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**Mechanisms of Nitrogenous Waste Excretion During the Complex Life Cycle of the  
Sea Lamprey (*Petromyzon marinus*)**

By

James Neal

(B.Sc. - Honors Specialization in Biology, University of Western Ontario, 2009)

M.Sc. THESIS

Submitted to the Department of Biology

Faculty of Science

In partial fulfillment of the requirements for the

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James Neal ©2013

## ABSTRACT

Sea lampreys (*Petromyzon marinus*) are a phylogenetically ancient jawless fish, with a multi-staged life cycle characterized by a prolonged suspension-feeding larval stage, which is followed by metamorphosis into parasitic lampreys that feed on the protein-rich blood of fishes. The switch from a nutrient poor to protein-rich diet in the sea lamprey is associated with an increased capacity to deaminate excess amino acids and to excrete ammonia and urea following metamorphosis. The focus of this thesis was to determine if changes in nitrogenous waste transporter protein abundance facilitate ammonia and urea excretion during different stages of the sea lamprey life cycle.

To investigate the mechanisms by which nitrogenous waste excretion ( $J_{N\text{-waste}}$ ) occurs in sea lampreys, individuals of various lifestages (larval/adults) were exposed to environmental stressors (highly alkaline water and high external ammonia) that have been previously shown to affect  $J_{N\text{-waste}}$  in other fishes. Both ammocoete and adult sea lamprey were unable to tolerate highly alkaline (HA:  $\text{pH} \approx 9.5$ ) water for more than 24 h. However, exposure of ammocoetes and adult lamprey to high external ammonia (HEA;  $0.5 \text{ mmol} \cdot \text{L}^{-1}$  ammonia) resulted in the reversal of ammonia excretion ( $J_{\text{Amm}}$ ) and a net uptake of ammonia over 2 days. In adults, urea excretion ( $J_{\text{Urea}}$ ) increased significantly but remained unchanged in ammocoetes.

To determine whether there was a correlation between  $J_{N\text{-waste}}$  patterns and the transport proteins associated with ammonia and urea excretion in sea lamprey, western blot analysis of Rh glycoproteins (Rhcg2) and urea transporters (UT) was performed on the main lifestages of sea lampreys (ammocoete, unfed parasitic, fed parasitic, adults) within gill and skin tissues. The abundances of Rhcg2 in the gill were significantly higher

in fed parasites when compared to unfed juveniles and adult lamprey. Corresponding  $J_{\text{Amm}}$  and plasma ammonia concentrations in these individuals were also greater. Larval sea lamprey UT protein abundances were significantly greater in gill tissues than in fed parasites and adult animals. This was postulated to be a function of the burrowing nature of the larval sea lampreys, and their relatively high rates of urea excretion compared to other life stages.

The period of metamorphosis was also accompanied by marked changes in body condition factor (CF), along with  $J_{\text{Amm}}$  and  $J_{\text{Urea}}$ , which were initially depressed during the mid-stages of metamorphosis before increasing several-fold near the completion of metamorphosis. Both Rhcg2 and UT expression in the gills peaked at stage 4 of metamorphosis and declined to young adulthood, whereas Rhcg2 and UT expression in the skin of metamorphosing animals was below detectable levels.

It is concluded that the changes in diet, along with habitat and activity level lead to the observed changes in the nitrogenous waste excretion patterns of the sea lamprey, which is reflected by corresponding changes in the abundance of the Rh and UT proteins. Further research into the regulation and localization of these proteins will prove useful in completing the picture of N-waste excretion in these phylogenetically ancient vertebrates.

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## **Chapter 1**

### **Introduction**

*In depth sea lamprey life cycle:*

Sea lampreys (*Petromyzon marinus*) belong to a group of phylogenetically ancient jawless vertebrates, representing one of the two extant classes belonging to the superclass Agnatha. Fossil records suggest that body structure and presumably lifestyle in these organisms have remained unchanged for 350 million years (Forey and Janvier 1993; Gess et al. 2006). Sea lampreys are common in Atlantic Canada and in the Great Lakes, where the larvae, known as ammocoetes, begin their multi-staged lifecycle burrowed in the substrate of streams (Youson 1997). This larval phase typically lasts 3-7 years, before they enter a metamorphic period lasting approximately 3 months in which these relatively sedentary larvae transform into freshwater or marine parasites/predators that ingest large quantities of blood from the fishes that they attack (Beamish and Potter 1975; Wilkie 2011).

Lampreys undergo a “true metamorphosis” in which the animals undergo a complete transformation from a free-living larva to an adult, rather than the minor changes observed between larval and adult morphs of a species (Youson 2003). In animals that undergo a true metamorphosis, the larval and adult forms exhibit distinct phenotypes that are associated with changes in the lifestyle and habitat of the animals. This “true” metamorphosis is found in all families of Petromyzontiformes, to which the sea lamprey belongs (Youson 2003). The difference between ammocoetes and adult lampreys was first recognized in the middle of the 19<sup>th</sup> century and since then there has been a marked increase in studies dealing with sea lamprey metamorphosis (see Bartels and Potter 2004; Youson and Manzon 2012 for reviews).



The life cycle of the sea lamprey begins following fertilization, when the eggs are covered with loose substrate within the spawning beds. Free flowing water irrigates the eggs, and after a period of approximately 4-5 weeks pro-ammocoetes emerge from the substrate and move downstream to protected riverbanks where they burrow into silty areas. A definitive larval form is recognizable after about 5 weeks, and any subsequent movement of the ammocoete is related to flow conditions and habitat structure.

Ammocoetes prefer areas with slow moving water, in which there are large quantities of detritus, such as behind rocks, logs or in eddies (Potter 1980).

After 3-7 years burrowed in the substrate of streams (Hardisty and Potter 1971b; Youson 1980; 2003), metamorphosis is initiated within a synchronous two-week period beginning in early-mid July. The primary stages of sea lamprey metamorphosis (stages 1-4) are characterized by the early development of an eye and a pronounced reconfiguring of the oral hood (Youson and Potter 1979; Youson 1980; 2003). However, the mid-stages of metamorphosis (stages 2-5) lead to changes in internal body structure, which are needed to allow for the acclimatization to salt water and migration to seawater where young adults begin feeding on fishes. There is extensive restructuring of gross and fine architecture of the gills, characterized by the disappearance of ammocoete mitochondrial rich cells (MRCs) and the appearance of marine MRCs, which are needed to excrete  $\text{Na}^+$  and  $\text{Cl}^-$  in salt water (Peek and Youson 1979; Bartels and Potter 2004; Reis-Santos et al. 2008). Similarly, the kidney switches from a “bilge-pump” that offloads large quantities of dilute urine in freshwater, to an organ that is mainly involved in excreting toxic divalent ions such as  $\text{Mg}^{2+}$ ,  $\text{SO}_4^{2-}$  and  $\text{Ca}^{2+}$  in sea water (Zydlowski and Wilkie 2013). The later stages (5-7) are characterized by the complete development of eye and oral disc,

as well as a silvering of the body, which may help conceal the animal from potential predators and prey as it actively feeds on fishes in the open water column (Peek and Youson 1979; Youson et al. 1977; Youson and Potter 1979). Upon completion of metamorphosis, young adult sea lamprey migrate to lakes and oceans where they feed almost exclusively on teleost fishes, although they have occasionally been seen feeding on elasmobranch fishes (Wilkie et al. 2004), and even cetaceans (Nichols and Hamilton 2004). Upon completion of this active feeding period (about 20 months in length), adult sea lampreys return to freshwater rivers and streams, cease feeding and undergo sexual maturation. After building a nest (redd), the migrant animals mate and then die (Beamish et al. 1975; Potter et al. 1978).

### *1.2 Lampreys outside of North America:*

Although sea lampreys are seen as an invasive predator in the Great Lakes, where there is an extensive sea lamprey control program, their numbers have been in decline in other parts of the world where they have been part of the ecosystem for millions of years. River lampreys (*Lampetra fluviatilis*) and non-parasitic brook lampreys (*Lampetra planeri*) are becoming endangered and even extinct in some parts of Europe, especially in places where they are considered a delicacy (Kelly and King 2001). Overfishing in such locations has decimated populations of native lampreys and industrialization has brought with it pollution, and stream blockages (dams and weirs) which have further contributed to declining populations over the last 30 years (Thiel et al. 2009). As a result, implementation of conservation programs are needed to protect these ancient species from becoming extinct.

### *1.3 Changes in sea lamprey metabolism associated with feeding:*

The feeding habits of sea lamprey and their metabolism change drastically throughout their life cycle. These changes profoundly influence catabolic and anabolic processes in lampreys leading to marked changes in the patterns of nitrogenous waste production and excretion as they switch from the filter-feeding larval stage to the parasitic stage (Wilkie et al. 2006). In addition, the lamprey life style is punctuated by prolonged periods of starvation, with non-trophic periods lasting 3-4 months or more, both during/immediately following metamorphosis along with the protracted upstream migration/spawning phase of their life cycle (Youson 1980; 2003). During these periods numerous physiological cascades are switched on and off such that the primary form of energy is released through series of catabolic rather than anabolic reactions (Hardisty and Potter 1971b), which likely explains the pronounced differences in patterns of nitrogenous metabolism and waste excretion that have been reported (Wilkie et al. 2006).

The detritus, diatoms, and other nutrients are ingested via a unidirectional respiratory current, whereby the velum draws water in through the oral hood of the animal, and food particles are filtered out (Sutton and Bowen 1994; Rovainen 1996). These food items are then transported through the pharynx to the gut where they are broken down and absorbed. The water then crosses the gills and exits the pharynx via the lateral branchiopores (Rovainen 1996). Amino acids that arise from digested protein in the sea lamprey primarily serve as lipogenic precursors (stored as lipid), whereas glucose is largely stored as glycogen (Moore and Beamish 1973). The early months of summer represent the time of year in which digestive efficiency and food availability reach a maximum in larval sea lampreys, which corresponds to when the greatest amount of food

energy is absorbed, whereas it declines during the winter months (Moore and Beamish 1973) In the last year prior to metamorphosis, however, premetamorphic ammocoete sea lampreys markedly increase their body lipid stores from approximately 4% to 13.5% per unit body mass, which provides the needed energy during the long (2 to 3 month) non-trophic period of metamorphosis (Hardisty and Potter 1971b; O'Boyle and Beamish 1977).

It is generally accepted that larval sea lampreys require a condition factor (CF) of 1.50 or greater (minimum length of 120mm and weight of 3.0g) to enter metamorphosis, where CF is a relationship between weight and body length (Youson et al. 1993). During metamorphosis the CF, which is a combined measure of length and weight, decreases to approximately 1.3 or less when the process is completed (Potter et al. 1978). This results from depreciation in whole body lipid reserves, which fall from approximately 13-14% to 7-8% of total body mass at the completion of stage 7, the final stage of metamorphosis. However, at the tissue level, there seems to be a two-stepped reorganization of lipid reserves. The first phase, observed from ammocoete to stage 3/4 individuals, involves lipid accumulation within the kidney and liver by shunting reserves from the intestine. In the second phase, comprising stages 3/4 to stage 7, lipid is depleted from kidney and liver and accrued in the intestine (Kao et al. 1997a/b). This lipid is primarily used as energy in the form of triacylglycerol (Lowe et al. 1973; Sheriden and Kao 1998). The exact mechanism by which metamorphosis is initiated and how metabolism is controlled are not fully known, however there is direct evidence that several hormonal and environmental cues are involved (Youson 2003; Sheridan and Kao 1998). For example, prior to ammocoete sea lampreys undergoing metamorphosis, there is a marked increase

in circulating thyroid hormones (thyroxine (T4) and triiodothyronine (T3)) which then precipitously drop with the initiation of transformation (Youson et al. 1997).

Following metamorphosis, young adults do not start feeding for several months (up to 10 months in anadromous species; Potter 1980). During this period of emergence and subsequent migration downstream, the remaining lipid stores drop from approximately 7% whole body mass to about 1% (Beamish et al. 1979). This drastic depreciation in overall lipid stores is enough to promote proteolysis in the juvenile (post-metamorphic sea lampreys). In animals that have completed metamorphosis, but have not yet begun feeding, there are signs of muscle wasting indicating that protein has been used to meet the animals energy demands (Youson et al. 1979). Wilkie et al. (2006) noted a marked up-regulation of activity in enzymes associated with amino acid transamination and deamination in these juvenile sea lampreys. However, it is not known if this increase in amino acid deamination capacity is initiated prior to the completion of metamorphosis or during the post-metamorphic period when lipid reserves are nearing depletion.

Once parasitic juvenile lamprey reach open waters and begin feeding, they ingest the protein-rich blood of their hosts (Wilkie et al. 2006). As a result, protein catabolism drastically increases, providing the building blocks for growth and regeneration. Animals attach to hosts, penetrate the skin and body wall, and begin blood ingestion (Farmer 1980). With their newly restructured gills and tidal breathing patterns, young adults are able to stay attached to hosts for extended periods of time and may consume up to 30% their own wet body mass per day, quickly growing in size (Farmer et al. 1975). Protein rich blood meals ingested by parasitic lamprey are processed in the posterior portion of the intestine, with absorption of taking place across caveolated cells. Subsequential

movement of absorbed proteins to the liver allows for transamination and the release of amino acids (Langille and Youson 1984).

After about 20 months at sea, or in lakes for land-locked populations, full-grown adult lampreys migrate up freshwater streams, where they sexually mature and spawn. During this period of atrophy, lampreys once again are relying on internal lipid reserves, which drop from approximately 10-11% to about 4% whole body mass (much of this being phospholipid rather than neutral hydrolysable lipid) and protein which drops by approximately 26% (Beamish et al. 1979).

While there is a good general understanding of the role that lipids and protein play in fueling the sea lampreys activities at different stages in its life cycle, we know little about how these processes are regulated or how the animals deal with changing patterns of metabolic waste production or changes in diet. The goal of this thesis was to relate the unique nature of the sea lamprey lifecycle and its variation in feeding patterns to life specific changes in metabolic waste production and excretion patterns, with a focus on how nitrogenous wastes are excreted at different stages in the sea lamprey's life cycle.

#### *1.4 Nitrogenous wastes and roles of Rh and UT:*

The catabolism of protein by animals produces excess amino acids that can serve as a valuable fuel to drive physiological processes due to the presence of a C-skeleton that can be utilized for intermediary metabolism via ATP synthesis. However, the catabolism of amino acids leads to the generation of ammonia (NH<sub>3</sub>), which is highly toxic and must be excreted or converted to a less toxic nitrogenous waste (N-waste) such as urea, and uric acid (Wright 1995; Ip et al. 2001; Wilkie 2002). Build-ups of internal

concentrations of  $\text{NH}_4^+$  may lead to depolarization of neurons and subsequent influx of  $\text{Ca}^{2+}$  into the cells resulting in neurotoxic damage in the central nervous system (Randall and Tsui 2002).

The type of waste excreted is strongly influenced by environmental factors. Most fishes mainly excrete ammonia due to its highly solubility in water and low production costs, but semi-terrestrial animals such as amphibians excrete urea during the adult phases of their life cycle due to the absence of sufficient water in which to excrete the more soluble, but more toxic ammonia. Terrestrial animals, such as mammals and birds/reptiles, excrete the majority of their N-waste as urea and uric acid, respectively (Wood 1993; Wright 1995).

The majority of ammonia produced in fishes and lampreys occurs within the liver through the deamination of amino acids, but other organs such as intestine, muscle and kidney also contribute (Mommsen and Walsh 1992; Wilkie et al. 2006). The majority of amino acids, which are L-amino acids, are transaminated by transaminase enzymes to form  $\alpha$ -ketoglutaric acid and L-glutamate. Successive oxidation of  $\alpha$ -ketoglutaric acid and L-glutamate via glutamate dehydrogenase in the mitochondrial matrix forms constituents that fuel the Krebs' cycle (pyruvate and acetyl CoA), but generate as  $\text{NH}_4^+$  as a by-product (Fig. 1.1; Forster and Goldstein 1969; Randall and Wright 1987).

Although many physiological processes such as gas exchange and ion balance are well understood in lampreys (Fange 1972; Morris 1972; Randall 1972; Beamish 1980; Wilson and Laurent 2002), the modes of nitrogenous waste excretion within this species remain unclear and have only recently been studied in detail (Blair 2012).

Ammonia exists as either un-ionized  $\text{NH}_3$  or ionized  $\text{NH}_4^+$ . Depending on external water chemistry, the  $\text{NH}_3/\text{NH}_4^+$  equilibrium shifts between  $\text{NH}_3$  and  $\text{NH}_4^+$ , but at physiological pH the majority of total ammonia exists as  $\text{NH}_4^+$  (Fig. 1.2; Wilkie 2002). In fishes, it was originally thought that ammonia passively diffuses out of the gills down the blood-to-water partial pressure  $\text{NH}_3$  ( $P_{\text{NH}_3}$ ) gradient (Wilson et al. 1994), and not ionized  $\text{NH}_4^+$ . It is now widely accepted that  $\text{NH}_3$  crosses the gills via gas channels called Rhesus glycoproteins (Rh glycoproteins), and it is trapped as  $\text{NH}_4^+$  in the gill-water boundary layer by  $\text{H}^+$  arising from the hydration of  $\text{CO}_2$  in that region, or  $\text{H}^+$  excretion by apical  $\text{H}^+$ -ATPases (Weihrauch et al. 2009; Wright and Wood 2009; Fig. 1.3, Fig. 1.4).

The first observations that Rh glycoproteins were associated with ammonia transport occurred in yeast and *Arabidopsis* (Mep1 and Amt1 respectively) (Marini et al. 1994; 1997). Sequence alignment of these  $\text{NH}_4^+$  transporters identified very high similarity between Mep1 and Amt1 and the well-known Rh50 family of human erythrocyte proteins (Ridgewell et al. 1992; Marini et al. 1997). Further analysis revealed that vertebrates possess a common gene family consisting of *RhAG*, *RhBG*, *RhCG*, and that fish contain an extra copy of *RhCG* (Huang and Peng 2005). Subsequent studies on rats and mice indicated that Rh glycoprotein protein expression was localized to organs involved in N-waste excretion (Liu et al. 2000; Liu et al. 2001).

In aquatic organisms, similar Rh glycoproteins were found to be up-regulated in instances where diffusion alone could not keep up with the pace of ammonia production and/or uptake from the environment (Nawata et al. 2007; Hung et al. 2008; Sashaw et al. 2010). It is now recognized that the paralogues Rhag, Rhbg, and Rhcg, are thought to work in series, facilitating movement of ammonia across gills (Wright and Wood 2009).



Rhag is found on erythrocyte membranes and facilitates the movement of ammonia into the plasma. Rhbg is located on the basolateral side of gill epithelial cells and promotes the uptake of ammonia from the plasma into the gill. Finally, Rhcg proteins are situated on the apical surface of the gill epithelial cells enabling the transport of ammonia from inside the gill cells to the surrounding water. Expression of these proteins has been observed in several diverse aquatic organisms including crab (*Carcinus maenas*), mangrove killifish (*Kryptolebias marmoratus*), puffer fish (*Takifugu rubripes*), zebra fish (*Danio rerio*) larvae and rainbow trout (*Oncorhynchus mykiss*) (Weihrauch et al. 2004; Perry et al. 2010; Hung et al. 2007; Nakada et al. 2007a; Nakada et al. 2007b; Nawata et al. 2007).

Another strategy to cope with increased levels of ammonia is to convert it into a less toxic waste product such as urea. In vertebrates, there are two main pathways by which urea is produced; the ornithine-urea cycle (OUC), which leads to the hydrolysis of arginine, or the uricolytic pathway. Since sea lampreys do not contain all of the necessary components for the OUC, the majority of urea they produce is through uricolysis, in which uric acid arising from purine degradation is converted to urea (Goldstein and Forster 1965; Wilkie et al. 1999), or from the hydrolysis of dietary arginine (Fig. 1.5; Wilkie et al. 2004; 2006). Once thought to readily move across membranes passively, it is now known that urea movement across epithelia takes place using specialized transporters or channels (Sands 1999).

The dipolar structure of the urea molecule, along with its low olive oil-water partition gradient, suggests that it cannot easily pass through the phospholipid bilayers of gill epithelial cells (Wilkie 2002). Recent work indicates that many fish use facilitated

urea transporters (UTs), to excrete the urea ( $J_{\text{Urea}}$ ) across the gills (Smith and Wright 1999; Walsh et al. 2001). This excretion of urea in gill epithelia depends on the  $\text{Na}^+/\text{K}^+$  ATPase to set up an ion gradient allowing for the co-transport of urea and  $\text{Na}^+$  out of the fish and into the surrounding water (Wilkie 2002; Fig. 1.6). UTs have been observed in a wide variety of marine and fresh water aquatic species, including European eel (*Anguilla anguilla*), Lake Magadi tilapia (*Alcolapia grahami*), and rainbow trout (Mistry et al. 2001; Walsh 1997; McDonald and Wood 1998), but not in lampreys. A central goal of this thesis was to determine if basal vertebrates such as sea lamprey also use UTs and Rh glycoproteins to rid their bodies of N-waste and to determine if excretion patterns are related to changes in life stage and diet.

### *1.5 Effects of changes in water chemistry, life stage and feeding on nitrogenous waste production and excretion:*

The amount of ammonia produced and excreted by fish is subject to both extrinsic and intrinsic factors. The external environments lampreys are exposed to are not static. Situations may arise, such as an increase in environmental ammonia or pH, that inhibit or prevent ammonia excretion by decreasing the  $\text{NH}_3$  diffusion gradient down which ammonia exits the gill (Wright and Wood 1985; Wilkie and Wood 1991; Sashaw et al. 2007). Moreover, the life stage of *P. marinus* can also greatly influence the amount of nitrogenous waste produced and excreted. During the parasitic phase of sea lampreys, high rates of blood ingestion (up to 30% body weight/day) increase protein uptake, and therefore the rate of amino acid deamination (Farmer et al. 1975; Wilkie et al. 2004;

Wilkie et al. 2006). Thus, *P. marinus* likely employ physiological strategies to tolerate or prevent build-ups of internal ammonia concentrations when feeding at such high rates.

Current evidence suggests that the sea lampreys' capacity to excrete ammonia greatly increases following metamorphosis, when they are likely to be ingesting large amounts of protein as they prey on teleosts, and even elasmobranchs (Wilkie et al. 2004) and cetaceans (Nichols and Hamilton 2004). Indeed, the activity of glutamate dehydrogenase increases several-fold following metamorphosis (Wilkie et al. 2006). More impressively, ammonia excretion rates increase by 15-25 fold following feeding by lampreys (Wilkie et al. 2004). Another goal of the present thesis was to determine if these increases in ammonia production and excretion rates following metamorphosis were accompanied by increases in Rh glycoprotein expression in the gills and skin of lampreys. These measurements were coupled with experiments designed to determine how ammonia is being excreted across the gills following feeding, and in response to environmental challenges such as high external ammonia (HEA) and alkaline pH.

Lampreys also exhibit a very high capacity to excrete urea, which increases 10-15 fold following feeding on rainbow trout (Wilkie et al. 2004). Increases in  $J_{\text{Urea}}$  of up to 450 times in lampreys have been observed when animals are removed from basking sharks (*Cetorhinus maximus*: Wilkie et al. 2004), which retain high levels of urea in their blood to maintain osmotic balance in sea water (Hammerschlag 2006). Urea transport proteins have not yet been identified in lampreys, although fragments of putative UT genes have been uncovered through searching the lamprey genome. Another major goal of this thesis was to therefore determine if UT expression increases in the lamprey

following metamorphosis, and after the fish experience increased urea loads due to feeding.

### *1.6 Hypothesis and Research Objectives:*

Parasitic sea lampreys ingest greater amounts of protein than do their larval, metamorphosing and upstream migrant counterparts. It was therefore hypothesized that their capacity to tolerate and excrete nitrogenous wastes arising from protein catabolism was greater compared to other life stages (Wilkie et al. 2004). To test this hypothesis, a wide array of experiments to elucidate both the capacity and mechanisms used by several key life stages of sea lampreys (larval, metamorphic, parasitic, and up-stream migrant) to excrete ammonia and urea were undertaken. The specific objectives of this thesis were to:

1. Determine the mechanisms by which nitrogenous wastes are excreted ( $J_{\text{Amm}}$  and  $J_{\text{Urea}}$ ) at different stages of the sea lamprey life cycle.
2. Ascertain how the ammonia and urea excretion capacity change during the life cycle of the sea lamprey.
3. Determine the role of Rh glycoprotein and UT abundances with respect to N-waste excretion during the life cycle of the sea lamprey.
4. Investigate how N-waste accumulation and excretion changes during the metamorphic period of the sea lamprey life cycle.

To investigate the following objectives, several experimental and analytical techniques were employed. Firstly, both  $J_{\text{Amm}}$  and  $J_{\text{Urea}}$ , as well as internal concentrations of ammonia and urea, were determined using both

colorimetric and enzymatic based assays on sea lamprey of different life stages, and in sea lampreys exposed to chemically modified water conditions. These experiments were intended to provide insight into the observed changes in excretion patterns and capacity during several life stages, and provided a physiological and mechanistic basis for the observed changes in  $J_{\text{Amm}}$  and  $J_{\text{Urea}}$  in sea lamprey. Secondly, protein quantification of Rh glycoprotein and UT were done to shed light on the mechanism(s) used by lampreys to excrete urea and ammonia. Western blot analysis of Rh and UT proteins, along with some sequence analysis of an amplified cDNA of the UT were used to determine how the expression patterns of these excretory proteins changed over the complex life cycle of the sea lamprey, and following feeding by juvenile, parasitic lampreys.

## **FIGURES:**

Fig.1.1. Deamination of amino acids in fish, which primarily occurs in the liver. The ingestion of protein rich foodstuffs likely increases the rates of transamination and deamination in lampreys.

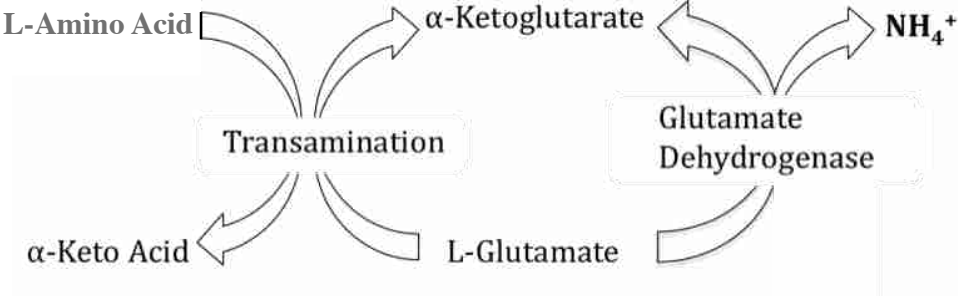


Fig.1.2. Equilibrium of ammonia (pKa of 9.5 at 15°C). Under normal conditions (15 °C and pH= 7.6) this equation is pushed far left.



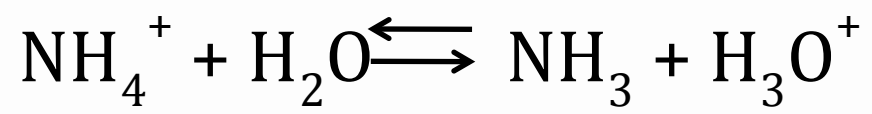


Fig. 1.3. Diffusion of ammonia out of freshwater fish. Normally, the  $\text{CO}_2$  excreted across the gill is hydrated to  $\text{HCO}_3^-$  and  $\text{H}^+$ , which acidifies the unstirred boundary layers next to the apical membrane of the gill. The intracellular enzyme, carbonic anhydrase (CA), catalyzes the hydration of  $\text{CO}_2$  in the gill cytosol, also leading to the generation of  $\text{H}^+$  in the cytosol, which is then thought to be pumped across the apical membrane by a  $\text{H}^+$ -ATPase, which further acidifies the gill boundary layer. As a result,  $\text{NH}_3$  passively diffuses down its  $P_{\text{NH}_3}$  gradient via Rh glycoproteins, and  $J_{\text{Amm}}$  is sustained due to  $\text{H}^+$  trapping of the  $\text{NH}_3$  in the gill boundary layer, where the  $\text{NH}_3$  is converted to  $\text{NH}_4^+$ .

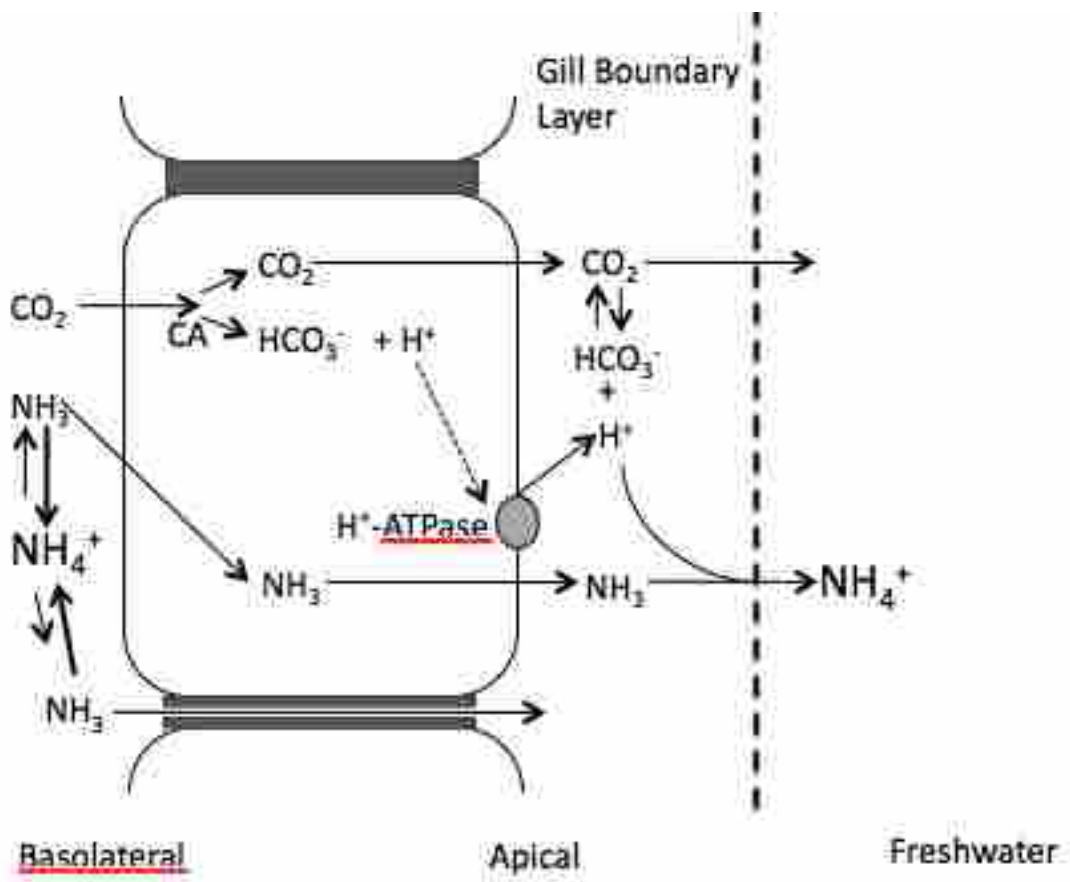
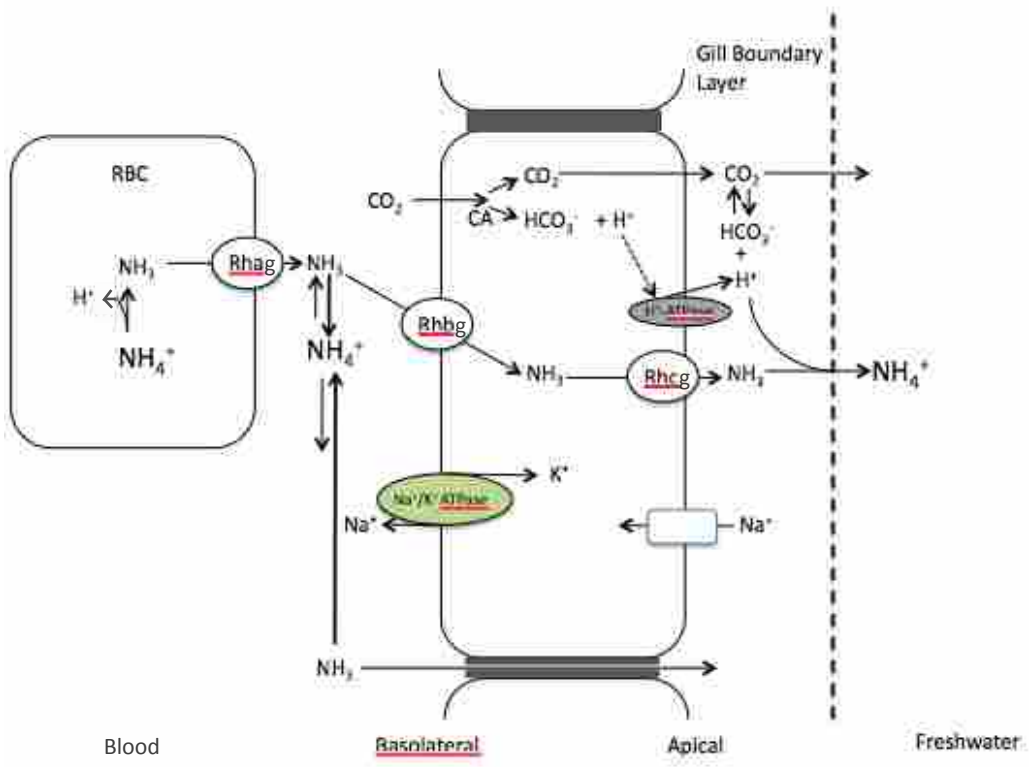


Fig. 1.4. Model illustrating how ammonia is thought to be transported across the fish gills. Rh proteins – Rhag (in erythrocyte membranes), Rhbg (in the basolateral membranes of branchial epithelial cells), and Rhcg (in the apical membranes of branchial epithelial cells) – facilitate the excretion of ammonia from blood to water via the gills of freshwater fishes. Based on Wright and Wood (2009) and Weihrauch et al. (2009).



Blood

Basolateral

Apical

Freshwater

Fig. 1.5. Production of urea by arginase. This process can arise following ingestion of dietary arginine and/or during times of muscle proteolysis.

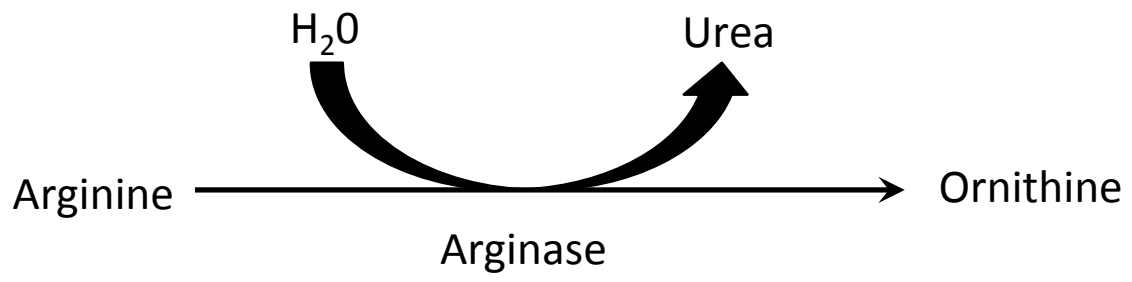
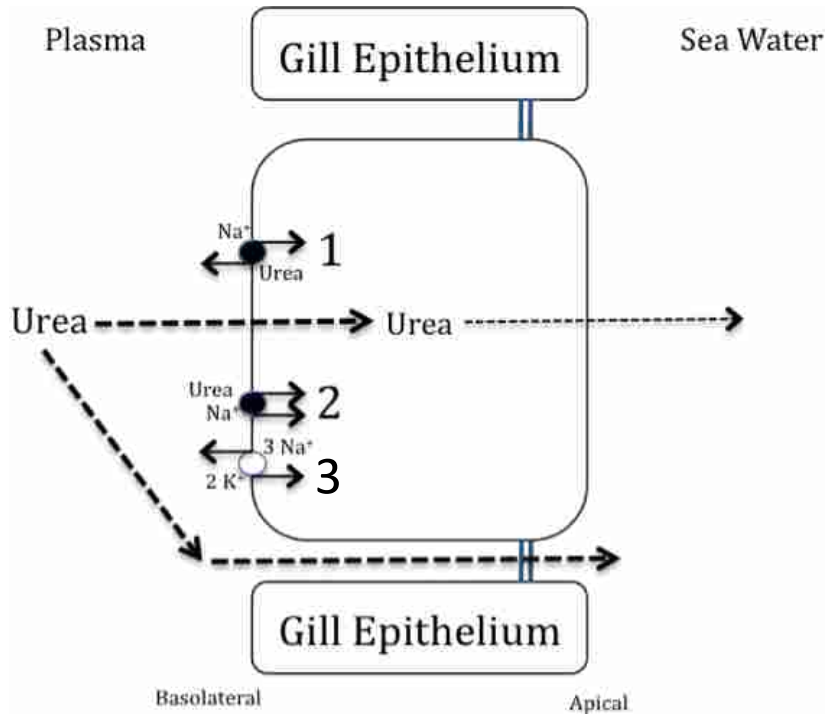


Fig. 1.6. Urea handling within a fish gill. This diagram outlines two possible urea transporters: (1)  $\text{Na}^+$ : urea antiporter or (2) a  $\text{Na}^+$ - urea facilitated transporter. The latter is more likely involved in the excretion of urea and is dependent on (3) a  $\text{Na}^+/\text{K}^+$ -ATPase to set up an ion gradient such that  $\text{Na}^+$  and urea can be transported across the basolateral membrane.





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## **Chapter 2:**

### **Plasticity of Urea Excretion and Urea Transporter Abundance During the Complex Life Cycle of the Sea Lamprey (*Petromyzon marinus*)**

## ABSTRACT

Ammonia excretion ( $J_{\text{Amm}}$ ), and urea excretion rates ( $J_{\text{Urea}}$ ) were determined in several distinct lifestages of sea lamprey (*Petromyzon marinus*) exposed to both highly alkaline water (pH 9.5) and high external ammonia (HEA:  $0.5 \text{ mmol}\cdot\text{L}^{-1}$ ). Though ammocoete and adult sea lamprey were unable to tolerate the highly alkaline water for greater than 24 h, each readily survived HEA. In both ammocoete and adult lampreys, respective plasma ammonia levels increased by 2-fold and 6-fold after 2d. These increases in plasma ammonia were due to a complete reversal of  $J_{\text{Amm}}$ , resulting in a net uptake of ammonia by both ammocoetes and adult lampreys during HEA. Ammocoetes, but not adults, were able to gradually restore  $J_{\text{Amm}}$  to control levels after 2 days.  $J_{\text{Urea}}$  on the other hand was significantly different only in adult lamprey, where it remained elevated by 2-3 fold during the entire 48 h exposure. Using primers for a conserved region of urea transport proteins (UT), amplification of a 109bp fragment was accomplished with sea lamprey gill tissue. Subsequent western blot analysis with zebrafish (*Danio rerio*) UT antibody revealed expression of the UT protein in several tissues of sea lamprey including; gill, skin, kidney and muscle. Life stage differences in UT protein were observed with ammocoetes having the greatest proportion of UT localization in the gills, where UT Abundance was 2-6 fold greater than in the other life stages. However, UT was also expressed in the skin, with UT abundance being greatest in the skin of the adults, where abundance was 8-fold greater than in the skin of other life stages. For the first time, this study provides evidence for UT proteins in sea lampreys. Moreover, UT abundance was influenced by life stage, suggesting that in activity level,

body plan, environmental conditions and feeding habits influence the sites and mechanisms of N-waste excretion in sea lampreys.

## INTRODUCTION

Over the last decade, our understanding of how nitrogenous wastes (N-wastes) are produced and handled by aquatic vertebrates and invertebrates has revealed that the two main products, ammonia and urea, are excreted using specialized Rh glycoproteins and urea transporters (UT), respectively (Weihrauch et al. 2009; Wright and Wood 2009). The UT appears to be a  $\text{Na}^+$ :Urea co-transporter located on the basolateral membrane of the branchial epithelium that facilitates the outward diffusion of urea (Walsh 1997; McDonald et al. 2006). The Rh glycoproteins on the other hand are thought to function as gas channels, through which  $\text{NH}_3$  diffuses down favorable partial pressure gradients across the gills and into the water (Wright and Wood 2009).

There are three types of Rh glycoproteins in the fish gill. The first, Rhag is found on erythrocytes, and facilitates  $\text{NH}_3$  unloading from the blood, whereas Rhbg and Rhcg are found on the basolateral and apical membranes of the gills (Weihrauch et al. 2009; Wright and Wood 2009). It has recently been proposed that Rhcg works in close association with apical  $\text{H}^+$ -ATPases,  $\text{Na}^+$  channels and possibly the  $\text{Na}^+/\text{H}^+$ -exchanger (NHE), as part of a metabolon in which the  $\text{NH}_3$  that passes through the Rhcg, is trapped as  $\text{NH}_4^+$  by  $\text{H}^+$  extruded by V-ATPases (proton pumps) associated with the metabolon (Wright and Wood 2009).

Alterations in UT and Rh glycoprotein abundance and distribution in fishes have been noted when ammonia excretion is inhibited in response to changes in water chemistry (greater pH), high external ammonia exposure (HEA) or air exposure (Sashaw et al. 2010; Braun et al. 2009; Zimmer et al. 2010; Nawata et al. 2010). However, less is known about the plasticity of urea and ammonia excretion and transporter abundance and



distribution following feeding, although Zimmer et al. (2010) demonstrated that natural increases in plasma ammonia, such as those encountered post-feeding, were observed to increase Rh mRNA expression. Early development is also a period in which nitrogenous waste excretion and associated protein expression patterns have recently been investigated. Braun et al. (2009) demonstrated that UT-mediated urea excretion predominated in embryonic zebrafish (*Danio rerio*), until hatching when there was a surge in ammonia excretion that was associated with greater Rhag, Rhbg and Rhcg1 expression. Few other studies, however, have examined the ontogeny of UT and Rh glycoprotein mediated nitrogenous waste excretion in fishes. Moreover, there is uncertainty about how conserved these mechanisms of nitrogenous waste excretion are in the vertebrates, in particular the extant jawless fishes, represented by the hagfishes (Myxinae) and lampreys (Petromyzontidae).

Ammonia and urea excretion ( $J_{\text{Amm}}$  and  $J_{\text{Urea}}$ ) patterns have been well studied in the sea lamprey, *Petromyzon marinus* (Wilkie et al. 1999; Wilkie et al. 2004; Wilkie et al. 2006), however the mechanism by which N-wastes are excreted is still unclear. The Rh glycoproteins have been isolated in a variety of tissues in lampreys including the gills and skin (Blair 2011), but the possible presence of UT transport proteins has not been investigated. However, UT and Rh glycoproteins were recently described in the Pacific hagfish (*Eptatretus stoutii*; Braun and Perry 2010). The goal of the present study was to relate the changes in  $J_{\text{Amm}}$  and  $J_{\text{Urea}}$  and the mechanisms of excretion to the pronounced changes in habitat, diet and internal and external anatomy that characterize the complex life cycle of the lampreys.

Sea lampreys begin their multi-staged lifecycle as larvae called ammocoetes that

live burrowed in the substrate of streams for 3- 7 years feeding on detritus and suspended matter (Sutton and Bowen 1994). The ammocoete phase is then followed by a true metamorphosis lasting approximately 3-4 months (Youson 1980; 2003), which culminates in the transformation of the ammocoetes into juvenile lampreys that subsequently enter a parasitic phase in which they attach themselves to, and feed on the blood of teleost fishes (Beamish and Potter 1975; Farmer 1980; Renaud et al. 2009), elasmobranchs (Wilkie et al. 2004), and even cetaceans (Nichols and Hamilton 2004). After approximately 12-20 months, the adults cease feeding, sexually mature, and migrate upstream to spawn and eventually die (Beamish and Potter 1975; Wilkie 2011).

Due to the marked changes in morphology, feeding habits, and habitat of the sea lamprey, we hypothesized that changes in N-waste production and excretion patterns would be reflected by changes in  $J_{\text{Urea}}$  and UT protein abundance, and to increases or decreases in internal ammonia and urea concentration in the tissues of lampreys at each of the 4 major stages of their life cycle, ammocoetes, juvenile, parasitic juvenile, and adult. Because ammocoetes excrete physiologically relevant levels of urea (Wilkie et al. 1999), the major goal of this study was to test the hypothesis that UT protein abundance would be greatest during this burrow-dwelling life-stage when they would likely have greater difficulty excreting ammonia due to a lack of water flow through the burrows. Indeed, the sand particles comprising the wall of the ammocoete burrow are thought to be cemented together by mucus to prevent collapse of the burrow (Sterba 1962; Beamish and Jebbink 1994). For this reason irrigation of the gills with food-laden, oxygenated water can only take place when the oral hood of the animals extends above the substrate-water column interface (Hardisty and Potter 1971), with the expired water being ejected

into the surrounding burrow. As a result, N-wastes including ammonia may be higher in this microenvironment, resulting in greater difficulty off-loading ammonia via the gills.

Previously, Wilkie et al. (1999) demonstrated that urea production was stimulated in ammocoetes during HEA, but they did not look at other life stages of the sea lamprey. A stimulation of  $J_{\text{Urea}}$  has also been reported in some teleosts including goldfish (*Carassius auratus*) and rainbow trout (*Oncorhynchus mykiss*) during HEA (Fromm and Gillette 1968; Wilkie et al. 2011) and at alkaline pH (Wilkie and Wood 1991; Walsh et al. 1990). To determine the relative importance of urea production in ammocoetes and adult lampreys, we first measured basal  $J_{\text{Amm}}$  and  $J_{\text{Urea}}$  in each life stage, followed by HEA or highly alkaline water (pH 9.5) exposures to determine if the lamprey's reliance on  $J_{\text{Urea}}$  increased when  $J_{\text{Amm}}$  was inhibited.

The sites of ammonia and urea excretion were previously determined using a divided flux chamber for ammocoetes, and by renal catheterization for the much larger adult lampreys (Wilkie Lab 1999). The potential role of the UT in facilitating urea excretion at different life stages was also determined by generating a partial clone of the lamprey UT, and using a UT-specific antibody to localize and quantify UT abundance in ammocoetes, juvenile (non-feeding), parasitic juvenile and adult sea lampreys.

## **MATERIAL AND METHODS**

### ***Experimental Animals and Holding***

Adult sea lampreys (*Petromyzon marinus*), were obtained courtesy of R. McDonald, at the Department of Fisheries and Oceans Canada (DFO) Sea Lamprey Control Center (Sault St. Marie, Ontario). Ammocoetes were collected by pulsed DC electrofishing from streams flowing into the North Humberland Strait, New Brunswick (DFO scientific collection permit SG-NBT-12-110 and SG-NBT-11-098). A subset of the New Brunswick animals underwent metamorphosis in the laboratory, which provided a pool of juvenile and parasitic juvenile sea lampreys that were used to determine how UT abundance in the gills changed with life stage in these animals. The ammocoetes were held in 70 L aquaria filled with a layer (4-5 cm deep) of sand that provided burrowing substrate for animals, and the ammocoetes were fed baker's yeast (1 g/larvae; Holmes and Youson 1994) on a weekly basis (Fleischmanns, St. Louis, MO). Metamorphosing lampreys were held in static and aerated 50-60 L aquaria in groups of 15-20, in which the water was changed weekly, and without feeding. Following the completion of metamorphosis (3-4 months), the newly transformed juvenile parasitic lampreys were allowed to feed on rainbow trout weighing 100-500 g that were purchased from Rainbow Springs Hatchery (Thamesford, ON), and held in 100-500 L flow-through tanks, which received a constant flow of aerated well-water (dissolved O<sub>2</sub> of approximately 80-100% saturation). These rainbow trout were fed three times a week to satiation using sinking pellets (3.0 Corey Feed Mills, Elmira, ON). Upstream migrant lampreys do not feed, and were housed in 700 L Living Streams (Frigid Units Inc., Toledo, OH) receiving aerated well-water on a flow through basis. Animals were all held under a 12 h light/12 h dark

photoperiod. All experiments were conducted in accordance with the guidelines of the Canadian Council on Animal Care and were approved by the Wilfrid Laurier University Animal Care Committee.

### ***Experimental Protocols***

#### *Experiment 1: Effect of high pH and high environmental ammonia (HEA) on $J_{Amm}$ and $J_{Urea}$*

To investigate whether the inhibition of  $J_{Amm}$  in sea lampreys resulted in a greater reliance on  $J_{Urea}$ , animals (n=10 for ammocoetes; n=8 for adults) were exposed to either an elevated pH ( $\approx 9.5$ ) or high external ammonia (HEA;  $0.5 \text{ mmol}\cdot\text{L}^{-1} \text{ NH}_4\text{Cl}$ ) for 2 days. It was hypothesized that nitrogenous waste excretion would decrease as external pH and ammonia increased if passive  $\text{NH}_3$  diffusion was the major mechanism of  $J_{Amm}$ , and that this would be followed by compensatory increases in  $J_{Urea}$ . Accordingly, ammocoetes and adult sea lampreys were held in opaque flux chambers with volumes of 0.200 L for larval lamprey (1.5-3.0 g) and 5.0 L for the much larger (100-200 g) adult lampreys. The chambers holding the ammocoetes received a constant flow of water near  $0.1 \text{ L}\cdot\text{min}^{-1}$ , but flow to the larger chambers holding adults was  $0.5\text{-}1.0 \text{ L}\cdot\text{min}^{-1}$  of well-water (pH =8; T=11°C; DO<sub>2</sub> 80-100%) draining from a head tank, and distributed to each chamber using a flow-splitter. After an overnight acclimation period, control  $J_{Amm}$  and  $J_{Urea}$  were determined over 4 h, followed by exposure to water of pH 9.5, or  $0.5 \text{ mmol}\cdot\text{L}^{-1}$  external ammonia. Prior to each  $J_{Amm}$  and  $J_{Urea}$  measurement period, water flow to each chamber was cut -off, and the volume of each chamber was adjusted (to approximately 20-30

times the mass of the larvae (100 mL) or adult sea lamprey (3.0 L)), after which water samples (5 mL) were taken at 0 h, 2 h, and 4 h of each flux measurement period.

Maintenance of water pH for the high pH experiment was achieved using an autotitrator (TT80 Autotitrator, Radiometer, Copenhagen, DM) connected to a PHM84 pH meter fitted with a GK2401C pH Electrode (Radiometer). When water pH fell below pH 9.5, the autotitrator activated a solenoid valve that opened, leading to the drop-wise addition of 0.2M KOH into the head-tank from a 10 L polypropylene carboy.

Maintenance of pH within the chambers during the flux periods was achieved by independently measuring the pH using an external pH meter and electrode (Oakton 11 series, Eutech instruments, Singapore) at regular intervals and manually adding drops of 1M KOH using a disposable, polypropylene Pasteur pipet. Between flux periods water flow was then re-established to each flux chamber.

The elevation of water ammonia concentration in the HEA series was achieved through the addition of the appropriate amounts of  $\text{NH}_4\text{Cl}$  ( $0.1 \text{ mol}\cdot\text{L}^{-1}$ ) to the water, and water pH was maintained at pH 8.0 using the autotitrator system. It should be noted that the addition of  $\text{Cl}^-$  ion through dissociation from ammonia had a negligible effect on the overall system water quality.

Upon completion of all the flux periods (2 d), each sea lamprey was lightly anesthetized using  $0.5 \text{ g}\cdot\text{L}^{-1}$  MS222 buffered with  $1.0 \text{ g}\cdot\text{L}^{-1}$   $\text{NaHCO}_3$ . After approximately 5 minutes in the anaesthetic dose, lampreys were then euthanized with an overdose of MS222 ( $1.5 \text{ g}\cdot\text{L}^{-1}$  MS222 buffered with  $3.0 \text{ g}\cdot\text{L}^{-1}$   $\text{NaHCO}_3$ ). Blood samples were collected from larval lampreys in a drip wise fashion after making an incision through the heart and collecting drops of blood into a 500  $\mu\text{L}$  centrifuge tube. Blood

collection from the adults was via caudal puncture, using a 28 G needle connected to a 1 mL disposable syringe pre-rinsed with heparin ( $\text{Na}^+$  heparin; SigmaAldrich, St. Louis, MO) syringe (18G). The blood collected was centrifuged at 10,000xg for 3 minutes in a microcentrifuge (Eppendorf 5415D, Hamburg, Germany), and the plasma transferred to 0.5 mL centrifuge tubes, and frozen in liquid nitrogen. An incision down the mid-ventral line exposed the liver, kidney and intestine, which were removed and quickly snap frozen. The remaining skin and muscle from the trunk of the animal were separated and snap frozen. Gill filaments were dissected under slight magnification by an initial anteroventral incision, removal of the filamentous tissue, and snap freezing in liquid  $\text{N}_2$ . All tissue samples were stored at  $-80^\circ\text{C}$  until analyzed. Water samples were frozen at  $-20^\circ\text{C}$  until analyzed for water ammonia and urea concentration.

*Experiment 2: Effects of Sea Lamprey Life Stage and Parasitism of Fishes on UT Expression and Nitrogen Excretion*

To determine how UT abundance changed with life stage in the sea lamprey, basal rates of  $J_{\text{Urea}}$  and  $J_{\text{Amm}}$  were measured in ammocoetes, juvenile, and adult sea lampreys, followed by blood and tissue collection as described above. However, an accompanying feeding study was undertaken to determine how increased postprandial (following feeding) ammonia and urea-loads influenced the abundance of UT proteins in parasitic juvenile lampreys. In this study, the parasitic juvenile lampreys were fed rainbow trout (~150-200 g trout per parasitic juvenile) for 2-3 weeks, during which time the lamprey grew from approximately 2.5-3.0 g to approximately 10.0 g following the feeding period. At the conclusion of the feeding period, the trout with the lamprey still

attached, were gently transferred to 10 L of aerated water, containing  $0.1 \text{ g}\cdot\text{L}^{-1}$  of tricaine methanesulfonate (MS222) buffered with  $0.2 \text{ g}\cdot\text{L}^{-1}\text{NaHCO}_3$ . Almost immediately, the parasitic lampreys detached from the animal and were used for either determinations of post-feeding  $J_{\text{Amm}}$  and  $J_{\text{Urea}}$ , or euthanized with a lethal dose of anaesthetic ( $1.0 \text{ g}\cdot\text{L}^{-1}$  tricaine methanesulfonate). Blood, gills, kidney, muscle, liver, intestine and skin of the parasitic lampreys were then collected, and feeding was verified by examining the gut contents for the presence of partially digested blood, which appeared as a black emulsate in the intestine. The blood was collected into  $100 \mu\text{L}$  hematocrit tubes, and centrifuged at  $3,500 \times g$  for 5 minutes, to pack the red blood cells. The plasma was then drawn off, transferred to  $500 \mu\text{L}$  centrifuge tubes and, along with the tissues, snap-frozen in liquid  $\text{N}_2$  and then stored at  $-80^\circ\text{C}$  until analyzed. Subsets of the gill, skin, kidney and intestine were also stored in 70% ethanol to be used in a separate study for immunohistochemistry analysis.

## ***Analytical Methods***

### *Ammonia and Urea Determination*

Water ammonia concentration was determined spectrophotometrically using the salicylate hypochlorite colorimetric assay, which in the presence of ammonia forms a blue indophenol with an optimal absorbance at  $650\text{nm}$  (Verdouw et al. 1978). Plasma ammonia concentrations were determined enzymatically using glutamate dehydrogenase, which catalyzed the oxidation of NADH at  $340\text{nm}$  (Sigma Aldrich, AA0100). Water and plasma urea concentrations were determined colorimetrically using ferric chloride, diacetyl monoxime and thiosemicarbazide, which produces a pink chromogen in the



presence of urea, that is quantified at 525nm (Rahmatullah and Boyde 1980). Each of these spectrophotometric assays was conducted using a Molecular Devices Spectramax190 plate spectrophotometer (Molecular Devices, Sunnyvale, CA). To ensure confluence throughout each assay, linearity of standard curves were only accepted with and  $r^2 > 95\%$ . All absorbances were taken at equal time intervals with water ammonia measured 90 min following reagent mixing, whereas plasma ammonia was measured at 0 min and 15 min following mixture of reagents. Urea absorbance's were taken following a 30 min cooling period of the samples after 15 min of boiling the sample and reagent mixture. In all cases if samples fell out of the range of the standard curve, appropriate dilution of the samples (10x or 25x) were done to ensure absorbance's fell within experimental range.

#### *RNA isolation and cDNA synthesis*

Gill tissue from sea lamprey was ground with a mortar and pestle under liquid nitrogen prior to RNA extraction. The total RNA was then obtained from the ground tissue (100 mg) using 1 mL TRIzol Reagent (Invitrogen) according to the manufacturer's protocol, and then dissolved in nuclease-free water and quantified using a NanoDrop ND 1000, and then stored at  $-80^{\circ}\text{C}$ . Before synthesis of cDNA, 1 $\mu\text{g}$  of sample was treated with amplification grade DNase I (Invitrogen, 18068-015, Grand Island, NY) according to the manufacturers protocol, and used to make cDNA using MultiScribe Reverse Transcriptase (Invitrogen, 4311235, Grand Island, NY) in conjunction with an Oligo-dT primer (Invitrogen, AM5730G, Grand Island, NY).

### *Polymerase chain reaction*

Degenerate primers (Fig.2.1), designed according to conserved regions of *UT* sequences, were used to amplify a target sequence of approximately 110 bp. The polymerase chain reaction (PCR) used 15  $\mu$ L in a gradient cycler (Eppendorf, 5331, Hamburg, DE), with PCR reagent concentrations for all reactions of 2 mmol L<sup>-1</sup> MgSO<sub>4</sub>, 200  $\mu$ mol L<sup>-1</sup> dNTP mix, 200 nmol L<sup>-1</sup> of each primer and 1 unit of *Taq* DNA polymerase. The thermal cycling parameters included initial incubation at 95°C (5 min) allowing activation of *Taq* polymerase followed by 35 cycles of 95°C (1 min), 60.5°C (1 min), 72°C (1 min), with a final extension time of 72°C for 5 min. Products were held at 4°C until analysed using gel electrophoresis (1.2% agarose in 1 × TAE buffer, 40 mM Tris acetate, 1 mM EDTA + 10  $\mu$ L RedSafe DNA Stain) and visualized under UV light. Subsequently, bands were excised from the gel, purified and sequences were then determined at the University of Guelph Bioinformatics lab (Guelph, Ontario).

### *Sequence Alignment and Identities*

cDNA sequence alignment and identifications were achieved using ClustalX, whereas determination of amino acid sequence was done using ExPasy (<http://www.expasy.org/proteomics>). Subsequent alignment and scoring of amino acid sequences against known UT fragments (Accession numbers: AF278537.1; AF165893.2; NM\_001020519.1; AB470074.1; AF257331.1; AB049726.1) was done using Clustal Omega (<http://www.ebi.ac.uk/Tools/msa/clustalo/>).

### *Antibodies and Western Blot Analysis*

Affinity purified polyclonal antibodies to *Danio rerio* (Zebrafish) UT (accession no. AY788989.1; amino acids 48-69) were a gift from SF Perry (U. of Ottawa), and used for western blot analysis to localize and quantify UTs in ammocoetes, juveniles and adult lampreys.

Snap frozen tissues were homogenized in ice-cold buffer solution containing 50 mM Tris pH 7.5 with protease inhibitor (Roche, Laval, QC) using a hand held probe sonicator, briefly (> 10 s) at 80% power (Fisher Scientific, XL2000-350R, Ottawa, ON). Homogenized samples were centrifuged at 16,000xg for 3 minutes at 4°C, and the supernatant transferred to a clean 1.5 mL centrifuge tube from which protein quantification was determined using the bicinchoninic acid (BCA) protein assay (Sigma Aldrich, BCA1; Smith et al. 1985). For samples that contained excess lipid, a secondary centrifugation period was needed to allow for preliminary removal of lipid layer. In all cases, protein from zebrafish gill was used as a positive control. Exactly 20 µg of zebrafish gill protein and 40 µg of lamprey tissue protein were loaded into each lane of a 12% sodium dodecyl sulfate (SDS) polyacrylamide gel and electrophoresed for 40 minutes at 200V (Biorad 165-5052; 165-3301, Hercules, CA). The protein size marker used was obtained from GeneDirex (PM007-0500, Las Vegas, NV). Following electrophoresis, both gel and nitrocellulose membranes were incubated for 30 minutes in 20% methanol transfer buffer. Transfer of protein from the gel to the nitrocellulose membrane was achieved using a semi-dry unit run at 20 V for 27 minutes (Biorad 170-3940, Hercules, CA). Membranes were then treated with Ponceau's stain for 5 minutes to determine transfer efficiency, followed by subsequent washing (3 washes, 10 minutes per wash)

with 1x Tris-buffered saline containing 0.1% Tween 20 (1x TTBS). Blocking of the membranes was done using 5% skim milk with 0.05% sodium azide in 1x TTBS. After membrane blocking, a 2 h incubation with the primary UT antibody was done (1:500 UT dilution), followed by washing with 1x TTBS (3 washes, 10 minutes per wash), and incubation in peroxidase-conjugated secondary anti-rabbit Ig (1:3000 dilution; Bio-Rad, 170-6515, Mississauga, ON) for 1 hour. After discarding the secondary antibody, membranes were washed with 1x TTBS (3 washes, 10 minutes per wash) and detection using enhanced chemiluminescence was performed (GE Healthcare, RPN2132, Baie d'Urfe, QC). Following detection of UT, blots were washed with 1x TTBS (3 washes, 10 minutes per wash). Each blot was then incubated for 1h in the dark using a primary  $\beta$ -actin antibody conjugated with Cy3 fluorescence tag (C5838; Sigma Alderich; St. Louis, MO). Relative quantification of protein expression was performed by scanning in images of nitrocellulose on Quantity One software (Bio-Rad, Hercules, CA), and size and density of the bands were analyzed using GelEval software (FrogDance software, Dundee, UK). To control for any variation in banding due to protein loading, densities of the UT bands were normalized to those of  $\beta$ -actin.

### *Calculations*

Whole body and extra-renal nitrogenous waste excretion rates were calculated using the following equation:

$$J_{\text{N-waste}} = \frac{([\text{N-waste}]_{\text{initial}} - [\text{N-waste}]_{\text{final}})(V)}{(M)(\Delta T)}$$

Where  $J_{\text{N-waste}}$  refers to rate of ammonia or urea excretion in  $\text{nmol N}\cdot\text{g}^{-1}\cdot\text{h}^{-1}$ ,  $[\text{N-waste}]_{\text{initial}}$  is the concentration of ammonia or urea in water at the beginning of the flux determination period, and  $[\text{N-waste}]_{\text{final}}$  is the concentration of ammonia or urea at the end of the flux determination period; V is the volume of water in the flux chamber; M is the mass of each lamprey;  $\Delta T$  is the duration of the flux determination period.

### *Statistical Analysis*

Paired data, as with the HEA exposures, was analyzed using repeated measures ANOVA, followed by a Student Newman-Keuls post-hoc test. Unpaired data were analyzed using a standard ANOVA, followed by a Tukey's post-test. If a given data set did not meet all of the assumptions of standard ANOVA, a nonparametric ANOVA was used, followed by a Dunnett's post-test. The level to which all data was analyzed was  $P < 0.05$ , and all data are presented as the mean  $\pm 1$  SEM. Statistical analysis was performed using a commercially available software package (GraphPad InStat 3.0 or SPSS ver. 20.0).

## RESULTS

### *Effect of high pH and high external ammonia (HEA) on $J_{amm}$ and $J_{urea}$ in ammocoete and upstream migrant sea lampreys*

Ammocoetes and adult lamprey were unable to tolerate exposure to pH 9.5, which led to unexpected mortality in 24 h or less. As a result, no additional animals were exposed to pH 9.5 due to the lamprey's inability to tolerate the more alkaline water.

Ammocoetes readily tolerated exposure to HEA (nominal =  $0.5 \text{ mmol}\cdot\text{L}^{-1}$  ammonia; actual [ammonia] =  $0.56 \pm 0.017 \text{ mmol}\cdot\text{L}^{-1}$  ammonia) for 52h. Under control (pre-exposure to HEA) conditions  $J_{Amm}$  was  $-147 \text{ nmol N}\cdot\text{g}^{-1}\cdot\text{h}^{-1}$ , but following exposure to HEA there was net uptake of ammonia at all time periods (Fig. 2.1A). At 24 h and 48 h, however, the rates of net ammonia uptake were not significantly different from zero or the controls, suggesting that the fish were in net ammonia balance. There was no significant difference in  $J_{Urea}$  between control rates of approximately  $-30 \text{ nmol N}\cdot\text{g}^{-1}\cdot\text{h}^{-1}$  and those observed during the 52h exposure to HEA, which accounted for approximately 20 % of total nitrogenous waste excretion (Fig. 2.1B). This inhibition of  $J_{Amm}$  was accompanied by a marked increase in plasma ammonia, which increased 2-fold during HEA reaching  $450 \text{ }\mu\text{mol N}\cdot\text{L}^{-1}$  (Fig. 2.3A).

Similarly, adult sea lamprey exposed to HEA (nominal =  $0.5 \text{ mmol}\cdot\text{L}^{-1}$  ammonia; actual =  $0.52 \pm 0.013 \text{ mmol}\cdot\text{L}^{-1}$  ammonia) underwent a net uptake of ammonia over the entire 2 day exposure period. In control animals,  $J_{Amm}$  was approximately  $-300 \text{ nmol N}\cdot\text{g}^{-1}\cdot\text{h}^{-1}$ , but after exposure to HEA, there was a net uptake of ammonia at all time periods (Fig. 2.2A).  $J_{Urea}$  significantly increased by 3- to 4-fold following HEA exposure at all time periods compared to control rates of approximately  $-6 \text{ nmol N}\cdot\text{g}^{-1}\cdot\text{h}^{-1}$  (Fig.

2.2B). The corresponding increase in plasma ammonia was approximately 6-fold after 2 days, stabilizing near 1400  $\mu\text{mol N}\cdot\text{L}^{-1}$  (Fig. 2.3A).

*Sequence analysis of amplified UT fragment and its distribution in P. marinus tissues*

A 109bp cDNA amplification product was obtained using a primer set designed to flank highly conserved regions of previously sequenced UT proteins from aquatic vertebrates (Fig. 2.4A). The corresponding amino acid sequence of this amplicon shared a high similarity to several UTs, including 64% identity with the spiny dogfish, *Squalus acanthias* UT (Fig. 2.4B).

Western blot analysis using an antibody specific to zebrafish yielded a single band at approximately 38 kDa in the gill, skin, kidney, intestine, and muscle of adult sea lamprey, compared to a single band at approximately 48 kDa for zebrafish (Fig. 2.5).

*Relative abundance of urea transport protein (UT) in the gill and skin of ammocoete, unfed juvenile, fed parasite and upstream migrant sea lamprey*

As the sea lamprey transformed, entered the parasite phase and then senesced as adults, the relative abundance of UT proteins within the gill tissue decreased by more than 90% by the adult stage (Fig. 2.6A). The abundance of UT protein in ammocoete gills was found to be 6-10 fold higher than that of both fed parasitic juveniles and adult sea lamprey, whereas in unfed juveniles, UT abundance was not significantly different than ammocoetes, fed parasites or adults (Fig. 2.6B).

As with the expression of UT protein in gill tissue, there were differences observed in UT abundance within the skin of ammocoete, unfed juvenile, fed parasites

and adult sea lamprey. Adult individuals were found to have 4-8 fold more UT protein abundance in their skin when compared to ammocoetes, unfed juvenile, and fed juveniles (Fig. 2.7B).



## FIGURES

Fig. 2.1. The influence of a 2 day exposure to HEA ( $0.5 \text{ mmol}\cdot\text{L}^{-1}$  ammonia) on (A)  $J_{\text{Amm}}$  and (B)  $J_{\text{Urea}}$  of ammocoete *P. marinus* (n=10). Negative values indicate excretion, while positive values indicate net ammonia uptake. Control bars are open, whereas treatment indicated by closed bars. Significant differences from control values are denoted by an asterisk ( $P<0.05$ ).

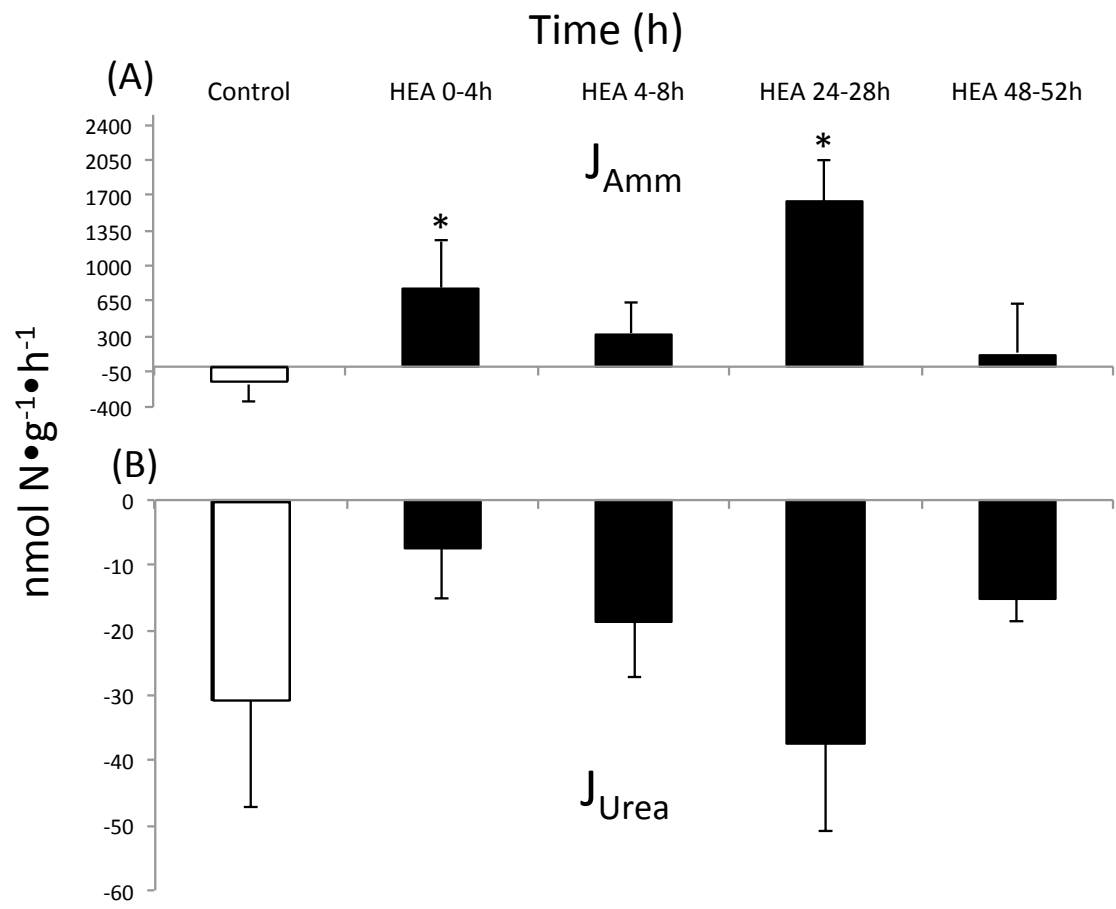


Fig. 2.2. The influence of a 2 day exposure to HEA ( $0.5 \text{ mmol}\cdot\text{L}^{-1}$  ammonia) on (A)  $J_{\text{Umm}}$  and (B)  $J_{\text{Urea}}$  of adult *P. marinus* (n=6). Negative values indicate excretion, while positive values indicate net ammonia uptake. Control bars are open, whereas treatment indicated by closed bars. Significant differences from control values are denoted by an asterisk ( $P<0.05$ ).

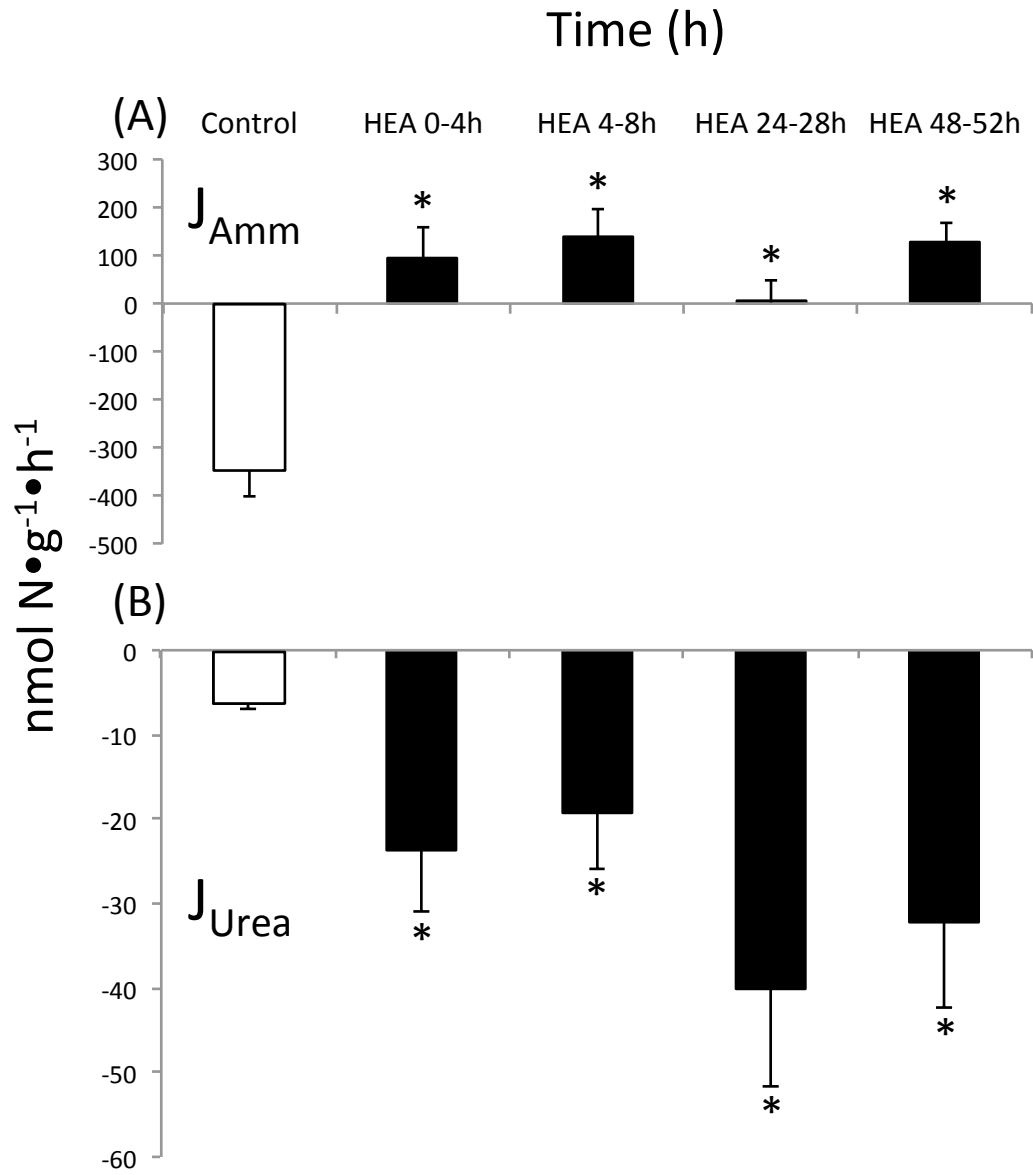


Fig. 2.3. Plasma ammonia concentrations ( $\mu\text{mol N}\cdot\text{L}^{-1}$ ) in ammocoete and adult *P. marinus* exposed to HEA ( $0.50 \text{ mmol}\cdot\text{L}^{-1}$  ammonia;  $n= 6$  or  $8$ )  $\pm$ SE. Control bars are open, whereas treatment indicated by closed bars. Significant differences from control values are denoted by an asterisk ( $P<0.05$ ).

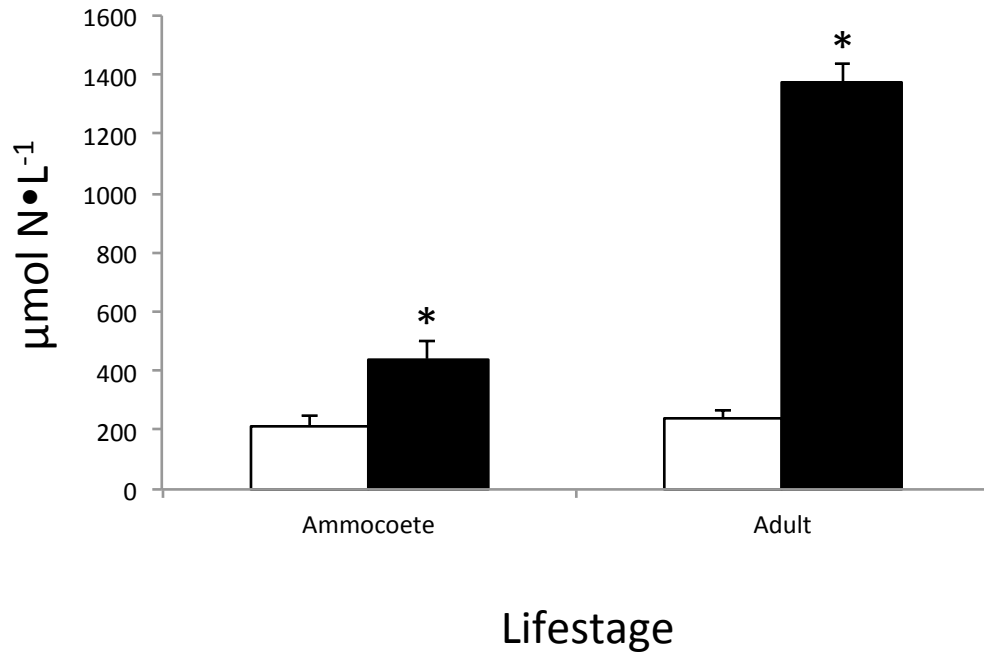


Fig. 2.4. (A) Amplicon (109bp) of putative sea lamprey UT protein along with associated primer set and their melting point (T<sub>m</sub>). (B) Alignment of part of the amino acid sequenced yielded from the amplification product with several known aquatic UT proteins, with the highest fragment identity coming from *Squalus acanthias* (64%). Letters underscored by an asterisk are all identical amino acids, whereas stacked dots refers to similar amino acids. Accession numbers are as follows: AF278537.1; AF165893.2; NM\_001020519.1; AB470074.1; AF257331.1; AB049726.1.

(A) 5-GCGACGACGACACAAAGAGCGGTGGCTGCCGCTGCAGTTCTTCGACTGGAGCCTGCGAGGA  
ACGGCGCAGGTGAACTTCGTCAACAACCTCTGAGCGGTGTCCTCATC-3'

Primers	Tm	Sequence
UT2AiF	53.2 °C	5' GCGACGACGACACAAAGAG 3'
UT2AiR	53.2 °C	5' TCTGAGCGGTGTCCTCATC 3'

(B)

<i>Alcolapia grahami</i>	LLRLLDWVLRGAAQVMFVNNPLSGLVIFA
<i>Opsanus beta</i>	LLQLLDWVLRGASQVMFVNNPLSGLIIFA
<i>Danio rerio</i>	LLQIMDWVLRGAAQVMFVNNPLSGLIIFA
<i>Callorhynchus milii</i>	IFQFLDWILRGAAQVMFVNNPLSGLVILF
<i>Squalus acanthias</i>	IFQFIDWILRGAAQVMFVNNPLSGLIILV
<i>Anguilla japonica</i>	LIQILDWVLRGAAQVMFVNNPLSGLITFG
<i>Petromyzon marinus</i>	PLQFFDWSLRGTAQVMFVNNPLSGVLIIT
	1211** **211** *****11*2



Fig. 2.5. Distribution of UT protein in several tissues of adult sea lamprey probed with zebrafish UT antibody (accession no. AY788989.1; amino acids 48-69; 1:500 dilution). Tissues labeled are as follows; M: Muscle, G: Gill, S: Skin, K: Kidney, L: liver, I: Intestine.

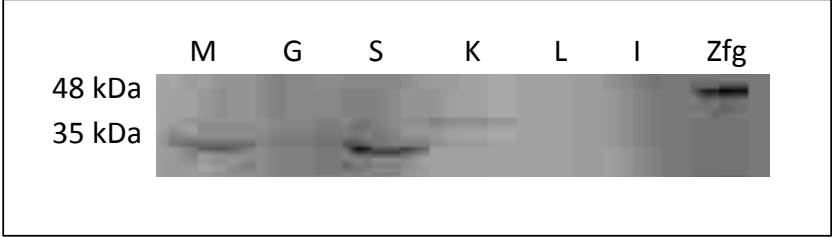


Fig. 2.6. (A) Representative western blot illustrating differences in Urea Transport (UT) protein abundance in gill tissue between ammocoetes, unfed juveniles, fed parasites, and adult *P. marinus* (A, U, P, Ad; approximately 35 kDa). (B) Relative normalized density of UT expression in gill tissue of ammocoete, unfed juvenile, fed parasite, and adult *P. marinus* (n=4). Bars sharing the same letter are not significantly different from one another ( $P < 0.05$ ). Samples normalized to  $\beta$ -actin. Antibody used was that of zebrafish (accession no. AY788989.1; amino acids 48-69; 1:500 dilution).

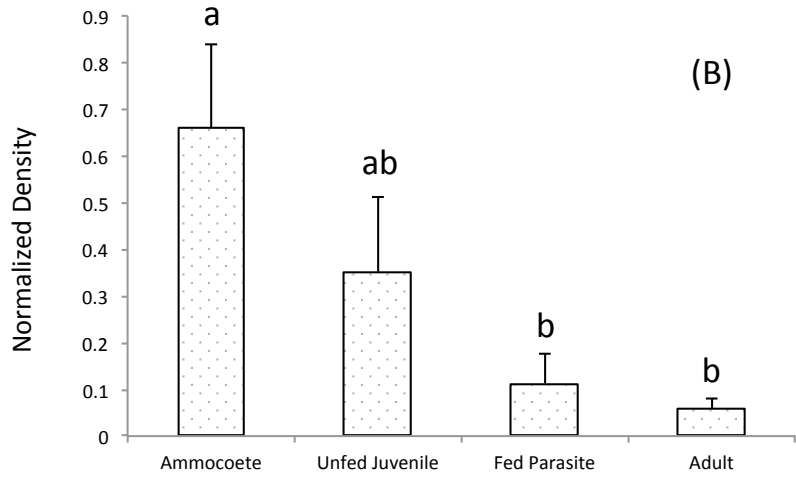
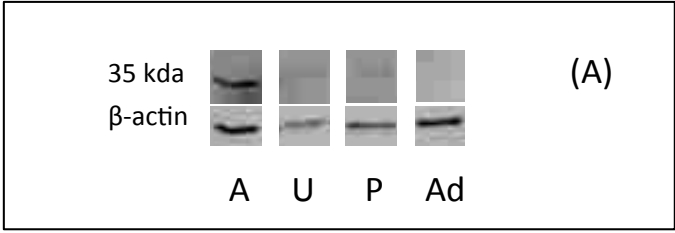
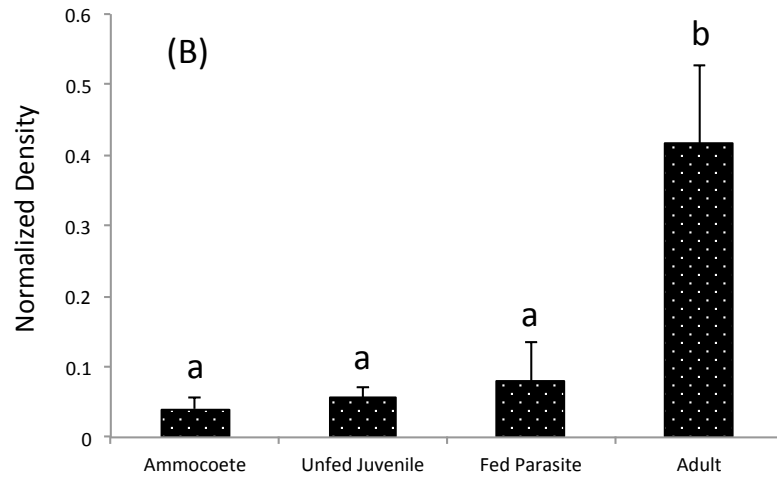
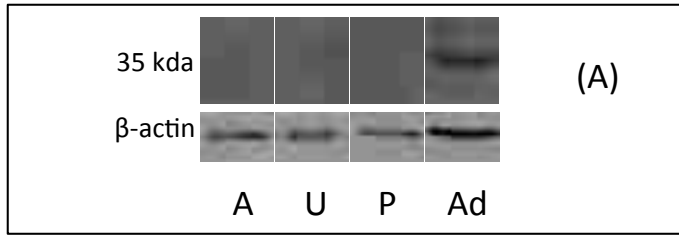


Fig. 2.7. (A) Representative western blot illustrating differences in skin Urea Transporter (UT) protein abundance in ammocoete, unfed juvenile, fed parasite, and adult *P. marinus* (A, U, P, Ad; approximately 35 kDa). (B) Relative normalized density UT expression in skin tissue of ammocoete, unfed juvenile, fed parasite, and adult *P. marinus* (n=4). Bars sharing the same letter are not significantly different from one another (P<0.05). Samples normalized to  $\beta$ -actin. Antibody used was that of zebrafish (accession no. AY788989.1; amino acids 48-69; 1:500 dilution).



## DISCUSSION

### *Evidence for a UT protein in P. marinus*

Amplification and subsequent sequence analysis of a small, 109 base pair fragment of sea lamprey cDNA confirm for the first time, that sea lamprey express UT-like mRNA (Fig. 2.4). Though this amplicon is only a small portion of the transporter, which is normally 1700-2000 base pairs long, the high sequence identity with the spiny dogfish, and UTs reported in other aquatic organisms and mammals are strong evidence that this transcript likely codes for a UT in the lampreys. Western blot analysis, using an antibody to the zebrafish UT, of several tissues of adult sea lamprey tissue (gill, skin, muscle, kidney, intestine, liver), revealed a single band at approximately 38 kDa, with signals appearing most consistently in the gill and skin, as well as in the kidney and the muscle (Fig. 2.5). The molecular weight of this lamprey UT protein was found to be smaller than that reported in another extant agnathan, the Pacific hagfish, in which the UT band was approximately 48 kDa (Braun and Perry, 2010), suggesting that lamprey may have a smaller isoform of the UT protein.

### *Relative abundance of urea transport protein (UT) in the gill and skin of ammocoete, unfed juvenile, fed parasite and upstream migrant sea lamprey*

It was postulated that the changes in habitat, feeding and life style with life stage would influence the tissue distribution patterns of the sea lamprey UT. Indeed, western blot analysis (Fig. 2.6A) of four distinct life stages revealed that the gill tissue of ammocoetes had significantly higher amounts of UT protein compared to both fed parasitic juvenile and adult sea lampreys (Fig. 2.6B). The present study and earlier work by our laboratory indicates that ammocoetes excrete a greater percentage of their total N-wastes (15-30 %) as urea than do the free-swimming parasitic

juvenile and adult life stages (Wilkie et al. 1999; Wilkie et al. 2006). This greater reliance on urea excretion in the burrow-dwelling larval phase may therefore explain why the abundance of lamprey UT in the gill is greater in this life stage, compared to the later free-swimming phases of the sea lamprey's life cycle. It is also notable that many fish rely on  $J_{\text{Urea}}$  during larval development (Depeche et al. 1979; Wright et al. 1995; Korsgaard et al. 1995; Chadwick and Wright 1999). In addition, mRNA expression of UT has been shown to be greater in the larval phase of zebrafish, and is increased further following ammonia exposure (Braun et al., 2009).

The burrow-dwelling life-style of ammocoetes likely impairs ammonia excretion by reducing the blood-water  $\text{NH}_3$  diffusion gradient across the gills (Wilkie et al. 1999). Ammocoetes prefer to burrow into fine sandy substrate, and it has been postulated that the secretion of mucus by this life stage stabilizes the burrow by cementing the sand particles together (Sterba 1962; Beamish et al. 1994). Such a mucus lining might also slow water passage through the burrow, and potentially lead to higher concentrations of ammonia (Randall et al., 2004; Swink and Neff, 2008). This would be particularly true in regions where ammocoetes tend to aggregate, often including regions with large amounts of decaying organic matter (Hardisty 1944; Potter 1980; Sutton and Bowen 1994; Smith et al. 2011).

Although urea is expensive to produce (4-5 mol ATP per urea; Campbell 1991) depending upon whether the urea is produced by the ornithine urea cycle (OUC) or derived from excess purines through uricolysis (Wood 1993; Wright 1995; Wilkie 1999), it is much less toxic than ammonia. Accordingly the burrowing lifestyle of ammocoetes may present conditions that favor the detoxification of ammonia to urea. The limited evidence on ammocoetes suggests, however, that urea production in ammocoetes is mainly through uricolysis (Wilkie et al. 1999), and possibly arginine hydrolysis (Wilkie et al. 2006). It may be fruitful to examine earlier stages of the larval



development to determine whether or not the OUC play a role in ammonia detoxification during the very early stage of embryonic development or during metamorphosis. Indeed, Neal (2013; Chapter 3) has shown that the contribution of urea to N-waste excretion approaches 50 % during the latter stages of metamorphosis in the sea lamprey.

A major difference between ammocoetes and post-metamorphic juvenile lampreys is gill morphology and breathing mechanics. In ammocoetes, respiration is unidirectional in which water currents generated by the velum draw in water via the oral hood, which then passes across the gills, before exiting the pharynx through the laterally located branchiopores (Rovainen 1996; Bartels and Potter 2004; Wilkie 2011). Following metamorphosis, however, the gills lie in gill pouches that are tidally ventilated via the branchial musculature (Peek and Youson 1979; Rovainen 1996; Bartels and Potter 2004). This change allows the parasitic lamprey to breath while attached to its prey/host, however, it could also be a less efficient means of N-waste excretion due to mixing of residual expired waste-laden water with the inspired clean water that is pumped into the gill pouches. This could also be an important factor contributing to the greater reliance on renal urea excretion in parasitic and adult sea lampreys, which has been observed (M.P. Wilkie, unpublished observations).

A single UT band at 38 kDa was also found in the skin of ammocoetes, juveniles, fed parasites and adult sea lampreys (Fig. 2.7A), where UT abundance was found to be greatest in the skin of adult sea lampreys compared to the other lifestages (Fig. 2.7B). It is well documented that the African lungfish (*Protopterus annectens*), and many amphibians use UTs to excrete urea via the skin (Hung et al. 2009; Ussing and Johansen 1969; Garcia-Romeu et al. 1981; Lacoste et al. 1991). The mRNA of lungfish UTs (lfUT) is markedly upregulated following several weeks of terrestrialization (air exposure) to presumably facilitate lfUT protein synthesis, and the offloading

of urea, which builds-up in the animals when they are out of the water (estivation; Hung et al. 2007). As sea lampreys do not have scales (Pfeiffer and Pletcher 1964), it is possible that physiologically relevant amounts of urea excretion take place via the integument at some point following metamorphosis, particularly in adults. Urea excretion across the skin could be seen in adult sea lampreys because they have a well-vascularized dermis (Potter et al. 1995), which would promote urea (and ammonia) delivery to epithelial UTs and Rh glycoproteins. In contrast, ammocoetes lack vascularization in the dermis, which could preclude  $J_{\text{Urea}}$  by this route.

We cannot explain why adult lampreys fitted with indwelling renal catheters excreted more than 90 % of urea via the urine (M.P. Wilkie, unpublished observations). The kidney also possessed a UT, but slightly larger than the 38 KD protein focused on here. Further experiments are required to tease out the differences if any in the properties of the UTs in lampreys, and their relative importance in nitrogenous waste excretion.

Further investigation of the life stage differences in sea lamprey kidney expression of UT proteins would provide much needed insight on the possibility of a transition from branchially-mediated excretion in ammocoetes, to a mainly renal urea excretion in the adults. Indeed, western blots of the kidney of adult sea lampreys exhibited significant banding, but at a slightly higher molecular weight, possibly suggesting the presence of another UT isoform (Fig. 2.6). It is possible that adult sea lampreys are using a system closely related to the one found in the proximal tubules of the Japanese eel (*Anguilla japonica*) eUT-C which has a molecular weight of approximately 40 kDa (Mistry et al. 2005).

*Life stage differences in  $J_{Amm}$  and  $J_{Urea}$  when exposed to highly external ammonia (HEA: 0.5 mmol·L<sup>-1</sup> ammonia)*

Exposure of ammocoetes and adult sea lampreys to HEA (0.5 mmol·L<sup>-1</sup> ammonia) resulted in a complete reversal of  $J_{Amm}$ , with a net uptake of ammonia observed in the first 24 h of HEA (Fig. 2.1A and 2.2A), which was most likely due to a reversal of the blood-water  $P_{NH_3}$  gradient. It was notable, however, that in ammocoetes there was no longer a difference in net-uptake of ammonia by 48 h compared to ammocoete excretion rates, whereas in adults there continued to be net ammonia uptake at this time. Given that the ammocoetes and adults were exposed to identical concentrations of ammonia, these findings suggest that ammonia excretion by the ammocoetes is more efficient than in adults. Indeed, plasma ammonia concentrations were 3-fold higher in the adults under the same conditions. The difference could be because flow across the ammocoete gill is unidirectional which would make it easier for the animals to establish and maintain blood-water  $P_{NH_3}$  diffusion gradients at HEA. Indeed, Wilkie et al. (1999) reported that ammocoetes were able to restore and maintain  $J_{Amm}$  during exposure to 2 mmol L<sup>-1</sup> total ammonia after 3 d, at which time the animals had achieved sufficient build-ups of internal ammonia needed to re-establish blood-water  $P_{NH_3}$  diffusion gradients across the gills. In adults, the gill is tidally ventilated which would lead to higher concentrations of ammonia next to the gill and less favourable blood-water  $P_{NH_3}$  gradients, due to mixing of ammonia-loaded exhalant water, with fresh inhalant water. Such differences could also explain why Rhcg abundance on the skin increases following metamorphosis in the sea lamprey, which would provide an accessory route for  $J_{Amm}$  but this requires further investigation (Blair 2011; Neal 2013, Chapter 3). Ammocoetes can also withstand very high concentrations of external ammonia, with a 96-h LC<sub>50</sub> of approximately 3 mmol·L<sup>-1</sup> total

ammonia, which is about 3-fold higher than other ammonia tolerant fish (Wilkie et al. 1999; United States Environmental Protection Agency, 1995).

Unlike earlier studies (Wilkie et al. 1999), the  $J_{\text{Urea}}$  of ammocoetes exposed to HEA did not significantly increase in the present study. It should be noted, however, that  $J_{\text{Urea}}$  in the control (unexposed) animals were already substantially higher compared to rates measured in the earlier studies (Wilkie et al. 1999). This may have been a reflection of how long the animals had been in captivity and/or the time of year. Unlike Wilkie's previous (1999) study, these were freshly caught summer animals that were used within 2-4 weeks of capture, and which were actively feeding on their natural diet of detritus, algae and diatoms (Moore and Beamish 1973; Sutton and Bowen 1994) prior to capture and not kept in the lab for several months being fed yeast before experimentation. Another factor is that sufficient time may not have elapsed during HEA to stimulate greater reliance on  $J_{\text{Urea}}$ . For instance, Wilkie et al. (1999) reported that 3 d of HEA exposure was needed to stimulate increased  $J_{\text{Urea}}$  in ammocoetes exposed to 2 mmol L<sup>-1</sup> ammonia. Braun et al. (2009) noted that larval zebrafish exposed to 0.5 mmol•L<sup>-1</sup> ammonia only increased  $J_{\text{Urea}}$  after 120 h of HEA. Similarly, in the hagfish,  $J_{\text{Urea}}$  did not increase following HEA exposure, but only after direct ammonia injections (Braun and Perry 2010). In other words, internal ammonia concentrations may not have increased sufficiently to promote increased  $J_{\text{Urea}}$  in ammocoetes. Wilkie et al. (1999) reported that plasma ammonia had risen to more than 2 mmol L<sup>-1</sup> when  $J_{\text{Urea}}$  was stimulated in ammocoetes exposed to HEA. In the present study, plasma ammonia concentrations were about 1/5 this value after 2 days of HEA.

In adult sea lampreys exposed to HEA,  $J_{\text{Urea}}$  was significantly higher immediately following exposure to HEA and plasma ammonia was 4 times greater than that observed in the ammocoetes, at approximately 1.4 mmol L<sup>-1</sup>. The higher plasma ammonia concentrations in the adults, despite

being exposed to the same concentration of ammonia, over a similar time course (and water pH), further suggests that the adult sea lampreys were much less tolerant to ammonia than burrow-dwelling ammocoetes. Such findings may also at least partially explain why Rh glycoprotein abundance is less in the gills of ammocoetes compared to latter stages (Neal et al. Chapter 3). As gas channels, the movement of  $\text{NH}_3$  is bi-directional through Rh glycoproteins, and dependent upon the  $\text{NH}_3$  partial pressure gradient. In the burrow-dwelling environment of the ammocoetes, build-ups of ammonia would impair  $J_{\text{Amm}}$  via this route. Thus, greater reliance on extrabranchial routes for  $J_{\text{Amm}}$ , presumably via the kidneys, would allow the lamprey to minimize ammonia uptake across the gills in the event of increases in external ammonia. The greater reliance on  $J_{\text{Urea}}$  could also provide the ammocoetes with an accessory means to rid the body of nitrogenous wastes. Future studies should test these hypotheses by quantifying both UT and Rh glycoprotein abundance in the gills, skin and kidneys of ammocoetes, juvenile and adult lampreys following ammonia exposure.

Some fishes, including the gulf toadfish (*Opsanus beta*), Magadi tilapia (*Alcolapia grahami*), lungfishes, along with several larval stages of fish produce urea using the ornithine urea cycle (OUC; Mommsen and Walsh 1989; Randall et al. 1989; Loong 2005; Depeche et al. 1979, Wright et al. 1995). This seems unlikely in the sea lampreys, even during HEA, because key OUC enzymes are absent or only present at low levels (Wilkie et al. 2006). Sea lampreys do contain the machinery to produce urea via uricolysis (Wilkie et al. 1999; Wilkie et al. 2004), however. Thus, it is likely that this increased ammonia load stimulated the uricolytic pathway in adults, leading to the increased  $J_{\text{Urea}}$  observed. Arginine arising from proteolysis in this terminal life stage, may have also also lead to the generation of urea, due to the presence of arginase in the liver and muscle of juvenile and adult lampreys and the high amounts of arginine generated in the muscle due to

proteolysis in the adult sea lampreys (Wilkie et al. 2006). Skin UT abundance may therefore be a function of urea generation in the closely situated white muscle, with subsequent dermal offloading due to proximity of formation, and the relatively short distance to excrete urea through the skin.

*Perspectives:*

Taking into account their ancient origins (over 350 million years old), the presence of a sea lamprey UT underscores the primitive origins of urea transport proteins in vertebrates (Forey and Janvier 1993; McDonald et al. 2012). Owing to the broad spectrum of their habitat, feeding and lifestyle throughout the sea lamprey life cycle, it is not surprising that dramatic changes in N-waste production (Wilkie et al. 2006) and excretion mechanisms change as well. Presently, the fragment of lamprey UT presented is not sufficient to develop a credible evolutionary pattern for the UTs in the vertebrates. However, our data do suggest that the UT studied here is closely related to UT-C found in Japanese eel (McDonald et al. 2006). Elongation of this gene fragment, along with immunohistochemical analyses of gill, skin and kidney of the primary stages of sea lamprey, will go a long way in resolving both the evolutionary and functional gaps in our present knowledge of lamprey UTs. Nevertheless, it does appear that at least in sea lampreys, UTs may play an important role in allowing lampreys to live in burrow-dwelling habitats where ammonia excretion could be more challenging than in the open water column, which characterizes the sea lamprey's habitat following metamorphosis.

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### **Chapter 3:**

**Effects of Metamorphosis and Feeding on Nitrogenous Waste Excretion Patterns, Rh glycoprotein and Urea Transporter Abundance in Sea Lampreys (*Petromyzon marinus*)**

## ABSTRACT

The phylogenetically ancient sea lamprey (*Petromyzon marinus*) begins its complex lifecycle as a burrow-dwelling, suspension feeding larval ammocoete, and following a 3-4 month metamorphic period, free swimming juveniles emerge from their burrows and swim downstream where they feed on the blood of fishes. During the non-trophic metamorphic period, sea lamprey rely on the breakdown of internal lipid and then protein reserves to meet their energy requirements. It was predicted that during metamorphosis, changes in feeding habits would be accompanied by changes in both ammonia and urea excretion rates ( $J_{\text{Amm}}$  and  $J_{\text{Urea}}$ ), along with changes in the abundance of Rh glycoproteins and urea transport (UT) proteins which facilitate excretion. There was a slight decrease in  $J_{\text{Amm}}$  and  $J_{\text{Urea}}$  through most of metamorphosis, but rates of excretion substantially increased in late stage 7 and in juvenile animals. Interestingly, animals experienced a period of urotely (<50%  $J_{\text{N-waste}}$  is urea) in late stage 7. Rhcg2 and UT protein abundances were observed to be the greatest during the mid-stages of metamorphosis and corresponded to internal buildups of both plasma ammonia and urea, respectively. Post-feeding Rhcg2 and UT protein abundance in gill tissue was also investigated in parasitic sea lamprey. A two-fold increase in Rhcg2 was observed in feeding parasites, whereas there was a decrease in UT abundance in actively feeding parasite gill tissue. It is postulated that post-metamorphic sea lampreys rely more on renal routes of  $J_{\text{Urea}}$  than gill-mediated  $J_{\text{Urea}}$ , and that restructuring of the kidneys during metamorphosis aids in the removal of urea in the urine of these animals.



## INTRODUCTION

The Rhesus glycoprotein (Rh) family facilitates the movement of  $\text{NH}_3$  across the gill epithelium of fishes and aquatic crustaceans (Weihrauch et al. 2009; Wright and Wood 2009). According to this model, paralogues of the Rh glycoproteins Rhag, Rhbg, and Rhcg work in series, with Rhag promoting hepatically-produced ammonia offloading by red blood cells at the gills, and Rhbg and Rhcg promoting ammonia excretion across the basolateral and apical membrane of the gills, respectively (Weihrauch et al. 2009; Wright and Wood 2009). The apically located Rhcg proteins are thought to be the focal point of an  $\text{NH}_3$  metabolon, in which  $\text{NH}_3$  excreted across the gills is trapped as  $\text{NH}_4^+$  due to acidification of the gill boundary layers by other transporters including apically located  $\text{H}^+$ -ATPases coupled to  $\text{Na}^+$ -channels, and possibly apical  $\text{Na}^+/\text{H}^+$ -exchangers (Wright and Wood 2009). The Rh glycoproteins have been observed in many aquatic organisms including crab (*Carcinus maenas*), mangrove killifish (*Kryptolebias marmoratus*), puffer fish (*Takifugu rubripes*), zebra fish (*Danio rerio*) larvae and rainbow trout (*Oncorhynchus mykiss*) (Weihrauch et al. 2004; Perry et al. 2010; Hung et al. 2007; Braun et al. 2009; Nakada et al. 2007a; Nakada et al. 2007b; Nawata et al. 2007).

In the extant jawless fishes, Rhbg and Rhcg have been partially characterized in hagfish (Braun et al. 2010), but it remains unclear what role they play in the lampreys. The goal of the present study was to determine how Rh glycoprotein abundance in the gills was influenced during metamorphosis in the sea lampreys (*Petromyzon marinus*) and following the ingestion of protein-rich blood during the parasitic phase of the sea lampreys' complex life cycle.

The anadromous sea lamprey undergoes a true metamorphosis that results in the animal transforming from a blind, relatively sedentary filter-feeding larvae called an ammocoete, into a free-swimming parasitic juvenile. The parasitic juvenile phase last 12-20 months, before the

animals return to freshwater streams as adults, spawn and die (Beamish and Potter 1975; Youson 2003). During the 3-4 month non-trophic period of metamorphosis, sea lampreys proceed through seven distinct stages characterized by the formation of an oral disc and rasping tongue which allows the parasitic lamprey to attach to the substrate or their host, the appearance of eyes, a switch from unidirectionally ventilated to tidally irrigated gills, and “silvering” of the body (Beamish and Potter 1975; Youson and Potter 1979; Youson 2003). In addition to these external changes there is a restructuring of internal body structure, an increase in overall metabolic rate, and a greater capacity to deaminate the excess amino acids derived from the protein rich blood the lampreys ingest from their hosts/prey (Wilkie et al. 2004; 2006). This period following metamorphosis is likely characterized by changes in the ammonia and urea excretion machinery of sea lampreys, which includes the urea transporters (UTs) and the Rh glycoproteins in the gills. One goal of the present study was to test the hypothesis that changes in internal ammonia and urea concentrations, and altered ammonia and urea excretion ( $J_{\text{Amm}}$  and  $J_{\text{Urea}}$ ) patterns that accompany metamorphosis are matched by quantitative changes in UT and Rhcg abundance in the gills.

The ingestion of large quantities of protein rich blood in parasitic juvenile sea lampreys can also result in marked increases in internal ammonia and urea leading to elevated post-feeding  $J_{\text{Urea}}$  and  $J_{\text{Amm}}$  as occurs in sea lampreys following feeding on basking sharks (*Cetorhinus maximus*) and rainbow trout (*Oncorhynchus mykiss*; Wilkie et al. 2004; 2006). Thus, the final goal of the present study was to test the hypothesis that post-feeding surges in ammonia excretion in parasitic lampreys are reflected by corresponding increases in the abundance of Rhcg in the gills of sea lampreys, the main site of ammonia excretion following metamorphosis (Chapter 2). In the present study, we specifically focused on Rhcg 2, which has recently been localized immunohistochemically to the apical membrane of sea lamprey gills (Blair 2011).

## **METHOD AND MATERIALS**

### ***Experimental Animals and Holding***

In the first part of this study, anadromous sea lampreys were collected by pulsed DC electrofishing in New Brunswick, Canada from streams flowing into the Northumberland Strait (DFO scientific collection permit SG-NBT-11-098 and SG-NBT-12-110). A subset of these animals underwent metamorphosis in the laboratory, which provided a pool of metamorphosing and parasitic sea lampreys that were used to determine how life stage affects Rh glycoprotein and UT abundance in the gills. The larval and metamorphosing sea lamprey (ammocoetes) were then held in 70 L aquaria filled with a 4-5 cm deep layer of sand that provided burrowing substrate for these burrow-dwelling animals. The larvae were fed bakers yeast (1g/larvae; Holmes and Youson 1994) on a weekly basis (Fleischmann's yeast), whereas the metamorphosing animals were not fed during this non-trophic period of their life cycle (O'Boyle and Beamish 1977). Juvenile, parasitic lampreys fed on rainbow trout (*Oncorhynchus mykiss*) weighing 100-500 g that were purchased from Rainbow Springs Hatchery (Thamesford, ON), and held in 100-500 L flow-through tanks, which received a constant flow of aerated well-water (dissolved O<sub>2</sub> of approximately 80-100% saturation). These rainbow trout were fed three times a week to satiation using sinking pellets (3.0 Corey Feed Mills, Elmira, ON).

Adult sea lampreys, captured in the initial stages of their upstream migration, were provided courtesy of Rod McDonald, Sea Lamprey Control Center, Fisheries and Oceans, Canada (DFO) in Sault Ste. Marie, Ontario. The adults were housed in 700 L living streams receiving aerated well-water (dissolved O<sub>2</sub> approximately 80-100% saturation) on a flow through basis. It was not necessary to feed the adult lampreys, which naturally do not feed at this life stage.

All animals were held under a 12 hour light/12 hour dark photoperiod, and all experiments completed were approved by the Wilfrid Laurier University Animal Care Committee, in accordance with the guidelines of the Canadian Council on Animal Care.

### ***Experimental Protocols***

#### *Experiment 1: Nitrogenous Waste Metabolism, UT and Rh Glycoprotein Abundance in Metamorphosing Sea Lampreys*

To identify the factors that might explain the greater capacity to excrete N-waste after metamorphosis and feeding in the parasitic stage, the patterns of N-waste excretion along with Rh and UT protein abundance were followed through 6 of the 7 stages of metamorphosis. The animals used in these experiments were collected as large ammocoetes in June 2012, and brought back to the aquatic holding facilities at Wilfrid Laurier University in well-aerated containers where they were sorted into 2 groups. The first group were those most likely to enter metamorphosis, which included animals with a condition factor (CF) greater than 1.5, and respective minimum mass and length of 3.0 g and 120 mm, due to the presence of large lipid stores (Holmes and Youson, 1994). The second group was comprised of smaller animals, unlikely to enter metamorphosis, which were used in measurements focusing on the ammocoete life stage. After sorting, the large ammocoetes were distributed to 50-60 L aquaria in groups of 15-20, containing well water at 15 °C and a 4-5 cm deep layer of sand lining the bottom of the aquaria since metamorphosing lampreys remain burrowed until the late stages of metamorphosis (Youson and Potter 1979). The water in the aquarium was changed weekly (50 % replacement) during the experiment, and no mortalities were noted. A separate sentinel tank containing 15 large ammocoetes burrowed in cotton allowed us to

follow the progress of metamorphosis with greater accuracy, without disturbing the animals that were burrowed in the sand of the other aquaria.

Staging was mainly based on changes in dentition, eye development, and development of the oral disc as described by Youson and Potter (1979). The stage of metamorphosis was determined by one investigator, the animals photographed and the stage of metamorphosis then verified by a qualified second investigator (Figures 1, 2). Between June and October, animals from stages 2 to 7 (n=9 for each stage) were removed from the aquaria, and transferred one at a time to individual opaque, polyethylene flux chambers (approximately 200 ml volume) receiving a constant flow (0.1 to 0.5L·min<sup>-1</sup>) of well-water (pH =8; T=11°C; dissolved O<sub>2</sub> 80-100%) draining from an aerated, head tank. Each aerated container contained approximately 0.2 g of aquarium cotton into which the animals could burrow. After an overnight acclimation period, control rates of J<sub>Amm</sub> and J<sub>Urea</sub> were determined at pH 8.0 over 8 h. Prior to each flux measurement period, water flow to each chamber was cut off, and the volume adjusted to 0.100L after which water samples (5mL) were collected at 0, 2, 4, 6, and 8 h. After 8 h, each sea lamprey was lightly anesthetized using 0.5 g·L<sup>-1</sup> tricaine methanesulfonate (MS222; Syndel Laboratories, Nanaimo, British Columbia) buffered with 1.0 g·L<sup>-1</sup> NaHCO<sub>3</sub>, followed by a lethal overdose of MS222 (1.5 g·L<sup>-1</sup> MS222 buffered with 3.0 g·L<sup>-1</sup> NaHCO<sub>3</sub>). Blood samples were then collected by making an incision through the heart, and collecting drops of blood into a 500uL centrifuge tube, which was then centrifuged at 10,000xg for 3 minutes in a microcentrifuge (Eppendorf, Model 5415D, Hamburg, Germany), the plasma collected, and frozen in liquid nitrogen. An incision down the mid-ventral line exposed the liver, kidney and intestine, which were removed and quickly snap frozen in liquid N<sub>2</sub>. The remaining skin and muscle tissue from the trunk of the animal were separated and snap frozen in the same manner. Gill filaments were dissected under slight

magnification by an initial antero-ventral incision, and subsequent excision of the filamentous gill tissue with fine tweezers followed by snap freezing. All tissue samples were stored at  $-80^{\circ}\text{C}$ , until analyzed within 6 months for plasma ammonia and urea concentration, and gill and skin UT and Rhcg2 abundance using western blotting. Water samples were frozen at  $-20^{\circ}\text{C}$ , and then thawed when water ammonia and urea concentration were quantified for the determination of  $J_{\text{Urea}}$  and  $J_{\text{Amm}}$  by the sea lampreys.

### *Experiment 2: Feeding in Parasitic Juvenile Sea Lampreys*

To investigate the plasticity and mechanisms of  $J_{\text{Amm}}$  and  $J_{\text{Urea}}$  in juvenile lampreys, a feeding study was undertaken to determine how increased ammonia and urea-loads influence the abundance of both the UT protein and Rhcg glycoprotein. Accordingly, post-metamorphic juvenile lampreys were given access to 100-500 g rainbow trout upon which they were able to feed for 2-3 weeks, during which time the lamprey grew from approximately 3.0-3.5 g to a mean size of approximately 10 g. Following feeding both the trout and the lampreys were anesthetized with  $0.5 \text{ g}\cdot\text{L}^{-1}$  MS222 buffered with  $1.0 \text{ g}\cdot\text{L}^{-1}$   $\text{NaHCO}_3$ , while the lamprey was still attached to the trout. In most cases the lamprey readily detached from the trout on its own volition, but in some instances removal required careful manipulation of the oral disc to break the “vacuum seal” holding the lamprey to the side of the trout. The lampreys were then either transferred to flux chambers for measurement of  $J_{\text{Amm}}$  and  $J_{\text{Urea}}$ , or transferred to a lethal dose of MS222 followed by blood and tissue collection as described above.

## ***Analytical Techniques***

### *Ammonia and Urea Determination*

Water ammonia concentration was determined spectrophotometrically using the salicylate hypochlorite colorimetric assay, which in the presence of ammonia forms a blue indophenol with an optimal absorbance at 650nm (Verdouw et al. 1978). Plasma ammonia concentrations were determined enzymatically via a glutamate dehydrogenase catalyzed reaction to oxidize NADPH at 340nm (Procedure AA0100; Sigma Aldrich Chemical Co. St. Louis, MO). Water and plasma urea concentrations were determined colorimetrically using ferric chloride, diacetyl monoxime and thiosemicarbizide, which produces a pink chromogen in the presence of urea, that is quantified at 525nm (Rahmatullah and Boyde 1980). Each of these spectrophotometric assays was conducted using a Molecular Devices Spectramax190 plate spectrophotometer (Molecular Devices, Sunnyvale, CA). To ensure confluence throughout each assay, linearity of standard curves were only accepted with an  $r^2 > 95\%$ .

### *Antibodies and Western Blot Analysis*

Affinity purified polyclonal antibodies to *Danio rerio* (Zebrafish) UT (accession no. AY788989.1; amino acids 48-69) were a gift from SF Perry (Department of Biology, University of Ottawa, Ottawa, Ontario), and used for western blot analysis to localize and quantify UTs in ammocoetes, juveniles and adult lampreys. Antibodies raised against amino acid sequence fragments encoding a part of the COOH terminus of Rhcg2 in *Takifugu rubripes* (NM\_001027934.1; amino acids 420–481) were attained from S Hirose (Tokyo Institute of Technology), courtesy of S. Edwards (Appalachian State University, Boone, NC).

Snap frozen tissues were homogenized in ice-cold buffer solution containing 50 mmol L<sup>-1</sup> Tris pH 7.5 with protease inhibitor (Roche, Laval, QC) using a hand-held sonicator probe, (~ 10 s pulse time per sample) at 80% power (Fisher Scientific, XL2000-350R, Ottawa, ON). Homogenized samples were centrifuged at 16,000xg for 3 minutes at 4°C, and the supernatant transferred to a clean 1.5 mL centrifuge tube from which protein quantification was determined using the bicinchoninic acid (BCA) protein assay (Procedure B9643; Sigma Aldrich, BCA1; Smith et al. 1985). For samples that contained excess lipid, a secondary centrifugation period was needed to allow for preliminary removal of lipid layer. In all cases, protein from zebrafish gill was used as a positive control. Exactly 20 µg of zebra fish gill protein and 40 µg of lamprey tissue protein were loaded into each lane of a 12% sodium dodecyl sulfate (SDS) polyacrylamide gel and electrophoresed for 40 minutes at 200V (Biorad 165-5052; 165-3301, Hercules, CA). The protein size marker used was obtained from GeneDirex (PM007-0500, Las Vegas, NV). Following electrophoresis, both gel and nitrocellulose membranes were incubated for 30 minutes in 20% methanol transfer buffer. Transfer of protein from the gel to nitrocellulose membrane was achieved using a semi-dry unit run at 20V for 27 minutes (BioRad 170-3940, Hercules, CA). Membranes were then treated with Ponceau's stain for 5 minutes to determine transfer efficiency, followed by subsequent washing (3 washes, 10 minutes per wash) with 1x Tris-buffered saline containing 0.1% Tween 20 (1x TTBS). Blocking of the membranes was done using 5% skim milk with 0.05% sodium azide in 1x TTBS. Following membrane blocking, a 2h (UT) and overnight (Rhcg2) incubation with the primary antibodies was done (1:500 UT; or 1:1000 Rhcg2). Washing with 1x TTBS (3 washes, 10 minutes per wash) was then followed by incubation in peroxidase-conjugated secondary anti-rabbit Ig (1:3000 dilution; Bio-Rad catalogue number 170-6515; Mississauga, ON)



for 1 h. After discarding the secondary antibody, membranes were washed with 1x TTBS (3 washes, 10 minutes per wash) and detection using enhanced chemiluminescence was performed (GE Healthcare, RPN2132, Baie d'Urfe, QC). Following detection of either Rhcg2 or UT, blots were washed with 1x TTBS (3 washes, 10 minutes per wash). Each blot was then incubated for 1h in the dark using a primary  $\beta$ -actin antibody conjugated with Cy3 fluorescence tag (C5838; Sigma Alderich; St. Louis, MO). Relative quantification of protein expression was performed by scanning in images of nitrocellulose on Quantity One software (Bio-Rad, Hercules, CA), and size and density of the bands were analyzed using GelEval software (FrogDance software, Dundee, UK). To control for any variation in banding due to protein loading, densities both Rhcg2 and UT bands were normalized to those of  $\beta$ -actin.

#### *Calculations and Statistics*

Nitrogenous waste excretion rates were calculated using the following equation:

$$J_{\text{N-waste}} = \frac{([\text{N-waste}]_{\text{initial}} - [\text{N-waste}]_{\text{final}})(V)}{(M)(\Delta T)}$$

Where  $J_{\text{N-waste}}$  refers to the rate of ammonia ( $J_{\text{Amm}}$ ) or urea excretion ( $J_{\text{Urea}}$ ) in  $\text{nmol N}\cdot\text{g}^{-1}\cdot\text{h}^{-1}$ ,  $[\text{N-waste}]_{\text{initial}}$  is the concentration of ammonia or urea at the beginning of the flux determination period, and  $[\text{N-waste}]_{\text{final}}$  is the concentration of ammonia or urea at the end of the flux determination period; V is the volume of water in the flux chamber; M is the mass of each lamprey;  $\Delta T$  is the duration of the flux determination period.

Condition factors were calculated using the following equation:

$$CF = (W/L^3) \times 10^6$$

Where CF refers to condition factor of sea lamprey, W is the weight of each fish in grams, and L is the length of each fish in millimeters (Youson et al 1993).

All data were presented as the mean  $\pm$ 1 standard error of the mean (SEM). Unpaired data between different groups of metamorphosing sea lamprey were analyzed using a non-parametric ANOVA, followed by a Student Newman-Keuls post-test. It should be noted that metamorphic  $J_{\text{Amm}}$  and  $J_{\text{Urea}}$  data were logarithmically transformed prior to statistical analysis because the data were either not normally distributed or there were significant differences in the standard deviations between each group of animals compare. All statistical significance was determined at the  $P < 0.05$  level (GraphPad InStat 3.0 or SPSS ver. 20.0).

## RESULTS

### *Diagnostic Features of Metamorphosis in the Sea Lamprey*

Using the criteria of Youson and Potter (1979), a number of key features were used to identify each stage of metamorphosis in the sea lamprey which included changes in oral disc formation, development of the eyes, and changes in colouration (Fig. 3.1, Fig. 3.2). Metamorphosis, likely began in mid-late June, earlier than previously reported (Youson and Potter 1979). As a result, the first stage defined was stage 2 when there was a more definitive eyespot (ES) than seen in the ammocoetes, and there was the formation of a papilla (P) in the oral hood. As the animals entered stage 3, a pupil (P) and iris (I) began to develop, which became more distinct as metamorphosis proceeded. By stage 4, there was a continuous ring of tissue around the presumptive oral disc, along with oval-shaped branchiopores (B) compared to the triangular slits that defined the ammocoete stage. Stage 5 was characterized by the formation of tooth precursors (TP) and a clearly visible piston in the buccal funnel, not seen in the earlier stages. By stage 6, the eyes (E) laterally protruded from each side of the head when the animal was viewed ventrally, and a distinct lateral line (LL) was visible for the first time. By stage 7, the pupil (P) and iris (I) of the eye were clearly defined, and the teeth (TO) of the oral disc had become whitish yellow at their points. The post-metamorphic animals, which had just emerged from their burrows, had characteristic yellow teeth bearing sharp tips (TO) and a fully developed eye (E).

### *Changes in condition factor during metamorphosis*

The condition factor in non-metamorphosing ammocoetes averaged 1.27. In all animals that underwent metamorphosis, the pre-metamorphic CF was greater than 1.5 and was stable near this value from stages 2 to 4, but then increased by stage 5, when the average CF was

approximately 1.65. This increase in CF was the result of a significant shortening of the animals, rather than any gain in body mass. Thereafter, CF significantly declined to approximately 1.45 by stage 6, reaching a minimum of 1.25 at the completion of metamorphosis (Fig. 3.3).

#### *Changes in $J_{\text{Amm}}$ and $J_{\text{Urea}}$ during metamorphosis*

As the sea lampreys proceeded from ammocoetes, through the initial stages of metamorphosis there was a 50% decrease in  $J_{\text{Amm}}$ , from approximately  $-100 \text{ nmol N}\cdot\text{g}^{-1}\cdot\text{h}^{-1}$  to  $-50 \text{ nmol N}\cdot\text{g}^{-1}\cdot\text{h}^{-1}$ , through to stage 6 (Fig. 3.4A). By early stage 7, however,  $J_{\text{Amm}}$  began to increase, so that by the juvenile stage, it was two-fold higher than rates measured in ammocoetes (Fig. 3.4A).

The changes in  $J_{\text{Amm}}$  were matched by similar reductions in  $J_{\text{Urea}}$  during metamorphosis. By stage 2,  $J_{\text{Urea}}$  was 50% lower than  $J_{\text{Urea}}$  in ammocoetes, falling from approximately  $-45 \text{ nmol N}\cdot\text{g}^{-1}\cdot\text{h}^{-1}$  to  $-20 \text{ nmol N}\cdot\text{g}^{-1}\cdot\text{h}^{-1}$  (Figure 3.5A). By stage 5,  $J_{\text{Urea}}$  was approximately 75% lower than measured in ammocoetes, at  $-15 \text{ nmol N}\cdot\text{g}^{-1}\cdot\text{h}^{-1}$ , where it remained until early stage 7 (Fig. 3.5A). In late stage 7, however,  $J_{\text{Urea}}$  increased markedly, reaching  $-70 \text{ nmol N}\cdot\text{g}^{-1}\cdot\text{h}^{-1}$ , exceeding the rates of  $J_{\text{Amm}}$ , indicating that the fish were ureotelic at this time (Fig. 3.5A). By the end of metamorphosis,  $J_{\text{Amm}}$  again exceeded  $J_{\text{Urea}}$ , but  $J_{\text{Urea}}$  still accounted for approximately 35% of total  $J_{\text{N-waste}}$  (Fig. 3.4A, 3.5A).

Plasma ammonia concentrations fluctuated throughout metamorphosis, peaking during stage 5 at approximately  $325 \text{ }\mu\text{mol N}\cdot\text{L}^{-1}$  (Fig. 3.6A). Due to the variability in the data set, there were no significant differences from the ammonia concentrations found in ammocoetes.

Plasma urea concentrations were relatively low during the early stages of metamorphosis at  $300\text{-}400 \text{ }\mu\text{mol N}\cdot\text{L}^{-1}$  (Fig. 3.6B). By stage 5, however, plasma urea concentrations were about two-

fold greater, and remained higher through to the completion of metamorphosis, fluctuating between 600-900  $\mu\text{mol N}\cdot\text{L}^{-1}$  (Fig. 3.6B).

#### *Abundance of Rhcg2 and UT in gill tissue during metamorphosis*

Western blot analysis using the Rhcg2 antibody specific to *Takifugu rubripes* yielded a single band at approximately 50 kDa in the gill tissue of sea lamprey (Fig. 3.4C). During metamorphosis, Rhcg2 abundance gradually increased, peaking at stage 4, where it was approximately 3.5-fold greater than observed in stage 2 (Fig. 3.4B). Thereafter, Rhcg2 abundance in the gill declined to levels not significantly different from amounts observed in stage 2 (Fig. 3.4B).

Western blot analysis of metamorphosing sea lamprey (stages 2-7) with UT antibody specific to *Danio rerio* yielded a single band at approximately 38 kDa for gill tissue in sea lamprey (Fig. 3.5C). As metamorphosis progressed, gill UT protein abundance also peaked at stage 4, before decreasing to levels that were 75% below those measured in the early stages of metamorphosis and approximately 80 % lower than measured in ammocoetes (Fig. 3.5B).

There was little UT or Rhcg2 protein found in the skin of metamorphosing sea lampreys in stages 2-7 (data not shown).

#### *Relationship between postprandial ammonia and urea excretion and Rhcg2 and UT abundance in the gills*

After ingesting trout blood,  $J_{\text{Amm}}$  increased by approximately 5-fold, from  $-185 \text{ nmol N}\cdot\text{g}^{-1}\cdot\text{h}^{-1}$  to  $-1080 \text{ nmol N}\cdot\text{g}^{-1}\cdot\text{h}^{-1}$  (Fig. 3.7A). This increase in  $J_{\text{Amm}}$  was related to 3-fold greater plasma ammonia concentrations, which averaged  $316 \mu\text{mol N}\cdot\text{L}^{-1}$  in the unfed juveniles, but  $943 \mu\text{mol}$

$\text{N}\cdot\text{L}^{-1}$  in the feeding juvenile lampreys, 4 h after they were removed from the trout (Fig. 3.7B). These changes were associated with 3-fold greater Rhcg2 abundance in the gill of the fed compared to the unfed juvenile and adult sea lampreys (Fig. 3.7C, D). Feeding had no significant effect on expression of Rhcg2 in the skin of juvenile, parasitic and adult lampreys (data not shown).

A similar 5-fold greater  $J_{\text{urea}}$  from  $56 \text{ nmol N}\cdot\text{g}^{-1}\cdot\text{h}^{-1}$  to  $256 \text{ nmol N}\cdot\text{g}^{-1}\cdot\text{h}^{-1}$  was observed in the fed compared to the non-fed juvenile lampreys (Figure 3. 8A) and accompanied by a 2-fold greater plasma urea to  $1900 \mu\text{mol N}\cdot\text{L}^{-1}$  (Fig. 3.8B). Despite this increase, however, there was no effect on UT abundance in the gill (Fig.3.8C, D).

## FIGURES

Fig. 3.1. Lateral view showing the anterior region of an ammocoete (Amm), metamorphic stages 2 to 7, and a juvenile (Juv) anadromous *P. marinus*. Abbreviations are as follows: B, branchiopore; E, developed eye; H, domed oral hood; ES, eye spot; F, furrow; I, iris; L, lateral lip of oral hood; LL, Lateral line; NN, non-existent notch; OB, ovoid branchiopore; P, pupil; T, transverse lip of oral hood; B, triangular branchiopore. From ammocoete to stage 2, the eye-spot (ES) of the animals becomes more pronounced. By stage 3, a distinct pupil (P) and iris (I) can be seen, which is well-defined by stage 5. In addition, there is a loss of the transverse notch in the oral hood (NN) and the branchiopores become ovoid in shape as the animals proceed from stage 3 to 4. Stage 5 is also characterized by “silvering” of the latero-ventral portions of the body. By stage 6, the lateral line (LL) is evident for the first time and development and differentiation of the eye continues into young adult hood.

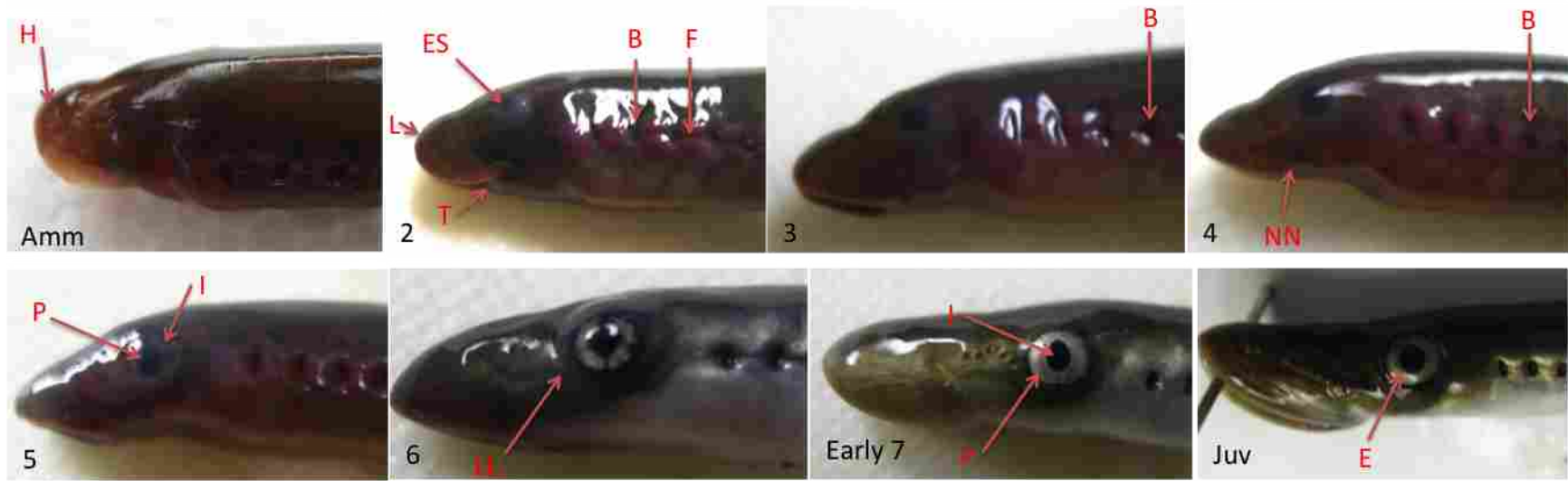




Fig. 3.2. Ventral view of the anterior regions of ammocoete (Amm), metamorphic stages 2 to 7, and juvenile (Juv) *P. marinus*.

Abbreviations are as follows: E, eye; I, infraoral lamina; NL, notched lip; NP, no papilla; PA, papilla; PC, posterior oral cirrhi; PD, premature oral disc; TO, teeth of oral disc; TP, tooth precursor. As animals begin transformation, the presences of papilla (PA) in the early stages are found within the oral hood. There is a loss of the notched lip (NL) from stages 3 to 4 and the formation of a premature oral disc (PD). Tooth precursors (TP) are prevalent in stage 5, and the eyes (E) can be seen protruding laterally from the body in stage 6. Through stage 7 the teeth of the oral disc (TO) have now been formed, and by young adulthood these sharp teeth are now bright yellow in color (TO).

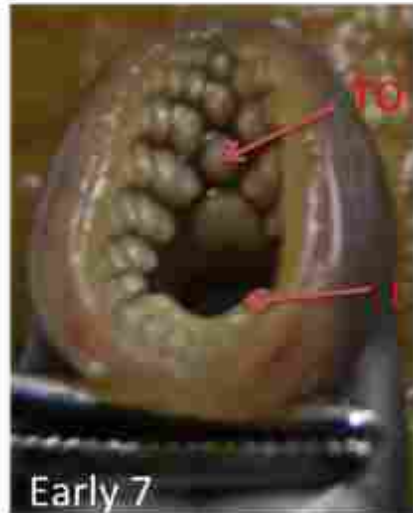


Fig. 3.3. Changes in the condition factor of sea lampreys before (ammocoete phase), during and after metamorphosis (juvenile phase). Bars sharing the same letter are not significantly different from one another ( $P < 0.05$ ). All values are shown as the mean  $\pm$  1 SEM, N = 6-10 per life stage.

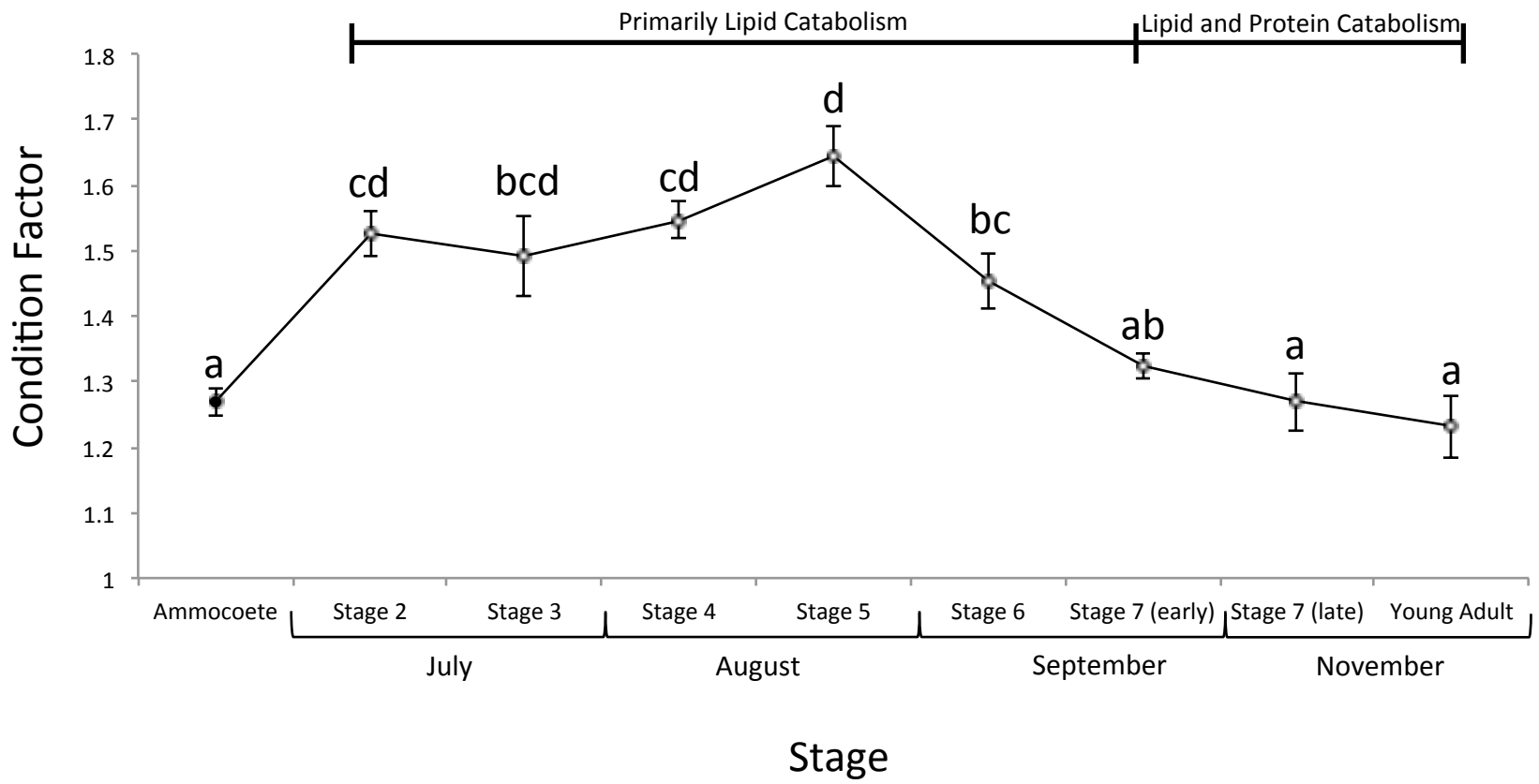


Fig. 3.4. Changes in (A)  $J_{\text{Amm}}$ , and (B,C) relative quantity of Rhcg2 in the gills of ammocoetes, metamorphosing and unfed juvenile sea lampreys. The quantity of Rhcg2 in the gill (B) was based on the relative density of bands normalized to  $\beta$ -actin in western blots of Rhcg2 protein in the gills (C). The stages of metamorphosis are as follow; S2: stage 2, S3: stage 3, S4: stage 4, S5: stage 5, S6: Stage 6, and S7: stage 7. Values shown are the mean  $\pm$  1 SEM, and N= 9 for measurements of  $J_{\text{Amm}}$ , and N = 4 for the relative quantify of Rhcg2 present in the gill at each stage of metamorphosis. Bars sharing the same letter are not significantly different from one another ( $P < 0.05$ ). Asterisks denote significant differences from measurements made in ammocoetes ( $P < 0.05$ ). Antibody used was that of *Takifugu rubripes* (accession no. NM\_001027934.1; amino acids 420–481; 1:1000 dilution).

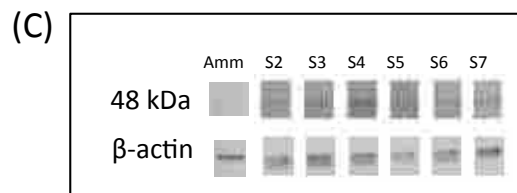
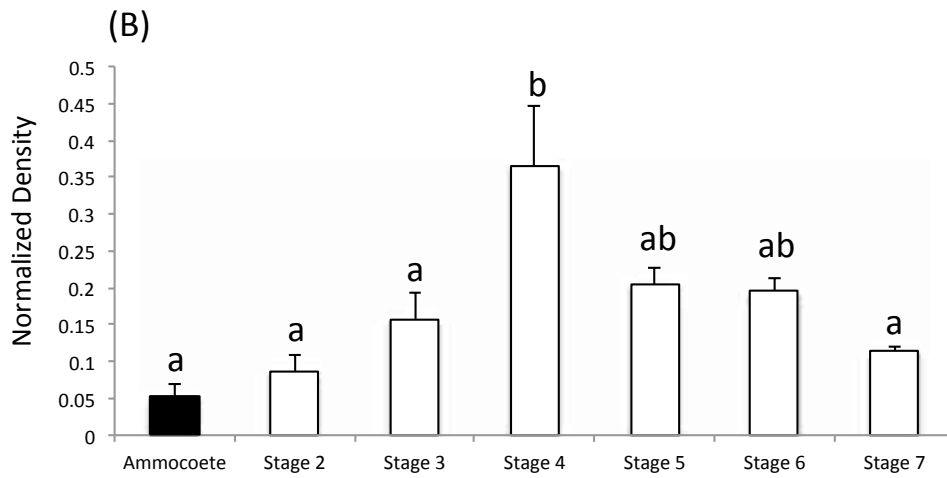
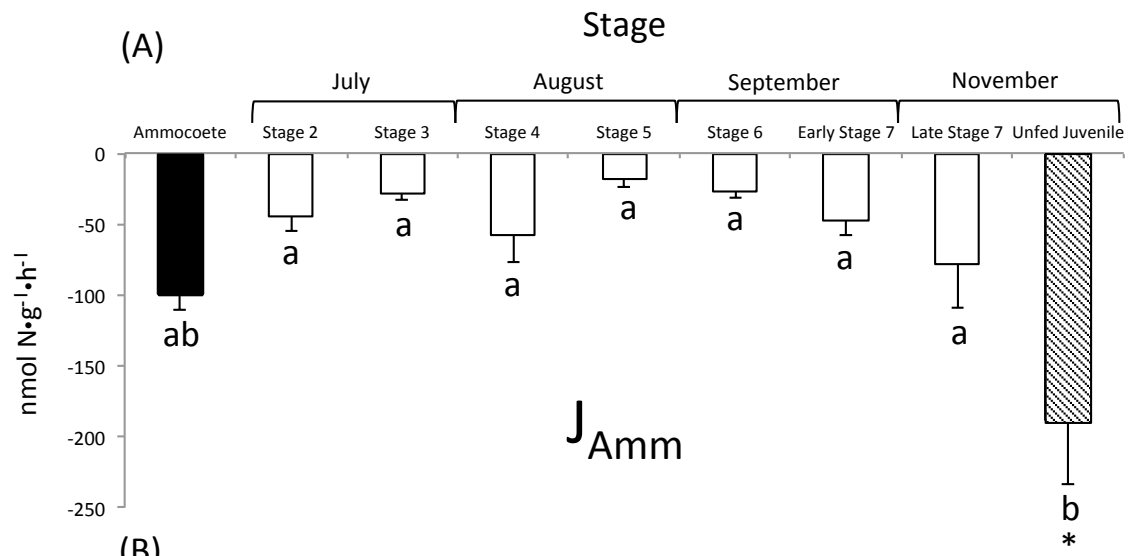


Fig. 3.5. Changes in (A)  $J_{\text{Urea}}$  and (B,C) relative quantity of UT in the gills of ammocoetes, metamorphosing and unfed juvenile sea lampreys. The quantity of UT in the gill (B) was based on the relative density bands normalized to  $\beta$ -actin in western blots of UT protein in the gills (C). Stages are as follows; S2: stage 2, S3: stage 3, S4: stage 4, S5: stage 5, S6: Stage 6, and S7: stage 7. Values shown are mean  $\pm$  1SE, and N= 9 for measurements of  $J_{\text{Urea}}$  and N = 4 for the relative quantify of UT present in the gill at each stage of metamorphosis. Bars sharing the same letter are not significantly different from one another ( $P < 0.05$ ). Asterisks denote significant differences from ammocoetes ( $P < 0.05$ ). Antibody used was that of *Danio rerio* (accession no. AY788989.1; amino acids 48-69; 1:500 dilution).

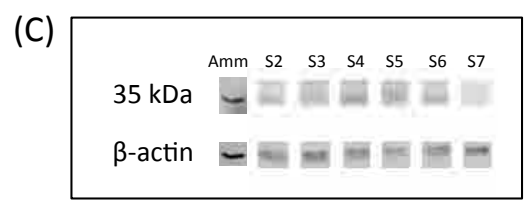
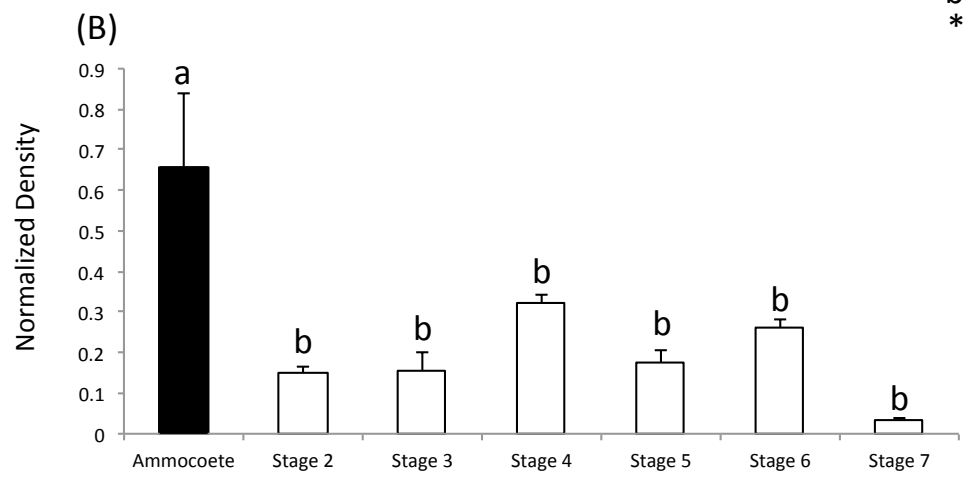
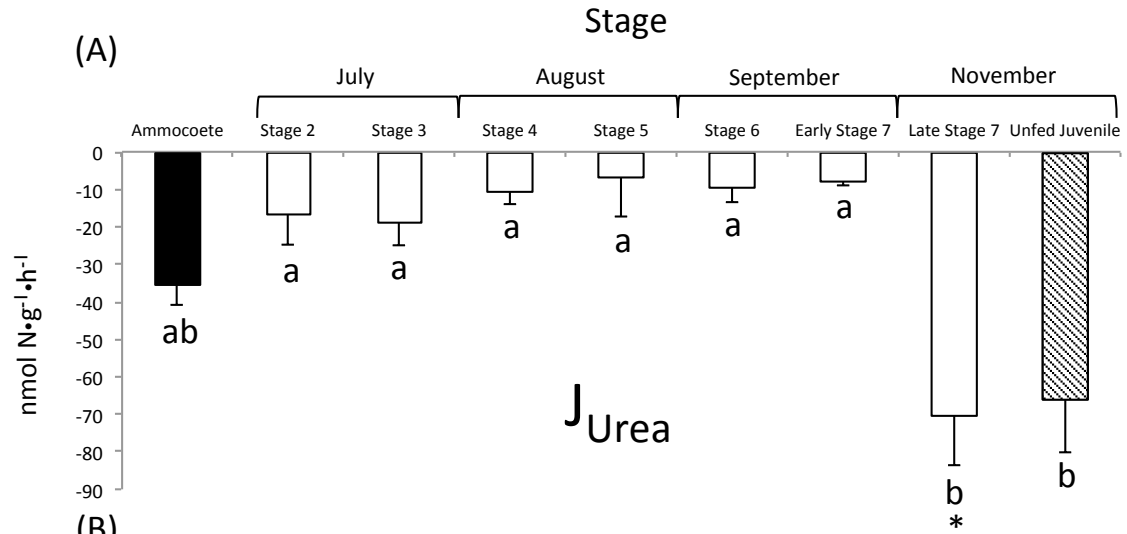




Fig. 3.6. Changes in (A) plasma ammonia and (B) urea concentrations in metamorphosing sea lampreys. Values are shown as the mean  $\pm$  1 SEM, and N =10 per group. Bars sharing the same letter are not significantly different from one another ( $P<0.05$ ).

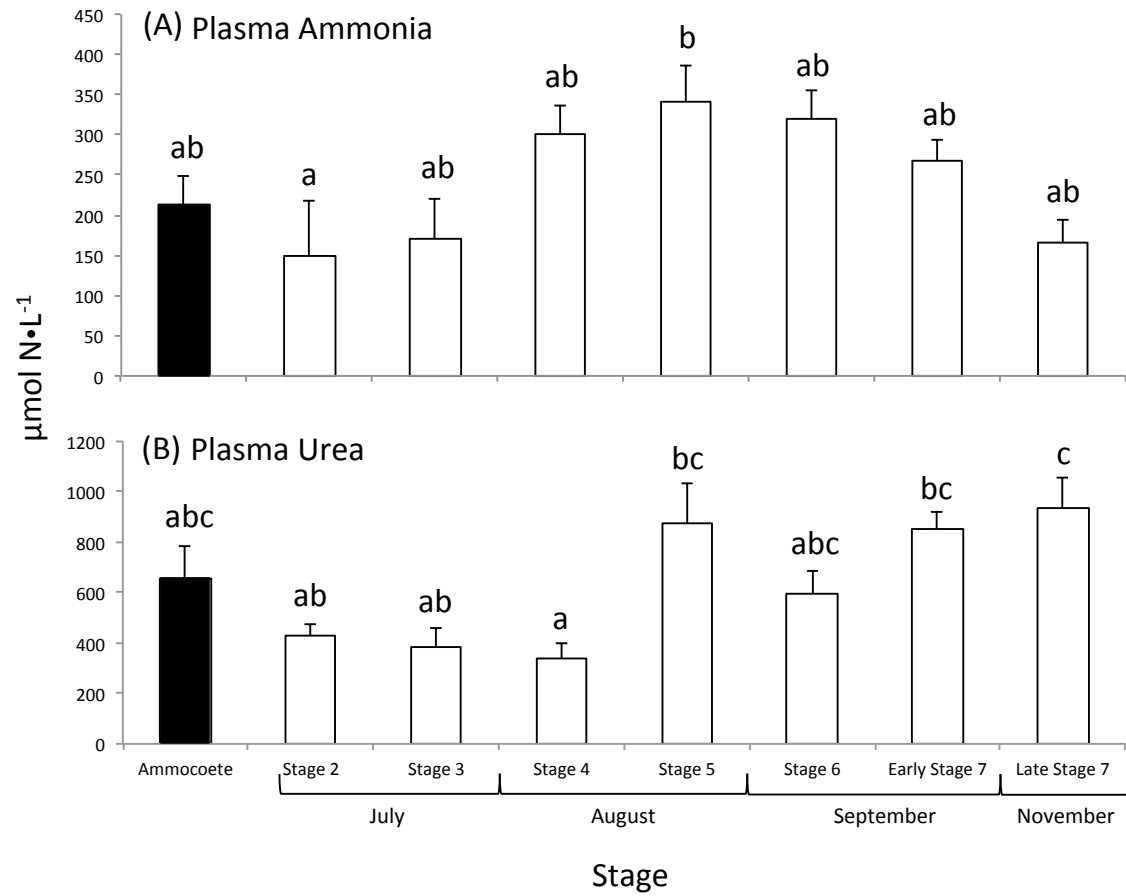


Fig. 3.7. Differences in (A)  $J_{\text{Amm}}$ , (B) plasma ammonia, and (C,D) the relative quantity of Rhcg2 in the gills of unfed juvenile, fed parasitic juvenile, and adult sea lampreys. The quantity of Rhcg2 in the gill (C) was based on the relative density of bands normalized to  $\beta$ -actin in western blots of Rhcg2 protein in the gills (D). Values shown represent the mean  $\pm$  1 SEM, and N= 6-8 for measurements of  $J_{\text{Amm}}$ , N > 6 depending on blood availability for plasma ammonia concentration, and N = 4 for the relative quantify of Rhcg2 present in the gill at each stage of metamorphosis. Bars with the same letter are not significantly different from one another (P<0.05). Antibody used was that of *Takifugu rubripes* (accession no. NM\_001027934.1; amino acids 420–481; 1:1000 dilution).

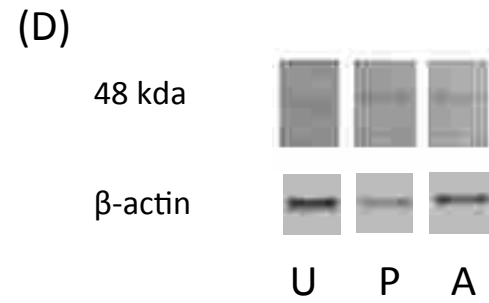
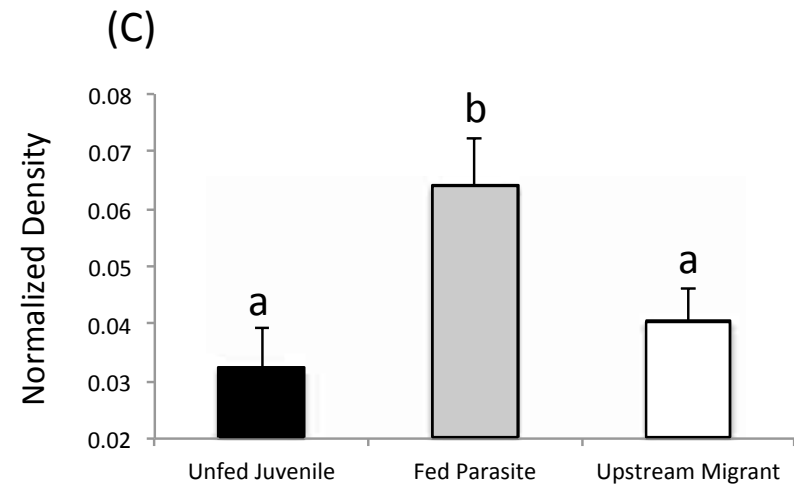
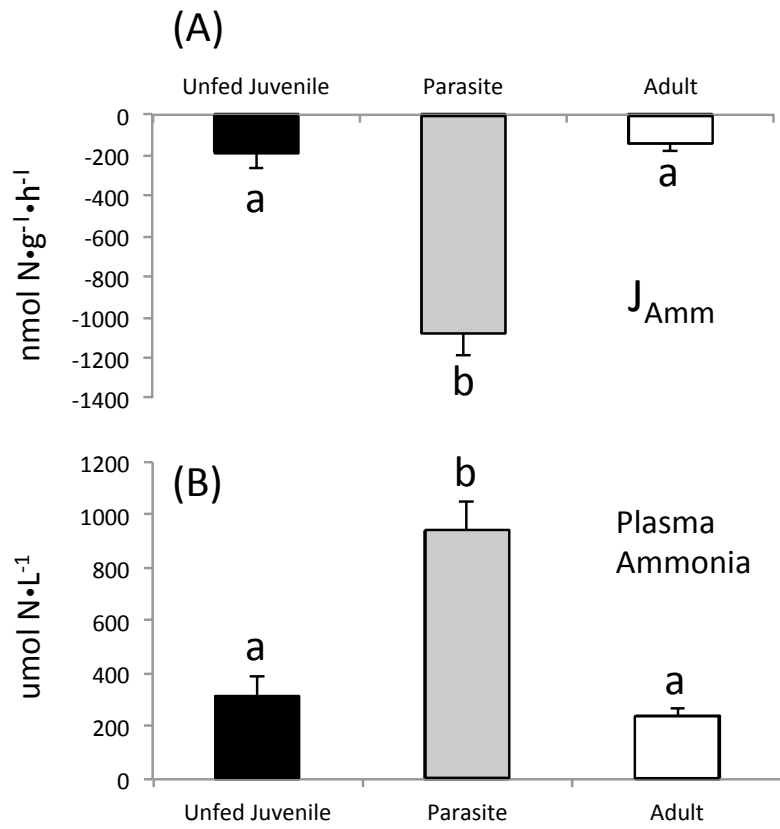
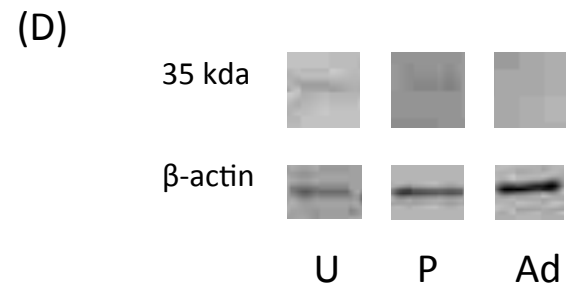
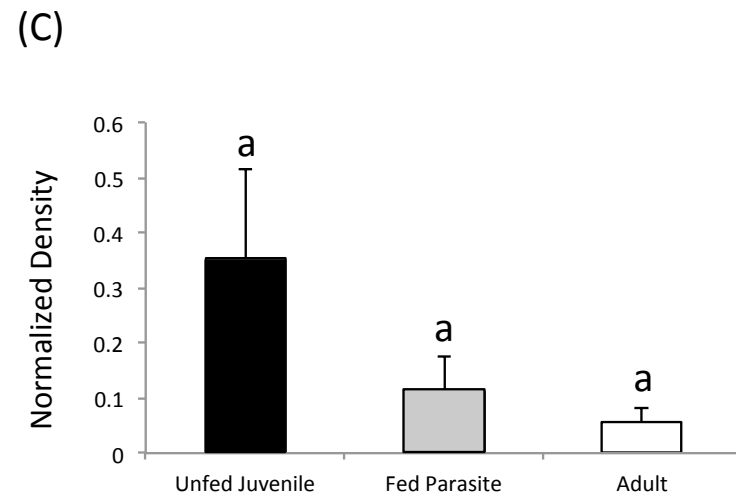
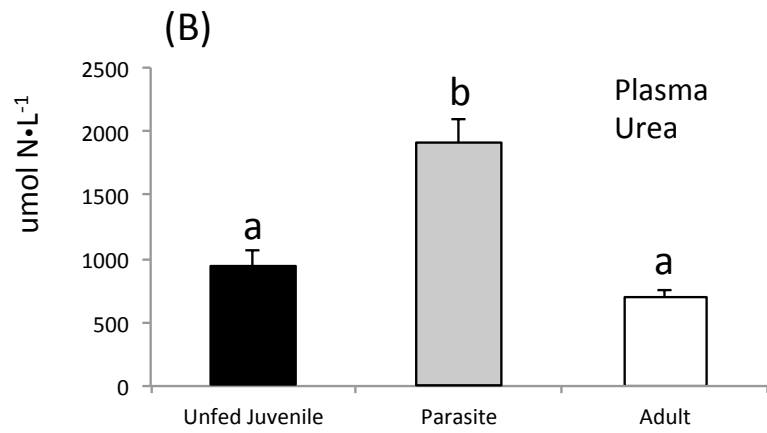
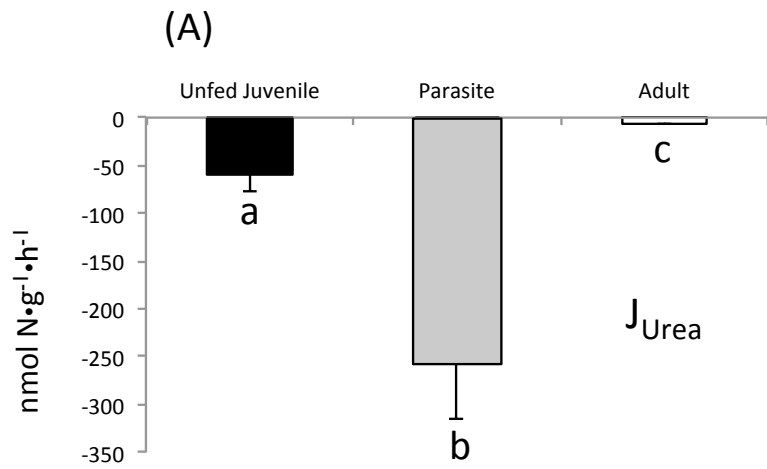


Fig. 3.8. Differences in (A)  $J_{\text{Urea}}$ , (B) plasma urea, and (C,D) the relative quantity of UT in the gills of unfed juvenile, fed parasitic juvenile, and adult sea lampreys. The quantity of UT in the gill (C) was based on the relative density of bands normalized to  $\beta$ -actin in western blots of UT protein in the gills (D). Values shown represent the mean  $\pm$  1 SEM, and N= 6-8 for measurements of  $J_{\text{Urea}}$ , N > 6 depending on blood availability for plasma urea concentration, and N = 4 for the relative quantify of UT present in the gill at each stage of metamorphosis. Bars with the same letter are not significantly different from one another ( $P < 0.05$ ). Antibody used was that of *Danio rerio* (accession no. AY788989.1; amino acids 48-69; 1:500 dilution).



## DISCUSSION

### *Relationship between $J_{Amm}$ and $J_{Urea}$ in metamorphosing sea lampreys*

In the months preceding metamorphosis, pre-metamorphic ammocoetes accumulate the large reserves of lipid needed to sustain the animals through this 3-4 month non-trophic period (Hardisty and Potter 1971b; O'Boyle and Beamish 1977). This reliance on lipid metabolism likely explains the 50-60 % reduction on in  $J_{Amm}$  observed during stages 2 to 6 of metamorphosis (Fig. 3.4A). It is unlikely that lower basal metabolic demands accounted for the reduced rates of  $J_{Amm}$  because oxygen consumption rates have been found to be stable or increase slightly during this period of metamorphosis (Lewis and Potter 1977). Further evidence can be seen in the changes in CF as the animals progressed through metamorphosis. Initially, body mass decreased but CF was relatively stable due to progressive shortening of the body with metamorphosis due to the development of the oral disc in stages 1-4. Indeed, by stage 5 CF had actually increased. Kao et al. (1997b) reported that, metamorphosing sea lampreys upregulated enzymes associated with lipolysis such as triacylglycerol lipase, but decreased the activity of lipogenic enzymes such as acetyl-CoA carboxylase and diacylglycerol acyltransferase. Sheridan and Kao (1998) reported that rates of lipolysis increase and lipogenesis decreases in the liver of metamorphosing sea lampreys from stages 3 to stage 6 renal and hepatic rates of lipolytic activities have been found to increase, whereas lipogenesis simultaneously declined in these same tissues. However in the intestine, the opposite was found, with an increase in triacylglycerols and total lipid content, which suggests that the reallocation of lipids during this metamorphic period is likely need to aid in the digestion

and the absorption of protein in the blood meals following metamorphosis (Sheridan and Kao 1998).

The precipitous decline in CF that occurs in the late stages of metamorphosis was likely related to an increase in basal metabolic demands as reflected by increases in oxygen consumption (Lewis and Potter 1977) and greater reliance on protein catabolism as lipid reserves were lowered. As a result, there was an increase in  $J_{Amm}$  and  $J_{Urea}$  that was likely due to greater reliance on proteolysis. Indeed, Youson et al. (1979) provided evidence of muscle wasting as the total volume of muscle is reduced in juvenile lampreys and that lipid comprised only 1% of whole animal wet weight in post-metamorphic juvenile lampreys. Moreover, most of this was phospholipid, rather than neutral lipids such as triacylglycerols (Youson et al. 1979), which are the preferred substrate for fatty oxidation (Metcalf and Gemmel 2005). Wilkie et al. (2006) also observed an up-regulation of enzymes associated with amino acid transamination and deamination following metamorphosis in sea lampreys, further illustrating the likelihood that lampreys are primarily relying on on-board protein reserves to satisfy their energetic needs prior to the parasitic phase. This switch to protein catabolism may in fact, be permanent because the vast bulk of the sea lampreys diet following metamorphosis will be protein-rich blood, which is ingested during the juvenile parasitic phase. Indeed, the amount of protein ingested by lampreys is 2-16% dry weight, which is approximately 5 times more than ingested by an intensively feeding salmon (Wood 2001; Wilkie et al. 2006).

As with  $J_{Amm}$ , there were significant changes in  $J_{Urea}$  throughout the transformation period in sea lamprey (Fig. 3.5A). Animals excreted relatively low levels of urea from stages 2 to early stage 7, which then spiked in early stage 7, and remained



elevated through to the juvenile phase. One free amino acid generated by proteolysis of white muscle is arginine (Mommsen et al. 1980), which yields urea when it is hydrolyzed via arginase. Although sea lampreys do not likely produce physiologically relevant amounts of urea using the ornithine urea cycle, sea lampreys do have significant amounts of arginase, suggesting that a major source of urea in post-metamorphic sea lampreys is arginine (Read, 1968; Wilkie et al. 2006).

Along with the changes in metabolism occurring throughout metamorphosis, there is also a drastic restructuring of the gills allowing for transition from fresh to saltwater (Peek and Youson, 1976). An up-regulation in several gill proteins (notably  $\text{Na}^+/\text{K}^+$ -ATPase) occurs in metamorphosing sea lamprey, and there is improved saltwater ionoregulatory capacity during the latter stages of metamorphosis (Reis-Santos et al. 2008). The up-regulation of  $\text{Na}^+/\text{K}^+$ -ATPase activity in the saltwater MRC begins in stages 3-5, and the animals are able to tolerate full seawater by stage 6 (Reis-Santos et al. 2008). Since multiple ion transport proteins are implicated in both Rh and UT performance in gill cells (Braun and Perry 2010), the increased abundance of ion transporters associated with downstream migration to marine environments may play a role in the facilitation of N-waste excretion using Rh and UT proteins.

A notable observation was that metamorphosing sea lamprey briefly exhibited ureotely ( $J_{\text{Urea}} > J_{\text{Amm}}$ ) during the late stage 7 of metamorphosis. We propose that the sea lamprey may have up-regulated urea production at this time to detoxify ammonia, which was being generated at high rates due to proteolysis of the muscle. Another intriguing possibility is that the lamprey used urea as a cloaking agent to mask ammonia excretion in order to decrease the likelihood of predation during this vulnerable period when the

starved, relatively emaciated animals first emerge from the substrate in the initial phases of their open water phase. Indeed, Barimo and Walsh (2006) provided convincing evidence that the gulf toadfish (*Opsanus beta*) produces urea for this purpose to avoid detection by predators in shallow marine waters.

#### *Rhcg2 and UT protein expression in metamorphosing seal lamprey*

The patterns of Rhcg2 expression closely followed those of plasma ammonia concentration, suggesting that Rhcg2 expression was a direct result of internal ammonia concentration in the metamorphosing sea lampreys (Fig. 3.4B). Braun et al. (2009) reported that increased external ammonia concentrations and associated build-ups of plasma ammonia increased the mRNA expression of Rhcg2 in zebrafish larvae. It was also notable that  $J_{\text{Amm}}$  was lowest during stages 4 and 5, suggesting that the restructuring of the gills at this time in preparation for the FW-SW transition impaired ammonia excretion, and necessitated an increase Rhcg2 abundance to prevent greater reductions in the excretion of ammonia.

In terms of UT expression in gill during metamorphosis, it was again seen that the mid to late stages of metamorphosis (stages 4 and 6) had the greatest abundance of UT (Fig. 3.5B). Much the same as found in the changes occurring with plasma ammonia, plasma urea was elevated from stages 5 onward (Fig. 3.6B). It was also during this time when  $J_{\text{Urea}}$  was at its lowest point, until late stage 7 where it increased markedly. This suggests that gill UT expression may directly reflect plasma urea concentration, as could occur if  $J_{\text{Urea}}$  were hindered during the gill-restructuring that takes place in late metamorphosis. McDonald et al. (2009) observed that increased urea concentrations in *Opsanus beta* (Gulf Toadfish) caused a subsequent increase in tUT mRNA transcription.

However, the authors were unable to equivocally state if this transcriptional response was due to an increase in urea or an increase in cortisol levels, or both.

Sea lampreys are the earliest known vertebrate to use corticosteroid hormone control (Close et al. 2010). Rather than cortisol, lampreys use 11-deoxycortisol (11-DOC), a precursor to cortisol and corticosterone in higher vertebrates. Like cortisol in other fishes, 11-DOC serves as a glucocorticoid (GC) and mineralcorticoid (MC), showing high specificity to corticosteroid receptors (CR). Released in response to stress, 11-DOC has been linked to ion transport ( $\text{Na}^+/\text{K}^+$ -ATPase) activity in the gills of sea lamprey. Activation of  $\text{Na}^+/\text{K}^+$ -ATPase is important in metamorphosing sea lamprey and the osmoregulatory challenges they face during migration to seawater. Moreover, corticosteroids are lipolytic, and have been observed to play a crucial role in lipid metabolism and kinetics in aquatic species that metamorphose (Sheridan, 1986; Gunesch, 1974). Since high circulating cortisol results in decreased lipogenesis and increased lipolysis (Sheridan and Kao 1998), it seems likely that 11-DOC plays a role in the metamorphosis of sea lamprey,  $\text{Na}^+/\text{K}^+$ -ATPase abundance, and lipid metabolism. However, it should be noted that increased circulating corticosteroid levels have been known to decrease Rh glycoprotein and UT expression in *Opsanus beta* (Rodela et al. 2009; Rodela et al. 2012). Thus, even though total lipids and triacylglycerol content decrease following stage 3 (Kao et al. 1998), and 11-DOC is likely rising, why too is Rhcg2 and UT protein expression at its peak (Rodela et al. 2009; Rodela et al. 2012)? Further investigation into the dynamics of 11-DOC and corticosteroid receptor abundance and distribution is clearly needed to establish the relationships between nitrogen excretion, and Rhcg and UT function in metamorphosing sea lampreys.

*The onset of feeding and its effects on  $J_{Amm}$  and  $J_{Urea}$  along with Rhcg2 and UT abundance*

As sea lampreys complete metamorphosis and become juveniles they travel downstream to open waters where they begin feeding (Beamish and Potter 1975; Farmer 1980; Bergstedt and Swink 1995). In the present study, feeding led to 5-fold increases in  $J_{Amm}$  and a marked 3-fold upregulation of Rhcg2 in the gills. The very high scope for  $J_{Amm}$  in parasitic juvenile lampreys is related to their high capacity to deaminate amino acids from their protein-rich blood meals (Wilkie et al. 2004; Wilkie et al. 2006). In addition to increased activities of the transaminases alanine aminotransferase and aspartate aminotransferase, the activity of the glutamate dehydrogenase (GDH), increases 6-fold following sea lamprey parasitism on teleosts (Wilkie et al. 2006). The greater Rhcg2 abundance in gills was likely driven by postprandial increases in plasma ammonia, which was 3-fold higher immediately following feeding (Fig. 3.7C). In rainbow trout, Rhcg2 mRNA expression is increased in response to increased plasma ammonia following feeding (Zimmer et al., 2010) and after ammonia infusion (Nawata and Wood 2009).

The Rhcg proteins may work in conjunction with branchial  $H^+$ -ATPase proteins as part of an ammonia transport metabolon (Wright and Wood 2009). However,  $H^+$ -ATPase was actually down-regulated in anadromous lampreys preparing for sea water entry (Reis-Santos et al. 2008). For this reason, and because the gill is likely more permeable to  $NH_4^+$  in marine waters (Wilkie 2002; Evans et al. 2005), it is quite possible that any feeding-induced increase in Rhcg2 would be less than in landlocked sea

lampreys. Future studies will be needed to address how the Rhcg2-H<sup>+</sup>-ATPase-Na<sup>+</sup>/H<sup>+</sup>-exchange metabolon functions in both the landlocked and anadromous populations of sea lampreys.

As with  $J_{\text{Amm}}$ ,  $J_{\text{Urea}}$  increased markedly following feeding in anadromous sea lampreys (Fig. 3.8A), but there was no difference observed in branchial UT abundance (Fig. 3.8C). As with some other fishes, UT abundance in sea lamprey was greatest in the larval stages of development, and to a lesser extent in adults (Wright et al. 1995; Chadwick and Wright, 1999; Braun et al. 2009; Chapter 2). A greater reliance on renal excretion of urea in the later stages of the sea lamprey lifecycle could also explain the relatively low abundance of gill UT in post-metamorphic sea lamprey.

### *Perspectives*

The dramatic metamorphosis of the anadromous sea lamprey as it prepares for an active lifestyle in seawater occurs over a 3-4 month non-trophic period where lampreys rely on internal body reserves to meet their energy demands (Youson et al. 1980). During this period N-waste production and excretion change in response to the switch from a diet of primarily detritus in ammocoetes, to starvation during metamorphosis, and then to protein-rich blood following metamorphosis. Restructuring of gills during metamorphosis could affect the sea lamprey's ability to excrete ammonia and urea during metamorphosis and it is proposed that corresponding changes in internal N-waste products led to increases in Rhcg2 and UT abundance during this period. Research to determine the exact location of these proteins within the gill tissue, along with their transport properties is needed to fully understand how Rh and UT proteins function in sea lamprey.

Nevertheless, the present study clearly shows that the patterns of ammonia and urea excretion during metamorphosis are dynamic, and reflected by changes in Rh glycoprotein and UT protein abundance in the gills of sea lampreys. Further investigations into other factors that may be modulated during metamorphosis, such as corticosteroids, should prove useful in building a complete picture of metamorphosis and metabolism in this ancient vertebrate.

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## **Chapter 4**

### **General Discussion and Integration**

There were 4 overarching goals to the present thesis; I) Determine the mechanisms by which N-wastes are excreted ( $J_{\text{Amm}}$  and  $J_{\text{Urea}}$ ) at different stages of the sea lamprey life cycle, II) Ascertain how the ammonia and urea excretion capacity changes during the life cycle of the sea lamprey, III) Quantify the role that Rh glycoproteins and UT proteins play in the excretion of N-wastes during different stages of the sea lamprey life cycle, and IV) Relate how changes in metabolism during the non-trophic period of metamorphosis influences patterns of N-waste excretion in sea lampreys.

Beginning on a larger scale with whole fish experiments,  $J_{\text{Amm}}$  and  $J_{\text{Urea}}$  were investigated in both ammocoete and adult sea lamprey in the presence of environmental stressors such as highly alkaline water and high external ammonia (HEA). Unfortunately fish were unable to survive the increased pH for greater than 24 h, however the HEA experiments did uncover life stage differences in N-waste excretion patterns in the presence of external ammonia. In both ammocoete and adult sea lampreys, HEA exposures resulted in a complete reversal of  $J_{\text{Amm}}$  and a net uptake of ammonia from the environment for the first 24 h of the experiment (Fig. 2.1A, 2.2A). However, during the later time periods of the exposure, ammocoetes were observed to exhibit  $J_{\text{Amm}}$  indicating that ammonia excretion in this stage may be more efficient than that of the migratory adults. Conversely,  $J_{\text{Urea}}$  for ammocoetes was found to remain unchanged throughout the duration of the HEA exposure, whereas  $J_{\text{Urea}}$  for adult lamprey significantly increased approximately 4-fold or greater at all time periods (Fig. 2.1B, 2.2B). It was postulated that this increase in  $J_{\text{Urea}}$  is a function of environmental ammonia stimulating the uricolytic pathway in adults, resulting in an increased concentration of urea produced and excreted by this life stage. Further examination of HEA tissues (such as gill, skin,

intestine and kidney) may reveal differential patterns of expression and abundances of mRNA and protein (Rh and UT) associated with N-waste excretion.

For the first time, amplification of an albeit small, but conserved fragment (109 bp) of UT like sea lamprey cDNA is a strong indicator that these transport proteins are present in this phylogenetically ancient jawless vertebrate (Fig. 2.5). Subsequent western blot analysis, using a well-characterized antibody to the zebrafish (*Danio rerio*) UT, resulted in banding at 38 kDa in several tissues of adult sea lamprey tissue (gill, skin, muscle, kidney, intestine, liver; Fig. 2.6). Elongation of the fragment found in the lamprey genome would prove useful in determining where exactly sea lamprey UT fits from an evolutionary standpoint, expanding upon the current phylogenetic tree of Anderson et al. (2011).

The research presented above aimed to investigate the effects of active parasitism on N-waste metabolism, capacity, and excretion mechanisms (UT and Rh) when compared to filter-feeding ammocoetes and non-trophic intermediates (unfed juveniles and adults; Fig. 4.1). Parasites excreted far greater amounts of urea and ammonia (approximately 5-fold) than did both unfed juvenile and adult lampreys (Fig. 3.7A, 3.8A). However, western blot analysis (Fig. 2.7A) of four distinct life stages revealed that the gill tissue of ammocoetes had significantly higher amounts of UT protein compared to both fed parasitic juvenile and adult sea lampreys (Fig. 2.7B). It is possible that branchial urea excretion in burrow-dwelling ammocoetes favored, with renal excretion becoming more important in post-metamorphic individuals (M.P. Wilkie, unpublished findings). Since the ammocoete is a burrow-dwelling animal, and the majority of water replacement in the burrow occurs around the gills, it is therefore much more likely that if ammocoetes



excreted large quantities of urea as urine their burrows would become fouled with urea. Through branchial excretion, the animals expel the majority of urea out of their burrows and into the surrounding water column.

Investigation of Rhcg2 abundance in unfed juveniles, parasites and adults on the other hand revealed that there was a much greater preponderance of this protein in the gill tissues of parasitic sea lamprey (Fig. 3.7A), indicating the importance of branchial ammonia excretion in these individuals. Since greater quantities of protein are ingested in the parasitic stage, the  $\text{NH}_3$  produced through deamination drastically increases plasma ammonia concentrations (Fig. 3.7B). This increase results in greater Rhcg2 abundances and therefore increases the ability for  $J_{\text{Amm}}$ . Furthermore, divided chamber studies confirmed that branchial excretion of ammonia is the preferred route for other stages of the sea lamprey life cycle (M.P. Wilkie, unpublished observations). On model that attempts to explain this life stage dependent transition in excretion patterns, Fig. 4.1, outlines the switch from N-waste excretion that is almost exclusively gill-based in ammocoete individuals, to a combination of gill (ammonia) and renally (urea) driven excretion. As the animals mature, and switch their dietary intake, it seems that filtration and subsequent removal of urea in the form of urine is paramount to  $J_{\text{Urea}}$ . An in depth looking into the changes of the ultrastructure of the kidneys as the animals mature, as well as pre- and post-feeding, would go a long way in determining the exact mechanism by which the sea lamprey produce the concentrated urine as they mature. On the flip side, despite the changes in breathing patterns in the gills following metamorphosis, sea lamprey, like many other fishes, remain using the gills as the primary site of  $J_{\text{Amm}}$ .

Another major goal of this thesis was to investigate the changes in N-waste production and excretion during the 7 distinct stages of metamorphosis. Six of the 7 stages of metamorphosis were identified, during which the CF of the animals sharply declined following stage 4 of metamorphosis likely due to the reorganization and depletion of lipid stores utilized to fuel this non-trophic 3-4 month transformation period (Fig. 3.3). Measurements of  $J_{\text{Amm}}$  and  $J_{\text{Urea}}$  revealed that both decrease upon initiation of metamorphosis and remain relatively low until late stage 7, where there is a sharp increase in  $J_{\text{Amm}}$  and  $J_{\text{Urea}}$  through into the juvenile stage (Fig. 3.4A; 3.5A).

Analysis of Rhcg2 and UT protein abundances in the gills of metamorphic sea lampreys stages 2-7 showed that of the amount of Rhcg2 and UT was greatest during mid metamorphosis (Stage 4; Fig 3.4B, 3.5B), and it was postulated that these heightened abundances may be a combination of an inability to excrete N-wastes due to extensive gill restructuring during this time and corresponding internal buildups of ammonia and urea respectively (Fig. 3.6A, B). Interestingly, corticosteroid hormones, which have been implicated in the modulation of Rh and UT expression (Rodela et al. 2009; Rodela et al. 2012), would likely be increased in animals undergoing metamorphosis (Fig. 4.2). This model may explain the decrease occurring with Rhcg2 and UT abundance following stage 4, and may explain the decreases observed in  $J_{\text{Amm}}$  and  $J_{\text{Urea}}$  along with the corresponding decreases in lipid content of these animals (Kao et al. 1998), along with the increases in reported in  $\text{Na}^+/\text{K}^+$ -ATPase activity (Reis-Santos et al. 2008).

Further research is needed to determine the exact location and distribution of both Rh and UT proteins within the excretory organs of sea lamprey. Immunohistochemical analysis of tissues such as gill, skin and kidney may also reveal differences in N-waste

associated proteins, and their relationships to excretion and the possibility of accessory functions these proteins may have. For example, urea is an important osmolyte in several marine species (Hammerschlag 2006), therefore, modulation in UT abundances could play a role in urea retention, like in elasmobranchs (Fines et al. 2001) possibly aiding in ion balance while sea lamprey are at sea. Likewise, Rh proteins have been postulated to be involved in a dual role as CO<sub>2</sub> carriers (Endeward et al. 2006; Kusta et al. 2006). Therefore, under conditions of hypercapnia (increased arterial CO<sub>2</sub>), Rh expression may increase, which was the case with Rhag in rainbow trout exposed to high CO<sub>2</sub> (Nawata and Wood 2008).

On a larger scale, sea lamprey research can aid in both the control of and protection of this species, along with other lamprey species. In the Great Lakes, lampreys are invasive predators that have had, and continue to have, pronounced ecological and economic impacts through their parasitism/predation of several game fishes. Indeed, sea lampreys decimated Great Lakes' fish populations in the early 20<sup>th</sup> century (Smith and Tibbles 1980), affecting both commercial fishing and sport fishing. Due the sea lampreys' economic and social impact, an ongoing sea lamprey control program was initiated in the Great Lakes systems. Through a partnership between Canada and the United States, the Great Lakes Fishery Commission (GLFC) now oversees a wide range of strategies to control sea lampreys. For example, treatment of entire streams with chemical compounds such as 3-trifluoromethyl-4-nitrophenol (TFM) is one of the most widely used methods of sea lamprey control. More recently, however, new avenues of treatment, such as the use of lamprey pheromones are being tested (Johnson et al. 2009). Other strategies, such as barriers to prevent migration of adults (Lavis et al. 2003), and

the release of sterile male-adults (Twohey et al. 2003) have also been used to help control sea lamprey populations..

However, sea lampreys, and closely related species are threatened or endangered in other parts of the world where they have been part of the ecosystem for millions of years. Species such as the brook lamprey (*Lampetra planeri*) and river lampreys (*Lampetra fluviatilis*) are becoming endangered and even extinct in some parts of Europe, especially where they are considered a delicacy (Kelly and King 2001). Moreover, pollution, and the construction of dams/weirs have been a large contributor to the declining populations of the last 30 years (Thiel et al. 2009). Implementation of conservation programs is needed to protect these ancient species from only being found in the fossil record. Therefore, in both circumstances outlined above, control and protection of lamprey species, the need for integrative approach to researching all aspects of their natural history such as their environment, physiology, biochemistry and molecular makeup is essential in attaining solutions.

## FIGURES

Fig. 4.1 Detailed look at (A) the differences in diet, lifestyle, habitat, and environment in ammocoete, unfed juvenile, parasitic, and adult sea lampreys. (B) Relative abundances of Rhcg2 and corresponding  $J_{Amm}$  in ammocoete, unfed juvenile, parasitic, and adult sea lampreys. (C) Relative abundances of UT and corresponding  $J_{Urea}$  in ammocoete, unfed juvenile, parasitic, and adult sea lampreys. In panels B and C, the hatched dashed lines represent to protein abundances whereas the solid lines refer to excretion rates.

(A)

	<b>Ammocoete</b>	<b>Unfed Juvenile</b>	<b>Parasite</b>	<b>Adult</b>
<i>Diet</i>	- Filter-feeder	- Non-trophic	- Protein rich	- Non-trophic
<i>Lifestyle</i>	- Sedentary	- Active	- Active	- Active
<i>Habitat</i>	- Sediment	- Open water	- Open water	- Open water
<i>Environment</i>	- Freshwater	- Fresh/ Seawater	- Seawater	Fresh/ Seawater

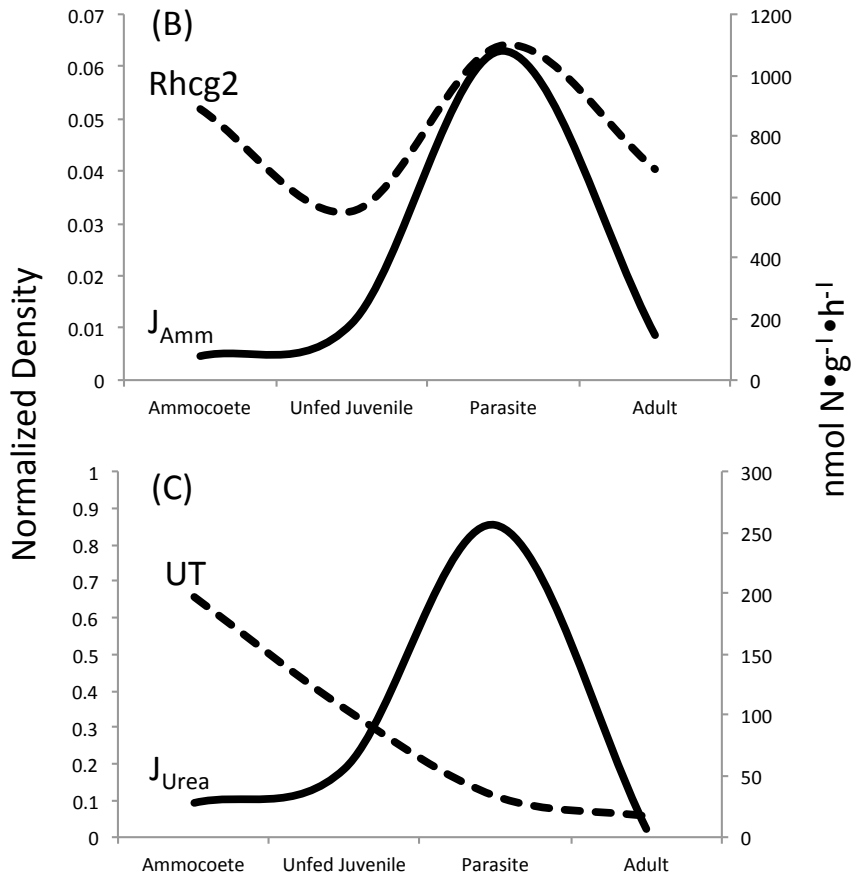
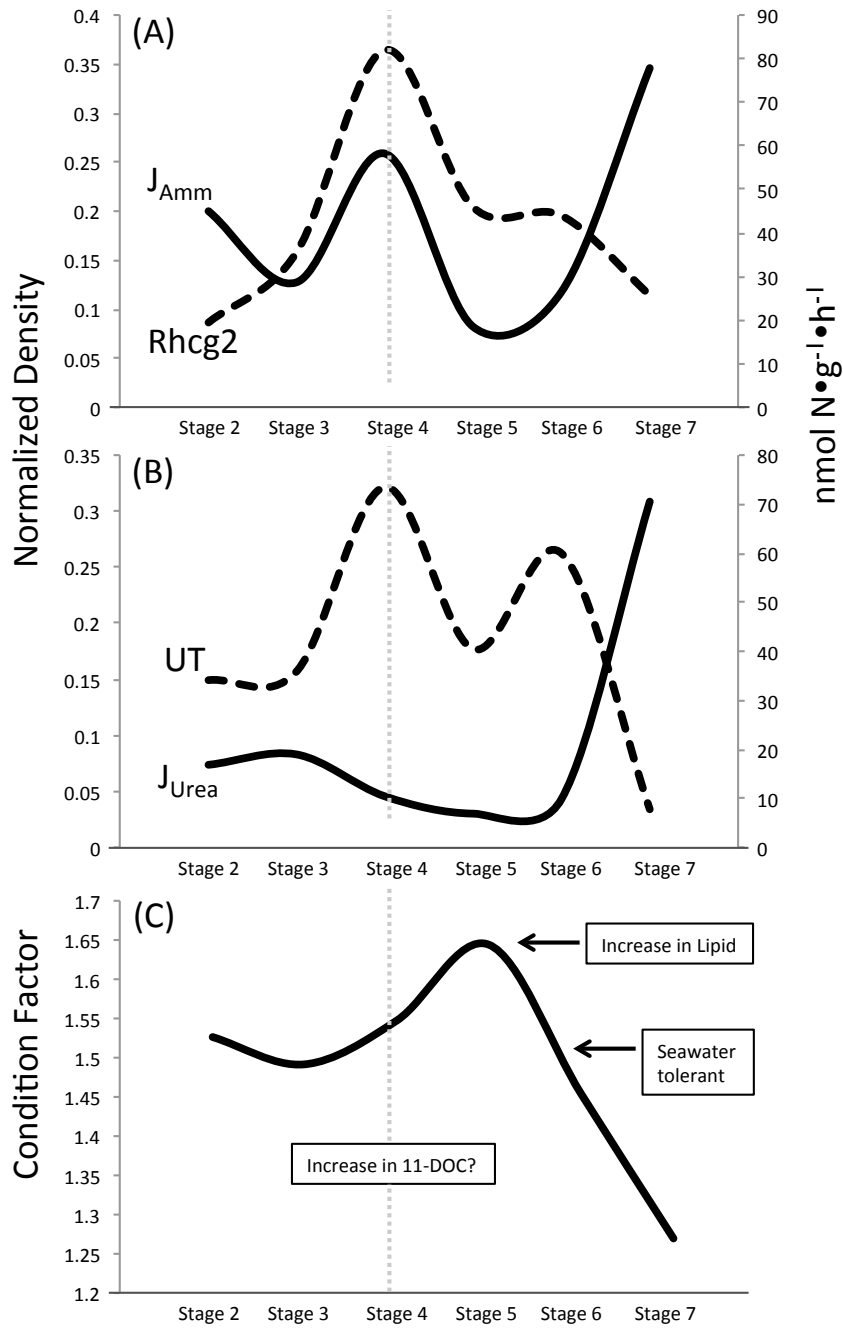


Fig. 4.2. Relative abundances of (A) Rhcg2 and (B) UT along with corresponding  $J_{\text{Amm}}$  and  $J_{\text{Urea}}$  in metamorphosing sea lampreys stages 2-7. (C) Condition factor and proposed timing in which 11-DOC could play a role in lipid metabolism and  $\text{Na}^+/\text{K}^+$ -ATPase regulation in lampreys readying for sea. In panels A and B, dashed lines represent to protein abundances, whereas solid lines refer to excretion rates.





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