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Rh glycoprotein expression is modulated in pufferfish (*Takifugu rubripes*) during high environmental ammonia exposure

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SUMMARY

Rhesus (Rh) protein involvement in ammonia transport processes in freshwater fish has received considerable attention; however, parallel investigations in seawater species are scant. We exposed pufferfish to high environmental ammonia (HEA; 1 and 5 mmol l^{-1} NH_4HCO_3) and evaluated the patterns of ammonia excretion and gill Rh mRNA and protein expression. Gill *H^+-ATPase*, *NHE1*, *NHE2*, *NHE3*, *Na^+/K^+-ATPase* (*NKA*), *Na^+/K^+/$2Cl^-$ co-transporter* (*NKCC1*) mRNA, H^+-ATPase activity, NKA protein and activity, were also quantified. Activation of NKA by NH_4^+ was demonstrated *in vitro*. The downregulation of *Rhbg* mRNA and simultaneous upregulations of *Rhcg1*, *H^+-ATPase*, *NHE3*, *NKA*, *NKCC1* mRNA, H^+-ATPase activity, and NKA protein and activity levels suggested that during HEA, ammonia excretion was mediated mainly by mitochondria-rich cells (MRCs) driven by NKA with basolateral NH_4^+ entry *via* NKA and/or NKCC1, and apical NH_3 extrusion *via* Rhcg1. Reprotonation of NH_3 by NHE3 and/or H^+-ATPase would minimise back flux through the Rh channels. Downregulated *Rhbg* and *Rhag* mRNA observed in the gill during HEA suggests a coordinated protective response to minimise the influx of external ammonia *via* the pavement cells and pillar cells, respectively, while routing ammonia excretion through the MRCs. Exposure to hypercapnia (1% CO_2 in air) resulted in downregulated gill and erythrocyte *Rhag* mRNA. Surprisingly, Rhag, Rhbg, Rhcg1 and Rhcg2 proteins responded to both hypercapnia and HEA with changes in their apparent molecular masses. A dual NH_3/CO_2 transport function of the pufferfish Rh proteins is therefore suggested. The results support and extend an earlier proposed model of pufferfish gill ammonia excretion that was based on immunolocalisation of the Rh proteins. Passive processes and/or Rhbg and Rhcg2 in the pavement cells may maintain basal levels of plasma ammonia but elevated levels may require active excretion *via* NKA and Rhcg1 in the MRCs.

Key words: fugu, gills, Rhesus proteins, ammonia transport, NHE3, Na^+/K^+-ATPase, NKCC1, H^+-ATPase.

INTRODUCTION

As the major end product of nitrogen metabolism for many teleost fish, ammonia must be excreted in order to avoid toxic accumulation, and this is accomplished mainly *via* the gills. Ammonia excretion in fish has been studied extensively over the years (for reviews, see Walsh, 1998; Wilkie, 2002; Wood, 1993); however, our understanding of the mechanism(s) involved is far from complete. In freshwater fish, it has been generally believed that the main mode of excretion occurs by diffusion of NH_3 down a favourable gradient across the gill with the subsequent formation of NH_4^+ in an acidified gill boundary layer. In seawater fish, in addition to NH_3 diffusion, NH_4^+ diffusion is thought to occur due to the shallow tight junctions between the mitochondria-rich cells (MRCs) that increase the cation and therefore NH_4^+ permeability (Evans et al., 2005). These views have been modified recently due to the discovery that Rhesus (Rh) genes and proteins are expressed in fish gills (reviewed by Weihrauch et al., 2009; Wright and Wood, 2009).

Based on their sequence similarity to the Mep/Amt family of ammonia transporters, it was originally postulated that the Rh proteins would have a similar function (Marini et al., 1997). Indeed, Marini et al. were able to show that RhAG and RhCG expressed in Mep-deficient yeast mediated the uptake of ammonia as well as the efflux of the ammonium analogue, methylamine (Marini et al., 2000). This was followed by numerous other functional studies that provided evidence for the transport of ammonia or methylamine through mammalian (reviewed by Javelle et al., 2007) and fish (Nakada et al., 2007; Nawata et al., 2010) Rh proteins expressed in heterologous systems. Very recently, it was demonstrated that purified RhCG reconstituted into liposomes functions as an NH_3 channel (Mouro-Chanteloup et al., 2010). However, because Amt and Rh coexist in a number of organisms (Huang and Ye, 2010) the question has been raised as to why these two proteins would perform the same function. Indeed there is some evidence suggesting that Rh proteins transport CO_2 (Li et al., 2007; Soupene et al., 2002; Soupene et al., 2004) or function as dual CO_2/NH_3 channels (Endeward et al., 2007).

The first comprehensive study of fish Rh proteins was in the marine pufferfish (*Takifugu rubripes*) where Rhag, Rhbg, Rhcg1 and Rhcg2 were localised to specific regions of cells in the gill epithelium (Nakada et al., 2007). Based entirely on localisation by immunohistochemistry and *in situ* hybridisation, Nakada et al. proposed a preliminary model for gill ammonia excretion (Nakada et al., 2007). Rhag in the pillar cells functioned in cooperation with basolateral Rhbg and apical Rhcg2 in the gill pavement cells (PVCs) to facilitate ammonia efflux out of the gill. Additional support from the MRCs was suggested as an auxiliary route of excretion *via* the basolateral Na^+/K^+-ATPase (NKA) and apical Rhcg1. This study was followed by numerous other investigations, which all corroborate the view that Rh proteins are involved in ammonia transport in fish but provide somewhat different models for other species (reviewed by Weihrauch et al., 2009; Wright and Wood, 2009).

Exposure of fish to high environmental ammonia (HEA) initially impairs ammonia excretion but over time the fish are able to re-establish excretion against the unfavourable gradient (Cameron, 1986; Cameron and Heisler, 1983; Claiborne and Evans, 1988; Nawata et al., 2007; Wilson et al., 1994; Zimmer et al., 2010). Recent studies have shown that HEA exposure also triggers an upregulation of Rh mRNA expression in tissues of the mangrove killifish (Hung et al., 2007), adult and juvenile rainbow trout (Nawata et al., 2007; Zimmer et al., 2010), and zebrafish embryos and larvae (Braun et al., 2009). In adult trout exposed to HEA, the upregulation of *Rhbg* and *Rhcg2* mRNA levels in the PVCs coincided with the resumption and enhancement of ammonia excretion (Nawata et al., 2007). Increasing the plasma ammonia levels in trout *via* ammonia infusion, which enhances the ammonia excretion rate, also resulted in upregulations of gill *Rhbg* and *Rhcg2* mRNA (Nawata and Wood, 2009). Similarly, mRNA levels of *Rhbg* and *Rhcg2* were elevated in cultured trout gill cells after pre-exposure to high ammonia (Tsui et al., 2009). Naturally elevated plasma ammonia levels after feeding also increase Rhcg2 transcript levels in juvenile trout gills (Zimmer et al., 2010). Therefore, at least in the freshwater rainbow trout, it appears that when plasma ammonia levels are elevated, Rhbg, Rhcg2 and PVCs may be key players in ammonia excretion.

While substantial work has been performed on freshwater fish species there is a lack of direct experimental evidence linking the Rh proteins to ammonia excretion in seawater fish. The focus of our study therefore was to use a combined physiological and molecular approach to evaluate the model proposed by Nakada et al. (Nakada et al., 2007) for ammonia excretion across the gills of the pufferfish. We employed HEA as an experimental tool, and measured ammonia flux rates, plasma ammonia concentrations and mRNA expression changes by real-time polymerase chain reaction (PCR) of Rh genes and other potential transporters [H^+-ATPase (V-type, B-subunit), NHE1, NHE2, NHE3, NKA (Na^+/K^+-ATPase) (α-subunit) and NKCC1 (Na^+/K^+/$2Cl^-$ co-transporter)] in the pufferfish gills, erythrocytes and skin during this HEA challenge. H^+-ATPase and NKA enzyme activities were measured and the ability of NH_4^+ to activate pufferfish gill NKA was evaluated *in vitro*. Changes in the Rh and NKA proteins were examined by western blotting. Additionally, as Rh proteins could potentially have a dual role as NH_3 and CO_2 channels, pufferfish were exposed to hypercapnic conditions and the Rh mRNA and protein responses in the gill, erythrocytes and skin were analysed.

MATERIALS AND METHODS

Experimental animals

Pufferfish [*Takifugu rubripes* (Temminck and Schlegel)] weighing 162–407 g, purchased from the Numazu Aquaculture Cooperative, Shizuoka, Japan, were held in 150 l glass aquaria (5 fish per tank) in artificial seawater (Rohtomarine, Rei-Sea, Tokyo, Japan), 34‰, pH 7.8 at 19–20°C. Fish were allowed to settle for a minimum of two days before experimentation and during this time food was withheld. Holding tank water ammonia levels averaged 113±14 μmol l^{-1}, significantly below control ammonia levels in the plasma (see Results). All procedures used were approved by the Tokyo Institute of Technology Institutional Animal Care and Use Committee.

High environmental ammonia (HEA) and hypercapnia exposures

Three experimental series were performed. For all exposures, individual fish were transferred immediately from the holding tank into a darkened box filled with 4 l of aerated, artificial seawater at 19–20°C. Control fish were treated identically to the experimental fish with no addition of NH_4HCO_3 or CO_2 to the external water.

In Series 1, fish were exposed to HEA comprising of a nominal total ammonia (T_{Amm}) concentration of 1 mmol l^{-1} NH_4HCO_3 (pH 7.75±0.05) for 6, 24 or 48 h. In Series 2, an additional batch of fish was exposed to a higher level of HEA, 5 mmol l^{-1} NH_4HCO_3 (pH 7.75±0.05), for 12 h. In Series 3, pufferfish were exposed to hypercapnia; a 1% CO_2 in air mixture was bubbled into the water over the course of 24 h. This reduced the water pH by approximately 0.6 pH units.

Ammonia analysis and tissue sampling

During all exposure periods, water samples (10 ml) were taken every 3 h, frozen at –20°C and later assayed in triplicate for T_{Amm} using a modified salicylate–hypochlorite method (Verdouw et al., 1978). Net excretion rates of total ammonia (J_{Amm}, μmol kg^{-1} h^{-1}) were calculated as previously described (Nawata et al., 2007). A negative J_{Amm} indicates a net excretion of ammonia to the water and a positive J_{Amm} indicates a net uptake of ammonia into the fish.

After the exposure periods, the fish were terminally anaesthetised with neutralised MS222 (0.1 g l^{-1}). Blood was drawn into a heparinised syringe from the hepatic vein and samples were then centrifuged to separate plasma from erythrocytes (2 min, 14,000 ***g***), and both fractions (with the buffy coat excluded) were snap frozen in liquid nitrogen and stored at –80°C. Erythrocytes for protein analysis were processed as described below. Immediately following blood sample removal, the gills were perfused free of blood with ice-cold phosphate buffered saline through the bulbus arteriosus. Gills and skin sections were quickly excised and flash frozen in liquid nitrogen and stored at –80°C until processing. Plasma was later analysed enzymatically for T_{Amm} (Raichem, Cliniqa Corp., San Marcos, CA, USA), an assay that measures the formation of L-glutamate catalysed by L-glutamate dehydrogenase. This assay is linear up to 600 μmol l^{-1} and therefore more concentrated samples were appropriately diluted with ammonia-free water. Reproducibility was ±5%. Although the method of blood sampling used here could potentially alter the blood chemistry due to stress-related factors, we wanted to avoid the more confounding issue of Rh gene expression alteration associated with surgically implanted dorsal aortic catheters, as previously reported for rainbow trout (Nawata and Wood, 2009).

RNA extraction and real-time quantitative PCR (qPCR)

Total RNA was extracted from blood and tissues by the acid guanidine isothiocyanate–phenol–chloroform method with Isogen (Nippon Gene Co., Toyama, Japan) and quantified spectrophotometrically. First strand cDNA was synthesised from 1 μg total RNA (DNaseI-treated; Invitrogen, Carlsbad, CA, USA) with an oligo(dT_{17}) primer and Superscript II reverse transcriptase (Invitrogen). Rh mRNA expression was assessed in the gill, skin and erythrocytes, and *H^+-ATPase* (V-type, B-subunit), *NHE1*, *NHE2*, *NHE3*, *NKA* (α-subunit) and *NKCC1* mRNA expressions were analysed in the gill using the above-described cDNA. Rh primers were designed from their known sequences (fRhag, AB218979; fRhbg, AB218980; fRhcg1, AB218981; fRhcg2, AB218982) and all other primers were designed based on the fugu genomic database (http://uswest.ensembl.org). Primer sets (listed in Table 1) were tested and products were sequenced. qPCR reactions were performed using Sybr Premix ExTaq II (Takara Bio, Otsu, Japan) on a Thermal Cycler Dice Real-Time System (Takara Bio). Melt-curve analysis verified production of a single product, and no-template controls and non-reversed-transcribed controls were run

Table 1. Primer list for qRT-PCR

Name	Forward / reverse (5′–3′)
Rhag	cggtaatcacacccaggaag / attttgatcttgccgtcctc
Rhbg	tgcctatcacctgtttcatcc / caacgctaccgaagccatac
Rhcg1	catggtgcccaccaagc / ctctcctcatcttcaggcag
Rhcg2	caagtacgcagacaggtcg / gtcctggtgcttgtggatc
H^+-ATP	ccgatcaaccctcagtgtc / tgggtattttctggcctctg
NHE1	agaacctggggacaatcctc / aaacaatggagccaaacagc
NHE2	catgtctcctcatcagcatc / tctcaaagaaagccctggtg
NHE3	atgctttggcttcattctgg / cccaggttgctgaagaagag
NKA	gtgtgtctgaccctgactgc / gcctcatggatctggttgtc
NKCC1	aggctgctgctaaagaatcg / cgtacaaatccattggttgc
β-actin	ggctgtgctgtccctgtatg / gaaggagtacccacgctctg

in parallel. Values were extrapolated from standard curves generated by serial dilution of one randomly selected control sample. *Beta actin* (*β-actin*) mRNA, *elongation factor-1α* mRNA and 18S rRNA were tested for stability as normalising genes using geNorm software (Vandesompele et al., 2002). *β-actin* was identified as the most stable gene within tissues and across treatments and was therefore chosen for normalisation. The mean cycle threshold (C_t) ± s.d. of β-actin was 17.93±0.94 in the gill, 18.31±0.78 in the skin and 19.56±1.42 in the blood.

Western blot analysis

Analyses were performed on gill samples taken from the last time point in all three experimental series (48 h for 1 mmol l^{-1} NH_4HCO_3, 12 h for 5 mmol l^{-1} NH_4HCO_3 and 24 h for hypercapnia exposure). Whole gill arches as well as skin samples were homogenised in buffer (25 mmol l^{-1} Tris-HCl, 250 mmol l^{-1} sucrose, 1 mmol l^{-1} EDTA, and protease inhibitor cocktail, pH 7.4) and centrifuged at 4500 ***g*** for 15 min at 4°C. The supernatant was then collected and centrifuged at 200,000 ***g*** for 1 h at 4°C. The resulting pellets were resuspended in homogenisation buffer, adjusted to 1 μg μl^{-1} and then boiled for 15 min at 70°C. Erythrocytes from 24 h exposures to 1 mmol l^{-1} NH_4HCO_3 or hypercapnia were separated from plasma and the buffy coat by centrifugation as described earlier, then washed three times in isotonic phosphate buffer and resuspended in an equal volume of buffer. Erythrocyte membrane protein was isolated by lysing 1 ml washed erythrocytes in 30 ml of ice-cold hypotonic phosphate buffer (5 mmol l^{-1} sodium phosphate, 0.5 mmol l^{-1} EGTA and protease inhibitor cocktail, pH 8.0) and centrifugation at 14,000 ***g*** for 15 min at 4°C. The pellet was washed several times in ice-cold buffer until free of haemoglobin, solubilised in 1% SDS, and then adjusted to 1 μg μl^{-1} and boiled for 15 min at 70°C.

Protein concentrations were determined using a BCA assay (Thermo Fisher Scientific Inc., Rockford IL, USA). In some tests, denatured protein samples were deglycosylated prior to blotting using Peptide:*N*-glycosidase F (PNGase F; New England BioLabs, Pickering, ON, Canada) according to manufacturer's instruction. Samples were separated by 10% SDS-PAGE and transferred onto polyvinylidene difluoride membranes. Non-specific binding was blocked with 5% non-fat skimmed milk in TBST (100 mmol l^{-1} Tris-HCl, pH 7.5, 150 mmol l^{-1} NaCl and 0.1% Tween 20) for 1 h at room temperature. The membranes were incubated with anti-fRhag (1:30,000), anti-fRhbg (1:10,000), anti-fRhcg1 (1:30,000), anti-fRhcg2 (1:30,000) (Nakada et al., 2007) or anti-eel NKA (1:30,000) (Mistry et al., 2001) antiserum at room temperature for 2–3 h. After washing with TBST, membranes were then reacted with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:60,000; Cell Signaling, New England Biolabs) for 1 h at room temperature. The bound secondary antibody was visualised by enhanced chemiluminescence detection using Immobilon Western HRP Substrate (Millipore Corporation, Billerica, MA, USA) and band density was measured using ImageJ software (http://rsb.info.nih.gov/ij/). One randomly chosen sample was used on every gel for normalisation of different immunoblots.

Enzyme analysis

NKA activity and *N*-ethylmaleimide-sensitive H^+-ATPase activity were measured in crude gill homogenates of control fish and those exposed to either 1 mmol l^{-1} NH_4HCO_3 for 48 h or 5 mmol l^{-1} NH_4HCO_3 for 12 h, following the methodology of McCormick (McCormick, 1993) as described previously (Nawata et al., 2007). To assess NH_4^+ activation of NKA, the assay was performed under optimised conditions containing either 10 mmol l^{-1} KCl or 10 mmol l^{-1} NH_4Cl (replacing KCl) in the reaction medium. Activities were then measured in the absence and presence of ouabain.

Statistical analysis

Data are presented as means ± s.e.m., *N*=number of fish. Enzyme activities and the effect of 1 mmol l^{-1} NH_4HCO_3 exposure on mRNA expression levels were analysed by one-way analysis of variance (ANOVA) followed by Fisher's Least Significant Difference *post-hoc* test. Student's unpaired *t*-test was used to analyse the effect of 5 mmol l^{-1} NH_4HCO_3 and hypercapnia exposure on mRNA expression, differences in protein levels and K^+ *vs* NH_4^+ activated ATPase activity. Significance was set at α=0.05.

RESULTS

Experimental series 1: HEA – 1 mmol l^{-1} NH_4HCO_3

Under control conditions, pufferfish excreted ammonia at a rate of about 378 μmol kg^{-1} h^{-1}. During the first 3 h of exposure to 1 mmol l^{-1} NH_4HCO_3, there was a large net uptake of ammonia (~1157 μmol kg^{-1} h^{-1}) into the fish. After 3 h, excretion resumed with a magnitude about 2.5-fold over that of the control rate (Fig. 1). Thereafter, the excretion stabilised at a rate not significantly different from that of the control. Plasma ammonia increased 2.5-fold from a control level of about 350 μmol l^{-1}, reaching a steady state across time during this exposure, averaging 795 μmol l^{-1}

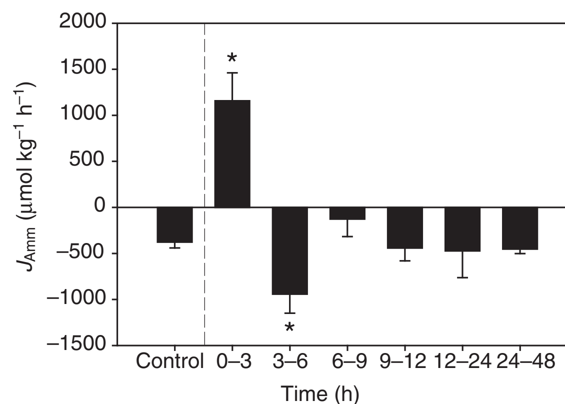

Fig. 1. Ammonia excretion rate (J_{Amm}) of pufferfish exposed to 1 mmol l^{-1} NH_4HCO_3. The control value represents the 24 h excretion rate in untreated fish. Negative values indicate net excretion and positive value indicates net uptake. Asterisks represent significant differences from the control value (*P*>0.05). Data are means ± s.e.m. (*N*=6). The vertical broken line separates the control value from the time points.

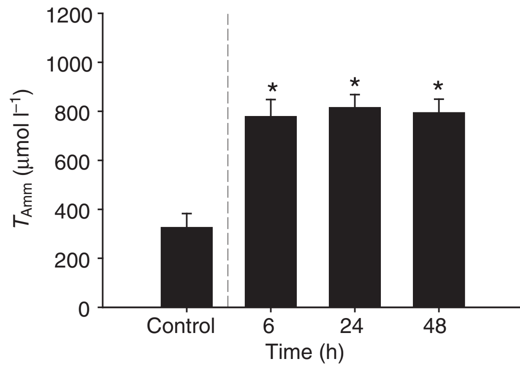

Fig. 2. Plasma ammonia levels (T_{Amm}) in pufferfish exposed to 1 mmol l^{-1} NH_4HCO_3. Asterisks represent significant differences from the control (untreated) fish (P>0.05). Data are means ± s.e.m. (N=6). The vertical broken line separates the control value from the time points.

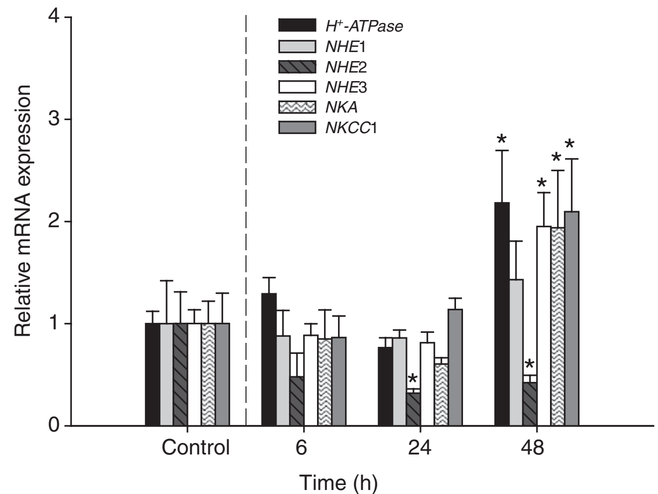

Fig. 4. Expression of H^+-*ATPase*, *NHE1*, *NHE2*, *NHE3*, Na^+/K^+-*ATPase* (*NKA*) and *NKCC* mRNA relative to *β-actin* in the gills of pufferfish exposed to 1 mmol l^{-1} NH_4HCO_3. Asterisks represent significant differences from the corresponding control (untreated) fish (P>0.05). Data are means ± s.e.m. (N=6). The vertical broken line separates the control value from the time points.

(Fig. 2) and significantly below the mean water ammonia concentration of 936 µmol l^{-1}.

Analysis of gill mRNA expression levels revealed a 5-fold upregulation of *Rhcg1* over the control level at 48 h (Fig. 3). Levels of *Rhag* mRNA were significantly downregulated by about 50% at all time points and similarly, *Rhbg* mRNA levels were reduced to about half of the control levels at 24 and 48 h. By contrast, *Rhcg2* mRNA levels remained unchanged across time. While there was a significant 50% decrease in *NHE2* mRNA levels at 24 and 48 h, there was a significant 2-fold increase in H^+-*ATPase*, *NHE3*, *NKCC1* and *NKA* mRNA at 48 h, and no change in *NHE1* mRNA levels (Fig. 4). In the erythrocytes, there was a 75% decrease in *Rhag* mRNA expression (Fig. 5). *Rhbg* mRNA was decreased in the skin but not significantly (P=0.06) and similarly no significant changes were seen in skin *Rhcg2* mRNA levels (Fig. 5).

Experimental series 2: HEA – 5 mmol l^{-1} NH_4HCO_3

To raise the plasma ammonia, without using an invasive infusion procedure, to a level higher than that achieved after exposure to 1 mmol l^{-1} NH_4HCO_3, fish were exposed to 5 mmol l^{-1} NH_4HCO_3 for 12 h. No signs of toxicity were observed at this ammonia level but in a preliminary trial 8 mmol l^{-1} proved to be toxic. Similar to the HEA exposure at 1 mmol l^{-1} NH_4HCO_3, during the first 3 h of exposure to 5 mmol l^{-1} NH_4HCO_3, there was a net uptake of ammonia (1556 µmol kg^{-1} h^{-1}) into the fish. After 3 h, excretion resumed at a magnitude 4-fold over that of the control rate (Fig. 6).

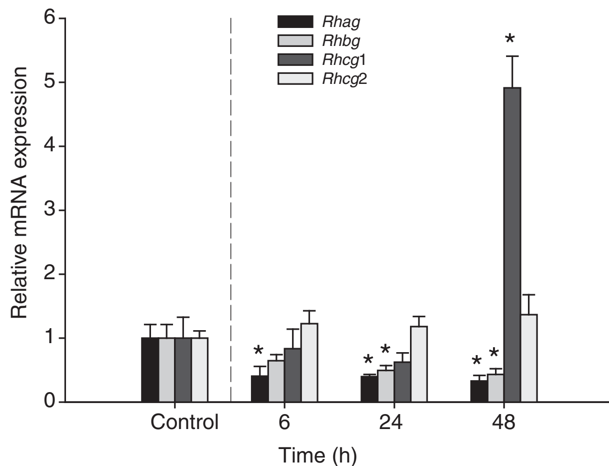

Fig. 3. Expression of *Rhag*, *Rhbg*, *Rhcg1* and *Rhcg2* mRNA relative to *β-actin* in the gills of pufferfish exposed to 1 mmol l^{-1} NH_4HCO_3. Asterisks represent significant difference from the corresponding control (untreated) fish (P>0.05). Data are means ± s.e.m. (N=6). The vertical broken line separates the control value from the time points.

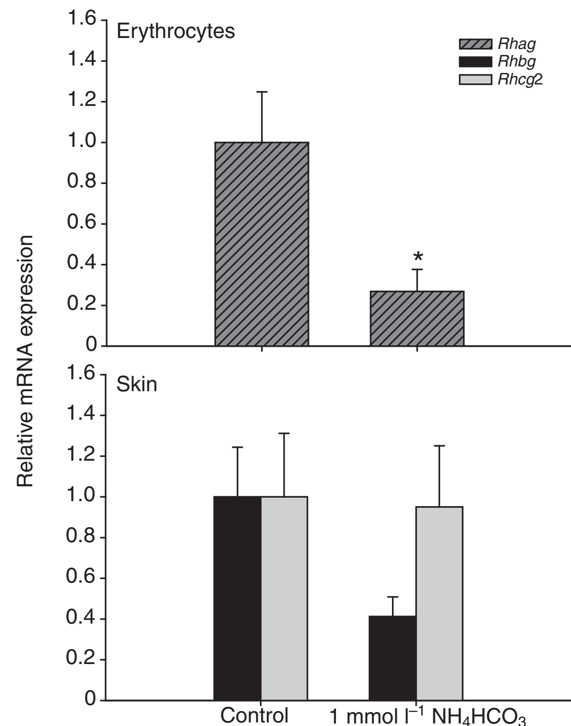

Fig. 5. Expression of erythrocytic *Rhag* mRNA and skin *Rhbg* and *Rhcg2* mRNA relative to *β-actin* in pufferfish exposed to 1 mmol l^{-1} NH_4HCO_3. Asterisk represents significant difference from the control (untreated) fish (P>0.05). Data are means ± s.e.m. (N=6).

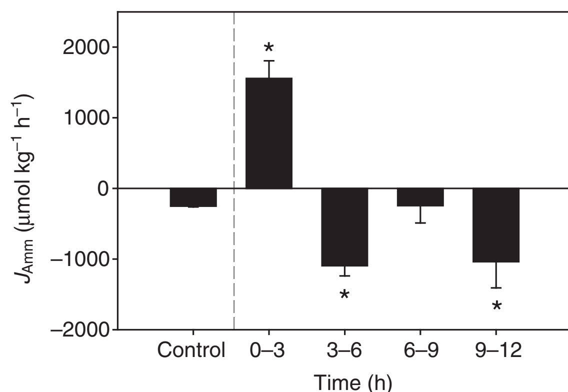

Fig. 6. Ammonia excretion rate (J_{Amm}) of pufferfish exposed to 5 mmol l^{-1} NH_4HCO_3. The control value represents the 12 h excretion rate in untreated fish. Negative values indicate net excretion and positive values indicate net uptake. Asterisks represent significant differences from the control value (P>0.05). Data are means ± s.e.m. (N=5). The vertical broken line separates the control value from the time points.

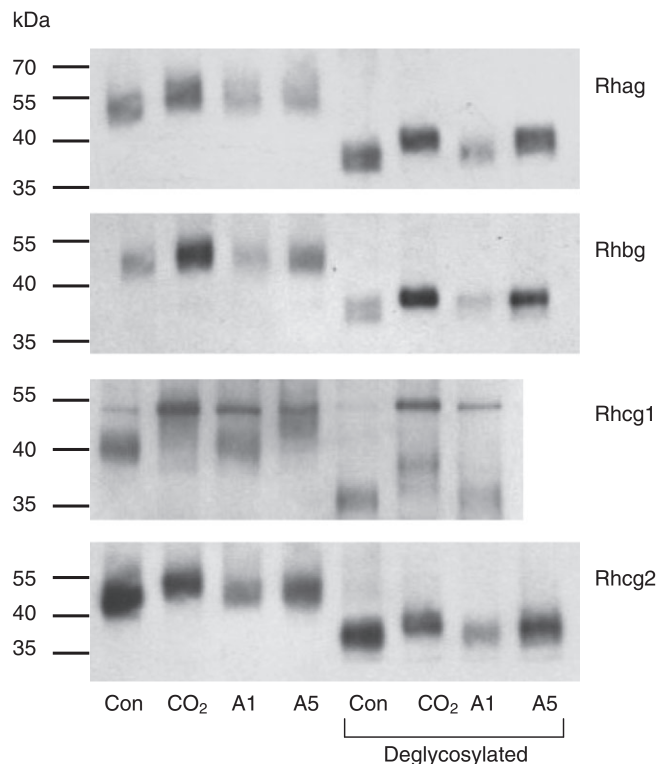

Fig. 7. Representative western blots of Rhag, Rhbg, Rhcg1 and Rhcg2 in the gills of pufferfish that were untreated (Con), exposed to 1% CO_2 in air for 24 h (CO_2), exposed to 1 mmol l^{-1} NH_4HCO_3 for 48 h (A1), or exposed to 5 mmol l^{-1} NH_4HCO_3 for 12 h (A5). Due to sample loss, the last lane (A5) is missing from the Rhcg1 blot; however, preliminary results showed similar band migration as that seen for A1.

Excretion continued at this elevated rate at 9–12 h. The plasma ammonia at 12 h was increased 7.8-fold over the control (2520±79 µmol l^{-1} *vs* 325±58 µmol l^{-1}) (data not shown) but remained far below the water level (4552±63 µmol l^{-1}).

Exposure to 5 mmol l^{-1} NH_4HCO_3 resulted in the upregulation of expression levels in the gill of both *Rhcg1* and *Rhcg2* mRNA (5.4- and 1.8-fold, respectively) at 12 h. However, mRNA levels of *Rhag* and *Rhbg* were not significantly altered (Table 2). Analysis of other gill transporters revealed a significant 1.9-fold increase in *H^+-ATPase* and a 2.2-fold increase in *NKCC1* mRNA but no significant changes in the mRNA levels of *NHE1*, *NHE2*, *NHE3* or *NKA* (Table 3). As with exposure to 1 mmol l^{-1} NH_4HCO_3, there was a significant 80% decrease in *Rhag* mRNA expression in the erythrocytes (Table 2). In the skin, both *Rhbg* and *Rhcg2* mRNA levels were significantly elevated by 2.3- and 2.8-fold, respectively (Table 2).

Experimental series 3: hypercapnia

With the exception of a significant 1.7-fold increase in the ammonia excretion rate over the control rate at 6–9 h, excretion rates remained unchanged during exposure to 24 h of hypercapnia (data not shown). Likewise, the plasma ammonia level after 24 h of hypercapnia (259±36 µmol l^{-1}) was not significantly different from the control value (358±57 µmol l^{-1}) (data not shown). Analysis of mRNA levels in the gill showed a 77% decrease in *Rhag* but no significant changes in the mRNA levels of *Rhbg*, *Rhcg1* or *Rhcg2* (Table 2). Similarly, in the skin, there were no changes in the *Rhbg* or *Rhcg2* mRNA levels (Table 2). *Rhag* mRNA levels in the erythrocytes, however, were significantly decreased by 93% (Table 2).

Protein analyses

Western blotting of whole gill homogenates failed to provide clear bands; therefore, we analysed the membrane protein enriched fraction, which by contrast, produced distinct bands. The Rhag band in the experimental gill samples from the HEA- and hypercapnia-exposed fish migrated at a higher apparent molecular mass (~8 kDa)

Table 2. Relative mRNA expression of Rh genes in the gills, skin and erythrocytes of pufferfish exposed to 5 mmol l^{-1} NH_4HCO_3 and hypercapnia (1% CO_2 in air)

Treatment	*Rhag* (gill)	*Rhbg* (gill)	*Rhcg1* (gill)	*Rhcg2* (gill)	*Rhbg* (skin)	*Rhcg2* (skin)	*Rhag* (erythrocytes)
Untreated	1.00±0.21	1.00±0.21	1.00±0.33	1.00±0.11	1.00±0.24	1.00±0.31	1.00±0.25
5 mmol l^{-1} NH_4HCO_3	0.54±0.16	1.19±0.30	5.40±1.21*	1.84±0.33*	2.33±0.51*	2.81±0.74*	0.18±0.07*
1% CO_2 in air	0.23±0.071*	0.44±0.08	0.64±0.18	0.88±0.11	1.57±0.40	0.50±0.14	0.07±0.02*

Data are normalised to beta actin. Values are means ± s.e.m. (N=5–6). *Significant difference from the untreated control (P<0.05).

Table 3. Relative mRNA expression of transporter genes in the gills of pufferfish exposed to 5 mmol l^{-1} NH_4HCO_3

Treatment	*H^+-ATPase*	*NHE1*	*NHE2*	*NHE3*	*NKA*	*NKCC1*
Untreated	1.00±0.14	1.00±0.42	1.00±0.31	1.00±0.14	1.00±0.22	1.00±0.3
5 mmol l^{-1} NH_4HCO_3	1.89±0.24*	2.71±0.79	0.58±0.17	1.57±0.35	2.16±0.52	2.25±0.14*

Data are normalised to beta actin. Values are means ± s.e.m. (N=5–6). *Significant difference from the untreated control (P<0.05).

than that in the control sample, which appeared at the predicted molecular mass of 47 kDa (Fig. 7). Two Rhcg1 bands were detected in the control and experimental samples (~39 and 54 kDa). Heavier staining of the 54 kDa band (the predicted molecular mass of Rhcg1) was observed in the experimental samples. The Rhbg and Rhcg2 bands in the control samples had lower apparent molecular masses than those in the corresponding experimental samples, which migrated closer to their predicted molecular masses (49 and 53 kDa, respectively, for Rhbg and Rhcg2). These mobility shifts prevented reliable quantification of Rh protein expression changes during the treatments but prompted us to investigate the cause of this phenomenon. When the proteins were experimentally deglycosylated, all samples displayed the expected decrease in molecular mass but a mobility shift persisted in samples from the HEA and hypercapnia treatments. In the case of Rhcg1, the 54 kDa-band persisted (Fig. 7).

While we were unable to obtain good visualisation of Rhbg in the skin, Rhcg2 appeared as a doublet in samples from both control and HEA treatments at around the same molecular mass as the control gill Rhcg2 band (~42–47 kDa). An additional band was detected at the predicted molecular mass of 53 kDa (Fig. 8).

There were no molecular mass differences between control and experimental erythrocyte Rhag samples either before or after deglycosylation, thereby allowing us to quantify these proteins. Erythrocyte Rhag levels did not change in response to 24 h of hypercapnia or HEA (1 mmol l^{-1} NH_4HCO_3) when compared with the control (Fig. 9). Analysis of NKA protein levels in the gill revealed a significant increase in expression with 48 h of 1 mmol l^{-1} NH_4HCO_3 exposure but a significant decrease after exposure to 12 h of 5 mmol l^{-1} NH_4HCO_3 (Fig. 10).

Enzyme activity

A significant 1.4-fold increase in NKA activity was noted in the gills of fish exposed to 1 mmol l^{-1} NH_4HCO_3 for 48 h when compared with the control fish (Fig. 11). Activities in the gills when K^+ was substituted with NH_4^+ were lower but not significantly different from the corresponding activities when K^+ was present. H^+-ATPase activity was significantly elevated by about 2-fold in the gills of fish exposed to 5 mmol l^{-1} NH_4HCO_3 when compared with the activity in the gills of control fish (Fig. 11).

DISCUSSION

In the present study we have provided the first experimental evidence linking Rh proteins to ammonia excretion in a seawater fish species. Some important differences in ammonia handling between freshwater and seawater species have been revealed, and an earlier model (based only on immunohistochemistry and *in situ* hybridisation) for ammonia excretion across the pufferfish gill (Nakada et al., 2007) has been largely supported by a combined molecular and physiological approach.

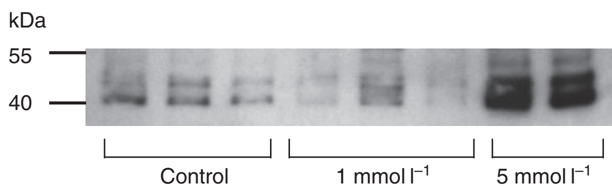

Fig. 8. Western blot of Rhcg2 in the skin of pufferfish that were untreated (Control) or exposed to 1 mmol l^{-1} NH_4HCO_3 for 48 h or to 5 mmol l^{-1} NH_4HCO_3 for 12 h.

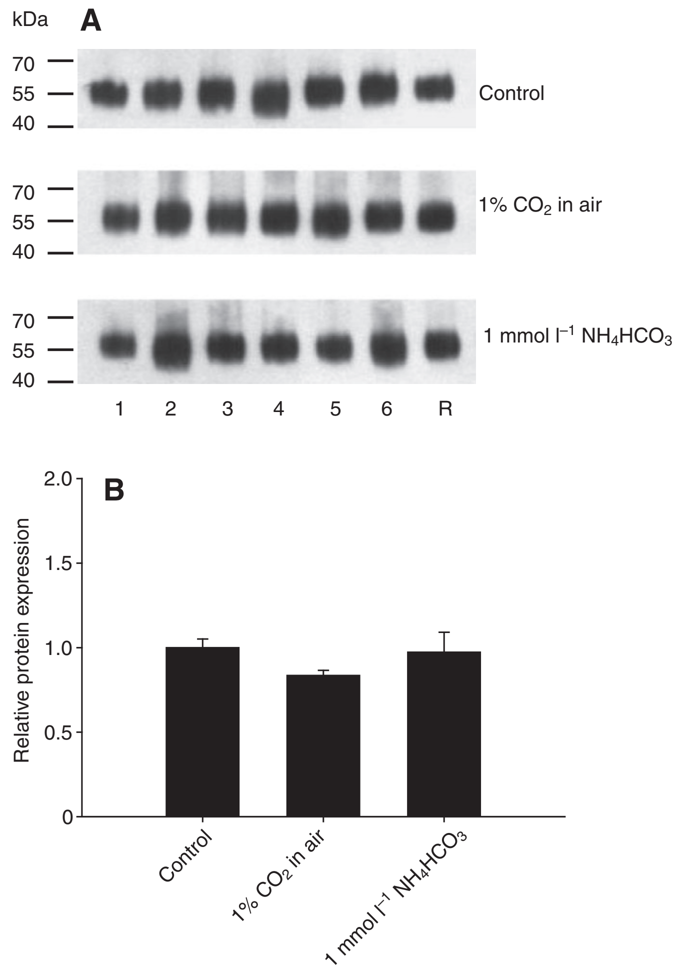

Fig. 9. (A) Western blots and (B) quantification of Rhag protein levels in isolated erythrocyte membranes of control (untreated) pufferfish and those exposed to 24 h of hypercapnia (1% CO_2 in air) or 1 mmol l^{-1} NH_4HCO_3. A reference sample (R) was used to equalise signal intensities from different blots. There were no significant differences in protein levels between the experimental samples and the control (untreated) samples. Data are means ± s.e.m. (N=6).

The main goal of our study was to determine the patterns of ammonia excretion and Rh expression in pufferfish gills after HEA exposure. Fish exposed to HEA are not only challenged with the influx of ammonia but, over time, are also faced with accumulating levels of endogenous ammonia. During exposure to HEA, pufferfish were able to maintain plasma ammonia levels constant at about 2.5-fold above the control levels and below those in the external water (Fig. 2). By contrast, rainbow trout exposed to HEA in freshwater had plasma ammonia levels elevated almost 10-fold over the control by 48 h (Nawata et al., 2007). Pufferfish, therefore, appear to regulate plasma ammonia levels more quickly than the rainbow trout during HEA.

It is important to note that our calculations of trans-gill ammonia gradients are based on measurements of total ammonia concentrations in blood plasma and water. Traditionally, it has been assumed that ammonia flux across fish gills is driven by the partial pressure of ammonia (P_{NH_3}) or NH_4^+ electrochemical gradients (e.g. Cameron and Heisler, 1983; Evans et al., 2005). However, it is unclear whether or not ammonia moves across the gill as NH_3, NH_4^+ or both in seawater fish during HEA and therefore total ammonia

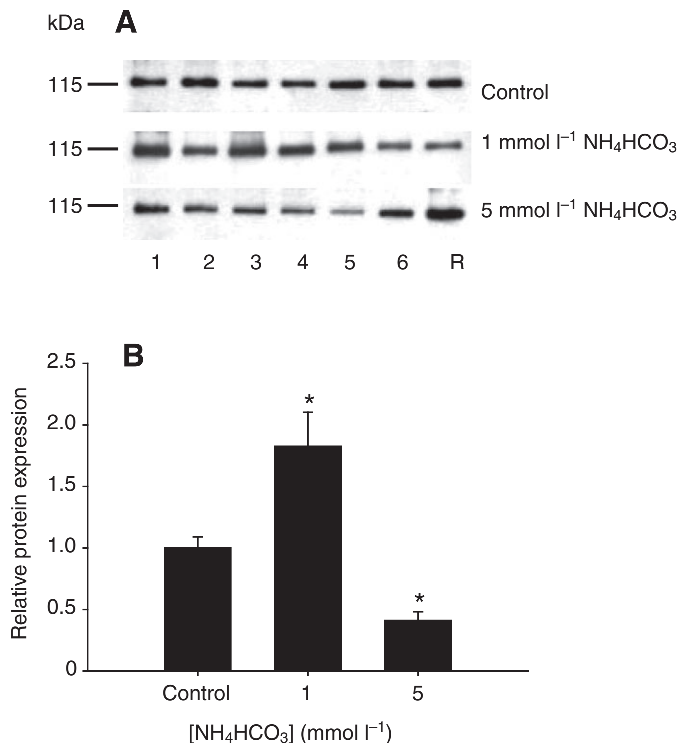

Fig. 10. (A) Western blots and (B) quantification of Na^+/K^+-ATPase protein levels in the gills of control (untreated) pufferfish and those exposed to NH_4HCO_3 (1 mmol l^{-1} NH_4HCO_3 for 48 h or 5 mmol l^{-1} for 12 h). A reference sample (R) was used to equalise signal intensities from different blots. Asterisks represent significant differences from the control (untreated) fish (P<0.05). Data are means ± s.e.m. (N=5–6).

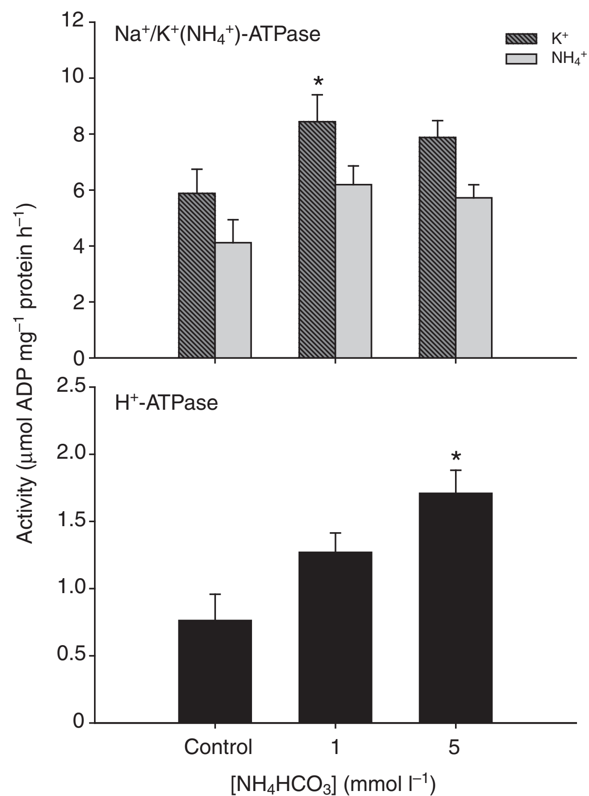

Fig. 11. Enzyme activities of Na^+/K^+-ATPase (with K^+ present and with K^+ replaced by NH_4^+ in the reaction) and H^+-ATPase in the gills of control (untreated) pufferfish and those exposed to NH_4HCO_3 (1 mmol l^{-1} NH_4HCO_3 for 48 h or 5 mmol l^{-1} for 12 h). Asterisks represent significant differences from activity levels in control (untreated) fish (P<0.05). Activities when K^+ was present and when K^+ was replaced by NH_4^+ were not significantly different from each other within each treatment group. Data are means ± s.e.m. (N=5-6).

gradients remain informative. For the purposes of our study, we were interested in determining whether or not pufferfish were able to maintain total plasma ammonia levels lower than those in the external water. Future investigations should include accurate determinations of P_{NH_3} gradients across the gill that would include both blood plasma and gill boundary layer pH, and NH_4^+ electrochemical gradients that would take into account transepithelial potential. These would require cannulation – an approach we wished to avoid in the present study.

Recovery of ammonia excretion in pufferfish after exposure to HEA occurred very rapidly at an elevated rate after 3 h, stabilising thereafter to control levels over 48 h (Fig. 1). Resumption of excretion in freshwater rainbow trout occurred only after 12 h at a rate not significantly different from that of the control during the first 12–36 h but enhanced by almost 3-fold at 36–48 h (Nawata et al., 2007). The recovery and enhancement of excretion in the trout coincided with the upregulations of *Rhbg* and *Rhcg2* mRNA in the PVCs, suggesting that these Rh proteins as well as the PVCs have a major role in ammonia excretion during HEA in this freshwater species (Nawata et al., 2007). In the pufferfish, the Rhcg gene that eventually responded after 48 h of exposure to 1 mmol l^{-1} NH_4HCO_3 was *Rhcg1* rather than *Rhcg2*, and Rhbg expression never increased (Fig. 3), suggesting a very different system than in the freshwater trout.

Some fish are able to detoxify ammonia by converting it into urea (Mommsen and Walsh, 1992). However, this process was ruled out as a significant factor in the pufferfish because plasma urea as well as amino acid levels in control and HEA-exposed fish were not significantly different from each other (data not shown). Additionally, urea transporter mRNA expression levels in the gill and liver were unaffected by HEA exposure while *Rhcg2* mRNA expression was significantly increased in the liver after exposure to 5 mmol l^{-1} NH_4HCO_3 (data not shown).

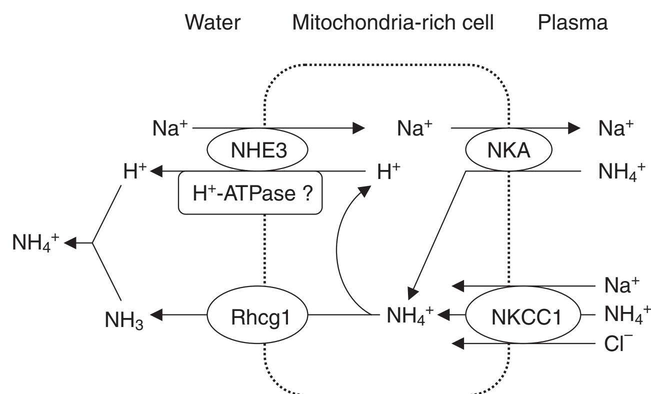

Fig. 12. Preliminary working model of Rhcg1-mediated ammonia transport in the mitochondria-rich cell of the pufferfish. NH_4^+ enters basolaterally *via* NKCC1 or Na^+/K^+-ATPase (NKA) by substituting for K^+. On the apical side, NH_3 that exits the Rhcg1 channel combines with a H^+ ion released from NHE3 (and/or H^+-ATPase) to form NH_4^+ in the gill boundary layer.

While our study was focused mainly on the acute response to HEA at a level comparable with those used previously in other fish species (i.e. 1 mmol l^{-1}), it would be interesting in a future investigation, to conduct a chronic study over several days. 5 mmol l^{-1} NH_4HCO_3 represents a very high ammonia concentration that pufferfish are unlikely to encounter in the natural environment. However, because we observed a response with this treatment, it would be interesting to make closer comparisons between this treatment and the 1 mmol l^{-1} NH_4HCO_3 exposure.

A role for MRCs during HEA

Because the PVCs comprise over 90% of the cell population in the fish gill epithelium (Evans et al., 2005), it is reasonable to assume that these cells facilitate the majority of the ammonia excretion under basal conditions in both freshwater and seawater fish. In the pufferfish, Rhbg is limited to the basolateral region of the PVCs, while Rhcg1 is limited to the apical region of the MRCs (Nakada et al., 2007). HEA exposure to pufferfish resulted in the downregulation of *Rhbg* and upregulation of *Rhcg1* mRNA levels in the gill over time (Fig. 3). This suggests that the persistence of elevated plasma ammonia may have triggered the recruitment of MRCs into the excretion process. Ammonia entry into the PVCs *via* Rhbg would be reduced and redirected to the MRCs where increased Rhcg1 levels would favour apical exit through this route. Only when plasma ammonia levels were very high (after exposure to 5 mmol l^{-1} NH_4HCO_3), did we observe an upregulation of *Rhcg2* as well as *Rhcg1* and a restoration of *Rhbg* to control levels (Table 2). In this extreme situation where ammonia excretion was enhanced over the control rate (Fig. 6), additional support from the PVCs may be necessary.

The role of Rhag

Rhag has been detected in the gills of zebrafish (Braun et al., 2009) and in the longhorn sculpin (Claiborne et al., 2008) and has been localised to the basolateral and apical regions of the pillar cells in the gills of pufferfish (Nakada et al., 2007). Gill *Rhag* mRNA levels were significantly downregulated throughout the HEA exposure period (Fig. 3). Under low environmental ammonia conditions, Rhag in the pufferfish pillar cells may facilitate the unidirectional movement of ammonia from the blood to the gill for excretion into the external water. However, it is known that Rh proteins transport ammonia and methylamine in a bi-directional manner (Marini et al., 2000; Mayer et al., 2006; Nawata et al., 2010; Weidinger et al., 2007; Westhoff et al., 2004) and therefore during HEA, ammonia could also pass from the water into the fish *via* Rh channels. The downregulation of *Rhag* mRNA levels in the gill during HEA suggests a barrier mechanism to minimise the entry and/or back flux of external ammonia into the general circulation of the pufferfish. The downregulation of *Rhbg* may serve as a similar barrier mechanism to reduce the flux of external ammonia into the fish through the PVCs which make up the vast majority of the gill surface.

Rhag mRNA but not protein was also downregulated in the erythrocytes (Figs 5 and 9). A similar downregulation of *Rhag* mRNA in freshwater trout exposed to HEA was also reported (Nawata et al., 2007). The reason for this response is not clear; however, it may be a protective mechanism to prevent uptake of excess NH_3, which would result in water uptake and swelling. Also, the buffering action of NH_3 would interfere with the Bohr effect, disrupting O_2 uptake and release from haemoglobin (Bruce, 2008).

H^+-ATPase

In the freshwater rainbow trout gill, H^+-ATPase has been localised to the apical region of both PVCs and MRCs (Wilson et al., 2000a; Perry and Fryer, 1997). During HEA, gill *H^+-ATPase* mRNA expression and activity increased in the rainbow trout, suggesting that proton excretion may be important for trapping NH_3 as it exits the PVCs through Rhcg2 (Nawata et al., 2007). A similar acid-trapping mechanism for NH_3 was demonstrated in the skin of freshwater zebrafish larvae when H^+-ATPase was knocked down (Shih et al., 2008). Also, in the euryhaline crab, *Carcinus maenas*, H^+-ATPase is thought to trap cytoplasmic NH_3 as NH_4^+ in vesicles for transport and exocytosis from the gill (Weihrauch et al., 2002). While H^+-ATPase may have little involvement in ammonia excretion from the mudskipper gill (Randall et al., 1999), it was apparent from the current study that *H^+-ATPase* mRNA expression (Fig. 4) and activity (Table 3) increased in response to HEA in the pufferfish gill. It is tempting to speculate that, similar to the rainbow trout, H^+-ATPase may function in cooperation with Rhcg2 in the PVCs because the mRNA expression and activity of H^+-ATPase increased simultaneously with the increase in *Rhcg2* mRNA expression after 5 mmol l^{-1} NH_4HCO_3 exposure (Table 3). Indeed, an increase in acid secretion would facilitate NH_3 trapping and reduce entry of NH_3 from the water through the Rh channels during HEA. The localisation of H^+-ATPase and its role in ammonia excretion in the pufferfish gill, however, have yet to be determined.

NHE

While eight isoforms of the Na^+/H^+ exchangers (NHEs) have been cloned in the zebrafish (Yan et al., 2007), NHE1, NHE2 and NHE3 are the most characterised isoforms in fish (Edwards et al., 2005). Although a clear apical or basolateral gill orientation has not been determined for NHE1 (Edwards et al., 2005), both NHE2 and NHE3 have been localised to the apical region (Evans, 2008). NHEs are thought to play a role in acid–base regulation in both freshwater and seawater fish (Edwards et al., 2005), and in the proximal tubules of the mammalian kidney, NHE3 is thought to be involved in ammonia excretion with NH_4^+ substituting for H^+ (Weiner and Hamm, 2006). Experimental evidence suggests that under low environmental ammonia conditions, little or no ammonia excretion occurs *via* apical Na^+/NH_4^+ exchange in seawater fish (Evans, 1982; Evans, 1984; Evans and More, 1988; Evans et al., 1979; Evans et al., 1989); however, under HEA conditions, apical NHE is thought to facilitate NH_4^+ excretion in the mudskipper (Randall et al., 1999). Although the results from our study did not reveal a change in *NHE1* mRNA expression, *NHE3* mRNA levels were greatly elevated, suggesting a role for this transporter during HEA (Fig. 4). In addition, the downregulation of *NHE2* and the upregulation of *NHE3* mRNA suggest that these two transporters may serve different functions in the pufferfish gill during HEA. Increased Na^+ loading due to upregulated levels of NHE3 would also necessitate the downregulation of NHE2 to limit or reduce the overall uptake of Na^+.

NKA

Due to a similar hydrated radius, NH_4^+ is known to substitute for K^+ in the NKCC and the NKA in a variety of different tissues (Kinne et al., 1986; Kurtz and Balaban, 1986; Towle and Holleland, 1987; Wall et al., 1995). NKA has been implicated in the basolateral uptake of ammonia in the collecting duct of the mammalian kidney (Kurtz and Balaban, 1986; Wall and Koger, 1994) and similarly, basolateral gill Na^+/NH_4^+ exchange *via* NKA has been suggested from studies

on the toadfish (Claiborne et al., 1982; Mallery, 1983). In fact, active excretion of ammonia against a gradient *via* NKA was demonstrated in mudskippers exposed to HEA (Randall et al., 1999). Ouabain treatment reduced ammonia excretion, but only when the mudskippers were exposed to HEA, suggesting that NKA-mediated NH_4^+ transport becomes important when the ammonia gradients are reversed and/or when plasma ammonia levels are elevated. Similarly, in isolated perfused gills of the marine crab, *Cancer pagurus*, active ammonia excretion was reduced in the presence of ouabain (Weihrauch et al., 1999). In the current study, NKA clearly responded to HEA in the pufferfish gills. *NKA* mRNA expression and activity were significantly elevated after exposure to 48 h of NH_4HCO_3 (Figs 4 and 11), and, furthermore, we have demonstrated that NH_4^+ was a functional substrate, capable of activating the enzyme in the absence of K^+ (Fig. 11), as was previously demonstrated with toadfish and mudskipper gill NKA (Mallery, 1983; Randall et al., 1999). Although there was a delay between the transcription and translation of NKA after 12 h exposure to 5 mmol l^{-1} NH_4HCO_3 (Table 3, Fig. 8), mRNA and protein levels were both upregulated after 48 h of exposure to 1 mmol l^{-1} NH_4HCO_3 (Figs 4 and 8).

NKCC1

Mammalian NKCC2 is a kidney-specific isoform with an apical orientation while the NKCC1 isoform is basolaterally located in the membranes of secretory cells (Haas and Forbush, 1998). NKCC1 is thought to be involved in the basolateral uptake of NH_4^+ in the kidney (Weiner and Hamm, 2006) as well as in colonic crypt cells together with Rh proteins (Worrell et al., 2008), and has also been implicated in ammonia-induced astrocyte swelling in the brain (Jayakumar et al., 2008). In seawater fish gills, NKCC1 is involved in salt secretion and has been detected in the basolateral regions of the MRCs in a number of species (Hwang and Lee, 2007). Although some ammonia may be excreted *via* NKCC in the dogfish shark pup (Evans and More, 1988), the same was not found to be true in the case of toadfish (Evans et al., 1989). Those studies, however, were carried out under low external ammonia conditions. Because NKCC1 is driven by the Na^+ gradient maintained by NKA, NKCC1 may become important during HEA conditions, when plasma ammonia levels are elevated and NKA is upregulated. Indeed the simultaneous upregulations of *NHE3*, *NKA* and *NKCC1* mRNA levels at 48 h of exposure to 1 mmol l^{-1} NH_4HCO_3 (Fig. 4) and the sustained upregulation of *NKCC1* with exposure to 5 mmol l^{-1} NH_4HCO_3 (Table 3) reinforce the importance of NKCC1 under HEA conditions.

Proposed model of ammonia excretion during HEA

Overall, our results are concordant with the original model proposed by Nakada et al. (Nakada et al., 2007). Under control conditions, there is a favourable P_{NH_3} gradient for passive ammonia diffusion from blood to water. Some or all of this may be facilitated by Rh proteins with pillar cell Rhag working in cooperation with basolateral Rhbg and apical Rhcg2 in the PVCs to facilitate ammonia efflux out of the gill. Additional support from the MRCs was suggested by Nakada et al. (Nakada et al., 2007) as an auxiliary route of excretion *via* the basolateral Na^+/K^+-ATPase (NKA) and apical Rhcg1. The present data suggest that this route comes into play during HEA. However, we provide evidence for the involvement of additional transporters (H^+-ATPase, NKCC1, NHE3) not implicated in the original model. Indeed the presence of NHE3 and NKCC1 in MRCs has been previously demonstrated (Choe et al., 2005; Hirata et al., 2003; Hiroi and McCormick, 2007; Inokuchi et al., 2008; Inokuchi et al., 2009; Ivanis et al., 2008; Yan et al., 2007), suggesting that the colocalisation of these transporters with Rhcg1 and NKA may serve to regulate ammonia excretion in a coordinated fashion from the MRCs. Additional mechanisms to reduce the entry or back flux of ammonia from the external water (downregulation of Rhag in pillar cells, and Rhbg in PVCs) are also suggested from the present study.

Seawater fish may face a greater challenge during HEA than the freshwater fish. With a more permeable gill, the influx of ammonia during HEA would be greater than that in the freshwater gill (Wilson and Taylor, 1992). Therefore, the first line of defence in the pufferfish when plasma ammonia levels are elevated under HEA conditions may be to try to reduce ammonia entry and/or back flux *via* downregulation of Rhbg in PVCs and Rhag in pillar cells, while switching over to the MRCs, where excretion could be driven by NKA. NH_4^+ would enter basolaterally *via* NKA and/or NKCC exiting as NH_3 through the apical Rhcg1 after being deprotonated. NH_3 would then combine with a H^+ ion released from NHE3, thus minimising the back flux of NH_3 though the Rh channels (Fig. 12). This is in contrast to the recently proposed model for freshwater fish (Wright and Wood, 2009) where Rhbg, Rhcg2, NHE2 and H^+-ATPase may work in a coordinated manner to facilitate gill ammonia excretion. The pufferfish model also differs slightly from the active ammonia excretion model proposed earlier for the mudskipper (Wilson et al., 2000b), before the discovery of Rh proteins in fish. In the mudskipper model, NH_4^+ is excreted *via* apical NHEs. Although we cannot exclude the possibility that some NH_4^+ may leave the gill by this route in the pufferfish, the inclusion of Rhcg1 into the pufferfish model suggests that NHE3 may be more important for H^+ excretion. While the distribution of pufferfish gill H^+-ATPase has not yet been determined and its localisation in the gills of different marine fish species appears to be inconsistent (Evans et al., 2005), in our preliminary working model, we have included it on the apical membrane where it may participate in NH_3 reprotonation (Fig. 12).

Effects of hypercapnia

Because some studies have provided evidence for Rh protein involvement in CO_2 transport (Endeward et al., 2007; Li et al., 2007; Soupene et al., 2002; Soupene et al., 2004), the possibility of a dual NH_3/CO_2 transport function for Rh proteins in fish should not be overlooked. An earlier study on freshwater trout did not reveal any significant changes in gill Rh transcript levels after exposure to 12 h of hypercapnia (Nawata and Wood, 2008). In the present study, 24 h of hypercapnia resulted in a downregulation of *Rhag* mRNA levels in the gill (Table 2), a similar response observed with HEA exposure (Fig. 3). However, unlike HEA, hypercapnia exposure did not raise the plasma ammonia levels and therefore this change must have been in direct response to the high CO_2. In fact, at the protein level, all of the Rh proteins (Rhag, Rhbg, Rhcg1 and Rhcg2) in the gill responded to hypercapnia as evidenced by changes in molecular mass when compared with the control (Fig. 7). Additionally, there was a significant decrease in *Rhag* mRNA but not protein levels in the erythrocytes with hypercapnic exposure (Table 2, Fig. 9). It is well known that hypercapnic conditions result in swelling of teleost erythrocytes (e.g. Irving et al., 1941; Nikinmaa, 1992) and, therefore, if indeed Rhag functions as a CO_2 channel, then downregulation of Rhag would reduce CO_2 entry thus protecting the erythrocytes from excessive swelling. Together, these observations support a possible role for Rh proteins as dual NH_3/CO_2 transporters.

A role for the skin?

An emerging picture is that the skin may be an auxiliary site of ammonia excretion *via* Rh proteins in fish. In the trout, Rh mRNA levels in the skin increased after branchial excretion was blocked by HEA or Hepes exposure, and after ammonia infusion (Nawata et al., 2007; Nawata and Wood, 2008). In the mangrove killifish, which volatilises ammonia through the skin, Rh mRNA levels were upregulated in the skin after HEA and also aerial exposure (Hung et al., 2007). The most convincing evidence of Rh involvement in skin ammonia excretion, however, was provided by Shih et al. (Shih et al., 2008) in zebrafish larvae and by Wu et al. (Wu et al., 2010) in medaka larvae, both of whom demonstrated, using the scanning ion electrode technique, that Rhcg1 facilitated ammonia excretion across MRC-type cells in the skin. In the present study, exposure of pufferfish to 1 mmol l^{-1} NH_4HCO_3 for 48 h did not result in significant changes of skin Rh mRNA levels (Fig. 5); however, exposure to 5 mmol l^{-1} NH_4HCO_3 resulted in an upregulation of both *Rhbg* and *Rhcg2* mRNA (Table 2). As previously noted in trout exposed to hypercapnia (Nawata and Wood, 2008), no changes of skin Rh mRNA levels were observed in pufferfish exposed to hypercapnia (Table 2). This suggests that Rh mRNA in the skin may be responding to plasma ammonia levels. The significance of Rh proteins in the skin of freshwater and seawater fish would be worthy of future investigation.

Post-translational modifications of the Rh proteins

This study has revealed that both HEA and high CO_2 exposure resulted in post-translational modifications (PTMs) of the Rh proteins in the membrane protein enriched fraction of the gill as indicated by the molecular mass shifts (Fig. 7). These changes were independent of glycosylation and of relatively large molecular mass, which would exclude PTMs such as phosphorylation or methylation. This suggests conjugation or complexation to other proteins. One possibility, especially in the case of Rhag, may be monoubiquitination (~8 kDa), which acts as an endocytic signal controlling the turnover and downregulation of membrane proteins (Mukhopadhyay and Riezman, 2007; Schnell and Hicke, 2003). Interestingly, although Rhag appeared to undergo modification in the gill after hypercapnia, a similar modification did not occur in the erythrocyte (Figs 7 and 9). In the case of Rhcg1, it may be that under control conditions, the majority of these transporters are in a quiescent state, requiring stimuli such as high ammonia or CO_2 to induce the changes necessary for full functionality. Further studies are required to identify the PTMs and to determine their functional significance in the modulation of gill Rh proteins during HEA and high CO_2 exposure.

Conclusions

The results of this study provide molecular and physiological evidence to support the model of ammonia excretion proposed earlier for pufferfish. A difference in the handling of high plasma ammonia *via* Rh proteins between freshwater and seawater fish has also been revealed. Under basal conditions, ammonia excretion in the pufferfish probably occurs *via* passive diffusion and/or *via* Rhbg and Rhcg2 in the PVCs, facilitated by a favourable gradient and the high permeability of the gill epithelium. When plasma levels become elevated, however, a more costly active excretion mechanism may be implemented. A switch-over to the MRCs, powered by NKA, with NKCC, NHE3 and Rhcg1 working in cooperation, would be more efficient at lowering plasma ammonia levels and countering the passive influx of ammonia during HEA. The roles of Rhbg in PVCs and Rhag in the pillar cells have also been highlighted as important regulators of ammonia transport to and from the blood.

LIST OF ABBREVIATIONS

HEA	high environmental ammonia
J_{Amm}	ammonia excretion rate
MRCs	mitochondria-rich cells
NHE	Na^+/H^+ exchangers
NKA	Na^+/K^+-ATPase
NKCC1	$Na^+/K^+/2Cl^-$ co-transporter
PCR	polymerase chain reaction
PTMs	post-translational modifications
PVCs	pavement cells
qPCR	quantitative PCR
Rh	Rhesus
T_{Amm}	total ammonia

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