NRC Research Press

ARTICLE

Rh glycoprotein immunoreactivity in the skin and its role in extrabranchial ammonia excretion by the sea lamprey (*Petromyzon marinus*) in fresh water

S.D. Blair, M.P. Wilkie, and S.L. Edwards

Abstract: Aquatic organisms employ various strategies to excrete ammonia across the gills, skin, and (or) renal routes. During three different stages of their life cycle, we hypothesized that the basal vertebrate sea lamprey (*Petromyzon marinus* L., 1758) used the skin as a route for ammonia excretion. Measurements of ammonia excretion using divided flux chambers revealed that extrabranchial sites (skin plus renal) of ammonia excretion were quantitatively more important in larval sea lampreys, but following metamorphosis, the gills became the dominant route of excretion in juvenile sea lampreys. Despite the greater relative importance of the skin in the larval stage, Rh glycoprotein isoforms Rhbg, Rhcg1, and Rhcg2 were detected in the skin in all three sea lamprey life stages examined, but the patterns of expression were dependent on the life stage. We conclude that, during the relatively sedentary filter-feeding larval stage, extrabranchial routes play an equally important role as the gill in facilitating ammonia excretion. However, the gills by virtue of their extensive branchial vasculature become the dominant route of ammonia excretion following metamorphosis because of the need to offload greater amounts of ammonia arising from higher rates of basal ammonia production and the potential to excrete higher amounts of ammonia following ingestion of protein-rich blood in the parasitic stage.

Key words: sea lamprey, ammonia, Rh glycoprotein, *Petromyzon marinus*, feeding, skin.

Résumé : Les organismes aquatiques emploient différentes stratégies pour excréter l'ammoniac par les branchies, la peau ou les voies rénales. Nous avons postulé que, durant trois étapes distinctes de son cycle biologique, la lamproie (*Petromyzon marinus* L., 1758), un vertébré basal, utilise la peau comme voie d'excrétion de l'ammoniac. Des mesures de l'excrétion d'ammoniac obtenues en utilisant des chambres de flux divisées révèlent que les sites extrabranchiaux (peau plus reins) d'excrétion d'ammoniac sont quantitativement plus importants chez les larves de lamproie, mais qu'après la métamorphose, les branchies deviennent la voie dominante d'excrétion chez les lamproies juvéniles. Malgré la plus grande importance relative de la peau à l'étape larvaire, les isoformes de glycoprotéine Rh Rhbg, Rhcg1 et Rhcg2 ont été détectés dans la peau pour les trois étapes du cycle biologique des lamproies examinées, mais les motifs d'expression dépendaient de l'étape. Nous en concluons que, durant l'étape relativement sédentaire des larves filtreuses, les voies extrabranchiales jouent un rôle tout aussi important que les branchies dans la facilitation de l'excrétion d'ammoniac. Cependant, grâce à leur vaste système vasculaire, les branchies deviennent la voie dominante d'excrétion d'ammoniac après la métamorphose en raison de la nécessité d'évacuer les plus grandes quantités d'ammoniac découlant de taux accrus de production basale d'ammoniac et du potentiel d'excréter de plus grandes quantités d'ammoniac après l'ingestion de sang riche en protéines à l'étape parasitique. [Traduit par la Rédaction]

Mots-clés : lamproie, ammoniac, glycoprotéine Rh, *Petromyzon marinus*, alimentation, peau.

Introduction

Ammonia is the toxic end product of amino acid catabolism that is either excreted as such or following its conversion to less toxic urea or uric acid in higher vertebrates (Wright 1995; Ip et al. 2001). Due to its high solubility in water, fishes predominantly excrete the majority of their nitrogenous wastes (N-wastes) as ammonia, along with lesser amounts of urea (Wood 1993). In most fishes, ammonia is mainly excreted across the gills. However, extrabranchial routes such as the skin and kidneys may serve as additional or alternate sites for ammonia excretion (Read 1968; Sayer and Davenport 1987; Smith et al. 2012; Zimmer et al. 2014*a*), particularly in amphibious air-breathing fishes that are capable of volatilizing NH_3 across the skin (Morii et al. 1978; Tsui et al. 2002; Frick and Wright 2002; Litwiller et al. 2006; Hung et al. 2007). The skin of larval fishes also appears to play a key role in ammonia excretion prior to full development of the gills (Braun et al. 2009).

Early work by Smith (1929) and Krogh (1938) linked ammonia excretion to the uptake of Na^+ in the gills of freshwater fishes. Since that time, numerous studies have been conducted suggesting two possible mechanisms of ammonia excretion. The weight of evidence now suggests that branchial ammonia excretion usually involves NH_3 diffusion down partial pressure gradients via

Received 19 May 2016. Accepted 17 November 2016.

S.D. Blair.* Department of Biology, Appalachian State University, Boone, NC 28608, USA; Department of Biological Sciences, University of Alberta, Edmonton, AB T6G 2E9, Canada.
M.P. Wilkie. Department of Biology, Wilfrid Laurier University, Waterloo, ON N2L 3C5, Canada; Department of Biology, Mount Allison University, Sackville, NB E4L 1G7, Canada.
S.L. Edwards. Department of Biology, Appalachian State University, Boone, NC 28608, USA.
Corresponding author: Salvatore D. Blair (email: sdblair@ualberta.ca).
*Present address: Department of Biological Sciences, University of Alberta, Edmonton, AB T6G 2E9, Canada.

Published at www.nrcresearchpress.com/cjz on 13 December 2016.

nonerythroid Rhesus (Rh) glycoproteins in aquatic invertebrates and vertebrates (Weihrauch et al. 2004; Hung et al. 2007; Nakada et al. 2007*a*, 2007*b*; Nawata et al. 2007; Nawata and Wood 2008; Wright and Wood 2009). Molecular, immunohistochemical, and physiological studies on Japanese pufferfish (*Takifugu rubripes* (Temminck and Schlegel, 1850)) first demonstrated that ammonia excretion was mediated by three types of Rh glycoproteins: (1) Rhag, which was localized to red blood cells (RBCs); (2) Rhbg had a basolateral distribution on branchial ionocytes or specialized ion-transporting cells; (3) Rhcg was apically located on the ionocytes (Nakada et al. 2007*a*). A role for Rh glycoproteins in ammonia excretion was definitively demonstrated using morpholino knockdowns in developing zebrafish (*Danio rerio* (Hamilton, 1822)), in which the absence of Rhbg and Rhcg protein expression greatly reduced ammonia excretion (Braun et al. 2009). The Rh glycoproteins likely act as facilitated diffusion NH_3 channels, in which NH_3 is unloaded from RBCs via Rhag, subsequently entering ionocytes via basolaterally located Rhbg, before being excreted to the water via apical Rhcg (Wright and Wood 2009). An indirect coupling between Rhcg-mediated NH_3 diffusion and H^+ extrusion via apical V-ATPases and (or) NHEs likely leads to the trapping of NH_3 as NH_4^+ to maintain favourable NH_3 diffusion gradients (Weihrauch et al. 2009; Wright and Wood 2009).

A role for Rh-mediated ammonia excretion in the skin and yolk sac of zebrafish embryos was also demonstrated, where the apical localization of Rhcg1 was associated with H^+-pump-rich cells (HRCs; Shih et al. 2008). Braun et al. (2009) subsequently revealed that Rhbg and Rhcg were diffusely distributed across the skin of larval zebrafish, and they also localized Rhbg and Rhcg in the gill of Pacific hagfish (*Eptatretus stoutii* (Lockington, 1878)) (see Braun and Perry 2010). More recently, Rhcg was shown to play a prominent role in ammonia excretion in the Atlantic hagfish (*Myxine glutinosa* L., 1758) in both the skin and the gills (Edwards et al. 2015). The first possible evidence of ammonia excretion via the skin in lampreys was provided by Read (1968), who partitioned the gills, body, and kidneys from one another using divided flux chambers and renal catheters surgically implanted in freshwater Pacific lamprey (*Entosphenus tridentatus* (Richardson, 1836)). Although the freshwater acclimated, adult Pacific lamprey appeared to excrete mainly ammonia via the gills, Read (1968) also noted that there was appreciable ammonia excretion across the skin (~8% of the total), with lesser amounts of ammonia in the urine.

Despite these observations, the underlying mechanism(s) of ammonia excretion by lampreys via either the skin and (or) the gills remains unresolved. Wilkie et al. (1999) demonstrated that larval sea lampreys (*Petromyzon marinus* L., 1758) could excrete ammonia against large inwardly directed NH_3- and NH_4^+-concentration gradients, but the mechanism of ammonia excretion has not been elucidated.

The sea lamprey has a highly complex life cycle, characterized by a prolonged larval (ammocoete) phase in which the animals live burrowed in the substrate of streams as relatively sedentary filter feeders before undergoing a complex metamorphosis characterized by major changes in both internal and external body structures. The process is characterized by the full development of eyes, formation of an oral disc and rasping tongue, along with a switch from unidirectionally to tidally ventilated gills (Youson 1980; Wilkie et al. 2001). These changes prepare the sea lamprey for the free-swimming parasitic phase in which they latch onto the skin of aquatic vertebrates including fishes, sharks, and even cetaceans, and then feed on their blood (e.g., Potter and Beamish 1977; Farmer 1980; Nichols and Hamilton 2004; Wilkie et al. 2004).

Earlier work by Wilkie et al. (2004) demonstrated that the impact of the altered life stage on ammonia excretion was most evident in parasitic sea lampreys in which J_{Amm} was 15- to 25-fold higher and J_{Urea} was up to 450-fold higher following the ingestion of urea-rich blood from basking sharks, and was 25-fold greater following their removal from ammoniotelic rainbow trout (*Oncorhynchus mykiss* (Walbaum, 1792)). Moreover, the basal rates of ammonia and urea excretion increase as metamorphosis concludes in the sea lampreys, suggesting that sea lampreys upregulate their capacity to rid the body of ammonia and urea when they are entering a life stage where they are more likely to ingest protein-rich blood (Wilkie et al. 2006). Indeed, recent experiments revealed that blood ammonia in parasitic lampreys was elevated 5- to 10-fold immediately following feeding on trout (M.P. Wilkie, unpublished observation).

The aim of the present study was to test the hypotheses that the skin was a significant route for ammonia excretion in the sea lamprey and that its relative importance reflected the stage of development of sea lampreys. Accordingly, divided flux chambers were used to isolate the gills from extrabranchial routes (skin plus renal routes) of excretion in larval and postmetamorphic juvenile sea lampreys, and Western blot and immunohistochemistry (IHC) were used to determine if the presence of Rh glycoproteins in the skin provide a possible route for ammonia excretion by larval, postmetamorphic juvenile, and parasitic juvenile sea lampreys.

Materials and methods

Experimental animals and holding

For physiological experiments, anadromous larval sea lampreys, some of which subsequently underwent metamorphosis, were captured by pulsed-DC electrofishing in tributaries of the Richibucto River, New Brunswick, Canada, and transported back to Mount Allison University, where they were held under a 12 h light : 12 h dark photoperiod in flowing well water at 12 °C. Larval sea lampreys were fed weekly with a slurry of baker's yeast (2 g/animal; Holmes et al. 1994), but postmetamorphic juvenile sea lampreys were not fed because they do not feed until they begin the parasitic phase of their life cycle (Youson 1980, 2003). Previous work by our group has demonstrated that the patterns of ammonia excretion in the landlocked populations compared with anadromous populations of sea lamprey are similar during the freshwater phases of the sea lamprey life cycle (Wilkie et al. 1999, 2006).

For protein-expression studies, three stages of the landlocked Great Lakes sea lampreys were utilized based on animal availability. Larval, postmetamorphic juvenile, and parasitic juvenile lampreys were obtained from N. Johnson at the Hammond Bay Biological Station (Millersburg, Michigan, USA). The larval sea lampreys (ammocoetes) and postmetamorphic juvenile sea lampreys were collected via pulsed-DC electrofishing from local tributaries of Lake Huron in Michigan (e.g., Wilkie et al. 2006), whereas parasitic juvenile sea lampreys were collected from local commercial fisherman, following their removal from lake trout (*Salvelinus namaycush* (Walbaum, 1792)) hosts. The parasitic sea lampreys were allowed to continue feeding on lake trout at Hammond Bay Biological Station, where along with the larval and juvenile sea lampreys, they were housed in continuously flowing lake water at 10 °C under a 12 h light : 12 h dark photoperiod.

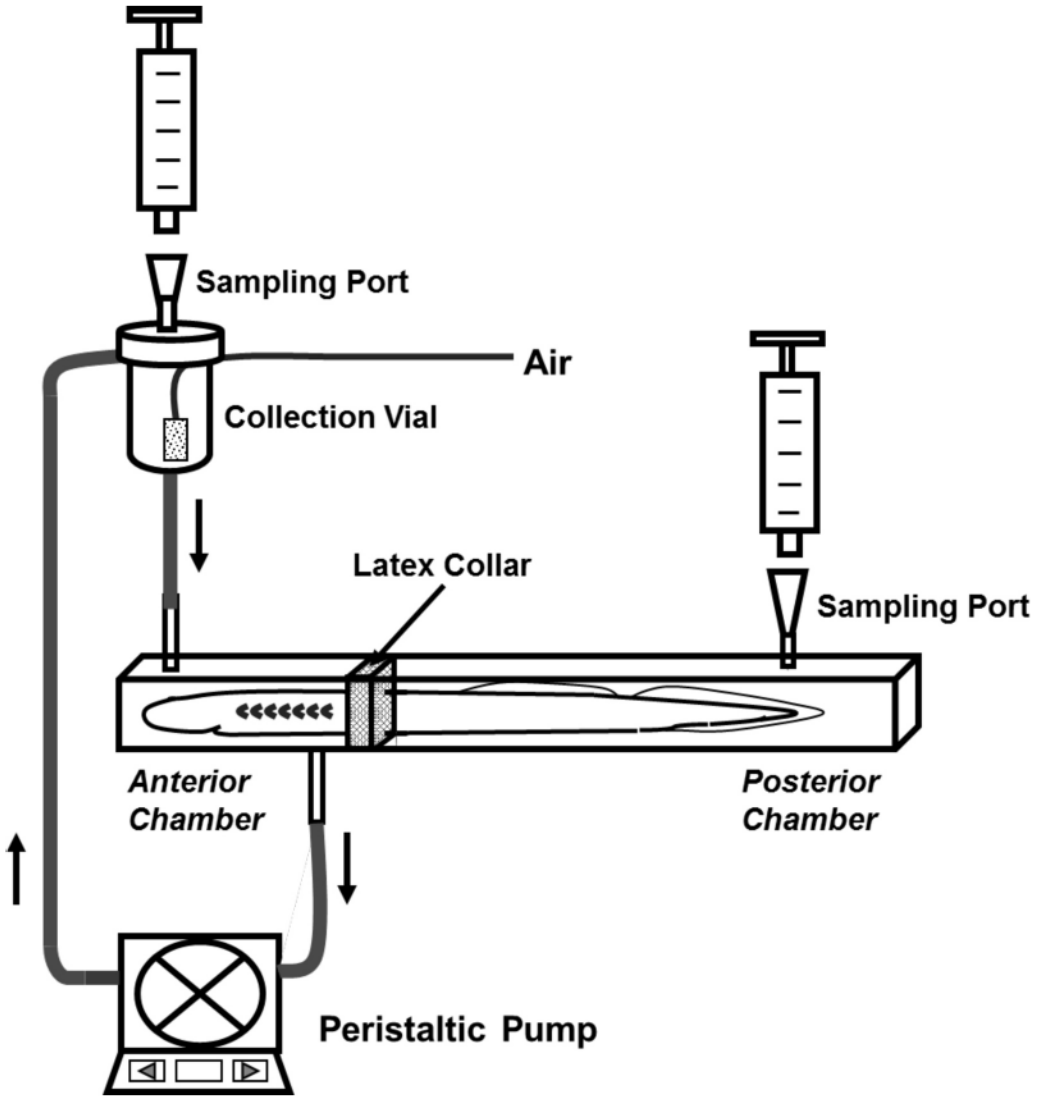
All experiments were conducted with the approval of the Animal Care committees at Appalachian State University, Mount Allison University, and the Hammond Bay Biological Station.

Experimental protocols

Partitioning the sites of excretion before and after metamorphosis in the sea lamprey

To characterize the sites of excretion in larval lampreys (n = 7; mass (mean ± SE) = 2.56 ± 0.22 g; range = 1.8–3.2 g) and postmetamorphic lampreys (n = 4; mass (mean ± SE) = 3.08 ± 0.37 g; range = 1.8–3.6 g), lightly anaesthetized animals (0. 1 g·L^{-1} tricaine methanefulfonate (TMS; Syndel Labs, Nanaimo, British Columbia, Canada) buffered with 0.2 g·L^{-1} $NaHCO_3$) were transferred to a divided flux chamber (Fig. 1) constructed from two plexiglass plates (dimensions: 20 cm long × 5 cm wide × 1 cm thick) in which a tapered, central groove (dimensions: 16 cm long × 1 cm wide) was

Fig. 1. Schematic diagram depicting the divided flux chambers used to partition branchial (gills) and extrabranchial (skin plus renal) routes of ammonia excretion from one another in a sea lamprey (*Petromyzon marinus*). Rates of branchial ammonia excretion were measured in the anterior compartment by isolating the branchiopores from the rest of the body using a latex collar, sealed with a light layer of denture adhesive, immediately behind the seventh (most posterior) branchiopore. Aerated water was recirculated through the anterior chamber from an overhead reservoir using a peristaltic pump. It was not necessary to aerate water in the posterior chamber, which was replenished with water at each sample period (every 4 h). To measure the accumulation of ammonia in each chamber, water samples were collected from the overhead reservoir (anterior) or the posterior chamber at 4 h intervals. See the text for further details.



carved out of the middle of each. When the plates were fastened together (using 4 × 3/16 inch (1 inch = 25.4 mm) stainless steel bolts at each corner), it created a compartment in which to hold the animals (Fig. 1). As burrowing animals, the confinement likely helped calm the animals ("thigmokinesis"; Rovainen and Schieber 1975). The head, including the branchial region, was separated from the body via a latex collar sealed with denture adhesive (Poligrip®) to guard against leaks between the anterior and the posterior regions of the chamber. The anterior chamber was continually supplied with recirculating water, which was pumped into the chamber from a 20 mL reservoir (polypropylene scintillation vial) via PE200 surgical tubing using a peristaltic pump (Gilson Minipuls3; Gilson SAS, Villiers-le-Bel, France). This arrangement allowed us to measure the rates of ammonia excretion in a relatively small volume of water (~30 mL total volume including the scintillation vial, the anterior chamber, and the connecting tubing) and to ensure that the water was continuously aerated using small air lines running into the reservoir. Ammonia excretion rates were then measured after the animals had recovered from the anaesthesia (~30 min), as characterized by regular pumping of the branchiopores, at which time water samples (2 mL) were drawn from the reservoir vial at 4 h intervals over 8 h. Using this closed recirculation setup, we were therefore able to calculate ammonia excretion from the changes in the water ammonia concentration in the sample water collected at each time interval (see calculations below). Water from the posterior chamber (~10 mL) was sampled at the end of each 4 h interval to determine rates of ammonia excretion via extrabranchial routes, which reflected the contribution of the skin plus renal pathways to excretion. The gills of larval lampreys are highly developed and virtually identical to those of teleost fishes, with gill filaments being extensively ornamented with lamellae to promote gas exchange (Lewis 1980). We therefore surmised that most oxygen consumption would take place across the gills and that the posterior (extrabranchial) regions would not require aeration during the 4 h measurement period.

At the end of each experiment, the animals were left in the divided chamber and "leak tests" were performed using water-soluble blue food colouring that was added to the anterior chamber and left to circulate for 1 h. After 1 h, a water sample was collected from the posterior chamber and its absorbance compared with deionized water at a wavelength of 650 nm, indicating negligible leakage between chambers. Because ammonia is highly soluble in water, this precaution ensured that the ammonia measured was an accurate depiction of the true amounts of ammonia excreted into each chamber and were not the result of water leakage between chambers.

Tissue sample collection from larval and juvenile sea lampreys

Sea lampreys, which included larval (n = 6), postmetamorphic juvenile (n = 6), and parasitic juvenile sea lampreys (n = 6), were removed one at a time from their holding tanks at the Hammond Bay Biological Station and immediately euthanized with an overdose of anaesthetic 1.0 g·L^{-1} tricaine methanesulfonate (Western Chemical, Inc., Ferndale, Washington, USA). When the animals were no longer responsive to a tail pinch, an ~1 cm^2 section of skin was excised from the lateral side of the animals along the midline. Half of the tissue was snap frozen in liquid nitrogen and stored at −80 °C for Western-blot analysis, with the remainder fixed in 4% PFA overnight for IHC.

Analytical methods

Measurements of water ammonia concentration

The respective water ammonia concentrations collected from the divided flux chamber experiments were quantified using the salicylate hypochlorite assay (Verdouw et al. 1978).

Antibodies

Polyclonal antibodies Rhbg, Rhcg1, and Rhcg2 (rabbit anti-*Fugu* Rh glycoprotein) were a gracious gift courtesy of S. Hirose (Tokyo Institute of Technology). These antibodies have previously been shown to be successful markers of Rh glycoproteins in the Pacific hagfish and a number of teleost species (Nakada et al. 2007*a*, 2007*b*; Shih et al. 2008; Braun and Perry 2010).

Western blot

Tissues were homogenized in ice-cold homogenization buffer (250 mmol·L^{-1} sucrose, 1 mmol·L^{-1} EDTA, 30 mmol·L^{-1} Tris, 100 μg·mL^{-1} phenylmethanesulfonyl fluoride (Sigma–Aldrich, St. Louis, Missouri, USA) and 5 mg·mL^{-1} protease inhibitor cocktail (Sigma–Aldrich). The homogenized samples were then transferred to 1.5 mL microcentrifuge tubes, centrifuged at 10 000*g* at 4 °C for 10 min, and the supernatants collected for protein concentration analysis using a Pierce® BCA Protein Assay Kit (Thermo Fisher Scientific, Rockford, Illinois, USA). Total protein from zebrafish gill and skin was used as positive controls for Rhbg, Rhcg1, and Rhcg2 primary antibody binding (Fig. 3). Exactly 25 μg of lamprey total skin protein and 25 μg of zebrafish total gill and skin protein (as a control) were loaded into NuSep 4%–20% LongLife gels (NuSep, Bogart, Georgia, USA) and separated by SDS–PAGE (sodium dodecyl sulfate – polyacrylamide gel electrophoresis). Separated proteins were then transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad, Hercules, California, USA), after which they were incubated overnight at 4 °C in 5% Blotto (5% nonfat dry milk powder in 0.1 mol·L^{-1} Tris-buffered saline with 0.2% Tween-20) (TBST). Membranes were then incubated in primary antibody diluted in blotto (1:1000 Rhbg, Rhcg1, and Rhcg2) at 4 °C on a rocker table overnight. The following day, the membranes were washed three times in 0.1 mol·L^{-1} TBST, then incubated with HRP (horseradish peroxidase) conjugated goat antirabbit antibody (1 : 10 000) and Precision Protein StrepTactin-HRP conjugate (Bio-Rad, Hercules, California, USA) in TBST at room temperature for 1 h. Excess secondary antibody was removed by consecutive washes in 0.1 mol·L^{-1} TBST, with a final wash in TBS in the absence of Tween-20™ to remove residual detergent. Following washes, the membranes were incubated with either Immun-Star™ AP Substrate or Immun-Star™ HRP Luminol/Enhancer (Bio-Rad) and peroxide buffer for 5 min at room temperature to undergo the chemiluminescent reaction. Antibody binding was detected by exposing the membrane to Hyperfilm™ ECL (Amersham Biosciences, Piscataway, New Jersey, USA).

Immunohistochemistry

Skin and gill tissue from larval, postmetamorphic juvenile, and parasitic juvenile sea lamprey were fixed in 4% PFA overnight at 4 °C and then prepared for cryosectioning. First, the PFA-fixed tissues were washed with several changes of 0.1 mol·L^{-1} PBS and with final overnight cryoprotection incubation in 30% sucrose in 0.1 mol·L^{-1} PBS. The tissues were then frozen in Tissue-Tek® OCT medium using liquid nitrogen and sectioned at 7 μm by cryostat (Leica CM 11000). The tissue sections were finally mounted on HistoBond® microscope slides and stored at room temperature for IHC analysis. Slides underwent an initial 10 min wash in 1× PBS with 0.1% Triton X-100 (Sigma–Aldrich), followed by three 5 min washes in 1× PBS to clear residual OCT compound. Nonspecific binding was blocked by incubating sections in 5% normal goat serum in 1× PBS for 20 min. Tissue sections were incubated overnight at room temperature in a humidified chamber in primary antibody (Rhbg, Rhcg1, Rhcg2 — 1:500) diluted in blocking solution and then rinsed in 1× PBS the following day. Tissue sections were then incubated in secondary antibody (1: 2000 Alexa Fluor® 568 goat antirabbit IgG or 1:2000 Alexa Fluor® 488 donkey antirabbit IgG) diluted in 1× PBS at room temperature for 1 h. Following washes in 1× PBS, the slides were coverslipped using ProLong® Gold antifade reagent (Invitrogen™, Eugene, Oregon, USA) and then visualized using scanning laser confocal microscopy (Zeiss LSM 5; Carl Zeiss). Negative controls were conducted in the absence of primary antibody in conjunction with experimental samples and viewed under identical conditions as the experimental sections using confocal imaging.

Calculations and statistics

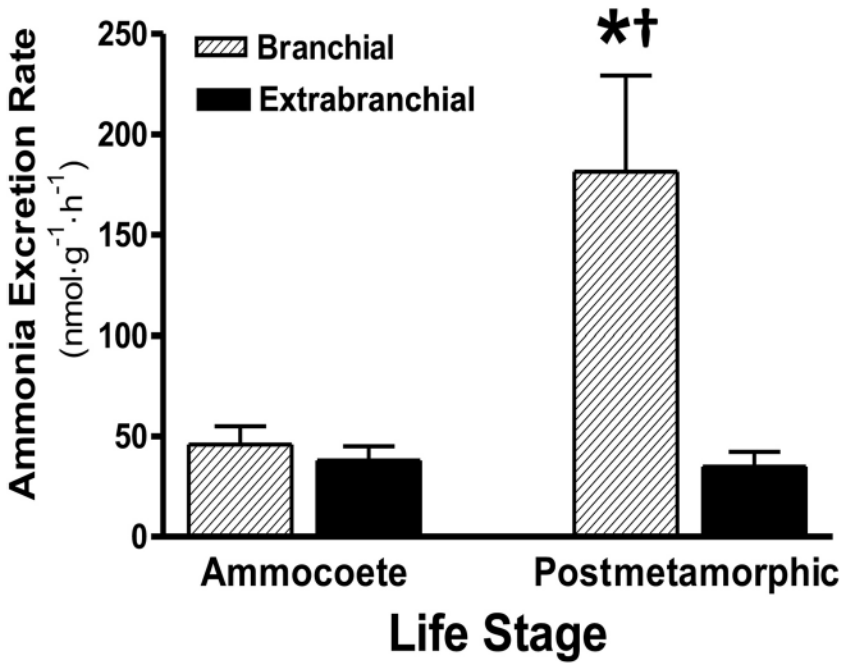
Ammonia excretion rates in the anterior and posterior chambers of the divided flux chamber apparatus were calculated separately based on the observed changes in water ammonia concentration taking place in the anterior or posterior chamber over each measurement period (2 × 4 h) using the following formula:

$$J_{\mathrm{Amm}} = \frac{([\mathrm{Amm}]_i - [\mathrm{Amm}]_f)\cdot V}{M\cdot \Delta T}$$

where $[\mathrm{Amm}]_i$ and $[\mathrm{Amm}]_f$ are the initial and final concentrations (nmol·L^{-1}), respectively, measured in the corresponding chamber at the beginning and end of an ammonia excretion measurement period; V is the volume (mL) of the anterior or posterior chamber of the divided flux chamber; M is the mass (g) of the lamprey; and ΔT is the duration of the measurement period (h).

All quantitative data were presented as the mean + 1 SE. Significant differences between rates of ammonia excretion in larval compared with postmetamorphic sea lamprey were determined using an unpaired t test; differences in the rates of ammonia excretion between branchial and extrabranchial sites of excretion within the same animal were determined using a paired t test. For other quantitative data, statistical significance was determined using one-way analysis of variance (ANOVA), followed by a Tukey post hoc test to determine significant differences between the mean values. Statistical analysis was completed using SigmaPlot (Systat Software Inc., San Jose, California, USA) or InStat 3 (GraphPad Inc., San Diego, California, USA) at the $P < 0.05$.

Fig. 2. Differences in the rates of ammonia excretion via branchial (hatched bars) and extrabranchial routes (solid bars) in larval (ammocoetes) and postmetamorphic, juvenile sea lampreys (*Petromyzon marinus*). Asterisk (*) denotes significant differences in the rates of branchial ammonia excretion between ammocoetes and postmetamorphic sea lamprey. Dagger (†) denotes a significant difference between rates of ammonia excretion via branchial and extrabranchial routes in postmetamorphic sea lamprey. All data presented as the mean + 1 SE at $P < 0.05$. $n = 7$ ammocoetes; $n = 4$ postmetamorphic sea lampreys.



Results

In vivo ammonia excretion

Experiments using the divided chambers revealed that the overall ammonia excretion rate (sum of branchial and extrabranchial routes) in larval sea lampreys was ~60 nmol·g⁻¹·h⁻¹ and is more or less equally split between branchial and extrabranchial routes in larval sea lampreys (Fig. 2). Following metamorphosis, however, overall ammonia excretion rates were about 4-fold higher, approaching 250 nmol·g⁻¹·h⁻¹, and the gills became the predominant site of ammonia excretion, accounting for ~80% of the total (Fig. 2). Thus, in both larval and postmetamorphic sea lampreys, there was appreciable ammonia excretion via extrabranchial routes, but these routes appeared to be proportionately more important in the larval sea lampreys.

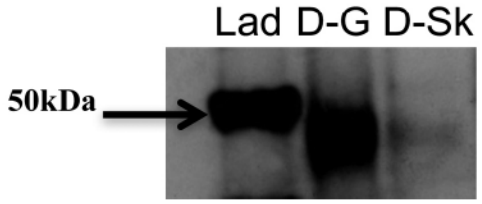
Relative Rh glycoprotein expression in skin of sea lamprey

To test the hypothesis that the skin played a significant role in ammonia excretion, Western blots were completed to visualize presence of Rhbg, Rhcg1, and Rhcg2 expression in the skin of sea lampreys. Immunoreactivity of the Rh glycoprotein antibodies was first confirmed using zebrafish skin and gill tissue as a positive control and in both cases demonstrated an immunoreactive band of the expected size of ~50 kDa (Fig. 3).

Rh glycoprotein expression (Rhbg, Rhcg1, Rhcg2) was detected in the skin of larval, postmetamorphic, and parasitic sea lampreys. A pronounced 50 kDa immunoreactive band for Rhbg was detected in the parasitic stage of the sea lamprey life cycle, but the same protein had a barely detectable signal in the larval and postmetamorphic stages (Fig. 4).

Although direct quantitative comparisons of expression between different antibodies were not possible, with normalized loading of exactly 25 μg of protein for each sample, inspection of the bands generated for the three Rhesus glycoproteins revealed that Rhcg1 and Rhcg2 were much more strongly displayed in the skin than Rhbg. Moreover, skin samples from larval, postmetamorphic, and parasitic sea lampreys each demonstrated clear immunoreactivity to a 50 kDa protein with the Rhcg1 antibody, but the signal was much stronger in the skin of the parasitic sea lampreys.

Fig. 3. Western-blot control. Quantified protein from zebrafish (*Danio rerio*) gill and skin tissue was used. Immunolabeling with Rh glycoprotein antibodies acted as positive control for analyzing the sea lamprey (*Petromyzon marinus*) protein, as Rh glycoproteins have successfully been identified in the zebrafish (Nakada 2007*a*). Lane 1 (Lad) is our standard, lane 2 (D-G) is *Danio* gill protein, and lane 3 (D-Sk) is *Danio* skin protein. Rhcg1 is detected with dark bands ~50 kDa.



Localization of Rh glycoproteins in sea lamprey skin

Further evidence of a role for Rhcg in the skin of larval sea lampreys (ammocoete) was provided by IHC analysis demonstrating that Rhcg2 was diffusely distributed in the apical regions of the skin, with additional concentration in club cells (Fig. 5D). On the other hand, Rhbg was only localized to club-like cells (Fig. 5B), whereas Rhcg1 had a diffuse basal and apical location in the skin (Fig. 5C).

Rh glycoprotein expression persists through the transformation from the larval ammocoete into the postmetamorphic stage. Immunoreactivity of Rhgb was observed throughout the epidermal layer of the skin (Fig. 6B), whereas Rhcg2 was again localized to the apical cell layer (Fig. 6D). Rhcg1 immunoreactivity was minimal in the postmetamorphic sea lampreys (Fig. 6C).

Fig. 4. Relative abundance of Rhesus glycoproteins in the skin of larval, postmetamorphic and parasitic juvenile sea lampreys (*Petromyzon marinus*). Columns or lanes designate quantified protein from larval skin (A), postmetamorphic skin (PM), and parasite skin (P). Rows of Rhbg (b), Rhcg1 (c1), and Rhcg2 (c2) detection is shown by the presence or absence of a band (~50 kDa) from top to bottom, respectively. Rh expression was observed in all samples with limited detectable amounts of Rhbg in the skin of the ammocoetes. Figure appears in colour on the Web.

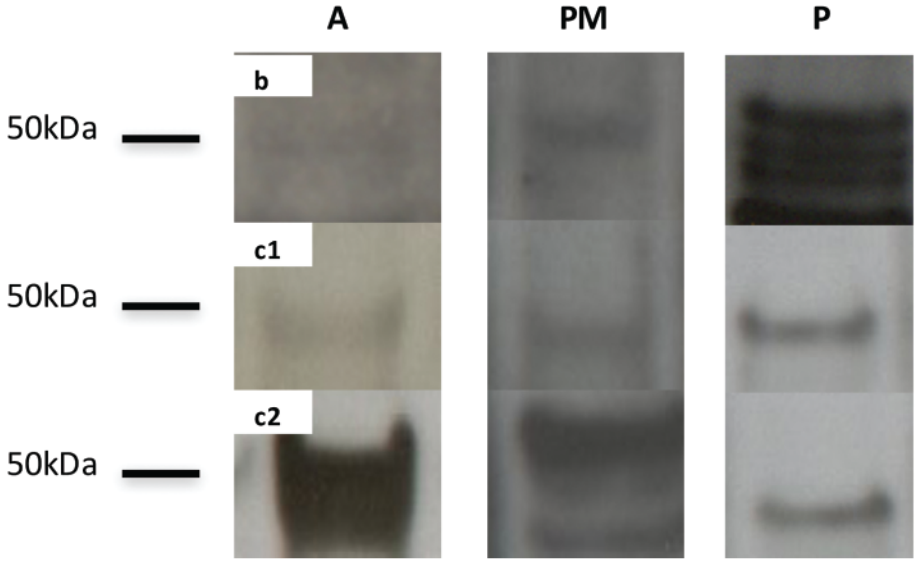


Fig. 5. Immunohistochemical visualization of Rhesus glycoprotein distribution in the skin of larval sea lampreys (*Petromyzon marinus*; ammocoetes). (A) Negative control (NC) showing no staining in skin of larval sea lamprey, stained (anti-RB 488 nm) with no primary antibody. (B) Localization of Rhbg within club cells (cc) in larval sea lamprey. (C) Localization of Rhcg1 of epithelial cells in the skin. (D) Rhcg2 localized to both the club cells and the apical zone of epithelial cells in the skin of larval sea lampreys. Scale bars = 50 μm. Figure appears in colour on the Web.

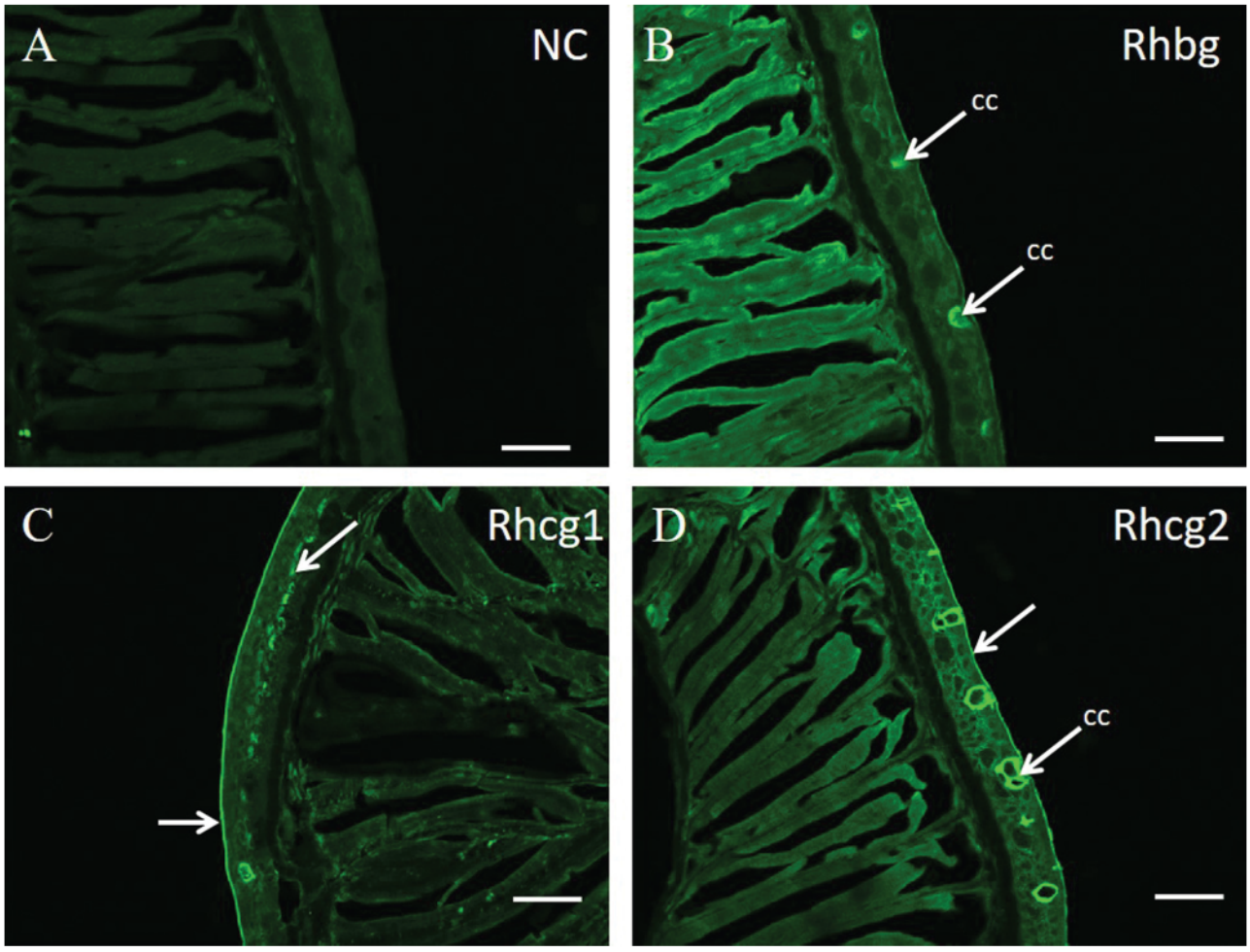
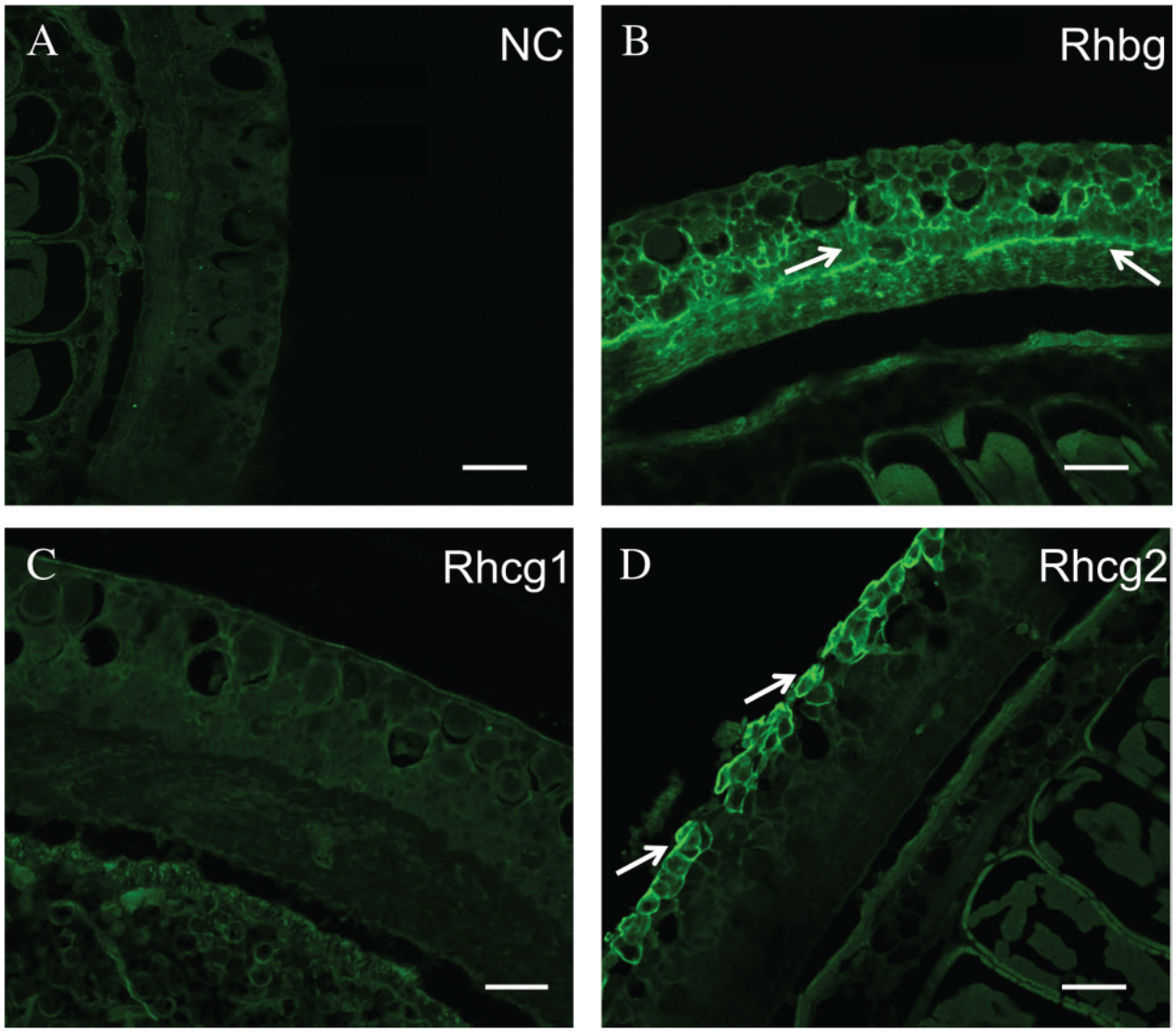


Fig. 6. Immunohistochemistry of postmetamorphic sea lamprey (*Petromyzon marinus*) skin sections. (A) Negative control (NC) showing no staining in skin of larval sea lamprey, stained (anti-RB 488 nm) with no primary antibody. (B) Rhbg expression is ubiquitous from the basal layer to the mid-region and tapers off towards the apical cells in the skin. (C) Rhcg1 expression is minimal to absent in the skin. (D) Rhcg2 expression is located to cells on the apical side of the skin of postmetamorphic sea lampreys. Scale bars = 50 μm. Figure appears in colour on the Web.



In contrast to the larval and postmetamorphic lampreys, all three isoforms of Rhesus glycoprotein (Rhbg (Fig. 7B), Rhcg1 (Fig. 7C), Rhcg2 (Fig. 7D)) were expressed ubiquitously throughout the skin of parasitic sea lampreys, but Rhcg1 had a predominantly apical orientation (Fig. 7C). Additionally, all negative control sections stained without primary antibody showed no fluorescence.

Discussion

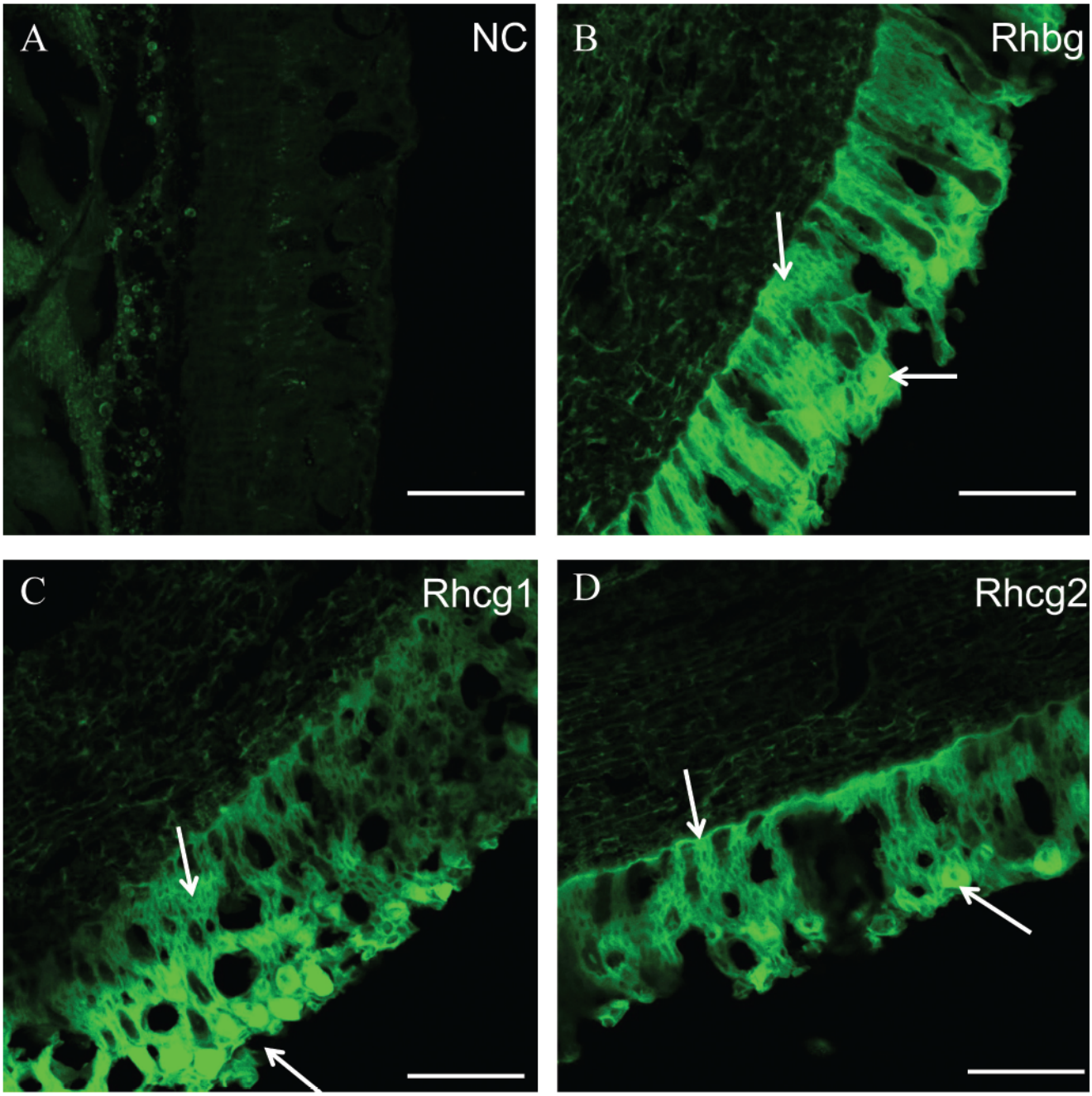
Using Western blotting and IHC, this study demonstrated that the Rh isoforms Rhbg, Rhcg1, and Rhcg2 were present in the skin of the sea lamprey. Together with the divided chamber ammonia excretion data, this study suggests that the skin of sea lampreys makes a significant contribution to ammonia excretion, particularly during the larval stages where ammonia excretion was more or less equally split between branchial and extrabranchial sites. Following metamorphosis, however, it appears that the skin takes on less relative importance because the gills become the predominant site of ammonia excretion, accounting for about 90% of the ammonia excretion in postmetamorphic juvenile sea lampreys (Fig. 2).

Compared with the early lamprey life stages characterized by isoform-specific cellular localization, the shift to homogeneous distribution of each of the three Rh isoforms in parasitic juvenile sea lampreys removed from lake trout in Lake Huron also suggests that the skin may play an important role as a route of ammonia excretion following the ingestion of protein-rich blood, which results in substantial increases in postprandial ammonia excretion (Wilkie et al. 2004, 2006).

Sites of ammonia excretion in larval sea lampreys and postmetamorphic juvenile sea lampreys

These results help to explain the findings of Read (1968), who first reported that significant amounts of ammonia were excreted via the skin in adult freshwater Pacific lamprey. However, the present findings also demonstrate that the skin is relatively more important in earlier stages of the sea lamprey life cycle, particularly in burrow-dwelling larval sea lampreys.

Fig. 7. Rh glycoprotein distribution in the skin of parasitic sea lampreys (*Petromyzon marinus*). (A) Negative control (NC) showing absence of Rh expression, stained (anti-RB 488 nm) with no primary antibody. (B) Rhbg, (C) Rhcg1, and (D) Rhcg2 localization in epithelial cells through the entire epidermal region of parasitic lampreys. Scale bars = 100 μm. Figure appears in colour on the Web.



The abundance of Rhcg2 in the larval sea lamprey skin (Fig. 4), along with a ubiquitous apical distribution, suggests that this facilitated transporter plays an important role in promoting ammonia excretion by the skin, which contributes almost as much as the gill to total ammonia excretion. At first glance, it seems difficult to explain why the skin would be an accessory ammonia excretory organ. However, Potter et al. (1995) speculated that the skin could be an important accessory gas exchange site if the animals were temporarily stranded on land as water rapidly receded following flooding or storms. Indeed, we have noted that larval sea lampreys can tolerate prolonged air exposure under humid conditions for several days, with no buildup of ammonia in the body (M.P. Wilkie, unpublished observations).

A significant contribution of the skin to ammonia excretion might be co-dependent, however, on adequate vascularization of the underlying dermal layers of the skin. The skin of postmetamorphic and adult sea lampreys, river lampreys (*Lampetra fluviatilis* (L., 1758)), and pouched lampreys (*Geotria australis* Gray, 1851) each has dermal capillaries, which may allow the animals to respire in air (Potter et al. 1995). It has been proposed that this could facilitate limited gas exchange across the skin of these animals, particularly if they were stranded in air while migrating upstream (Potter et al. 1995). The close association between the dermal capillaries and the skin of juvenile lampreys might also be sufficient to promote ammonia excretion via the Rhcg2 in these animals. However, with the relatively low levels and punctate distribution

of Rhbg, it remains unclear how ammonia would cross the basolateral epithelium of epidermal cells. Larval sea lampreys lack dermal capillaries, but the low diffusion distance due to the very thin epidermis might be sufficient to promote not only gas exchange (Potter et al. 1995), but the underlying vasculature might still be sufficiently near to facilitate Rh-glycoprotein-mediated ammonia excretion.

The skin has also been demonstrated to be a significant route of ammonia excretion in a number of aquatic and amphibious fishes (for reviews see Weihrauch et al. 2009; Glover et al. 2011). Using a combination of divided flux chambers and urinary catheters to isolate the gills, skin, and urinary routes of excretion from one another, Sayer and Davenport (1987) concluded that the skin was a quantifiably more important route of ammonia excretion in marine fishes including the smooth blenny (*Lipophrys pholis* (L., 1758)) and in the dab (*Limanda limanda* (L., 1758)). They also noted that skin took on an important role for ammonia excretion during air exposure in amphibious fishes such as the blenny, which can become stranded in tidal pools. Similar findings have been made in other amphibious fishes including the oriental weather loach (*Misgurnus anguillicaudatus* (Cantor, 1842)) (see Tsui et al. 2002) and the mangrove killifish (*Kryptolebias marmoratus* (Poey, 1880)) (see Frick and Wright 2002; Litwiller et al. 2006; Wright 2012) that are capable of ammonia volatilization while in air. The mangrove killifish appears to rely on a combination of Rhbg- and Rhcg1-mediated NH_3 transfer across the gills, along with NH_4^+ diffusion via paracellular routes while in brackish water (Cooper et al. 2013). However, the skin also displays Rhcg1 immunoreactivity that is co-localized with NKA, which is likely important for excreting ammonia in water and in air (Cooper et al. 2013).

A limitation of the divided chamber apparatus used in the present study was that we could not directly collect the urine of the animals because of their small size (2.5–4.0 g). Thus, the possibility that all of the extrabranchial ammonia was excreted via the kidneys cannot be ruled out. However, it seems highly unlikely that the sea lamprey could have sufficiently concentrated ammonia in the urine to explain all of the extrabranchial ammonia excretion. As with other freshwater hyperosmoregulators, the lampreys excrete copious dilute urine while in fresh water (Logan et al. 1980) and have very high urine flow rates (4–14 $mL \cdot kg^{-1} \cdot h^{-1}$; Logan et al. 1980; Cai et al. 2013). Thus, it seems unlikely that the concentration of ammonia in the urine could be sufficiently high to explain all of the ammonia excreted via extrabranchial routes. In the postmetamorphic sea lampreys, a urine ammonia concentration of approximately 3–10 $mmol \cdot L^{-1}$ would be required to account for all the ammonia excreted by the animal if its urine flow rates were between 4 and 14 mL. Such high concentrations of ammonia in the urine seem unlikely, given that the concentrations of ammonia in the urine of freshwater-acclimatized adult Pacific lamprey were in the micromoles per litre range (Read 1968).

Read (1968) also reported that extrabranchial routes of ammonia excretion (skin plus renal routes) accounted for ~12% of the total ammonia excreted by adult Pacific lamprey, with the majority (~8% of the total) excreted across the skin and 4% via the urine. Thus, in juvenile freshwater-acclimated sea lampreys and probably larval sea lampreys, it seems likely that much of the ammonia arising in the posterior chamber arose from the skin, with lesser amounts from urinary excretion. Postexperiment leak tests using food blue colouring also ruled out any leakage of water between the chambers.

Notably, the measured rates of extrabranchial ammonia excretion were similar between the larval and the postmetamorphic sea lampreys. However, the gills were the relatively more important route of excretion in the juvenile sea lampreys following metamorphosis, when ammonia production and excretion rates are known to increase (Wilkie et al. 2006). Indeed, these findings suggest that the skin could have an upper limit as a route for ammonia excretion, and that following metamorphosis, the gills by virtue of their extensive branchial vasculature offload the additional ammonia arising from higher rates of basal ammonia production (Wilkie et al. 2006) and following feeding (Wilkie et al. 2004).

These findings are very similar to those reported in larval rainbow trout, in which ammonia excretion is more or less equally partitioned between the skin and the gills up until about 15 days post hatch (dph) when the burden primarily shifts to the gills, which remain the major site of excretion (Zimmer et al. 2014*b*). Zimmer et al. (2014*b*) noted that this shift was tightly correlated with the development of a more functional gill, as indicated by an upregulation of the machinery thought to be involved in ammonia excretion and ion exchange. This included increases in Na^+/K^+-ATPase, V-ATPase, and carbonic anhydrase activity, as well as increased gene expression of Rhcg1 and Rhcg2 (Zimmer et al. 2014*b*). Their observation that the switch to primarily branchial ammonia excretion was also tied to increases in ammonia turnover time (production) is also very similar to the present observations in sea lamprey.

The findings in larval trout (Zimmer et al. 2014*b*) support our assertion that the capacity of the skin to serve as a route of ammonia excretion is finite, and that as metabolic ammonia production rates increase, the gills take on much greater importance. Such an adjustment would be crucial in sea lampreys when they switch from a suspension-feeding diet of detritus of low nutritive value as larvae to a protein-rich diet of blood following their metamorphosis into parasitic juvenile lampreys that ingest large quantities of blood (Wilkie et al. 2004, 2006). It seems less likely that the switch is related to structural changes in the gills following metamorphosis. The gills of lampreys do switch from a unidirectional ventilated gill in larval sea lampreys to a tidally ventilated gill in which water is actively pumped out of gill pouches in the re-arranged branchial apparatus of juvenile lampreys (Lewis 1980; Bartels and Potter 2004). However, the ornamentation of the gill filaments with lamellae and the functional surface area of the gills are thought to be similar before and after metamorphosis (Lewis 1980).

Rh protein expression during the sea lamprey life cycle

The presence of Rh glycoprotein expression in the skin, as demonstrated using Western blotting, provided further evidence that the skin plays an important role in ammonia excretion by sea lampreys. Rhcg glycoproteins were clearly present in the skin of larval sea lamprey, but Rhbg was barely detectable in Western blots. On the other hand, IHC clearly indicated that Rhbg was present but was restricted to club-like cells of the skin (Fig. 5B), which suggests that the overall amount of Rhbg was insufficient to be detected using standard Western-blot protocols. Nevertheless, the presence of the remaining isoforms (Rhcg1, Rhcg2) in the skin, particularly Rhcg2, provides additional evidence that the skin is an important route of ammonia excretion in the larval sea lamprey (Figs. 4, 5C (Rhcg1), 5D (Rhcg2)).

Western-blot experiments demonstrated expression of all three isoforms of the Rh glycoproteins in postmetamorphic juvenile sea lamprey skin, which was further suggestive of significant extrabranchial ammonia excretion during this life stage (Figs. 4, 6A–6D). This conclusion was borne out by IHC, which demonstrated that there was Rhbg (Fig. 6B) and Rhcg2 (Fig. 6D) immunoreactivity in the skin, further supporting the hypothesis that Rh glycoproteins mediate ammonia excretion via this route. In contrast to the larval lamprey, however, Rhbg had a wider distribution in the basal regions of the epithelium, while retaining its localization in the club-like cells (Fig. 6B). Such an arrangement is more consistent with the "metabolon" model proposed by Wright and Wood (2009) in which NH_3 crosses the basolateral membrane via Rhbg before being excreted via an apically located Rhcg loosely coupled to either NHE or V-ATPase, which trap NH_3 as NH_4^+ through H^+

extrusion. However, the possibility that the club-like cells serve as the vehicle for such a process requires further investigation.

The different patterns of Rhbg and Rhcg2 distribution implies that specific Rh glycoprotein isoform expression can likely be altered depending on the physiological needs of the animal at different points in their life cycle or perhaps in response to physiological challenges. Nawata et al. (2010) reported that high environmental ammonia resulted in an upregulation of the Rh isoform Rhcg1, whereas the expression of others (Rhag and Rhbg) were simultaneously downregulated in the gill epithelium. The differential localization of these isoforms is also consistent with the physiological evidence for landlocked postmetamorphic animals in which the relative amounts of extrabranchial excretion of ammonia was decreased compared with that of the larval sea lampreys, likely because of the higher rates of ammonia production and gill-mediated excretion that take place during the juvenile stage (Fig. 2).

Rh glycoproteins in the skin of parasitic sea lampreys

The extensive immunoreactivity to Rhbg (Fig. 7B), Rhcg1 (Fig. 7C), Rhcg2 (Fig. 7D) seen in the skin of parasitic sea lampreys strongly suggests that the skin could play an important supplementary role in promoting ammonia excretion following feeding. Following feeding, plasma ammonia concentrations increase markedly, accompanied by 10- to 25-fold increases in ammonia excretion (Wilkie et al. 2004). Wilkie et al. (2006) estimated that an actively feeding sea lamprey ingests up to five times the protein or nitrogen load of an intensively feeding salmon at a fish farm (Wood 2001). Moreover, parasitic sea lampreys tend to stay attached to their hosts for many hours or days, during which time their blood consumption could approach 30% of their body mass in a single day (Farmer et al. 1975; Farmer 1980). The gills of postmetamorphic (juvenile and adult) sea lampreys are also tidally ventilated, which we speculate could result in the accumulation of ammonia in the gill pouches because of the mixing of "fresh" inhalant water with ammonia-laden exhalant water. Thus, an ability to offload ammonia via the skin could supplement branchially mediated ammonia excretion, which would be less efficient with a tidally ventilated gill, particularly following feeding. However, follow-up experiments are needed to test this hypothesis.

Conclusions

We conclude that during the relatively sedentary, filter-feeding larval stage of the sea lamprey, extrabranchial routes play an equally important role as the gill in facilitating ammonia excretion and that the Rh glycoproteins play a central role in facilitating this process via the skin. The routes of ammonia excretion also reflect the ontogeny of the sea lampreys, with the gills becoming the predominant site of excretion following metamorphosis owing to the need to offload greater amounts of ammonia arising from higher rates of basal ammonia production and following the ingestion of large amounts of protein-rich blood in the parasitic stage. The presence of Rhbg and Rhcg1 in the parasitic stage suggests, however, that the skin may still serve as an accessory route of ammonia excretion when sea lampreys are ingesting vast quantities of blood during their parasitic phase.

The gills of larval sea lampreys are an important site of oxygen uptake (Lewis 1980) and ammonia excretion (Read 1968; present study). Nevertheless, as modern representatives of the jawless fishes, the present findings in sea lampreys support the hypothesis that the relative importance of gills versus skin increased as the gnathostome fishes evolved. With the increased feeding efficiency, increased mobility, and the corresponding increases in nitrogenous waste production in higher vertebrates, we suggest that the relative importance of the gills as a route of excretion increased in parallel with its increasing importance for gas exchange, relegating the skin to an accessory role in the excretion of ammonia.

Acknowledgements

This work was funded by the National Science Foundation IOS 1121369 to S.L.E., Natural Sciences and Engineering Research Council of Canada (NSERC) Discovery Grant to M.P.W., and supported by an Appalachian State University Office of Student travel grant to S.D.B. Our special thanks go to S. Hirose (Tokyo Institute of Technology) for the generous donation of antibodies and to N. Johnson (Hammond Bay Biological Station) for supplying the animals. We also thank S. Swindell and W. Anderson for their outstanding technical assistance and work at Mount Allison University.

References

Bartels, H.B., and Potter, I.C. 2004. Cellular composition and ultrastructure of the gill epithelium of larval and adult lampreys: implications for osmoregulation in fresh and seawater. J. Exp. Biol. 207: 3447–3462. doi:10.1242/jeb.01157. PMID:15339941.

Braun, M.H., and Perry, S.F. 2010. Ammonia and urea excretion in the Pacific hagfish *Eptatretus stoutii*: evidence for the involvement of Rh and UT proteins. Comp. Biochem. Physiol. A, 157(4): 405–415. doi:10.1016/j.cbpa.2010.08.020.

Braun, M.H., Steele, S.L., Ekker, M., and Perry, S.F. 2009. Nitrogen excretion in developing zebrafish (*Danio rerio*): a role for Rh proteins and urea transporters. Am. J. Physiol. Renal Physiol. 296(5): F994–F1005. doi:10.1152/ajprenal.90656.2008. PMID:19279128.

Cai, S.-Y., Lionarons, D.A., Hagey, L., Soroka, C.J., Mennone, A., and Boyer, J.L. 2013. Adult sea lamprey tolerates biliary atresia by altering bile salt composition and renal excretion. Hepatology, 57(6): 2418–2426. doi:10.1002/hep.26161. PMID:23175353.

Cooper, C.A., Wilson, J.M., and Wright, P.A. 2013. Marine, freshwater and aerially acclimated mangrove rivulus (*Kryptolebias marmoratus*) use different strategies for cutaneous ammonia excretion. Am. J. Physiol. Regul. Integr. Comp. Physiol. 304(8): R599–R612. doi:10.1152/ajpregu.00228.2012. PMID:23389109.

Edwards, S.L., Arnold, J., Blair, S.D., Pray, M., Bradley, R., Erikson, O., and Walsh, P.J. 2015. Ammonia excretion in the Atlantic hagfish (*Myxine glutinosa*) and responses of an Rhc glycoprotein. Am. J. Physiol. Regul. Integr. Comp. Physiol. 308(9): R769–R778. doi:10.1152/ajpregu.00355.2014. PMID:25715835.

Farmer, G.J. 1980. Biology and physiology of feeding in adult lampreys. Can. J. Fish. Aquat. Sci. 37(11): 1751–1761. doi:10.1139/f80-220.

Farmer, G.J., Beamish, F.W., and Robinson, G.A. 1975. Food consumption of the adult landlocked sea lamprey, *Petromyzon marinus*, L. Comp. Biochem. Physiol. A, 50(4): 753–757. doi:10.1016/0300-9629(75)90141-3. PMID:236136.

Frick, N., and Wright, P. 2002. Nitrogen metabolism and excretion in the mangrove killifish *Rivulus marmoratus* II. Significant ammonia volatilization in a teleost during air-exposure. J. Exp. Biol. 205(1): 91–100. PMID:11818415.

Glover, C.N., Bucking, C., and Wood, C.M. 2011. Adaptations to in situ feeding: novel nutrient acquisition pathways in an ancient vertebrate. Proc. R. Soc. B Biol. Sci. 278(1721): 3096–3101. doi:10.1098/rspb.2010.2784.

Holmes, J.A., Beamish, F.W.H., Seelye, J.G., Sower, S.A., and Youson, J.H. 1994. Long-term influence of water temperature, photoperiod, and food deprivation on metamorphosis of sea lamprey, *Petromyzon marinus*. Can. J. Fish. Aquat. Sci. 51(9): 2045–2051. doi:10.1139/f94-207.

Hung, C., Tsui, K., Wilson, J., Nawata, C., Wood, C., and Wright, P. 2007. Rhesus glycoprotein gene expression in the mangrove killifish *Kryptolebias marmoratus* exposed to elevated environmental ammonia levels and air. J. Exp. Biol. 210(14): 2419–2429. doi:10.1242/jeb.002568. PMID:17601945.

Ip, Y., Chew, S., and Randall, D. 2001. Ammonia toxicity, tolerance, and excretion. Fish Physiol. 20: 109–148. doi:10.1016/S1546-5098(01)20005-3.

Krogh, A. 1938. The active absorption of ions in some freshwater animals. Z. Vergl. Physiol. 25(3): 335–350.

Lewis, S.V. 1980. Respiration of lampreys. Can. J. Fish. Aquat. Sci. 37(11): 1711–1722. doi:10.1139/f80-217.

Litwiller, S., O'Donnell, M., and Wright, P. 2006. Rapid increase in the partial pressure of NH_3 on the cutaneous surface of air-exposed mangrove killifish, *Rivulus marmoratus*. J. Exp. Biol. 209(9): 1737–1745. doi:10.1242/jeb.02197. PMID:16621954.

Logan, A., Moriarty, R., and Rankin, J. 1980. A micropuncture study of kidney function in the river lamprey, *Lampetra fluviatilis*, adapted to fresh water. J. Exp. Biol. 85(1): 137–147.

Morii, H., Nishikata, K., and Tamura, O. 1978. Nitrogen excretion of mudskipper fish *Periophthalmus cantonensis* and *Boleophthalmus pectinirostris* in water and on land. Comp. Biochem. Physiol. A, 60(2): 189–193. doi:10.1016/0300-9629(78)90229-3.

Nakada, T., Hoshijima, K., Esaki, M., Nagayoshi, S., Kawakami, K., and Hirose, S. 2007*a*. Localization of ammonia transporter Rhcg1 in mitochondrion-rich cells of yolk sac, gill, and kidney of zebrafish and its ionic strength-dependent expression. Am. J. Physiol. Regul. Integr. Comp. Physiol. 293(4): R1743–R1753. doi:10.1152/ajpregu.00248.2007. PMID:17686885.

Nakada, T., Westhoff, C.M., Kato, A., and Hirose, S. 2007*b*. Ammonia secretion from fish gill depends on a set of Rh glycoproteins. FASEB J. 21(4): 1067–1074. doi:10.1096/fj.06-6834com. PMID:17218543.

Nawata, C.M., and Wood, C.M. 2008. The effects of CO_2 and external buffering on ammonia excretion and Rhesus glycoprotein mRNA expression in rainbow trout. J. Exp. Biol. **211**(20): 3226–3236. doi:10.1242/jeb.020396. PMID:18840656.

Nawata, C.M., Hung, C.C., Tsui, T.K., Wilson, J.M., Wright, P.A., and Wood, C.M. 2007. Ammonia excretion in rainbow trout (*Oncorhynchus mykiss*): evidence for Rh glycoprotein and H^+-ATPase involvement. Physiol. Genomics, **31**(3): 463–474. doi:10.1152/physiolgenomics.00061.2007. PMID:17712040.

Nawata, C.M., Hirose, S., Nakada, T., Wood, C.M., and Kato, A. 2010. Rh glycoprotein expression is modulated in pufferfish (*Takifugu rubripes*) during high environmental ammonia exposure. J. Exp. Biol. **213**(18): 3150–3160. doi:10.1242/jeb.044719. PMID:20802117.

Nichols, O.C., and Hamilton, P.K. 2004. Occurrence of the parasitic sea lamprey *Petromyzon marinus* on western North Atlantic right whales *Eubalaena glacialis*. Environ. Biol. Fishes, **71**(4): 413–417. doi:10.1007/s10641-004-0776-5.

Potter, I., and Beamish, F. 1977. The freshwater biology of adult anadromous sea lampreys *Petromyzon marinus*. J. Zool. (Lond.), **181**(1): 113–130. doi:10.1111/j.1469-7998.1977.tb04573.x.

Potter, I., Welsch, U., Wright, G.M., Honma, Y., and Chiba, A. 1995. Light and electron microscope studies of the dermal capillaries in three species of hagfishes and three species of lampreys. J. Zool. (Lond.), **235**(4): 677–688. doi:10.1111/j.1469-7998.1995.tb01777.x.

Read, L.J. 1968. A study of ammonia and urea production and excretion in the fresh-water-adapted form of the Pacific lamphrey, *Entosphenus tridentatus*. Comp. Biochem. Physiol. A, **26**(2): 455–466. doi:10.1016/0010-406X(68)90638-5.

Rovainen, C.M., and Schieber, M.H. 1975. Ventilation of larval lampreys. J. Comp. Physiol. A Neuroethol. Sens. Neural Behav. Physiol. **104**(2): 185–203. doi:10.1007/BF01379459.

Sayer, M., and Davenport, J. 1987. The relative importance of the gills to ammonia and urea excretion in five seawater and one freshwater teleost species. J. Fish Biol. **31**(4): 561–570. doi:10.1111/j.1095-8649.1987.tb05258.x.

Shih, T.-H., Horng, J.-L., Hwang, P.-P., and Lin, L.-Y. 2008. Ammonia excretion by the skin of zebrafish (*Danio rerio*) larvae. Am. J. Physiol. Cell Physiol. **295**(6): C1625–C1632. doi:10.1152/ajpcell.00255.2008. PMID:18815227.

Smith, A.A., Zimmer, A.M., and Wood, C.M. 2012. Branchial and extra-branchial ammonia excretion in goldfish (*Carassius auratus*) following thermally induced gill remodeling. Comp. Biochem. Physiol. A, **162**(3): 185–192. doi:10.1016/j.cbpa.2012.02.019.

Smith, H.W. 1929. The excretion of ammonia and urea by the gills of fish. J. Biol. Chem. **81**(3): 727–742.

Tsui, T., Randall, D., Chew, S., Jin, Y., Wilson, J., and Ip, Y. 2002. Accumulation of ammonia in the body and NH_3 volatilization from alkaline regions of the body surface during ammonia loading and exposure to air in the weather loach *Misgurnus angutllicaudatus*. J. Exp. Biol. **205**(5): 651–659. PMID:11907054.

Verdouw, H., Van Echteld, C., and Dekkers, E. 1978. Ammonia determination based on indophenol formation with sodium salicylate. Water Res. **12**(6): 399–402. doi:10.1016/0043-1354(78)90107-0.

Weihrauch, D., Morris, S., and Towle, D.W. 2004. Ammonia excretion in aquatic and terrestrial crabs. J. Exp. Biol. **207**(26): 4491–4504. doi:10.1242/jeb.01308. PMID:15579545.

Weihrauch, D., Wilkie, M.P., and Walsh, P.J. 2009. Ammonia and urea transporters in gills of fish and aquatic crustaceans. J. Exp. Biol. **212**(11): 1716–1730. doi:10.1242/jeb.024851.

Wilkie, M.P., Wang, Y., Walsh, P.J., and Youson, J.H. 1999. Nitrogenous waste excretion by the larvae of a phylogenetically ancient vertebrate: the sea lamprey (*Petromyzon marinus*). Can. J. Zool. **77**(5): 707–715. doi:10.1139/z99-038.

Wilkie, M.P., Bradshaw, P.G., Joanis, V., Claude, J.F., and Swindell, S.L. 2001. Rapid metabolic recovery following vigorous exercise in burrow-dwelling larval sea lampreys (*Petromyzon marinus*). Physiol. Biochem. Zool. **74**(2): 261–272. doi:10.1086/319656. PMID:11247745.

Wilkie, M.P., Turnbull, S., Bird, J., Wang, Y.S., Claude, J.F., and Youson, J.H. 2004. Lamprey parasitism of sharks and teleosts: high capacity urea excretion in an extant vertebrate relic. Comp. Biochem. Physiol. A, **138**(4): 485–492. doi:10.1016/j.cbpb.2004.06.001.

Wilkie, M.P., Claude, J.F., Cockshutt, A., Holmes, J.A., Wang, Y.S., Youson, J.H., and Walsh, P.J. 2006. Shifting patterns of nitrogen excretion and amino acid catabolism capacity during the life cycle of the sea lamprey (*Petromyzon marinus*). Physiol. Biochem. Zool. **79**(5): 885–898. doi:10.1086/505998. PMID:16927235.

Wood, C.M. 1993. Ammonia and urea metabolism and excretion. *In* The physiology of fishes. *Edited by* D.H. Evans. CRC Press, Boca Raton, Fla. pp. 379–425.

Wood, C.M. 2001. Influence of feeding, exercise, and temperature on nitrogen metabolism and excretion. Fish Physiol. **20**: 201–238. doi:10.1016/S1546-5098(01)20007-7.

Wright, P.A. 1995. Nitrogen excretion: three end products, many physiological roles. J. Exp. Biol. **198**(2): 273–281. PMID:7699310.

Wright, P.A. 2012. Environmental physiology of the mangrove rivulus, *Kryptolebias marmoratus*, a cutaneously breathing fish that survives for weeks out of water. Integr. Comp. Biol. **52**(6): 792–800. doi:10.1093/icb/ics091. PMID:22693260.

Wright, P.A., and Wood, C.M. 2009. A new paradigm for ammonia excretion in aquatic animals: role of Rhesus (Rh) glycoproteins. J. Exp. Biol. **212**(15): 2303–2312. doi:10.1242/jeb.023085. PMID:19617422.

Youson, J.H. 1980. Morphology and physiology of lamprey metamorphosis. Can. J. Fish. Aquat. Sci. **37**(11): 1687–1710. doi:10.1139/f80-216.

Youson, J.H. 2003. The biology of metamorphosis in sea lampreys: endocrine, environmental, and physiological cues and events, and their potential application to lamprey control. J. Gt. Lakes Res. **29**: 26–49. doi:10.1016/S0380-1330(03)70476-6.

Zimmer, A.M., Brauner, C.J., and Wood, C.M. 2014*a*. Ammonia transport across the skin of adult rainbow trout (*Oncorhynchus mykiss*) exposed to high environmental ammonia (HEA). J. Comp. Physiol. B, **184**(1): 77–90. doi:10.1007/s00360-013-0784-x. PMID:24114656.

Zimmer, A.M., Wright, P.A., and Wood, C.M. 2014*b*. What is the primary function of the early teleost gill? Evidence of for Na^+/NH_4^+ exchange in developing rainbow trout (*Oncorhynchus mykiss*). Proc. R. Soc. B Biol. Sci. **281**: 20141422. doi:10.1098/rspb.2014.1422. PMID:25274361.