Mechanism of ammonia excretion in the freshwater leech *Nephelopsis obscura:* characterization of a primitive Rh protein and effects of high environmental ammonia

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Quijada-Rodriguez AR, Treberg JR, Weihrauch D. Mechanism of ammonia excretion in the freshwater leech *Nephelopsis obscura:* characterization of a primitive Rh protein and effects of high environmental ammonia. *Am J Physiol Regul Integr Comp Physiol* 309: R692–R705, 2015. First published July 15, 2015; doi:10.1152/ajpregu.00482.2014.—Remarkably little is known about nitrogen excretion in freshwater invertebrates. In the current study, the nitrogen excretion mechanism in the carnivorous ribbon leech, *Nephelopsis obscura,* was investigated. Excretion experiments showed that the ribbon leech is ammonotelic, excreting $166.0 \pm 8.6$ nmol·grams fresh weight (gFW)$^{-1}$·h$^{-1}$ ammonia and $14.7 \pm 1.9$ nmol·gFW$^{-1}$·h$^{-1}$ urea. Exposure to high and low pH hampered and enhanced, respectively, ammonia excretion rates, indicating an acid-linked ammonia trapping mechanism across the skin epithelia. Accordingly, compared with body tissues, the skin exhibited elevated mRNA expression levels of a newly identified Rhesus protein and at least in tendency the Na$^+$/K$^+$-ATPase. Pharmacological experiments and enzyme assays suggested an ammonia excretion mechanism that involves the V-ATPase, Na$^+$/K$^+$-ATPase, and carbonic anhydrase, but not necessarily a functional microtubule system. Most importantly, functional expression studies of the identified Rh protein cloned from leech skin tissue revealed an ammonia transport capability of this protein when expressed in yeast. The leech Rh-ammonia transporter (NoRhp) is a member of the primitive Rh protein family, which is a sister group to the common ancestor of vertebrate ammonia-transporting Rh proteins. Exposure to high environmental ammonia (HEA) caused a new adjustment of body ammonia, accompanied with a decrease in NoRhp and Na$^+$/K$^+$-ATPase mRNA levels, but unaltered ammonia excretion rates. To our knowledge, this is only the second comprehensive study regarding the ammonia excretion mechanisms in a freshwater invertebrate, but our results show that basic processes of ammonia excretion appear to also be comparable to those found in freshwater fish, suggesting an early evolution of ionoregulatory mechanisms in freshwater organisms.

primitive Rhesus proteins; cutaneous ammonia excretion; high environmental ammonia; ammonia trapping

THE MAJORITY OF AMMONIA (in this study, NH$_3$ refers to nonionic ammonia, NH$_4^+$ refers to ionic ammonia, and ammonia refers to the sum of both) in an organism is synthesized through the metabolic process of deamination, while uricolytic or ureolytic pathways usually account for a small portion of ammonia produced (17, 38). In solution, ammonia will be present in a pH-dependent equilibrium of the nonionic, membrane-permeable, NH$_3$ form and the ionic form, NH$_4^+$ (11). As the pKa of ammonia is relatively high (pKa = 9.2 to 9.8), the vast majority of ammonia present in the environment or body fluids will typically be in the ionic NH$_4^+$ form, unless fairly alkaline environments or body fluid conditions are present.

While inherently produced by all cells through protein metabolism, ammonia is toxic, exhibiting various detrimental effects. For example, ammonia is capable of affecting energy metabolism (13, 44, 71) and ion transport capabilities in vertebrates, as well as invertebrates (7, 21, 40). Furthermore, as extensively studied in mammals, ammonia has various detrimental effects on the central nervous system, such as cerebral edema, cerebral atrophy, and disturbances of neuronal growth and signal transduction pathways (8). Because of the toxicity of ammonia, all animals must have an efficient mechanism by which ammonia is detoxified into less toxic nitrogenous waste products or rapidly excreted to maintain body fluid levels within a tolerable range. Typically, nonmammalian aquatic organisms excrete the majority of their nitrogenous waste as ammonia (30, 76).

Various studies have shown that ammonia transport occurs often via tissues also responsible for gas exchange and/or osmoregulation such as skin (14, 51, 63), intestine (49, 68, 72), nephridial systems (29, 32), gills (66, 75), kidneys (59), and anal papillae (18, 64). Early work on ammonia excretion suggested that transport of NH$_3$ was purely passive because of its assumed permeability in lipid bilayers (28) and the lack of identification of an NH$_3$-transporting protein. The discovery that Rhesus glycoproteins (Rh proteins) in humans function as ammonia transporters (33) and the subsequent identification of Rh proteins expressed in ionoregulatory tissues of aquatic organisms (65) have prompted further studies into the mechanism of ammonia transport in a variety of aquatic organisms. Mechanistic studies of ammonia excretion in aquatic organisms have mainly been focused on teleost fish, with a few studies conducted on marine decapod crabs (65, 66, 75). It is important to note that freshwater invertebrates from at least seven different phyla comprise ~85% of all described freshwater species (5) and to date, only a single comprehensive mechanistic study has been conducted on a freshwater invertebrate, the planarian *Schmidtea mediterrnea* (63). With freshwater invertebrates remaining a highly understudied, but highly diverse, group of organisms, further investigations are critical to gain an understanding of the evolution of ammonia excretion mechanisms and determine whether there are general ammonia excretion strategies functioning in freshwater environments.

The present study focuses on the freshwater ribbon leech *Nephelopsis obscura* (also called *Eropobella obscura*) commonly found distributed throughout various freshwater bodies...
in Canada and northern United States (15). Unlike the more widely known blood-feeding leeches, this species is carnivorous, feeding on a protein-rich diet comprising mainly chironomids and small oligochaetes (16). Although little is known about the nitrogen physiology of *N. obscura*, a number of studies have investigated the sodium transport across the skin in other freshwater leeches (12). From studies on the blood-feeding medicinal leech *Hirudo medicinalis*, the skin has been identified as a site of sodium uptake (60, 61), which may also potentially serve as the site of ammonia excretion due to the similarity in transporters typically utilized for both processes (30, 35, 45). In addition to skin, other potential sites of ammonia transport include the metanephridia, where urine formation and salt balance occur, as well as the intestine where nutrient uptake and food breakdown occur (12).

This study aims to examine the mechanism of ammonia excretion in the integument of the freshwater ribbon leech *N. obscura* and the effects of elevated environmental ammonia concentrations, utilizing a combination of excretion experiments and gene expression analysis. If general freshwater ammonia excretion mechanisms exist, then an acid-trapping mechanism is likely mediating cutaneous ammonia excretion under normal environmental ammonia conditions. Moreover, the ammonia transport capability of an identified and cloned primitive Rhesus glycoprotein was determined, utilizing a yeast complementation assay.

**METHODS**

**Animals.** Leeches (*N. obscura*) obtained from a local bait shop (Manny’s Live Bait, Winnipeg, MB, Canada) were maintained at 10°C under natural lighting conditions (12:12-h light-dark) in 100 liters of dechlorinated tap water (DTW; pH 8.3) and constantly aerated via air stones. Tank ammonia concentrations were maintained below 10 µmol/l. Leeches were fed frozen bloodworms twice a week for 2 h ad libitum. Aquarium water was replaced 6 h after each feeding period. All experiments were performed at 10°C on leeches starved for a minimum of 2 days.

**Whole animal excretion experiments.** For whole animal excretion experiments, individual leeches [−0.5–3.7 g fresh weight (gFW), 2.54–12.7 cm in length] were placed into 100-ml beakers containing 30 ml of DTW (pH 8.3), aerated by means of air stones. After a 1-h equilibration period, the media were replaced with a fresh volume of DTW for the initial sampling period. At the end of each sampling period, a 10-ml sample was taken and frozen (−20°C) for later analysis of ammonia and urea. After each sampling period, the fluid in the beakers was discharged, and animals were subsequently rinsed with 50 ml of DTW. A no-animal control enriched with 20 µmol/l NH₄Cl was run and confirmed that no ammonia loss occurred during the 1-h experimental sampling period.

**Excretion experiments under the influence of various inhibitors and pH regimes.** To determine ammonia excretion rates in media buffered to various pH regimes, a control sampling period (1 h DTW, pH 8.3 unbuffered) was followed by a 1-h sampling period in DTW buffered to one of three pH values (pH 5, 8.3, and 9.5) by either 5 mmol/l MES (pH 5), which has an effective buffer range at a low pH, or 5 mmol/l Tris (pH 8.3 and 9.5), which has an effective buffer range at higher pH ranges. The pH of the experimental media was adjusted with HCl or NaOH. Buffering of the environmental media was used to prevent the leeches from manipulating the pH of the unstirred boundary layer above the epithelial skin surface. The low- and high-pH exposures were utilized to simulate a high-proton and low-proton environment, respectively, which would promote and hamper, respectively, any acid trapping of ammonia over the skin surface.

To determine whether exposure to a buffered acidic environment is causing an increased ammoniagenesis, total ammonia (body ammonia content + ammonia excreted during treatment) was measured. In brief, a control sampling period (1 h DTW, pH 8.3 unbuffered) was followed by a 1-h treatment sampling period in either DTW buffered to pH 5 with 5 mmol/l MES or unbuffered DTW pH 8.3. Following treatment sampling, leeches were frozen at −80°C, and then body ammonia content was measured (see **Body ammonia and urea content**).

In another set of experiments, the effect of pharmacological agents on ammonia excretion rates was investigated. The control-sampling period (1 h) was followed by a 30-min preincubation period, during which the leeches were enriched with a specific inhibitor. Experiments were performed in 30 ml of media. A 5-ml sample was taken after the preincubation period, and a 1-h experimental sampling period in the remaining media (25 ml) followed. Acetazolamide, concanamycin C, and 5-(N-ethyl-N-isopropyl)amiloride (EIPA) were dissolved in DMSO at a final concentration of 0.5% in the experimental solution. DMSO was also added to the respective control experimental solutions for experiments involving these inhibitors. Inhibitors used in this study were applied at concentrations shown to be effective in other invertebrates (62, 63, 67, 68). The inhibitors used in this study were (target protein) 0.005 mmol/l concanamycin C (V-ATPase), 1 mmol/l acetazolamide (carbonic anhydrase), 0.1 mmol/l EIPA (Na⁺/H⁺-exchangers), 5 mmol/l ouabain (Na⁺/K⁺-ATPase), and 2 mmol/l colchicine (microtubule networks). Following inhibitor experiments, no mortalities due to inhibitor treatments were observed in leeches monitored for 1 wk.

**Feeding experiments.** Feeding experiments were performed to investigate regulation of an internal ammonia load, which leeches would regularly experience in nature. When feeding experiments were performed, leeches were starved for 5 days, and then a feeding period (frozen bloodworms, ad libitum) of 1 h in 80 ml of DTW followed the control measurement. After the feeding period, beakers were rinsed with DTW, and 30 ml of fresh DTW was added for the first 1-h sampling period after feeding. As performed for the first sampling period, five consecutive 1-h sampling periods postfeeding followed. After the sixth 1-h sampling period postfeeding, the integument of the leeches was dissected (see **Tissue preparation**). Only when bloodworm exoskeletons were macroscopically observed in the leech intestine during the skin dissection (6 h after feeding), samples were used for ammonia analysis. Typically, bloodworm exoskeletons can be observed in fecal matter within 12 h after feeding. In parallel, after feeding, another set of leeches was maintained in 500 ml of DTW for 24 h. After 24 h postfeeding, leeches were transferred to 30 ml of DTW for a 24-h postfeeding sampling period.

**High environmental ammonia experiments.** For high environmental ammonia (HEA) experiments, a control-sampling period (1 h ammonia-free DTW, pH 8.3) was followed by two consecutive 1-h sampling periods. The first sampling period occurred in DTW enriched with 1 mmol/l NH₄Cl, while the second sampling period was in ammonia-free DTW. Following this, leeches were placed in a three-liter container of DTW enriched with 1 mmol/l NH₄Cl for 1 day. After a 1-day HEA exposure, leeches underwent three consecutive 1-h sampling periods. The first and last sampling periods were done in DTW containing 1 mmol/l NH₄Cl, and the second sampling period was done in ammonia-free DTW. Following these sampling periods, leeches were returned to the three-liter container with 1 mmol/l NH₄Cl for six more days. After 7 days of HEA exposure, three consecutive 1-h sampling periods identical to that of 1-day HEA-acclimated leeches were performed. The 1 mmol/l NH₄Cl solution for HEA acclimation was replaced daily. A timeline of this experimental procedure is demonstrated in Fig. 8A.

**Ussing chamber experiments.** Ussing chamber experiments were used to investigate the capacity of the skin to transport ammonia and clarify the role of the Na⁺/K⁺-ATPase in cutaneous ammonia excretion by providing direct access to the basolateral side of the skin for...
pharmacological studies. Leech skin was isolated (see Tissue preparation) and mounted with Vetbond tissue adhesive (3M, St. Paul, MN) in a modified Ussing chamber (P2300; Physiologic Instruments, San Diego, CA) with a 0.45 cm² aperture custom-made tissue holder. Experiments were performed at 10°C with experimental solutions maintained constantly aerated. Leech skin was bathed with unbuffered DTW (pH 8.3) containing 10 mmol/l theophylline on the apical side and leech Ringer solution on the basolateral side. The leech Ringer solution (pH 7.4) contained (in mmol/l) 115 NaCl, 1.8 CaCl2, 2 MgCl2, 4 KCl, and 10 HEPES (54). The leech Ringer solution was further supplemented with 10 mmol/l glucose to provide an energy substrate for the tissue and 10 mmol/l theophylline, which has been shown to activate ammonia excretion in frog skin epithelia mounted in an Ussing chamber (14). Chambers were filled with 4 ml of the corresponding experimental solutions as stated above. Prior to all Ussing chamber experiments, the mounted tissue was given a 1-h period to equilibrate to the experimental solutions indicated above. For metabolic ammonia experiments, during the equilibration period, there was no ammonia on the basolateral side. Conversely, during pharmacological experiments, the basolateral side was enriched with 500 μmol/l NH4Cl for the equilibration period. Unless otherwise mentioned, the experimental solutions during the incubation, washout, and equilibration periods were replaced with fresh solutions between each period.

For determination of metabolic ammonia released toward the apical side of the leech skin, a 1-h equilibration period was followed by three consecutive 1.5-h sampling periods, with no ammonia on either the basolateral or apical side. Following each sampling period, 4-ml samples were taken from both sides of the tissue and immediately frozen for later ammonia analysis, and fresh experimental solutions were added to the chambers.

For pharmacological studies examining the effects of ouabain, an inhibitor of Na⁺/K⁺-ATPase, on ammonia excretion, the leech Ringer solution was further enriched with 300 μmol/l NH4Cl, while the apical DTW contained no ammonia to provide an in vivo-like ammonia gradient across the tissue. Pharmacological Ussing chamber experiments consisted of six experimental time points: 1) 1-h equilibration period, 2) 1-h control sampling period, 3) 30-min inhibitor incubation period (Ringer enriched with 5 mmol/l ouabain), 4) 1-h inhibitor incubation (Ringer enriched with 5 mmol/l ouabain), 5) 30-min inhibitor washout period to remove excess ouabain from experimental chambers and tissue, and 6) 1-h tissue integrity sampling period to verify that the tissue was still active by reestablishing near-control excretion levels. The measured apically directed metabolic ammonia release (see RESULTS, Ammonia excretion mechanism) by the tissue was subtracted from flux rates in pharmacological experiments to obtain values for ammonia excretion due to the active transport of basal ammonia.

Long-term HEA exposure experiments. Groups of leeches (six leeches in three liters) were maintained in 1 mmol/l NH4Cl for 1 or 7 days with experimental solutions changed daily. After the 1- or 7-day exposure time, leeches were cut in half, and either the anterior or posterior half of the leeches were frozen for determination of body ammonia, whereas the integument of the corresponding other half was dissected (see Tissue preparation) for gene expression analysis.

Body ammonia and urea content. Deproteinization of leech body tissue samples for ammonia/urea analysis was modified after Veauvy et al. (58) with the entire procedure performed on ice or in a refrigerated centrifuge. Diced frozen (−80°C) leeches were homogenized (Powermax AHS 200, VWR International, Radnor, PA) in 10 volumes of ice-cold 6% perchloric acid (assuming a 1 g/ml density of tissue). Homogenates were incubated on ice (10 min) for deproteinization of the samples to occur. Homogenates were then centrifuged (5 min, 4°C, 5,000 g), and the supernatant was neutralized with 0.6 volumes 2.5 mol/l KHCO3. Neutralized samples were centrifuged (5 min, 4°C, 16,000 g) and immediately analyzed for ammonia (see Ammonia and urea measurement).

Following deproteinization, body urea was analyzed using the colorimetric diacetyl monoxime/thiosemicarbazide assay (48). In summary, one volume of neutralized body homogenate or urea standard was mixed with 0.5 volumes of urea assay reagent and incubated in a water bath (100°C, 10 min). Cooled samples were spectrophotometrically (Abs450nm) analyzed on a microplate reader (Powerwave, BioTek, Winoski, VT). The composition of urea assay reagent was 1.2 mol/l H2SO4, 0.33 mol/l H2PO4, 1.37 × 10−4 mol/l FeCl3, 6.1 × 10−3 mol/l thiosemicarbazide, and 2.74 × 10−3 mol/l diacetyl monoxime. Urea standards (0, 20, 40, 80, 100, and 120 μmol/l urea) were prepared in deionized water, and a minimum R² value of 0.98 was required for standard curves.

Ammonia and urea measurement. Total ammonia was measured using a gas-sensitive NH3 electrode (Thermo Orion, Beverly, MA) connected to a digital pH meter, as previously described in detail (62). The electrode can account for ±1 μmol/l in the 4–50 μmol/l ammonia range and ±1.5 μmol/l in the 50–200 μmol/l ammonia range. Measurement of total urea by the gas-sensitive NH3 electrode was performed as described previously (14). Briefly, prior to urease treatment (10 U/ml, 25°C, 30 min) for the measurement of urea, the ammonia concentration of the samples was measured to determine the background ammonia in the sample. Total urea in urease-treated samples was calculated according to Eq. 1, where $S_b$ is the sample total ammonia after urease treatment, and $S_p$ is background ammonia prior to urease treatment. Standard curves were prepared from the corresponding experimental solutions. The analysis of four separate samples with known urea concentrations (17.3 ± 0.4 μmol/l urea, n = 4, colorimetric urea assay) validated that urease treatment in combination with a gas-sensitive NH3 electrode produces comparable results (17.8 ± 0.2 μmol/l urea, n = 4) to the widely utilized colorimetric diacetyl monoxime/thiosemicarbazide assay for urea determination in tissue, plasma, and water samples.

$$\text{Urea} = \frac{S_b - S_p}{2}$$

Na⁺/K⁺-ATPase activity. A Na⁺/K⁺-ATPase enzyme assay was used to determine whether this enzyme is capable of accepting ammonia as a substrate in place of potassium. Na⁺/K⁺-ATPase activity of the leech skin was measured spectrophotometrically at 20°C based on established protocols (19, 37). The skin was homogenized in 15 volumes of ice-cold buffer containing: 150 mmol/l sucrose, 10 mmol/l EDTA, 50 mmol/l imidazole, and 0.1% (wt/vol) sodium deoxycholate, and subsequently centrifuged at 5,000 g for 1 min at 4°C. The assay procedure was the same as described in Cruz et al. (14), with the exception of using 5 mmol/l ouabain for inhibiting the Na⁺/K⁺-ATPase in the current study. Activities were determined using either 10 mmol/l KCl or 10 mmol/l NH4Cl. Protein concentrations were determined using the Biuret assay using BSA for standard curve preparation.

Tissue preparation. To isolate the skin for RNA isolation and Ussing chamber experiments, leeches were placed on ice for 20–30 min. The skin was dissected under RNase-free conditions by making a ventral incision from head to tail. Subsequently, the dorsal skin was removed from the internal organs and the muscle layers by carefully scraping the skin with a scalpel until it became transparent. For body tissue samples, a dorsoventral cross section was taken in the middle of the leech to account for the majority of organs present. Dissected skin and body sections were stored in RNA later (Applied Biosystems, Austin, TX) at −80°C until RNA isolation.

Quantitative PCR. Total RNA was isolated from leech skin, and the whole body with TRI Reagent (Sigma-Aldrich, St. Louis, MO). Following phase separation, the RNA-containing phase was purified with E.N.Z.A. tissue RNA kit (Omega Bio Tek, Winoski, VT) and spectrophotometrically quantified (NanoDrop 2000c, Thermo Scientific, Wilmington, DE). Before synthesis of CDNA, 0.5 μg of total RNA was treated with DNase (DNase I; Invitrogen, Carlsbad, CA), and
purity was verified by PCR using the primer pair No RPS2 F1/R1 (Table 1) targeting the ribosomal protein S2 (GenBank accession no.: KM923910). Complementary DNA (cDNA) was synthesized from 0.5 μg DNA 1-treated RNA using iScript cDNA synthesis kit (Bio-Rad, Mississauga, ON, Canada). The cDNA quality was evaluated by PCR using the primer pair No RPS2 F1/R1 (Table 1). PCR products were assessed by ethidium bromide-stained agarose gel electrophoresis.

Partial sequences utilized as a base for designing species-specific primers to be employed in quantitative PCR were obtained through degenerate primers. Degenerate primers targeting the Rh protein, V-ATPase (subunit B), Na+/K+-ATPase (α subunit), and ribosomal protein S2 (Table 1) were designed on the basis of conserved regions in published amino acid sequences of the respective genes. PCR products of the predicted size were purified (E.N.Z.A gel extraction kit, Bio Tek) and sequenced (Robarts Research Institute, London, ON, Canada). A GenBank search with BLAST was used to verify the identity of the isolated PCR products. Primers for quantitative PCR were then generated using the obtained and verified sequences for the respective target genes. Primers employed in amplification of NoRhp, V-ATPase B subunit, Na+/K+ ATPase (α subunit) and ribosomal protein S2 (GenBank accession nos.: KM923907, KM923908, KM923909, and KM923910, respectively). For all primers used in quantitative PCR (qPCR; Table 1), a standard PCR was performed to verify generation of a single PCR product of the expected size.

For quantitative PCR (MiniOpticon, Bio-Rad, Mississauga, ON, Canada), standard curves were generated using a dilution series with known quantities of DNA (10⁻¹, 10⁻², 10⁻¹, 10⁻⁰, 10⁰, 10¹, 10² fg) from purified PCR products (E.N.Z.A gel extraction kit, Bio Tek) and sequenced (Robarts Research Institute, London, ON, Canada). A GenBank search with BLAST was used to verify the identity of the isolated PCR products. Primers for quantitative PCR were then generated using the obtained and verified sequences for the Rh protein, V-ATPase (subunit B), Na+/K+-ATPase (α subunit) and ribosomal protein S2 (GenBank accession nos.: KM923907, KM923908, KM923909, and KM923910, respectively). For all primers used in quantitative PCR (qPCR; Table 1), a standard PCR was performed to verify generation of a single PCR product of the predicted size.

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The ribosomal protein S2 served as the internal control for all mRNA expression experiments because it remained stable under all experimental conditions as tested by the NormFinder algorithm (3) for both skin and body sections of N. obscura.

### Table 1. Primers employed in amplification of NoRhp, V-ATPase B subunit, Na+/K+ ATPase α subunit, and ribosomal protein S2

<table>
<thead>
<tr>
<th>Primer</th>
<th>Nucleotide Sequence (5′ → 3′)</th>
<th>Annealing Temperature, °C</th>
<th>Product size, bp</th>
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<tr>
<td>Rh-protein</td>
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<tr>
<td>DegRh F2</td>
<td>CAYGCNTTYGNGCNGCATTATTY</td>
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Primers employed in yeast expression contain restriction enzymes SpeI and XhoI in the primer name, while all other primers containing the prefix "No" were utilized for quantitative PCR. Y, replaces C/T; N, replace A/T/G/C; R, replace A/G; D, replaces A/G/T.

**Molecular cloning and analysis of full-length N. obscura Rhesus protein.** To clone the full-length cDNA of the N. obscura Rh protein (NoRhp), a BLAST-verified partial sequence obtained through degenerate primers (Table 1) was used to design NoRhp-specific primers to be used in RLM-RACE (FirstChoice RLM-RACE kit, Ambion, Austin, TX). 5’ and 3’ RLM-RACE products of the predicted size were cloned into pGEM T-easy vector (Promega, Madison, WI) and sequenced (Robarts Research Institute). To obtain a proofread full-length cDNA of NoRhp, specific primers flanking the 5’ and 3’ of the open reading frame (ORF) were designed (Table 1). The proofread sequence was amplified using Phusion high-fidelity DNA polymerase (Thermo Scientific, Ottawa, ON, Canada) and sequenced (Robarts Research Institute). Alignment of NoRhp with published Rhesus proteins was performed by MUSCLE alignment of amino acid sequences on MEGA 5 (53). Generation of predicted transmembrane domains was done with Phyre 2.0 (26).

**Yeast complementation assays.** To determine whether NoRhp is capable of transporting ammonia, a yeast complementation assay was used with the Saccharomyces cerevisiae strain 31019b, which is deficient in endogenous ammonia transporters. The ORF of NoRhp cDNA (nt 6 - to +1,482, where +1 is A of the ATG start codon) was amplified (Phusion High-Fidelity DNA Polymerase, Thermo Scientific) using specific primers containing SpeI and XhoI restriction sites on the 5’ and 3’ ends, respectively. Amplified PCR products were then purified, digested, and ligated into the SpeI and XhoI restriction sites of the yeast expression vector pRS426-MET25.

The expression systems used in this study was Saccharomyces cerevisiae strains 23344c (ura3) (Grenson M, unpublished data) and 31019b (mep1Δ, mep2Δ, and ura3) (34), which are isogenic with the wild-type Σ1278b (6). Transformation of yeast strains with expression vectors was achieved by heat shock, as described previously (20). Transformed yeast cells were grown in buffered minimal medium containing 3% glucose as a carbon source (25). The nitrogen sources used were 3 mmol/l (NH₄)₂SO₄ or 0.1% (6.8 mmol/l) glutamine. The yeast strain 31019b transformed with the Human RhCG was used as a positive control (33). Yeast cells transformed with
NoRhp were verified for the correct insert by PCR on yeast colonies using NoRhp qPCR primers (Table 1).

Phylogenetic analysis of rhesis glycoproteins. The Rh protein data set contained 49 full-length cDNA sequences. Amino acid sequences of Rh protein data set were aligned by MUSCLE alignment in MEGA 5 (53). Phylogenetic analysis of MUSCLE-aligned sequences was done in MEGA 5 using maximum likelihood method with the Jones-Taylor-Thornton + four categories of gamma substitution rates + invariable sites model and Nearest Neighbor Interchange heuristic method. Bootstrap values were determined from 1,000 bootstrap replicates. The maximum likelihood (ML) tree was rooted with the prokaryotic Rh protein from Nitrosomonas europaea.

Chemicals. Sodium thiosulfate, ammonium chloride, Tris hydrochloride, and MES were purchased from Fisher Scientific (Ottawa, Ontario, Canada). All other chemicals (unless otherwise stated) were of analytical grade and were purchased from Sigma-Aldrich.

Statistics. Data are presented as means ± SE. All data were tested for homogeneity of variance with Levene’s test and normal distribution with Shapiro-Wilk test. Differences between two means were tested with a Student’s t-test, while differences between more than two means were tested with a one-way ANOVA. In the case that data were not normally distributed or did not show homogenous variance, nonparametric tests were used to assess statistical significance. When nonparametric tests were required, the Mann-Whitney U-test was used to determine differences between two means, and the Kruskal-Wallis test was utilized to assess difference between more than two means. P values ≤ 0.05 were considered significant. Statistics employed in each experiment are also provided in the figure legends or in text.

RESULTS

Basics. Under control conditions, ammonia excretion rates in N. obscura were 166.0 ± 8.6 nmol·gFW⁻¹·h⁻¹ (n = 96), and body ammonia content was 0.88 ± 0.06 μmol/gFW (n = 12). Urea excretion rates were measured to 14.7 ± 1.9 nmol·gFW⁻¹·h⁻¹ (n = 29), and body urea content was 0.51 ± 0.05 μmol/gFW (n = 12).

Transporter mRNA expression body vs. skin. Relative mRNA expression of NoRhp, V-ATPase, and Na⁺/K⁺-ATPase in skin and whole body sections of N. obscura revealed a three-fold higher expression level of the Rh protein in the skin (Fig. 1). In addition, the Na⁺/K⁺-ATPase (α-subunit) also had, at least a tendency (P = 0.07), for greater expression in the skin, while the V-ATPase (subunit B) was equally expressed (Fig. 1).

Ammonia excretion mechanism. Disruption of the V-ATPase and carbonic anhydrase after application of concanamycin C (0.005 mmol/l) and acetazolamide (1 mmol/l), respectively, reduced ammonia excretion rates (Fig. 2). Conversely, disruption of microtubule networks by colchicine (2 mmol/l) and inhibition of Na⁺+/H⁺-exchangers by EIPA (0.1 mmol/l) resulted in no change in ammonia excretion (Fig. 2). Whole animal exposure to ouabain, an inhibitor of the Na⁺+/K⁺-ATPase, did not affect ammonia excretion (Fig. 2).

Utilizing the Ussing chamber setup, applying an outwardly directed gradient (300 μmol/l: 0 μmol/l NH₄Cl) over the isolated theophylline-activated skin of the leech resulted in a flux of 61.5 ± 12.7 nmol·cm⁻²·h⁻¹ (n = 6). Here, basolateral application of ouabain resulted in a 39% decrease in ammonia excretion (Fig. 3). For correctional purposes, also the apically released metabolic ammonia from theophylline-activated N. obscura was measured. The apical ammonia release remained stable over three consecutive 1.5-h time periods at 22.5 ± 0.8 nmol·cm⁻²·h⁻¹, 21.1 ± 0.6 nmol·cm⁻²·h⁻¹, and 19.9 ± 1.7 nmol·cm⁻²·h⁻¹ (n = 5), and the respective values were subtracted for the calculation of the transepithelial fluxes. Furthermore, an enzyme assay of isolated leech skin revealed that the Na⁺+/K⁺-ATPase accepts ammonium as a substrate. The assay confirmed that the ouabain-sensitive ATPase activity in the skin homogenates did not change whether ammonium (2.32 ± 0.13 nmol·ADP·min⁻¹·mg-protein⁻¹; n = 4) or potassium was provided as a substrate (2.96 ± 0.37 nmol·ADP·min⁻¹·mg-protein⁻¹; n = 4).

Ammonia excretion in N. obscura was highly dependent on environmental pH (Fig. 4). When leeches were exposed to pH 5, ammonia excretion increased approximately twofold relative to the control excretion rates (pH 8.3 unbuffered DTW). While excretion rates increased when buffered pH 5, the total ammonia (body ammonia content + ammonia excreted) remained unchanged with a total ammonia of 1.49 ± 0.12 μmol/gFW in control leeches and 1.63 ± 0.12 μmol/gFW in
leeches exposed for 1 h to buffered pH 5 DTW (n = 6 or 7, Student’s unpaired t-test). When leeches were exposed to high pH (pH 9.5 buffered), hampering acid trapping over the epithelial surface, ammonia excretion rates decreased substantially to 13% of control levels (Fig. 4). Buffering of the control media with 5 mmol/l Tris, and thus preventing the leech from manipulating the pH in its unstirred integumental boundary layer, did not affect the rate of ammonia excretion (Fig. 4).

Following buffered pH manipulations, ammonia excretion rates returned to control levels when placed in unbuffered pH 8.3 DTW (data not shown).

Identification and characterization of a primitive Rh protein in N. obscura. Employing isolated mRNA from skin tissues, degenerate primers, and subsequently the RACE technique, the full-length (1,482 bp) mRNA for a primitive Rh protein now named NoRhp (GenBank accession no.: KM923907) was obtained.

Translation of the NoRhp ORF resulted in a 493-amino acid glycoprotein with a 49%, 46%, and 43% identity to verified ammonia transporters from trout (Rhbg, GenBank accession no.: NP_001118134), human (RhCG, GenBank accession no.: AAP81044), and pufferfish (Rhcg2, GenBank accession no.: BAE96344.1), respectively. Employing the Phyre 2.0 protein structure predictor (26), 12 transmembrane domains for NoRhp were predicted. In addition, alignment of NoRhp with known ammonia-transporting Rh proteins from vertebrates (33, 39, 43) and proposed ammonia-transporting Rh proteins of invertebrates (36, 68), demonstrated that the NoRhp contains the set of conserved amino acid residues crucial for conduction of ammonia transport (77), with the exception of amino acid 266 in NoRhp, where now a leucine sits instead of isoleucine. However, both amino acids bear nonpolar aliphatic residues. It was further predicted that the NH₂ terminus of NoRhp is localized extracellularly and that the first intracellular loop contains three N-glycosylation sites: N53, N59, and N67 (data not shown).

To further characterize NoRhp, a yeast functional complementation assay was conducted to determine the ammonia transport capability of NoRhp employing the Saccharomyces cerevisiae strain 31019b, which is incapable of growing in media with a sole nitrogen source of less than 5 mmol/l ammonia due to a deficiency of endogenous ammonia transporters (34). In contrast, the wild-type yeast strain 23344c is capable of uncompromised growth in media with a sole nitrogen source of less than 5 mmol/l ammonia (Fig. 5). Complementation of this growth deficiency in the S. cerevisiae strain 31019b by expression of NoRhp and, as a positive control, human RhCG (33) demonstrates the ammonia transport capability of NoRhp protein when expressed in yeast (Fig. 5). In the positive growth control, all strains exhibited growth with 0.1% glutamine as a nitrogen source (Fig. 5).

Phylogenetic analysis of Rhesus glycoproteins. Reconstruction of MUSCLE-aligned Rh proteins utilizing ML likelihood tree method separated the 49 Rhesus proteins used into five clear gene clusters, namely the vertebrate nonammonia-transporting Rh30, the “primitive” invertebrate Rhp, and the three vertebrate ammonia-transporting gene clusters Rhag, Rhbg, and Rhcg (Fig. 6). The first node of the tree divides the Rh30s from the remaining gene clusters. Following divergence of the Rh30 proteins, the next gene cluster to diverge is the primitive Rh proteins (Rhp). This divergence leaves the invertebrate Rhp proteins as a sister group to vertebrate ammonia-transporting Rh proteins (Rhag, Rhbg, and Rhcg).

The effects of feeding. Feeding caused a significant increase in ammonia excretion rates (Fig. 7) but did not significantly increase urea excretion rates (data not shown). Ammonia excretion rates increased by 1.9-fold 1 h after feeding and reached a peak excretion rate 5 h after feeding (~3-fold increase, Fig. 7). A parallel experiment showed that excretion rates went back to control rates 24 h after feeding (data not shown; n = 6). Interestingly, 6 h after feeding, when excretion rates were still elevated, relative mRNA expression levels of NoRhp, V-ATPase, and Na⁺/K⁺-ATPase in the skin remained unchanged, compared with controls (data not shown; n = 6).

The effect of high environmental ammonia exposure. Short-term (1 h) exposure of control leeches (DTW, pH 8.3) to HEA (1 mmol/l NH₄Cl) led immediately to elevated ammonia excretion rates (Fig. 8B). Upon returning control leeches to DTW, the rate of ammonia excretion decreased but remained elevated relative to the ammonia excretion rates in DTW prior
to the HEA exposure (Fig. 8A). Following a 1- and 7-day acclimation to HEA, *N. obscura* demonstrated an ammonia excretion rate in DTW enriched with 1 mmol/l NH₄Cl equal to that of control leeches in DTW (Fig. 8, C and D). In animals acclimated for 1 day to HEA, ammonia excretion rates increased when transferred from high ammonia into DTW and returned to normal control levels when reexposed to DTW enriched with ammonia (Fig. 8C). In contrast, 7-day acclimated leeches exhibited a significant increase in ammonia excretion rates when placed in DTW; however, reexposure to DTW enriched with high ammonia led to an ammonia influx (Fig. 8D). In addition to changes seen in ammonia excretion with HEA exposure, long-term HEA exposure (1 mmol/l NH₄Cl) led to a significant increase (unpaired *t*-test, *P* ≤ 0.05) in body ammonia levels from 0.88 ± 0.06 µmol/gFW (*n* = 12) in control leeches to 2.31 ± 0.16 µmol/gFW (*n* = 6) and 6.35 ± 0.24 µmol/gFW (*n* = 6) in 1- and 7-day HEA-acclimated leeches, respectively.

Moreover, a one-day exposure to HEA had no effect on the relative mRNA expression of NoRhp, V-ATPase B subunit, or Na⁺/K⁺-ATPase α-subunit in the skin (Fig. 9). However, following a 7-day exposure to HEA, NoRhp was significantly downregulated, and Na⁺/K⁺-ATPase (*P* = 0.07) was trending toward downregulation (Fig. 9). As seen in 1-day HEA-acclimated leeches, 7-day HEA leeches showed no change in mRNA expression of V-ATPase B subunit (Fig. 9).

**DISCUSSION**

**Basics.** On the basis of measured body content and the excretion rates for both ammonia and urea, the carnivorous ribbon leech *Nephelopsis obscura* is ammonotelic, excreting 92% of the measured nitrogen as ammonia, similar to the closely related blood-feeding leech *H. medicinalis* and other aquatic invertebrates (30, 57, 76). While ammonia was the most abundantly excreted nitrogenous waste product measured, it must be noted that other nitrogenous waste products such as uric acid, guanine, allantoin, allantoic acid, or trimethylamine oxide (10, 76) have not been tested. Theophylline-activated *N. obscura* skin mounted in an Ussing chamber generated a considerable amount of apically released metabolic ammonia (about 20 nmol·cm⁻²·h⁻¹), which is slightly higher but still comparable to that measured in frog skin, where ~14 nmol·cm⁻²·h⁻¹ of apical release was measured (14). It is important to note that in the frog skin study, the tissue was not activated by theophylline during the metabolic ammonia release experiment unlike in this study, which may account for the slight difference in apically directed metabolic ammonia release observed in the leech skin.

The observed ammonia transport capability of *N. obscura* skin (Fig. 3) and high mRNA expression levels of NoRhp and Na⁺/K⁺-ATPase (Fig. 1) in the leech skin relative to the rest of the body suggest that at least a portion of the animal’s metabolically produced ammonia is excreted by this tissue. It should be noted that in Ussing chamber experiments, the tissue was theophylline-activated, and concentrations of glucose and ammonia used in the study might not be at physiological levels, as these levels are currently unknown for this leech. However, the Ussing chamber experimental setup does demonstrate that the skin has a substantial capacity for ammonia transport and may, indeed, be the main site of ammonia excretion. Although more thoroughly investigated for its role in water and salt balance, it cannot be excluded that metanephridia (70) and the intestine, as shown for fish (49), may also play a role in ammonia transport in leeches.
Fig. 6. Maximum likelihood tree rooted employing the microbe Nitrosomonas europaea Rh protein as the outgroup. Numbers beside branches represent bootstrap values from 1,000 replicates. The tree branches are drawn to scale, with the scale bar representing the number of amino acid substitutions per site. *Significant sequences with a confirmed ammonia transport capability. $ denotes invertebrate sequences with suggested ammonia transport capability. The black box indicates the sequence functionally characterized in this study as an ammonia transporter. GenBank accession numbers are given in brackets following the species name and gene, Nitrosomonas europaea Rh (AAR24581.1), Nephelopsis obscura Rhp (KM923907), Geodia cydonium Rh (CAA73029.1), Caenorhabditis elegans Rhr-1 (AAF97864.1), Metacarcinus magister Rh (AAE41159.1), Carcinus maenas Rh (AAX50057.2), Manduca sexta Rh (AB120766.1), Aedes aegypti Rh50-1 (AAX19513.1), Aedes aegypti Rh50-2 (AY926464), Drosophila melanogaster Rh (AF193812), Stronglyocentrotus purpuratus Rh (XP_798738.3), Helobdella robusta Rh (XP_009016010.1), Takifugu rubripes Rh (AAV28817.1), Danio rerio Rhag (AAQ10011.1), Ambystoma tigrinum Rhag (AAV28818.1), Xenopus laevis Rhag (BAB13345.1), Mus musculus Rhag (AA25155.1), Bos taurus Rhag (AAG38955.1), Homo sapiens RhAG (AAC25155.1), Macaca mulatta Rhag (AAO3037.1), Oncorhynchus mykiss Rhag (ABV24962.1), Danio rerio Rhbg (AAQ09527.1), Takifugu rubripes Rhbg (AA48577.1), Tetraodon nigroviridis Rhbg (AAY41906.1), Xenopus laevis Rhbg (AAR08675.1), Mus musculus Rhbg (AF19371.1), Canis familiaris Rhbg (AAV40851.1), Homo sapiens RhBG (AAO1086.1), Oncorhynchus mykiss Rhbg-1 (AB016485.1), Sus scrofa Rhbg (AAK14651.1), Oncorhynchus mykiss Rhcg (AAU89494.1), Danio rerio Rhcg (AAM90586.1), Takifugu rubripes Rhcg2 (AAM48579.1), Oryctolagus cuniculus Rhcg (AAK14653.1), Xenopus laevis Rhcg (AAH84943.1), Bos taurus Rhcg (AAK14650.1), Homo sapiens RhCG (AAO19572.1), Takifugu rubripes Rhcg1 (AAM48578.1), Mus musculus Rhcg (AAF19373.1), Canis familiaris Rh30 (AAX39718.1), Bos taurus Rh30 (AAD11526.1), Homo sapiens Rh30 (CAA38401.1), Oncorhynchus mykiss Rh30 (AAM48567.1), Mus musculus Rh30 (AACC5123.1), Xenopus laevis Rh30 (AAR00222.1), Danio rerio Rh30 (AAU81655.1), Takifugu rubripes Rh30 (AAM48580.2), and Salmo salar Rh30 (AAR01778.1). The Rhp2 proteins are not included.
Mechanism of ammonia excretion. In terms of basolateral ammonia transport into the cytoplasm of the skin, the reduction of ammonia excretion in theophylline-activated leech skin in an Ussing chamber upon basolateral application of ouabain demonstrated that Na+/K+-ATPase in leech skin plays an important role in ammonia excretion. Furthermore, the fact that the Na+/K+-ATPase from leech skin does accept NH4+ as a substrate, as also observed for this enzyme in other ammonia-transporting epithelia (14, 41, 56) provides further indication that this pump mediates the entrance of ammonia from the body fluids into the excretory epithelial cells.

The lack of inhibitory effects in whole animal ouabain experiments could be explained by poor diffusion of the inhibitor across the tight apical membrane or by studies investigating the “insect ouabain paradox”. These studies have suggested that OATPs (organic anion-transporting polypeptides), colocalized with the Na+/K+-ATPase, actively excrete ouabain (55). With the ouabain binding site in the Na+/K+-ATPase being localized at the intracellular end of an ion permeation pathway (50), excretion of ouabain would prevent this pharmacological agent from reaching the basolateral-localized Na+/K+-ATPase at inhibitory concentrations, thus creating the appearance of an ouabain insensitivity.

Ammonia excretion in N. obscura was dependent on ambient pH (Fig. 4), suggesting an ammonia-trapping mechanism, by which NH3 is transported from the epithelial cytoplasm into the apical unstirred boundary layer of the skin, likely mediated by the identified and functional NoRhp protein. Demonstration that total ammonia (body ammonia + ammonia excreted) remains unchanged when exposed to pH 5 buffered media suggests that ammoniagenesis is not occurring in this scenario. This supports the notion that dependence of ammonia excretion on low pH is likely due to an ammonia-trapping mechanism. It should be noted that under normal environmental conditions, ammonia trapping is likely driven by an acid secretion, as supported by whole animal pharmacological evidence (Fig. 2) showing the involvement of the V-ATPase and carbonic anhydrase in the ammonia excretion process, where protons are likely secreted in parallel with NH3 to drive an ammonia excretion. Cutaneous ammonia excretion via ammonia-trapping (also referred as acid-trapping) is a common excretion strategy in freshwater animals and has been shown for the planarian S. mediterranea (63), zebrafish larvae D. rerio (51), rainbow trout gills (74), and the skin of the African clawed frog X. laevis (14). While pharmacological evidence supports the role of the V-ATPase and carbonic anhydrase, the data of the current study do not support a participation of a microtubule-dependent vesicular ammonia transport found in the crucianace gill (30) or Na+/H+ exchangers, as suggested for the skin of freshwater planarians (63). Although not directly shown in this study, it can be assumed that in N. obscura, the V-ATPase is localized in the apical membrane of the transport epithelium, as shown for the skin and gills of various freshwater organisms (27, 31, 73). Furthermore, while the carbonic anhydrase appears to play a role in ammonia excretion, likely by the generation of protons during CO2 hydration, it remains unclear as to whether this occurs intracellularly or at the surface of the tissue via a membrane-bound carbonic anhydrase.

Interestingly, unlike as shown for the planarian S. mediterranea (63), rainbow trout O. mykiss (47), and the African clawed frog X. laevis (14), buffering the environmental media to the pH of control nonbuffered water did not affect ammonia excretion. This suggests that the leech may not be manipulating the pH of its unstirred boundary layer at the epithelial cell surface to promote ammonia trapping. However, the poorly ventilated crypts of the mucus-secreting cells embedded in the skin of leeches (2) could provide a microenvironment for the acidification of an unstirred boundary layer. Therefore, similar to the V-ATPase-bearing rhabdites in the planarians (63), it is likely that the V-ATPase is also abundant in the mucus-secreting cells of N. obscura. Future studies investigating the cellular localization of the V-ATPase in the skin of N. obscura could identify the specific site of the apical acidification and acid trapping within the leech skin.

Characterization of N. obscura skin Rh protein, NoRhp. Members of the Rhesus protein family are found in various organisms ranging from the microbe N. europaea to humans. Proteins of this family have been grouped into six gene clusters: Rhag, Rhsb, Rhcg, Rhp1, Rhp2, and Rho30. Of these six gene clusters, only one cluster, the Rhp1, is found in invertebrates, whereas the other five are found in vertebrates, with the Rhp2 being found only in nonmammalian vertebrates (22). In general, 12 transmembrane domains have been predicted for Rh-glycoproteins (24). Similarly, an analysis of NoRhp (a primitive Rh protein) with Phyre 2.0 (26) also predicted 12 transmembrane domains for this functional transporter.

Phylogenetic analysis of the Rh protein family by reconstruction of ML tree (Fig. 6) groups the Rhag, Rhsb, and Rhcg clusters closely together, while the Rhp group remains further away, and the nonammonia-transporting Rho30 cluster is situated furthest away. The ML-tree positioning of the microbe N. europaea Rh protein, a member of the prokaryotic Rh proteins, which are suggested to be a common ancestor from which eukaryotic Rh proteins derive (23), implies that the primitive invertebrate Rhp proteins are a sister group to the possibly more derived vertebrate ammonia-transporting Rhag, Rhsb, and Rhcg. While the ammonia-transporting capabilities of the vertebrate Rh proteins have received considerable attention (69), the role of Rhp genes in ammonia transport has been poorly studied. However, Rhp mRNA expression studies in the planarian S. mediterranea (63), yellow fever mosquito Aedes aegypti (64), and Dungeness crab Metacarcinus magister (36)
have demonstrated that these primitive Rh proteins do respond to ammonia stress.

Moreover, an amino acid alignment of the NoRhp with vertebrate Rh proteins demonstrated that amino acid residues critical for conducting ammonia transport in the human RhCG (77), are also conserved in the N. obscura primitive Rh protein. Indeed, the current study demonstrated clearly that NoRhp, the primitive Rh protein expressed in the skin of N. obscura, is capable of ammonia transport when expressed in yeast (Fig. 5). Beside CeRhr-1, the ubiquitously expressed Rh protein in the soil nematode Caenorhabditis elegans (1), and the Rh50s of the mosquito Anopheles gambiae (46), N. obscura is now the third invertebrate known to have a protein in the “primitive Rh-protein” cluster that has verified ammonia transport capabilities (Fig. 5). This underlines the notion that ammonia transport capabilities of Rh proteins may be an ancestral trait in the invertebrate Rh-protein (Rhp1 gene cluster) and the vertebrate ammonia-transporting Rh-protein (Rhag, Rhbg, and Rhcg) sister groups.

High mRNA expression levels of NoRhp in the skin and characterization of this protein as an ammonia transporter implicate NoRhp as a potential key player in the mechanism of cutaneous ammonia excretion in the leech. However, the cellular localization of NoRhp is not known to date. The obtained full open reading frame of NoRhp will help to develop antibodies for future localization studies necessary...
to gain clarity of the role of this transporter in the ammonia excretion mechanism in the skin and possibly other excretory sites in the leech.

Effects of feeding. Feeding resulted in a two- to three-fold increase in ammonia excretion rates over 6 h postfeeding (Fig. 7), likely due to an internal ammonia load caused by an increase in protein catabolism. This has been observed in the rainbow trout O. mykiss, where blood ammonia levels increased 2–4 h after feeding (9). However, in the current study, blood samples could not be taken to determine whether blood ammonia levels were elevated after feeding. Unlike trout and planarians, where elevated ammonia excretion rates in response to feeding were paralleled with increased transcript levels of V-ATPase and Rh-protein (63, 78), in N. obscura, transcript levels of the Na⁺/K⁺-ATPase α-subunit, V-ATPase B-subunit, and NoRhp remained unchanged. It is assumed that the ammonia excretion mechanism in the skin of the leech has the capacity to deal with moderately elevated ammonia loads. However, it cannot be excluded, that the metanephridial system and intestine, which have not been evaluated for differential mRNA expression levels or ammonia influx, as the animals are incapable of sufficiently excreting against the inwardly directed ammonia gradient (41, 78). Conversely, a short-term HEA (1 h) exposure in N. obscura caused a large increase in ammonia excretion rates (Fig. 8B). This enhanced excretion in response to HEA could potentially be a result of a stress response, elevating metabolic rates. In a comparable study, marine Dungeness crabs Metacarcinus magister were able to maintain an unaltered ammonia excretion across both anterior and posterior gills in the presence of HEA (36). It should be noted that these experiments on crabs were done on perfused gills; therefore, a possible ammonia excretion enhancement due to whole animal elevated metabolic rates cannot be excluded. Following HEA stress, when animals were placed back in ammonia-free DTW, ammonia excretion began to decrease perhaps in an attempt to establish control excretion rates (Fig. 8B), as also seen in the pufferfish T. rubripes (41).

One- and seven-day HEA-acclimated leeches, exhibited excretion rates similar to values observed in control animals when exposed to DTW enriched with 1 mmol/l ammonia (Fig. 8, C and D). This is likely due, in part, to an elevation of blood and tissue ammonia, reducing the strength of the inwardly directed ammonia gradient. This proposed elevation of blood ammonia is further supported by the observed elevation in body ammonia levels in leeches acclimated to HEA, as well as the observed excretion rates when the animals were placed from ammonia-enriched DTW to ammonia-free DTW. Similar results have been observed in fish (41, 42), in which recovery of control excretion rates coincided with elevations in blood ammonia.

Moreover, 1-day HEA-acclimated leeches may have maintained an uncompromised active ammonia excretion mechanism as mRNA expression of Na⁺/K⁺-ATPase α-subunit, V-ATPase B subunit, and NoRhp remained unchanged. It should be noted that the lack of a response at the mRNA level does not necessarily mean that there is no response on the protein level. While elevated blood ammonia may play a role in recovery of control excretion rates, 1-day HEA-acclimated leeches appear to rely more heavily on the maintenance of their active ammonia excretion mechanism. This is evident as reexposure to HEA resulted in the rapid reestablishment of control
excretion rates, even though blood ammonia was likely lost during the ammonia-free water exposure.

In contrast, 7-day HEA-acclimated leeches appeared to be more dependent on maintaining elevated blood ammonia levels for ammonia excretion, as reexposure to HEA following a short exposure to ammonia-free DTW, resulted in an ammonia influx. It is likely that the active mechanism of ammonia excretion has been depressed, as indicated by the reduced mRNA expression of NoRhp and trendin decreasing Na+/K+-ATPase ($P = 0.07$). Therefore, to retain an ammonia efflux, leeches acclimated for 7 days to HEA likely have elevated blood ammonia levels near or above environmental concentrations to promote a somewhat compromised active ammonia excretion. This notion is further supported by the observed seven-fold increase in body ammonia levels of long-term exposed leeches. Similar elevations of blood ammonia levels to near environmental levels have also previously been observed in decapod crabs and fish (36, 41, 42).

Although this study used the skin of the leech to determine how the leech handles an environmental ammonia challenge, it is conceivable that alternative routes and/or mechanisms may be employed, which include possibly the metanephridia and the intestine. Both of these tissues would not directly interact with the environmental media; therefore, inwardly directed ammonia gradients would not hamper the ability of these tissues to excrete ammonia as expected for the skin. Therefore, in 7-day HEA-acclimated leeches, the observed downregulation of NoRhp and decreasing trend in Na+/K+-ATPase ($P = 0.07$) may be a result of active ammonia excretion in the skin being decreased and with a concomitant compensatory increase in ammonia clearance by the intestine or metanephridia that could be accounting for a portion of the observed ammonia excretion. In addition to alternative routes for ammonia excretion, it is plausible that other mechanisms are in play to reduce internal ammonia loads, such as elevation of glutamine synthetase activity to drive the conversion of ammonia into glutamine as seen in fish exposed to elevated environmental ammonia (4). Further, similar to the sulfatides in mammalian kidneys (52), it is possible that a similar compound may be binding ammonia in the skin or other tissue allowing the leech to cope with the high ammonia levels present. It is evident that further investigation into the role of the skin, intestine, and metanephridia under HEA conditions are required to clarify how the leech compensates for HEA. Future avenues of research on this topic include the use of isolated skin mounted in Ussing chambers to determine the capacity of active ammonia transport by this tissue following HEA acclimation. Further, evaluation of differential mRNA expression of the intestine and metanephridia following HEA acclimation could provide some indication as to whether either organ could be compensating for deficiencies in skin-mediated ammonia transport.

Perspectives and Significance

In summary, this study provides a comprehensive investigation of the mechanism of ammonia excretion in a single member of the large and highly diverse group of freshwater invertebrates, where there is a paucity of mechanistic data. Utilizing the open reading frame of the identified Rh-protein from *N. obscura* (NoRhp), we provided data that further clarifies the ammonia transport capability of primitive Rh proteins of invertebrates. The evidence provided for the ammonia transport capability of NoRhp, its higher expression in the skin of the leech, and ammonia transport capability of the skin mounted in an Ussing chamber, strongly suggests the skin as a likely site for ammonia excretion. The obtained data suggest a role of the Na+/K+-ATPase in transporting ammonia from the blood into the cytoplasm of the epithelial cells of the skin. In addition, the V-ATPase and, indirectly, the carbonic anhydrase are potentially involved in acidifying the unstirred boundary layer of the skin/crypts of skin (mucus). This, in turn, could generate a P$\text{H}_3\text{O}^+$ over the apical membrane of the epithelial cells, driving NH$_3$ out of the cytoplasm through Rh proteins, possibly via NoRhp. While a substantial amount of data supports that the skin has a capacity for ammonia excretion, further investigations are required to determine whether this is the primary organ involved in ammonia excretion and whether acid trapping is truly the only mechanism at work.

The ammonia excretion mechanism predicted for *N. obscura* shows many similarities to the mechanisms proposed for the gills of freshwater fish (66, 75), suggesting thereby an early evolution of the general freshwater ammonia excretion mechanisms.

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS


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29. R704 AMMONIA EXCRETION IN FRESHWATER LEECHES


