($p>0.05$) in relative content of Rhcg-like protein among ammocoete, early metamorphosing lamprey, and juvenile sea lamprey (Fig. 2.5). Meanwhile, relative NKA content was low in ammocoetes and during metamorphosis (<0.5), before increasing more than six-fold beginning at stage 7 of metamorphosis, and peaking in the freshwater juvenile at levels greater than 15-fold that of NKA observed in ammocoete (Fig. 2.6). The elevation in relative NKA protein content was sustained

in the juvenile sea lampreys through seawater acclimation, but there were no significant changes in relative NKA expression amongst the juvenile lamprey ($p>0.05$).

The distribution of the proton-pump, V-ATPase B subunit, was widespread on the lamellae and within the interlamellar spaces of the filament in sea lamprey ammocoetes (Fig. 2.7A). Based on the flattened nature of the cells binding the V-ATPase antibody, it is likely that the V-ATPase was restricted to pavement cells. By late metamorphosis, V-ATPase expression began to decrease, and was below detection by the juvenile phases (Fig. 2.7).

Changes in the Distribution and Expression of Urea Transporter Protein (UT)

UT was diffusely distributed on the lamellae of ammocoetes and during early metamorphosis (Fig. 2.8A,B). By late metamorphosis, however, UT had virtually disappeared and was not detected in freshwater, seawater acclimated, and parasitic juvenile sea lamprey, never staining stronger than the non-specific secondary antibody staining observed in the negative control (Fig. 2.8D to F inset; see Appendix B for example null comparisons). Western blot analysis revealed a single band at the expected molecular weight of 35 kDa for UT (Fig. 2.9). There was a downward trend in UT abundance beginning in the mid-late stages of metamorphosis. At the completion of metamorphosis, UT was barely detectable in the juvenile lamprey, and significantly lower compared to all other groups ($p<0.05$), with the exception of the SW acclimated juveniles (Fig. 2.9).

Figures

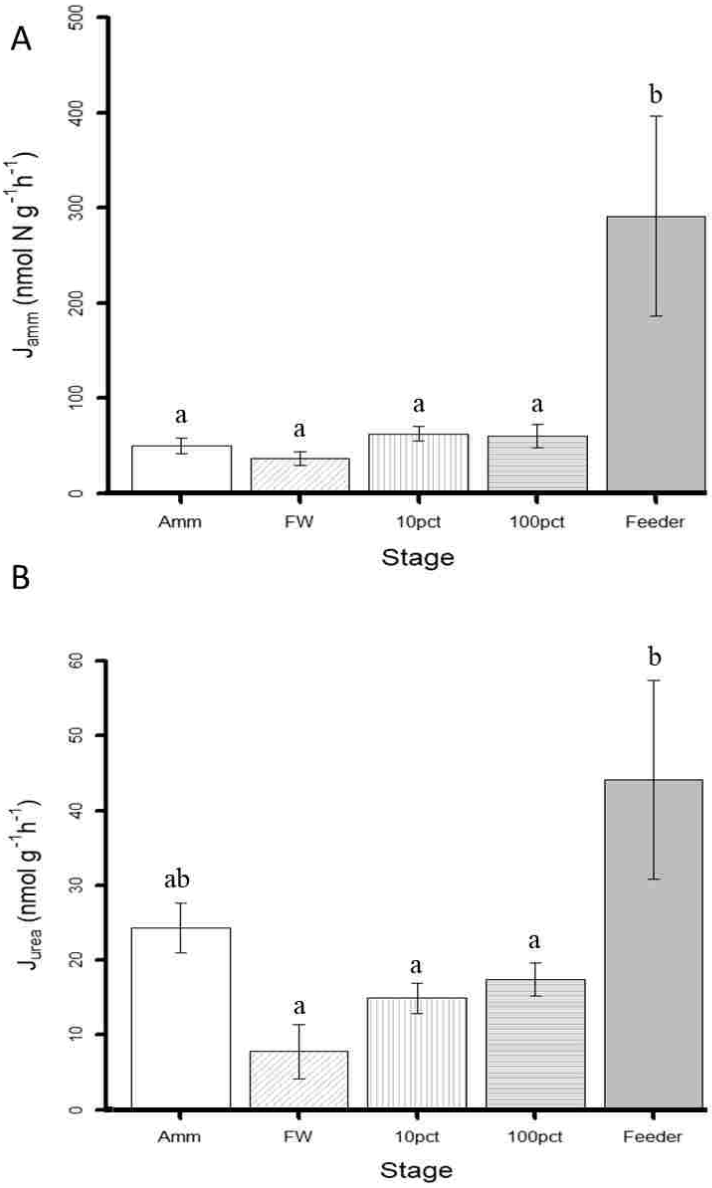

Figure 2.1. Changes in (A) ammonia (J_{amm}) and (B) urea (J_{urea}) excretion rates of larval (ammocoetes), and juvenile (post-metamorphic) sea lamprey in fresh water (FW), and following acclimation of the juvenile lamprey to 10 % SW, 100 % SW, and following feeding in 10 % SW. Data presented as the mean ± 1 SEM, N = 13 in ammocoetes, N = 10 juveniles in fresh water and seawater acclimations, and N = 4 juvenile lamprey following feeding. Bars sharing the same letters are not significantly different.

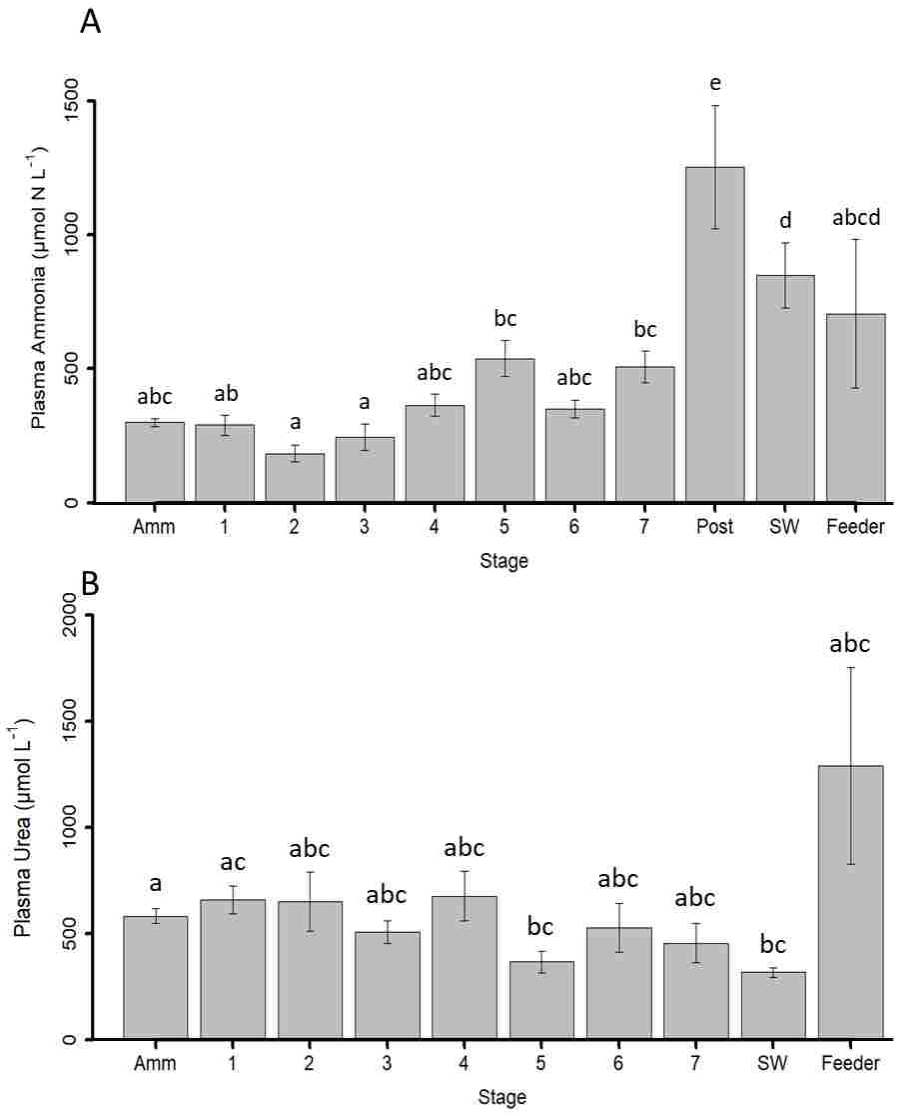

Figure 2.2. Differences in plasma ammonia (A) and urea (B) concentrations of sea lamprey during their larval (ammocoete) phase, during stages 1- 7 of metamorphosis, and in post-metamorphic juvenile sea lamprey acclimated to FW, 100 % SW, and after 2 weeks of feeding on rainbow trout in 10% SW. Bars sharing the same letter are not significantly different from one another ($p>0.05$). No plasma urea data was available for post-metamorphic FW animals (Post).

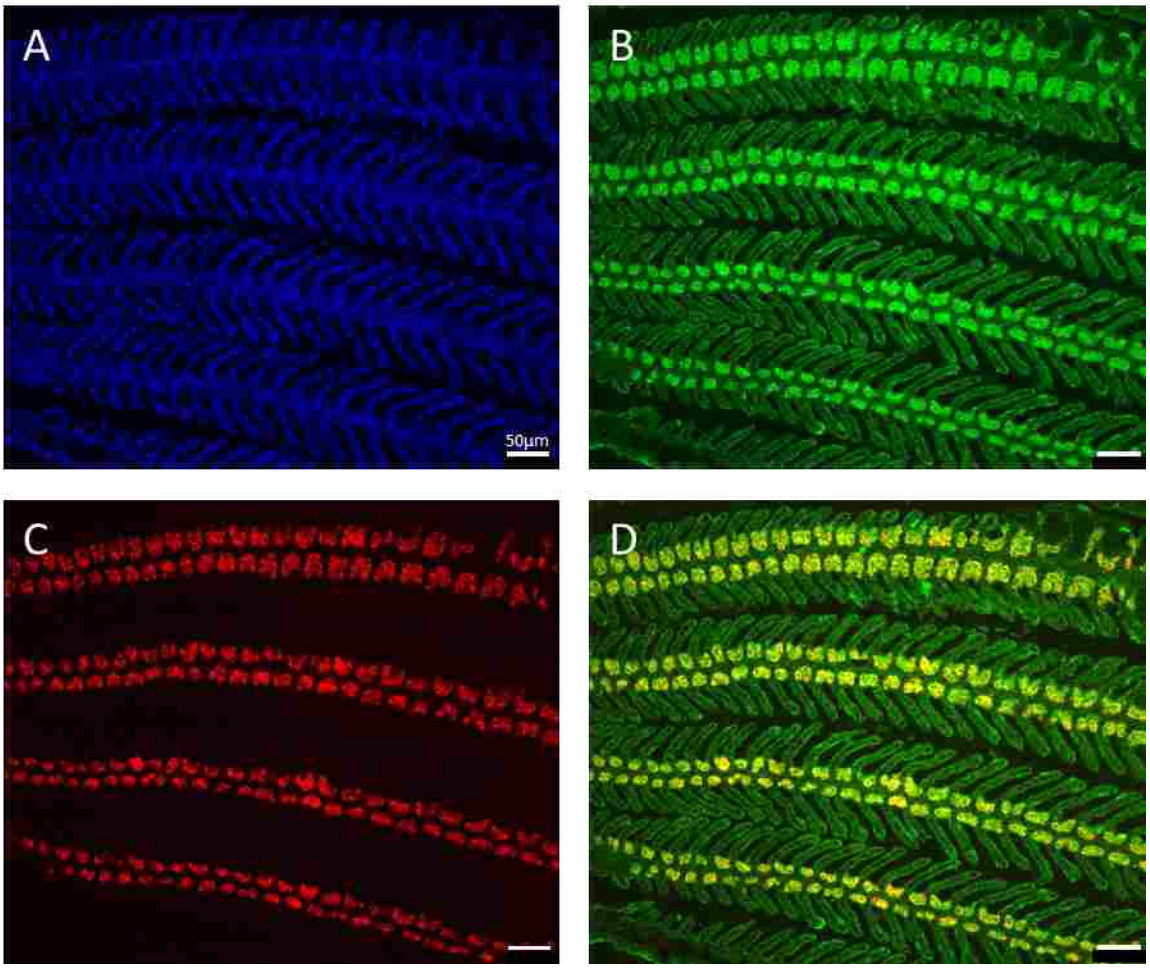

Figure 2.3. Patterns of immunohistochemical staining, using juvenile sea lamprey as an example, to illustrate different staining of the sea lamprey gills. A) DAPI staining stains cell nuclei blue, B) Rhcg-like protein stains green C) Na^+/K^+-ATPase (NKA), stains red. In D), double labelling for Rhcg-like protein (green) and NKA (red) demonstrates that these proteins colocalized in the interlamellar regions of the gill of juvenile sea lamprey. Scale bar = 50 µm.

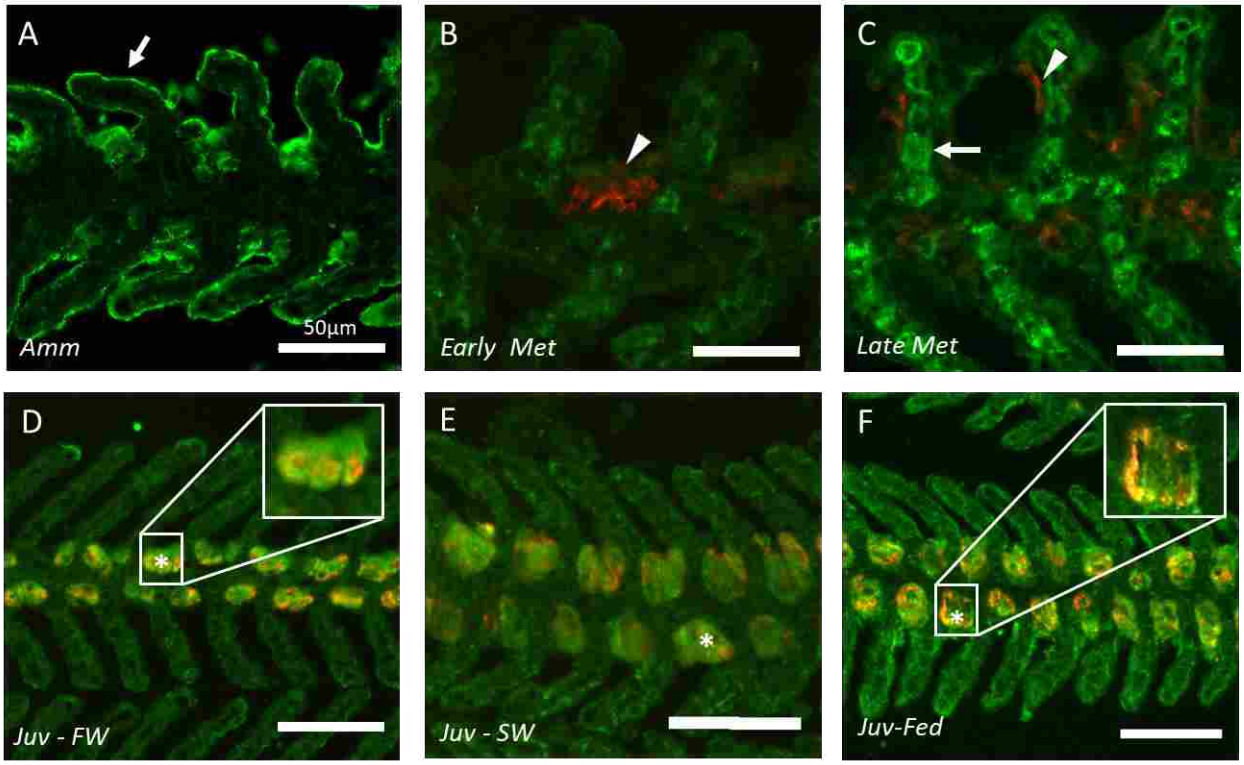

Figure 2.4. Immunohistochemical labelling of Rhcg-like protein (green) and NKA (red) in the gills of (A) larval (ammocoetes; Amm), (B) early (Early Met.), and (C) late metamorphosis (Late Met.), and in (D) post-metamorphic juvenile sea lamprey in FW (Juv - FW), E) after acclimation to 100% SW (Juv - SW), and F) following feeding in 10% SW (Juv - Fed). Arrows demonstrate the changing localization of Rhcg-like proteins throughout metamorphosis until eventual localization within seawater-type mitochondrion-rich cells (SW MRC; *). Arrowhead shows the increasing appearance of NKA during metamorphosis, in SW MRC within the interlamellar regions of juvenile lamprey gills. Scale bar = 50 µm.

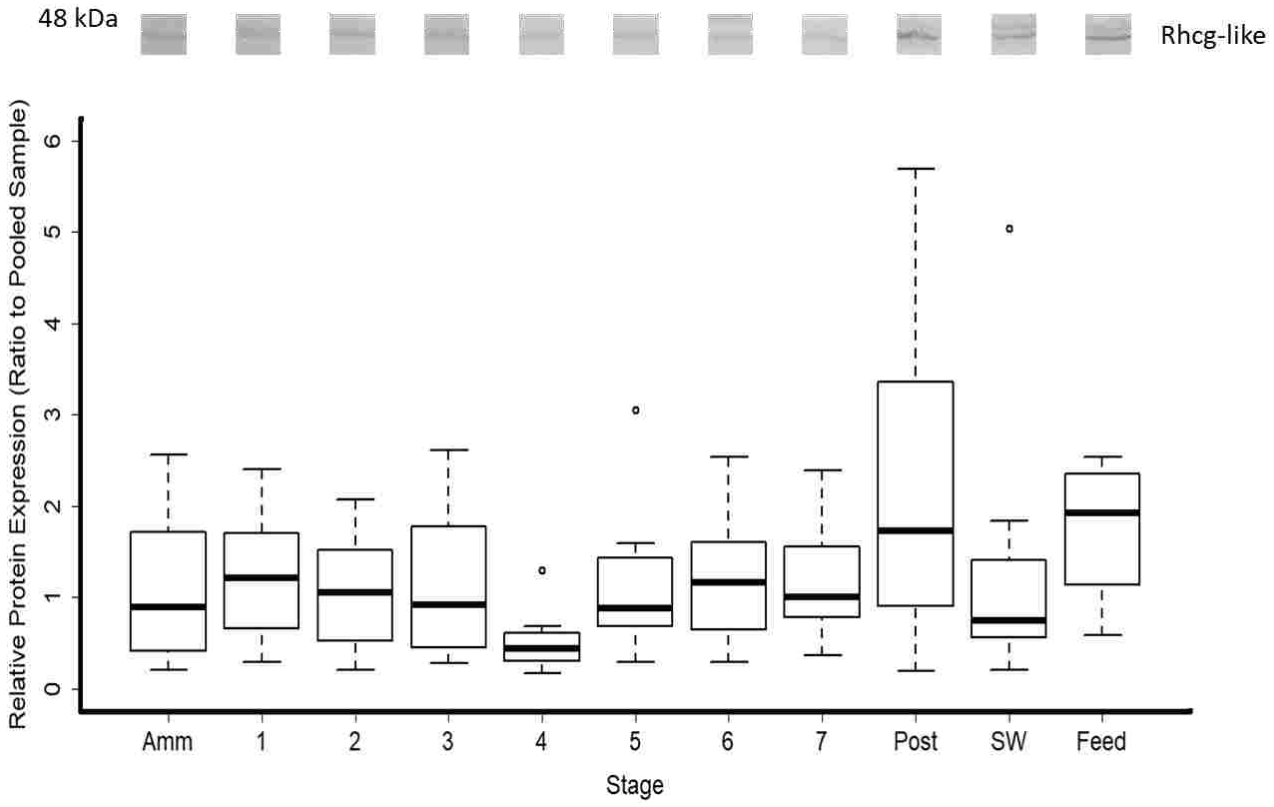

Figure 2.5. Western blot analysis demonstrating the variation in the relative abundance of Rhcg-like proteins in the gills of sea lamprey throughout metamorphosis and following SW acclimation and feeding of juvenile lamprey. The quantity of Rhcg-like protein in the gill was based on the relative density of bands (48 kDa) normalized to a pooled sample. Values shown are the mean ± 1 SD (box) and 95% confidence interval (dashed lines). No significant differences were detected between groups. N = 8 for all groups with the exception of ammocoetes (n=11) and feeding juveniles (n=4). Dots indicate outliers.

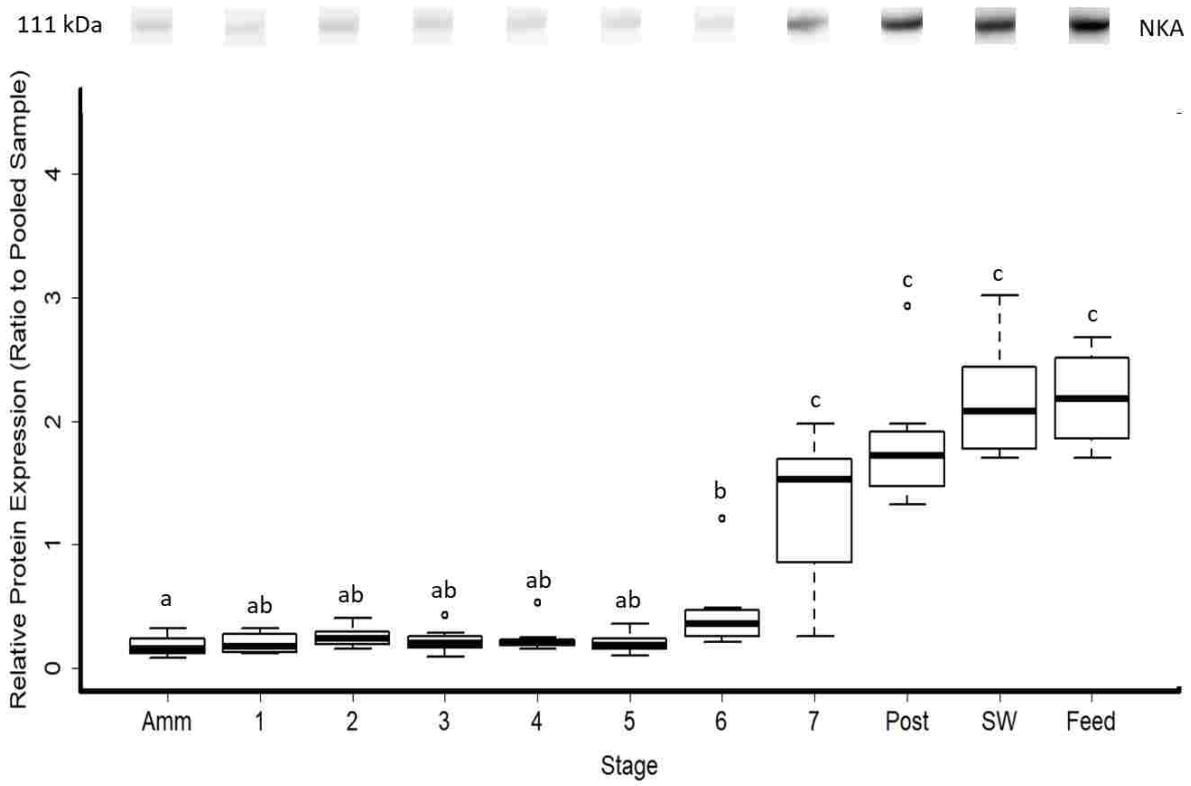

Figure 2.6. Western blot analysis illustrating the differences in the relative abundance of Na^+/K^+-ATPase (NKA) protein in the gills of sea lamprey during the larval (ammocoete) phase, stages 1-7 of metamorphosis, and in juveniles (post-metamorphic) in FW, following SW acclimation and feeding. The quantity of NKA in the gill was based on the relative density of bands at 110 kDa normalized to a pooled sample. Values shown are the mean ± SD (box) and 95% confidence interval (dashed lines). Boxes sharing the same letter or not significantly different from one another ($p<0.05$). N = 8 for all groups with the exception of ammocoetes (n=11) and feeding juveniles (n=4). Dots indicated outliers.

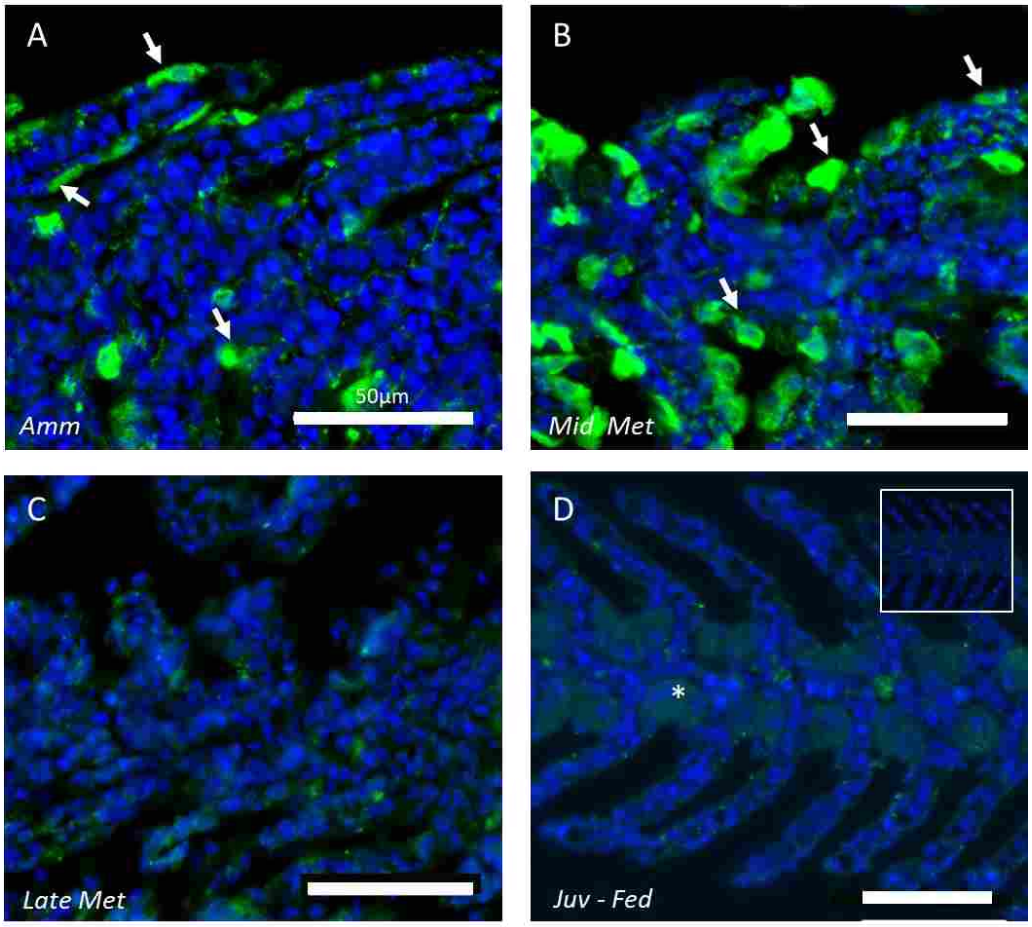

Figure 2.7. Representative images showing immunohistochemical labelling of V-type H^+-ATPase (V-ATPase; green) and cell nuclei using DAPI (blue) in the gills of (A) larval (ammocoetes; Amm), (B) early (Early Met.), and (C) late metamorphosis (Late Met.), and in (D) parasitic juvenile (Juv - Fed) sea lamprey. Arrows demonstrate the localization of V-ATPase on the lamellar epithelium in ammocoetes (A) and mid-metamorphosing (B). The asterisk (*) indicates the location of SW MRCs in the interlamellar region of the juvenile gill epithelium. Scale bar = 50 µm.

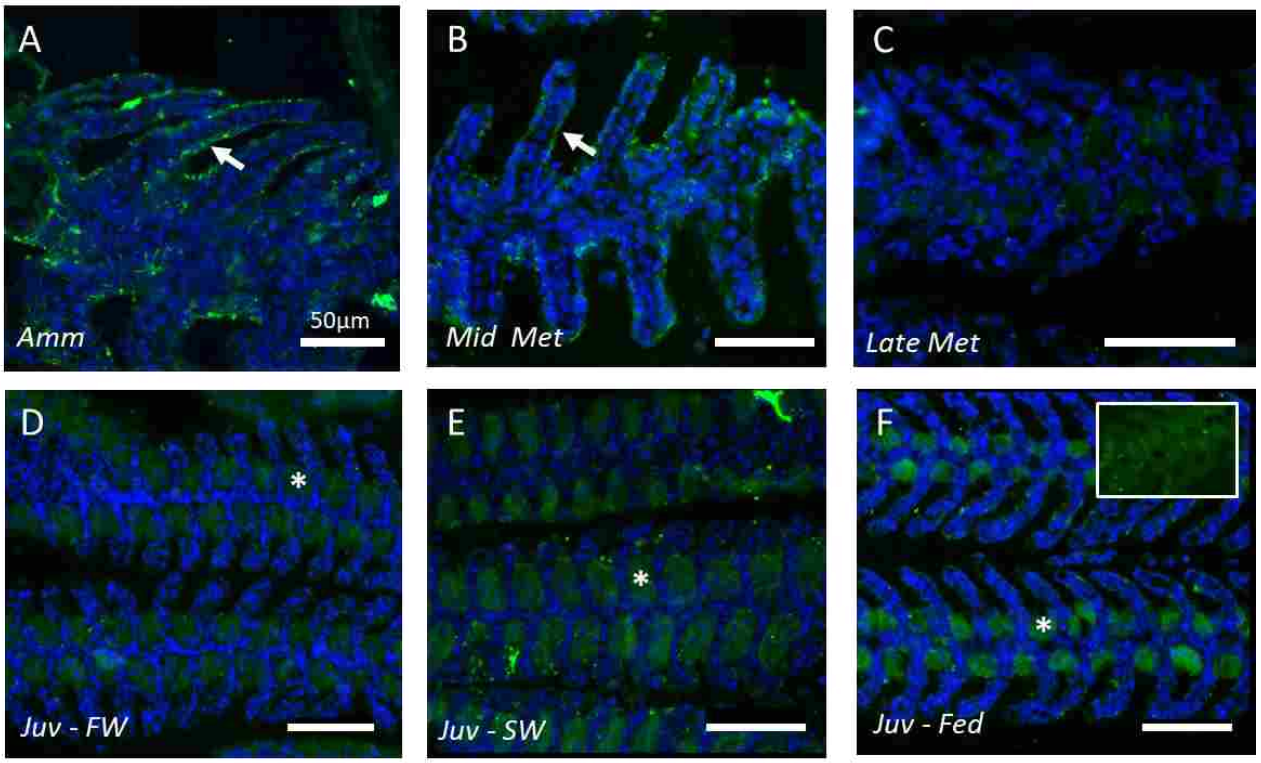

Figure 2.8. Immunohistochemical localization of urea transporters (UT; green) doubly labelled with DAPI (blue) to show cell nuclei in the gills of metamorphosing sea lamprey. Images show localization patterns typical of A) ammocoetes (Amm), B) early stages of metamorphosis (Early Met.), C) late metamorphosis (Late Met.), D) post-metamorphic juveniles in FW (Juv - FW), E) 100% SW acclimated juveniles (Juv - SW), and F) parasitic juveniles (Juv - Fed). The inset depicted in panel (F) provides the null staining of the gill examined from the same animal for reference. Arrows indicate UT coating the lamellae in ammocoetes and early metamorphosis (A-B) before fading in mid-late metamorphosis (C-D). Seawater-type mitochondrion-rich cells are indicated with an asterisks (*). Scale bar = 50 µm.

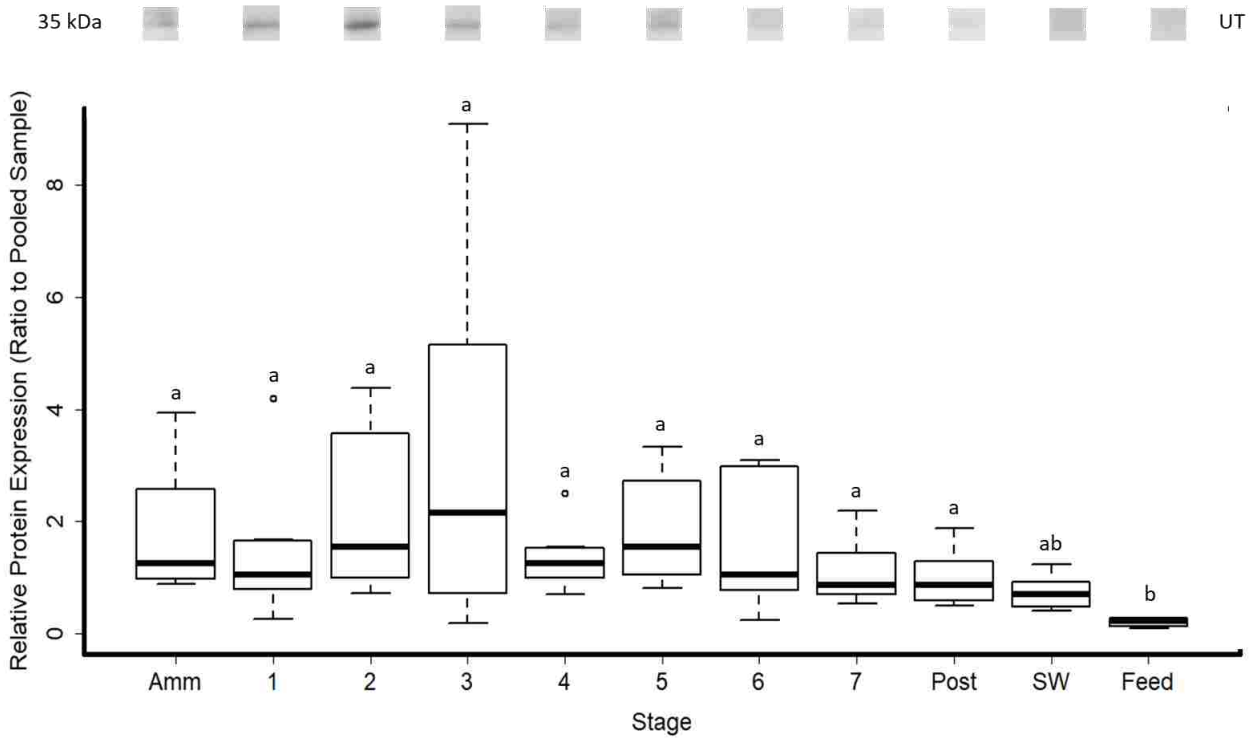

Figure 2.9. Western blot analysis illustrating the relative quantity of urea transporter (UT) in the gills of sea lamprey throughout metamorphosis and following SW acclimation and feeding of juvenile animals. The quantity of UT in the gill was based on the relative density of bands at 35 kDa normalized to a pooled sample. Values shown are the mean ± SD (box) and 95% confidence interval (dashed lines). Boxes sharing the same letter or not significantly different from one another ($p<0.05$). N = 8 for all groups with the exception of ammocoetes (n=11) and feeding juveniles (n=4). Dots indicate outliers.

Discussion

This study demonstrates that the complex reorganization of the gills that takes place during metamorphosis in sea lamprey is accompanied by changes in the distribution and abundance of the transport machinery required to excrete N-wastes during the parasitic life stage, when juvenile sea lampreys ingest large quantities of protein rich blood. Wilkie et al. (2006) showed that ammonia and urea production increases during the juvenile phase, as demonstrated by increased activities of key amino acid catabolizing enzymes such as glutamate dehydrogenase, and by marked elevations in J_{amm} and J_{urea} after metamorphosis. Further, feeding by juvenile parasitic lampreys resulted in substantial 10 to 15-fold increases in postprandial ammonia excretion, likely due to the very high protein intake associated with their haematophagous (blood) diet (Wilkie et al. 2004). Postprandial J_{urea} was shown to increase by up to 25-fold in lamprey feeding on elasmobranchs (Wilkie et al. 2004), likely due to the ingestion of large amounts of dietary urea and arginine. Arginine is hydrolyzed to L-ornithine and urea via the enzyme arginase in the liver and gut and is present at higher levels following metamorphosis (Wilkie et al. 2004). Similar changes in J_{amm} and J_{urea} were observed in the present study, but the changes were not as pronounced as reported by Wilkie et al. (2004, 2006) This is likely due to differences in the feeding regime between studies. In the present study, the sea lamprey only fed sporadically on trout for 1-2 weeks with some estimated to have been fasting for up to 12 hours prior to measurements of J_{amm} and J_{urea}. This means that the J_{amm} and J_{urea} measured in this study may have been closer to basal excretion rates compared to other studies where sea lamprey had been feeding more consistently for several weeks, and postprandial rates of J_{amm} and J_{urea} were measured in the hours immediately following removal from the host fish (Wilkie et al. 2004).

This would result in the measurement of much higher rates of excretion (e.g. Brett and Zala 1975; Wood et al. 2001). My results, along with earlier studies on postprandial J_{amm} by sea lamprey (Wilkie et al. 2004; 2006), support the hypothesis that the re-organization of the gill that prepares juvenile lamprey for the FW-SW transition occurs in parallel with complementary changes in branchial mechanisms of J_{amm}. The excretion of urea on the other hand, appears to be less dependent upon branchial routes in juvenile, parasitic sea lamprey despite increases in net urea excretion (Swindell 1999).

Reorganization of N-waste Excretion Mechanisms in the Gills Facilitate Marked Increases in J_{amm}

The most notable change in the gills was the re-distribution of Rhcg-like protein. In ammocoetes, Rhcg-like protein was diffusely distributed along the lamellar epithelium and within the interlamellar spaces of the gill epithelium. However, during metamorphosis, Rhcg-like protein immunoreactivity on the lamellae decreased, but exhibited intense immunoreactivity within the interlamellar spaces at the conclusion of metamorphosis (Fig. 2.5). The loss of Rhcg-like protein from the lamellae during metamorphosis would have coincided with the loss of ammocoete type MR cells (AMRCs), for which the precise function remains unknown (Bartels and Potter 2004). The present data is circumstantial evidence that aMRCs facilitate ammonia excretion via Rhcg-like proteins as suggested by Bartels et al. (2004). Higher resolution immunofluorescence microscopy and/or electron microscopy are needed to help to test this hypothesis.

Cellular localization of Rhcg-like protein after metamorphosis was much clearer, colocalizing with Na^+/K^+-ATPase (NKA) within seawater-type mitochondrion-rich cells (SW MRCs) found in the interlamellar spaces of the gill epithelium. The SW MRCs of sea lamprey are arranged side-by-side within the interlamellar spaces (Bartels and Potter 2004; Reis-Santos et al. 2008), exhibiting intense immunoreactivity to NKA, an electrogenic pump which transports Na^+ into the paracellular spaces. This generates an electrochemical gradient that favors the excretion of Na^+ via cation selective epithelial tight junctions (Marshall and Edwards 2013). The evenly distributed immunoreactivity of NKA within the cells likely reflects the basolateral localization of NKA on the complex tubular network associated within SW MRCs. The accompanying colocalization of Rhcg-like protein immunoreactivity with NKA suggests that this protein is also associated with this tubular network. In other words, Rhcg-like protein is found on the basolateral membrane of SW MRCs, rather than apically on the gill epithelium as proposed in FW teleost models (Braun et al. 2009a; Wright and Wood 2009; Nawata et al. 2010). Thus, SW MRCs may play a dual role in hyposmoregulatory and ammonia excretory processes in SW acclimated juvenile sea lampreys. Coupling ammonia excretion with ionoregulation could lower the metabolic costs of these processes. For instance, the Rhcg-like protein could facilitate NH_3 loading of SW MRCs, most of which would be converted to NH_4^+ in the more acidic cytosol of the cell. In turn, the NH_4^+ could be excreted by the cell by substituting for H^+ on apical Na^+/H^+ exchangers (NHE), in exchange for Na^+. Such an arrangement would facilitate not only ammonia excretion, but also acid-base regulation by excreting NH_4^+, a weak acid.

Despite the conspicuous changes in Rhcg-like protein distribution and greater rates of ammonia excretion that characterize the juvenile life stages (Wilkie et al. 2006), Western blot

analysis revealed that there was no net increase in Rhcg-like protein content within the gills (Fig. 2.6). In freshwater fishes, Rh glycoproteins are gas channels found in gill MR cells, that conduct NH_3 down its partial gradient. There are four types of Rh glycoproteins that have been reported in teleosts, Rhag, Rhbg, and Rhcg1 and Rhcg2, which are all thought to facilitate ammonia excretion via branchial epithelial cells in both FW and SW (Braun et al. 2009; Wright and Wood 2009; Hwang et al. 2011). Some have also been recently characterized in the branchial epithelium and skin of sea lamprey (Blair et al. 2016). Notably, Rhag and Rhbg could not be localized in sea lamprey in this study, despite repeated attempts to refine the immunodetection protocol for these proteins. The absence of Rhag on the gill epithelium was not surprising, given that its presence was only expected on erythrocytes (Wright and Wood 2009). However, Blair (2011) detected very weak Rhag immunoreactivity in the gills of juvenile lamprey using cryopreserved specimens but was unable to detect the protein using Western blot techniques. He was, however, able to detect Rhbg, Rhcg1 and Rhcg2 in the skin of larval and juvenile sea lamprey but was unsuccessful in quantifying Rhbg-like protein using Western blotting (Blair et al. 2016). Additionally, an investigation of the sea lamprey genome localized both Rhag-like and Rhbg-like protein transcripts to the Rhcg-annotated region of the genome. These findings suggest that either these isoforms have not yet been identified in the lamprey genome, or that these proteins are not differentiated in the lamprey. Together these results suggest that Rh glycoprotein expression in the gills may be limited to the Rhcg-like protein isoform(s). Future transcriptomic analyses along with the subsequent development of more specific antibodies for immunohistochemistry will be needed to confirm these hypotheses.

A reorganization in any of these other Rh glycoproteins may explain some of the observed changes in internal ammonia levels that occurred during and following metamorphosis. The decrease in Rhcg-like protein expression seen in mid-metamorphosis could be associated with the increase in internal ammonia observed in the same stage. Peek and Youson (1979) report the degeneration of many cells within the gills during the mid-stages of metamorphosis (stages 1-5), which may explain the decrease in Rhcg-like protein expression reported in my study. Also puzzling is the spike in internal ammonia at the onset of the post-metamorphic, juvenile phase. Not only did Rhcg-like protein levels increase at this time, but the lamprey had not yet begun feeding and thus the source of ammonia is likely internal. While changes in the gill can explain the decreases in Rhcg-like protein expression, the catabolism of internal energy stores may have caused the changes in internal ammonia levels observed here. During metamorphosis, sea lamprey consume lipid stores to fuel metabolic processes, as reflected by weight loss observed in this study, which are particularly high during this non-feeding phase (Lowe et al. 1973). While protein content did not change significantly in the body of the metamorphosing sea lamprey in a previous study b Lowe et al. (1973), it does not necessarily rule out a shift to protein consumption in the present study. Indeed, the juvenile animals studied in the present study were starved until January, while the exact period of starvation remains unknown in the Lowe et al. (1973) study. Thus, given the marked depletion of lipid stores from initial levels over the 4-month metamorphic period, it is plausible that the animals had switched to protein catabolism at this time to survive the non-feeding period preceding parasitic feeding. A study of protein and lipid catabolism throughout metamorphosis in concert with plasma N-waste levels and transporter abundance would be necessary to confirm this explanation.

Decrease of Branchial V-ATPase Expression Prior to Seawater Migration

While Rhcg-like protein distribution changes during metamorphosis, simultaneous changes in ionoregulatory transporters associated with the ammonia excretion pathway provide further insight into N-waste excretion mechanisms. Immunohistochemical analysis indicated that there was a marked decrease branchial V-type H^+-ATPase (V-ATPase) abundance, which was distributed on the lamellae and gill filaments in ammocoetes and the early stages of metamorphosis, but virtually absent in juvenile lamprey following SW acclimation. These observations are in agreement with those of Reis-Santos et al. (2008) who observed similar reductions in V-ATPase immunoreactivity with seawater acclimation in sea lamprey. In freshwater fishes, V-ATPase helps generate the electrochemical gradient across the apical membrane of MR cells that is required to promote Na^+ uptake via an apically-located, epithelial Na^+ channel (ENaC; Edwards and Marshall 2013). However, V-ATPase is also hypothesized to play a critical role in promoting Rhcg-like protein mediated ammonia excretion, by trapping NH_3 as NH_4^+ as it is excreted across the apical membrane (Wright and Wood 2009). Following FW-SW transfer, the osmotic challenges faced by a euryhaline fish would be reversed, making Na^+ uptake unnecessary, and by extension, V-ATPase. Not only would this necessitate different modes of H^+ excretion for the purposes of acid-base regulation, it would also necessitate a different mode of ammonia excretion in SW as described above.

The presence of Rhcg-like protein and V-ATPase in ammocoetes, and during the early stages of metamorphosis, so far suggest that the fundamental mechanism of ammonia excretion by sea lamprey in freshwater is largely consistent with the model proposed by Wright and Wood (2009). However, the basolateral location of Rhcg-like protein in SW acclimated juvenile

lamprey suggests that the mechanism of ammonia excretion is much different during the SW life stages. Based on these findings, I propose that in SW, ammonia is transported into the cell cytosol of SW MRCs as NH_3 via a basolateral Rhcg-like protein transporter. Simultaneously, NKA pumps Na^+ from the cytosol into the plasma, maintaining relatively low concentrations of Na^+ in the cytosol. This in turn augments the inwardly directed Na^+ electrochemical gradient across the apical membrane of the SW MRC, resulting in the inward movement of Na^+ in exchange for H^+ via an apical Na^+/H^+ exchanger (NHE) (Weihrauch et al. 2009). If an NHE is indeed present, it raises the possibility that ammonia that accumulates in the SW MRC via the RHcg-like protein could be excreted via the NHE by substituting H^+ for NH_4^+ as proposed by a number of investigators (Weihrauch et al. 2009; Edwards and Marshall 2013.

Paracellular routes of ammonia excretion should not be discounted. It is well known that Na^+ accumulates in the paracellular channels between accessory cells and SW MRCs in teleost fishes, due to NKA pumping on the basolateral membrane (e.g. Edwards and Marshall 2013). The epithelial junctions between are often referred to as "leaky" in marine fishes, allowing Na^+ to diffuse across the gills to the external environment. In fact, these epithelial junctions are selective to Na^+ and other cations, suggesting that NH_4^+ could also leave via this route. Thus, a significant portion of the observed increase in J_{amm} in SW may be the result of paracellular ammonia transport via this route. In freshwater, this would not likely be possible as the epithelial junctions are much tighter than in SW, to help the fish minimize osmotic influx across the gills (Bartels and Potter 2004).

Branchial Urea Transport Mechanisms

Feeding by juvenile lampreys was associated with an increase in plasma urea, likely arising from the breakdown of arginine and the conversion of ammonia to urea via the process of uricolysis (Wilkie et al. 2006). Urea is normally thought to be a product of ammonia detoxification, but it too needs to be excreted. In zebrafish and trout, urea transport is facilitated by branchial urea transport proteins (UT; LeMoine and Walsh 2015). Immunohistochemical analysis pointed to a decrease in urea transporter (UT) expression in the gills of lamprey during mid-metamorphosis. This was supported by western blotting which also displayed a decrease in UT beginning in latter stages of metamorphosis. Somewhat surprisingly, these changes corresponded to a substantial increase in urea excretion rates following seawater acclimation and the onset of feeding. These findings suggest that there may be a shift in the site of urea excretion following metamorphosis. The most likely alternative urea excretion route is the kidneys. Although urine flow decreases markedly following the FW-SW transition (Pickering and Morris 1970), the urine produced is much more concentrated than in FW, and thus may have higher concentrations of urea than in FW. Indeed, experiments using divided chambers demonstrated that while urea excretion is roughly excreted in equal proportions between the gills and extra-branchial routes in ammocoetes, most urea excretion takes place via renal routes following metamorphosis in FW acclimated sea lamprey suggesting that the gills' role in this process is greatly reduced during the parasitic phases that follow (Swindell 1999). Small amounts of urea may also be excreted across the skin, though this possibility needs further investigation (Blair et al. 2016).

Another possibility is that the urea permeability of the gills increases following seawater acclimation, making specialized urea transport unnecessary. As described above, freshwater fishes have a "relatively tight" gill epithelium to prevent water influx (Bartels and Potter 2004), which could also restrict the passive diffusion of urea via this route. In sea water, it could be that the "leakier" cells between adjacent SW MRCs are also permeable to urea. It would therefore be informative to measure the permeability of the gills to urea in FW and SW acclimated lamprey. Additional work is also needed both in lamprey and ureotelic fish species to determine if other transporters may be associated with the excretion of urea.

Perspectives

My results for both Rhcg-like protein and NKA during metamorphic phases of the sea lamprey life cycle indicate an important role for the seawater-type mitochondrion-rich cells (SW MRCs) in both ionoregulation and ammonia excretion in SW environments. Continued study of these transporters and SW MRCs in freshwater and non-parasitic lamprey species would further determine the importance of SW MRCs for both its ionoregulatory and N-waste excretory roles. Additionally, further study may elucidate the benefits of the association between ionoregulatory and N-waste excretion pathways. Meanwhile, my results for UT and V-ATPase suggest that these transporters may be more important for branchial N-waste excretion in the freshwater life stages. Further work is needed to both confirm the increased role of V-ATPase subunits for N-waste in FW, and determine other transporters which may facilitate the excretion of urea across the gills via UT.

Chapter 3

Differences in nitrogenous waste excretion mechanisms in the gills of parasitic and non-parasitic lamprey species

Abstract

Lamprey (*Petromyzontiformes*) are an ancient group of jawless fishes comprising 41 species. Among these are closely related 'paired' species derived from a common ancestral lamprey but have parasitic and non-parasitic existences. One example of a 'paired' species is the non-parasitic northern brook lamprey (*Ichthymyzon fossor*) and the parasitic silver lamprey (*Ichthymyzon unicuspis*). These speciesare indistinguishable until the completion of metamorphosis. Although the external features of these species differ substantially after metamorphosis, little is known about differences in their internal physiology. In this study, I investigated how the mechanisms of nitrogenous waste (N-waste) excretion across the gills differs between the two species and compared them to juvenile sea lamprey (*Petromyzon marinus*; Chapter 2), not yet exposed to sea water, as an outgroup. Using immunohistochemical techniques, I examined Rhesus c-like glycoproteins (Rhcg-like), implicated in ammonia excretion, urea transporter (UT), and associated ionoregulatory transporters, Na^+/K^+-ATPase (NKA) and V-type H^+-ATPase (V-ATPase). UT was distributed on the lamellae and within interlamellar regions of the gill epithelium and appeared more abundant in the silver lamprey than in the northern brook lamprey. Rhcg-like protein was also sparsely distributed in specific cells on the lamellae in northern brook lamprey but was restricted to the interlamellar regions of the silver lamprey, in which the Rhcg-like protein appeared to colocalize with NKA. This Rhcg-like protein distribution, and association with NKA in the silver lamprey, suggests that Rhcg-like protein is likely restricted to the apical membrane of intercalated mitochondrion-rich cells (IMRCs) in the gill epithelium. There was a strong signal for V-ATPase in both the northern brook and silver lamprey, which was widely distributed in the gill epithelium. The presence of

V-ATPase in juvenile northern and silver lamprey, but its decreased abundance in juvenile sea lamprey supports the hypothesis that V-ATPase is of particular importance for ionoregulation and N-waste excretion in freshwater. My results suggest that preparation for an anadromous life style greatly affects the mechanisms of N-waste excretion and ionoregulation in lamprey species, and perhaps their evolution in more recently derived fish species.

Introduction

Lamprey (*Petromyzontiformes*) are a phylogenetically ancient group of jawless vertebrates (agnathans) that diverged from their jawed relatives at least 360 million years ago (Green and Bronner 2014). There are at least 41 species of lampreys (Potter and Gill 2003; Renaud et al. 2009; Potter et al. 2015), whose body plan and external features have remained largely unchanged for at least 360 million years (Gess et al. 2006; Potter et al. 2015). Despite the similarity between modern and fossil record lamprey species, this group has continuously been evolving for millions of years. All species begin as burrow-dwelling ammocoetes, feeding on detritus, algae and other suspended organic matter within freshwater rivers and streams before undergoing a complex metamorphosis (Youson 1980). Metamorphosis is characterized by a profound re-organization of the internal and external body structure. Changes include the transformation of the oral hood of ammocoetes, which is used to direct water and food items into the pharyngeal region, into an oral disc and rasping tongue, used to facilitate the attachment of parasitic species to their hosts/prey and to ingest blood. Other changes include a re-organization of the gills from a unidirectionally ventilated gill in ammocoetes, into a tidally ventilated gill, which allows the juvenile lamprey to irrigate the gills while attached to substrate or host fishes, and the development of distinct eyes and changes in body coloration (see Youson 1980; 2003 for review).

Even though all lamprey species undergo metamorphosis and are thought to have descended from a common parasitic ancestor in sea water, not all modern species of lamprey are parasitic (Youson and Sower 2001; Potter and Gill 2003; Gess et al. 2006; Renaud et al. 2009;

Docker et al. 2012). In fact, the majority (23 species) by-pass the parasitic phase following metamorphosis, and transform directly into non-feeding adults, which reproduce and then die (Docker et al. 2012; Bartels et al. 2016). Most of these species are 'paired' or satellite species, comprising of parasitic species and non-parasitic variants, each descended from a common anadromous, parasitic ancestor (Artamonova et al. 2011; Potter et al. 2015). Often, these paired species are indistinguishable from each other as ammocoetes and during the early stages of metamorphosis (Leach, 1940; Docker et al., 2012; Ren et al. 2016). An example of such paired species is the non-parasitic northern brook lamprey (*Ichthyomyzon fossor*), and the closely related parasitic silver lamprey (*Ichthyomyzon unicuspis*), which are virtually indistinguishable from each other as ammocoetes (Potter 1980; Renaud et al. 2009; Docker et al. 2012). Recent mitochondrial DNA studies suggest that northern brook and silver lamprey may in fact represent a single species of lamprey, which can proceed to either a non-parasitic or parasitic ecotype (Ren et al. 2016). Although these species are of great interest, their habitat and populations are imperiled, making them a species of special concern in Canada (COSEWIC 2007; 2011).

The distinctions between northern brook and silver lamprey are much clearer following metamorphosis, reflecting the two distinct lifestyles of the juvenile animals (Scott and Crossman, 1973; Potter et al. 2015). Typically, non-parasitic species, in this case the northern brook, possess a less well-developed gut than their parasitic counterpart, the silver lamprey, as well as relatively smaller eyes and oral disc. The diameter of the oral disc of juvenile parasitic silver lamprey is wider than the maximum width of the head, while juvenile northern brook lamprey have an oral disc that is considered "degenerate", with a diameter less than or equal to the maximum width of the head (Scott and Crossman 1973; Potter et al. 2015). Non-parasitic species

also have less well-developed dentition than their parasitic relative (Hardisty and Potter 1971; Vladykov and Kott, 1979; Potter et al. 2015). However, it is not known if the presence or absence of a blood diet in silver and northern brook lamprey are reflected by functional differences in the gills, particularly in the processes involved in nitrogenous waste (N-waste) excretion and ionoregulation. The over-arching goal of the following study was to compare the distribution of urea and ammonia transporters, along with the ionoregulatory and acid-base excretion machinery, in the gills of parasitic juvenile silver lamprey to their non-parasitic counterpart, the northern brook lamprey.

The sea lamprey, which has a parasitic juvenile phase in marine environments, served as an outgroup for my comparisons. The sea lamprey increases its capacity to produce and excrete ammonia following metamorphosis as demonstrated by marked increases in basal and postprandial ammonia and urea excretion (Wilkie et al. 2004, 2006), and by the pronounced re-distribution of Rhesus c-like glycoprotein (Rhcg-like) in the gills after metamorphosis (Chapter 2). These changes include the basolateral localization of Rhcg-like proteins within the seawater-type mitochondrion-rich (SW MR) cells in the interlamellar regions of the gills. Given the exclusively freshwater (FW) nature of these 'paired' species, I expected that the distribution of Rhcg-like proteins and urea transport proteins (UT) would differ from juvenile sea lamprey. Further, I expected the abundance and distribution of acid-excreting H^+-ATPases (V-ATPase) and NKA to differ from their anadromous, parasitic counterpart. To test these hypotheses, gills were collected from juvenile silver and northern brook lamprey that had recently completed metamorphosis and were fixed and processed for immunohistochemical examination. Branchial Rhcg-like protein, NKA, V-ATPase and UT distribution were examined, to determine if the

presence or absence of a juvenile feeding stage affected the distribution of these transport proteins.

Material and Methods

Experimental Animals and Holding

Northern brook and silver lamprey were collected in the summer of 2015, using pulsed DC electrofishing, by Department of Fisheries and Oceans Canada (DFO) personnel from Coldwater Creek, Hog Creek and Mad River near Simcoe County, Ontario, Canada. Lamprey were then transported to the Animal Care Facility at Wilfrid Laurier University in Waterloo in September 2015, where they were housed in 30 L aquaria, filled with aerated well water (pH ~8.1, alkalinity ~250mg/L $CaCO_3$), and immersed in a water bath to maintain temperature at 17-18˚C under a 12:12-hour light-dark cycle. The sampling and holding protocols were all approved by the Wilfrid Laurier Animal Care Committee and followed Canadian Council of Animal Care (CCAC) guidelines.

Experimental Protocols

After approximately 2 weeks in the laboratory, the juvenile northern brook (N = 8) and silver lamprey (N = 4) were euthanized with an overdose of anesthetic (MS-222) at $1.5g \cdot L^{-1}$ buffered with $3.0g \cdot L^{-1}$ $NaHCO_3$ (Birceanu et al. 2009). Tissues including the liver, brain, and muscle were collected for a related study on protein catabolism and N-waste production. Gills were collected by making cross sections (~4mm wide) through the branchial (gill) region, which were immediately fixed in paraformaldehyde (10% phosphate-buffered saline PBS, pH 7.4), and stored in 20 mL glass scintillation vials at 4˚C for approximately 24 hours. The slices were then

rinsed twice and stored in 70% ethanol at 4˚C, before being processed for embedding using Citadel 1000 tissue processor on a 16-hour dehydration and infiltration cycle. This process included two changes of 70% ethanol, one change of 95% ethanol, three changes of 100% ethanol, and three changes of xylene to dehydrate the tissues, followed by two changes in paraffin wax to infiltrate the tissues. Sections were then embedded in paraffin blocks prior to sectioning. Ammocoete and juvenile sea lamprey (*Petromyzon marinus*), held in fresh water (Chapter 2), were sampled in December 2017 and processed as outlined above for comparison to the northern brook and silver lamprey.

Analytical Methods

Slices of embedded gill tissue were sectioned into 5μm sections on a microtome, placed on aminopropyltriethoxysilane (APS) coated slides and dried at room temperature. All tissues were processed following the protocol outlined by Wilson et al. (2007). Briefly, slides were dewaxed in an oven at 60˚C for 20 minutes, followed by three changes of xylene and three changes of 100% ethanol, each for five minutes. A hydrophobic circle (Super PAP pen, Sigma-Aldrich, St. Louis, Missouri, USA) was drawn around each section before being rinsed with de-ionized (DI) water, then rinsed with 1% sodium dodecyl sulfate (SDS) in PBS, pH 7.3 (Brown et al. 1996) for five minutes to unmask antibody epitopes made unavailable by the fixation process (Shi et al. 2007). The SDS was rinsed away with DI water before the slides were treated with PBS with 0.05% TWEEN 20 (TPBS) for five minutes. A fluorescent blocker (BLØK, MilliporeSigma, Burlington, Massachusetts, USA) was applied within each hydrophobic circle and left at room temperature for 20 minutes.

Various antibodies were used to examine each protein under study. Rhesus c-like glycoproteins (Rhcg-like) were visualized using a polyclonal rabbit antibody raised against *Takifugu rubripes* Rhcg1 (Nakada et al., 2007; provided courtesy of Professor S. Hirose, Tokyo Institute of Technology, Japan) and applied at a concentration of 1:200 in BLØK solution. Immunolocalization of V-type H^+-ATPase was carried out using an antibody to the B subunit of the protein (Wilson et al. 2007) at a dilution of 1:200 in BLØK. Urea transporter (UT) detection was done using a polyclonal rabbit antibody developed against the *Danio rerio* UT (Braun et al. 2009; provided courtesy of Dr. S.F. Perry, U. of Ottawa, Ontario, Canada) applied at a concentration of 1:200 in BLØK. Finally, a polyclonal mouse antibody for Na^+/K^+-ATPase (α5-subunit) was used with all antibodies to examine the distribution of Na^+/K^+-ATPase (NKA) at a concentration of 1:100. The α5 was deposited to the DSHB by Fambrough, D.M. (Developmental Studies Hybridoma Product α5). Alexa Fluor 488 anti-rabbit and 555 anti-mouse (Thermo Fischer Scientific, Waltham, Massachusetts, USA) were used as secondary antibodies, at a concentration of 1:500 in BLØK and incubated for one hour at 37˚C. The stain, 4',6-diamidino-2-phenylindole (DAPI) was applied in TPBS for 10 minutes to detect cell nuclei within the sectioned gill tissue. Negative control was applied to one section on each slide by applying BLØK in place of primary antibodies, and repeating all other steps as described above.

Sequential labelling was then performed on a selected subset of tissues, to determine if any colocalization occurred between V-ATPase and Rhcg-like protein in the branchial epithelium of lampreys. Sections for sequential labelling were processed and mounted onto APS coated slides as described above. Initial rinsing, unmasking, and blocking steps were also performed as for other slides in this study. V-ATPase (Wilson et al. 2007) primary antibody was

applied to half of the slides tested, and *Takifugu rubripes* Rhcg1 (Nakada et al., 2007; provided courtesy of Professor S. Hirose, Tokyo Institute of Technology, Japan) applied to the other half, and incubated overnight at 4˚C. The following morning, sections were rinsed in TPBS, then Alexa Fluor 555 anti-mouse diluted 1:500 (Thermo Fischer Scientific, Waltham, Massachusetts, USA), and a conjugated Fab fragment of goat anti-rabbit IgG (H +L) CL647 diluted 1:500 (Thermo Fischer Scientific, Waltham, Massachusetts, USA) secondary antibodies were applied and incubated for 1 hour at 37˚C. This was followed by another rinse series in TPBS before slides were blocked for 1 hour with goat rabbit Fab fragment diluted 1:100 (Jackson ImmunoResesarch Inc. West Grove, Pennsylvania, USA) in BLØK solution. Slides were rinsed again in TPBS and primary antibodies were applied. On slides initially incubated for V-ATPase B2, one section per slide received the *Takifugu rubripes* Rhcg1 antibody, and vice-versa for those initially incubated with *Takifugu rubripes* Rhcg1 primary antibody. The slides were again left to incubate overnight and rinsed the following morning in TPBS. Alexa Fluor 488 anti-rabbit secondary antibody (Thermo Fischer Scientific, Waltham, Massachusetts, USA) diluted 1:500 was applied for 1 hour at 37˚C. The slides were finally rinsed in TPBS and stained with DAPI as described previously.

All stained sections were analyzed for Rhcg-like protein, NKA, V-ATPase, and UTs based on the detection of fluoresecent antibodies bound to the tissues. Photos were captured with a Leica DM5500 fluorescence microscope using a Hamamatsu C11440 ORCA-Flash 4.0 digital camera and analyzed in Leica Application Suite X (LASX) for analysis (Leica Microsystems 2016, Wetzlar, Germany). Figures were compiled using GIMP GNU Image Manipulation Program (2.8.22; 2017).

Results

Gross Features

The juvenile northern brook lamprey used in this study averaged 4.67 ± 44 g and 126.95 ± 16.9 mm while silver lamprey weighed 5.21 ± 0.83 g and were 143.63 ± 7.59 mm, showing no significant difference in size between the two species ($p>0.05$). Northern brook lamprey were distinguished from silver lamprey primarily based on the size of the oral disc relative to the head. Those with an oral disc diameter greater than the maximum head width were deduced to be silver lamprey while those with a diameter smaller than the head were northern brook lamprey (Figure 3.1; Scott and Crossman 1973; Potter et al. 2015). As noted previously, there was also a much greater degree of dentition development, characterized by numerous sharp teeth and a more distinct rasping tongue, in the parasitic silver lamprey compared to the more degenerate dentition of the northern brook lamprey (Figure 3.1). Juvenile silver lamprey also appeared to have more speckling in their dorsal region compared to the northern brook lamprey (Potter et al. 2015).

Differences in N-waste Transporter Distribution in the Gills of Northern Brook Lamprey and Silver Lamprey Compared to Sea Lamprey

Comparisons to sea lamprey juvenile and ammocoetes (Chapter 2) are included in all figures for comparative purposes. Urea transporter (UT) showed concentrations of staining on both the lamellae and within the interlamellar regions on the gill filaments (Fig. 3.2). The signal for UT was both stronger and more abundant in the silver lamprey (Fig. 3.2B) than in the northern brook lamprey (Fig. 3.2A). While the UT was widely distributed on the lamellae of

larval sea lamprey (Fig. 3.2C), it was completely absent from the gills of juvenile sea lamprey (Fig. 3.2D).

The distribution of Rhesus c-like glycoproteins (Rhcg-like) differed notably between the northern brook and silver lamprey. Rhcg-like protein was localized primarily on the lamellae, with minimal presence within the interlamellar regions in the northern brook lamprey (Fig. 3.3A). In contrast, Rhcg-like protein was primarily present within the interlamellar regions of the filament of silver lamprey (Fig. 3.3B). In both cases, Rhcg-like protein was sparse in its distribution, and localized to the apical membrane of the cells in which it was found (Fig. 3.3A inset). The distribution and abundance of Rhcg-like protein was markedly different in sea lamprey. In larval sea lamprey, Rhcg-like protein was distributed broadly in the lamellae and within the interlamellar regions of the gill epithelium (Fig. 3.3C). Following metamorphosis, Rhcg-like protein was concentrated, with a high abundance and uniform distribution within cells found in the interlamellar regions of the gills (Fig. 3.3D). These cells corresponded to seawater-type mitochondrion-rich cells (SW MRCs), which have abundant NKA in the juvenile sea lamprey (Figure 3.4D).

Relative Abundance of NKA Transporters in Freshwater and Anadromous Lamprey Species

In northern brook lamprey NKA was mainly found on the lamellae but the signal was relatively weak along with some NKA detected in the interlamellar regions (Fig. 3.4A). In silver lamprey, NKA was consistently localized in the interlamellar regions of the gill filaments, with a stronger signal, observed in the same regions, than in the northern brook lamprey (Fig. 3.4B). The distribution of NKA of northern brook lamprey was similar in signal strength and

distribution to that of larval sea lamprey (Fig. 3.4C). These observations were in stark-contrast to the juvenile sea lamprey, in which NKA was highly concentrated in SW MRCs of the interlamellar region (Figure 3.4D).

Relative Distribution of Rhcg-like Protein and NKA in the Gills

The pattern of Rhcg-like protein and NKA distribution markedly differed between northern brook, silver, and sea lamprey. In northern brook lamprey, Rhcg-like protein and NKA were located in separate cells, both sparsely distributed along the lamellae and within the interlamellar spaces on the gill epithelium (Fig. 3.5A). While Rhcg-like protein had broad lamellar and interlamellar distribution in larval sea lamprey, the lack of NKA on the gills suggests that Rhcg-like protein distribution was independent from NKA (Fig. 3.5C). In contrast, Rhcg-like protein and NKA were colocalized with one another in the interlamellar spaces of the gill filament in the juvenile silver lamprey and juvenile sea lamprey (Fig. 3.5B, D). However, unlike in the juvenile sea lamprey, the staining for NKA and Rhcg-like protein were distinct from one another, and did not overlap. From this observation and the complete overlap of Rhcg-like protein with V-ATPase (Fig. 3.6C,D), it was deduced that the Rhcg-like protein in the juvenile silver lamprey had an apical distribution, rather than the basolateral distribution described for juvenile sea lamprey (Fig. 3.5D; see Chapter 2 for further details).

Relative Distribution and Abundance of V-ATPase

Vacuolar-type H^+-ATPase (V-ATPase) distributed on the gill lamellae and interlamellar regions of northern brook and silver lamprey (Fig. 3.6). The interlamellar distribution of V-ATPase within the silver lamprey appeared similar to that of Rhcg-like protein and NKA in the

same species (Fig. 3.6B). Similarly, V-ATPase was widely distributed throughout the gills of larval sea lamprey (Fig. 3.6C) but was virtually absent in the gills of the juvenile sea lamprey (Fig. 3.6D). Further investigation showed a distinct colocalization between Rhcg-like protein and V-ATPase in both the northern brook and silver lamprey (Fig. 3.7A-D), a result not found in the juvenile sea lamprey (Fig. 3.7G-H). As noted above, Rhcg-like protein and V-ATPase also completely overlapped in some cases in the northern and silver lamprey, which was consistent with an apical location or each transporter. The sea lamprey ammocoete did show some association between V-ATPase and Rhcg-like protein, however the proteins appear to localize in different parts of the cell, or within different cells that are closely associated with each other (Fig. 3.7.E to F).

Figures

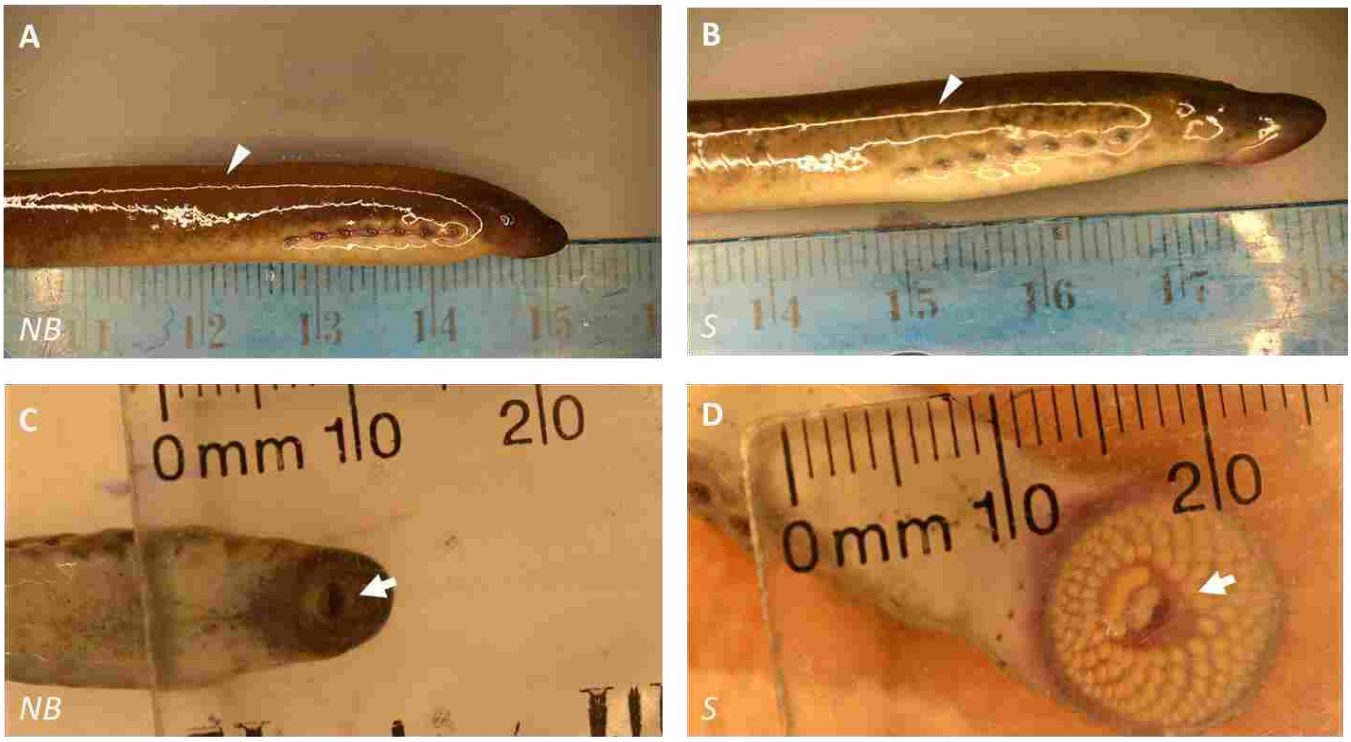

Figure 3.1. Representative photographs demonstrating the lateral and ventral views of northern brook (A, C) and silver (B, D) lamprey and the key characteristics used to differentiate the two species. Arrowheads show differences in pigmentation (A-B) as the silver lamprey show some speckling and no visible myomere indentations. White arrows show the difference in the development of dentition (C-D), with much more developed dentition and a larger oral disc compared to head size in the silver lamprey compared to the northern brook lamprey.

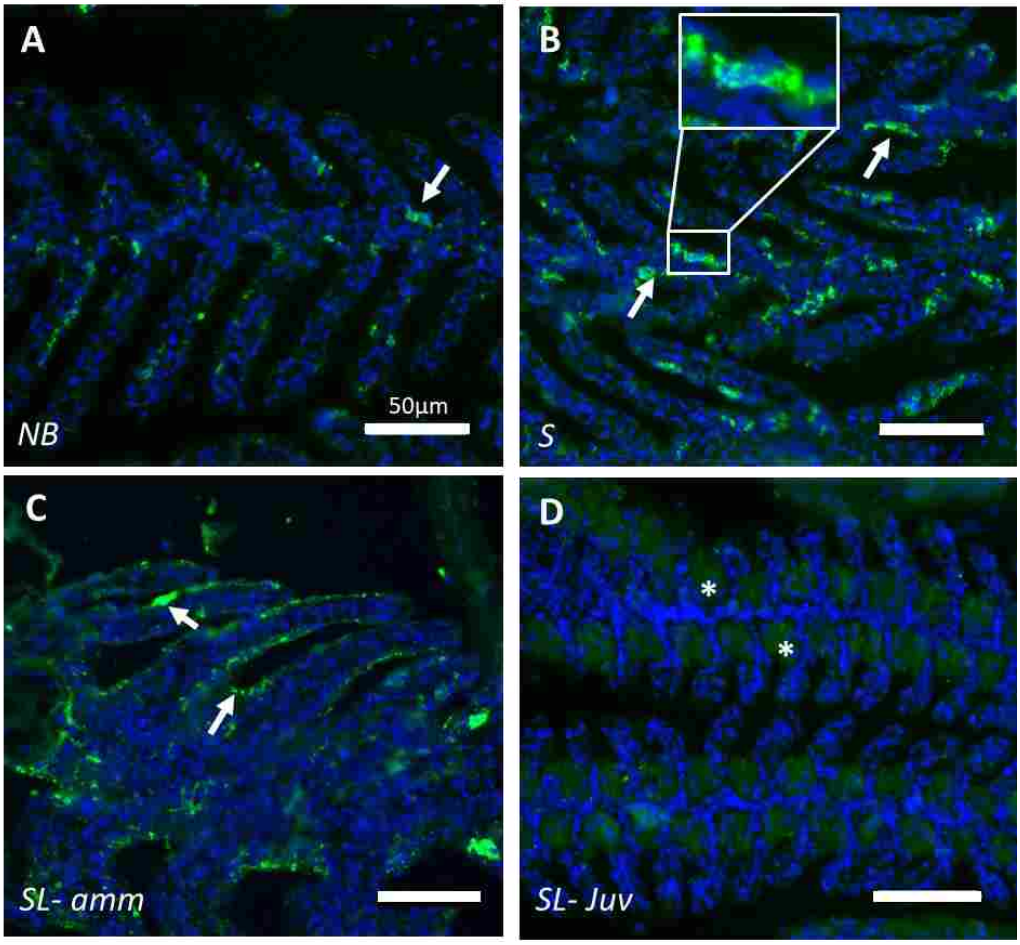

Figure 3.2. Distribution of UT (green) in gill filaments doubly-labelled with DAPI showing cell nuclei (blue) in A) juvenile northern brook lamprey (NB), B) juvenile silver lamprey (S), C) larval sea lamprey (SL-amm), and D) juvenile sea lamprey (SL-Juv). Note the distribution of UT on the lamellae (arrows) and interlamellar spaces of the gill filament of the sea lamprey ammocoete, the northern brook and silver lamprey. In contrast, UT was not detected in the gills of juvenile sea lamprey. Asterisk (*) denotes the location of seawater-type mitochondrion-rich cells (SW MRC). Scale bar = 50 µm.

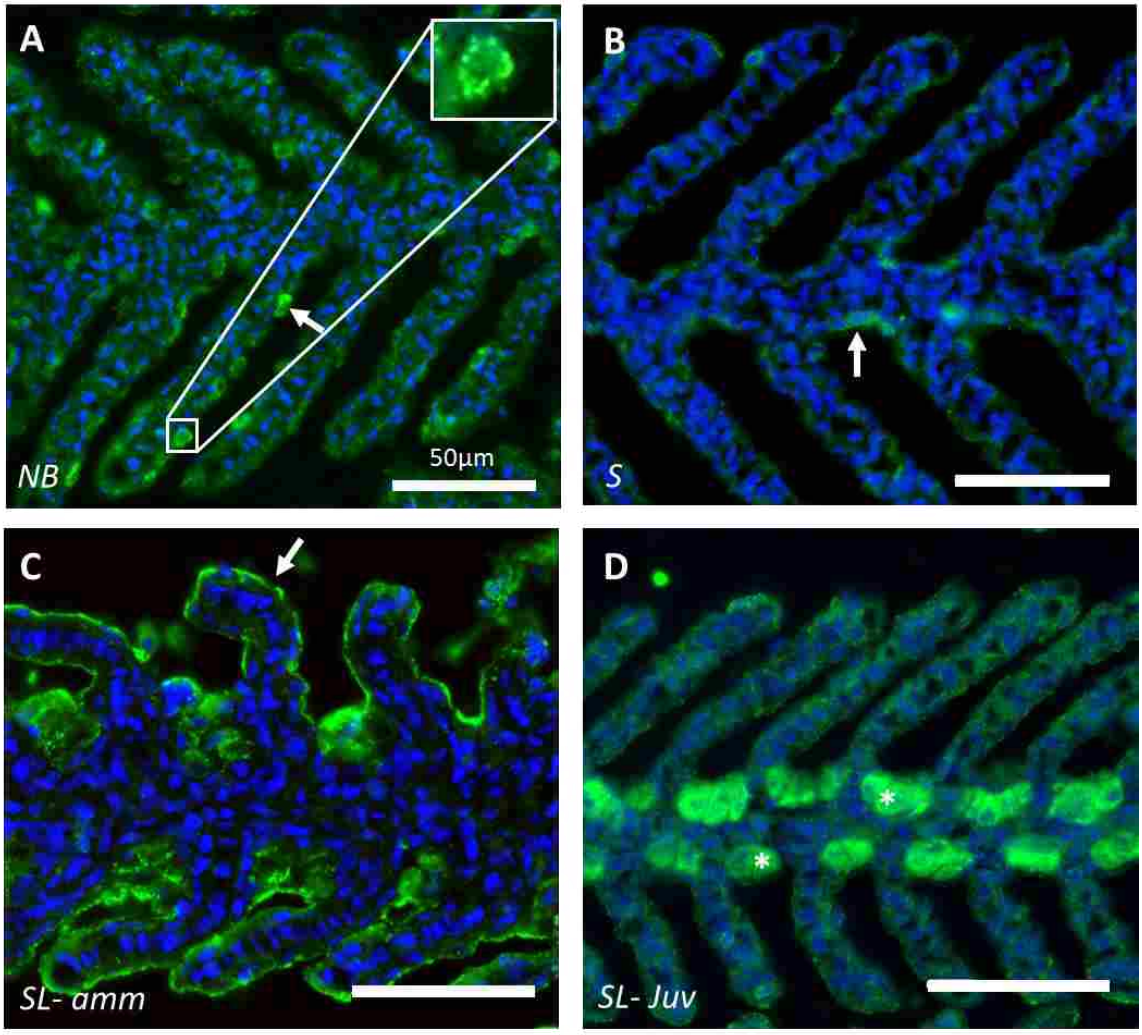

Figure 3.3. Immunohistochemical staining of Rhcg-like protein expression (green) doubly-labelled with DAPI (blue) to stain cell nuclei in the gill of A) juvenile northern brook lamprey (NB), B) juvenile silver lamprey (S), C) larval sea lamprey (SL-amm), D) juvenile sea lamprey (SL-Juv). In northern brook lamprey, Rhcg-like protein was distributed in specific cells on the lamellae but, in the closely related juvenile silver lamprey, was restricted to the interlamellar. Scale bar = 50 µm.

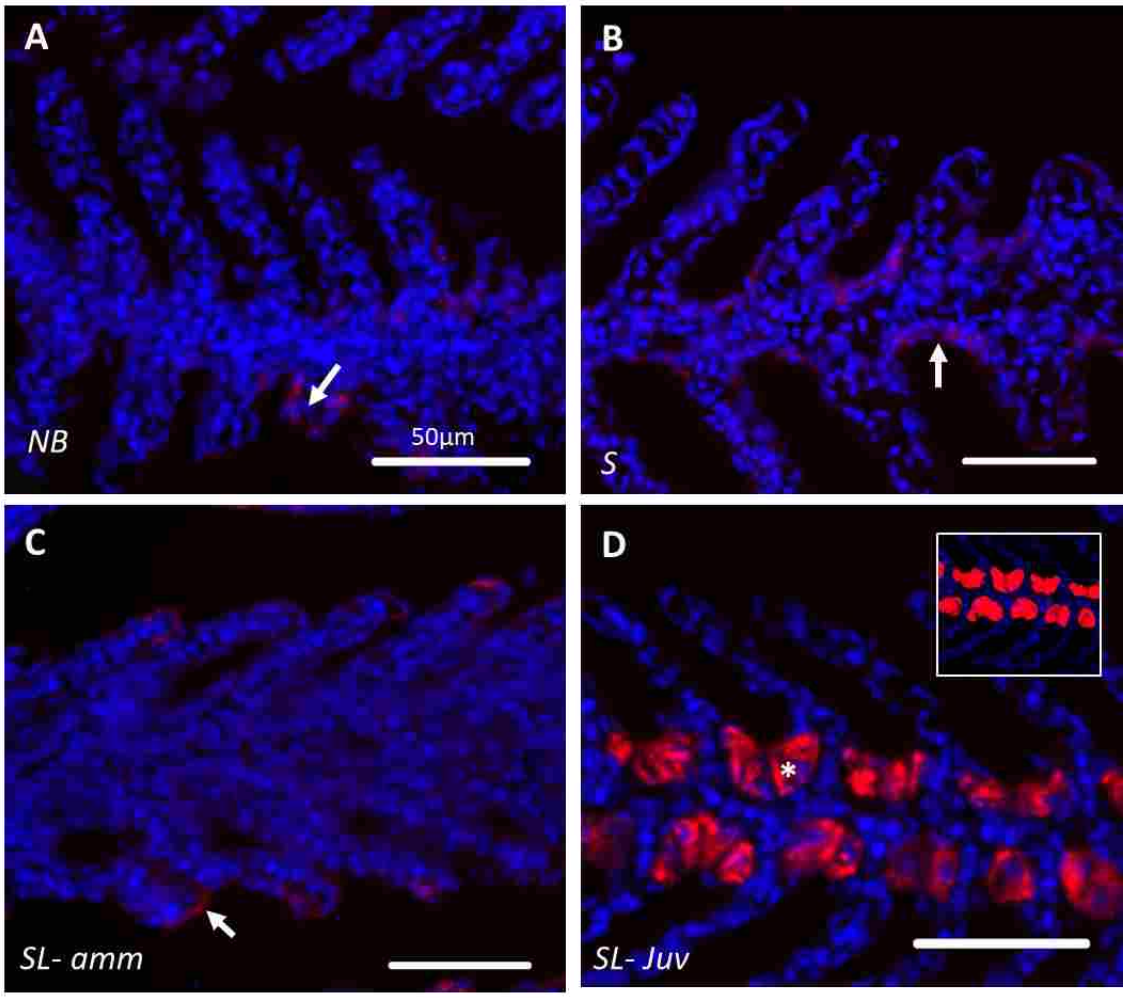

Figure 3.4. Immunohistochemical staining of Na^+/K^+-ATPase (NKA; red) double labelled with DAPI (blue) to stain cell nuclei in the gills of A) juvenile northern brook lamprey (NB), B) juvenile silver lamprey (S), C) sea lamprey ammocoete (SL-amm), D) juvenile sea lamprey (SL-Juv). In the non-parasitic northern brook, and larval sea lamprey, NKA localizes largely on the lamellae. In contrast, in the parasitic silver and juvenile sea lamprey, NKA appears in the interlamellar regions, specifically within the seawater-type mitochondrion-rich cells (SW MRC; Asterisk; *) in the juvenile sea lamprey. Scale bar = 50 µm. Capture settings for panel D are adjusted to show detail, inset shows intensity of NKA localization at capture settings identical to panels A-C.

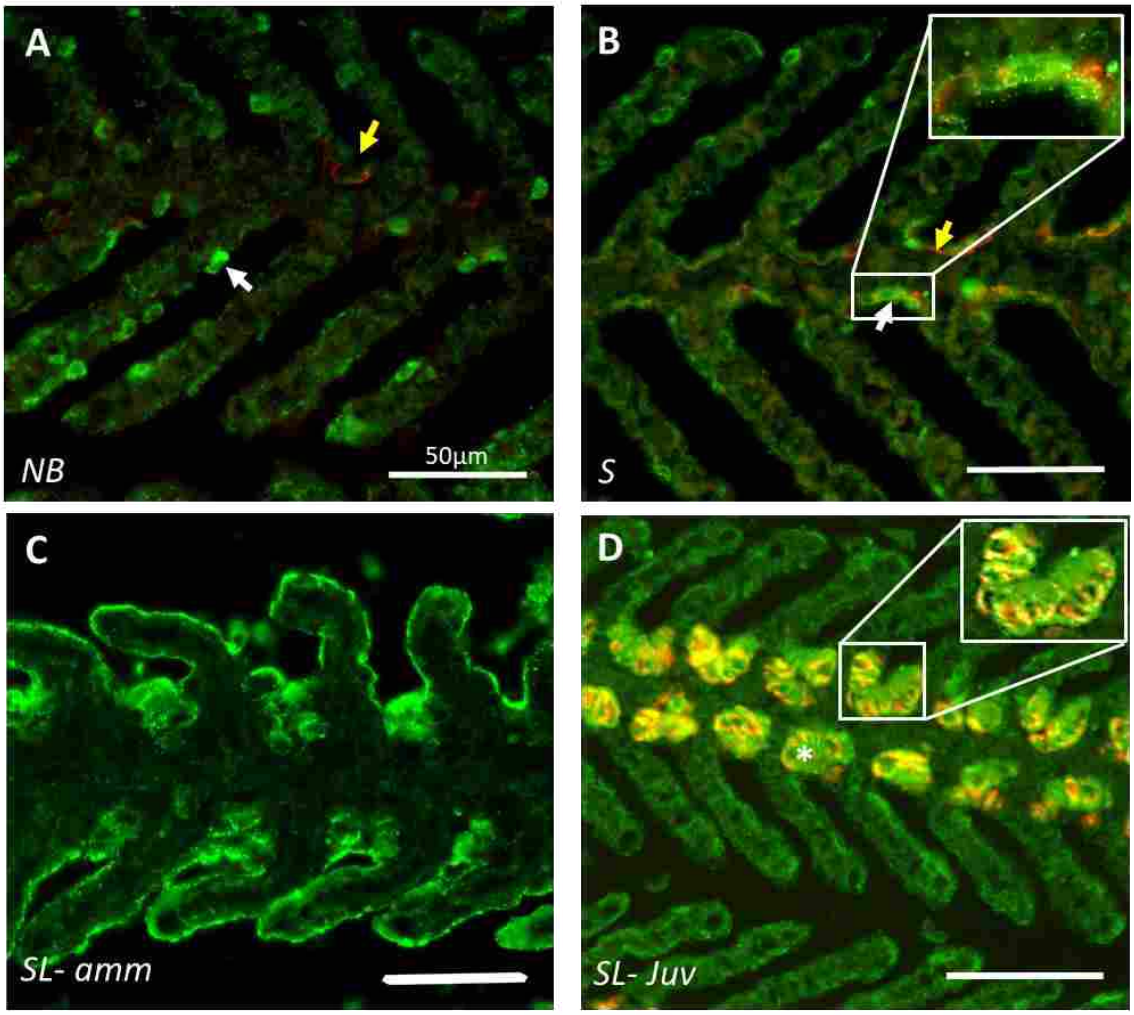

Figure 3.5. Double labelling immunohistochemistry of Rhcg-like protein and NKA in the gills of A) juvenile northern brook lamprey (NB), B) juvenile silver lamprey (S), C) larval sea lamprey (SL-amm), and D) juvenile sea lamprey (SL-Juv). Note the separate localization of Rhcg-like protein (white arrow) and NKA (yellow arrow) in the northern brook lamprey in contrast to the colocalization in the interlamellar region of the silver lamprey (B inset). Scale bar = 50 µm.

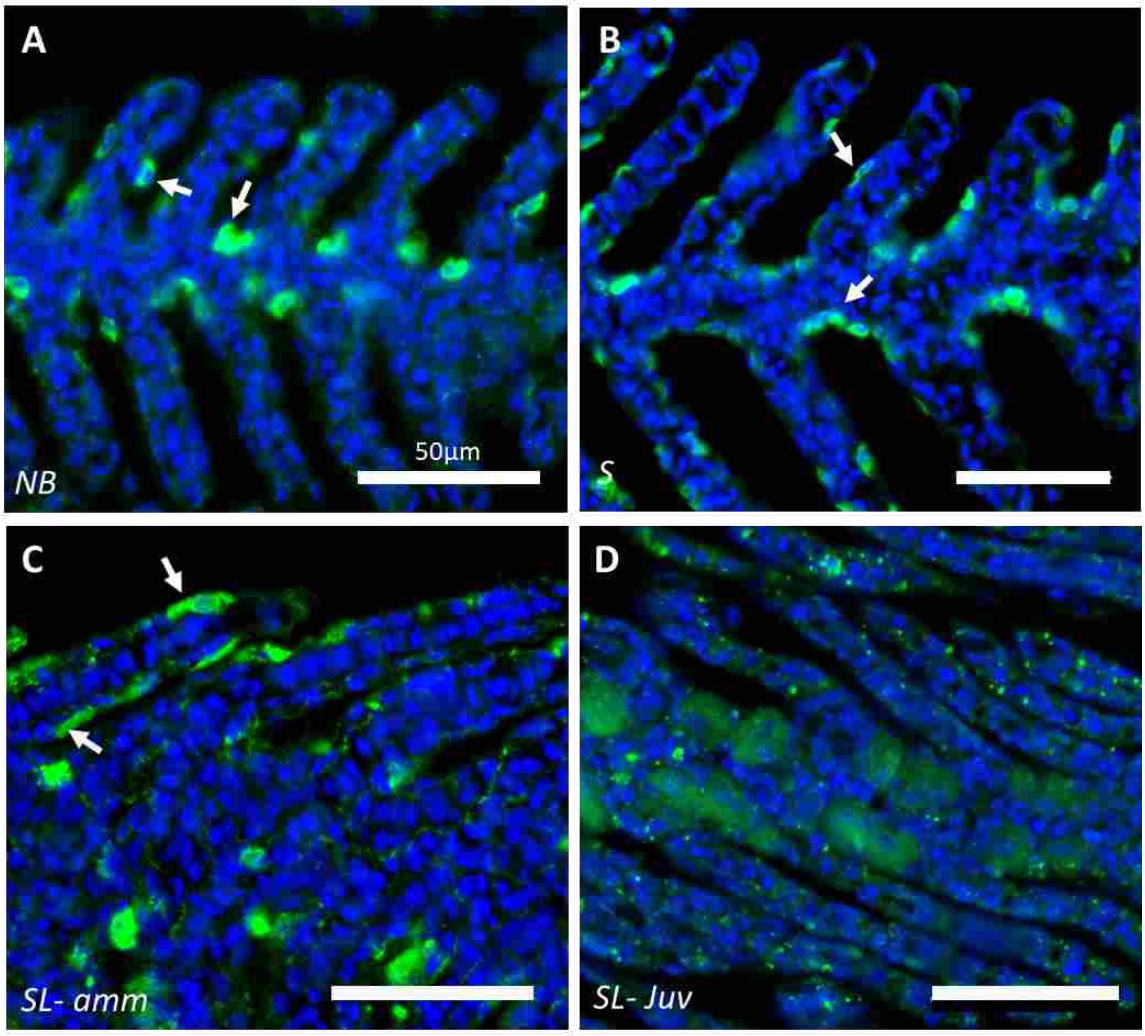

Figure 3.6. Immunohistochemical staining of V-type H^+-ATPase (V-ATPase; green) doubly-labelled with DAPI (blue) to stain cell nuclei in the gills of A) juvenile northern brook lamprey (NB), B) juvenile silver lamprey (S), C) larval sea lamprey (SL-amm), and D) juvenile sea lamprey (SL-Juv). Note the whole cell staining distribution on the lamellae in all examples except for the juvenile sea lamprey. Scale bar = 50 µm.

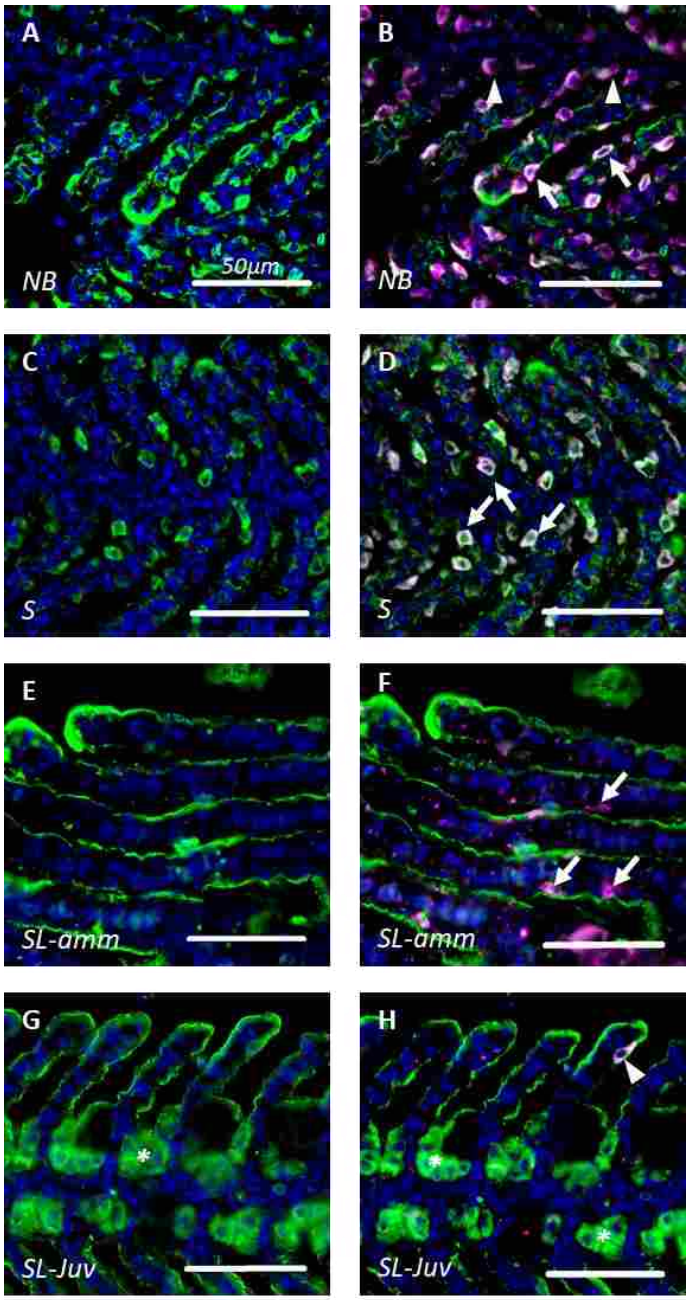

Figure 3.7. Sequential labelling of Rhcg-like protein (green) with V-ATPase (pink) shown with DAPI labelling (blue) to stain cell nuclei in northern brook lamprey (A-B), silver lamprey (C-D), sea lamprey ammocoetes (E-F) and juvenile sea lamprey (G-H). Left hand figures are shown without V-ATPase labelling. Arrows indicate locations where V-ATPase and Rhcg-like protein colocalize (appears more white) and arrowheads indicate areas where V-ATPase does not appear to be associated with Rhcg-like protein. Scale bar = 50 µm.

Discussion

Functional Differences in Gill Structure and Function in Parasitic and Non-parasitic Lamprey

Given the marked differences between larval and juvenile sea lamprey in the distribution of N-waste associated transporters in the gills (Chapter 2) and the pronounced differences in N-waste excretion rates following feeding, I predicted that there would be significant differences in the patterns of UT, Rhcg-like protein, Na^+/K^+-ATPase (NKA) and V-type H^+-ATPase (V-ATPase) between the gills of the non-parasitic northern brook lamprey and the parasitic silver lamprey. These 'paired' lamprey species, virtually indistinguishable from one another as ammocoetes, have starkly different life styles following metamorphosis, as characterized by a total absence of feeding in northern brook lamprey and a parasitic phase in the silver lamprey (Renaud 2011; Docker et al. 2012). The different life styles were clearly reflected in the gross anatomy of the northern brook and silver lamprey species. The larger oral disk and well-developed dentition of the silver lamprey are better suited to attaching to and parasitizing fishes (Renaud et al., 2009). Although no differences in body size were observed here, silver lamprey would likely grow much larger than the non-feeding northern brook lamprey (Scott and Crossman 1973).

These differences in external morphology were also accompanied by changes in gill physiology which may facilitate differences in nitrogenous waste (N-waste) excretion between parasitic and non-parasitic species. Increased feeding rates and increased relative protein content of meals increases N-waste excretion rates in many fish species and are thus expected to do the same for these species (Boucher-Rodoni and Mangold 1985; Ramnarine et al. 1987; García et al.

2011; Wu and Gatlin III 2014). Similar, but greater increases in N-waste excretion following feeding have also been observed in juvenile, parasitic sea lamprey but comparisons to other lamprey species have not yet been made (Wilkie et al. 1999; 2004; 2006). My results suggest that differences in the distribution of various N-waste excretory proteins, could be related to differences in N-waste excretion rates due to the absence of a feeding phase in the northern brook lamprey. A study of N-waste excretion rates in juvenile northern brook and silver lamprey would help to validate this hypothesis.

Urea Transporter Distribution

Urea transporters (UT) were more abundant in the juvenile northern brook and silver lampreys than in the juvenile sea lamprey. The abundance appears similar to that of the larval sea lamprey, suggesting that urea excretion across the gills may have a larger role in N-waste excretion of these species than in the sea lamprey (Neal 2013). The results also suggest that UT was present in greater amounts on the gills of silver lamprey than in northern brook lamprey. This is likely a reflection of the demand for silver lamprey to unload urea due to its parasitic life style, created by the ingestion of proteins from host fishes and the hydrolysis of dietary arginine to urea (Wilkie et al. 2006). These differences would need to be confirmed with western blotting to properly quantify the amount of UT within these closely related species, as well as excretion experiments to relate urea excretion rates (J_{urea}) to the relative expression of UT described here. These differences between parasitic and non-parasitic species in UT distribution suggest perhaps J_{urea} and its excretion mechanism are influenced by the dietary patterns of lamprey. Further, the increased UT expression in silver lamprey over the juvenile sea lamprey suggests that branchial

urea excretion is of increased importance in freshwater (FW) environments as discussed in Chapter 2.

Rhesus Glycoprotein Distribution and Association with NKA

It has been suggested that fish living in different salinities employ different strategies for N-waste excretion (Evans et al. 2005; Weihrauch et al. 2009). The salinity of the external environment can even have dramatic effects on osmoregulatory and N-waste excretion mechanisms within life stages of a single individual as they prepare to transition between fresh water (FW) and sea water (SW)(Chapter 2). Briefly, ionoregulation in SW results in fish needing to take up water while excreting ions to prevent dehydration. To achieve this, different transporters are upregulated (e.g. NKA) in SW while others are downregulated (e.g. V-ATPase) within the gills of fish (Evans et al. 2005; Reis-Santos et al. 2008; Zydlewski and Wilkie, 2013). In this study, I expected that NKA expression would be lower in the FW species examined here than in the sea lamprey, while V-ATPase expression would be higher. This is due to the increased role of NKA in SW environments to assist in the excretion of Na^+ ions, and the importance of V-ATPase for excreting protons in FW environments to create an electrochemical gradient for facilitated Na^+ uptake via an apical channel (Marshall 2002; Evans et al. 2005).

The signal strength of NKA in the gills of northern brook and silver lamprey was similar to that of the larval sea lamprey, but slightly more abundant in the gills of the silver lamprey (Fig. 3.4). Using identical capture settings, NKA was oversaturated in the juvenile sea lamprey (Chapter 2; Fig. 3.4D), localizing specifically within the seawater-type mitochondrion-rich cells (SW MRCs; Fig. 3.4D inset). SW MRCs are also absent from sea lamprey ammocoetes and FW

lamprey species (Bartels et al. 1998; 2015; 2017; Reis-Santos et al. 2008). Not only are these SW MRCs important for ionoregulation in SW largely via NKA, they also appear to be essential in the excretion of ammonia across the gills of sea lamprey in SW environments (Chapter 2). The action of NKA in the SW MRC maintains a favourable gradient for the exchange of Na^+ ions for H^+ across the apical epithelium. As NH_4^+ is thought to substitute for H^+ on this transporter, the action of NKA may also facilitate the exchange of Na^+ for NH_4^+ across the apical epithelium of the gill (see Chapter 2; Weihrauch et al. 2009). Thus, the basolateral location of the Rhcg-like protein in the juvenile sea lamprey could be the first step in the excretion of NH_4^+ via the NHE in a future salt water environment.

In fresh water, such a set-up may not work as the inwardly directed Na^+ electrochemical required to drive the NHE would be absent (Wilkie 1997). This may explain the different distribution of Rhcg-like protein between the parasitic and non-parasitic FW lamprey species studied here. In the non-parasitic northern brook lamprey, Rhcg-like protein appears on the lamellae, but in the interlamellar regions of the parasitic silver lamprey. Given the sparse Rhcg-like protein distribution of the northern brook lamprey, similar to the larval sea lamprey, and its exclusively FW lifestyle, it is probable that the cells in which Rhcg-like protein are found are intercalated mitochondrion-rich cells (IMRC; Bartels and Potter 2004). Bartels and Potter (2004) report that these IMRCs are found between pavement cells in the FW stages of the sea lamprey life cycle, thus it is reasonable to assume that these cells would also be present in exclusively FW lamprey species. In the parasitic morph, the juvenile silver lamprey, the interlamellar distribution of the Rhcg-like protein as more similar to that of the juvenile sea lamprey, but less pronounced. This could be a reflection of a prolonged ancestry in FW as compared to the anadromous sea

lamprey. Thus, it could be argued that the localization of Rhcg-like protein in the silver lamprey is intermediate between that of its northern brook lamprey cousin, and the anadromous sea lamprey.

Meanwhile, in both the parasitic silver lamprey and the juvenile sea lamprey, Rhcg-like protein localizes in the interlamellar regions of the gills, colocalizing with NKA in the region. In sea lamprey, these are the SW MRC, essential for both ionoregulation and N-waste excretion in SW (Chapter 2). As NKA is known to be basolateral within epithelial cells, Rhcg-like protein also appears basolateral in the SW MRC of sea lamprey (Reis-Santos et al. 2008). These cells are absent in the FW silver lamprey but the association between Rhcg-like protein and NKA is still observed. The association is different however, as Rhcg-like protein appears to localize apically in the silver lamprey basolaterally with as in the juvenile sea lamprey. This is further evidence that the arrangement of Rhcg-like protein is intermediate between the closely related northern brookk lamprey, and the more distant sea lamprey.

V-ATPase in Freshwater and Saltwater Species of Lamprey

V-ATPase, or H^+-ATPase, is a proton pump that contributes to ionoregulation and acid-base regulation in freshwater fishes (Evans et al. 2005). In FW environments, V-ATPase pumps H^+ ions across the gills into the bulk water, generating a transmembrane potential allowing the uptake of essential Na^+ into the organism (Marshall 2002; Evans et al. 2005). Given this role for V-ATPase, it is also expected to decrease in environments where external Na^+ concentrations are high (ie. SW; Reis-Santos et al. 2008). The V-ATPase on the gills of larval sea lamprey, juvenile northern brook, and silver lamprey was similarly distributed on the gill lamellae and in the

interlamellar regions. It was virtually absent, however, from the gills of juvenile sea lamprey. This is likely due to the replacement of V-ATPase by Na^+/H^+-exchanger (NHE) in SW, as Na^+ is now directed inwardly by the Na^+ gradient, rendering a V-ATPase unnecessary for proton excretion (Marshall 2002; Evans et al. 2005).

In addition to its ionoregulatory role, V-ATPase is also thought to play an important role in ammonia excretion (Weihrauch et al. 2009; Wright and Wood 2009). The colocalization of V-ATPase with Rhcg-like proteins in the interlamellar regions of the silver lamprey, and on the lamellae of northern brook lamprey (Fig. 3.7A to D) suggests that V-ATPase is involved in ammonia excretion in freshwater lampreys. Similar to the model proposed by Wright and Wood (2009), ammonia excretion by larval sea lamprey, northern brook and silver lamprey likely involves coupling of V-ATPase proton (H^+) pumping across the apical membrane, which traps NH_3 as NH_4^+ in the gills' external boundary layers to sustain the outward NH_3 diffusion gradient. It is likely that proton pumping also helps to sustain the inwardly directed electrochemical gradient for Na^+ (discussed above) which likely enters via epithelial Na^+ channels (ENaC) which have not yet been characterized in lamprey. Furthermore, the near absence of V-ATPase in the juvenile sea lamprey, and its lack of colocalization with Rhcg-like protein further supports the hypothesis that V-ATPase is not involved in the mechanism of ammonia excretion in SW (Weihrauch et al. 2009).

To complete the investigation of the role of external salinity on N-waste excretion mechanisms, it would be beneficial to apply the techniques in this study to the landlocked sea lamprey of the Great Lakes, which have been isolated from their anadromous population only

recently in evolutionary terms. This could inform at which point the SW MRCs, essential in SW environments, may be lost in exclusively FW populations and species. To this effect, it would also be of interest to investigate the regulation and expression of related genes in these species throughout metamorphosis and salinity acclimations. This would provide insight both into the conservation of the related genes, and their expression in different environments and life styles.

Perspectives

Closely related 'paired' lamprey species provide a unique opportunity to explore differences in N-waste excretion mechanisms between groups where the primary difference is the presence of parasitism in the life cycle. My results suggest that although parasitism may have an effect on N-waste excretion mechanisms, salinity of the external environment may be the dominant factor in determining the distribution of transporter proteins within the gills. Whether this is an evolutionary effect remains to be determined. If the northern brook and silver lamprey diverged from their common parasitic ancestor more recently than from an anadromous ancestor, this could cause the differences due to salinity to be more prominent than those due to parasitism. Perhaps these species have had enough time to diverge from their anadromous ancestor, and to adapt their N-waste and ionoregulatory mechanisms to an exclusively freshwater life style. Another possibility however, is that the anadromous life style is simply a stronger influencer of N-waste excretion mechanism than parasitism. Additionally, the possibility that these are two ecotypes of the same species cannot be ignored, and thus there may be genetic limitations to the differences in N-waste excretion mechanisms between the parasitic and non-parasitic ecotypes. Further work is needed to quantify the results reported here via genetic or

western blotting techniques. These results would also need to be compared with excretion rates to relate structural differences to the function of these branchial transporters. These results, and the continued study of this topic will provide insight into both the evolutionary radiation of lamprey species, and the evolution of N-waste excretion in different environments.

Chapter 4

General Discussion and Integration

Introduction

Lamprey (*Petromyzontiformes*) live a very complex life cycle, metamorphosing from detritus, filter-feeding ammocoetes into free-swimming, juveniles before maturing into non-feeding adults (Youson 1980; Potter and Gill 2003). The progression of metamorphosis differs between species with some consisting of a parasitic juvenile phase while others proceed directly to the non-feeding adults (Potter 1980; Potter and Gill 2003). Additionally, some species live their lives entirely in freshwater (FW) environments, while others migrate to marine environments during their juvenile phase (Beamish 1980; Potter 1980). As a result, anadromous species must deal with the additional iono- and osmoregulatory challenges associated with the transition to sea water (SW; Reis-Santos et al. 2008). Parasitic lamprey also must increase excretion of nitrogenous wastes (N-wastes) to avoid internal accumulation of toxic ammonia produced by the digestion of protein-rich blood meals (Ramnarine et al. 1987; Wilkie et al. 2006). Given the different life styles of lamprey species, it is of interest to understand how different species and life stages prepare for each of the challenges associated with their life histories.

The overall objective of my study was to characterize the mechanisms of N-waste excretion in multiple lamprey species and to understand the influence salinity and parasitism have on these processes. Specifically, I aimed to assess the changes that take place in the N-waste and associated ion transporters within the gills throughout the metamorphosis of sea lamprey (*Petromyzon marinus*) and relate this to the onset of feeding and acclimation to SW. I also then compared my findings of sea lamprey gill structure and function to the observations I made on exclusively FW, parasitic silver lamprey (*Ichthyomyzon unicuspis*) and the closely

related non-parasitic northern brook lamprey (*Icthyomyzon fossor*), providing further opportunity to better understand how parasitism and salinity influence N-waste excretion mechanisms in lampreys. As very little is known about the mechanisms of N-waste excretion in lamprey, I based my early investigations on known pathways in teleost fishes. My study of ammonia excretion in multiple lamprey species supports the hypothesis that different mechanisms are used to excrete N-wastes in freshwater (FW) compared to seawater (SW) environments.

Conservation of V-type H^+-ATPase in the Freshwater Mechanism of N-waste Excretion

Based on the abundance of V-ATPase in FW lamprey life stages and species, and its colocalization with Rhcg-like protein, the FW mechanism of N-waste excretion in lamprey (Fig. 4.1) was largely consistent with findings in other fish species (Weihrauch et al. 2009; Wright and Wood 2009). In this model, Rhesus c-like glycoproteins (Rhcg-like) were located apically on the gill, transporting ammonia from the cytosol of gill epithelial cells into the environment. Simultaneously, the action of an apical V-type H^+-ATPase (V-ATPase) actively excreted H^+ into the boundary layer. These protons then trap ammonia (NH_3) as ammonium (NH_4^+) which diffuse into the bulk water and maintain a favourable partial pressure gradient for the continued facilitated diffusion of ammonia (Fig. 4.1). These protons are sourced either from the breakdown of internal ammonium into the ammonia that is transported across the apical membrane, or from the hydration of CO_2 into HCO_3^- and H^+. However, the component of this mechanism that transports ammonia from the extracellular fluid into the cell cytosol remains unknown, suggesting that it is possible that another Rh glycoprotein or ionoregulatory transporter is involved in this step of the ammonia excretion mechanism.

This mechanism for FW ammonia excretion was well supported in my study, as V-ATPase was abundant in the FW lamprey species and sea lamprey life stages studied but was virtually absent in the SW transitioning juvenile sea lamprey. I therefore conclude that the lamprey V-ATPase is important for ionoregulation and N-waste excretion in the FW environment. The colocalization of V-ATPase with Rhcg-like protein also supported the hypothesis that the ammonia excretion and acid-base regulatory mechanisms are closely linked in FW environments. As also observed by Reis-Santos et al. (2008) in sea lamprey, there was a decrease in V-ATPase expression with increasing salinity. Thus, I conclude that there is a conservation of FW N-waste and ionoregulatory mechanisms both within the lamprey lineage, and in more recently derived fish species.

A Dual-role for Seawater-type Mitochondrion-rich Cells in the Anadromous Sea Lamprey

The mechanism for N-waste excretion in SW transitioning sea lamprey differed greatly from the FW mechanism observed in all three lamprey species. Sea lamprey demonstrated a reorganization of Rhcg-like proteins in the gills during metamorphosis, culminating in the localization of Rhcg-like proteins within the interlamellar seawater-type mitochondrion-rich cells (SW MRCs) in juvenile phases and a distinct colocalization with Na^+/K^+-ATPase (NKA) on the basolateral membranes of these cells (Fig. 4.2). Based on these observations, I propose that Rhcg-like protein transports ammonia from the plasma and across the basolateral membrane into the cytosol of the SW MRC. Meanwhile, NKA, also involved in ionoregulation, pumps Na^+ from the SW MRC cytosol into the plasma. This keeps internal Na^+ concentrations sufficiently low to allow an apical Na^+/H^+ exchanger (NHE) to take up Na^+ and to excrete H^+ into the bulk water. However, it is unlikely that ammonia passes through the apical membrane of the SW MRC

without the use of a transporter. If another Rh glycoprotein is not present apically, I propose that ammonia could be excreted as NH_4^+ by substituting for H^+ on the apical NHE (Hu et al. 2014). With a smaller unhydrated radius than Na^+, it is also possible that some NH_4^+ passively diffuses rom the extracellular fluid to the bulk water via paracellular pathways through cation selective epithelial junctions (aka "leaky" tight junctions) between SW MRCs. This pathway is the route used by seawater fishes to excrete Na^+ which builds up in the paracellular pathways between cells due to the action of the basolateral Na^+/K^+-ATPase (Fig. 4.2). Further investigation is needed to determine more precisely how ammonia is excreted into the bulk water in SW, and the relative contribution of each of these proposed pathways to overall ammonia excretion rates.

The presence of both Rhcg-like protein and NKA in the basolateral membrane of SW MRCs along with the total absence of SW MRCs in FW lamprey species and life stages studied here and in other studies (Bartels and Potter 2004), suggest that the SW MRCs play a crucial role in both SW ionoregulation and ammonia excretion. These marked differences in the mechanisms of ammonia excretion and presence of SW MRCs between these species suggest that preparation for an anadromous life style may be a key factor in determining the mechanism of ammonia excretion in the lamprey gill. Further, salinity has been found previously to upregulate the expression of NKA in the gills of both lamprey (Reis-Santos et al. 2008; Lança et al. 2015) and other fish species (Wilson et al. 2007; Zydlewski and Wilkie 2012). Together, these results suggest that salinity of the environment is a major influencer of transporter distribution within the gills of fishes.

Implications of Urea Transporter Abundance for Branchial Urea Transport in FW and SW

My study provides evidence that urea excretion across the gills of lampreys is more prominent in FW environments, as displayed by the UT distribution in the FW phases of the sea lamprey and the exclusively FW species studied here (Fig. 4.1). UT abundance decreased in juvenile sea lamprey despite an increase in urea excretion and plasma urea levels with parasitic feeding (Wilkie et al. 2004). These results, and those reported by Swindell (1999), suggest that a larger proportion of urea is being excreted through pathways other than across the branchial epithelium in the SW phases of the sea lamprey life cycle, as compared to the larval and early metamorphic stages of this species. This excretion most likely occurs via renal routes, though it is also possible that "some urea excretion takes place across the gills without the aid of a urea specific transporter via paracellular pathways (Fig. 4.2) (Bartels and Potter 2004; Zydlewski and Wilkie 2012). In the FW silver and northern brook lamprey, UT was abundant in the gills of juvenile phases, with the highest abundance in the parasitic silver lamprey. This suggests that UT expression may be upregulated to facilitate higher rates of urea excretion when lampreys are ingesting large amounts of protein-rich blood, which may also contain large amounts of the amino acid arginine which can lead to elevated concentrations of urea when it is hydrolyzed via the enzyme arginase. To understand urea excretion in lamprey, much more work is needed including an assessment of excretion rates in FW species, divided chamber work to elucidate the role of extra-branchial routes for urea excretion in anadromous and FW lamprey species, and an investigation of transporters associated with branchial UT.

The Evolution of Lampreys and Mechanisms of N-waste Excretion and Ionoregulation

Current evidence suggests that the lampreys originated in a marine environment and that all extant non-parasitic lamprey species were derived from a parasitic ancestor (Potter and Hilliard 1987; Potter and Gill 2003; Gess et al. 2006). Evidence from my study supports the hypothesis that extant species were derived from parasitic, anadromous ancestors. Assuming that the gill structure and transporter abundance of the anadromous, parasitic sea lamprey is similar to the ancestral lamprey, differences in the northern brook and silver lamprey species, namely the absence of SW MRCs, would indicate that these changes in transporter abundance were derived as the animals adapted to their exclusively FW environment. Further, the silver lamprey condition appears to be intermediate between the juvenile sea lamprey, and northern brook lamprey, with NKA colocalizing with Rhcg-like protein in the interlamellar regions, but not in SW MRCs.

Should these animals have diverged from an anadromous ancestor long ago in evolutionary terms, it would not be surprising if salinity played the predominate role in the evolution of their modes of N-waste excretion mechanism rather than their parasitic lifestyle. Although Rh glycoproteins appear to be highly conserved in the vertebrate lineage, they are present in hagfishes, lampreys, elasmobranchs, and most teleost species, it appears that the mechanisms of ammonia excretion in SW dwelling sea lamprey are unique (Weihrauch et al. 2009; Braun et al. 2009a; Braun et al. 2010; Ip and Chew 2010). In contrast, the marked similarity in the FW N-waste excretion mechanisms of lamprey to those of other fish species suggest a high degree of conservation of these ammonia excreting mechanisms in vertebrates. In other words, the Na^+/NH_4^+ exchange complex proposed by Wright and Wood (2009), in which

membrane transporters such as the Rh glycoproteins, V-type H^+-ATPases and Na^+-channels form a "metabolon" to promote ammonia excretion, represents an ancient strategy of ammonia excretion. Indeed, given the long, 360 million year separation of the agnathan (jawless fishes) and gnathostome (jawed vertebrate) lineages, the results of the present study suggest that this arrangement may very well have been used by the earliest vertebrates to excrete ammonia following their invasion of freshwater environments.

Future Directions

The results gathered in this study suggest that the onset of parasitic feeding influences the rate of N-waste excretion, while an anadromous life history is the larger influencer on excretion mechanism (Wilkie et al. 1999; 2004; 2006). As the absence of a SW dwelling phase appears to have a strong influence on differences between anadromous and FW species that have been separated for an evolutionary significant time period, investigating populations that have been separated relatively recently in evolutionary time could shed light into the regulation and adaptation of SW and FW N-waste excretion mechanisms. The landlocked population of sea lamprey in the Great Lakes of North America would provide an ideal opportunity for this investigation given their recent separation (~200 years) from the anadromous population on an evolutionary scale (Larson et al. 2003). Thus further studies, perhaps using paleolimnological approaches to more accurate determine when sea lampreys entered the Great lakes, would be valuable.

Although my study has begun to elucidate the mechanisms used by lamprey for N-waste excretion in FW and SW, components of these mechanisms, namely the basolateral transport of

ammonia in FW and the apical transport of ammonia in SW, require further investigation. Experiments using known inhibitors of the ionoregulatory and N-waste associated transporters tested here (e.g. ouabain for NKA, bafilomycin for V-ATPase, floretin for UT) would further our understanding of the individual roles of each transporter and could provide insight into the missing components of these pathways. I was unable to obtain results for Rhag-like and Rhbg-like glycoproteins, thus their role in either excretion model cannot be confirmed. Despite their presence being reported in other studies of sea lamprey epithelium (Reis-Santos et al. 2008, Blair et al. 2016), these proteins have not been annotated on the sea lamprey genome. Further genetic study and phylogenetic analysis is needed to determine whether these proteins have differentiated in the sea lamprey, or if the lamprey possess some form of precursor to these Rh glycoprotein isoforms.

Finally, whether the distribution of Rhcg-like protein differs between ammocoetes and juveniles in the freshwater northern brook and silver lamprey species is unknown as changes throughout their metamorphosis were not studied here. A study of metamorphosis in these exclusively FW species and associated changes in transporter distribution and abundance is needed to fully understand how these species may differ from the sea lamprey. Investigation of the excretion rates of these FW species as ammocoetes and juveniles, would also improve our understanding of the mechanisms of N-waste excretion and how different mechanisms may improve excretion efficiency when protein consumption and therefore ammonia production is high. Studies on the role that different endocrine cues play in triggering these changes in gill structure and function would also be informative. For instance, the role of thyroid hormone, involved in triggering metamorphosis (Youson 2003), in the regulation of gill restructuring and

function would be very useful. As would a better understanding of the role that 11-deoxycortisol, which was recently found to play a key role in regulating ion balance in sea lamprey (Close et al. 2010).

Integrative Biology

My study has related whole animal functional studies with internal physiology and quantitative investigation of protein expression. Using these techniques, I have been able to form hypotheses about the biochemical processes of N-waste excretion and ionoregulation within the gills of multiple lamprey species. These results then allow me to form hypotheses about the evolution of the lamprey lineage and more recently derived fish species. Although not studied here, these data would be well supplemented by bioinformatic and phylogenetic analyses to further understand both the regulation of these mechanisms at a molecular and genetic level, while also contributing to our understanding of the conservation of these processes among vertebrates and the evolution of gill structure and function in vertebrates. Finally, as northern brook and silver lamprey, both of which are listed as species of special concern in Canada share habitat with the invasive sea lamprey, understanding how their physiology differs in sensitivities to their external environment may allow the development of sea lamprey control techniques that have a reduced effect on these native FW species. Current methods to control the invasive sea lamprey in the Great Lakes include a chemical control method, 3-trifluoromethyl-4-nitrophenol (TFM), which selectively targets lamprey due to their lower levels of enzymes necessary to detoxify this compound as compared to other fish (Larson et al. 2003). Perhaps an understanding of the differences in lamprey physiology may allow this method to be refined to selectively

target specific lamprey species, thus protecting native species while controlling sea lamprey populations.

Figures

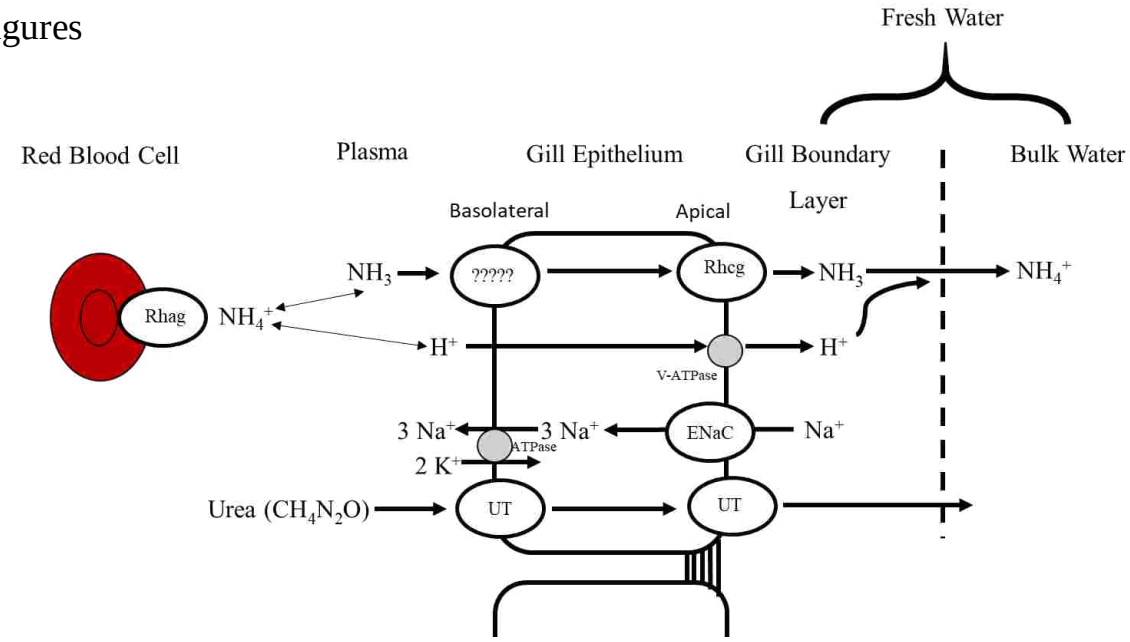

Figure 4.1. Proposed model of ammonia excretion across the gill epithelium of lamprey in fresh water. As in other fish species, ammonia excretion by lampreys is dependent on Rhesus c-like glycoprotein (Rhcg-like) located apically on the gill epithelium. Ammonia excretion involves V-type H^+-ATPase (V-ATPase) which acidify the gill boundary layer, protonating NH_3 into NH_4^+. This process helps to sustain the cytosolic-gill boundary layer NH_3 diffusion gradient needed to drive the process. The action of V-ATPase also generates a favourable electrochemical gradient that likely promotes the uptake of Na^+ via an apically-located epithelial Na^+ channel (ENaC) which has yet to be characterized in lampreys. The action of the basolateral Na^+/K^+-ATPase (NKA) is critical for facilitating the uptake of Na^+ across the basolateral membrane into the plasma. Other potential Rh glycoproteins are not shown though their presence cannot be ruled out at this time. For urea transport, urea transporter (UT) protein is likely involved but whether it is located apically or basolaterally remains to be determined. Transport via the paracellular junctions is unlikely in fresh water.

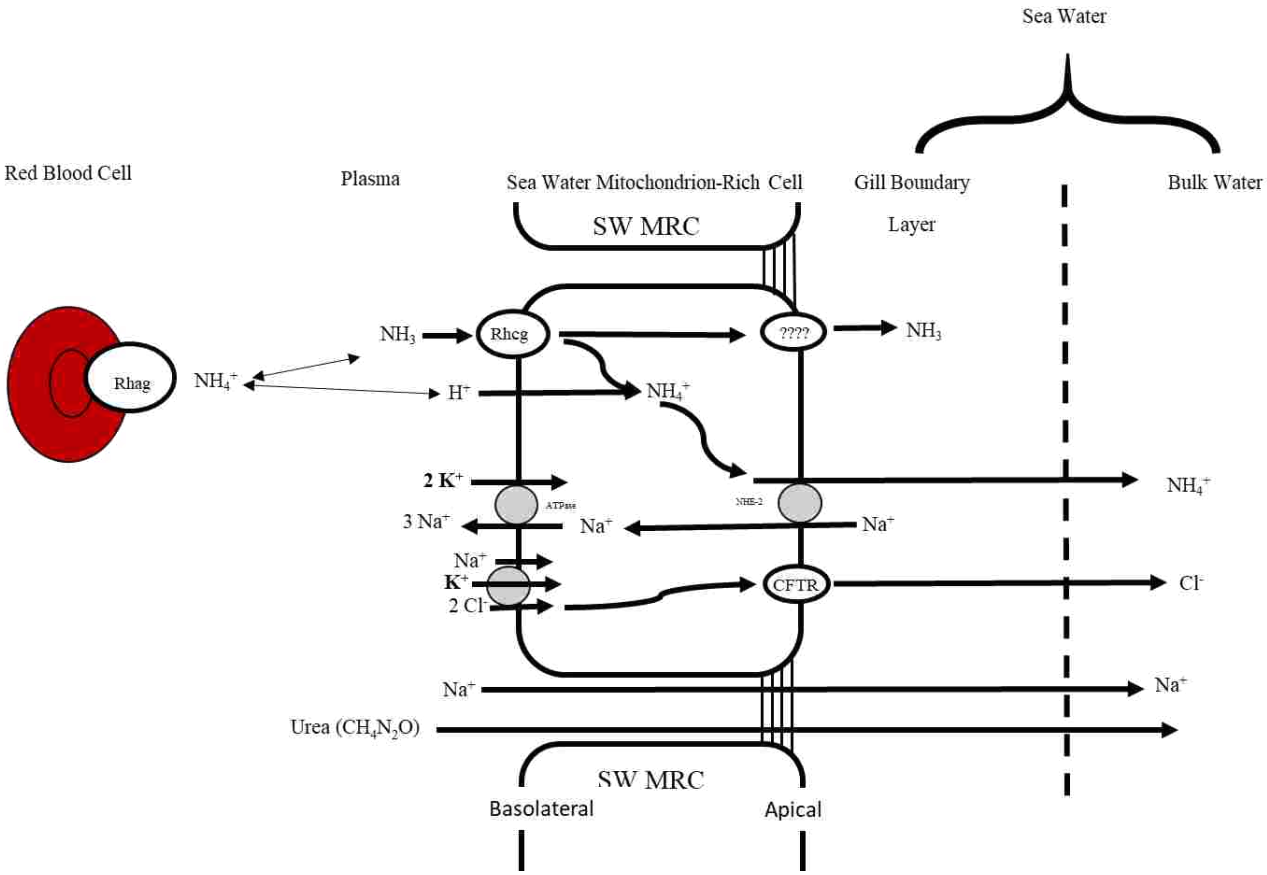

Figure 4.2. Proposed mechanism(s) of ammonia excretion across the gill epithelium of a sea lamprey in sea water, based on the findings presented in this study. This mechanism relies heavily on Rhesus c-like glycoprotein (Rhcg-like) and Na^+/K^+-ATPase (NKA) colocalized basolaterally on the seawater-type mitochondrion-rich cells (SW MRCs), arranged in rows within the interlamellar regions of the gill epithelium. NH_3 is likely protonated into NH_4^+ internally due to the lower pH of the gill cytosol compared to the extracellular fluid (Weihrauch et al. 2009). It is proposed that the NH_4^+ is excreted by substituting for H^+ on an apical Na^+/H^+ exchanger (NHE), which is driven by large inwardly directed Na^+ gradients between the sea water and cytosolic interior of the cell. In the absence of evidence of a urea transporter protein (UT), urea may be excreted via the paracellular route as the tight junctions between epithelial cells are "leakier" in sea water compared to fresh water gills. Other potential Rh glycoproteins are not shown though their presence cannot be ruled out at this time.

Appendix A
Compendium of P-Values

Weight Change During Metamorphosis (data not shown)

Two-Sample T-test

Comparison	P-value
Transformer vs. Non-transformer	0.03086 *

Influence of Feeding on J_{amm} (Fig 2.1A)

One-Way Analysis of Variance and Tukey's Honestly Significant Difference

Comparison	P-value
ANOVA	1.11×10^{-6}*
Ammocoete - FW	0.85897
Ammocoete – 10pct	0.95734
Ammocoete – 100pct	0.99404
Ammocoete - Feeder	6.6×10^{-6} *
FW – 10pct	0.39894
FW – 100pct	0.58184
FW - Feeder	3.00×10^{-7} *
10pct – 100pct	0.99811
10pct – Feeder	1.98×10^{-5} *
100pct - Feeder	9.7×10^{-6} *

Influence of Feeding on J_{urea} (Fig 2.1B)

One-Way Analysis of Variance and Tukey's Honestly Significant Difference Post-hoc

Comparison	P-value
ANOVA	7.04×10^{-5} *
Ammocoete - FW	0.10791
Ammocoete – 10pct	0.60749
Ammocoete – 100pct	0.83853
Ammocoete - Feeder	0.41586 •
FW – 10pct	0.60637
FW – 100pct	0.33407
FW - Feeder	3.83×10^{-5} *
10pct – 100pct	0.98655
10pct – Feeder	0.00699*
100pct - Feeder	0.02612 *

Changes in Plasma Ammonia with Metamorphosis (Fig 2.3A)

One-Way Analysis of Variance and Games-Howell Post-hoc

Comparison	P-value
ANOVA	1.53×10^{-5} *
Ammocoete – 1	1.000
Ammocoete – 2	0.115
Ammocoete – 3	0.990
Ammocoete – 4	0.896
Ammocoete – 5	0.101
Ammocoete – 6	0.926
Ammocoete – 7	0.108
Ammocoete – Post-metamorphic	0.084 ·
Ammocoete – SW	0.077 ·
Ammocoete – Feeder	0.868
1 – 2	0.530
1 – 3	1.000
1 – 4	0.950
1 – 5	0.115
1 – 6	0.976
1 – 7	0.138
1 – Post-metamorphic	0.079 ·
1 – SW	0.068 ·
1 – Feeder	0.861
2 – 3	0.989
2 – 4	0.108
2 – 5	0.008 *
2 – 6	0.042 *
2 – 7	0.006 *
2 – Post-metamorphic	0.051 ·
2 – SW	0.033 *
2 – Feeder	0.718
3 – 4	0.750

Changes in Plasma Ammonia with Metamorphosis (Fig 2.3A) continued

One-Way Analysis of Variance and Games-Howell Post-hoc

Comparison	P-value
3 – 5	0.064 ·
3 – 6	0.786
3 – 7	0.078 ·
3 – Post-metamorphic	0.065 ·
3 – SW	0.046 ·
3 – Feeder	0.809
4 – 5	0.534
4 – 6	1.000
4 – 7	0.670
4 – Post-metamorphic	0.110
4 – SW	0.121
4 – Feeder	0.938
5 – 6	0.363
5 – 7	1.000
5 – Post-metamorphic	0.251
5 – SW	0.535
5 – Feeder	0.999
6 – 7	0.465
6 – Post-metamorphic	0.104
6 – SW	0.108
6 – Feeder	0.924
7 – Post-metamorphic	0.216
7 – SW	0.411
7 – Feeder	0.998
Post-metamorphic – SW	0.886
Post-metamorphic – Feeder	0.873
SW - Feeder	1.000

Changes in Plasma Urea with Metamorphosis (Fig 2.3B)

One-Way Analysis of Variance and Games-Howell Post-hoc

Comparison	P-value
ANOVA	1.208×10^{-4} *
Ammocoete – 1	0.990
Ammocoete – 2	1.000
Ammocoete – 3	0.964
Ammocoete – 4	0.996
Ammocoete – 5	0.053 ·
Ammocoete – 6	1.000
Ammocoete – 7	0.925
Ammocoete – SW	<0.001 *
Ammocoete – Feeder	0.821
1 – 2	1.000
1 – 3	0.741
1 – 4	1.000
1 – 5	0.043 *
1 – 6	0.989
1 – 7	0.741
1 – SW	0.002 *
1 – Feeder	0.877
2 – 3	0.986
2 – 4	1.000
2 – 5	0.675
2 – 6	0.999
2 – 7	0.963
2 – SW	0.475
2 – Feeder	0.888
3 – 4	0.913
3 – 5	0.687
3 – 6	1.000
3 – 7	1.000

Changes in Plasma Urea with Metamorphosis (Fig 2.3B) continued

One-Way Analysis of Variance and Games-Howell Post-hoc

Comparison	P-value
3 – SW	0.102
3 – Feeder	0.768
4 – 5	0.410
4 – 6	0.992
4 – 7	0.866
4 – SW	0.245
4 – Feeder	0.898
5 – 6	0.946
5 – 7	0.994
5 – SW	0.994
5 – Feeder	0.668
6 – 7	1.000
6 – SW	0.727
6 – Feeder	0.796
7 – SW	0.864
7 – Feeder	0.738
SW - Feeder	0.631

Changes in Expression of Rhcg-like protein through Metamorphosis (Fig. 2.5)
One-Way Analysis of Variance and Tukey's Honestly Significant Difference

Comparison	P-value
ANOVA	0.234
Ammocoete – 1	1.000
Ammocoete – 2	1.000
Ammocoete – 3	1.000
Ammocoete – 4	0.964
Ammocoete – 5	1.000
Ammocoete – 6	1.000
Ammocoete – 7	1.000
Ammocoete – Post-metamorphic	0.351
Ammocoete – SW	0.999
Ammocoete – Feeder	0.992
1 – 2	0.999
1 – 3	1.000
1 – 4	0.954
1 – 5	1.000
1 – 6	1.000
1 – 7	1.000
1 – Post-metamorphic	0.635
1 – SW	1.000
1 – Feeder	0.998
2 – 3	1.000
2 – 4	0.994
2 – 5	0.999
2 – 6	1.000
2 – 7	1.000
2 – Post-metamorphic	0.401
2 – SW	0.999
2 – Feeder	0.987
3 – 4	0.980

Changes in Expression of Rhcg-like protein through Metamorphosis (Fig. 2.5) continued
One-Way Analysis of Variance and Tukey's Honestly Significant Difference

Comparison	P-value
3 – 5	1.000
3 – 6	1.000
3 – 7	1.000
3 – Post-metamorphic	0.522
3 – SW	0.999
3 – Feeder	0.995
4 – 5	0.970
4 – 6	0.972
4 – 7	0.972
4 – Post-metamorphic	0.051 ·
4 – SW	0.892
4 – Feeder	0.638
5 – 6	1.000
5 – 7	1.000
5 – Post-metamorphic	0.655
5 – SW	1.000
5 – Feeder	0.998
6 – 7	1.000
6 – Post-metamorphic	0.502
6 – SW	0.999
6 – Feeder	0.996
7 – Post-metamorphic	0.567
7 – SW	0.999
7 – Feeder	0.997
Post-metamorphic – SW	0.772
Post-metamorphic – Feeder	0.999
SW - Feeder	0.999

Changes in Expression of Na^+/K^+-ATPase through Metamorphosis (Fig. 2.6)
One-Way Analysis of Variance and Games-Howell Post-hoc

Comparison	P-value
ANOVA	2.2×10^{-16}*
Ammocoete – 1	1.000
Ammocoete – 2	0.334
Ammocoete – 3	0.990
Ammocoete – 4	0.789
Ammocoete – 5	0.999
Ammocoete – 6	0.031*
Ammocoete – 7	0.001*
Ammocoete – Post-metamorphic	<0.001*
Ammocoete – SW	<0.001*
Ammocoete – Feeder	<0.001*
1 – 2	0.999
1 – 3	1.000
1 – 4	0.993
1 – 5	1.000
1 – 6	0.134
1 – 7	0.001*
1 – Post-metamorphic	<0.001*
1 – SW	<0.001*
1 – Feeder	<0.001*
2 – 3	0.992
2 – 4	1.000
2 – 5	0.960
2 – 6	0.530
2 – 7	0.005*
2 – Post-metamorphic	<0.001*
2 – SW	<0.001*
2 – Feeder	<0.001*
3 – 4	1.000

Changes in Expression of Na^+/K^+-ATPase through Metamorphosis (Fig. 2.6) continued
One-Way Analysis of Variance and Games-Howell Post-hoc

Comparison	P-value
3 – 5	1.000
3 – 6	0.275
3 – 7	0.002*
3 – Post-metamorphic	<0.001*
3 – SW	<0.001*
3 – Feeder	<0.001*
4 – 5	0.998
4 – 6	0.521
4 – 7	0.004*
4 – Post-metamorphic	<0.001*
4 – SW	<0.001*
4 – Feeder	<0.001*
5 – 6	0.208
5 – 7	0.002*
5 – Post-metamorphic	<0.001*
5 – SW	<0.001*
5 – Feeder	<0.001*
6 – 7	0.074
6 – Post-metamorphic	<0.001*
6 – SW	<0.001*
6 – Feeder	<0.001*
7 – Post-metamorphic	0.774
7 – SW	0.387
7 – Feeder	0.391
Post-metamorphic – SW	0.843
Post-metamorphic – Feeder	0.871
SW - Feeder	1.000

Changes in Expression of Urea Transporter (UT) through Metamorphosis (Fig. 2.9)
One-Way Analysis of Variance and Tukey's Honestly Significant Difference

Comparison	P-value
ANOVA	1.91×10^{-4}*
Ammocoete – 1	0.986
Ammocoete – 2	0.999
Ammocoete – 3	1.000
Ammocoete – 4	0.999
Ammocoete – 5	1.000
Ammocoete – 6	1.000
Ammocoete – 7	0.937
Ammocoete – Post-metamorphic	0.808
Ammocoete – SW	0.282
Ammocoete – Feeder	0.0001*
1 – 2	0.963
1 – 3	0.981
1 – 4	0.999
1 – 5	0.990
1 – 6	0.998
1 – 7	1.000
1 – Post-metamorphic	0.999
1 – SW	0.971
1 – Feeder	0.008 *
2 – 3	1.000
2 – 4	0.998
2 – 5	1.000
2 – 6	0.999
2 – 7	0.890
2 – Post-metamorphic	0.747
2 – SW	0.276
2 – Feeder	0.0001*
3 – 4	0.999

Changes in Expression of Urea Transporter (UT) through Metamorphosis (Fig. 2.9) continued

One-Way Analysis of Variance and Tukey's Honestly Significant Difference

Comparison	P-value
3 – 5	1.000
3 – 6	0.999
3 – 7	0.931
3 – Post-metamorphic	0.816
3 – SW	0.343
3 – Feeder	0.0002 *
4 – 5	0.999
4 – 6	0.999
4 – 7	0.999
4 – Post-metamorphic	0.997
4 – SW	0.873
4 – Feeder	0.003 *
5 – 6	0.990
5 – 7	0.960
5 – Post-metamorphic	0.877
5 – SW	0.439
5 – Feeder	0.0004 *
6 – 7	0.989
6 – Post-metamorphic	0.947
6 – SW	0.552
6 – Feeder	0.0005 *
7 – Post-metamorphic	1.000
7 – SW	0.994
7 – Feeder	0.015 *
Post-metamorphic – SW	0.999
Post-metamorphic – Feeder	0.0311 *
SW - Feeder	0.146

Appendix B
Negative Control Images

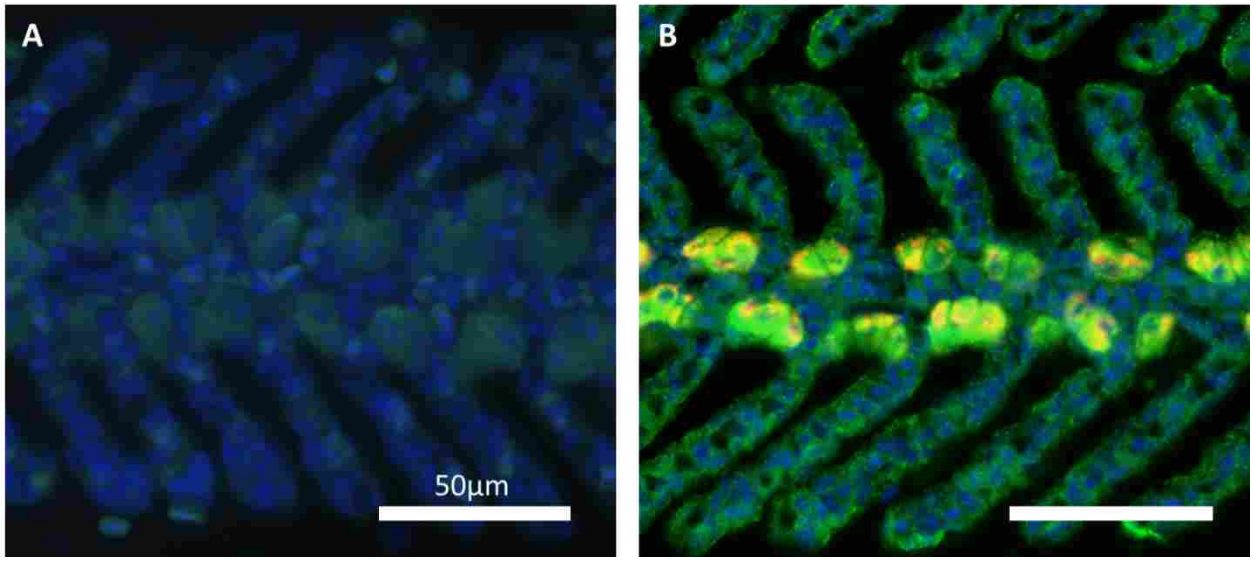

Figure A1. Comparison of null (A) and Rhcg/NKA double staining (B) in the gills of a juvenile sea lamprey. DAPI is used to stain cell nuclei to allow visual comparison between stains. Scale bar = 50µm. See figure 2.9.

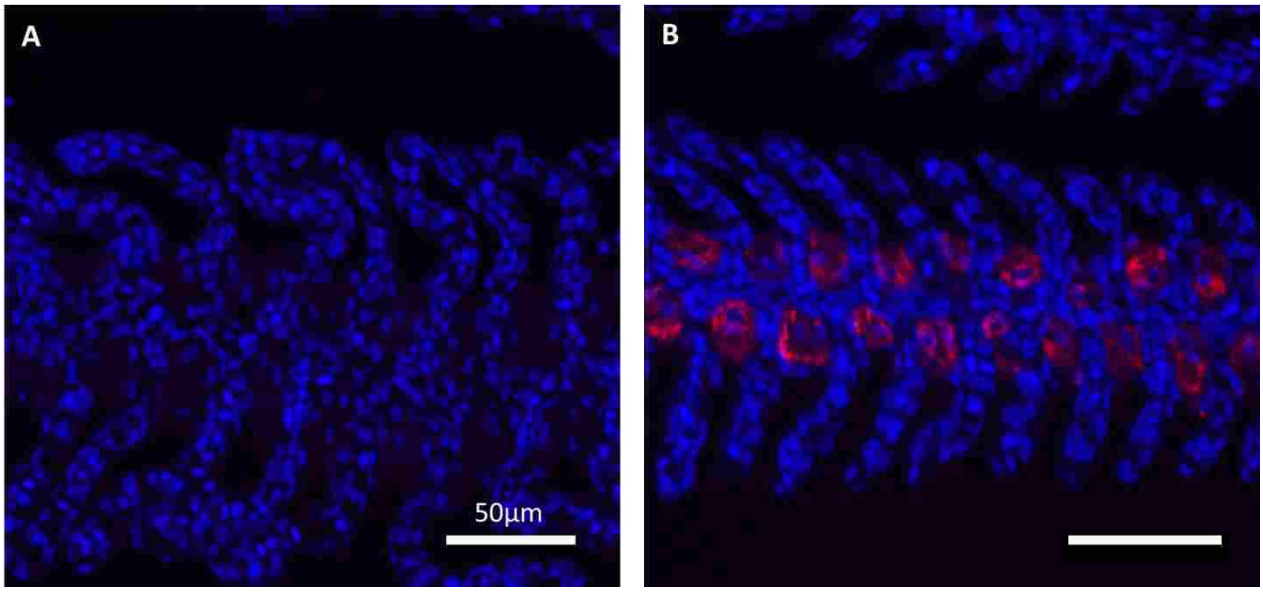

Figure A2. Comparison of null (A) and NKA double staining (B) in the gills of a parasitic sea lamprey. DAPI is used to stain cell nuclei to allow visual comparison between stains. Scale bar = 50µm. See figure 2.9.

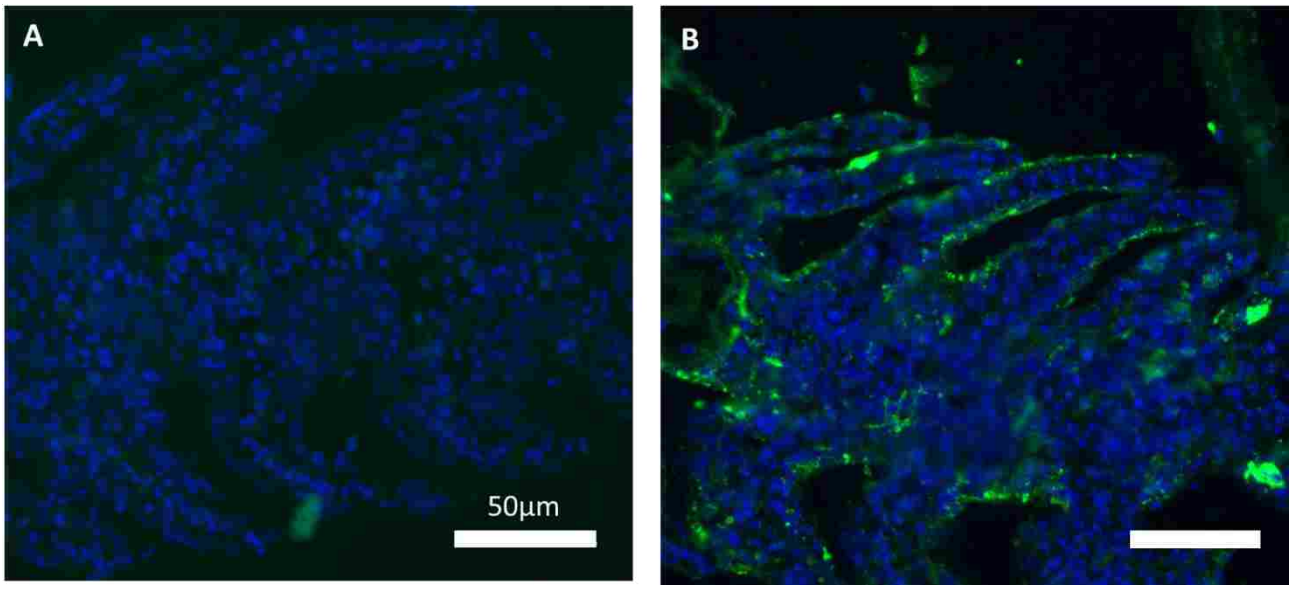

Figure A3. Comparison of null (A) and UT staining (B) in the gills of a sea lamprey ammocoete. DAPI is used to stain cell nuclei to allow visual comparison between stains. Scale bar = 50µm. See figure 2.14.

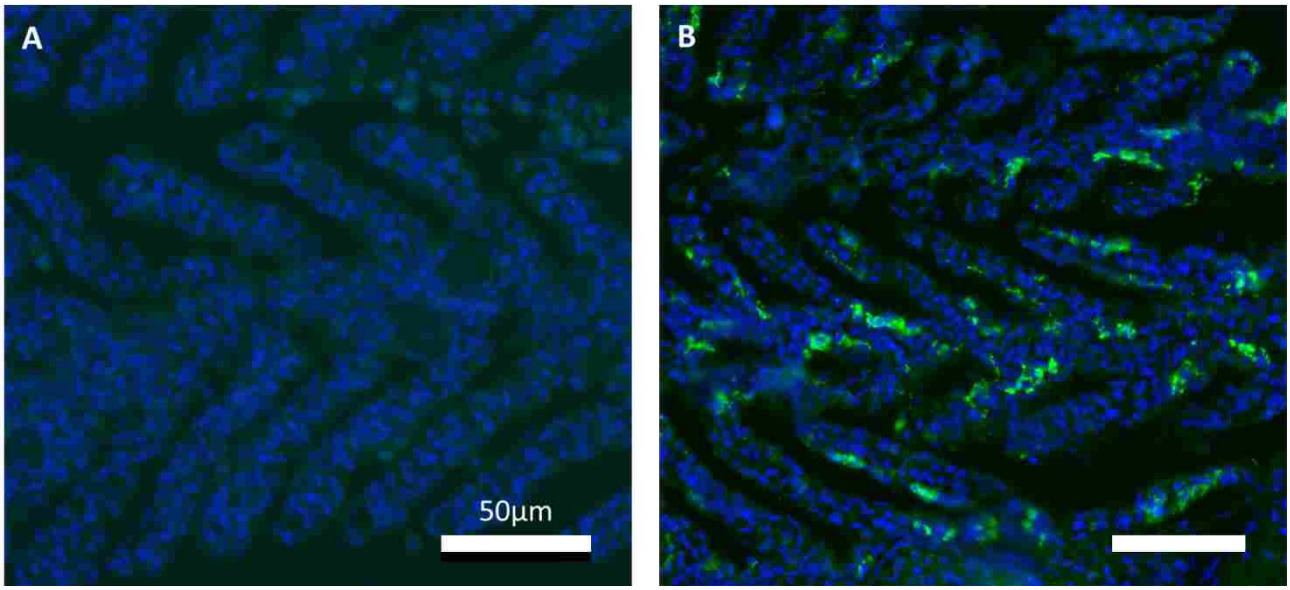

Figure A4. Comparison of null (A) and UT staining (B) in the gills of a juvenile silver lamprey. DAPI is used to stain cell nuclei to allow visual comparison between stains. Scale bar = 50µm. See figure 3.2.

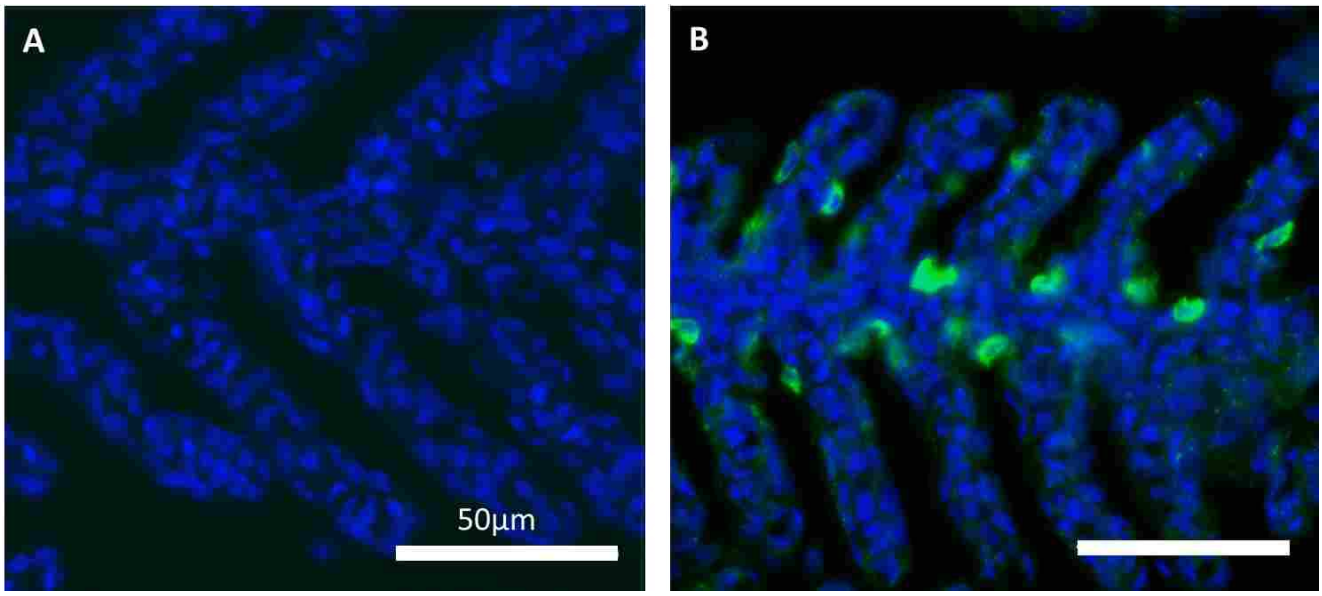

Figure A5. Comparison of null (A) and V-ATPase staining (B) in the gills of a juvenile northern brook lamprey. DAPI is used to stain cell nuclei to allow visual comparison between stains. Scale bar = 50µm. See figure 3.5.

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