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Ammonia excretion in rainbow trout (*Oncorhynchus mykiss*): evidence for Rh glycoprotein and H^+-ATPase involvement

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Nawata CM, Hung CC, Tsui TK, Wilson JM, Wright PA, Wood CM. Ammonia excretion in rainbow trout (*Oncorhynchus mykiss*): evidence for Rh glycoprotein and H^+-ATPase involvement. *Physiol Genomics* 31: 463–474, 2007. First published August 21, 2007; doi:10.1152/physiolgenomics.00061.2007.—Branchial ammonia transport in freshwater teleosts is not well understood. Most studies conclude that NH_3 diffuses out of the gill and becomes protonated to $NH_4{}^+$ in an acidified gill boundary layer. Rhesus (Rh) proteins are new members of the ammonia transporter superfamily and rainbow trout possess genes encoding for Rh30-like1 and Rhcg2. We identified seven additional full-length trout Rh cDNA sequences: one Rhag and two each of Rhbg, Rhcg1, and Rh30-like. The mRNA expression of Rhbg, Rhcg1, and Rhcg2 was examined in trout tissues (blood, brain, eye, gill, heart, intestine, kidney, liver, muscle, skin, spleen) exposed to high external ammonia (HEA; 1.5 mmol/l NH_4HCO_3, pH 7.95, 15°C). Rhbg was expressed in all tissues, Rhcg1 was expressed in brain, gill, liver, and skin, and Rhcg2 was expressed in gill and skin. Brain Rhbg and Rhcg1 were downregulated, blood Rh30-like and Rhag were downregulated, and skin Rhbg and Rhcg2 were upregulated with HEA. After an initial uptake of ammonia into the fish during HEA, excretion was reestablished, coinciding with upregulations of gill Rh mRNA in the pavement cell fraction: Rhcg2 at 12 and 48 h, and Rhbg at 48 h. NHE2 expression remained unchanged, but upregulated H^+-ATPase (V-type, B-subunit) and downregulated carbonic anhydrase (CA2) expression and activity were noted in the gill and again expression changes occurred in pavement cells, and not in mitochondria-rich cells. Together, these results indicate Rh glycoprotein involvement in ammonia transport and excretion in the rainbow trout while underscoring the significance of gill boundary layer acidification by H^+-ATPase.

ammonia transport; gene expression; gill; Rhesus proteins

THE CURRENT MODEL OF AMMONIA excretion from the freshwater teleost gill links NH_3 diffusion to CO_2 hydration (62). Expired CO_2 is converted to HCO_3^- and H^+ by carbonic anhydrase (CA) in the gill mucus, and the resulting acidified gill boundary layer then traps NH_3 to NH_4^+ (68, 69). In addition, this layer could also be acidified by H^+ extruded from an apical vacuolar H^+-ATPase (29, 30). However, as it is acidified, this boundary layer creates a microenvironment for a favorable transbranchial (blood-to-water) P_{NH3} gradient that facilitates ammonia excretion. This is especially advantageous during exposure to high external ammonia (HEA), which reverses the normally positive ammonia gradient. Indeed, abolishing this layer with HEPES buffer during HEA reduces J_{Amm} (64).

Despite the dominance of this NH_3 diffusion-trapping theory, many past studies have supported the possibility of apical Na^+/NH_4^+ exchange (e.g., Refs. 26, 70). It has been argued, however, that freshwater cannot provide the Na^+ gradients necessary to drive this exchange (62). Likewise, basolateral entry of NH_4^+ into the gill via an Na^+/NH_4^+ exchanger (NHE) is unlikely since the electromotive force for Na^+ is directed into the gill cytosol and NH_4^+ cannot out-compete the much more abundant Na^+ in the plasma for access to the NHEs (62). Also, no evidence exists to support basolateral ammonia transport via NH_4^+ substitution for K^+ on the Na^+/K^+-ATPase in the freshwater gill, although there are reports of this occurring in seawater fish (37, 50).

Ammonia transport has been studied for decades in microorganisms, but the molecular identity and function of potential transporters involved were not elucidated until recently. The genes and corresponding proteins were first identified as methylammonium permease (Mep) in yeast (41) and the ammonium transporter (Amt) in plants (48). Based on sequence similarity, mammalian homologs to the Mep/Amt proteins were identified as the Rh blood group-related proteins: Rh30 and the glycoproteins RhAG/Rhag, RhBG/Rhbg, and RhCG/Rhcg (19). The terminologies RhAG, RhBG and RhCG refer to human tissue, while Rhag, Rhbg, and Rhcg indicate nonhuman tissue (59). Rh30 and RhAG/Rhag are restricted to erythrocytes (RBCs), whereas RhBG/Rhbg and RhCG/Rhcg are expressed in various other tissues (19, 34, 35). The three glycoproteins conduct ammonia when expressed in heterologous systems, although disagreement exists as to whether NH_3 or NH_4^+ is transported (22). The nonglycosylated Rh30 proteins form part of the Rh complex with RhAG on the RBC membrane, but their function is unknown (60, 61).

Rh proteins have been identified in different fish species (20, 21, 47), and functionally, it has been shown that pufferfish *Takifugu rubripes* Rh glycoproteins, like their mammalian counterparts, mediate the transport of the ammonium analog, methylammonium, when expressed in *Xenopus* oocytes (47). Recently we identified three Rh cDNA sequences in the unexposed and HEA-exposed mangrove killifish *Kryptolebias marmoratus* (21). HEA exposure resulted in upregulated Rh mRNA expression in the gill, liver, muscle, and skin. Two rainbow trout Rh genes (Rh30-like1 and Rhcg2) were identified in a previous study (20), but their tissue expression and potential involvement in ammonia transport have not been investigated.

With this background in mind, the objectives of this study were to identify and sequence Rh orthologs in the rainbow trout, to examine their expression pattern in different trout tissues under control conditions and when exposed to HEA,

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and to quantify the HEA-exposed gill expression of Rh orthologs as well as other proteins thought to be involved in the ammonia transport process.

MATERIALS AND METHODS

Animals

Rainbow trout [*Oncorhynchus mykiss*, Walbaum; 178–260 g], obtained from Humber Springs Trout Hatchery, Ontario, Canada were used for all experiments. The fish were held in dechlorinated Hamilton tap water (moderately hard: $[Na^+] = 0.6$ mequiv/l, $[Cl^-]=0.8$ mequiv/l, $[Ca^{2+}] = 0.8$ mequiv/l, $[Mg^{2+}] = 0.3$ mequiv/l, $[K^+] = 0.05$ mequiv/l; titration alkalinity 2.1 mequiv/l; pH ~8.0; hardness ~140 mg/l as $CaCO_3$ equivalents; temperature 12–16°C), and food was withheld for at least 1 wk prior to experimentation to stabilize the endogenous fraction of nitrogenous waste excretion (15). All fish were transferred to individual, darkened acrylic flux boxes supplied with aerated, continuously flowing dechlorinated tap water at 15 ± 0.5°C and allowed to recover overnight prior to experimentation. All procedures used were approved by the McMaster University Animal Research Ethics Board and are in accordance with the Guidelines of the Canadian Council on Animal Care.

HEA Experiment

Six fish were exposed to a nominal total ammonia (T_{Amm}) concentration of 1.5 mmol/l NH_4HCO_3 (pH 7.95 ± 0.05, 15.0 ± 0.5°C) for 12 or 48 h. Fluxes were conducted during which time the water flow was stopped and the box volume was set to 4 l. For the 48-h flux, a period of flow-through flushing was performed every 12 h to prevent toxic build-up of ammonia. Control fish (six each) were treated identically for 12 and 48 h without the addition of ammonia.

Water samples (10 ml) were taken at 12-h intervals, frozen at −20°C and later assayed in triplicate for T_{Amm} using a modified salicylate-hypochlorite method (58). After the flux periods, the fish were anesthetized with MS222 (~0.1 g/l; Sigma, St. Louis, MO), and caudal blood was drawn into a heparinized syringe. Samples were centrifuged to separate plasma from RBCs (2 min, 20,000 *g*), immediately frozen in liquid nitrogen, stored at −70°C, and later analyzed enzymatically for plasma T_{Amm} (Raichem; Hemagen Diagnostics, San Diego, CA). Net flux rates of total ammonia (J_{Amm}, $\mu mol \cdot kg^{-1} \cdot h^{-1}$) were calculated as: $J_{Amm} = (T_{Ammi} - T_{Ammf}) \times V/(t \times M)$, where *i* and *f* refer to initial and final concentration (μmol/l), *V* is water volume (L) in the box, *t* is the time elapsed (h), and *M* is the fish mass (kg). A negative J_{Amm} indicates a net excretion of ammonia to the water. A positive J_{Amm} indicates a net uptake of ammonia into the fish.

Sequencing and Characterization of Rainbow Trout Rh cDNAs

Tissue sampling. Gill and blood samples for cloning purposes were removed from fish that were exposed to 1.5 mmol/l NH_4HCO_3 for 24 h. Control samples for all subsequent analyses were collected from fish that were held in individual flux boxes with flowing dechlorinated tap water for 24 h. Blood samples were collected (as described above) immediately after anesthetization and prior to tissue collection.

Preliminary studies indicated Rhbg expression on RBCs, so all analyses subsequent to cloning were performed on saline-perfused tissues to minimize the contribution of RBC mRNA to tissue levels. Samples of brain, eye, gill, heart, intestine, kidney, liver, muscle, skin, and spleen were removed after the fish were anaesthetized, placed on ice, and perfused free of blood with chilled, heparinized (50 IU/ml lithium heparin, Sigma) Cortland saline (66). Perfusions were performed by cannulation of the bulbus arteriosus with a 23-gauge butterfly needle attached to a peristaltic pump, and the ventricle was severed immediately after initiation of perfusion to allow for drainage. Tissue samples were immediately frozen in liquid nitrogen and stored at −70°C until analysis.

RNA extraction and reverse-transcriptase PCR amplification. Total RNA was extracted from blood and tissues using TRIzol (Invitrogen, Burlington, ON, Canada), quantified spectrophotometrically, and electrophoresed on 1% agarose gels stained with ethidium bromide to verify integrity. First strand cDNA was synthesized from 1 μg total RNA using an oligo(dT_{17}) primer and Superscript II reverse transcriptase (Invitrogen). Samples were stored at −70°C.

Identification of Rh orthologs. Partial Rh30-like, Rhag, Rhbg, and Rhcg1 sequences were obtained from cDNA of HEA-exposed blood or gill samples using primers (Table 1) designed from the trout Rh30-like1 sequence and pufferfish Rhag, Rhbg, and Rhcg2 sequences (GenBank: AY207445, AY618933, AY116074, AY116076, respectively). PCR were carried out in a PTC-200 MJ Research thermocycler with Taq DNA polymerase (Invitrogen) at 94°C (2 min) followed by 45 cycles of 94°C (30 s), 52°C (30 s), and 72°C (1 min). Products were electrophoresed on ethidium bromide-stained 1% agarose gels, and bands of appropriate size were excised and extracted using the Qiaquick gel extraction kit (Qiagen, Mississauga, ON, Canada). Purified gel products were ligated to a pGEM-T easy vector (Promega; Fisher Scientific, Nepean, ON, Canada), transformed into heat-shock competent *Escherichia coli* (XL-Blue; Stratagene, Mississauga, ON, Canada), and then grown on ampicillin LB agar plates. Colonies containing the ligated product were inoculated into liquid media and grown overnight. Plasmids were harvested using GeneJet Plasmid Miniprep Kit (Fermentas Canada, Burlington, ON, Canada), and sequencing was performed on an ABI 3100 Gene Analyzer at the MOBIX Lab, McMaster University (Hamilton, ON, Canada). Primers designed specific to the Rh orthologs (Table 1) were used to obtain full-length cDNA sequences by 5′- and 3′-5-rapid amplification of cDNA ends (Smart RACE cDNA amplification kit; BD Bioscience Clontech, Mississauga, ON, Canada). Several clones of the 3′- and 5′-ends were sequenced in both directions, and full-length transcripts were determined by majority-rule consensus with BioEdit (17). Sequences have been deposited into GenBank (Table 2).

Phylogenetic, amino acid sequence identity, hydropathy, and glycosylation analysis. Phylogenetic analyses were conducted using ClustalW (57) and MEGA 3.1 software (27) by the neighbor-joining method with support for each node using 500 bootstrap replicates. Amino acid sequence analyses were performed using BioEdit (17), hydropathy profiles were determined using EMBOSS (52) and SPLIT 4.0 (23), and N-glycosylation sites were predicted using NetNGlyc 1.0 Server (http://www.cbs.dtu.dk/services/NetNGlyc/).

Tissue distribution (PCR). Following the methods described above, total RNA and cDNA were prepared from the RBCs and saline-perfused tissues collected from the initial HEA experiment. Distribution of Rh orthologs in control and HEA-exposed (48 h) tissues was determined using ortholog-specific primers (Table 1) with the PCR protocol described earlier. Products were electrophoresed on ethidium bromide-stained 1.5% agarose gels.

Tissue expression (quantitative real-time PCR). Rh expression in tissues exposed to HEA for 12 and 48 h was compared with control tissues by quantitative real-time PCR (qPCR) using the cDNA prepared above and ortholog-specific primers (Table 1). Primer specificity was confirmed in preliminary experiments, and qPCR analyses were performed on an Mx3000P QPCR System (Stratagene, Cedar Creek, TX). Reactions (20 μl) containing 4 μl of DNaseI-treated (Invitrogen) cDNA, 4 pmol of each primer, 10 μl of Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen), and 0.8 μl of ROX (1:10 dilution) were performed at 50°C (2 min), 95°C (2 min), followed by 40 cycles of 95°C (15 s) and 60°C (30 s). Melt-curve analysis confirmed production of a unique product, and gel electrophoresis verified presence of a single band. A nonreversed-transcribed sample controlled for possible genomic DNA contamination. Elongation factor-1α (EF-1α, GenBank AF498320) for gill and β-actin (GenBank AF157514) for other tissues had the most stable expression

Table 1. *Primers used for cloning, PCR, and qPCR analysis*

Name	Application	Sequence (5′-3′)	Name	Application	Sequence (5′-3′)
Rh30-like	cloning-forward	ccagacattcgtaaaggtgcag	Rhag	qPCR-forward	ctggcggccaatgatgttg
Rh30-like	cloning-reverse	catagctacccacggcaaatg	Rhag	qPCR-reverse	atggcgaagaggtcagagtg
Rhag	cloning-forward	caacgcagacttcagcac	Rhbg	qPCR-forward	cgacaacgacttttactaccgc
Rhag	cloning-reverse	ccacaggtgtcctggatg	Rhbg	qPCR-reverse	gacgaagccctgcatgagag
Rhbg	cloning-forward	catcctcatcatcctctttggc	Rhcg1	qPCR-forward	catcctcagcctcatacatgc
Rhbg	cloning-reverse	cagaacatccacaggtagacg	Rhcg1	qPCR-reverse	tgaatgacagacggagccaatc
Rhcg1	cloning-forward	gggattcaacttcctgatcg	Rhcg2	qPCR-forward	cctcttcggagtcttcatc
Rhcg1	cloning-reverse	accaaaggtggagatgatgc	Rhcg2	qPCR-reverse	ctatgtcgctggtgatgttg
Rh30-like	3′ RACE	gggacattccggtttcgcgtagaagg	CA	qPCR-forward	gccagtctcccattgacatc
Rh30-like	5′ RACE	gcagctctcacaggcagtttctctcttc	CA	qPCR-reverse	cctgtacgtccctgaaatgg
Rhag	3′ RACE	ccgatcatggcgaagaggtcagagtgg	HATP	qPCR-forward	tcagccttggttgtgagatg
Rhag	5′ RACE	gacaatttcacagcactggccatcctgg	HATP	qPCR-reverse	caacattggtgggaaacagg
Rhbg	3′ RACE	ggtccagtacgacgatgagacagacgccaag	NHE	qPCR-forward	tatggccattgtgacctgtg
Rhbg	5′ RACE	ctctattccgacatggatcttgcccccgtgc	NHE	qPCR-reverse	caggcctctccacactaagg
Rhcg1	3′ RACE	cggtgctaggcaaggtgagcccagtc	NKA1a	qPCR-forward	ttgacctggatgaccacaag
Rhcg1	5′ RACE	gactgggctcaccttgcctagcaccg	NKA1a	qPCR-reverse	ggatctccttagcccgaac
Rhbg	PCR-forward	cgacaacgacttttactaccgc	NKA1b	qPCR-forward	tataagctggtggcgacctc
Rhbg	PCR-reverse	agaacacgagccaccatcag	NKA1b	qPCR-reverse	ggtcatccaggtcaacttcc
Rhcg1	PCR-forward	catcctcagcctcatacatgc	NKA1c	qPCR-forward	aggccatgaagaaggagagg
Rhcg1	PCR-reverse	gtttctgtccagcaggcgtc	NKA1c	qPCR-reverse	taaggtcggtgccgtatttg
Rhcg2	PCR-forward	gcacactgttcctgtggatg	NKA3	qPCR-forward	gcaaatcttctcccagcaag
Rhcg2	PCR-reverse	cagcaggatctccccaga	NKA3	qPCR-reverse	tcctgcaacactcctctatgg
Rh30-like2	qPCR-forward	gctgctgcactgatatcaac	b-actin	qPCR-forward	actgggacgacatggagaag
Rh30-like3	qPCR-forward	attgcccatgggctttcac	b-actin	qPCR-reverse	aggcgtatagggacaacacg
Rh30-like2,3	qPCR-reverse	cgcccgttgtgagatcatc	EF-1a	qPCR-forward	ggaaagtcaaccaccacag
			EF-1a	qPCR-reverse	gataccacgctccctctcag

qPCR, quantitative real-time PCR.

across samples and were used as endogenous standards to calculate relative mRNA expression by the standard curve method. Standard curves were generated by serial dilution of a random mixture of control samples. To discriminate between erythroid and nonerythroid Rhbg mRNA expression, unperfused samples of brain, kidney, and liver were analyzed and compared with samples that had been perfused free of blood, by the same methods as those described earlier.

Pavement cell and mitochondria-rich cell expression. Pavement and mitochondria-rich (MR) cells were isolated from gill epithelial cells using a technique modified from Galvez et al. (16). Whole branchial baskets were removed from control and HEA-exposed fish, and gill filaments were trypsin-digested, passed through 96-μm filters, treated with RBC lysis buffer and layered onto a discontinuous Percoll density gradient. Pavement cells were harvested from the 1.03–1.05 g/ml interface, and MR cells were harvested from the 1.05–1.09 g/ml interface. Total RNA and cDNA were processed as described above from both cell fractions, and the mRNA expression of Rh was measured relative to β-actin.

Enzyme/Transporter Expression in the Gill

Following the qPCR protocol outlined earlier, expression of H^+-ATPase (V-type, B subunit; GenBank AF14002), carbonic anhydrase (cytoplasmic, CA2; GenBank AY514870), four isoforms of Na^+/K^+-ATPase (α1a, α1b, α1c, α3; GenBank: AY319391, AY319390, AY319387, AY319388), and NHE2 (GenBank EF446605) was quantified in control and HEA-exposed whole gill samples relative to EF-1α, and in control and HEA-exposed pavement and MR cells relative to β-actin. Expression of Na^+/K^+-ATPase isoforms α1a and α1b were quantified in the gill cell fractions, but as the results were similar, only the results of α1a are reported here.

Table 2. *Accession number and size of each Rh cDNA identified*

Ortholog	GenBank Accession Number	5′-UTR	Coding Region	3′-UTR
Rh30-like2	EF062577	83	1263	265
Rh30-like3	EF062578	83	1050	551
Rhag	EF667352	140	1305	358
Rhbg-a	EF051113	188	1386	1080
Rhbg-b	EF051114	188	1386	607
Rhcg1-a	DQ431244	73	1463	562
Rhcg1-b	EF051115	73	1463	215

UTR, untranslated region.

Enzyme Activity in the Gill

Enzyme assays were performed in triplicate on homogenates of gill tissues that were perfused free of blood, extracted, and frozen from the previous HEA experiment. Protein assays were performed using Bradford Reagent (Sigma) and BSA standards.

ATPase activity. Protocols for Na^+/K^+-ATPase (44) and H^+-ATPase (32) assays were modified and adapted to measure activity levels in control and HEA-exposed fish gills. Activities are reported as control activity (no inhibitor) minus inhibitor-treated activity [ouabain for Na^+/K^+-ATPase and sodium azide with N-ethylmaleimide (NEM) for V-type H^+-ATPase], measured at 340 nm in a kinetic microplate reader (SpectraMAX Plus; Molecular Devices, Menlo Park, CA) at 15-s intervals for 30 min. We obtained the same results for H^+-ATPase activity using 10 μM bafilomycin as an inhibitor but are only reporting results obtained using NEM.

CA. CA activity was determined using the electrometric ΔpH method (18). Briefly, samples were assayed in a pH 7.4 buffer (225 mmol mannitol, 75 mmol sucrose, 10 mmol Tris) held at 4°C, and the reaction was initiated with 200 μl of CO_2-saturated water. The reaction velocity was measured, using hydrogen and reference electrodes (Kwik-Tip; World Precision Instruments, Sarasota, FL) attached to an Accumet XL15 pH meter (Fisher Scientific), over a 0.15 pH change, and the activity is reported as the observed rate minus the uncatalyzed rate.

Statistical Analysis

All data are presented as means ± SE (*n*, number of fish). Values at each time point were compared with control values using one-way analysis of variance with Fisher's least significant difference post hoc test. Student's *t*-test was used for comparisons of T_{Amm} and J_{Amm} values and for comparisons between unperfused and perfused tissues. Significance was set at $\alpha = 0.05$.

RESULTS

HEA Experiment

Exposure to HEA initially resulted in net uptake of ammonia from the water into the fish during the first 12 h. Thereafter, net excretion of ammonia occurred and by 48 h, J_{Amm} exceeded the 12- and 48-h control rates, which remained unchanged (Fig. 1). Plasma T_{Amm} levels in HEA-exposed fish were significantly higher than the control value (Fig. 2), but they remained lower than water levels throughout the time course. There is however, a noticeable upward trend toward 48 h approaching a toxic level (36), but no signs of stress were visible in these fish.

Identification of Rh Orthologs in Trout

Seven Rh ortholog cDNA sequences were identified in this study. Phylogenetic analysis (Fig. 3) showed that two sequences with different coding regions (CDS), isolated from HEA-exposed RBCs, fell into a cluster of fish sequences described as Rh30-like (designated Rh30-like2 and Rh30-like3). Another sequence, also isolated from RBCs, fell into the Rhag cluster (designated Rhag). Two sequences with identical CDS but different 3′-untranslated regions (3′-UTR) were cloned from HEA-exposed gill, and these fell into the Rhbg cluster (designated Rhbg-a and Rhbg-b). A further two sequences that differ only in the 3′-UTRs were identified in the HEA-exposed gill, and these fell into *subgroup 1* in the Rhcg cluster (designated Rhcg1-a and Rhcg1-b). Amino acid sequence analysis revealed that the Rh30-like2 sequence is 98.8% identical to the previously identified Rh30-like1 sequence, and all Rh30-like sequences are 44.5–96.4%, similar to their counterparts in other fish. Rhag is 78.5–83.7%, Rhbg is 76.1–80.6%, and Rhcg1 is 75.8–82.7% identical to their respective homologs in other fish (Fig. 4). The CDS of the identified sequences vary in length from 1050 to 1463 nucleotides (Table 2), and hydrophobicity analyses of all sequences

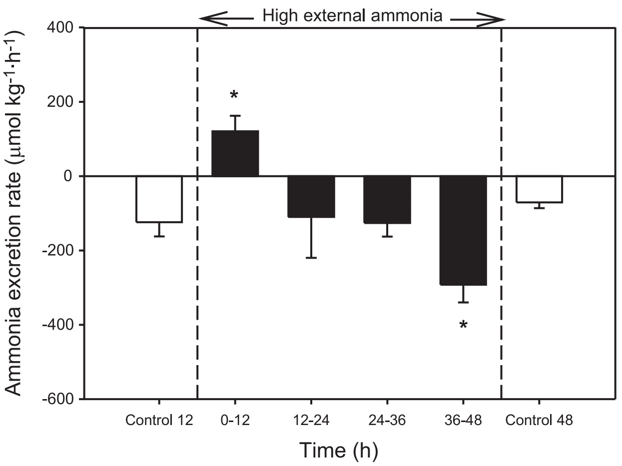

Fig. 1. The effect of 48 h of exposure to 1.5 mmol/l NH_4HCO_3 on net ammonia flux (J_{Amm}). Negative values indicate excretion; positive values represent uptake. *Significantly different from the 12- and 48-h controls (0 mmol/l NH_4HCO_3). Control values are not significantly different from each other ($P < 0.05$). Data are means ± SE (n = 6–12).

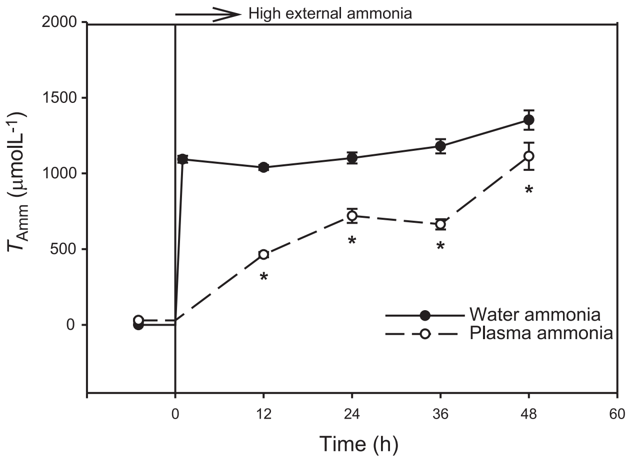

Fig. 2. The effect of 48 h of exposure to 1.5 mmol/l NH_4HCO_3 on water and plasma total ammonia (T_{Amm}). *Plasma values significantly different from water values ($P < 0.05$). Data are means ± SE (n = 6–12).

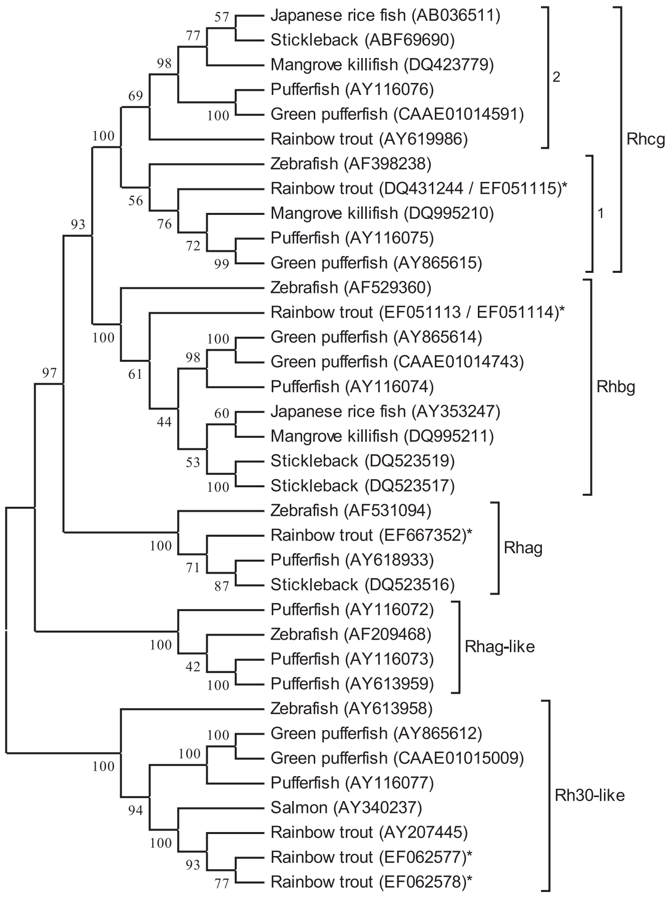

Fig. 3. An unrooted phylogenetic tree of Rh homolog sequences in fish. Numbers represent bootstrap values from 500 replicates. Accession numbers appear in parentheses. *Sequences identified in this study.

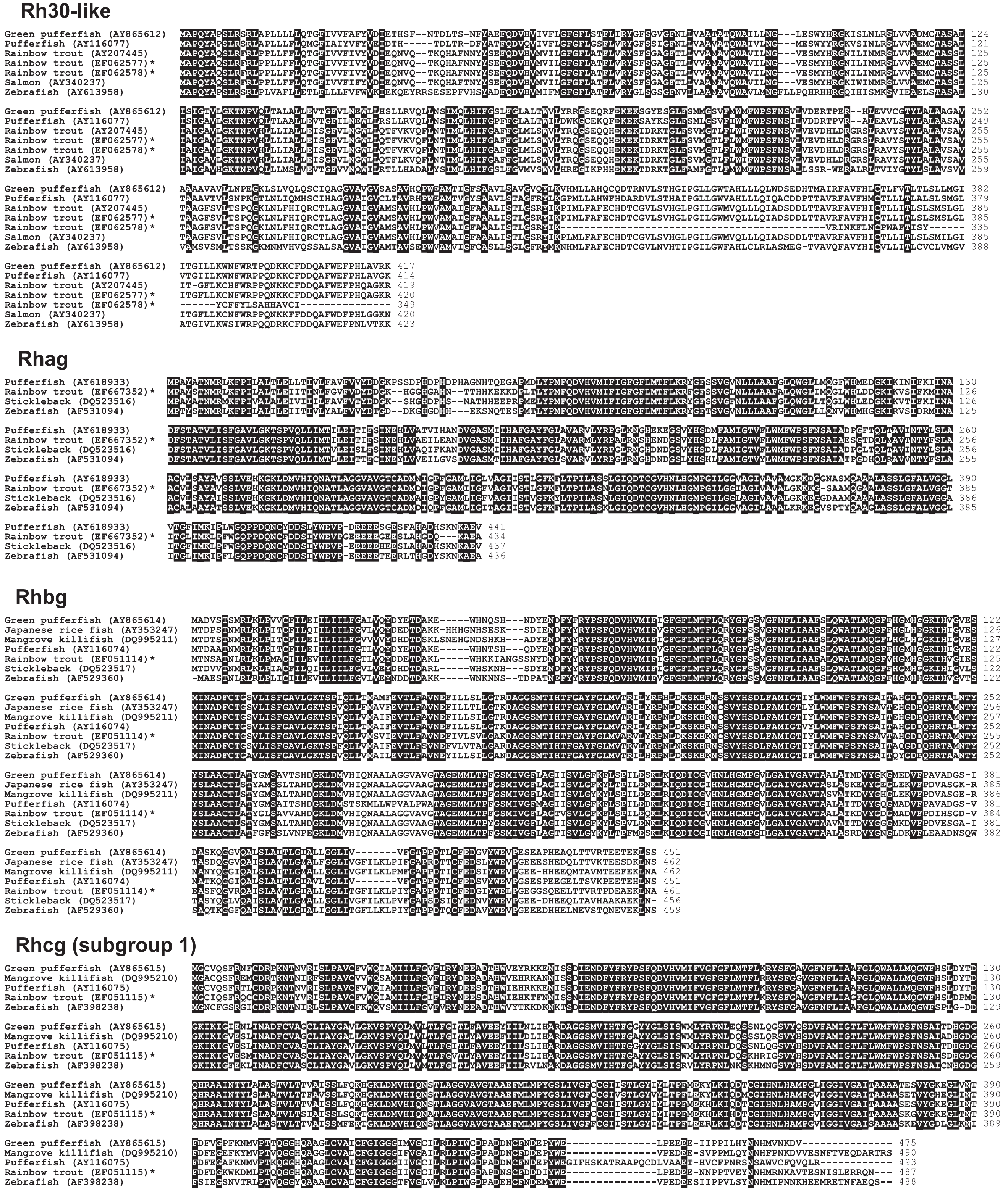

Fig. 4. Amino acid sequence alignments of fish Rh homologs. Accession numbers appear in parentheses. *Sequences identified in this study.

revealed 12 predicted transmembrane domains (Fig. 5). Rhag, Rhbg, and Rhcg1 have predicted N-glycosylation sites in the extracellular domain at Asp45, Asp48, and Asp60, respectively. No potential glycosylation sites were identified in the Rh30-like sequences.

Tissue Distribution and Expression of Rh

PCR analysis of the various trout tissues revealed the presence of Rhbg in all control and HEA-exposed tissues examined, including the blood (Fig. 6). Since Rhbg was expressed in

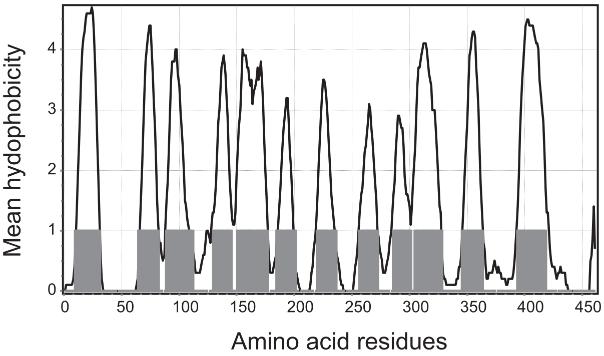

Fig. 5. Hydropathy profile of rainbow trout Rhbg. Gray boxes indicate predicted transmembrane regions.

RBCs, it was important to ensure that expression in tissues nominally cleared of blood was not due to residual RBCs. When mRNA copy number is taken into consideration, Rhbg was always higher in the unperfused tissues due to contribution from the blood. The contribution of Rhbg mRNA from the blood, however, was small compared with that of β-actin, and therefore the removal of blood in perfused tissues produced a more profound reduction in the relative amount of β-actin. On average, perfusion of brain, kidney, and liver reduced the Rhbg mRNA copy number by 1.7-fold, but β-actin copy number was reduced by sixfold. While this large reduction in β-actin expression represents a substantial elimination of contaminating blood, the smaller reduction in Rhbg indicates that Rhbg expression was originating from the tissues. As a result, Rhbg mRNA expression was always higher in the perfused tissues when normalized to β-actin (Fig. 7). Interestingly, Rhbg expression decreased significantly at 48 h of HEA in the perfused brain (Fig. 7), did not change in the HEA-exposed gill (Fig. 8), but increased by almost 200-fold at 48 h HEA in the skin (Fig. 9). Of particular note also is the increased Rhbg expression in the pavement cell fraction that is devoid of contaminating RBCs after lysis treatment (see below and Fig. 11*A*).

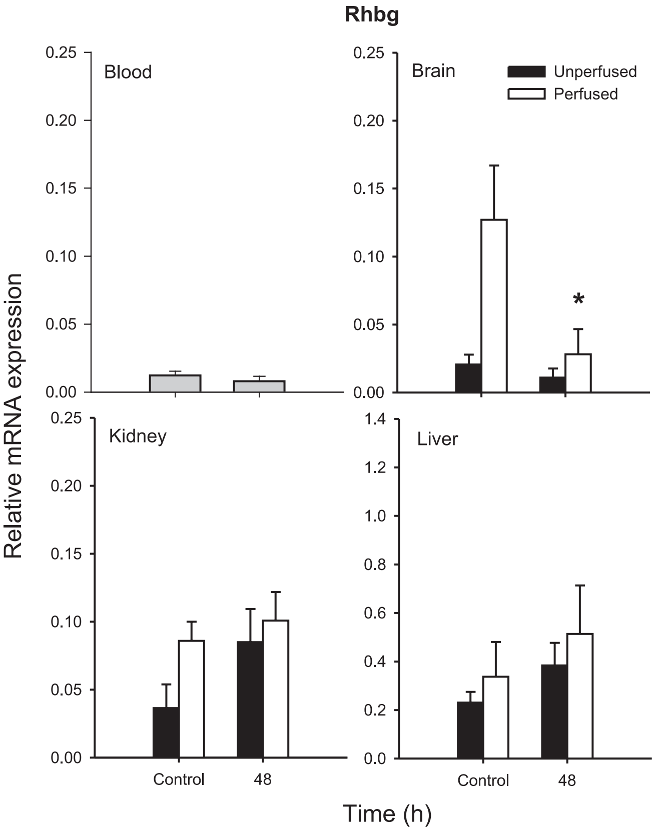

Fig. 7. mRNA expression of Rhbg relative to β-actin in the blood and the unperfused and perfused brain, kidney, and liver of rainbow trout exposed to 0 mmol/l NH_4HCO_3 (control) and 1.5 mmol/l NH_4HCO_3 for 48 h. *Significant difference between control and 48-h perfused brain ($P < 0.05$). Data are means ± SE ($n = 6$).

Rhcg1 was clearly detected in the gill and skin by PCR in both control and HEA conditions, and there was some evidence of expression in the brain and liver during HEA as well (Fig. 6). Analysis by qPCR confirmed that Rhcg1 mRNA was

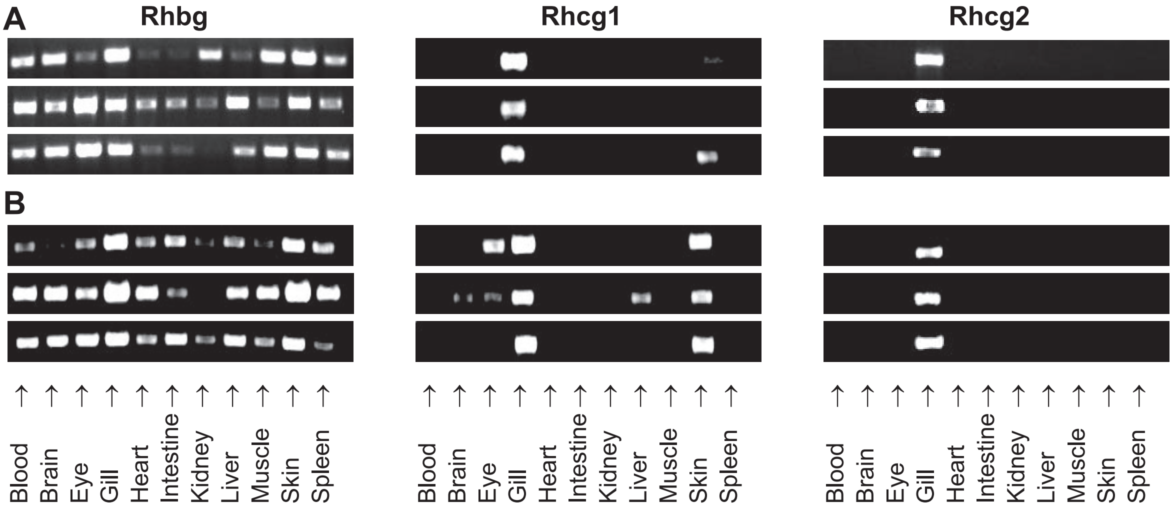

Fig. 6. Tissue distribution of Rh glycoproteins as determined by PCR in rainbow trout exposed to 0 mmol/l NH_4HCO_3 (*A*) and 1.5 mmol/l NH_4HCO_3 (*B*). These gels show qualitative rather than quantitative expression; $n = 3$ for each Rh ortholog and treatment.

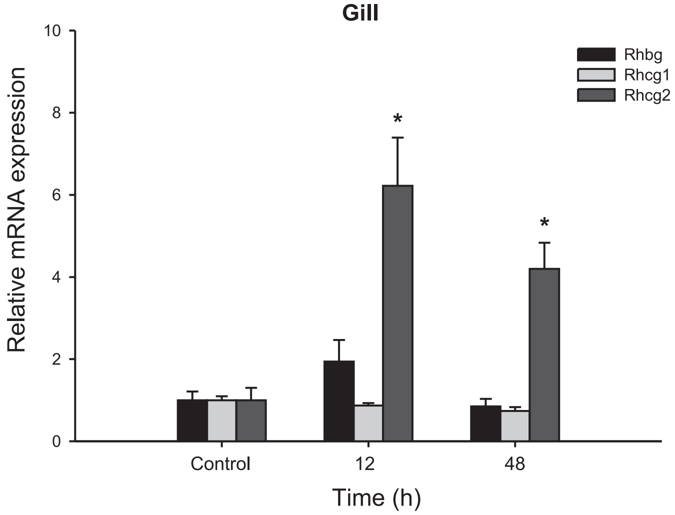

Fig. 8. mRNA expression of Rhbg, Rhcg1, and Rhcg2 relative to elongation factor (EF)-1α expression in the gills of rainbow trout exposed to 1.5 mmol/l NH_4HCO_3 for 12 and 48 h *Significant differences from the control ($P < 0.05$). Data are means ± SE ($n = 6$).

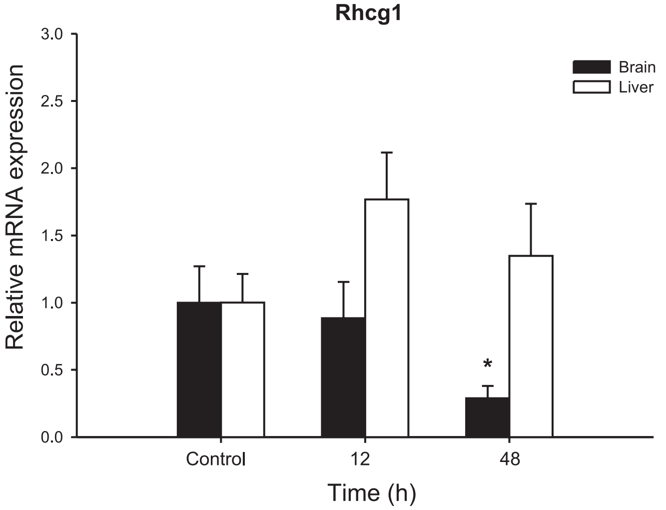

Fig. 10. mRNA expression of Rhcg1 relative to β-actin in the brain and liver of rainbow trout exposed to 1.5 mmol/l NH_4HCO_3 for 12 and 48 h. *Significant differences from the control ($P < 0.05$). Data are means ± SE ($n = 6$).

expressed in control and HEA conditions in brain, gill, liver, and skin, but the only significant change noted was a decrease in expression at 48 h of HEA in the brain (Fig. 10), parallel to that seen with Rhbg in the brain at this time (cf. Fig. 7).

Although PCR analysis revealed Rhcg2 expression in the gill only (Fig. 6), qPCR analysis also detected Rhcg2 in the skin, and its expression increased about ninefold with 48 h HEA (Fig. 9). In the HEA-exposed gill, Rhcg2 expression increased by 6.2-fold at 12 h and remained elevated by 4.2-fold at 48 h (Fig. 8).

Gill cell fractionation was performed to ascertain the cellular location of these changes in expression. In the pavement cell fraction, there was no change in Rhcg1 expression, but there was a 4.3-fold increase in Rhbg expression at 48 h of HEA and an 8.1 and 10.8-fold increase in Rhcg2 expression at 12 and 48 h of HEA, respectively (Fig. 11*A*). No significant changes in Rh mRNA expression were noted in the MR cell fraction, though Rhcg1 expression tended to fall (Fig. 11*B*).

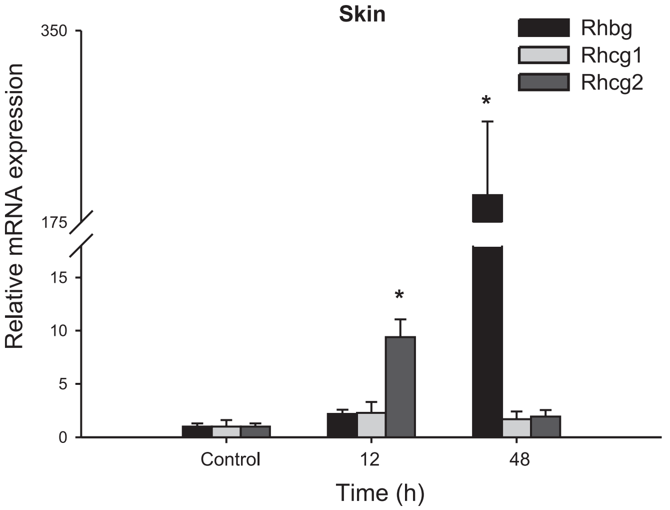

Fig. 9. mRNA expression of Rhbg, Rhcg1, and Rhcg2 relative to β-actin in the skin of rainbow trout exposed to 1.5 mmol/l NH_4HCO_3 for 12 and 48 h. *Significant differences from the control ($P < 0.05$). Data are means ± SE ($n = 6$).

With the Rh30-like1 and Rh30-like2 sequences being nearly identical, only the expression of Rh30-like2 and Rh30-like3 was analyzed. The expression of these as well as Rhag decreased significantly to less than half the control value in RBCs exposed to 12 and 48 h of HEA (Fig. 12)

Enzyme/Transporter Expression and Activity in the Gill

The expression of H^+-ATPase in the HEA-exposed gill increased by 2-fold at 12 and 48 h (Fig. 13*A*) and H^+-ATPase enzyme activity increased significantly by 1.4-fold at 48 h HEA (Fig. 13*D*). CA2 expression decreased significantly at 48 h to half of the control value (Fig. 13*B*), and this corresponded to a 1.7-fold decrease in CA enzyme activity at 48 h HEA (Fig. 13*E*). Expression of four α-isoforms of Na^+/K^+-ATPase in the gill did not change significantly with HEA exposure (Fig. 13*C*), and Na^+/K^+-ATPase enzyme activity also remained unchanged (Fig. 13*F*). Similarly, NHE2 expression did not change significantly with control, 12 and 48 h HEA values of 1.00 ± 0.21, 1.82 ± 0.41, and 0.72 ± 0.09, respectively ($n = 6$).

In the pavement cells, there was no change in NHE2 expression, but there was a 8.3-fold decrease in CA2 expression at 48 h, a 3-fold increase in H^+-ATPase expression at 12 h, and a 5.3-fold increase in Na^+/K^+-ATPase expression at 48 h HEA (Fig. 14*A*). In MR cells, Na^+/K^+-ATPase was significantly downregulated by 5.6-fold at 48 h HEA while CA2, H^+-ATPase, and NHE2 expression remained unchanged (Fig. 14*B*).

DISCUSSION

Marini et al. (39) first reported that RhAG and RhCG rescued the growth of Mep-deficient yeast and that RhAG mediated efflux of methylammonium. Since then, the role of Rh proteins as potential ammonia transporters has become an active area of investigation. Previous work by Huang and Peng (20) identified an Rh30-like1 and Rhcg2 gene in the rainbow

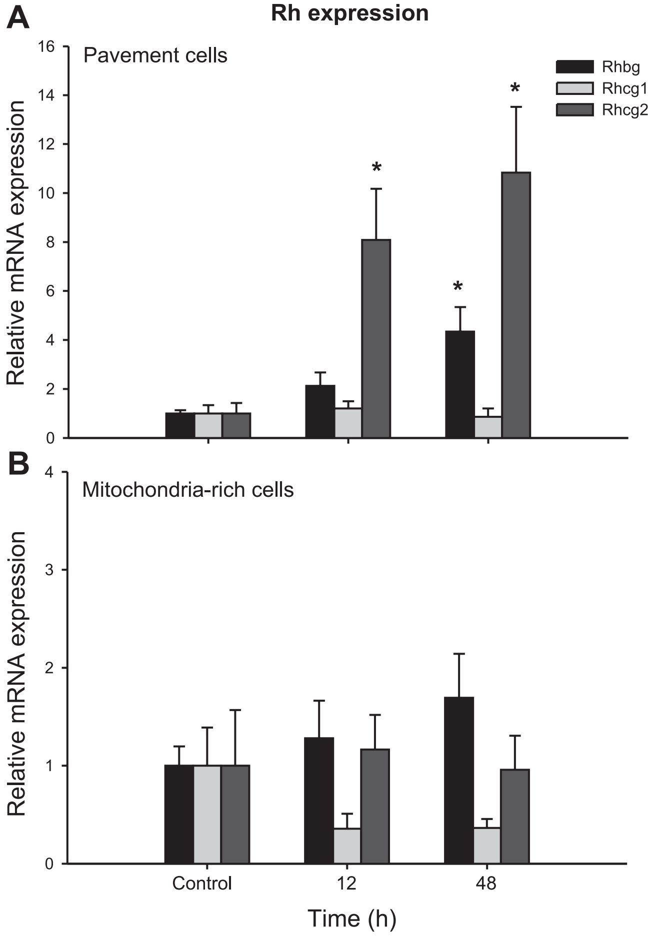

Fig. 11. mRNA expression of Rhbg, Rhcg1, and Rhcg2 relative to β-actin in pavement cells (*A*) and mitochondria-rich cells (*B*) isolated from the gills of rainbow trout exposed to 1.5 mmol/l NH_4HCO_3 for 12 and 48 h. *Significant differences from the control ($P < 0.05$). Data are means ± SE ($n = 6$).

trout, but the physiological significance of these genes has not been explored. In the present study, we identified additional cDNA sequences encoding for Rh30-like, Rhag, Rhbg, and Rhcg1 and examined their potential involvement in ammonia transport. Rh expression was studied exclusively at the mRNA expression level in the present investigation. An important next step in future studies will be to quantify expression at the protein level, once suitable antibodies have been developed for use in rainbow trout.

All sequences identified in this study have 12 predicted transmembrane domains, a highly conserved characteristic observed in the Rh protein family (19). The Rh30-like1 and Rh30-like2 sequences are identical, with the exception of a single amino acid deletion and four amino acid substitutions in Rh30-like1 (Fig. 4). Rh30-like2 and Rh30-like3 differ only at the 3′-end due to a 73-base pair (bp) deletion in Rh30-like2. We also identified a partial Rh30-like4 sequence with a more varied CDS as well as two variants of Rhag lacking the 12 predicted transmembrane spanning domains typical of the other Rh family members.

These multiple Rh30-like and Rhag sequences are likely the result of polyploidization events that occurred in the lineage leading to modern ray-finned fishes (65). Salmonids have further undergone a recent (25–100 million years ago) tetraploidization event (1), resulting in duplicated genes. Indeed, multiple Wilms' tumor suppressor (5) and glutamine synthetase (46) genes in trout are likely the result of both ancient and recent duplication events. It is possible, therefore, that additional (yet unidentified) Rh genes will be characterized in the future.

The cDNA sequences of Rhbg-a and Rhbg-b are identical with the exception of a 474-bp deletion in the 3′-UTR of Rhbg-b. Rhcg1-a and Rhcg1-b are also identical, except for a 299-bp deletion in the 3′-UTR of Rhcg1-b. Presumably the two Rhbg sequences are RNA splice variants from the same gene, and this is likely the case for the two Rhcg1 sequences. Although we cannot speculate about the function of the Rhbg and Rhcg1 variants, it is reasonable to conclude that they are differentially expressed given that 3′-UTRs are important for posttranscriptional regulation (43). Furthermore, preliminary results using primers designed specifically to Rhbg-a suggest that the Rhbg expression changes that we report here in the brain and skin (Figs. 7 and 9) likely reflect changes in Rhbg-b.

Both Rhbg and Rhcg1 are very similar (>75%) to their counterparts in other fish species, but there is more variability between the Rh30-like sequences (Fig. 4). The Rh30-like sequences fall into a cluster of homologs related to the Rh30 proteins (Fig. 3) that were first described in humans (3). There is ~60% amino acid similarity between trout Rhbg/Rhcg and human RhBG/RhCG, but trout Rh30-like sequences are <31% similar to human Rh30 (not shown). This agrees well with Huang and Peng (20), who showed that Rh30 is more functionally divergent across species than the other Rh genes. Rh30 proteins do not directly transport ammonia, but their membrane expression depends on the presence of RhAG (60). In the present study, the mRNA expression of Rh30-like and Rhag followed the same pattern of downregulation in HEA-exposed RBCs (Fig. 12), suggesting that these proteins could also be coexpressed together on the trout RBC membrane. Whether Rhag is additionally expressed in pillar cells of the trout gill, as they are in the pufferfish (47), remains to be determined.

Exposure to 1.5 mmol/l NH_4HCO_3 for 48 h resulted in an initial net uptake of ammonia into the fish with subsequent

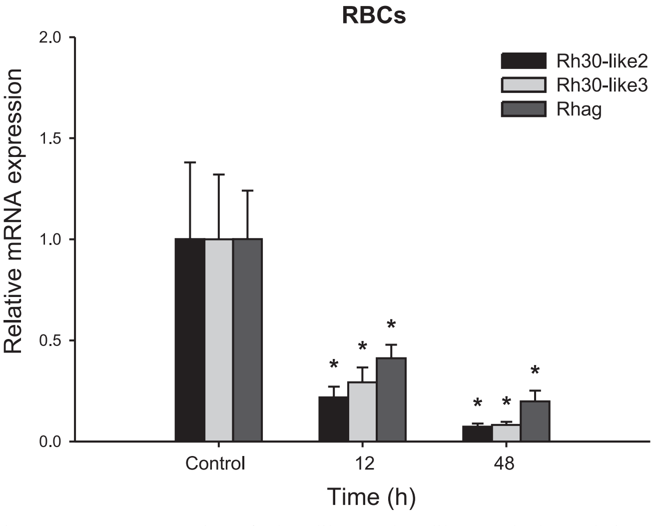

Fig. 12. mRNA expression of Rh30-like2 , Rh30-like3, and Rhag relative to β-actin in the red blood cells (RBCs) from rainbow trout exposed to 1.5 mmol/l NH_4HCO_3 for 12 and 48 h. *Significant differences from the control ($P < 0.05$). Data are means ± SE ($n = 6$).

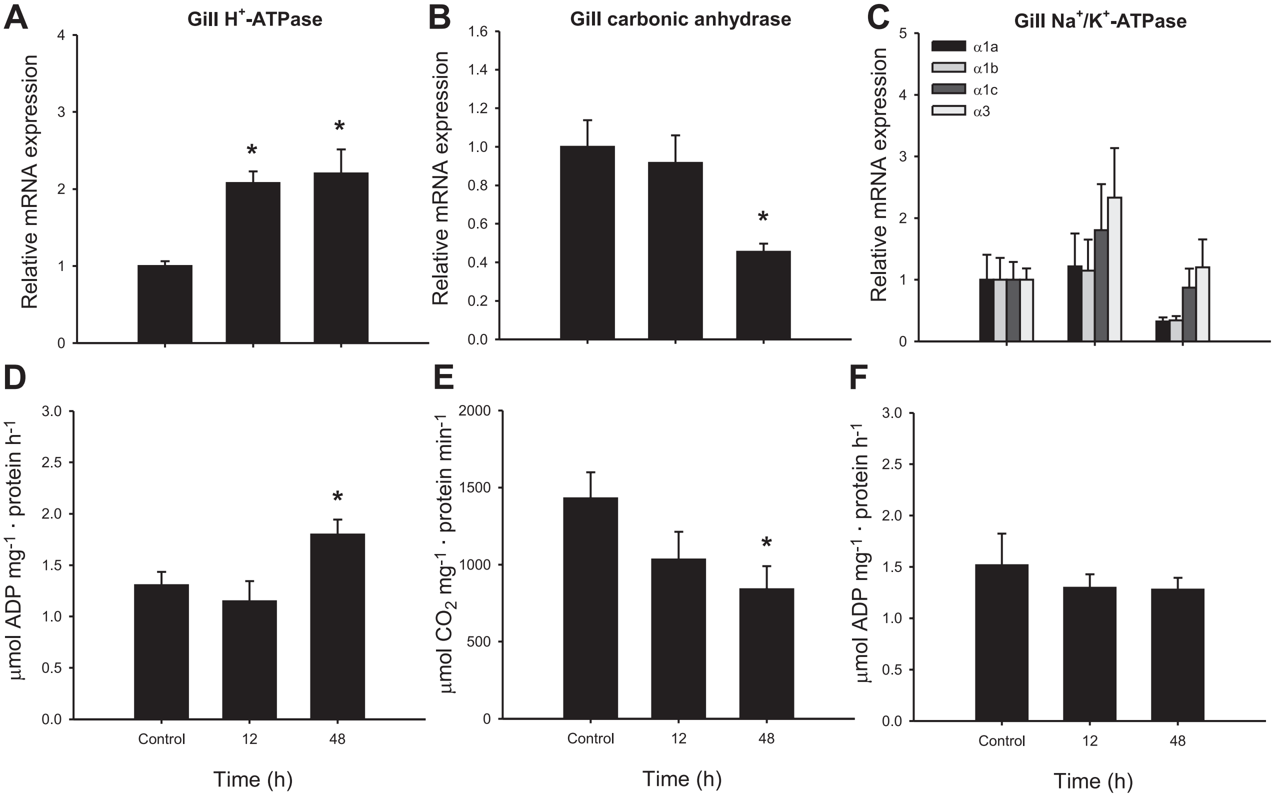

Fig. 13. mRNA expression relative to EF-1α of H^+-ATPase (*A*), carbonic anhydrase (CA2, *B*), and Na^+/K^+-ATPase α-subunit isoforms (*C*) and the enzyme activities of H^+-ATPase (*D*), carbonic anhydrase (*E*), and Na^+/K^+-ATPase (*F*) in the gills of rainbow trout exposed to 1.5 mmol/l NH_4HCO_3 for 12 and 48 h. *Significant differences from the control ($P < 0.05$). Data are means ± SE ($n = 6$).

recovery of excretion (Fig. 1). This is consistent with earlier investigations that reported the same phenomenon in both freshwater and seawater fish exposed to high (up to 1 mmol/l) external concentrations of NH_4Cl or $(NH_4)_2SO_4$ (6, 7, 8, 63). These studies hypothesized that an active NH_4^+ extrusion mechanism is induced to counteract the inward diffusion of NH_3, but active NH_4^+ exchange has not yet been demonstrated in the freshwater teleost gill, leaving NH_3 diffusion as the main route of excretion (54, 62).

The lipid solubility of NH_3 is, however, only moderate at best (13, 67), and a protein channel would greatly facilitate NH_3 passage even under routine conditions. This is particularly advantageous when potentially toxic concentrations of ammonia need to be transported out quickly. In HEA conditions, fish need to counter the influx of ammonia as well as eliminate endogenously produced ammonia. This became apparent at 48 h of HEA when plasma T_{Amm} approached water T_{Amm} (Fig. 2). Recovery of excretion after 12 h, and the increased J_{Amm} at 48 h (Fig. 1) was accompanied by the simultaneous upregulation of Rhcg2 at 12 and 48 h (Fig. 8). This suggests that Rhcg2 is upregulated in response to HEA, resulting in enhanced ammonia excretion.

Although Rhcg2 was upregulated during HEA, all three Rh mRNAs (Rhbg, Rhcg1, and Rhcg2) were expressed in the whole gill and gill cell fractions during control and HEA conditions (Figs. 8 and 11). Analysis of the gill cell fractions revealed that Rhcg2 upregulation at 12 and 48 h was accompanied by Rhbg upregulation at 48 h and that these changes were occurring in the pavement cells and not in MR cells (Fig. 11). Taken together, this suggests that while both cell types may be involved in routine ammonia transport via Rh glycoproteins during control and HEA conditions, pavement cells respond to HEA with an upregulation of Rhcg2, restoring excretion and elevating rates above basal levels. Rhbg may be involved in the maintenance or further regulation of this process. These ideas still need to be validated with trout-specific Rh protein studies, which will reveal whether changes in Rh protein levels or subcellular distribution occur as they do in rat renal cells during metabolic acidosis (55).

In the gill of the pufferfish, localization of Rh mRNAs and proteins suggests that at least under basal conditions, Rhcg1 is expressed apically and is confined to the MR cells while Rhcg2 and Rhbg are restricted to pavement cells in the apical and basolateral regions, respectively (47). In the gills of the rainbow trout, on the other hand, our findings show that the trout Rh mRNAs are expressed in both the MR and pavement cells under control conditions but respond differentially under HEA: Rhbg and Rhcg2 expression increases in pavement cells only, while Rhcg1 expression does not change in pavement cells but tends to fall in MR cells. Whether this discrepancy in findings is due to species differences and whether or not the same polarized localization of Rh glycoproteins occurs in the trout gill await further investigation.

Previous studies demonstrated that expired water passing over the gills of freshwater fish is acidified by up to 1.5 pH units, depending on initial water pH and buffer capacity (30, 49, 69). This acidified gill boundary layer traps excreted NH_3 to NH_4^+ (51, 64), but whether the acidification is due to H^+

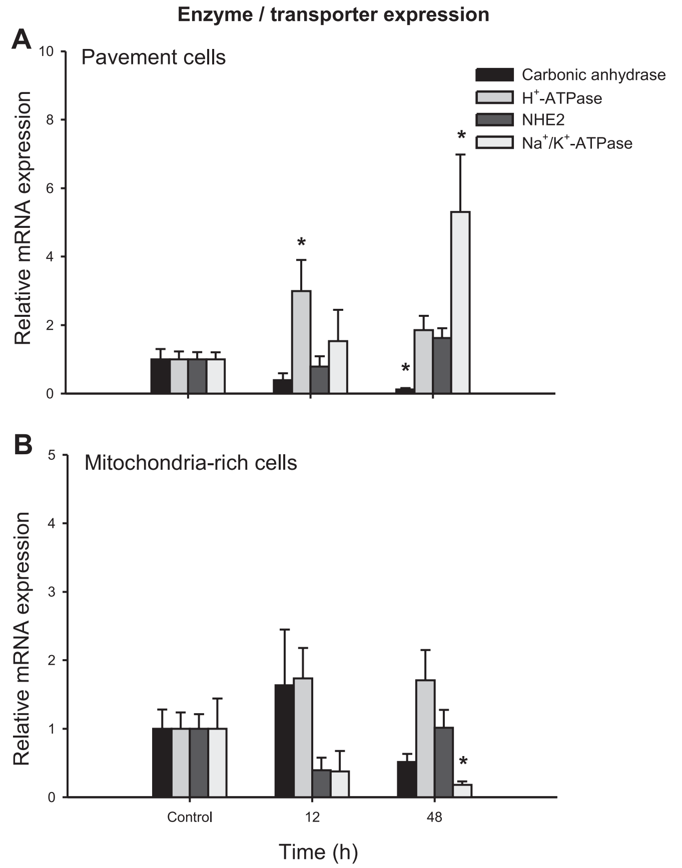

Fig. 14. mRNA expression relative to β-actin of carbonic anhydrase, H^+-ATPase, NHE2, and Na^+/K^+-ATPase (α1a) in pavement cells (*A*) and mitochondria-rich cells (*B*). *Significant differences from the control ($P < 0.05$). Data are means ± SE ($n = 6$).

arising from CO_2 hydration or is a result of H^+ extruded by H^+-ATPase is unknown (62). In our study, an increase in H^+-ATPase expression and activity (Fig. 13, *A* and *D*) accompanied the Rhcg2 upregulation and recovery of ammonia excretion during HEA, suggesting that H^+-ATPase is responsible for the acidification. Moreover, this upregulation of H^+-ATPase occurred in the pavement cells (Fig. 14), indicating a possible functional coupling with Rhcg2, which has also been hypothesized to occur in the intercalated cells of the rat nephron (11).

The CA-catalyzed hydration of CO_2 normally fuels the H^+-ATPase with H^+ in the trout gill (31), so we expected to see an increase in CA expression and/or activity in conjunction with the upregulated H^+-ATPase in HEA conditions. Instead, CA expression and activity were significantly decreased at 48 h (Fig. 13, *B* and *E*), a response that again appeared specific to the pavement cells (Fig. 14), in agreement with one study that reported inhibition of CA activity by HEA in trout (2). Downregulation or lowered CA activity suggests that CO_2 is no longer the main source of H^+ for the H^+-ATPase pump. Under ammonia-loaded conditions, NH_4^+ may be the major supplier of H^+. This correlates with the present model of the ammonium transporter (AmtB) of *E. coli,* which is structurally and functionally similar to Rh proteins (9, 38, 72). AmtB is a trimeric membrane-spanning complex (10, 24, 71) functioning as an NH_3 channel. The NH_4^+ is deprotonated before entering the channel so that it can pass through as NH_3 and reprotonate after exiting (24, 25). Under HEA, a continuous and abundant supply of H^+ stripped off from NH_4^+ entering the Rh channel may be fuelling the H^+-ATPase (Fig. 15), making elevated CA activity unnecessary.

Alternative routes of ammonia transport in the gill are via the NHE and Na^+/K^+-ATPase. So far, only the giant mudskipper, *Periophthalmodon schlosseri*, is documented to actively excrete NH_4^+ by a basolateral Na^+/K^+-ATPase and an apical NHE during HEA (50). The lack of evidence for ammonia transport via Na^+/K^+-ATPase in the freshwater fish gill may be related to higher Na^+/K^+-ATPase activity levels in seawater fish (62); however, not all seawater species display high enzyme activity (42). In our study, HEA did not result in changes in Na^+/K^+-ATPase at the transcript (Fig. 13*C*) or activity level (Fig. 13*F*) in whole gill samples. Analysis of mRNA expression in the gill cell fractions, however, revealed upregulation in the pavement cells and downregulation in the MR cells (Fig. 14). These opposing cell-specific differences may explain the absence of change in expression and activity in the whole gill. Therefore, we cannot rule out the potential for basolateral NH_4^+ transport via this route until more cell-specific studies are performed. No changes in NHE2 mRNA expression were seen in whole gill or the gill cell fractions (Fig. 14), so a role for this transporter during HEA is not apparent. NH_4^+ transport through the gill remains a possibility, but a mechanism has yet to be characterized.

Results from this study revealed a wide tissue expression of Rh mRNAs with a noted downregulation of Rhbg and Rhcg1 occurring in the brain with HEA exposure (Figs. 7 and 10). Since trout brain cells have only a limited capacity to detoxify ammonia (45), we could hypothesize that this downregulation of Rh was a protective response to prevent ammonia overloading into the cells. Downregulation of Rhbg did not occur in the mangrove killifish brain in response to similar HEA conditions (21), and this may reflect species differences: the mangrove killifish is very ammonia tolerant (14) compared with the trout (45). Interestingly, Rh glycoproteins were not detected in the pufferfish brain (47).

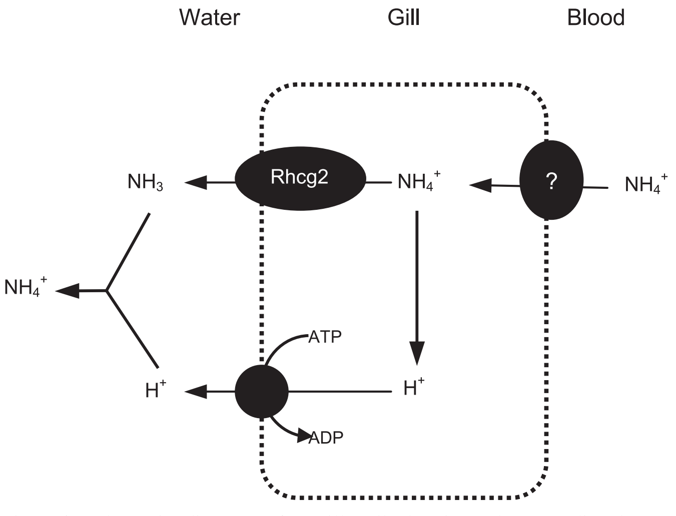

Fig. 15. Schematic diagram of a gill cell showing Rhcg2-mediated NH_3 transport coupled to active H^+ secretion. The result is a net excretion of NH_4^+ across the apical gill epithelium.

During HEA, Rhbg and Rhcg2 were upregulated in the skin (Fig. 9). This was surprising as the skin under normal conditions contributes very little to total ammonia excretion in freshwater teleosts compared with seawater species (67). Upregulation of Rhcg1 and Rhcg2 was also observed in the mangrove killifish skin exposed to HEA or air (21). This species, however, is known to volatilize ammonia via the skin during air exposure (33). Whether the trout skin also assumes an excretory role during HEA is not clear, but Rh proteins appear to play some role in this tissue during HEA.

An important controversy regarding the function of Rh proteins is whether or not they transport CO_2. *Chlamydomonas reinhardtii* is a green algae with Amt and Rh (Rh1) genes. When Rh1 is inhibited, ammonium uptake is unaffected but the growth increase that normally occurs in high CO_2 is impaired. Elevating the CO_2 content of the growth medium increases Rh1 expression (28, 56). Also, RBCs lacking RhAG (Rh_{null}) have reduced CO_2 permeability (12), and RhCG-expressing oocytes have increased CO_2 permeability (4). Contrary to this, however, Ripoche et al. (53) reported no change in CO_2 permeability in Rh_{null} ghost cell preparations. Future studies should include investigations into the potential CO_2 transport via Rh proteins.

In summary, we identified seven full-length Rh cDNA sequences in the rainbow trout and evaluated the gene expression of these as well as Rhcg2 at the transcript level in the rainbow trout challenged with HEA. Rh mRNAs were expressed in many tissues, and changes in expression upon HEA exposure were noted in some. In the gill, the main site of ammonia excretion, Rhcg2 expression as well as H^+-ATPase expression and activity were upregulated during HEA and this coincided with the net recovery of ammonia excretion. CA expression and activity were significantly downregulated at 48 h of HEA, suggesting that CA is no longer fuelling the H^+-ATPase pump at this point. Furthermore, pavement cells and not MR cells appear to be instrumental in the regulation of ammonia transport during HEA since clear expression changes in Rh and enzymes were occurring in this cell type only. The contribution of Rh glycoproteins and H^+-ATPase to enhanced ammonia excretion across the gill during HEA has been highlighted, adding to our current knowledge and understanding of branchial ammonia transport in the freshwater rainbow trout.

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REFERENCES

1. **Allendorf FW, Thorgaard GH.** Tetraploidy and the evolution of salmonid fishes. *In Evolutionary Genetics of Fishes* edited by Turner BJ. New York: Plenum Press, 1984, p. 1–46.
2. **ArasHisar S, Hisar O, Yanik T, Aras SM.** Inhibitory effects of ammonia and urea on gill carbonic anhydrase enzyme activity of rainbow trout (*Oncorhynchus mykiss*). *Env Tox Pharm* 17: 125–128, 2004.
3. **Avent ND, Reid ME.** The Rh blood group system: a review. *Blood* 95: 375–387, 2000.
4. **Bakouh N, Benjelloun F, Hulin P, Brouillard F, Edelman A, Chérif-Zahar B, Planelles G.** The challenge of understanding ammonium homeostasis and the role of Rh glycoproteins. *Tranfus Clin Biol* 13: 139–146, 2006.
5. **Brunelli JP, Robison BD, Thorgaard GH.** Ancient and recent duplications of the rainbow trout Wilms' tumor gene. *Genome* 44: 455–462, 2001.
6. **Cameron JN.** Responses to reversed NH_3 and NH_4^+ gradients in a teleost (*Ictalurus punctatus*), an elasmobranch (*Raja erinacea*), and a crustacean (*Callinectes sapidus*): evidence for NH_4^+/H^+ exchange in the teleost and the elasmobranch. *J Exp Zool* 239: 183–195, 1986.
7. **Cameron JN, Heisler N.** Studies of ammonia in the trout: physico-chemical parameters, acid-base behaviour and respiratory clearance. *J Exp Biol* 105: 107–125, 1983.
8. **Claiborne JB, Evans DH.** Ammonia and acid-base balance during high ammonia exposure in a marine teleost (*Myoxocephalus octodecimspinosus*). *J Exp Biol* 140: 889–105, 1988.
9. **Conroy MJ, Bullough PA, Merrick M, Avent ND.** Modelling the human rhesus proteins: implications for structure and function. *Br J Haematol* 131: 543–551, 2005.
10. **Conroy MJ, Jamieson SJ, Blakey D, Kaufmann T, Engel A, Fotiadis D, Merrick M, Bullough PA.** Electron and atomic force microscopy of the trimeric ammonium transporter AmtB. *EMBO Rep* 5: 1153–1158, 2004.
11. **Eladari D, Cheval L, Quentin F, Bertrand O, Mouro I, Cherif-Zahar B, Cartron JP, Paillard M, Doucet A, Chambrey R.** Expression of RhCG, a new putative NH_3/NH_4^+ transporter, along the rat nephron. *J Am Soc Nephrol* 13: 1999–2008, 2002.
12. **Endeward V, Cartron JP, Ripoche G, Gros G.** Red cell membrane CO_2 permeability in normal human blood and in blood deficient in various blood groups, and effect of DIDS. *Transfus Clin Biol* 13: 123–127, 2006.
13. **Evans DH, Cameron JN.** Gill ammonia transport. *J Exp Zool* 239: 17–23, 1986.
14. **Frick NT, Wright PA.** Nitrogen metabolism and excretion in the mangrove killifish, *Rivulus marmoratus*. I. The influence of environmental salinity and external ammonia. *J Exp Biol* 205: 79–89, 2002.
15. **Fromm PO.** Studies on renal and extra-renal excretion in a freshwater teleost, *Salmo gairdneri*. *Comp Biochem Physiol* 10: 121–128, 1963.
16. **Galvez F, Reid SD, Hawkings G, Goss GG.** Isolation and characterization of mitochondria-rich cell types from the gill of freshwater rainbow trout. *Am J Physiol Regul Integr Comp Physiol* 282: R658–R668, 2002.
17. **Hall TA.**BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symp Ser* 41: 95–98. 1999.
18. **Henry RP.** Techniques for measuring carbonic anhydrase activity in vitro: the electrometric delta pH and pH stat assay. In: *The Carbonic Anhydrases: Cellular Physiology and Molecular Genetics*, edited by Dodgson, SJ, Tashian RE, Gros G, Carter ND. New York: Plenum, 1991, p. 119–126.
19. **Huang CH, Liu PZ.** New insights into the Rh superfamily of genes and proteins in erythroid cells and nonerythroid tissues. *Blood Cells Mol Dis* 27: 90–101, 2001.
20. **Huang CH, Peng J.** Evolutionary conservation and diversification of Rh family genes and proteins. *Proc Natl Acad Sci USA* 102: 15512–15517, 2005.
21. **Hung CYC, Tsui KNT, Wilson JM, Nawata CM, Wood CM, Wright PA.** Rhesus glycoprotein gene expression in the mangrove killifish *Kryptolebias marmoratus* exposed to elevated environmental ammonia levels and air. *J Exp Biol* 210: 2419–2429, 2007.
22. **Javelle A, Lupo D, Li XD, Merrick M, Chami M, Ripoche P, Winkler FK.** Structural and mechanistic aspects of Amt/Rh proteins. *J Struct Biol* 158: 472–481, 2007.
23. **Juretic D, Zoranic L, Zucic D.** Basic charge clusters and prediction of membrane protein topology. *J Chem Inf Comput Sci* 42: 620–632, 2002.
24. **Khademi S, O'Connell J III, Remis J, Robles-Colmenares Y, Mierke LJW, Stroud RM.** Mechanism of ammonia transport by Amt/MEP/Rh: structure of AmtB at 1.35 Δ. *Science* 305: 1587–1594, 2004.
25. **Khademi S, Stroud RM.** The AmtB and the mechanism of ammonia gas conduction. *Physiology* 21: 419–429, 2006.
26. **Kirschner LB, Greenwald L, Kerstetter TH.** Effect of amiloride on sodium transport across body surfaces of fresh water animals. *Am J Physiol* 224: 832–837, 1973.

27. **Kumar S, Tamura K, Nei M.** MEGA3: Integrated software for molecular evolutionary genetics analysis and sequence alignment. *Brief Bioinform* 5: 150–163, 2004.
28. **Kustu S, Inwood W.** Biological gas channels for NH_3 and CO_2: evidence that Rh (Rhesus) proteins are CO_2 channels. *Transfus Clin Biol* 13: 103–110, 2006.
29. **Lin H, Pfeiffer DC, Wayne VA, Pan J, Randall DJ.** Immunolocalization of H^+-ATPase in the gill epithelia of rainbow trout. *J Exp Biol* 195: 169–183, 1994.
30. **Lin H, Randall DJ.** The effect of varying water pH on the acidification of expired water in rainbow trout. *J Exp Biol* 141: 14–160, 1990.
31. **Lin H, Randall DJ.** Evidence for the presence of an electrogenic proton pump on the trout gill epithelium. *J Exp Biol* 161: 119–134, 1991.
32. **Lin H, Randall DJ.** H^+-ATPase activity in crude homogenates of fish gill tissue: inhibitor sensitivity and environmental and hormonal regulation. *J Exp Biol* 180: 163–174, 1993.
33. **Litwiller SL, O'Donnell MJ, Wright PA.** Rapid increase in the partial pressure of NH_3 on the cutaneous surface of air-exposed mangrove killifish, *Rivulus marmoratus. J Exp Biol* 209: 1737–1745, 2006.
34. **Liu Z, Chen Y, Mo R, Hui C, Cheng JF, Mohandas N, Huang CH.** Characterization of human RhCG and mouse Rhcg as novel nonerythroid Rh glycoprotein homologues predominantly expressed in kidney and testis. *J Biol Chem* 275: 25641–25651, 2000.
35. **Liu Z, Peng J, Mo R, Hui C, Huang CH.** Rh type B glycoprotein is a new member of the Rh superfamily and a putative ammonia transporter in mammals. *J Biol Chem* 276: 1424–1438, 2001.
36. **Lumsden JS, Wright P, Derksen J, Byrne PJ, Ferguson HW.** Paralysis in farmed Arctic char (*Salvelinus alpinus*) associated with ammonia toxicity. *Vet Rec* 133: 422–423, 1993.
37. **Mallery CH.**A carrier enzyme basis for ammonium excretion in teleost gill. NH_4^+-stimulated Na-dependent ATPase activity in *Opsanus beta. Comp Biochem Physiol* 74A: 889–897, 1993.
38. **Marini AM, Boeckstaens M, Benjelloun F, Chérif-Zahar B, André B.** Structural involvement in substrate recognition of an essential aspartate residue conserved in Mep/Amt and Rh-type ammonium transporters. *Curr Genet* 49: 364–374, 2006.
39. **Marini AM, Matassi G, Raynal V, André B, Cartron JP, Chérif-Zahar B.** The human Rhesus-associated RhAG protein and a kidney homologue promote ammonium transport in yeast. *Nat Genet* 26: 341–344, 2000.
40. **Marini AM, Urrestarazu A, Beauwens R, André B.** The Rh (Rhesus) blood group polypeptides are related to NH_4^+ transporters. *Trends Biochem Sci* 22: 460–461, 1997.
41. **Marini AM, Vissers S, Urrestarazu A, André (1994) B.** Cloning and expression of the MEP1 gene encoding an ammonium transporter of *Saccharomyces cerevisiae*. *EMBO J* 13: 3456–3463, 1994.
42. **Marshall WS, Bryson SE.**Transport mechanisms of seawater teleost chloride cells: an inclusive model of a multifunctional cell. *Comp Biochem Physiol* 119A: 97–106, 1998.
43. **Mazumder B, Seshadri V, Fox PL.** Translational control by the 3′- UTR: the ends specify the means. *Trends Biochem Sci* 28: 91–98, 2003.
44. **McCormick SD.** Methods for the nonlethal gill biopsy and measurements of Na^+, K^+-ATPase activity. *Can J Fish Aquat Sci* 50: 656–658, 1993.
45. **Mommsen TP, Walsh PJ.** Biochemical and environmental perspectives on nitrogen metabolism in fishes. *Experientia* 48: 583–593, 1992.
46. **Murray BW, Busby ER, Mommsen TP, Wright PA.** Evolution of glutamine synthetase in vertebrates: multiple glutamine synthetase genes expressed in rainbow trout (*Oncorhynchus mykiss*). *J Exp Biol* 206: 1511–1521, 2003.
47. **Nakada T, Westhoff CM, Kato A, Hirose S.** Ammonia secretion from fish gill depends on a set of Rh proteins. *FASEB J* 21: 1–8, 2007.
48. **Ninnemann O, Jauniaux JC, Frommer WB.** Identification of a high affinity NH_4^+ transporter from plants. *EMBO J* 13: 3464–3471, 1994.
49. **Playle RC, Wood CW.**Water chemistry changes in the gill microenvironment of rainbow trout: experimental observations and theory. *J Comp Physiol* 159B: 527–537, 1989.
50. **Randall DJ, Wilson JM, Peng KW, Kwok TWK, Kuah SSL, Chew SF, Lam TJ, Ip YK.** The mudskipper, *Periophthalmodon schlosseri*, actively transports NH_4^+ against a concentration gradient. *Am J Physiol Regul Integr Comp Physiol* 277: R1562–R1567, 1999.
51. **Randall DJ, Wright PA.** The interaction between carbon dioxide and ammonia excretion and water pH in fish. *Can J Zool* 67: 2936–2942, 1987.
52. **Rice P, Longden I, Bleasby A.** EMBOSS: The European Molecular Biology Open Software Suite. *Trends Genet* 16: 276–277, 2000.
53. **Ripoche P, Bertrand O, Gane P, Birkenmeier Colin Y, Cartron JP.** Human role of RhAG and AQP1 in NH_3 and CO_2 gas transport in red cell ghosts: a stopped-flow analysis. *Transfus Clin Biol* 13: 117–122. 2006.
54. **Salama A, Morgan IJ, Wood CM.** The linkage between Na^+ uptake and ammonia excretion in rainbow trout: kinetic analysis, the effects of $(NH_4)_2SO_4$ and NH_4HCO_3 infusion and the influence of gill boundary layer pH. *J Exp Biol* 202: 697–709, 1999.
55. **Seshadri RM, Klein JD, Smith T, Sands JM, Handlogten ME, Verlander JW, Weiner ID.** Changes in subcellular distribution of the ammonia transporter, Rhcg, in response to chronic metabolic acidosis. *Am J Physiol Renal Physiol* 290: F1443–F1452, 2006.
56. **Soupene E, Inwood W, Kustu S.** Lack of the Rhesus protein Rh1 impairs growth of the green alga *Chlamydomonas reinhardtii* at high CO_2. *Proc Natl Acad Sci USA* 101: 7787–7792, 2004.
57. **Thompson JD, Higgins DG, Gibson TJ.** CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* 22: 4673–4680, 1994.
58. **Verdouw H, van Echteld CJA, Dekkers EMJ.** Ammonia determinations based on indophenol formation with sodium salicylate. *Water Res* 12: 399–402, 1978.
59. **Weiner ID, Hamm LL.**Molecular mechanisms of renal ammonia transport. *Annu Rev Physiol* 69: 3.1–3.24, 2006.
60. **Westhoff CM.** The structure and function of the Rh antigen complex. *Semin Hematol* 44: 42–50, 2007.
61. **Westhoff CM, Wylie DE.** Transport characteristics of mammalian Rh and Rh glycoproteins expressed in heterologous systems. *Transfus Clin Biol* 13: 132–138, 2006.
62. **Wilkie MP.** Ammonia excretion and urea handling by fish gills: present understanding and future research challenges. *J Exp Zool* 293: 284–301, 2002.
63. **Wilson RW, Taylor EW.** Transbranchial ammonia gradients and acid-base responses to high external ammonia concentration in rainbow trout (*Oncorhynchus mykiss*) acclimated to different salinities. *J Exp Biol* 166: 95–112, 1992.
64. **Wilson RW, Wright PM, Munger S, Wood CM.** Ammonia excretion in fresh water rainbow trout (*Oncorhynchus mykiss*) and the importance of gill boundary layer acidification: lack of evidence for Na^+/NH_4^+ exchange. *J Exp Biol* 191: 37–58, 1994.
65. **Wittbrodt J, Meyer A, Schartl M.** More genes in fish? *Bioessays* 20: 511–515, 1998.
66. **Wolf K.** Physiological salines for fresh water teleosts. *Progve Fish Cult* 25: 135–140, 1963.
67. **Wood CM.** Ammonia and urea metabolism and excretion. In: *The Physiology of Fishes,* edited by Evans D. Boca Raton, FL: CRC, 1993, p. 379–425.
68. **Wright PA, Heming T, Randall DJ.** Downstream pH changes in water flowing over the gills of rainbow trout. *J Exp Biol* 126: 499–512, 1986.
69. **Wright PA, Randall DJ, Perry SF.** Fish gill boundary layer: a site of linkage between carbon dioxide and ammonia excretion. *J Comp Physiol* 158: 627–635, 1989.
70. **Wright PA, Wood CM.** An analysis of branchial ammonia excretion in the freshwater rainbow trout: effects of environmental pH change and sodium uptake blockage. *J Exp Biol* 114: 329–353, 1985.
71. **Zheng L, Kostrewa D, Berneche S, Winkler FK, Li XD.** The mechanism of ammonia transport based on the crystal structure of AmtB of *Escherichia coli*. *Proc Natl Acad Sci USA* 101: 17090–17095, 2004.
72. **Zidi-Yahiaoui N, Ripoche P, Van Kim CL, Gane P, D'Ambrosio AM, Cartron JP, Colin Y, Mouro-Chanteloup I.** Ammonium transport properties of HEK293 cells expressing RhCG mutants: preliminary analysis of structure/function by site-directed mutagenensis. *Transfus Clin Biol* 13: 128–131, 2006.