

## Functional characterization of Rhesus glycoproteins from an ammoniotelic teleost, the rainbow trout, using oocyte expression and SIET analysis

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### SUMMARY

Recent experimental evidence from rainbow trout suggests that gill ammonia transport may be mediated in part *via* Rhesus (Rh) glycoproteins. In this study we analyzed the transport properties of trout Rh proteins (Rhag, Rhbg1, Rhbg2, Rhcg1, Rhcg2, Rh30-like) expressed in *Xenopus* oocytes, using the radiolabeled ammonia analogue [<sup>14</sup>C]methylamine, and the scanning ion electrode technique (SIET). All of the trout Rh proteins, except Rh30-like, facilitated methylamine uptake. Uptake was saturable, with  $K_m$  values ranging from 4.6 to 8.9 mmol l<sup>-1</sup>. Raising external pH from 7.5 to 8.5 resulted in 3- to 4-fold elevations in  $J_{max}$  values for methylamine;  $K_m$  values were unchanged when expressed as total or protonated methylamine. Efflux of methylamine was also facilitated in Rh-expressing oocytes. Efflux and influx rates were stimulated by a pH gradient, with higher rates observed with steeper H<sup>+</sup> gradients. NH<sub>4</sub>Cl inhibited methylamine uptake in oocytes expressing Rhbg1 or Rhcg2. When external pH was elevated from 7.5 to 8.5, the  $K_i$  for ammonia against methylamine transport was 35–40% lower when expressed as total ammonia or NH<sub>4</sub><sup>+</sup>, but 5- to 6-fold higher when expressed as NH<sub>3</sub>. With SIET we confirmed that ammonia uptake was facilitated by Rhag and Rhcg2, but not Rh30-like proteins. Ammonia uptake was saturable, with a comparable  $J_{max}$  but lower  $K_m$  value than for total or protonated methylamine. At low substrate concentrations, the ammonia uptake rate was greater than that of methylamine. The  $K_m$  for total ammonia (560 μmol l<sup>-1</sup>) lies within the physiological range for trout. The results are consistent with a model whereby NH<sub>4</sub><sup>+</sup> initially binds, but NH<sub>3</sub> passes through the Rh channels. We propose that Rh glycoproteins in the trout gill are low affinity, high capacity ammonia transporters that exploit the favorable pH gradient formed by the acidified gill boundary layer in order to facilitate rapid ammonia efflux when plasma ammonia concentrations are elevated.

Key words: ammonia transport, Rh glycoproteins, *Xenopus* oocytes, trout, gill.

### INTRODUCTION

Although ammonia is an important nitrogen source for the growth of bacteria, fungi and plants, it is the major end product of nitrogen metabolism in ammoniotelic animals. Transport of ammonia across membranes is therefore essential for the maintenance of homeostasis in these organisms. The classical view has been that the lipid soluble gas phase (NH<sub>3</sub>) of ammonia passes readily through membranes whereas the ionic phase (NH<sub>4</sub><sup>+</sup>) requires carriers in order to cross membranes (Kleiner, 1981). This view is now being challenged by the recent identification of genes for ammonia transporters in yeast (MEP) and plants (Amt), followed later by the discovery that Rhesus (Rh) blood group proteins are related to these transporters (Marini et al., 1994; Marini et al., 1997; Ninnemann et al., 1994).

The X-ray structure of the *Escherichia coli* ammonia transporter (AmtB) revealed that NH<sub>3</sub> and not NH<sub>4</sub><sup>+</sup> is the species that passes through the channel. NH<sub>4</sub><sup>+</sup> is deprotonated in the periplasmic vestibule of AmtB before NH<sub>3</sub> passes through the pore and reprotonates in the cytoplasmic vestibule (Khademi et al., 2004; Zheng et al., 2004). Comparison of the recently solved structure of the Rh protein (Rh50) from the bacteria *Nitrosomonas europaea* with AmtB, showed similarities in the pore but differences in the external vestibule which may reflect a lower affinity or a weaker sequestering capacity for NH<sub>4</sub><sup>+</sup> in the Rh proteins (Li et al., 2007; Lupo et al., 2007). Although a few reports have suggested that CO<sub>2</sub> could also pass through the Rh channels (Endeward et al., 2007; Kustu and Inwood, 2006; Li et al., 2007; Soupene et al., 2002;

Soupene et al., 2004), the numerous functional studies that have been performed to date support the view that both Amt and Rh proteins facilitate ammonia transport (Javelle et al., 2007).

In mammals, RhAG/Rhag proteins are mainly confined to erythrocytes but RhBG/Rhbg and RhCG/Rhcg are located in several key tissues related to ammonia metabolism such as the brain, liver, kidney and gastrointestinal tract (Handlogten et al., 2005; Huang, 2008; Liu et al., 2000; Liu et al., 2001). In fact, recent knock-down studies in mice revealed that Rhcg protein expression was necessary for renal ammonia excretion (Biver et al., 2008; Lee et al., 2009).

Unlike ureotelic mammals, most fish are ammoniotelic and excrete large amounts of ammonia, mostly through the gills rather than through the kidney. The first study that linked fish Rh proteins to ammonia excretion, which was carried out in pufferfish gills, identified apical Rhcg2 and basolateral Rhbg in the pavement cells, apical Rhcg1 in the mitochondria-rich cells, and apical and basolateral Rhag in the pillar cells (Nakada et al., 2007a). Rhbg, Rhcg1 and Rhcg2 have also been identified in the gills and skin of the air-breathing mangrove killifish (Hung et al., 2007). Shortly after this it was reported that Rhcg2 mRNA expression levels in the adult rainbow trout gill paralleled the restoration of ammonia excretion in the face of elevated external ammonia (Nawata et al., 2007), and levels of Rhcg2 mRNA in larval rainbow trout correlated with an increase in ammonia excretion rate over developmental time (Hung et al., 2008). Similarly, Rhcg1 mRNA expression in larval zebrafish coincided with increased ammonia excretion (Nakada et al., 2007b), while knockdown of Rhag, Rhbg and Rhcg1 in the same larval

species led to a decrease in ammonia excretion (Braun et al., 2009; Shih et al., 2008). Also, an *in vitro* cultured gill epithelium system demonstrated that increased ammonia permeability caused by pre-exposure to elevated ammonia and cortisol, as well as exposure to freshwater low in Na<sup>+</sup>, was associated with increased Rhcg2 mRNA (Tsui et al., 2009). These data have been further examined in several recent reviews (Perry et al., 2009; Weihrauch et al., 2009; Wright and Wood, 2009).

Functional studies of Rh proteins have been hampered by the lack of a specific inhibitor as well as a long-lived radiotracer for ammonia. Researchers have therefore relied on the heterologous expression of Rh proteins in cells or cell-preparations in conjunction with the radiolabeled ammonia analogue, [<sup>14</sup>C]methylamine, to study Rh protein function. The first detailed study of Rh proteins in fish showed that *Xenopus* oocytes expressing pufferfish Rh proteins exhibited an increased uptake of methylamine (Nakada et al., 2007a). Our goal was to further characterize the functional properties of these potentially important gill ammonia transporters of rainbow trout (*Oncorhynchus mykiss* Walbaum). Trout Rhag, Rhbg1, Rhbg2, Rhcg1, Rhcg2 and Rh30-like proteins were expressed in *Xenopus* oocytes and [<sup>14</sup>C]methylamine was used to characterize the transport properties of these proteins. In addition, we used the scanning ion electrode technique (SIET) (Ammann, 1986) to directly confirm that ammonia transport was also facilitated, and to characterize ammonia uptake kinetics in Rh-expressing oocytes and in H<sub>2</sub>O-injected (control) oocytes.

## MATERIALS AND METHODS

### Reagents and solutions

All chemicals and reagents used in this study were obtained from Sigma (St Louis, MO, USA) unless otherwise noted. The standard oocyte bath solution was ND96 containing (in mmol l<sup>-1</sup>): 96 NaCl, 2 KCl, 1.8 CaCl<sub>2</sub>, 1 MgCl<sub>2</sub>, 5 Hepes, pH 7.5. Sterile ND96 for long-term storage of oocytes contained 2.5 mmol l<sup>-1</sup> sodium pyruvate, 1 mg ml<sup>-1</sup> penicillin–streptomycin and 50 µg ml<sup>-1</sup> gentamicin (Gibco, Long Island, NY, USA). Low K<sup>+</sup> ND96 contained 0.2 mmol l<sup>-1</sup> KCl and Na<sup>+</sup>- and K<sup>+</sup>-free ND96 contained 98 mmol l<sup>-1</sup> *N*-methyl-D-glucamine chloride in place of NaCl and KCl. The acidification buffers adjusted to pH 6.8 or pH 6.4 contained (in mmol l<sup>-1</sup>): 55 NaCl, 60 sodium acetate, 1.8 CaCl<sub>2</sub>, 1 MgCl<sub>2</sub> and 10 Hepes.

### Plasmid constructs and cRNA synthesis

Rh cDNAs were isolated from rainbow trout gill and erythrocytes as previously described (Nawata et al., 2007; Nawata and Wood, 2008) and amplified with high fidelity *Taq* polymerase (Invitrogen, Burlington, ON, Canada) using primers (Table 1) flanking the coding region of each gene. Correct sequences were verified after cloning into a pGEM T-easy vector (Promega, Fisher Scientific, Nepean, ON, Canada). The Rh cDNAs were then subcloned by blunt-end ligation into the *Xho*I and *Spe*I restriction sites of a pXT7 vector containing *Xenopus* beta globin 3'- and 5'-UTR sequences flanking the cloning site (courtesy of G. Goss, University of Alberta).

In-frame insertion of cDNAs was confirmed by sequencing. Linearization with *Sma*I was followed by proteinase K treatment (1 mg ml<sup>-1</sup>; Invitrogen) and phenol–chloroform extraction. The linearized constructs were then transcribed and capped (Ambion, Austin, TX, USA) *in vitro* with T7 RNA polymerase (Fermentas, Burlington, ON, Canada). The resulting cRNAs were purified by phenol–chloroform extraction and quantified spectrophotometrically (Nanodrop, ND-1000, Wilmington, DE, USA) and assessed for quality on a 1% agarose gel.

### Preparation of oocytes

Stage V–VI oocytes were collected from adult female *Xenopus* sp. following an established protocol (Ceriotti and Colman, 1995). Briefly the frogs were anaesthetized in 0.1% MS-222 for approximately 20 min. Excised ovarian tissue was placed in Ca<sup>2+</sup>-free ND96 solution containing collagenase (1 mg ml<sup>-1</sup>) and gently agitated for 30 min. The oocytes were then rinsed three times in Ca<sup>2+</sup>-free ND96, three times with ND96, and then allowed to recover overnight at 18°C in sterile ND96. Frogs were humanely killed after the final oocyte collection. 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.

### Injection of oocytes

Oocytes isolated the previous day were injected with 36.8 nl of cRNA (0.5 ng nl<sup>-1</sup>) to provide a total of 18.4 ng, using a Nanoliter 2000 Injector (World Precision Instruments, Sarasota, FL, USA). Control oocytes were injected with 36.8 nl of RNase-free H<sub>2</sub>O. Experiments were performed 3- to 5-days post-injection.

### [<sup>14</sup>C]methylamine studies

Experiments were performed at room temperature in 200 µl of uptake buffer which contained: low K<sup>+</sup> ND96, 0.5 µCi ml<sup>-1</sup> [<sup>14</sup>C]methylamine (Dupont, New England Nuclear, Boston, MA, USA), and 20 µmol l<sup>-1</sup> of unlabeled methylamine. Incubation times ranged from 15 to 60 min depending on the experiment (described in more detail below). Three groups of three oocytes were assayed for each experimental point; each group was considered as one replicate. At the end of each assay, oocytes were washed three times with 2 ml of ice-cold, unlabeled uptake buffer and immediately solubilized in 200 µl of 5% SDS. Radioactivity was measured in 5 ml of Ultima-Gold AB scintillation cocktail (PerkinElmer, Toronto, ON, Canada) by liquid scintillation counting (Tri-Carb 2900 TR; PerkinElmer). H<sub>2</sub>O-injected (control) oocytes were run in parallel in all assays. The focus of our study was on trout Rhcg2 since according to previous studies, mRNA levels of this protein responded the most to elevated levels of ammonia (e.g. Nawata et al., 2007; Tsui et al., 2009). Other trout Rh genes were included in the assays to evaluate whether the same principles applied, and to ensure that the protocols were working properly, but not all genes were used in all tests.

Table 1. Primer list for cloning

Name	Forward/reverse sequence (5'–3')	Accession no.
Rhag	ggagactattaccacaagcc/ctcactttccatctctagc	EF667352
Rhbg1	gaccaactcatgtgtcagcttgag/gctgccacatcctggtgtac	EF051113
Rhbg2	cgacaacgacttttactaccgc/gtacaaccaggatgtggcagc	EU660221
Rhcg1	gccgtctttctccataaggacc/ccagcaggagtcctgttagtaggacc	DQ431244
Rhcg2	gtacttactcagcctccacc/gagtgccggtgtctgttg	AY619986
Rh30-like	gacattccggttccgctag/gattggtcattgctctctgac	EF062577

### Methylamine kinetics

The methylamine uptake rates measured over 20-min incubation periods in 0.02, 0.2, 1, 2, 10 and 15 mmol l<sup>-1</sup> concentrations of methylamine were used to determine the kinetic profile. Endogenous uptake rates in control oocytes were subtracted from the test oocyte uptake values. The concentration dependence of methylamine was described in terms of  $J_{\max}$  and  $K_m$  values by using non-linear regression to fit the Michaelis–Menten equation to the experimental data using Sigma Plot version 10.0.

### NH<sub>4</sub>Cl inhibition

Total ammonia concentrations used for NH<sub>4</sub>Cl inhibition studies were verified enzymatically by measuring the formation of L-glutamate catalyzed by L-glutamate dehydrogenase, an assay linear to 600 μmol l<sup>-1</sup> with reproducibility of ±5% (Riachem, Cliniqua Corp., CA, USA) and concentrations of NH<sub>3</sub> and NH<sub>4</sub><sup>+</sup> were calculated using the Henderson–Hasselbalch equation with a pK<sub>a</sub> value of 9.25 (Cameron and Heisler, 1983) for ammonia. A pK<sub>a</sub> value of 10.66 (CRC Handbook of Chemistry and Physics, 2005) for methylamine was used to determine the unprotonated (MA) and protonated (MA<sup>+</sup>) fractions of the  $K_m$  values. The inhibition constant ( $K_i$ ) of ammonia against methylamine uptake was determined using the equation:

$$K_i = IC_{50} / (1 + c / K_m), \quad (1)$$

where IC<sub>50</sub> is the concentration of NH<sub>4</sub>Cl, NH<sub>4</sub><sup>+</sup> or NH<sub>3</sub> that reduces the uptake by 50%,  $c$  is the substrate concentration, and  $K_m$  is the substrate concentration permitting half-maximal uptake of methylamine.

### Measurements of ammonia uptake using the scanning ion electrode technique (SIET)

Transport of ammonia into or out of an oocyte produces gradients in NH<sub>4</sub><sup>+</sup> concentration in the unstirred layer adjacent to the oocyte surface. These gradients can be calculated from the voltages recorded by an NH<sub>4</sub><sup>+</sup>-selective microelectrode moved between two points within the unstirred layer. Although the great majority (>95%) of the ammonia will exist as NH<sub>4</sub><sup>+</sup> at the pH value (7.5) set by the buffer in the present experiment, it may have moved across the oocyte membrane as NH<sub>3</sub>, or as NH<sub>4</sub><sup>+</sup> or both. Thus the microelectrode can be used to measure ‘apparent NH<sub>4</sub><sup>+</sup> flux’ which is approximately equivalent to total ‘ammonia flux’, the term used here. Ammonia flux can then be calculated from the NH<sub>4</sub><sup>+</sup> concentration gradients measured in the unstirred layer using Fick’s law, as described below. Measurement of fluxes in this way is the basis of the scanning ion electrode technique (SIET), which allows fluxes to be repeatedly measured in near real-time at multiple sites on the oocyte surface. Extensive descriptions of the use of SIET are reported by Rheault and O’Donnell (Rheault and O’Donnell, 2004) and Donini and O’Donnell (Donini and O’Donnell, 2005).

SIET measurements were made using hardware from Applicable Electronics (Forestdale, MA, USA) and automated scanning electrode technique (ASET) software (version 2.0) from Science Wares Inc. (East Falmouth, MA, USA). Each oocyte was placed in a 35 mm diameter Petri dish filled with 5 ml of Na<sup>+</sup>- and K<sup>+</sup>-free ND96. Reference electrodes were made from 10 cm lengths of 1.5 mm borosilicate glass capillaries that were bent at a 45° angle, 1–2 cm from the end, to facilitate placement in the sample dish. Capillaries had been filled with boiling 3 mol l<sup>-1</sup> KCl in 3% agar and were connected to the ground input of the Applicable Electronics amplifier through a Ag/AgCl half cell.

Micropipettes for NH<sub>4</sub><sup>+</sup>-selective microelectrodes were made from 1.5 mm unfilamented borosilicate glass capillaries pulled on a Flaming–Brown P-97 pipette puller (Sutter Instruments, Novato, CA, USA) to tip diameters of 3–5 μm. The micropipettes were backfilled with 100 mmol l<sup>-1</sup> NH<sub>4</sub>Cl and tip-filled with a 200-μm long column of NH<sub>4</sub><sup>+</sup> Ionophore I, Cocktail A (Fluka, Buchs, Switzerland). This ionophore is sensitive to interference from K<sup>+</sup> and Na<sup>+</sup> and the calibration and bathing solutions were therefore based on Na<sup>+</sup>- and K<sup>+</sup>-free ND96. NH<sub>4</sub><sup>+</sup>-selective microelectrodes for use with SIET were calibrated in 0.1, 1.0 and 10 mmol l<sup>-1</sup> NH<sub>4</sub><sup>+</sup> in Na<sup>+</sup>- and K<sup>+</sup>-free ND96 resulting in a Nernstian slope of 57.2±0.3 mV log unit<sup>-1</sup> ( $n=7$ ). The NH<sub>4</sub><sup>+</sup>-selective microelectrode was initially placed 5–10 μm from the surface of the oocyte. The microelectrode was then moved a further 50 μm away, perpendicular to the oocyte surface. The ‘wait’ and ‘sample’ periods at each limit of the 50 μm excursion distance were 5.5 and 0.5 s, respectively. Voltage differences across this excursion distance were measured three times at each of four sites located 25 μm apart over the surface of the oocyte. Voltage differences were corrected for electrode drift measured at a reference site 20 mm away from the oocyte. Voltage differences ( $\Delta V$ ) were converted to the corresponding NH<sub>4</sub><sup>+</sup> concentration difference using the following equation (Donini and O’Donnell, 2005):

$$\Delta C = C_B \times 10^{(\Delta V/S)} - C_B, \quad (2)$$

where  $\Delta C$  is the NH<sub>4</sub><sup>+</sup> concentration difference between the two limits of the excursion distance (μmol cm<sup>-3</sup>),  $C_B$  is the background NH<sub>4</sub><sup>+</sup> concentration in the bathing medium,  $\Delta V$  is the voltage gradient (mV), and  $S$  is the slope of the electrode between 0.1 and 1 mmol l<sup>-1</sup> NH<sub>4</sub><sup>+</sup>. Concentration differences were used to determine the ammonia flux using Fick’s law of diffusion:

$$J_{\text{Amm}} = D_{\text{NH}_4} (\Delta C / \Delta X), \quad (3)$$

where  $J_{\text{Amm}}$  is the net flux in μmol cm<sup>-2</sup> s<sup>-1</sup>,  $D_{\text{NH}_4}$  is the diffusion coefficient of NH<sub>4</sub><sup>+</sup> (2.09×10<sup>-5</sup> cm<sup>2</sup> s<sup>-1</sup>),  $\Delta C$  is the NH<sub>4</sub><sup>+</sup> concentration gradient and  $\Delta X$  is the excursion distance between the two points (cm). Ammonia uptake rates were determined immediately after oocytes were exposed to 0.1, 0.3, 1, 3 and 10 mmol l<sup>-1</sup> NH<sub>4</sub>Cl. Non-linear regression to fit the Michaelis–Menten equation was used to determine the  $J_{\max}$  and  $K_m$  values (Sigma Plot version 10.0). The endogenous uptake measured in the H<sub>2</sub>O-injected oocytes was not subtracted from the control uptake rate as it was in the methylamine uptake kinetic analysis.

### Data analysis

All data shown are means ± s.e.m. with  $N$ =number of replicates or for SIET,  $N$ =number of oocytes. Statistical significance was determined by Student’s unpaired  $t$ -test followed by Bonferonni adjustment using Systat version 10.0.  $\alpha$  was set at 0.05.

## RESULTS

### Methylamine uptake

Methylamine uptake rates were measured in 20 μmol l<sup>-1</sup> methylamine over a period of 60 min in control oocytes and in oocytes expressing Rhag, Rhcg2, Rbhg1 and Rh30-like proteins. The uptake rates of the Rh30-like-injected oocytes and the control oocytes were not significantly different from each other (Fig. 1). However, expression of Rhag, Rbhg1 or Rhcg2 enhanced the uptake rate when compared with those of the control and Rh30-like-expressing oocytes. Rhag-expressing oocytes maintained an uptake rate that was 6-fold greater than that of the control oocytes throughout the time course. The rate in Rbhg1-expressing oocytes

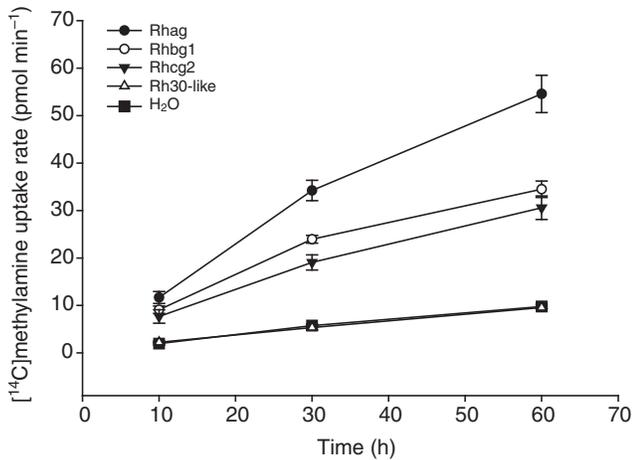


Fig. 1. Rh-facilitated methylamine uptake. Time course of [ $^{14}\text{C}$ ]methylamine uptake ( $\text{pmol oocyte}^{-1}$ ) measured in Rhag-, Rhbg1-, Rhcg2-, Rh30-like- and  $\text{H}_2\text{O}$ -injected control oocytes at pH 7.5. Uptake in Rh30-like-injected oocytes was not significantly different from that of control oocytes. In each case, the concentration of methylamine was  $20 \mu\text{mol l}^{-1}$ . Data shown are means  $\pm$  s.e.m. ( $N=3$ ) for groups of three oocytes.

was 4.5-fold higher than in the control oocytes at 10 min and 3.5-fold higher at 60 min. Similarly, the rate in Rhcg2-expressing oocytes was 4-fold higher than the control oocytes at 10 min and 3-fold higher at 60 min.

#### Kinetics of methylamine uptake

The rate of methylamine uptake was measured in oocytes expressing Rhag, Rhbg1, Rhbg2 and Rhcg2 over a range of methylamine concentrations ( $0.02\text{--}15 \text{ mmol l}^{-1}$ ) at pH 7.5. Endogenous uptake rates measured in control oocytes were subtracted from test oocyte

uptake rates. Uptake rates were saturable as a function of methylamine concentration. The  $J_{\text{max}}$  values (in  $\text{pmol oocyte}^{-1} \text{ min}^{-1}$ ) were:  $191.1 \pm 36.0$  (Rhag),  $106.1 \pm 15.0$  (Rhbg1),  $87.4 \pm 11.3$  (Rhbg2) and  $194.7 \pm 35.9$  (Rhcg2), with the values for Rhag and Rhcg2 being significantly greater than for the other two. The respective concentrations permitting half-maximal uptake ( $K_{\text{m}}$ ) were:  $7.8 \pm 3.4$  (Rhag),  $6.8 \pm 2.4$  (Rhbg1),  $4.6 \pm 1.7$  (Rhbg2) and  $8.9 \pm 3.6 \text{ mmol l}^{-1}$  (Rhcg2), none of which were significantly different from the others (Fig. 2). To test the effect of pH on the uptake kinetics of methylamine, we performed the same test on Rhbg1 and Rhcg2-expressing oocytes, at pH 8.5. There was some variability in the responses at the highest methylamine concentrations tested, but overall the results again indicated saturating relationships (Fig. 3). Compared to the  $J_{\text{max}}$  values obtained at pH 7.5, the values at pH 8.5 increased significantly by about 4-fold in Rhbg1-expressing oocytes ( $440.8 \pm 69.9 \text{ pmol oocyte}^{-1} \text{ min}^{-1}$ ) and about 3-fold in Rhcg2-expressing oocytes ( $663.6 \pm 102.7 \text{ pmol oocyte}^{-1} \text{ min}^{-1}$ ), whereas the  $K_{\text{m}}$  values did not change significantly when expressed as total or protonated methylamine (Fig. 3, Table 2). However,  $K_{\text{m}}$  values increased 8- to 10-fold (significant for Rhcg2 only) when expressed as the unprotonated form (Table 2).

#### pH-sensitive transport

The apparent sensitivity of methylamine uptake to pH led us to a more in-depth investigation of this observation. Methylamine uptake rates in control oocytes and those expressing Rhag, Rhbg1, Rhbg2, Rhcg1 and Rhcg2, were measured for 60 min in uptake buffers at a substrate level of  $20 \mu\text{mol l}^{-1}$  set at pH 6.5, 7.5 and 8.5. The substrate concentration of  $20 \mu\text{mol l}^{-1}$  was chosen since saturation of transport did not occur over time at this concentration (see Fig. 1). Uptake rates of all Rh-expressing oocytes decreased at pH 6.5 and increased at pH 8.5 when compared with rates at pH 7.5 (Fig. 4). Notably, the rates in Rhag- and Rhcg2-expressing oocytes were 3.5-fold higher at pH 8.5 than the rates at pH 7.5 and the rate in

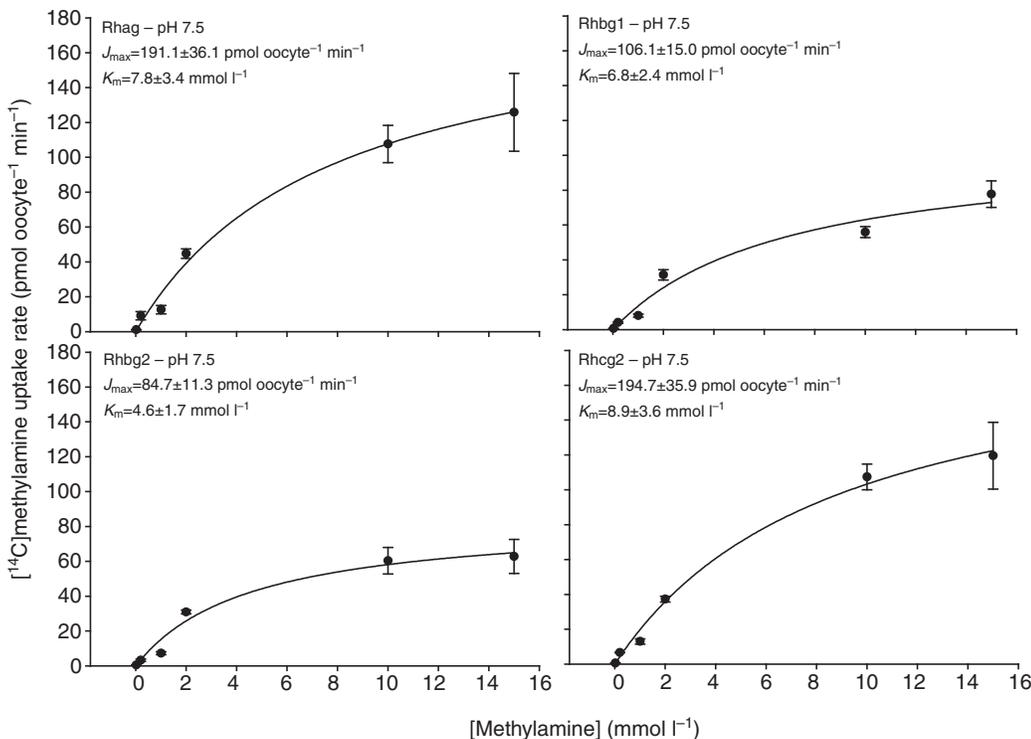


Fig. 2. Methylamine uptake kinetics at pH 7.5. Uptake rates ( $J_{\text{max}}$ :  $\text{pmol oocyte}^{-1} \text{ min}^{-1}$ ) of [ $^{14}\text{C}$ ]methylamine in Rhag-, Rhbg1, Rhbg2- and Rhcg2-expressing oocytes were measured over a concentration range of  $0.02\text{--}15 \text{ mmol l}^{-1}$  methylamine set at pH 7.5.  $\text{H}_2\text{O}$ -injected control oocytes were run in parallel and control uptake values were subtracted from test oocyte values. Values are means  $\pm$  s.e.m. ( $N=3$ ) for groups of three oocytes.

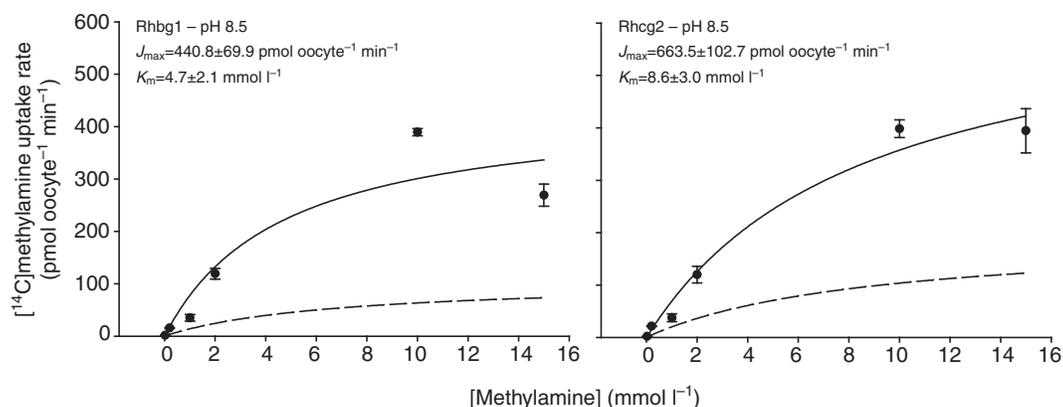


Fig. 3. Methylamine uptake kinetics at pH 8.5. Uptake rates ( $\text{pmol oocyte}^{-1} \text{min}^{-1}$ ) of  $[^{14}\text{C}]$ methylamine in Rbhg1- and Rhcg2-expressing oocytes were measured over a concentration range of 0.02–15  $\text{mmol l}^{-1}$  methylamine set at pH 8.5.  $\text{H}_2\text{O}$ -injected control oocytes were run in parallel and control uptake values were subtracted from test oocyte values. Dashed lines represent the corresponding uptake rates at pH 7.5. Values are means  $\pm$  s.e.m. ( $N=3$ ) for groups of three oocytes.

Rbhg1-expressing oocytes was over 4-fold greater at pH 8.5 than at pH 7.5. The rates were significantly higher at pH 7.5 than at pH 6.5 for Rhag-, Rbhg1-, Rbhg2- and Rhcg2-expressing oocytes.

To further test the pH dependence of methylamine transport, Rhag-, Rbhg2-, and Rhcg2-expressing oocytes were acidified following established methods (Tsai et al., 1995; Westhoff et al., 2002). Oocytes were incubated in sodium acetate at pH 6.8 or pH 6.4 for 25 min, washed once in ice-cold unlabeled uptake buffer and transferred to  $[^{14}\text{C}]$ methylamine uptake buffer (20  $\mu\text{mol l}^{-1}$  methylamine, pH 7.5) for 15 min. Uptake rates from controls were subtracted from the rates measured in the acidified and untreated oocytes. Control oocytes had an average intracellular pH of  $7.29 \pm 0.09$  and therefore the intracellular to extracellular pH difference in untreated oocytes was approximately 0.2. Intracellular acidification at both pH 6.4 and 6.8 resulted in a 2-fold increase in uptake in Rbhg2- and Rhcg2-expressing oocytes and a 4- to 5-fold increase in uptake in Rhag-expressing oocytes, when compared with the rate in unacidified oocytes (Fig. 5). Additionally, the uptake rate in Rhag-expressing oocytes acidified at pH 6.4 was significantly higher than those acidified at pH 6.8.

#### Efflux of methylamine

To determine whether or not Rh proteins facilitate bi-directional methylamine transport, we measured the efflux of methylamine from control oocytes and Rhag-, Rbhg1-, Rhcg2-expressing oocytes. Oocytes were incubated in  $[^{14}\text{C}]$ methylamine (20  $\mu\text{mol l}^{-1}$ ) uptake buffer at pH 8.5 for 60 min and then quickly washed with ice-cold, unlabeled uptake buffer three times before being transferred into unlabeled uptake buffer without methylamine at pH 8.5 or pH 6.5

for 15 min. Radioactivity in the oocytes and buffer was counted separately. Efflux rates were expressed as the percentage of the total initial radioactivity in the oocytes that appeared in the buffer during the 15 min efflux period. Control oocytes released a similar amount of methylamine (5% and 4%) at pH 6.5 and 8.5 (Fig. 6). Effluxes from Rhag-, Rbhg1- and Rhcg2-expressing oocytes at pH 6.5 (22%, 14% and 17%, respectively) and at pH 8.5 (7%, 8% and 9%, respectively) were significantly greater than those from the control oocytes. Additionally, significantly greater effluxes were observed at pH 6.5 than at pH 8.5 in all the Rh-expressing oocytes.

#### Inhibition by $\text{NH}_4\text{Cl}$

Next, we examined the kinetics of methylamine uptake in the presence of  $\text{NH}_4\text{Cl}$  in order to characterize the substrate specificity of the Rh proteins. Methylamine uptake was measured in Rbhg1- and Rhcg2-expressing oocytes at a constant methylamine concentration of 20  $\mu\text{mol l}^{-1}$  in the presence of increasing concentrations of total ammonia (80–3500  $\mu\text{mol l}^{-1}$ ), which takes into account the background total ammonia concentration of 80  $\mu\text{mol l}^{-1}$  in the oocyte bath medium (Fig. 7). External buffer pH was set to either 7.5 or 8.5 and control oocyte uptake rates were subtracted from test oocyte uptake rates. The  $K_i$  values measured at pH 7.5 were similar for Rbhg1- and Rhcg2-expressing oocytes at  $2.45 \pm 0.31$  and  $2.53 \pm 0.21$   $\text{mmol l}^{-1}$ , respectively (expressed as total ammonia). The values measured at pH 8.5 were also similar with  $1.61 \pm 0.04$   $\text{mmol l}^{-1}$  for Rbhg1 and  $1.55 \pm 0.06$   $\text{mmol l}^{-1}$  for Rhcg2 (Fig. 6). Compared with the values at pH 7.5, these  $K_i$  values at pH 8.5 were moderately reduced by 35–40% when expressed as total ammonia or  $\text{NH}_4^+$ ,

Table 2. Protonated and unprotonated fractions of  $K_m$  for methylamine and  $K_i$  values for ammonia as an inhibitor of methylamine uptake at pH 7.5 and 8.5

	pH	$K_m$			$K_i$		
		MA/MA <sup>+</sup> ( $\mu\text{mol l}^{-1}$ )	MA <sup>+</sup> ( $\mu\text{mol l}^{-1}$ )	MA ( $\mu\text{mol l}^{-1}$ )	$\text{NH}_3/\text{NH}_4^+$ ( $\mu\text{mol l}^{-1}$ )	$\text{NH}_4^+$ ( $\mu\text{mol l}^{-1}$ )	$\text{NH}_3$ ( $\mu\text{mol l}^{-1}$ )
Rbhg1	7.5	6822.0 $\pm$ 2368.8	6817.3 $\pm$ 2367.2	4.7 $\pm$ 1.6	2447.5 $\pm$ 30.5	2414.0 $\pm$ 30.5	38.1 $\pm$ 30.5
	8.5	4659.8 $\pm$ 2089.3	4627.8 $\pm$ 2075.0	32.0 $\pm$ 14.4	1607.1 $\pm$ 4.3*	1382.1 $\pm$ 4.3*	218.4 $\pm$ 4.3*
Rhcg2	7.5	8849.9 $\pm$ 3600.9	8843.8 $\pm$ 3598.4	6.1 $\pm$ 2.5	2524.8 $\pm$ 20.5	2478.7 $\pm$ 20.5	39.2 $\pm$ 20.5
	8.5	8566.5 $\pm$ 2969.1	8507.6 $\pm$ 2948.7	58.9 $\pm$ 20.4*	1552.8 $\pm$ 5.8*	1340.9 $\pm$ 5.8*	212.1 $\pm$ 5.8*

MA, unprotonated methylamine; MA<sup>+</sup>, protonated methylamine.

Asterisks indicate significant differences from the corresponding pH 7.5 values. Values are means  $\pm$  s.e.m. ( $N=3$ ) for groups of three oocytes.

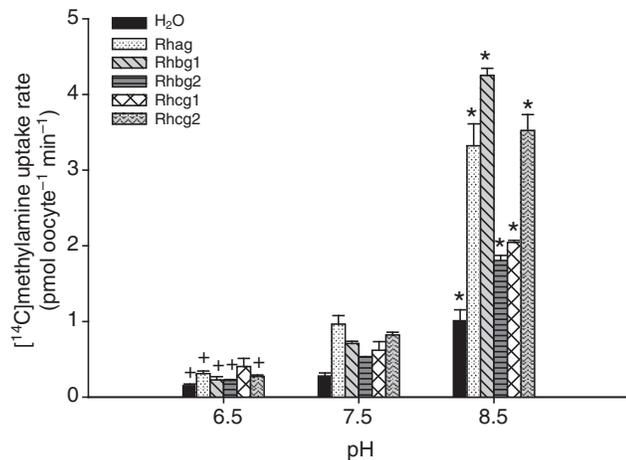


Fig. 4. The effect of external pH on methylamine uptake. [ $^{14}\text{C}$ ]methylamine uptake rates ( $\text{pmol oocyte}^{-1} \text{min}^{-1}$ ) were measured in  $\text{H}_2\text{O}$ -, Rhag-, Rhbg1-, Rhbg2-, Rhcg1- and Rhcg2-expressing oocytes for 60 min at an external pH of 6.5, 7.5 and 8.5. In each case, the concentration of methylamine was  $20 \mu\text{mol l}^{-1}$ . Asterisks indicate significant differences between the rates at pH 8.5 and those at pH 6.5 and pH 7.5. Crosses indicate significant differences between pH 6.5 and pH 7.5. Values are means  $\pm$  s.e.m. ( $N=3$ ) for groups of three oocytes.

but greatly raised, by 5- to 6-fold, when expressed as  $\text{NH}_3$  (Table 2). Both of these changes were significant.

#### Ammonia uptake measured by SIET

We used SIET to further verify whether or not ammonia is a true substrate of the Rh transporters. Ammonia uptake rates were measured in control oocytes and oocytes expressing Rhag, Rhcg2 and Rh30-like proteins exposed to  $100 \mu\text{mol l}^{-1}$   $\text{NH}_4\text{Cl}$  at pH 7.5. In Fig. 8, SIET rates have been expressed both in traditional units ( $\text{pmol cm}^{-2} \text{s}^{-1}$ ) and in  $\text{pmol oocyte min}^{-1}$  by taking into account the surface area of the oocytes ( $\sim 0.031 \text{ cm}^2$ ). All oocytes took up ammonia but the rates in Rhag- and Rhcg2-expressing oocytes ( $18.5 \pm 1.1$  and  $17.4 \pm 0.5 \text{ pmol oocyte min}^{-1}$ , respectively) were significantly higher, by 1.5-fold, than the rate in control oocytes ( $12.0 \pm 0.6 \text{ pmol oocyte min}^{-1}$ ). Notably, oocytes expressing Rh30-like protein exhibited no increase in ammonia uptake rate, consistent with the findings for methylamine uptake (Fig. 1). To compare ammonia uptake rates with those of methylamine, the [ $^{14}\text{C}$ ]methylamine uptake rates of  $\text{H}_2\text{O}$ -, Rhag- and Rhcg2-injected oocytes at a methylamine concentration of  $100 \mu\text{mol l}^{-1}$  were calculated from the Michaelis–Menten curves generated earlier (Fig. 2). Methylamine uptake rates were much lower than the ammonia uptake rates (10-fold lower in  $\text{H}_2\text{O}$ -injected oocytes and about 4-fold lower in Rhag and Rhcg2-expressing oocytes).

#### Kinetics of ammonia uptake measured by SIET

Finally, we measured the kinetics of ammonia uptake using SIET. Oocytes expressing Rhcg2 and control oocytes were exposed to increasing concentrations of  $\text{NH}_4\text{Cl}$  ( $0.1$ – $10 \text{ mmol l}^{-1}$ ) at pH 7.5 (Fig. 9). Uptake of ammonia by Rhcg2-expressing oocytes displayed saturation kinetics with a  $J_{\text{max}}$  of  $63.3 \pm 0.6 \text{ pmol cm}^{-2} \text{ s}^{-1}$ , or  $118 \pm 1 \text{ pmol oocyte min}^{-1}$ . The latter was not significantly different from the  $J_{\text{max}}$  value measured earlier for methylamine ( $195 \pm 36 \text{ pmol oocyte min}^{-1}$ ; Fig. 2).  $K_{\text{m}}$  values (in  $\text{mmol l}^{-1}$ ) were

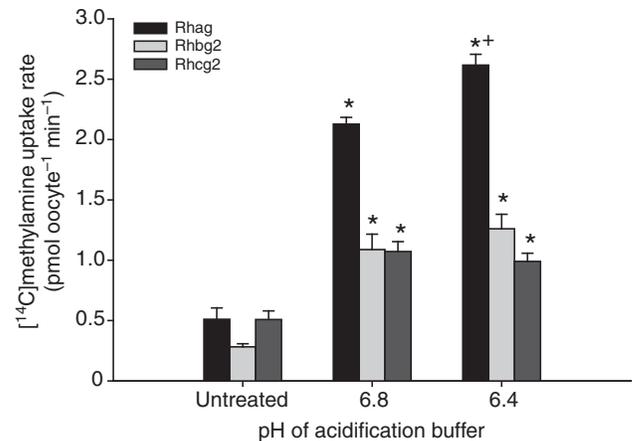


Fig. 5. The effect of intracellular acidification on methylamine uptake. [ $^{14}\text{C}$ ]methylamine uptake ( $\text{pmol oocyte}^{-1} \text{min}^{-1}$ ) was measured in Rhag-, Rhbg2-, Rhcg2-expressing oocytes that were acidified in sodium acetate (pH 6.4 or 6.8) for 25 min. Untreated oocytes (intracellular pH  $7.29 \pm 0.09$ ) were not incubated in sodium acetate. In each case, the concentration of methylamine was  $20 \mu\text{mol l}^{-1}$ . Asterisks indicate a significant increase in uptake rates in the acidified oocytes compared with the untreated oocytes. The cross indicates a significant difference between the uptake rates at pH 6.4 and pH 6.8 in Rhag-expressing oocytes.  $\text{H}_2\text{O}$ -injected control oocytes were run in parallel and control uptake values were subtracted from test oocyte values. Values are means  $\pm$  s.e.m. ( $N=3$ ) for groups of three oocytes.

$0.56 \pm 0.26$  for  $\text{NH}_4\text{Cl}$ ,  $0.55 \pm 0.26$  for  $\text{NH}_4^+$ , and  $0.010 \pm 0.005$  for  $\text{NH}_3$ . These were much lower than the respective values for total and protonated methylamine, but comparable for unprotonated methylamine (Table 2). Uptake by control oocytes saturated much later with a  $J_{\text{max}}$  of  $137.5 \pm 17.3 \text{ pmol cm}^{-2} \text{ s}^{-1}$  and  $K_{\text{m}}$  values (in  $\text{mmol l}^{-1}$ ) of  $5.6 \pm 1.5$ ,  $5.5 \pm 1.5$ , and  $0.09 \pm 0.03$  for  $\text{NH}_4\text{Cl}$ ,  $\text{NH}_4^+$ , and  $\text{NH}_3$ , respectively. The  $J_{\text{max}}$  value ( $137.5 \pm 17.3 \text{ pmol cm}^{-2} \text{ s}^{-1}$ ) was also significantly higher. There were no significant differences in the uptake rates between the control oocytes and the Rhcg2-expressing oocytes at 0.1 and 0.3  $\text{mmol l}^{-1}$ ; however, at 1  $\text{mmol l}^{-1}$ , the uptake rate was significantly higher (3-fold) in the Rhcg2-expressing oocytes than in the control oocytes. At 10  $\text{mmol l}^{-1}$ , the uptake rate in Rhcg2-expressing oocytes was significantly lower (1.8-fold) than that in the control oocytes.

#### DISCUSSION

The recent addition of the Rh proteins to the ammonia transporter superfamily has sparked renewed interest in the area of ammonia transport in fish (Perry et al., 2009; Weihrauch et al., 2009; Wright and Wood, 2009). Indeed there is increasing evidence that Rh proteins are involved in the gill ammonia transport mechanism(s). We observed earlier that experimental elevations in plasma ammonia in rainbow trout resulted in enhanced ammonia excretion as well as an upregulation of gill Rhcg2 mRNA levels (Nawata et al., 2007; Nawata and Wood, 2009). In light of these findings, the aim of our present study was to characterize the functional properties of trout Rh proteins in an effort to understand what role these proteins may play in gill ammonia transport.

With one exception (Rh30-like protein), all trout Rh proteins expressed in *Xenopus* oocytes facilitated uptake of the ammonia analogue, methylamine, with rates 3- to 6-fold greater than that seen in control oocytes (Fig. 1). The uptake was also saturable, suggesting a carrier-mediated process (Figs 2, 3). This is in accordance with previous studies which showed that human, murine and pufferfish

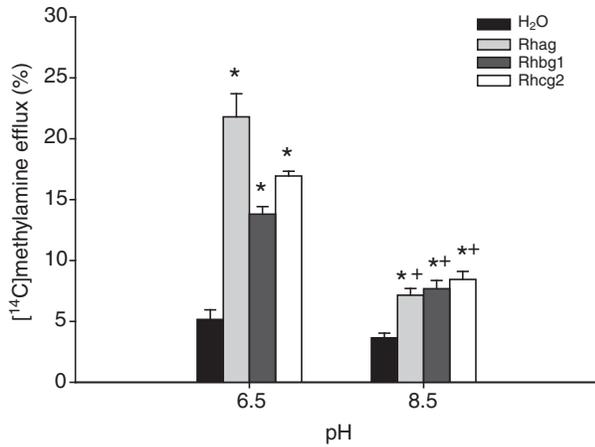


Fig. 6. Methylamine efflux. H<sub>2</sub>O-, Rhag-, Rhbg- and Rhcg2-injected oocytes were incubated for 60 min in buffer containing 20 μmol l<sup>-1</sup> [<sup>14</sup>C]methylamine at pH 8.5. Oocytes were then washed and added to fresh buffer containing 0 μmol l<sup>-1</sup> methylamine at pH 6.5 and 8.5. Radioactivity in the buffer and oocytes was counted separately. Results are expressed as the percentage radioactivity that appeared in the buffer after 15 min. Asterisks indicate significantly lower methylamine release from H<sub>2</sub>O-injected oocytes compared with Rhag-, Rhbg1- and Rhcg2-injected oocytes. Crosses indicate significantly lower efflux at pH 8.5 compared with pH 6.5. Values are means ± s.e.m. (N=3) for groups of three oocytes.

Rh proteins expressed in *Xenopus* oocytes also facilitated methylamine uptake (Ludewig, 2004; Mak et al., 2006; Mayer et al., 2006; Nakada et al., 2007a; Westhoff et al., 2002). Interestingly, all transport rates at 1 mmol l<sup>-1</sup> appeared to fall below the curve. Whether or not this is random variation or a true characteristic of the trout Rh proteins is not clear but this was not due to systematic error since experiments were conducted at different times with fresh solutions. However, it could represent an additional transport

system of lower affinity and higher capacity superimposed on a higher affinity, lower capacity system. Further experiments using a series of low methylamine concentrations would be needed to evaluate this possibility.

More importantly we showed, for the first time in any system, by direct measurement using SIET that these proteins also facilitated the uptake of ammonia, the purported natural substrate. Ammonia has also been demonstrated to be the natural substrate for RhAG expressed in MEP-deficient yeast (Marini et al., 2000). At least for the one protein (Rhcg2) tested in detail with this more difficult approach, the uptake was saturable with a *K<sub>m</sub>* (560 μmol l<sup>-1</sup>) within the physiological range for total ammonia in trout (Fig. 9), and considerably below the *K<sub>m</sub>* for the analogue methylamine (8850 μmol l<sup>-1</sup>; Fig. 2, Table 2). When compared at the same low substrate concentration, these proteins facilitated a greater transport rate of ammonia than of methylamine (Fig. 8). Furthermore NH<sub>4</sub>Cl inhibited methylamine uptake with *K<sub>i</sub>* values for total ammonia which were also considerably lower than the *K<sub>m</sub>* values for methylamine. Overall, we believe these data provide strong evidence that these Rh proteins are important in facilitating ammonia transport across cell membranes, such as those in the gill epithelium.

The trout Rh30-like protein did not enhance methylamine uptake (Fig. 1) or ammonia uptake (Fig. 8). Similarly, human Rh30 proteins (RhD and RhCE) which are present in the erythrocyte membrane do not facilitate methylamine or ammonia transport (Ripoche et al., 2004; Westhoff and Wylie, 2006). Homology modeling of the human Rh proteins and AmtB has shown that several residues in the channel differ between AmtB, RhAG, RhBG, RhCG and the Rh30 (RhD, RhCE) proteins, and could possibly explain the difference in transport properties (Zidi-Yahiaoui et al., 2009).

There was enhanced uptake of methylamine into Rh-expressing oocytes at an external alkaline pH and a reduction in uptake rate at an external acidic pH (Fig. 5). The same effect of pH on uptake rates has been seen in studies on human RhAG, RhBG, RhCG (Ludewig, 2004; Mayer et al., 2006; Westhoff et al., 2002), murine

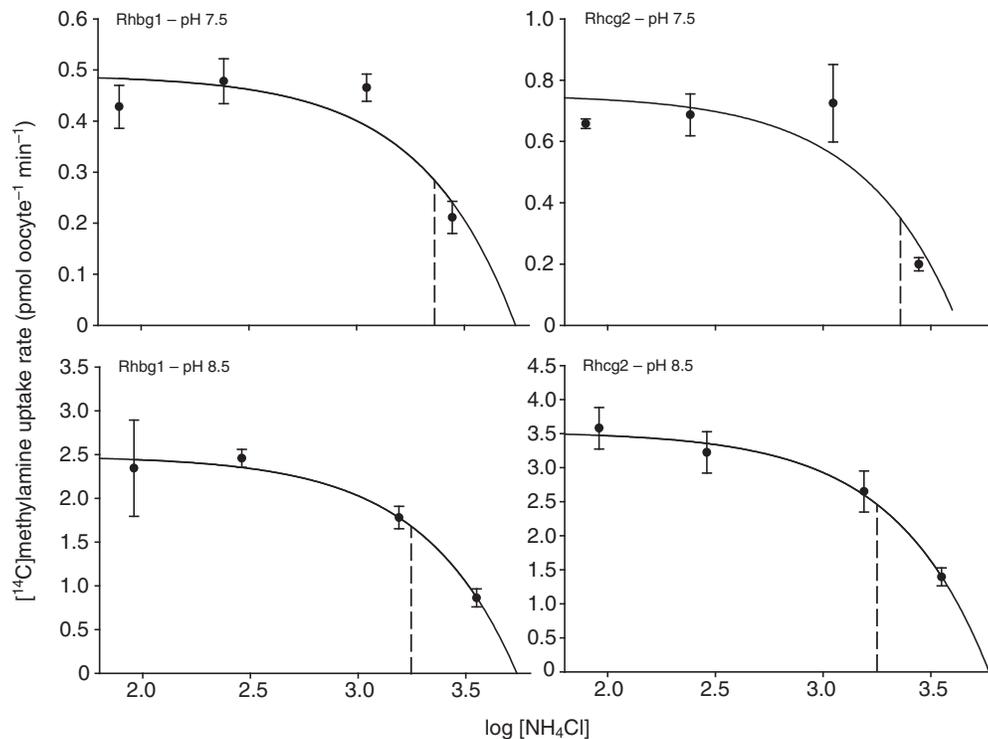


Fig. 7. Inhibition of methylamine uptake with NH<sub>4</sub>Cl (μmol l<sup>-1</sup>). [<sup>14</sup>C]methylamine concentration was held constant at 20 μmol l<sup>-1</sup>. Uptake rates (pmol oocyte<sup>-1</sup> min<sup>-1</sup>) of Rhbg1- and Rhcg2-expressing oocytes were measured in the presence of total ammonia concentrations ranging from 80 to 3500 μmol l<sup>-1</sup> at an external pH of 7.5 and 8.5. H<sub>2</sub>O-injected control oocytes were run in parallel and control uptake values were subtracted from test oocyte values. Dashed lines represent the IC<sub>50</sub> values. Corresponding *K<sub>i</sub>* values are listed in Table 2. Values are means ± s.e.m. (N=3) for groups of three oocytes.

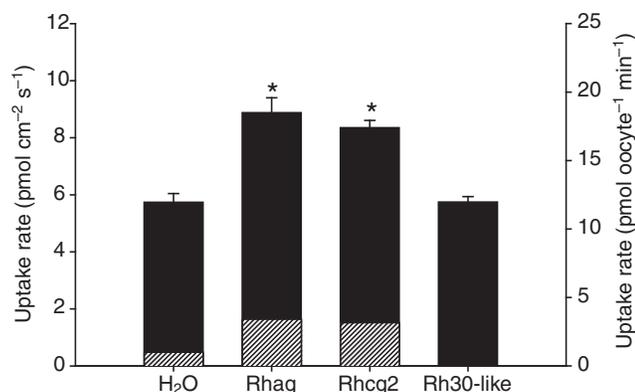


Fig. 8. Ammonia uptake. The uptake rate of ammonia in  $\text{pmol cm}^{-2} \text{s}^{-1}$  (left axis) and  $\text{pmol oocyte}^{-1} \text{min}^{-1}$  (right axis) measured with the scanning ion electrode technique (SIET) in  $\text{H}_2\text{O}$ -, Rhag-, Rhcg2- and Rh30-like-injected oocytes exposed to  $100 \mu\text{mol l}^{-1}$   $\text{NH}_4\text{Cl}$ . Asterisks indicate significant differences from the ammonia uptake rates in  $\text{H}_2\text{O}$ -injected oocytes. Data are means  $\pm$  s.e.m. ( $N=6-10$ ). Inset hatched bars represent corresponding [<sup>14</sup>C]methylamine uptake rates of  $\text{H}_2\text{O}$ -, Rhag-, Rhcg2-injected oocytes at a methylamine concentration of  $100 \mu\text{mol l}^{-1}$ .

Rhcg and Rhbg (Mak et al., 2006), as well as AmtB (Javelle et al., 2005). At least for Rhag, it appeared that the transport rate was not only sensitive to the direction of the pH gradient, but also to the magnitude of the gradient, with greater uptake observed at pH 6.4 than at pH 6.8 (Fig. 4). A simple explanation for these observations is that the transport rate depends upon the concentration of the unprotonated species (MA) since a one-unit pH change would increase or decrease the MA concentration by 10-fold. However, the situation is not that simple. If the transport rate depended only on the concentration of MA, we would expect to see approximately equivalent increases and decreases in rate, since these assays were run at a total methylamine concentration of  $20 \mu\text{mol l}^{-1}$ , right at the bottom of the kinetic uptake curves (Fig. 2). However, despite a 10-fold decrease in MA from pH 7.5 to pH 6.5 and 10-fold increase in MA from pH 7.5 to pH 8.5, the uptake rates observed in Rh-expressing oocytes did not change by a similar amount. Rather, the changes were in the order of 3- to 5-fold per pH unit (Fig. 4). The explanation for this probably lies in the fact that the position of the kinetic curves was not constant, but also shifted with pH (Fig. 3). Indeed, based on the observed changes in the  $K_m$  and  $J_{\text{max}}$  values (Fig. 3, Table 2), we can calculate for Rhbg1 and Rhcg2 that at a total methylamine concentration of  $20 \mu\text{mol l}^{-1}$ , the transport rate would have increased by 3- to 5-fold as pH was increased from 7.5 to 8.5, exactly as was observed (Fig. 3). It is important to note that this conclusion is independent of whether the substrate is considered to be MA or  $\text{MA}^+$ . If MA is the true substrate, the fact that the increase in transport rate was only 3- to 5-fold rather than 10-fold is because the affinity decreased markedly at higher pH (i.e.  $K_m$  expressed as MA increased 6- to 10-fold; Table 2). If  $\text{MA}^+$  is the true substrate, then  $K_m$  did not change appreciably at higher pH (Table 2), and the fact that transport rate increases 3- to 5-fold is entirely due to the observed increase in  $J_{\text{max}}$ . It is unclear why  $K_m$  should change with pH, so a conservative conclusion is that  $\text{MA}^+$  is the species that binds initially to the transporter, but the transport itself is sensitive to a pH gradient.

Similar conclusions may be drawn from the inhibition studies. When pH was increased from 7.5 to 8.5, the  $K_i$  for  $\text{NH}_3$  increased 5- to 6-fold, whereas the  $K_i$  for  $\text{NH}_4^+$  changed only moderately

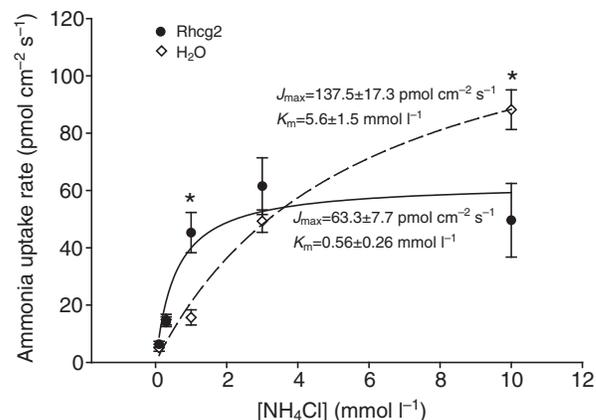


Fig. 9. Kinetics of ammonia uptake. The uptake of ammonia ( $\text{pmol cm}^{-2} \text{s}^{-1}$ ) was measured with the scanning ion electrode technique (SIET) in Rhcg2- and  $\text{H}_2\text{O}$ -injected oocytes over the concentration range of 0.1 to  $10 \text{ mmol l}^{-1}$   $\text{NH}_4\text{Cl}$ . Asterisks indicate significant differences between the uptake rates in the  $\text{H}_2\text{O}$ -injected oocytes and the Rhcg2-expressing oocytes at the corresponding  $\text{NH}_4\text{Cl}$  concentration. Data are means  $\pm$  s.e.m. ( $N=5-8$ ).

(decreases of 35–40%). While the  $K_i$  values for  $\text{NH}_4^+$  were lower than the  $K_m$  values of  $\text{MA}^+$ , the  $K_i$  values for  $\text{NH}_3$  were higher than the  $K_m$  values of MA (Table 2). Why the natural substrate should have a higher  $K_i$  than the analogue  $K_m$ , and why the value should change greatly with pH is again unclear, but supports the conclusion that the protonated species binds to the transporter. Importantly, however, this does not indicate whether it is the protonated or unprotonated form that is actually transported.

Overall, this argues against  $\text{NH}_3$  as the species binding to the Rh proteins, in accordance with conclusions made previously regarding mammalian RhAG, Rhbg and Rhcg (Ludewig, 2004; Mak et al., 2006; Mayer et al., 2006; Westhoff et al., 2002). Indeed in a physiological context this is reasonable, considering that the majority of ammonia (>95%) in fish plasma is present in the protonated form (Wood, 1993). If this is the case, then why does the pH gradient have a substantial influence on Rh protein-mediated methylamine transport in the oocyte expression system (Figs 3, 4, 5 and 6)? Similarly, why does the pH gradient influence ammonia efflux across cultured branchial epithelia *in vitro* (Kelly and Wood, 2001) and trout gills *in vivo* (Wright and Wood, 1985; Wilson et al., 1994; Wright et al., 1986; Wright et al., 1989)? We suggest that this is because the actual species moving through the Rh channel is the unprotonated form, such that both the upstream de-protonation reaction and the downstream re-protonation reaction ('acid trapping') are a function of the ambient pH. This is almost impossible to distinguish from  $\text{NH}_4^+/\text{H}^+$  exchange, and functionally the two processes would be the same in their net effect (Ludewig, 2004) and therefore we cannot rule out the possibility that  $\text{NH}_4^+$  or that both  $\text{NH}_3$  and  $\text{NH}_4^+$  pass through the channel. Indeed, from the numerous functional studies on Rh proteins there is considerable disagreement about the mechanism involved (see Javelle et al., 2007).

Not only was methylamine uptake facilitated in Rh-expressing oocytes, but enhanced efflux was also observed, and this was similarly dependent upon a pH gradient with the greatest efflux rate observed at a low external pH (Fig. 5). Bi-directionality has also been demonstrated in yeast expressing RhAG and RhCG (Marini et al., 2000; Mayer et al., 2006; Westhoff et al., 2004) or Rh50 from

*Nitrosomonas europaea* (Weidinger et al., 2007). This property would be particularly beneficial for erythrocytes. Rhag in erythrocytes could facilitate ammonia loading from the tissues and the subsequent unloading of ammonia at the gill where it can ultimately be eliminated into the external water, as depicted in the model of Wright and Wood (Wright and Wood, 2009). A bi-directional function, however, appears counterintuitive in the gill where unidirectional flow or excretion of ammonia is necessary. Nevertheless, the data of Tsui et al. (Tsui et al., 2009) suggest that the upregulation of Rh proteins in the cultured trout gill epithelium *in vitro* conferred a bi-directional increase in ammonia permeability. An acidified gill boundary layer has long been thought to aid in ammonia excretion *in vivo* (Wilson et al., 1994; Wright et al., 1986; Wright et al., 1989). This property would also create a favorable pH gradient for optimal transport by the Rh proteins and facilitate vectorial ammonia movement out of the gill. At the same time, this acidified layer would form an inwardly unfavorable  $H^+$  gradient that could potentially slow the influx of ammonia into the gill through the Rh channels when external ammonia concentrations are elevated. In fact, we demonstrated earlier that when the acidified boundary layer in trout was abolished with Hepes, there was a downregulation of Rhcg2 mRNA in the gill and a reduction in ammonia excretion (Nawata and Wood, 2008). Therefore, one possible regulatory mechanism conferring unidirectionality in trout gill Rh proteins may be the pH or a  $H^+$  gradient. Models have been recently proposed wherein functional coupling of Rh proteins to apical  $H^+$ -ATPase pumps and/or  $Na^+/H^+$  exchange mechanisms facilitate ammonia trapping in an acidic gill boundary layer (Tsui et al., 2009; Wright and Wood, 2009).

Amt and Rh proteins share a common ancestor and coexist in many organisms, but higher vertebrates have retained only the Rh genes. This suggests that the Amt and Rh proteins have evolved different functions (Huang, 2008). Indeed structural studies revealed differences between the Rh and Amt proteins, especially in the external vestibule where critical residues thought to be essential for  $NH_4^+$  binding are lacking or are not conserved in the Rh proteins (Lupo et al., 2007; Zidi-Yahiaoui et al., 2009). These differences may reflect different ammonia transport requirements. Bacteria need to assimilate ammonia from very low micromolar environmental concentrations, thus necessitating a high affinity  $NH_4^+$  trapping mechanism. Mammals and fish, on the other hand, must dispose of ammonia, which is normally present in the millimolar range in the mammalian renal system (Knepper et al., 1989) and in the high micromolar range in fish plasma (Wood, 1993), and therefore high affinity  $NH_4^+$  trapping would be less critical. In fact, AmtB proteins are only induced in bacteria when external ammonia concentrations are limiting (Javelle et al., 2004). However, in the rainbow trout, Rh transcripts were detectable under control conditions but were upregulated when plasma ammonia concentrations were elevated (Nawata et al., 2007; Nawata and Wood, 2009). These differences are also reflected in the different binding affinities for methylamine. For the trout Rh proteins the  $K_m$  values ranged from 4.6 to  $8.9 \text{ mmol l}^{-1}$ , whereas the  $K_m$  for AmtB was reported to be  $200 \mu\text{mol l}^{-1}$  (Merrick et al., 2001).

The concentration of  $NH_4Cl$  required to inhibit the uptake of methylamine was similar for both Rhbg and Rhcg2 ( $2.5 \text{ mmol l}^{-1}$  at pH 7.5 and  $1.6 \text{ mmol l}^{-1}$  at pH 8.5, Table 2). This suggests that these two proteins have a similar affinity for ammonia. Murine Rhbg, however, had a much higher affinity for both methylamine and ammonia than Rhcg, with  $K_i$  values for  $NH_4Cl$  of  $0.5 \text{ mmol l}^{-1}$  for Rhbg versus  $2.9 \text{ mmol l}^{-1}$  for Rhcg (Mak et al., 2006). It was proposed that in the mammalian kidney, a higher affinity basolateral

Rhbg and a lower affinity apical Rhcg in the collecting duct could facilitate the vectorial transport of ammonia from the interstitium to the lumen where there is an increasing ammonia gradient (Mak et al., 2006; Westhoff and Wylie, 2006). In fish, it is not certain whether all species and all types of gill cells have an apical Rhcg and a basolateral Rhbg. Although this is the case in pufferfish gill pavement cells, it is not the case in pufferfish gill mitochondria-rich cells (Nakada et al., 2007a). Moreover, it was reported recently that Rhcg is present both apically and basolaterally in the mouse kidney (Kim et al., 2009). In the trout gill, favorable plasma-to-water ammonia and pH gradients, plus the high intracellular ammonia levels and low extracellular ammonia levels (Wood, 1993), may be more important in regulating unidirectional flow than the differential binding affinities of basolateral and apical Rh proteins. Furthermore, we still cannot rule out passive diffusion of ammonia out of the gill as a significant mode of excretion under basal conditions. Indeed evidence from studies on the cultured trout gill epithelium indicates that there is a large diffusive component to ammonia excretion (Tsui et al., 2009). The relatively high  $K_m$  values of the trout Rh proteins suggest that these are low-affinity, high capacity transporters that would function optimally when plasma ammonia concentrations are elevated. In support of this idea, it has been proposed that above a certain threshold concentration of plasma ammonia ( $200 \mu\text{mol l}^{-1}$ ), a carrier-mediated process replaces passive ammonia transport out of the gill (Heisler, 1990).

The difficulty and discrepancies in determining how Rh proteins transport ammonia may be inherent in the different methodologies used and in the case of native *Xenopus* oocytes, a unique handling of ammonia. Although there are exceptions (e.g. Kikeri et al., 1989; Waisbren et al., 1994), most eukaryotic cells are more permeable to  $NH_3$  than  $NH_4^+$ . These cells, when exposed to ammonia, display a classic biphasic rise and fall of intracellular pH ( $pH_i$ ; basis of the pre-pulse method) caused by the influx of  $NH_3$  followed by a lower influx of  $NH_4^+$  (Boron and DeWeer, 1976). *Xenopus* oocytes are exceptional in that exposure to high ammonia concentrations ( $\geq 1 \text{ mmol l}^{-1}$ ) leads to a paradoxical fall in  $pH_i$ , depolarization, and an inward current (Bakouh et al., 2004; Boldt et al., 2003; Burckhardt and Burckhardt, 1997; Burckhardt and Fromter, 1992). These changes are thought to be caused by an initial rapid diffusion of  $NH_3$  into the oocyte that causes an alkalization close to the oocyte surface which subsequently activates nonselective cation channels through which  $NH_4^+$  could enter (Boldt et al., 2003; Cougnon et al., 1996). Low concentrations of ammonia ( $< 1 \text{ mmol l}^{-1}$ ), however, cause little change in  $pH_i$  and no inwardly induced current (Bakouh et al., 2004; Holm et al., 2005; Mayer et al., 2006).

We measured the uptake of ammonia into Rhcg2-expressing oocytes using SIET and observed saturation uptake kinetics with a  $K_m$  for  $NH_4Cl$  of  $560 \mu\text{mol l}^{-1}$  (Fig. 9). The early saturation of uptake in Rhcg2-expressing oocytes compared with the control oocytes suggests that the rapid influx of ammonia mediated by Rhcg2 (at  $1 \text{ mmol l}^{-1}$ ) resulted in the rapid accumulation of intracellular ammonia, which probably reduced the gradient for further uptake at higher external ammonia concentrations. Also, since Rh proteins function bi-directionally, efflux may have been enhanced when intracellular ammonia levels became elevated. Endogenous  $NH_4^+$  uptake pathways, triggered by external ammonia concentrations  $> 1 \text{ mmol l}^{-1}$ , probably explain why the ammonia uptake rate into the control oocytes was similar to that of the Rhcg2-expressing oocytes at  $3 \text{ mmol l}^{-1}$ , and surpassed the rate in Rhcg2-expressing oocytes at  $10 \text{ mmol l}^{-1}$ .

Although control oocytes demonstrated endogenous uptake of ammonia, the Rhcg2-expressing oocytes had a greater affinity for

ammonia and facilitated uptake more rapidly at a lower concentration. Plasma ammonia concentrations in fasted fish are typically under  $500\ \mu\text{mol l}^{-1}$  and closer to  $100\text{--}200\ \mu\text{mol l}^{-1}$  (Wood, 1993) but postprandially, levels can increase more than 3-fold (Bucking and Wood, 2008; Wicks and Randall, 2002). Concentrations approaching  $2\ \text{mmol l}^{-1}$  in salmonids cause toxicity (Lumsden et al., 1993) and it was around this concentration (between 1 and  $2\ \text{mmol l}^{-1}$ ) when ammonia uptake by Rhcg2 started to saturate.

Rhcg2-mediated ammonia influx followed the same kinetic profile as that reported for RhCG-expressing oocytes, where inward currents saturated as a function of ammonia concentration with a  $K_m$  of  $468\ \mu\text{mol l}^{-1}$  for  $\text{NH}_4\text{Cl}$  (Bakouh et al., 2004). Although these  $K_m$  values fall within the physiological range for trout, the  $K_i$  values for methylamine inhibition by  $\text{NH}_4\text{Cl}$  measured in this study, as well as in previous reports (Ludewig, 2004; Mak et al., 2006; Mayer et al., 2006), seem high ( $2\text{--}3\ \text{mmol l}^{-1}$ ), and the values also differed moderately between pH 7.5 and pH 8.5 (Table 2). Inhibition studies may be confounded by the endogenous uptake of ammonia by *Xenopus* oocytes, which appeared to be greater than the endogenous uptake of methylamine (see Fig. 8). Our results suggested that when the concentrations were low ( $0.1\text{--}0.3\ \text{mmol l}^{-1}$ ), ammonia entered the oocytes mainly via an endogenous pathway(s), whereas higher concentrations ( $1\text{--}10\ \text{mmol l}^{-1}$ ) were Rh-mediated (Fig. 9). Therefore, low concentrations of  $\text{NH}_4\text{Cl}$  would be relatively ineffective at reducing Rh-mediated methylamine uptake. Additionally, application of high concentrations of  $\text{NH}_4\text{Cl}$  acidify *Xenopus* oocytes (Bakouh et al., 2004; Burckhardt and Fromter, 1992; Cougnon et al., 1996; Nakhoul et al., 2005), a factor that would further stimulate methylamine uptake. The result would be an underestimation of the ability of  $\text{NH}_4\text{Cl}$  to inhibit methylamine uptake.

It was concluded recently, based on pH measurements on the oocyte surface, that  $\text{NH}_3$  rather than  $\text{NH}_4^+$  fluxes predominate in native oocytes and that expression of AmtB in oocytes enhances these  $\text{NH}_3$  fluxes (Musa-Aziz et al., 2009). Although it is still premature to make definitive conclusions about the transport mechanism of Rh proteins, the evidence seems to point more in favor of  $\text{NH}_3$  rather than  $\text{NH}_4^+$  transport through these channels. One possible interpretation of the dependence of transport on the pH gradient is that uptake is mediated by an exchange of  $\text{NH}_4^+$  with  $\text{H}^+$ , but a diffusion trapping mechanism is equally plausible. Regardless, both mechanisms are chemically equivalent to  $\text{NH}_3$  uptake. We suggest that  $\text{NH}_4^+$  is deprotonated before  $\text{NH}_3$  enters the Rh channel, however, we cannot rule-out the possibility that trout Rh proteins function as electroneutral  $\text{NH}_4^+/\text{H}^+$  exchangers as suggested for the mammalian Rh proteins (Ludewig, 2004; Mak et al., 2006). Our understanding of trout Rh protein function is far from complete, and structural studies would be informative.

The functional characteristics of trout Rh proteins we observed in this study agree well with the findings reported for humans, mice and pufferfish. Trout Rh proteins facilitated the movement of both methylamine and ammonia across the *Xenopus* oocyte membrane and the rates were dependent upon the concentration of the protonated species as well as upon the pH gradient. Therefore, the mechanism may involve binding of  $\text{NH}_4^+$ , but transport of  $\text{NH}_3$ . Using SIET, we obtained a  $K_m$  of  $560\ \mu\text{mol l}^{-1}$  for ammonia uptake in Rhcg2-expressing oocytes, a value that lies within the physiological range for trout. This suggests that Rhcg2 is a low affinity, high capacity ammonia transporter that could exploit the acidified gill boundary layer and facilitate rapid efflux of ammonia from the trout gill when plasma ammonia levels are elevated. Basal

plasma ammonia levels, by contrast, are probably maintained by passive diffusion of  $\text{NH}_3$  out of the gill and Rh proteins may have a lesser role under these conditions.

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