

Ammonia excretion in the Atlantic hagfish (*Myxine glutinosa*) and responses of an Rhc glycoprotein

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Edwards SL, Arnold J, Blair SD, Pray M, Bradley R, Erikson O, Walsh PJ. Ammonia excretion in the Atlantic hagfish (*Myxine glutinosa*) and responses of an Rhc glycoprotein. *Am J Physiol Regul Integr Comp Physiol* 308: R769–R778, 2015. First published February 25, 2015; doi:10.1152/ajpregu.00355.2014.—Hagfishes, the most ancient of the extant craniates, demonstrate a high tolerance for a number of unfavorable environmental conditions, including elevated ammonia. Proposed mechanisms of ammonia excretion in aquatic organisms include vesicular NH_4^+ transport and release by exocytosis in marine crabs, and passive NH_3 diffusion, active NH_4^+ transport, and paracellular leakage of NH_3 or NH_4^+ across the gills of fishes. Recently, an emerging paradigm suggests that Rhesus glycoproteins play a vital role in ammonia transport in both aquatic invertebrates and vertebrates. This study has identified an Rh glycoprotein ortholog from the gills of Atlantic hagfish. The hagfish Rhcg shares a 56–60% amino acid identity to other vertebrate Rhcg cDNAs. Sequence information was used to produce an anti-hagfish Rhcg (hRhcg) antibody. We have used hRhcg to localize protein expression to epithelial cells of the gill and the skin. In addition, we have quantified hRhcg expression following exposure to elevated plasma ammonia levels. Animals exposed to a 3 mmol/kg NH_4Cl load resulted in significantly elevated plasma ammonia concentrations compared with controls for up to 4 h postinjection. This correlated with net ammonia excretion rates that were also significantly elevated for up to 4 h postinjection. Rhcg mRNA expression in both the gill and skin was significantly elevated by 15 min and 1 h, respectively, and hRhcg protein expression in gills was significantly elevated at 2, 4, and 8 h postinjection. These results demonstrate a potential role for Rhcg in the excretion of ammonia in the Atlantic hagfish.

IN MOST FISHES, THE MAJOR END product of nitrogen metabolism is ammonia, which is toxic and must be excreted to avoid accumulation. More than 80% of ammonia in teleost fishes is excreted across the branchial epithelium of the gill (42). Recently, there has been a resurgence in exploration of the mechanisms associated with ammonia excretion in fishes, with most studies focusing on freshwater teleosts (19, 21–24), elasmobranchs (20), and, most recently, the Pacific hagfish (1). The modern hagfishes are the sole survivors of the jawless stage in vertebrate evolution; they are considered the most ancient of the agnatha, having diverged from the main vertebrate lineage more than 500 million years ago (14). Hagfishes are exclusively osmoconforming marine animals, with most living at considerable depths, and are the only living vertebrates to maintain their plasma NaCl concentration almost iso-osmotic to that of seawater (10). The unique osmoregulatory characteristics imply that hagfishes have always inhabited

a marine environment, making them a unique model to explore ammonia excretion (26, 27).

Hagfishes feed on dead and decaying carcasses located on the sea floor, often invading the carcass via an orifice and eating their way out. In such circumstances, the hagfish can be exposed to extreme conditions, including high CO_2 , low O_2 , low pH, and elevated ammonia concentrations (1, 38). Yet to date, we know very little about how these animals produce or rid themselves of nitrogenous wastes. A previous study on Pacific hagfish demonstrated that in the laboratory, these animals excrete nitrogenous waste primarily in the form of ammonia with only 6.9% excreted in the form of urea (35). To date, there has been very little attention given to the mechanisms associated with ionoregulation and osmoregulation in hagfishes. One possible explanation for this is that it has long been thought that if an organism iono- and osmo-conforms to its environment, it might not require highly developed and energetically expensive transport mechanisms (5). However, the hagfish gills are populated by numerous mitochondrion-rich cells (MRCs), the cells that associated with Na^+ uptake and Cl^- excretion in marine teleosts. The hagfish MRCs occur singly and are tightly bordered by pavement cells, and there is an absence of the leaky paracellular junction that exists in the branchial epithelium of marine teleosts. However, to the best of our knowledge, there is no physiological data on the transepithelial conductance of hagfish gill epithelium. The hagfish branchial epithelium has been shown to express a number of proteins associated with ammonia transport in both invertebrates and vertebrates; Na^+/K^+ -ATPase, V-Type H^+ -ATPase, carbonic anhydrase (CA), and Na^+/H^+ exchanger isoforms (NHE) (3, 6, 33, 34). Although the hagfishes may not use these transporters to regulate Na^+ and Cl^- , they have been shown to play a role in the maintenance of acid-base homeostasis (6). The presence of a functional Na^+/H^+ exchanger in the hagfish gill was first suggested by Evans as a mechanism associated with acid-base regulation in a marine environment (7). This suggestion was further supported by the demonstration of a significant increase in hagfish gill NHE mRNA expression following the induction of metabolic acidosis (6). An alternate role for the NHE would be to act as a $\text{Na}^+/\text{NH}_4^+$ exchanger; however, Evans (7) found no evidence for the presence of $\text{Na}^+/\text{NH}_4^+$ exchange in the Atlantic hagfish. This finding, along with recent evidence presented for Pacific hagfish, suggests the hagfish's ability to excrete ammonia following a perturbation may be via Rh glycoproteins (1).

The Rh glycoproteins are a family of transmembrane proteins that were first identified in erythroid cells and were mainly studied for their importance to human blood transfusion immunocompatibility. The broader Rh protein family com-

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prises the nonglycosylated proteins, the Rh-30, and the glycosylated Rh-50 proteins that are implicated in ammonia transport. On the molecular level, these proteins are related to the MEP/Amt (methyl ammonium permease/ammonia transport) proteins and are present in the kidney, liver, skin, and testis of mammals, all possible sites of ammonia excretion. A recent phylogenetic study found that Rh and Amt proteins were found together in a wide range of organisms from eukaryotic microbes to invertebrates; however, all vertebrates examined to date, while possessing multiple copies of Rh genes, lacked Amt genes (11).

The presence of Rh proteins in a range of organisms suggests a long evolutionary history. Phylogenetic analysis has given some insight into the origin and gene duplications of Rh genes. In aquatic crustaceans, only one Rh isoform has been identified and has been grouped into the more primitive cluster Rhp1 (11, 37). Some vertebrates possess additional paralogs; teleost fishes have two Rhcg genes and a novel gene defined as Rhp2. It is thought that Rhp2 is probably the most ancient of the Rh genes in vertebrates, as it is specifically expressed in the gut and in the shark kidney and, with the exception of *Danio rerio*, it lacks introns (20). Additionally, genome mining has identified the Rhp1 gene, which is present in a number of invertebrates and was initially assumed to be the most ancient cluster of the eukaryotes. It has five introns in all species examined; however, to date, no Rhp1 mRNA expression has been identified in vertebrate tissues (12). The identification of Rhag, Rhbg, Rhcg, and Rhp2, in the elasmobranchs, all of which are also seen in advanced vertebrates, suggests that these genes were present prior to the divergence of the elasmobranchs and teleost fishes.

Nakada et al. (19) presented evidence suggesting that the pufferfish (*Takifugu rubripes*) utilizes multiple Rh glycoproteins for ammonia excretion. The localization of Rh glycoprotein mRNA and protein expression in the gill lamellae allowed the development of a working model for Rh glycoprotein-mediated ammonia excretion in pufferfish. The evidence suggested that Rhag was located both apically and basolaterally on pillar cells, Rhbg was localized to the basolateral membrane of pavement cells, with Rhcg2 localized to the apical membrane. Finally, Rhcg1 was localized to the apical membrane of MRCs. A number of subsequent studies have identified Rh glycoprotein orthologs in multiple marine fish species (20, 28). In a review, Weihrauch et al. (38) presented two working models for ammonia excretion across the branchial epithelium of marine fishes, one associated with pavement cells and the other with MRCs. The pavement cell model involves basolaterally located Rhbg and apically located Rhcg2, as proposed by Nakada et al. (19), but adds that significant diffusion of NH_3 and NH_4^+ likely occurs across the shallow paracellular junction of gill epithelial cells in marine fishes. Weihrauch et al. (38) suggested that as pavement cells comprise the majority (greater than 90%) of the surface area of the marine fish gill, they most likely provide the dominant route of branchial ammonia excretion. Alternatively, the proposed MRC model is primarily focused on the transport of NH_4^+ into the MRCs via basolaterally located NKA and NKCC and excreted as NH_4^+ substituted for H^+ as the substrate of apically located Na^+/H^+ -exchanger (NHE-2) or as NH_3 by Rhcg1 (38). The localization of Rhcg variants 1 and 2 to the apical membrane of branchial epithelial cells suggests their possible involvement in

facilitating the outward diffusion of NH_3 from the branchial epithelium to the water. A recent study on the Pacific hagfish utilized heterologous antibodies raised against zebrafish Rh orthologs and broadly suggested that these proteins present a possible route for ammonia excretion in hagfish (1). That study set the stage for the current work, as the purpose of the present study was to focus specifically on the apical Rhcg as a possible excretion route for nitrogenous waste in the Atlantic hagfish. We hypothesized that if Rhcg were involved in ammonia excretion in Atlantic hagfish, after the induction of elevated plasma ammonia levels, there should be a corresponding increase in gill Rhcg mRNA and protein expression. This study used RT-PCR to identify a single Rhcg ortholog from hagfish tissues, from which we have produced and verified the first hagfish-specific, anti-Rhcg antibody. This study utilized these tools to determine Rhcg localization, mRNA, and protein expression following ammonia loading in the Atlantic hagfish.

MATERIALS AND METHODS

Animals

Atlantic hagfish (*Myxine glutinosa*) ($n = 80$; 40–115 g) were caught off the coast of Maine by commercial fishermen and transferred to large (20,000 liter) aquaria at the Mount Desert Island Biological Laboratory (MDIBL). Seawater (12–15°C) was pumped continuously from Frenchman Bay into the aquaria via the MDIBL running seawater system. All animals were held without feeding for 2 wk prior to commencement and during experimentation. Institutional Animal Care and Use Committees of MDIBL and the Appalachian State University approved the animal experimental procedures. In all terminal procedures, hagfish were anesthetized using 800 mg/l MS-222 (Argent Laboratories, Redmond, WA) prior to decapitation.

Molecular Cloning and Sequence Analysis

Total RNA was prepared from tissues homogenized in Tri-Reagent (Molecular Research Center, Cincinnati, OH), cDNA was generated in a reverse transcription reaction (SuperScript II; Invitrogen, Carlsbad, CA), and a PCR product was obtained using a degenerate primer pair (Table 1) aimed at conserved regions based on other vertebrate sequences. A BLAST search indicated that the resulting sequence of the ~700 bp RT-PCR fragment was most homologous to Rhcg, and a series of specific nested primers (Table 1) were then designed for use in 3' and 5' rapid amplification of cDNA ends (RACE) (Clontech, Mountain View, CA). RACE products were subcloned into a pGem vector (Promega, Madison, WI) and were sequenced at Genome Quebec (Montreal, Quebec). An open reading frame (ORF) was constructed from overlapping fragments MacVector (MacVector, Cary, NC).

Table 1. Primers used for cloning, PCR, and quantitative PCR analysis

Name	Application	Sequence (5'-3')
RhcgF1 deg	PCR	GAARYYTTSATYAACGCNGAYTT
RhcgR1 deg	PCR	TGNAYNCCACANGTRTCMTGGAK
HagRhcgF5	PCR	TGGTGGATTTTGGTCTCGC
HagRhcgB5	PCR	CAATGATACCTCCGCAGAAGCC
HFRhc3F	3'-RACE	CGGAGCAGTTGCGGTGGAACGG
HFRhc3F2	3'-RACE nested	CACCTTCATCAGCCTGCCCTGG
HFRhc5R	5'-RACE	CCAGGGCAGGCGTGATGAAGGTG
18S F2	qPCR	GCTCTGGATGAGTGTCCGTTG
18S R2	qPCR	TTCTTGGCAAATGCTTTCCG
Rhcg F1	qPCR	GGTGGCACTATTGTCGGTAT
Rhcg R1	qPCR	CCTCCCAATATGCTCTGTCTT

The predicted amino acid sequence from the hagfish Rhcg ORF was aligned with mammalian and fish Rh glycoprotein sequences downloaded from NCBI. The phylogenetic tree was constructed (MacVector) using the neighbor-joining method with Poisson correction and calculation of absolute differences and bootstrap confidence estimates (1,000 replications).

In Situ Hybridization

RNA probes. A 252-bp cDNA fragment of hagfish Rhcg (hRhcg) was inserted into RNA expression vector pGem TEasy (Promega). RNA expression vector was then linearized by restriction enzymes *Sac*II and *Spe*I to allow in vitro run-off synthesis of both sense and antisense RNA probes. Generation of both sense and antisense digoxigenin (DIG)-labeled RNA probes was accomplished using in vitro transcription as per the DIG-RNA-labeling kit (Roche Applied Science, Indianapolis, IN).

Tissue sections were postfixed in 4% paraformaldehyde in DEPC-treated PBS and then rinsed in PBS with 0.1% DEPC followed by 5× SSC (NaCl/Na citrate). Sections were then placed into prehybridization buffer [4× SSC containing 50% (vol/vol) deionized formamide] at 62°C for 2 h. Prehybridization buffer was replaced with hybridization buffer (40% deionized formamide, 10% dextran sulfate, 1× Denhardt's solution, 4× SSC, 10 mM dithiothreitol 1 mg/ml yeast t-RNA, 1 mg/ml salmon sperm DNA) containing 10 ng/ml labeled sense or antisense mRNA (generated from the PCR product, as above) and incubated in a humid chamber overnight at 62°C. Tissue sections were immersed in 2 × SSC in a shaking water bath at 37°C, then 1 × SSC. Sections were then equilibrated in washing buffer (Roche Applied Science, Indianapolis, IN) for 5 min at room temperature followed by an overnight incubation in anti-DIG antibody diluted 1:5,000 in blocking reagent at room temperature. Sections were washed in washing buffer (BM) and then equilibrated in detection buffer. Labeled hRhcg mRNA was visualized with nitro-blue tetrazolium/5-bromo-4-chloro-3'-indolyl phosphate (NBT/BCIP), and the reaction was stopped with Tris EDTA buffer (TE) pH 8.

Ammonia Injection and Water and Tissue Sampling

We chose to follow a similar ammonia-loading protocol, as established by Braun and Perry (1), who, in order to achieve a circulating plasma ammonia concentration of 10 mM NH_4Cl , assumed that the animals extracellular volume was 30% of the mass. Therefore, we calculated the volume of 100 mM NH_4Cl (diluted in buffered seawater, pH 7.8), which resulted a 3 mmol/kg infusion of ammonia. Control animals were infused with a sham (buffered seawater only). Animals were anesthetized, and injections were made to the posterior sinus.

The animals were then returned to the individual aerated chambers, water flow was ceased, and 2.5-ml water samples were taken at time 0, 0.25, 0.5, 1, 2, 4, 6, 8, 12, and 24 h and then frozen (−20°C). A parallel series of experiments was conducted using an identical protocol as above, and terminal tissue and blood samples were taken at time 0, 0.25, 0.5, 1, 2, 4, 6, 8, 12, and 24 h. Animals were killed, as previously mentioned, and blood, gills, and skin were sampled. The blood was immediately centrifuged and plasma was snap-frozen in liquid nitrogen for ammonia assay. Harvested tissues were preserved in 4% paraformaldehyde for immunohistochemistry or were snap frozen in liquid nitrogen and stored at −80°C.

Water Analysis

Water samples were analyzed in triplicate to determine total ammonia (T_{Amm}) using the phenol-hypochlorite method (36). Net flux rates ($\mu\text{mol}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$) of T_{Amm} were calculated as $J_{\text{Amm}} = ([T_{\text{Amm}}]_i - [T_{\text{Amm}}]_f) \times V/(\Delta t \times M)$, where $[T_{\text{Amm}}]_i$ and $[T_{\text{Amm}}]_f$ are initial and the final T_{Amm} concentration ($\mu\text{mol/l}$) in the water, V is the volume of water (l) in flux chamber, Δt is the time (in hours) elapsed in the flux period, and M is the fish mass (in kilograms). A positive J_{Amm} indicates net excretion, and negative value indicated net uptake.

Plasma Ammonia Profile

Plasma samples were deproteinized in two volumes of 8% perchloric acid, vortexed, and centrifuged at 16,000 g (4°C). The supernatant was neutralized with saturated KHCO_3 and centrifuged at 16,000 g (4°C). Using the final deproteinized supernatant, ammonia concentrations ($\mu\text{mol/l}$) were measured in triplicate using a microplate modification of the L-glutamate dehydrogenase assay (Sigma AA0100, St. Louis, MO). The absorbance of each sample was measured at 340 nm following incubation with the ammonia assay reagent and again after the addition of L-glutamate dehydrogenase enzyme with a VersaMAX plate reader (Molecular Devices, Sunnyvale, CA).

Real-Time Quantitative PCR

Relative expression of Rhcg mRNA was quantified in gill and skin tissues using homologous primers (Table 1). In both gill and skin, the ribosomal protein 18S was used as an endogenous control. Expression of mRNA was only quantified for individuals in experiments lasting up to 8 h after injection. Each sample was analyzed in triplicate using 25 ng of template cDNA, 70 nmol of each forward and each reverse primer, 0.5 μl of ROX reference dye diluted 1:10 and SYBR GreenER SuperMix-UDG (Invitrogen) in a total volume of 25 μl . Amplification of the desired product was confirmed using melt curve analysis. Relative mRNA expression in both gill and skin tissues from ammonia-injected individuals was normalized to that of the saline-injected individuals from the same experiments (time durations). Threshold cycle (C_t) was determined for each sample using ABI Systems 7500 software. Analysis of relative expression used the $2^{(-\Delta\Delta C_t)}$ method (25).

Antibody Production

Myxine glutinosa-specific, rabbit polyclonal antibody was raised against amino acid 419–434 specific to the hagfish Rhcg (hRhcg) sequence (5'-CYEDRAYWEVPEEEVTV-3'). The antibodies were produced in-house at Appalachian State University. Rabbits were initially inoculated intramuscularly with 1.5 mg of keyhole limpet hemocyanin-conjugated peptide (BioSource, Lewisville, TX) emulsified in 0.5 ml of Freund's complete adjuvant (Sigma-Aldrich). Animals received three booster injections of conjugated peptide (0.75–1 mg emulsified in Freund's incomplete adjuvant) 3 wk apart. Animals were bled prior to each booster injection, and antibody titers were determined by ELISA.

Western blot analysis

Hagfish gill and skin were weighed and placed in ice-cold homogenization buffer (250 mM sucrose, 1 mM EDTA, 30 mM Tris, 100 $\mu\text{g/ml}$ PMSF, and 5 mg/ml protease inhibitor cocktail). Tissues were placed in a buffer (0.18 g Tris-base, 4.28 g sucrose, 0.5 ml 100 mM EDTA, at pH 7.8) and were homogenized in polypropylene tubes on ice. The homogenate was centrifuged at 13,000 g at 4°C to remove debris. The supernatant was decanted, and its total protein concentration was quantified using a BCA (bicinchoninic acid) protein assay (Thermo Scientific, Rockford, IL). Protein samples (25 μg) were loaded in Criterion-TGX −20% acrylamide gels (Bio-Rad, Hercules, CA) and were separated by SDS-PAGE (sodium dodecyl sulfate, polyacrylamide gel electrophoresis). Separated proteins were transferred onto nitrocellulose membranes (Millipore, Billerica, MA). Membranes were blocked overnight at 4°C in 5% blotto (5% nonfat dry milk powder in 0.1 M TBS with 0.2% Tween-20). Membranes were then incubated in 5% blotto (negative control), hRhcg antibody (1:5,000) in 5% blotto (positive control), or hRhcg antibody preabsorbed 1:10 and 1:20 with purified hagfish Rhcg peptide (1:5,000) in 5% blotto overnight at room temperature. Following three washes [0.1 M Tris-buffered saline with 0.2% Tween-20 (TBST)], membranes were incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibody (1:10,000) and Precision Protein StrepTactin-

HRP conjugate (Bio-Rad) in TBST at room temperature for 1 h. Unbound secondary antibodies were removed with three additional washes in TBST and a final wash in TBS. Immunoblotted membranes were then developed using an enhanced chemiluminescence system (Bio-Rad). Visualization of the blots was done using Bio-Rad Chemidoc system, and densitometry analysis was conducted using image analysis software (Bio-Rad).

Immunohistochemistry

Gill filaments from hagfish were removed and placed in fixative (4% paraformaldehyde in 10 mmol/l PBS, pH 7.4) for 24 h at 4°C. After fixation, filaments were rinsed in three changes of 1× PBS and paraffin-processed. Tissues were sectioned at 7 µm on a Leitz microtome and mounted on positively charged slides (Fisher Scientific). Sections were blocked (5% normal goat serum and 0.1% Tween-20 in PBS at pH 7.4), then incubated with primary antibody, diluted in block: hRhcg (1/500) overnight at room temperature, in a humidified chamber. Unbound primary antibody was removed by washing in PBS. Sections were then incubated with Alexa Fluor goat anti-rabbit 488 (Molecular Probes, Grand Island, NY) secondary antibody diluted in block for 1 h at room temperature. After rinsing for 15 min in PBS, sections were incubated for 30 min with NucRed Dead 647 Ready-Probes reagent (Molecular Probes) and coverslipped using Prolong gold anti-fade reagent (Invitrogen, Grand Island, NY) and visualized using a Zeiss LSM510 confocal microscope.

Negative staining controls for hRhcg were processed, as above, in the absence of primary antibody and using preabsorbed antibody incubations. In preabsorbed controls, hRhcg was diluted to 1.25 µg/ml in block, which also contained 2.5 µg/ml of hRhcg antigen. The antibody and peptide mixture was allowed to incubate at room temperature for 30 min before addition to the tissue sections.

Statistical Analysis

Data are presented as means ± SE. Hypotheses were tested using a one-way repeated-measures ANOVA ($\alpha = 0.05$) followed by Fisher's least significant difference post hoc test for ammonia concentrations, excretion rates, and comparisons of mRNA and protein expression over time. Student's two-sample *t*-test ($P = 0.05$) was used as needed for the simple comparison of two means.

RESULTS

Molecular Properties of Hagfish Rhcg

The complete sequence obtained by 5' and 3' RACE was deposited into the GenBank database (accession no. GU733440). The isolated cDNA was 1,461 nucleotides in length, encoding

for a sequence of 486 amino acid residues. Like the other members of the Rh glycoprotein family, the protein contains a putative N-glycosylation site (ASN-239) that appears to be highly conserved among all members of this gene family. The deduced amino acid sequence of hagfish Rhcg shares high identity (64%) with other known vertebrate Rhcg homologs, indicating that the cDNA encodes a hagfish Rhcg (Fig. 1).

In Situ Hybridization and Immunohistochemical Localization of hRhcg

Sections incubated in the antisense hagfish Rhcg RNA probe labeled specific epithelial cells localized to the basal aspect of the filament epithelium along the region of the filament closest to the blood margin (Fig. 2A). Sense negative controls were devoid of staining (Fig. 2A, inset). The hagfish-specific Rhcg antibody labeled a similar population of epithelial cells localized to the basal aspect of the branchial epithelium, specifically hRhcg (green) was localized to epithelial cells along the innermost layer of the epithelium closest to the blood vessel margin (Fig. 2, B and C). No immunoreactivity was present in the negative controls (not shown). An interesting finding was that hRhcg immunoreactivity was also present in the skin of the hagfish, with hRhcg-immunoreactive cells (red) localized to regions surrounding the mucous glands (Fig. 3, A and B). No immunoreactivity was present in negative controls (not shown).

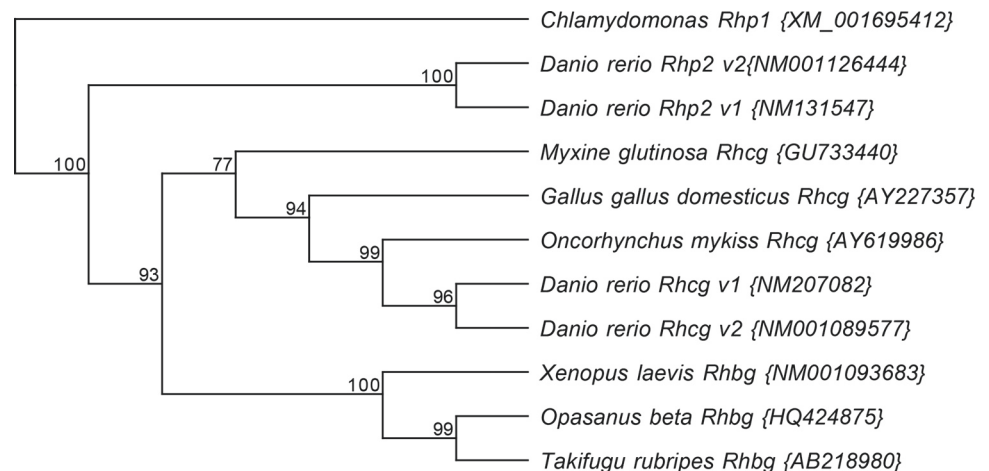
Water Ammonia Profile

Individuals injected with 3 mmol/kg NH_4Cl exhibited a significantly increased ($P = <0.05$) rate of ammonia efflux (relative to sham-injected individuals) up to 4 h postinjection (Fig. 4). There were no significant differences in ammonia efflux between sham and ammonia-injected groups throughout all subsequent time points.

Plasma Ammonia Profile

The concentration of total ammonia detected in the plasma of ammonia-injected hagfish was significantly higher than in sham-injected individuals at all times points prior to 8 h postinjection (Fig. 5). Plasma ammonia concentrations were most elevated at 0.25 h following injection but generally

Fig. 1. Phylogenetic reconstruction using neighbor-joining method with Poisson-correction (bootstrap with 10,000 replications) of selected Rh glycoproteins and the hagfish Rhcg sequence. The tree is rooted by *Chlamydomonas* Rhp1 (NCBI accession XM_001695412). Bootstrap confidence estimates are shown at each node and NCBI accession numbers are listed adjacent to each sequence in parentheses.



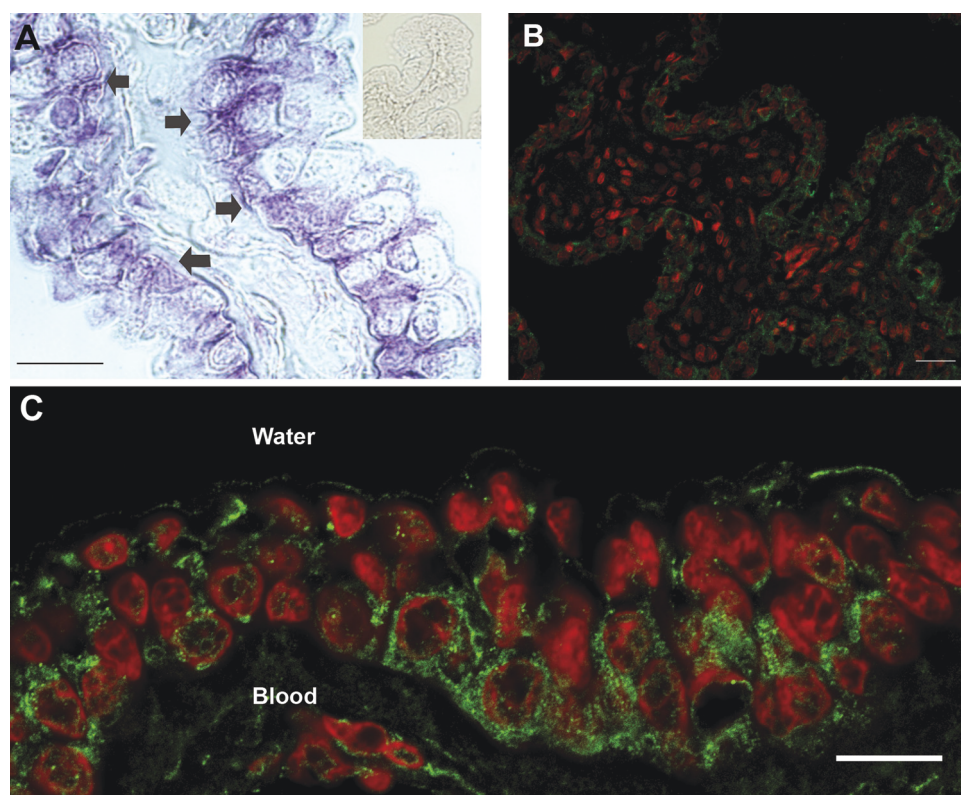


Fig. 2. Representative in situ hybridization and immunohistochemistry images of hagfish gills. *A*: in situ hybridizations that clearly shows hagfish Rhcg mRNA localized to the basal aspect of the filament epithelium. Arrows highlight the punctate staining pattern in the epithelial cells localized along the basal aspect of the gill epithelium. *Inset*: representative section incubated with the sense control, demonstrated no evidence of localization. *B* and *C*: immunohistochemistry representative confocal images at $\times 20$ and $\times 63$ magnification of hRhcg immunolocalized to the gill epithelium (green), nuclei visualized using Nuc Red 648 (red). Scale bars = 20 μm .

decreased over time relative to concentrations observed in saline-injected individuals.

Relative Quantification of hRhcg mRNA

The relative expression of Rhcg mRNA was significantly altered in the gill tissues of ammonia-injected individuals compared with sham at all time points (Fig. 6A). Hagfish gill Rhcg mRNA was significantly elevated in ammonia-injected animals relative to controls at 0.25, 0.5-h time periods ($P < 0.05$). In the time periods from 1 h postinjection onward to 12 h, Rhcg mRNA expression in ammonia-injected individuals was significantly lower than that of saline-injected individuals. Relative mRNA expression of hRhcg mRNA in skin samples demonstrated a significant increase in Rhcg mRNA expression in animals at 0.5 h after ammonia load compared with sham ($P = 0.002$). This was followed by a reduced Rhcg mRNA expression at all time points from 1 h onward (Fig. 6B).

Western Blot Analysis of hRhcg Expression in Gill

Western blots on gill tissue using the homologous hRhcg antibody demonstrated a distinct immunoreactive protein at 48 kDa (Fig. 7A). In negative controls, blots incubated in antibody preabsorbed with peptide (Fig. 7B, lanes 1–3) and in blots incubated in preimmune serum (Fig. 7, lane 4) the 48-kDa band is clearly absent. Protein concentration for each gill sample was quantified on the basis of a standardized load of 25 μg of total protein loaded into each well of each gel. The gill of 48-kDa hRhcg-immunoreactive protein demonstrated a significant increase in expression compared with controls at 2, 4, and 8 h postammonia injection (Fig. 7C).

DISCUSSION

There have been previous studies demonstrating the hagfish's ability to excrete ammonia (1, 7, 18); however, the

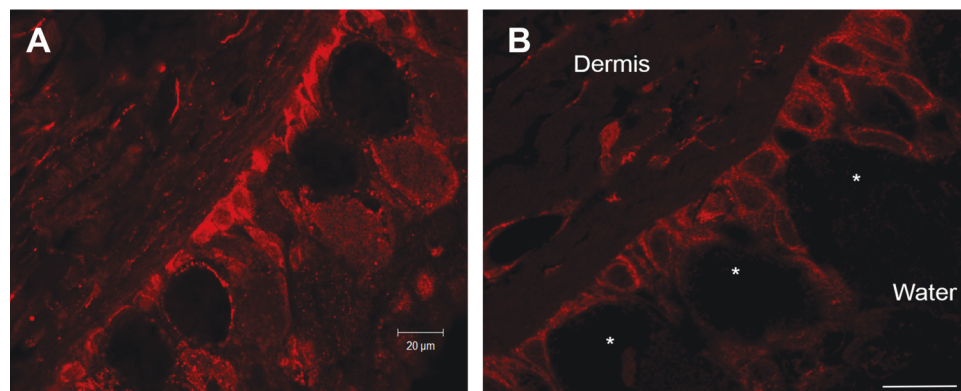


Fig. 3. Representative confocal images of hagfish-specific Rhcg immunolocalized to the skin of the Atlantic hagfish. *A*: localization of hRhcg in the epithelial cells (red) along the basement membrane of the epidermis. *B*: immunoreactive cells extend from the basement membrane and surround the large mucous cells (asterisks) located in the epidermis. Scale bars = 20 μm .

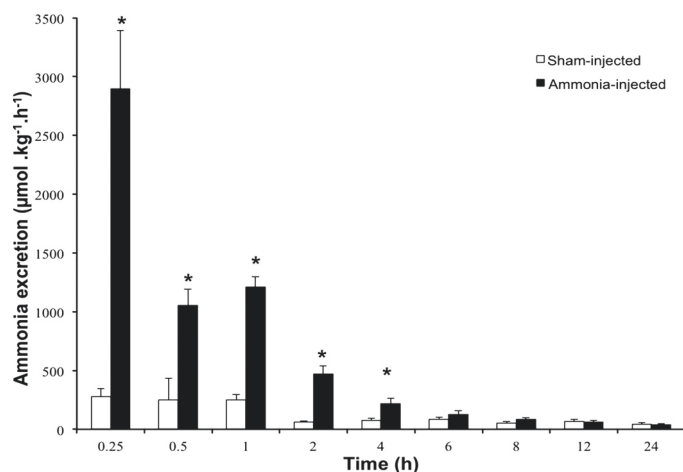


Fig. 4. Effect of 3 mmol/kg NH_4Cl load on net ammonia flux (J_{Am}). Ammonia excretion was significantly ($*P = <0.05$) elevated above that of controls for the first 4 h postinjection, by 6 h excretion was no longer significantly elevated. Data are expressed as means $\pm$ SE ($n = 5$).

current study is the first to identify and quantify Rhcg mRNA and protein expression in epithelial tissues of the Atlantic hagfish (*Myxine glutinosa*) following an ammonia load. This study has demonstrated that following an artificial elevation in plasma ammonia concentrations, Atlantic hagfish respond through increased ammonia efflux, which is correlated to an initial significant elevation in both gill and skin hRhcg mRNA expression and a later upregulation of hRhcg protein expression in the gill, which overlapped slightly with the elevated excretion period. Immunohistochemistry more specifically demonstrated that hRhcg-immunoreactive cells were, indeed, present in both the branchial epithelium, as well as in the dermis of the skin.

Phylogenetic reconstruction (Fig. 1) of Atlantic hagfish Rhcg lineages relative to Rhcg, Rhbg, Rhp2, and Rhp1 isoforms in a number of other organisms demonstrates that hagfish Rhcg forms a clade with other vertebrate Rhcg isoforms. A recent study examining the phylogenetic relationship of cyclostome Rh isoforms suggested that the our hagfish Rhcg and lamprey Rhcg-like genes are more than likely orthologs of

gnathostome Rh (32). The aforementioned study did not examine the primitive isoform Rhp2, although present in a teleost and elasmobranch fishes, the Rhp2 appears to be ancestral to the hagfish Rhcg. This result is to be expected given the results of Huang and Peng (11).

Prior to this study, it was known that Atlantic hagfish had the ability to excrete ammonia (7, 18, 35). However, little was known about the mechanisms by which this excretion was achieved. Given this species' known burrowing and feeding behaviors (17), the ability to rapidly excrete ammonia in less than favorable conditions is advantageous and, thus, not surprising. Atlantic hagfish spend the majority of their life burrowed in hypoxic and, at times, often anoxic ocean-floor substrate and may remain there for days at a time (31). Although gut-content analysis has shown that Atlantic hagfish may feed primarily on shrimp and other invertebrates (29), they are known to opportunistically feed on the carcasses of whales and teleost fishes, for which they have been shown to enter through an orifice to feed on the rotting flesh from the inside out (31). The invasion of a carcass to feed could result in a reduction in adequate gas exchange and, consequently, result in an acid-base imbalance (2). Conditions within a carcass likely include low O_2 , high CO_2 , low pH, and high environmental ammonia (5, 38). Hagfish have developed multiple physiological adaptations to combat extended exposure to hypoxic/anoxic conditions, including reduced metabolic rates and energetic requirements, as well as cutaneous respiration (15). It has been suggested that fishes that are tolerant of hypoxia are often ammonia-tolerant, as the result of the common mechanism of neurotoxicity that is shared between hypoxia and ammonia (glutamine excitotoxicity) (41). Pacific hagfish tolerate both high internal ammonia loads and high environmental ammonia concentrations, surviving more than 72 h of exposure to high environmental ammonia of 100 mM NH_4Cl (1). While in the current study, Atlantic hagfish were not exposed to high environmental ammonia, they were shown to be tolerant of a 3 mmol/kg infusion of ammonia as NH_4Cl , eliminating the internal ammonia load and surviving for 24 h postinfusion.

Following injection, hagfish plasma ammonia concentration was significantly elevated to 1,200 $\mu\text{mol/l}$ within 15 min and

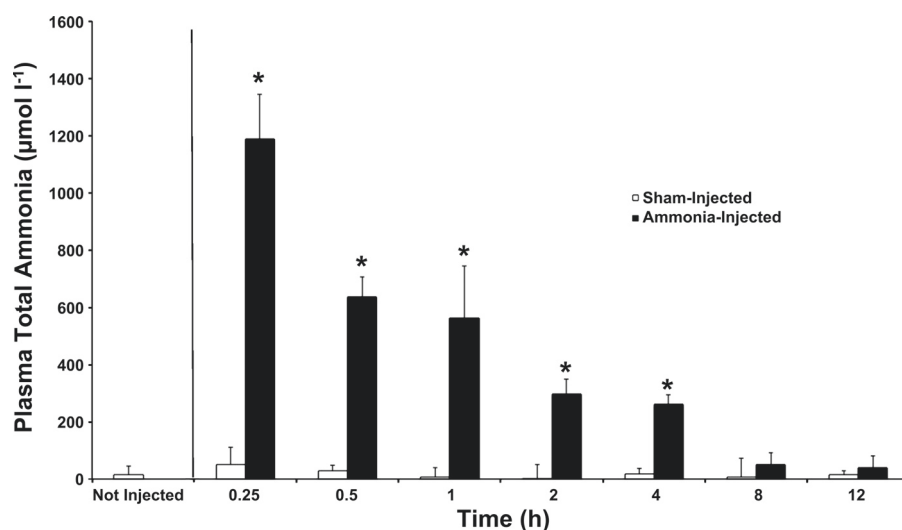


Fig. 5. The effect of 3 mmol/kg NH_4Cl load on plasma total ammonia (T_{Am}). Plasma ammonia concentrations were significantly ($*P = <0.05$) elevated for 4 h postammonia injection compared with sham controls. Data are expressed as means $\pm$ SE ($n = 5$).

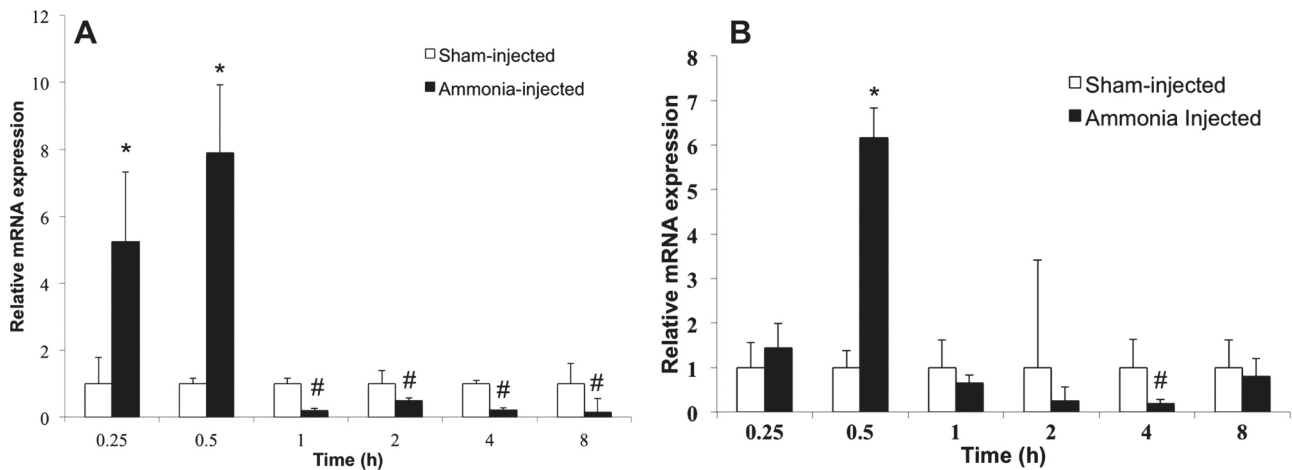


Fig. 6. A: mRNA expression of hagfish Rhcg in the gills of hagfish exposed to 3 mmol/kg NH_4Cl . Rhcg mRNA was significantly ($*P < 0.05$) elevated in the first 15 and 30 min postinjection. However, in subsequent time points gill Rhcg mRNA is significantly ($\#P = < 0.05$) downregulated compared with sham-injected controls. Data are expressed as means $\pm$ SE ($n = 5$). B: mRNA expression of hagfish Rhcg in the skin of hagfish exposed to 3 mmol/kg NH_4Cl . Rhcg mRNA expression was significantly ($*P = < 0.05$) elevated at 30 min postinjection. However, unlike the gill expression pattern, significant downregulation of skin Rhcg mRNA expression was only seen 4 h postinjection. Data are means $\pm$ SE ($n = 5$).

remained significantly elevated for up to 4 h postinjection (Fig. 5). Increased plasma ammonia concentrations corresponded with significantly increased rates of ammonia efflux over the same time period (Fig. 4), suggesting that Atlantic hagfish are either readily equipped for remarkable ammonia excretion (more the $10\times$ that of sham-injected fish) or that the necessary protein components may be preassembled and stored in intracellular vesicles to facilitate a rapid response to a stimulus, such as feeding, resulting in an increased ability to handle the associated ammonia load. This increase in excretion rate has been also reported in a previous study in Atlantic hagfish, in which ammonia excretion rates of nearly $1,400 \mu\text{mol}\cdot\text{N}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$ were observed in the first 0.5 h following 4 mmol/kg infusion of ammonia as $(\text{NH}_4)_2\text{SO}_4$; this was a seven-fold increase over resting ammonia excretion rates (18).

Ammonia excretion rates in ammonia-injected hagfish gradually decreased to levels similar to sham-injected animals by 6 h postinjection. The maximum accumulated total ammonia concentration in the environmental water of ammonia-injected hagfish, occurring at 24 h postinjection was 2.2 mmol/kg, which was 74% of the total 3 mmol/kg load introduced by injection. The net remaining 0.8 mmol/kg of total ammonia was not excreted into the environmental water, although the remaining ammonia was apparently eliminated from the plasma. There are several explanations that may account for this nonexcreted fraction of the ammonia load. In the sea lamprey (*Petromyzon marinus*), it has been suggested that in lamprey, the muscle may serve as a reservoir to reduce circulating plasma ammonia concentrations (40). It is possible that a similar mechanism could be present in the Atlantic hagfish; testing of this hypothesis requires further investigation. Alternatively, the remaining ammonia may be metabolized into amino acids/proteins or urea. Braun and Perry (1) demonstrated that in Pacific hagfish injected with NH_4Cl , there was a delayed response in urea excretion with a significant increase in the excretion of urea seen at 6–9 h postinjection reported to be $\sim 0.08 \mu\text{mol}\cdot\text{N}\cdot\text{g}^{-1}\cdot\text{h}^{-1}$. However, although it is not known whether Atlantic hagfish possess the enzymes associated with the metabolism of ammonia into urea, it is possible

that the remaining ammonia load in this study could have been converted to urea and excreted.

Our results have demonstrated an increase in Rhcg mRNA expression in ammonia-loaded fish prior to 1 h postinjection. This is not necessarily surprising, as elevated plasma ammonia may serve as a stimulus for the increased production of Rh glycoproteins and may suggest that the expression of Rh glycoprotein mRNA is tightly regulated in response to ammonia infusion. Evidence presented in teleost fishes supports the upregulation of branchial Rhcg1, Rhcg2, and Rhbg mRNA expression in response to ammonia infusion (22). However, those authors suggest that a combination of elevated plasma ammonia and a temporary pulse of cortisol may be key to Rh mRNA regulation. Atlantic hagfish plasma cortisol levels were not assessed in the current study due to a long-standing controversy regarding the identity of an active corticosteroid in hagfish (39). However, Atlantic hagfish gill and skin Rhcg mRNA expression was clearly diminished in later time points. The gills demonstrated significantly downregulated mRNA expression at all subsequent time points in ammonia-injected hagfish despite elevated plasma ammonia concentrations and ammonia excretion rates up to 4 h following ammonia injection (Fig. 6, A and B). This pattern of regulation suggests an immediate response to elevated plasma ammonia that is subsequently altered in response to additional factors. Among the possible stimuli inducing the downregulation of gill and skin Rhcg mRNA expression is the concentration of ammonia in the environmental water (24). It has been suggested that Rh glycoproteins can transport ammonia bidirectionally (16) and, thus, could potentially allow inward flux of ammonia, the result of a reversed diffusion gradient caused by high environmental ammonia. This hypothesis was evaluated in marine pufferfish (*Takifugu rubripes*) exposed to 1 mmol/l environmental ammonia in which was demonstrated a significant downregulation of Rhag mRNA expression after 6 h and a downregulation of Rhbg mRNAs after 24 h of exposure (24). However, no protein quantification of the Rh glycoproteins was conducted, because of the presence of multiple nonspecific bands in Western blot analysis. Interestingly, Rhcg1 mRNA expression, as well as

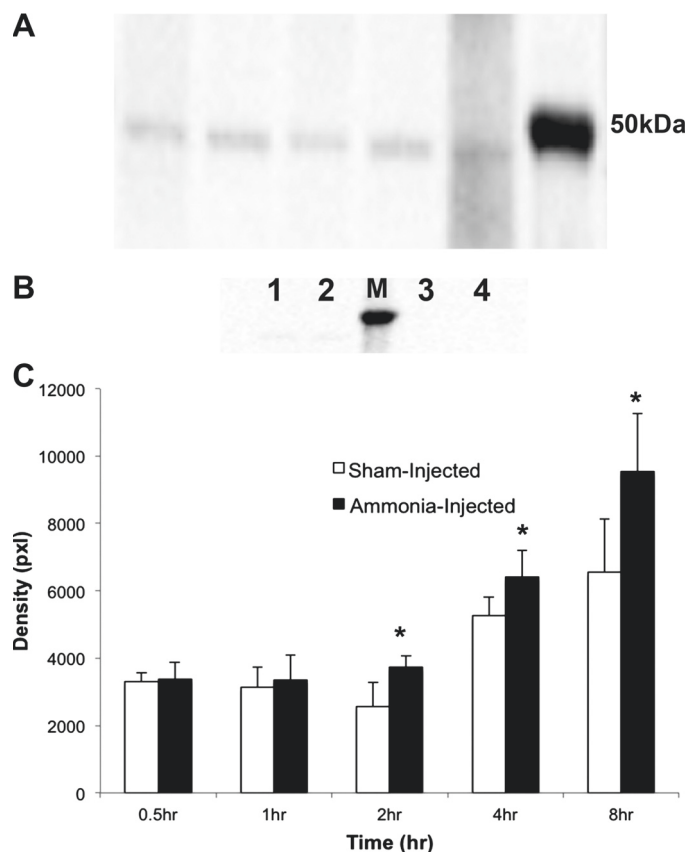


Fig. 7. Protein expression of hagfish Rhcg antibody in gill tissue. **A**: representative Western blot incubated in the hRhcg primary antibody demonstrated a strongly immunoreactive protein in all gill samples at ~48 kDa. **B**: merged image of individual Western blots incubated in hagfish-specific Rhcg primary antibody preabsorbed in hagfish-specific Rhcg peptide. Lane 1: 0.5 μ g/ml peptide-antibody mix, lane 2: 15 μ g/ml peptide-antibody mix, lane 3: 2.5 μ g/ml peptide-antibody mix, lane 4: Preimmune serum. M denotes marker at 50 kDa. **C**: quantification of hRhcg protein expression in gill tissue following exposure to 3 mmol/kg NH_4Cl load. Significant ($*P = <0.05$) elevation of hagfish-specific Rhcg ($P = 0.05$) was seen in animals at 2, 4, and 8 h after ammonia injection compared with sham-injected controls. Data are expressed as means $\pm$ SE ($n = 5$).

that of NKCC1, NKA, NHE3, and H^+ -ATPase, was upregulated in response to high environmental ammonia. This led the authors to propose that, following prolonged high environmental ammonia exposure, ammonia transport may shift from a dependence on passive transport via Rh glycoproteins to active transport by the recruitment of ion transporters in MRCs (24). It is conceivable that accumulated environmental ammonia concentrations could stimulate a subsequent downregulation of gill and skin Rhcg mRNA expression in ammonia-injected hagfish, even while plasma ammonia concentrations and ammonia excretion rates both remain elevated above sham-injected levels as a protective mechanism to reduce the backflow of ammonia from the environment.

Quantification of protein expression from individuals in this study, which was conducted using the hagfish-specific Rhcg antibody (hRhcg), demonstrated that hRhcg protein expression in the gill was significantly elevated ($P < 0.05$) by 2 h postinjection and continued to rise through 8 h postinjection in ammonia-injected hagfish (Fig. 7C). In ammonia-injected hagfish, gill hRhcg protein expression (relative to sham-injected

individuals) was seen at its highest in animals 8 h postinjection, after plasma ammonia and ammonia excretion rates were reduced to control levels at (Fig. 7C). The current study and the protein expression analysis discussed above provide the first insights into the regulation of a potential Rhcg-mediated ammonia excretion from the transcript to protein levels in the Atlantic hagfish. The findings that Atlantic hagfish excrete an ammonia load primarily within the first hour postinjection suggest that they may be equipped to immediately respond to elevated plasma ammonia by utilizing existing Rhcg glycoproteins that are possibly stored in subapical endosomes. The ammonia load immediately resulted in an increase of Rhcg mRNA transcripts, which could be used to transcribe Rhcg glycoprotein stores. The extreme rise in Rhcg protein levels after the bulk of the ammonia load has been cleared may be an acclimation response, where the initial elevated plasma ammonia levels utilize intracellular Rhcg stores, and the elevated expression is required to replenish and prepare the animals for future encounters with high ammonia.

Localization of hRhcg mRNA and immunoreactive protein in the gill tissue demonstrated that the presence of hRhcg-containing cells was seen predominantly along the basal aspect of the epithelium closest to the blood margin (Fig. 2, C and D). The structure of hagfish gills is unique compared with that of teleost gills, but like the gill filament of marine teleosts, the "filament" of the Atlantic hagfish has a high density of MRCs (3, 8). In this study, hRhcg expression was present in the multilayered epithelium of the primary fold, the structural equivalent of the gill filament in teleosts. The hRhcg immunolocalization was supported by localization of hagfish Rhcg mRNA expression in the same region of the branchial epithelium. The pattern of localization suggests that hRhcg may be responsible for the movement of ammonia from the blood into the branchial epithelium. Similar to results seen in teleost fishes (19), immunolocalization studies in the Pacific hagfish, using a heterologous teleost antibody raised against zebrafish Rhcg1, demonstrated that Rhcg1 was localized to the apical membrane of NKA-immunoreactive cells (1). We found no evidence of colocalization of the hRhcg and NKA in the gill epithelium of Atlantic hagfish. This contradiction in the findings may be the result of differences associated between the two genera of hagfish, of which there are many. Alternatively, the use of heterologous, as opposed to homologous, antibodies may be an issue; with no sequence data on Rhcg in Pacific hagfish, it is difficult to determine the homology of the amino acid sequence to that of the fugu to which the antibodies used were raised. We have clearly demonstrated hRhcg immunoreactivity is localized within the epithelium of the skin; the immunoreactive epithelial cells appear to be associated with the cutaneous epithelium surrounding large mucous cells (Fig. 3, A and B). This localization supports the results from a recent in vivo study on Pacific hagfish, which demonstrated that hagfish are capable of excreting large amounts of ammonia across the skin (4). To date, quantification of hRhcg protein expression in the skin of hagfish has proven to be inconsistent, primarily due to low protein yields associated with skin samples.

Among teleost fishes, the gill serves as the primary site of ammonia excretion (8). Rhcg mRNA expression data from this study suggest that cutaneous routes involving Rh glycoproteins may also play a role in ammonia excretion in the Atlantic

hagfish. Rh glycoproteins have been identified in the skin of rainbow trout (21), mangrove rivulus (13), zebrafish (30), and pufferfish (24); all experiments involved exposure to high environmental ammonia [see Glover et al. (9) for a recent review of fish skin as a transport epithelium]. It was not within the scope of this study to determine the specific contributions of branchial and extra-branchial routes toward overall ammonia excretion. However, we present evidence that Rhcg is expressed in the skin of the Atlantic hagfish and that cutaneous Rhcg mRNA expression may respond to elevated plasma ammonia.

Perspectives and Significance

The hagfishes possess the longest known evolutionary history of the extant craniates and are likely to offer exclusive insight into vertebrate origins. The Atlantic hagfish is physiologically adapted to spend much of its life burrowed in ocean-floor substrate and marine carcasses, where high environmental ammonia and unfavorable conditions for ammonia excretion are likely encountered. The current study presents evidence that suggests Atlantic hagfish are capable of reducing experimentally elevated plasma ammonia concentrations and eventually eliminating the ammonia load within 8 h. Following the injection of ammonia, elevated plasma ammonia concentrations paralleled elevated ammonia excretion rates and coincided with the initial significant upregulation of Rhcg in the gill and Rhcg in the skin, suggesting that the transcriptional regulation of Rh glycoproteins may respond, in part, to elevated plasma ammonia. The subsequent significant upregulation of hRhcg protein suggests Rh glycoproteins are involved in the regulation of ammonia excretion in Atlantic hagfish.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: S.L.E. conception and design of research; S.L.E., J.M.A., S.D.B., M.E.P., R.R.B., O.E., and P.J.W. performed experiments; S.L.E. and J.M.A. analyzed data; S.L.E. interpreted results of experiments; S.L.E. prepared figures; S.L.E. and J.M.A. drafted manuscript; S.L.E., S.D.B., and P.J.W. edited and revised manuscript; S.L.E., S.D.B., and P.J.W. approved final version of manuscript.

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