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The effects of CO_2 and external buffering on ammonia excretion and Rhesus glycoprotein mRNA expression in rainbow trout

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SUMMARY

Rhesus (Rh) proteins were recently characterized as ammonia gas (NH_3) channels. Studies indicate, however, that Rh proteins also facilitate CO_2 transport in a green alga and in human erythrocytes. Previously, we reported that Rh mRNA expression in various rainbow trout tissues responded to high environmental ammonia. To determine whether or not Rh proteins may also be involved in CO_2 transport in rainbow trout, we examined the effects of a 12 h exposure to external hypercapnia (1% CO_2 in air) on Rh mRNA expression in the gill, skin and erythrocytes. External hypercapnic conditions lowered the water pH and facilitated ammonia excretion; therefore, we also studied the effects of hypercapnia and normocapnia in the presence of 10 mmol l^{-1} Hepes-buffered water. Hepes treatment prevented water acidification, but resulted in elevated plasma ammonia levels and reduced ammonia excretion rates. Hypercapnia exposure without buffering did not elicit changes in Rh mRNA expression in the gill or skin. However, *Rhcg2* mRNA expression was downregulated in the gills and upregulated in the skin of both normocapnia- and hypercapnia-exposed fish in Hepes-buffered water. mRNA expression of a newly cloned *Rhbg2* cDNA was downregulated in the skin of fish exposed to buffered water, and *Rhag* mRNA expression in erythrocytes was decreased with exposure to normocapnia in buffered water but not with hypercapnia exposure in either buffered or unbuffered water. With the aid of Hepes buffering, we were able to observe the effects of both CO_2 and ammonia on Rh mRNA expression. Overall, we conclude that high CO_2 did not directly elicit changes in Rh mRNA transcription levels in the gill and skin, and that the changes observed probably reflect responses to high plasma ammonia, mirroring those in trout exposed to high environmental ammonia. Therefore a dual function for gill and skin Rh proteins in CO_2 and ammonia transport is not evident from these results. *Rhag* expression, however, responded differentially to high CO_2 and high ammonia, suggesting a possible dual role in the erythrocytes.

Key words: hypercapnia, Rhesus glycoproteins, *Oncorhynchus mykiss*, gills, skin, cortisol, Hepes, ammonia transport.

INTRODUCTION

Rhesus (Rh) proteins are most commonly known for their role in erythrocyte antigenicity (Avent et al., 2006). Several non-erythrocytic Rh proteins have now been identified in a variety of species ranging from bacteria to mammals (e.g. Chain et al., 2003; Huang and Peng, 2005; Weihrauch, 2006). Structural and functional studies have revealed that Rh proteins are transporters of ammonia, a property shared with AmtB, their *Escherichia coli* homologue (for reviews, see Planelles, 2007; Van Kim et al., 2006; Weiner and Hamm, 2007). There is some conflict as to whether the primary substrate is NH_3 or NH_4^+, but most evidence points to NH_3 (Javelle et al., 2007).

A number of different Rh orthologues have been characterized in fish (Huang and Peng, 2005; Hung et al., 2007; Nakada et al., 2007a; Nakada et al., 2007b; Nawata et al., 2007). While Rhag appears to be limited to erythrocytes in mammals (Huang and Peng, 2005), it occurs in both erythrocytes and pillar cells in the pufferfish *Takifugu rubripes* (Nakada et al., 2007b). Additionally, Nakada and colleagues showed in pufferfish that while Rhbg and Rhcg2 are situated basolaterally and apically, respectively, in the pavement cells, Rhcg1 is located apically in mitochondria-rich cells (Nakada et al., 2007b). Exactly how these Rh proteins function in ammonia transport in fish is not yet clear; however, Rh mRNA expression levels do change in response to high external ammonia in some fish species (Hung et al., 2007; Nawata et al., 2007), but not all (Nakada et al., 2007a).

The bulk of studies implicate Rh proteins in NH_3 transport, although there remains the possibility that they also facilitate transfer of CO_2, another readily hydrated gas of similar size (Endeward et al., 2006; Kustu and Inwood, 2006). Soupene and colleagues reported that the Rh1 protein of the green alga *Chlamydomonas reinhardtii* was upregulated by high CO_2 and that the absence of Rh1 impaired the growth of this alga under high CO_2, a condition that normally promotes rapid growth (Soupene et al., 2002; Soupene et al., 2004). Recently it was demonstrated, using ^{18}O and mass spectrometry, that both NH_3 and CO_2 pass through the RhAG protein of human erythrocytes (Endeward et al., 2006). These two substrates compete for entrance and passage through the RhAG channel, although the affinity appears to be greater for NH_3 than CO_2. Furthermore, structural studies performed on the Rh1 protein of the ammonia-oxidizing bacterium *Nitrosomonas europaea* revealed that it may have a much lower ammonium affinity than the Amt proteins (Lupo et al., 2007) and a possible CO_2 binding site at the COOH-terminal end has been described (Li et al., 2007).

A few studies suggest, however, that CO_2 is not transported through the Rh channel. Ripoche and colleagues, for example, found no difference in the permeability to CO_2 in human erythrocyte membranes lacking RhAG, using a stopped-flow method (Ripoche et al., 2006). More recently it was shown that both wild-type (Weidinger et al., 2007) and *Rh1* knockout mutants (Cherif-Zahar et al., 2007) of *N. europaea* were unresponsive to CO_2.

Like ammonia, CO_2 in fish is believed to pass through membranes mainly in its gaseous form (Henry and Heming, 1998), while the transport of CO_2 occurs in the plasma in its hydrated form, HCO_3^- (Perry and Gilmour, 2002). A small amount of CO_2 exits the apical gill epithelium as HCO_3^- in exchange for Cl^- after being hydrated in the gill by carbonic anhydrase and thus serves a role in acid–base regulation (Claiborne et al., 2002; Perry, 1986; Perry and Gilmour, 2006).

In the present study, our goal was to determine whether exposure to high external CO_2 levels could elicit changes in Rh mRNA expression in rainbow trout gill, skin and erythrocytes, which would indicate a possible dual role for the Rh glycoproteins as NH_3 and CO_2 channels. However, studying the effects of high CO_2 on Rh mRNA expression in fish is problematic in that hypercapnia causes an increase in endogenous ammonia production (Claiborne and Heisler, 1986). Increased ammonia excretion rates have also been associated with exposure to external hypercapnia (Claiborne and Heisler, 1986) and this may be due to the favourable plasma to water ammonia gradient created by the lowered water pH as a result of the hydration of CO_2 in the water (Claiborne and Heisler, 1986; Larsen and Jensen, 1997; Lloyd and Herbert, 1960; Wright et al., 1989). Buffering minimizes acidification of the water during hypercapnia; however, this introduces the confounding effect of abolishing the acidified boundary layer at the gill that normally facilitates ammonia excretion (Salama et al., 1999; Wilson et al., 1994; Wright et al., 1986; Wright et al., 1989).

We therefore examined the effect of three experimental conditions on the mRNA expression of Rh glycoproteins in trout: hypercapnia alone, hypercapnia with Hepes buffering, and normocapnia with Hepes buffering. The hypercapnia treatment chosen (1% CO_2 for 12 h) directly duplicated a previous study in our lab in identical water quality where internal acid–base and ion status (both extracellular and intracellular) were monitored (Wood and LeMoigne, 1991). Additionally we analysed the ammonia excretion rates, plasma cortisol levels and the mRNA expression of other gill transporters possibly involved in ammonia excretion in each experimental condition. Finally, we isolated three full-length *Rhbg2* cDNA variants and assessed the mRNA expression of these in the gill and skin.

MATERIALS AND METHODS

Animals

Rainbow trout (*Oncorhynchus mykiss*, Walbaum), weighing 170–240 g, were obtained from Humber Springs Trout Hatchery, Ontario, Canada, and held in dechlorinated Hamilton tap water (moderately hard: $[Na^+]$=0.6 mequiv.l^{-1}, $[Cl^-]$=0.8 mequiv.l^{-1}, $[Ca^{2+}]$=0.8 mequiv.l^{-1}, $[Mg^{2+}]$=0.3 mequiv.l^{-1}, $[K^+]$=0.05 mequiv.l^{-1}; titration alkalinity 2.1 mequiv.l^{-1}; pH ~8.0; hardness ~140 mgl^{-1} as $CaCO_3$ equivalents; temperature 12–16°C) and fed *ad libitum* until 1 week before experimentation, during which time food was withheld. Fish were transferred to individual, opaque boxes supplied with aerated, flowing dechlorinated tap water (15±0.5°C) and allowed to recover overnight. 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.

Experimental conditions

Effect of external hypercapnia

Water flow was closed off to each box and the box water volume was set to 4 l. Fish were exposed for 12 h to a 1% CO_2 in air mixture (hypercapnia), provided by a Wösthoff gas-mixing pump (Calibrated Instruments, Ardsley, NY, USA), bubbled into the water. Control fish were treated identically with a 12 h exposure to 100% air (normocapnia).

Effect of Hepes buffering

Bubbling of 1% CO_2 in air into the external water reduced the pH by approximately 1.22 pH units, a similar reduction to that reported earlier by other investigators (Larsen and Jensen, 1997; Wright et al., 1988a). In order to minimize acidification of the water while fish were exposed to hypercapnia, Hepes (Sigma, St Louis, MO, USA) was added to the external water to a nominal concentration of 10 mmoll^{-1} using a 1 moll^{-1} stock solution adjusted to pH 8.0 with KOH. This maintained the pH within the range of 7.76–7.39 during the course of 12 h. To examine the effects of Hepes buffering alone on ammonia excretion and mRNA expression, another set of fish were exposed to normocapnia for 12 h in the presence of 10 mmoll^{-1} Hepes, as above.

Effect of high environmental ammonia

In order to check some particular parameters highlighted in the results of the hypercapnia and Hepes-buffering exposures, a high environmental ammonia (HEA) exposure protocol identical to that used by Nawata and colleagues (Nawata et al., 2007) was repeated using an ammonia concentration similar to that used by previous investigators (Cameron, 1986; Cameron and Heisler, 1983; Claiborne and Evans, 1988; Wilson and Taylor, 1992; Wilson et al., 1994). Trout were exposed to a nominal total waterborne ammonia (T_{Amm}) concentration of 1.5 mmoll^{-1} NH_4HCO_3 (pH 7.95±0.05) for 12 h. During this period the water flow was stopped and the box volume was set to 4 l. Water samples were removed every 3 h and total water ammonia concentration remained virtually constant at 1.41±0.03 mmoll^{-1} over the 12 h period.

Analyses

Water samples (10 ml) were removed at 3 h intervals, stored at –20°C and later analysed in triplicate for total ammonia (T_{Amm}) using a modified salicylate-hypochlorite method (Verdouw et al., 1978). Because Hepes altered colour generation in this assay, additional ammonia standards containing 10 mmoll^{-1} Hepes were prepared and analysed in conjunction with the samples from the Hepes experiments. At the end of each experiment, fish were anaesthetized with 0.1 gl^{-1} MS222 (Sigma) and caudal blood samples were collected into a heparinized syringe. Blood cells were separated from plasma by centrifugation and both blood fractions were snap frozen in liquid nitrogen and stored at –70°C.

Net flux rates of total ammonia (J_{Amm}; μmolkg^{-1} h^{-1}) into the water were calculated as: $J_{Amm}=(T_{Amm,i}-T_{Amm,f})\times V/(t\times M)$ where i and f refer to the initial and final concentration (μmoll^{-1}), V is the box water volume (l), t is the time elapsed (h) and M is the fish mass (kg). A negative J_{Amm} indicates net excretion into the water. Plasma total ammonia was measured enzymatically (Raichem, Hemagen Diagnostics, San Diego, CA, USA) and reported in μmoll^{-1}.

External water pH was monitored with a Radiometer GK2401C (Copenhagen, Denmark) low ionic strength combination electrode thermostatically set to the experimental water temperature.

Tissue sampling

Prior to tissue extraction, fish were perfused free of blood using Cortland saline (Wolf, 1963) as previously described (Nawata et al., 2007). Samples of gill, skin and blood were removed, snap frozen in liquid nitrogen and stored at –70°C until later processing. Gill

Table 1. Primer list

Name	Forward/reverse sequence (5′–3′)	Application	GenBank accession no.
Rhbg2	cctggctgtgaccctcggcattg/ctccctggctgtgaccctcggcattg	RACE	EU660222
Rhbg1	gaagatacaggacacgtgtg/gatgcccagggttacggc	qPCR	EF051113
Rhbg2	ctgtgaccctcggcattgc/ggatctcaagtgtgtgtgtg	qPCR, RT-PCR	EU660221
Rhcg1	catcctcagcctcatacatgc/tgaatgacagacggagccaatc	qPCR	DQ431244
Rhcg2	cctcttcggagtcttcatc/ctatgtcgctggtgatgttg	qPCR	AY619986
Rh30-like2	gctgctgcactgatatcaac/cgcccgttgtgagatcatc	qPCR	EF062577
Rhag	ctggcggccaatgatgttg/atggcgaagaggtcagagtg	qPCR	EF667352
CA	gccagtctcccattgacatc/cctgtacgtccctgaaatgg	qPCR	AY514870
HAT	tcagccttggttgtgagatg/caacattggtgggaaacagg	qPCR	AF140002
NHE	tatggccattgtgacctgtg/caggcctctccacactaagg	qPCR	EF446605
NKA	ttgacctggatgaccacaag/ggatctccttagcccgaac	qPCR	AY319391
EF-1α	ggaaagtcaaccaccacag/gataccacgctccctctcag	RT-PCR	AF498320
v1	ggcagaccaactcatgtgtc/gttgccgaattcgtcatgg	qPCR	EU660222
v2	ggactccctgcactccca/gttcaactcagctccagagc	qPCR	EU660222
v3	ggactcccactctcccatc/gttgccgaattcgtcatgg	qPCR	EU660222
v4	ggactctcccatcgcatc/gttgccgaattcgtcatgg	qPCR	EU660222

CA, carbonic anhydrase; *HAT*, H^+-ATPase; *NHE*, Na^+/H^+ exchanger; *NKA*, Na^+/K^+-ATPase; *EF-1α*, elongation factor-1 α; v1–4, 3′-UTR variants of *Rhbg2*.

samples from control fish were used for cloning of *Rhbg2* and additional samples of brain, gill, intestine, kidney, liver, muscle and skin were removed for screening of *Rhbg2* mRNA expression.

Total RNA isolation and reverse-transcriptase PCR amplification

Total RNA was extracted from blood and tissues using Trizol (Invitrogen, Burlington, ON, Canada) and concentrations were quantified and checked for quality with a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA) and electrophoresed on 1% agarose gels stained with ethidium bromide, to verify integrity. First strand cDNA synthesis was performed using 1 μg of DNaseI-treated (Invitrogen) total RNA with oligo(dT_{17}) primer and Superscript II reverse transcriptase (Invitrogen).

Full-length cDNA of *Rhbg2* was obtained by 5′- and 3′-rapid amplification of cDNA ends (Smart RACE cDNA amplification kit, BD Bioscience Clontech, Mississauga, ON, Canada) using the RACE primers listed in Table 1 and the protocol described previously (Nawata et al., 2007). Sequence analyses were performed with BioEdit (Hall, 1999) and CLUSTAL W (Thompson et al., 1994). Hydropathy profile and *N*-linked glycosylation site predictions were made using SPLIT 4.0 (Juretic et al., 2002), and ScanProsite (de Castro et al., 2006), respectively.

Tissue distribution of Rhbg2 was determined by performing reverse transcriptase PCR (PCR) on the above-mentioned cDNA with the primer set listed in Table 1 at 35 cycles for *Rhbg2* and 25 cycles for elongation factor *EF-1α*. Products were electrophoresed on 1.5% agarose gels stained with ethidium bromide and sequenced to confirm identity.

mRNA expression

Quantitative real-time PCR (qPCR) was performed on the cDNA described above using the primers listed in Table 1. Rh mRNA expression was assessed in the gill, skin and erythrocytes and the expression of carbonic anhydrase (*CA2*; cytoplasmic), *H^+-ATPase* (v-type, B-subunit), Na^+/H^+ exchanger (*NHE*) and Na^+/K^+-ATPase (*NKA*; α1a-subunit) was analysed in the gill. Each 20 μl reaction contained 4 μl of cDNA, 8 pmol of each primer and 10 μl of RT^2 Real-Time SYBR Green/ROX PCR Master Mix (SuperArray, Bioscience Corp., Frederick, MD, USA). Analyses were performed at 95°C for 2 min, followed by 40 cycles of 95°C for 30 s and 60°C for 1 min. Melt-curve analysis and gel electrophoresis verified the presence of a single product. No-template controls and non-reverse-transcribed controls were run in parallel. Data were extrapolated from standard curves generated by serial dilution of one randomly selected control sample. Three housekeeping genes encoding β-actin, EF-1α and 18S rRNA proved to be unstable across treatments and data were therefore normalized to ng total RNA, another acceptable method of normalization (Bustin, 2000; Bustin, 2002; Nolan et al., 2006), as further elaborated in the Discussion.

Plasma cortisol

Plasma cortisol levels were measured in duplicate on 25 μl samples by radioimmunoassay (Cortisol ^{125}I RIA Kit, MP Biomedicals, Orangeburg, NY, USA) and values are reported in ng ml^{-1}.

Statistical analysis

Data are presented as means ± s.e.m. (*N*, number of fish). One-way analysis of variance (ANOVA) followed by Fisher's least significant difference *post-hoc* test was used to analyse both the effects of the experimental treatments and the relative abundance of *Rhbg2* mRNA in the skin. One-way repeated measures ANOVA was used to analyse ammonia excretion rates. Significance was set at α=0.05.

RESULTS

Identification of *Rhbg2*

Three *Rhbg2* cDNA sequences were identified in this study (GenBank accession nos: EU660221, EU660222 and EU660223). Two of these, designated *Rhbg2a* and *Rhbg2b*, have identical open reading frames (ORFs) encoding a protein 461 amino acid residues long. Analysis showed a 98% amino acid sequence identity with the ORF of *Rhbg1* (Fig. 1A). Although these *Rhbg2* variants are identical, *Rhbg2b* has a 414 base-pair deletion in the 3′-untranslated region (UTR). The ORF of the third cDNA, designated *Rhbg2c*, is a truncated variant of the full-length ORF, with 166 amino acids deleted from the COOH-terminal end. The last 18 amino acids form an alternative end (Fig. 1A). *Rhbg2b* and *Rhbg2c* additionally have four different 5′-UTR variants (herein termed variants 1, 2, 3 and 4, in order of decreasing length) with an upstream ORF (uORF) that would encode a polypeptide of six amino acids. The longest 5′-UTR variant (variant 1) has an additional uORF upstream to the common uORF that would encode a polypeptide of 26 amino acids (Fig. 1B). *Rhbg2a* has three of the four 5′-UTR variants described; the longest one was not detected by RT-PCR analysis (Fig. 1C).

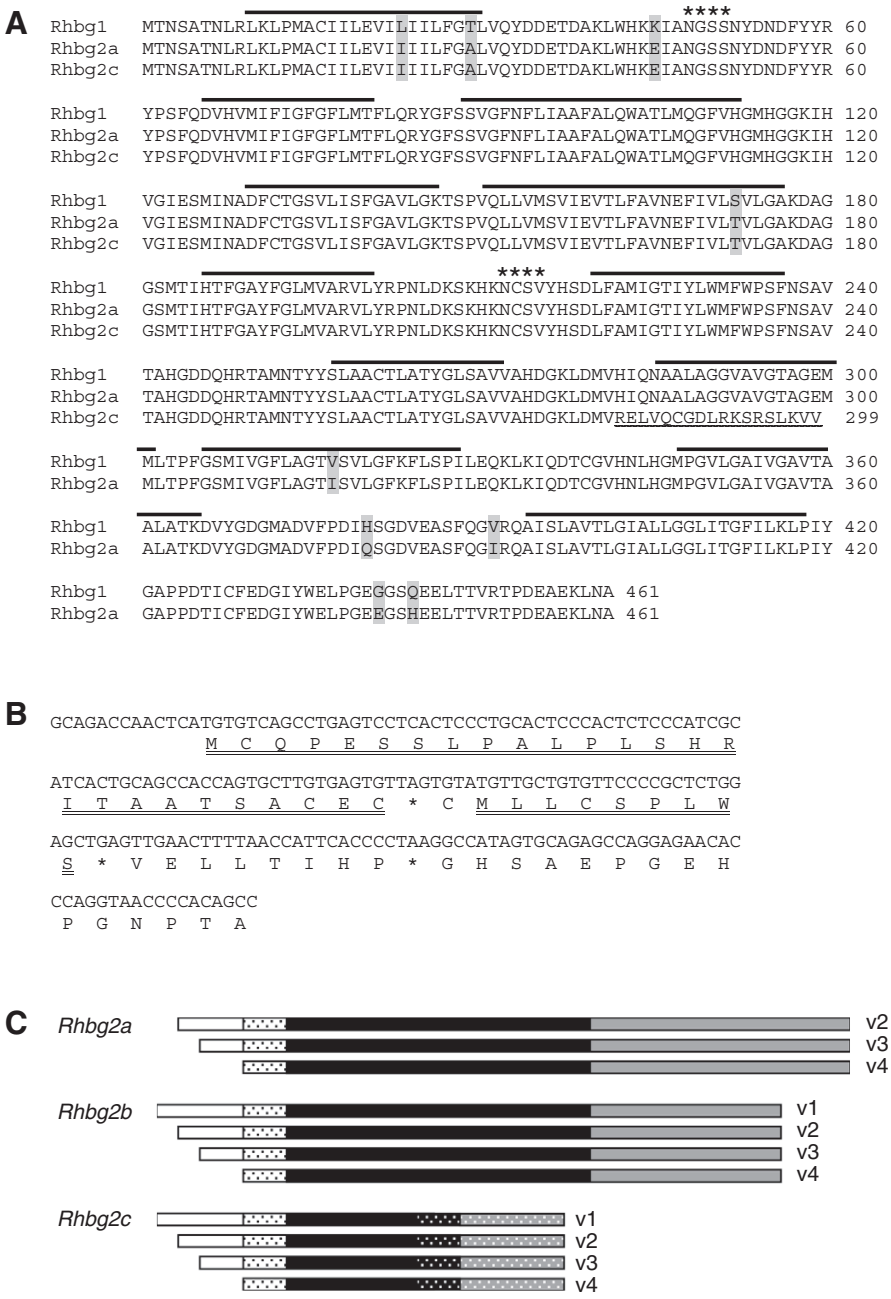

Fig. 1. (A) Predicted amino acid sequence alignment of Rhbg1, Rhbg2a and Rhbg2c. Lines above the sequences represent putative transmembrane domains and asterisks indicate predicted *N*-linked glycosylation sites. The alternative COOH-terminal end of Rhbg2c is underscored. Shading highlights amino acid differences between Rhbg1 and Rhbg2. (B) 5′-Untranslated region (UTR) of the longest *Rhbg2* variant. Two upstream open reading frames (ORFs) are underscored. (C) Schematic representation of the mRNA variants identified in *Rhbg2*. Shading and stippling identifies regions of identity between variants. White background represents the 5′-UTR, black background represents the ORF, and grey background represents the 3′-UTR. Four variants (v1, 2, 3, 4) were detected in the 5′-UTRs, three of which were detected in *Rhbg2a*.

Hydrophobicity analysis revealed 12 predicted transmembrane domains and two putative *N*-linked glycosylation sites for Rhbg2a and Rhbg2b (Asn48 and Asn212). The truncated variant, Rhbg2c retains the same predicted *N*-linked glycosylation sites as the full-length variants, but has only eight predicted transmembrane domains (Fig. 1A).

Tissue distribution and expression of *Rhbg2*

PCR analysis of the various trout tissues from control fish revealed that *Rhbg2* is expressed in the brain, gill, intestine, skin and possibly in the liver and muscle (Fig. 2). The truncated form, *Rhbg2c* was only detected in the gill (data not shown). qPCR analysis performed on control samples of gill and skin showed that the 5′-UTR variants were present in equal abundance in the gill. In the skin, however, variants 1 and 3 were significantly more abundant than the other two. To examine whether or not these variants respond differentially, we analysed the expression in the skin of fish exposed to hypercapnia in Hepes-buffered water. Expression of all four variants decreased such that they were no longer differentially expressed (Fig. 3).

External hypercapnia

Exposure to external hypercapnia resulted in ammonia excretion rates that were elevated significantly over both the 3 and 12 h control rates during the course of the exposure. The rates at 3 and 12 h were 69% and 54% higher than the control rates, respectively (Fig. 4). Plasma ammonia levels in these fish at 12 h were not significantly different from the control values (Fig. 5). There were no significant changes in Rh glycoprotein mRNA expression in the gill or skin, although *Rhcg2* mRNA was decreased by almost 50% (Figs 6 and 7). Similarly, no significant differences in *Rhag* and *Rh30-like2* mRNA expression were noted in the erythrocytes; however, *Rhag* was increased by about 1.7-fold (Fig. 8). Expression of *CA2*, H^+-

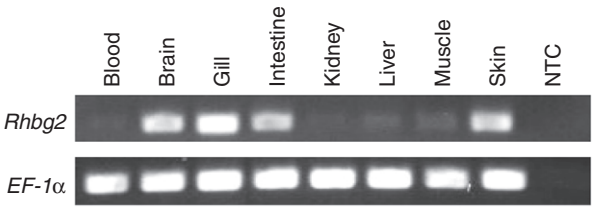

Fig. 2. Tissue distribution of *Rhbg2* mRNA in rainbow trout exposed to control conditions (100% air for 12 h) as determined by reverse transcriptase PCR. Elongation factor (EF-1α) was used as an internal control. NTC, no template control.

ATPase, *NHE2* and *NKA* mRNA in the gill was unchanged (Fig. 9). The plasma cortisol concentration was not significantly different from that of the control fish (Fig. 10).

External hypercapnia with Hepes buffering

Throughout the 12 h exposure to hypercapnia in Hepes-buffered water, trout exhibited ammonia excretion rates that were significantly lower than those in fish exposed to hypercapnia alone, with a 57% reduction at 3 h and a 42% reduction at 12 h. At 9 h, however, the rates were significantly increased above the 3 h control rates and by 12 h they were not different from the 12 h control rates (Fig. 4). Plasma ammonia levels were significantly increased 2.3-fold above the control value after 12 h of exposure (Fig. 5). In the gill, *CA2* and *NHE* mRNA levels were significantly increased (Fig. 9) and *Rhcg2* mRNA was a significantly decreased by 60% (Fig. 6). No significant changes were noted in the mRNA expression of *Rhag* or *Rh30-like2* in the erythrocytes, although expression of *Rhag* was 2-fold higher than the control (Fig. 8). In the skin, there was a 67% reduction in *Rhbg2* but a 30-fold increase in *Rhcg2*. *Rhcg1* was decreased, but not significantly (Fig. 7). Plasma cortisol was not significantly different from the control value at 12 h (Fig. 10).

External normocapnia with Hepes buffering

The ammonia excretion rate in fish exposed to normocapnia in Hepes-buffered water was not significantly different from the 3 h control rate, throughout the 12 h exposure. However, there was a trend towards decreased excretion and by 12 h the rate was reduced to 71% of the 12 h control value (Fig. 4). Plasma ammonia was significantly elevated over the control value at 12 h (Fig. 5). *Rhcg2* mRNA levels were significantly reduced in the gill to about half of the control level (Fig. 6), but no changes were noted in the other gill mRNAs (*CA2*, *H^+-ATPase*, *NHE2*, *NKA*; Fig. 9). In the skin, while there was a 2-fold reduction in *Rhbg1* and a 4-fold reduction in *Rhbg2* mRNA, *Rhcg1* and *Rhcg2* mRNA expression was increased by 4- and 58-fold, respectively (Fig. 7). In the erythrocytes, there was a 3-fold reduction in both *Rhag* and *Rh30-like2* mRNA expression (Fig. 8) and plasma cortisol levels were low, but not significantly different from control values (Fig. 10).

High environmental ammonia

Because of the high nucleotide sequence identity between the *Rhbg1* and the *Rhbg2* cDNAs (the highest being 91.5%, between the *Rhbg1a* and *Rhbg2a* variants), we designed a primer set more specific for *Rhbg1* than that used previously by Nawata and colleagues (Nawata et al., 2007), and assessed the expression of *Rhbg1* and *Rhbg2* in the gill and skin of HEA-exposed fish. Both *Rhbg1* and *Rhbg2* mRNA expression levels remained unchanged

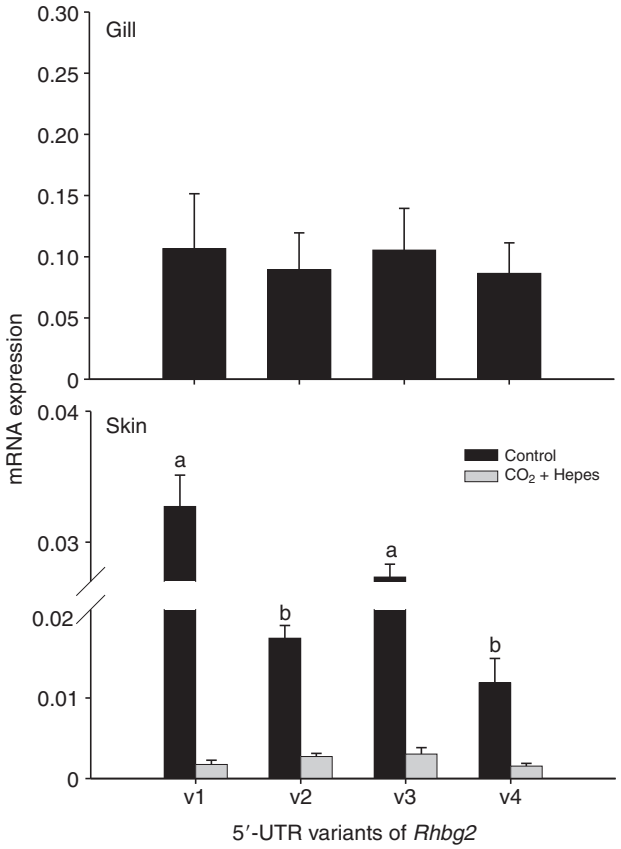

Fig. 3. Relative abundance of 5′-UTR variants (v1, 2, 3, 4) in the gill under control conditions (12 h exposure to 100% air) and skin during control conditions and after 12 h of exposure to hypercapnia (1% CO_2 in air) in 10 mmol l^{-1} Hepes-buffered water. Expression data were normalized to ng total RNA concentration. Different letters indicate significant differences in mRNA abundance between variants in the skin under control conditions. Data are means ± s.e.m. (*N*=6).

in the gill during HEA (Fig. 6). In the skin, *Rhbg1* mRNA levels were not significantly different from that of the control, but *Rhbg2* mRNA expression was significantly downregulated by about 4-fold (Fig. 7). Expression of *Rhcg1* and *Rhcg2* mRNA in the gill and skin, *Rhag* and *Rh30-like2* mRNA in the erythrocytes, and *CA2*, *H^+-ATPase*, *NHE2* and *NKA* mRNA in the gill was also measured and confirmed the results reported earlier by Nawata and colleagues (Nawata et al., 2007) and are therefore not reported here. Plasma ammonia and cortisol levels were both significantly elevated above the control values by 10- and 4-fold, respectively (Figs 5 and 10).

DISCUSSION

In the present study, quantitative expression data for mRNA by real-time PCR were normalized to total RNA rather than to reference control genes. Expression levels of endogenous reference genes such as β-actin or elongation factor (EF-1α) are frequently used as internal controls to normalize expression levels of target genes in real-time PCR studies (Guilietti et al., 2001; Nolan et al., 2006), as in our previous studies on Rh proteins (Hung et al., 2007; Nawata et al., 2007). Several recent reports, however, have shown that reference genes themselves may be modulated by experimental conditions, which in turn may result in inaccurate and misleading interpretations of data (Gibbs et al., 2003; Huggett et al., 2005; Ings and Van der Kraak, 2006; Kubista et al., 2006; Tricarico et al., 2002). Indeed,

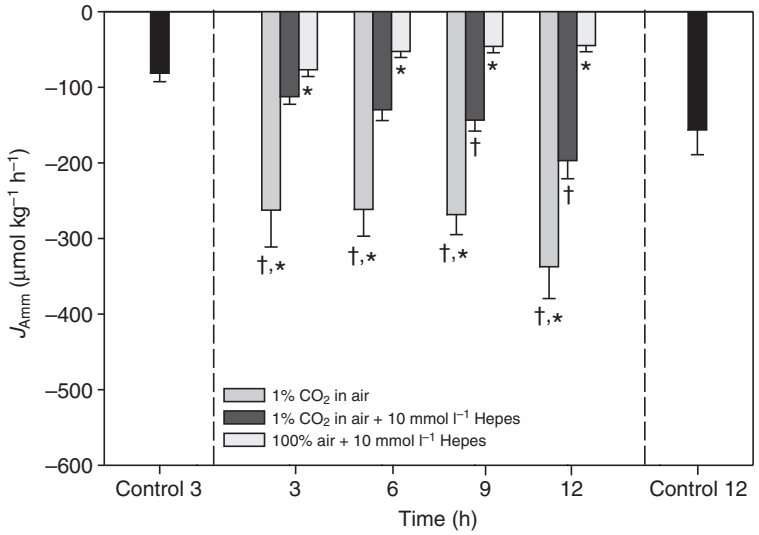

Fig. 4. The effect of 12 h exposure of rainbow trout to: hypercapnia (1% CO_2 in air) in unbuffered water, hypercapnia in 10 mmol l^{-1} Hepes-buffered water, and normocapnia (100% air) in Hepes-buffered water on the net ammonia flux (J_{Amm}). Control fish were exposed to normocapnia for 12 h in unbuffered water. The negative values indicate excretion into the water. Crosses represent values significantly different from the 3 h control value. Asterisks represent values significantly different from the 12 h control value. Control values are significantly different from each other (P<0.05). Data are means ± s.e.m. (N=6–10).

our initial experiments revealed that the expression levels of three reference genes (encoding β-actin, EF-1α and 18S rRNA) were elevated in tissues after exposure to Hepes. Currently, no universal method of normalization exists that accounts for all possible variables; however, an accepted method commonly used (e.g. Luqueti et al., 2005; Serrano et al., 2007; Tipsmark et al., 2008; Vera Cruz et al., 2006) is normalization against total RNA (Bustin, 2000; Bustin, 2002; Huggett et al., 2005; Nolan et al., 2006). Therefore, to eliminate any artificial bias in the data due to reference gene variability, we chose to normalize quantitative expression data to total RNA. In this regard it is important to note that our approach is validated by the target gene responses to HEA normalized against total RNA reported in the present study, which were qualitatively and quantitatively similar to those reported earlier by Nawata and colleagues (Nawata et al., 2007) in parallel but separate HEA experiments on trout where β-actin or EF-1α was used for normalization.

Recently, we established a link between Rh proteins and ammonia by showing that rainbow trout exposed to high environmental ammonia (HEA) responded with upregulated *Rhcg2* mRNA expression in the gill (Nawata et al., 2007). This response coincided with the re-establishment and enhancement of ammonia excretion that was initially inhibited by the reversed plasma to water ammonia gradient. In the present study, we used hypercapnia as a tool to assess the response of Rh mRNA transcription levels to high CO_2 in the rainbow trout. This proved problematic in that, like the study by Claiborne and Heisler (Claiborne and Heisler, 1986), hypercapnia caused an increase in internal ammonia production as evidenced by the elevated rate of ammonia excretion (Fig. 4) without alteration of plasma total ammonia concentration (Fig. 5). Bubbling of CO_2 into the water lowered the pH, creating a sink for NH_3, thus enhancing the rate of excretion. This increased excretion rate was sufficient to maintain plasma ammonia at control levels (Fig. 5) and may explain why changes in Rh mRNA levels were not observed. Any effects that elevated CO_2 may have had were not reflected as changes in mRNA transcription levels in the gill or skin.

Buffering of the external water effectively reduced the acidification caused by the CO_2, but it also probably abolished the acidified gill boundary layer. It has been well documented that the boundary layer, whether acidified by hydration of CO_2 by carbonic anhydrase in the mucus (Wright et al., 1986; Wright et al., 1989) or by release of protons from an apical H^+-ATPase (Lin et al., 1994; Lin and Randall, 1990), normally facilitates ammonia excretion. Indeed, the present study confirms previous reports (Salama et al., 1999; Wright et al., 1989) showing that there was a decline in the ammonia excretion rate when fish were exposed to normocapnia in Hepes-buffered water, with the rate reduced to a third of the control rate by 12 h (Fig. 4). As a consequence of the reduced excretion, plasma ammonia levels in these fish were elevated after 12 h of treatment (Fig. 5). Despite this elevation, and unlike HEA-exposed fish, which also have high plasma ammonia (Nawata et al., 2007), the *Rhcg2* mRNA transcription level was decreased in the gill (Fig. 6). Upregulated or enhanced ammonia transport capacity at the gill would probably prove futile in the presence of Hepes since any protons formed or released at the apical gill surface would be rapidly removed by the buffer, effectively eliminating a favourable ammonia gradient.

However, in the skin of fish exposed to Hepes in the presence of normocapnia, both *Rhcg1* and *Rhcg2* mRNA levels were highly elevated (Fig. 7). Rhcg2 exhibited the greatest fold increase in mRNA expression, suggesting that this protein may have a dominant role in the skin, but this requires further investigation because only

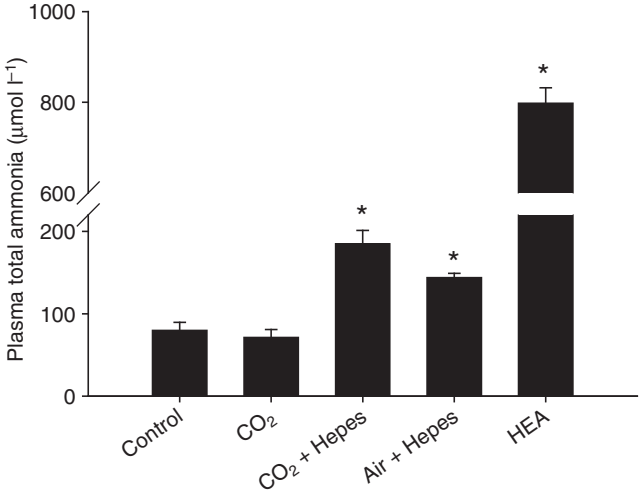

Fig. 5. Plasma total ammonia after 12 h of exposure of rainbow trout to 1% CO_2 in air (CO_2), 1% CO_2 in air with 10 mmol l^{-1} Hepes-buffered water (CO_2 + Hepes), 100% air with 10 mmol l^{-1} Hepes-buffered water (Air+Hepes) and high environmental ammonia (HEA; 1.5 mmol NH_4HCO_3). Control fish were exposed to 100% air for 12 h in unbuffered water. Asterisks represent plasma values significantly different from the control value (P<0.05). Data are means ± s.e.m. (N=6–10).

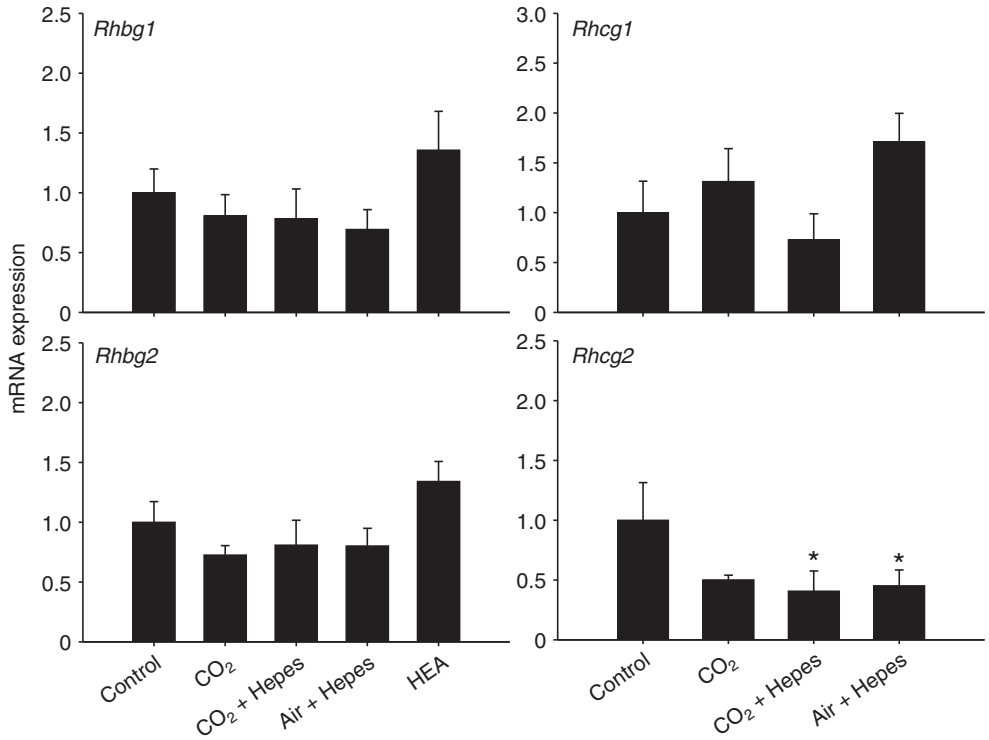

Fig. 6. Gill Rh mRNA expression in rainbow trout after 12 h of exposure to 1% CO_2 in air (CO_2), 1% CO_2 in air with 10 mmol l^{-1} Hepes-buffered water (CO_2+Hepes), 100% air with 10 mmol l^{-1} Hepes-buffered water (Air+Hepes) and high environmental ammonia (HEA; 1.5 mmol NH_4HCO_3). Control fish were exposed to 100% air for 12 h in unbuffered water. Expression data were normalized to ng total RNA concentration with the control value set to one. Asterisks indicate significant difference from the control (P<0.05). Data are means ± s.e.m. (N=6).

mRNA level and not protein function was measured. Upregulation of *Rhcg2* mRNA expression in the skin also occurred when excretion was blocked at the gill after 12 h of exposure to HEA (Nawata et al., 2007). Fish skin is generally thought to have low permeability (Fromm, 1968) and therefore its role in gas and ion exchange has been largely ignored. A few studies have measured ammonia excretion from fish skin (for a review, see Wood, 1993), but it appears that the skin has a secondary role to the gill, if any, in freshwater fish. This may be true especially under normal circumstances, but under conditions in which excretion is blocked at the gill, the skin may become an important alternative site of ammonia release and this possibility needs to be explored further in freshwater species. Indeed the skin becomes a site of ammonia excretion *via* volatilization during aerial exposure in the mangrove killifish *Kryptolebias marmoratus*, and skin Rh mRNA levels are also upregulated during this time (Hung et al., 2007).

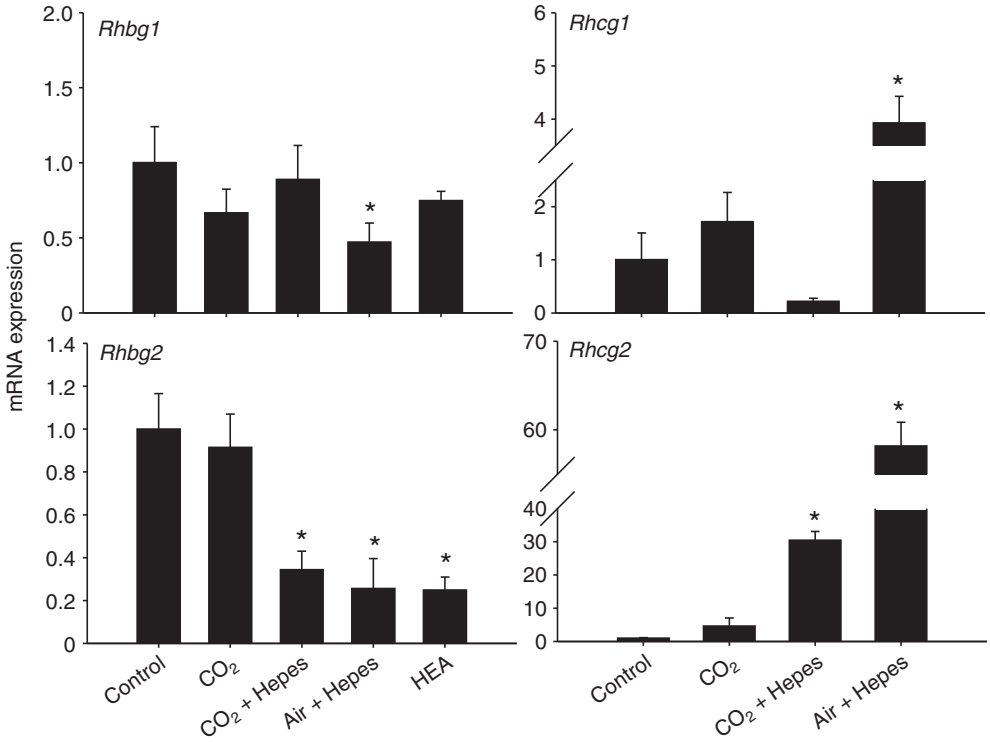

Fig. 7. Skin Rh mRNA expression in rainbow trout after 12 h of exposure to 1% CO_2 in air (CO_2), 1% CO_2 in air with 10 mmol l^{-1} Hepes buffered-water (CO_2+Hepes), 100% air with 10 mmol l^{-1} Hepes-buffered water (Air+Hepes) and high environmental ammonia (HEA; 1.5 mmol NH_4HCO_3). Control fish were exposed to 100% air for 12 h in unbuffered water. Expression data were normalized to ng total RNA concentration with the control value set to one. Asterisks indicate significant difference from the control (P<0.05). Data are means ± s.e.m. (N=6).

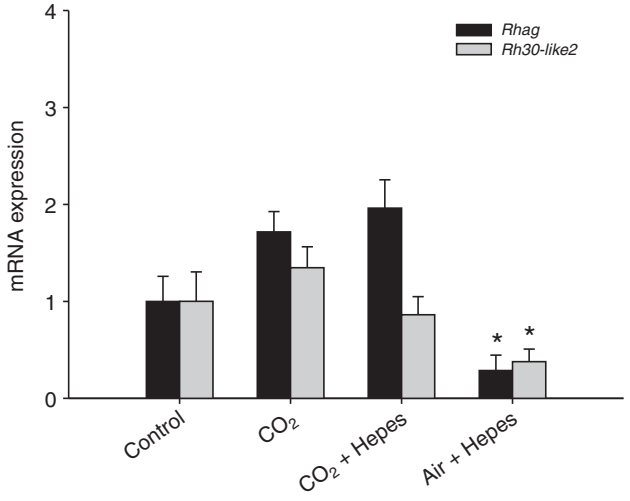

Fig. 8. Erythrocyte Rh mRNA expression in the rainbow trout exposed for 12 h to 1% CO_2 in air (CO_2), 1% CO_2 in air with 10 mmol l^{-1} Hepes buffered-water (CO_2+Hepes) and 100% air with 10 mmol l^{-1} Hepes-buffered water (Air+Hepes). Control fish were exposed to 100% air for 12 h in unbuffered water. Expression data were normalized to ng total RNA concentration with the control value set to one. Asterisks indicate significant difference from the control (P<0.05). Data are means ± s.e.m. (N=6).

The acid–base disturbance in response to hypercapnia has been well described in catheterized fish (for reviews, see Claiborne, 1998; Heisler, 1993). Catheterization was avoided in the present study, as our initial trials indicated that catheterization alone may alter Rh expression (C.M.N. and C.M.W., unpublished data). However, we used an exposure regime in which the acid–base effects had been documented previously. In an identical 1% CO_2 exposure protocol in Hamilton tap water, chronically catheterized rainbow trout exhibited a 0.3 unit depression in arterial pH at 3 h with partial recovery by 12 h [see figure 7 of Wood and LeMoigne (Wood and LeMoigne, 1991)]. Although gill cell intracellular ion levels (Na^+ and Cl^-) declined, gill intracellular pH did not change.

The associated increase in acid excretion which helps correct internal pH by building up internal HCO_3^- levels during hypercapnia involves the hydration of CO_2 by carbonic anhydrase within the gill (Henry and Heming, 1988; Perry, 1986; Perry and Gilmour, 2006). Apically located H^+-ATPase and/or NHE may then be involved in the release of protons into the water (Edwards et al., 2005). Although buffering during hypercapnia would cause an internal elevation of both CO_2 and ammonia, the results from our study suggest that fish in this treatment group were responding to an acidosis induced by the high CO_2. The upregulation of *NHE2* mRNA levels in the gill corresponds well with the findings of Edwards and colleagues, who reported an increase in NHE2-like protein expression in *Fundulus heteroclitus* exposed to external hypercapnia (Edwards et al., 2005). This suggests that, although H^+-ATPase is believed to play the major role in acid secretion in freshwater teleosts (Edwards et al., 2005), NHE2 may also have a role during acidosis in rainbow trout, especially in Hamilton tap water where Na^+ levels are relatively high (700 µmol l^{-1}). *H^+-ATPase* mRNA expression levels were not upregulated at 12 h (Fig. 9), but this does not necessarily mean that the corresponding protein is non-functional during acidosis. The expression levels of *H^+-ATPase* mRNA may in fact peak as early as 2 h after hypercapnia exposure (Perry et al., 2000), a response that could then result in a sufficient quantity of protein by 12 h. The increased *CA2* mRNA levels that occurred are also in agreement with previous studies that reported increased carbonic anhydrase activity (Dimberg and Hoglund, 1987) and increased *CA2* mRNA and protein expression (Georgalis et al., 2006) in the gills of hypercapnia-exposed trout.

Fish exposed to hypercapnia in Hepes-buffered water also experienced inhibition of ammonia excretion at the gill as evidenced

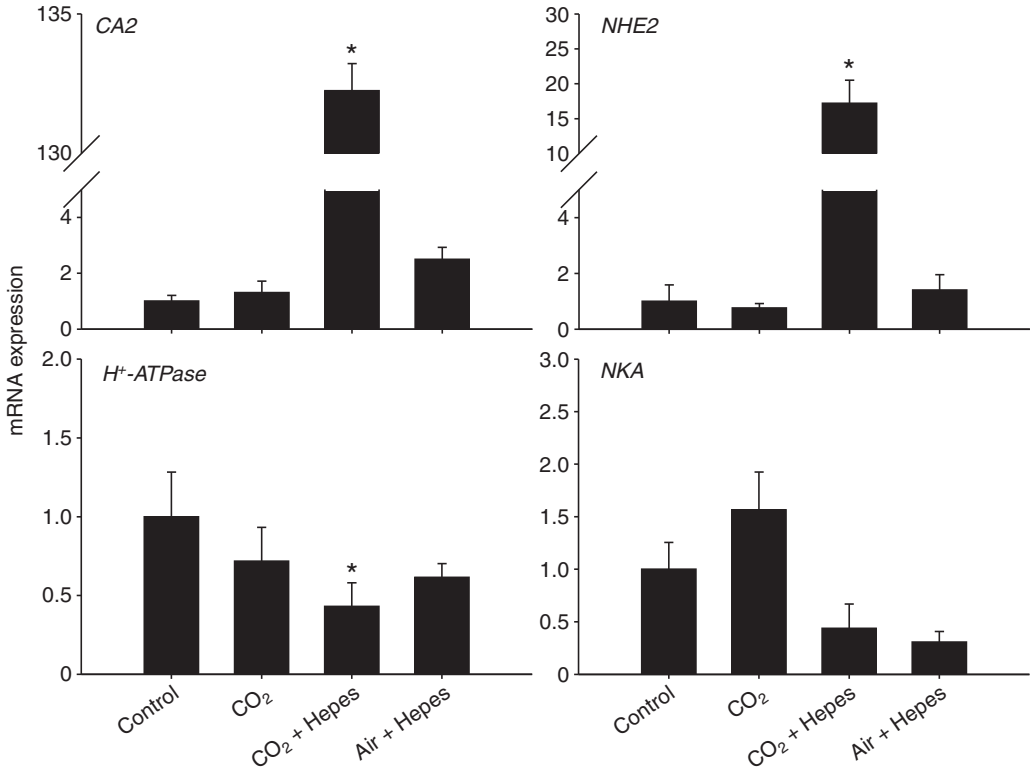

Fig. 9. The effect of 12 h exposure to 1% CO_2 in air (CO_2), 1% CO_2 in air with 10 mmol l^{-1} Hepes buffered-water (CO_2+Hepes), and 100% air with 10 mmol l^{-1} Hepes-buffered water (Air+Hepes) on the mRNA expression of carbonic anhydrase (*CA2*), *H^+-ATPase*, *NHE2* and Na^+/K^+-ATPase α-1a (*NKA*) in the gills of rainbow trout. Control fish were exposed to 100% air for 12 h in unbuffered water. Expression data were normalized to ng total RNA concentration with the control value set to one. Significant differences from the control are indicated by an asterisk (P<0.05). Data are means ± s.e.m. (N=6).

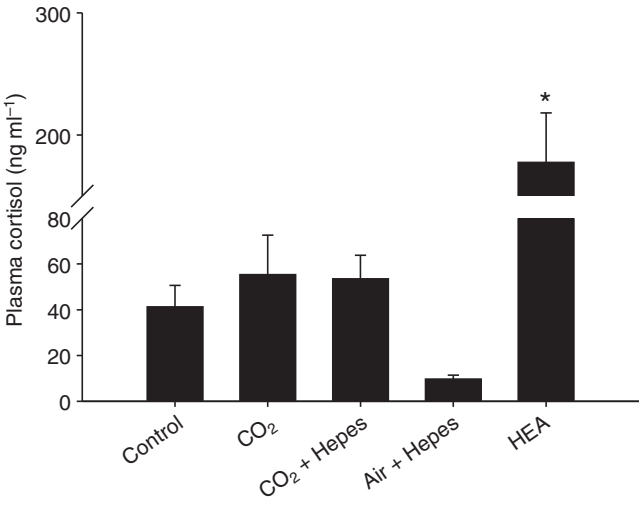

Fig. 10. Plasma cortisol levels after 12 h of exposure to 1% CO_2 in air (CO_2), 1% CO_2 in air with 10 mmol l^{-1} Hepes-buffered water (CO_2+Hepes), 100% air with 10 mmol l^{-1} Hepes-buffered water (Air+Hepes) and high environmental ammonia (HEA; 1.5 mmol NH_4HCO_3). Control fish were exposed to 100% air for 12 h in unbuffered water. Asterisk indicates significant difference from the control value (P<0.05). Data are means ±s.e.m. (N=6–10).

by the elevated plasma ammonia (Fig. 5) as well as excretion rates that were significantly lower than those of the hypercapnia-exposed fish in unbuffered water (Fig. 4). Although the excretion rates started to rise at 9 h (Fig. 4), the downregulation of *Rhcg2* and H^+-ATPase mRNA levels in the gill indicate that these fish were probably not using the same mechanism to enhance ammonia excretion as that used during HEA. Both treatments resulted in blockage of ammonia excretion from the gill, but it appears that in hypercapnic fish, the primary mRNA response in the gill was to CO_2 rather than high ammonia. Similar to the fish exposed to normocapnia in Hepes-buffered water, there was an upregulation of *Rhcg2* mRNA in the skin (Fig. 7), again reinforcing the idea that this may be an alternative route for ammonia excretion.

Previously we reported that *Rhag* as well as the Rh30-like mRNAs were downregulated during HEA, when plasma ammonia levels were high (Nawata et al., 2007). In the present study, both groups of fish exposed to Hepes-buffered water had elevated plasma ammonia levels, albeit lower than HEA-exposed fish (Fig. 5), so we would have expected a similar downregulation of *Rhag* expression in these two groups. Fish exposed to normocapnia did indeed exhibit lowered *Rhag* mRNA levels; however, the hypercapnia-exposed fish did not. Instead, the *Rhag* mRNA expression in the hypercapnia-exposed fish was increased 2-fold over that of the control fish, a response similar to that of the hypercapnia-exposed fish in unbuffered water (Fig. 8). This suggests that *Rhag* was responding to both high CO_2 and high ammonia, but in a differential fashion.

Ammonia accumulates in trout erythrocytes during hypercapnia (Wright et al., 1988b). If Rhag allows passage of NH_3, then downregulation when plasma ammonia levels are elevated may be a protective response. Transport of NH_3 with CO_2 and H_2O into the erythrocyte would produce NH_4^+ and HCO_3^- and once HCO_3^- is exchanged for Cl^-, the formation of NH_4Cl would lead to H_2O uptake and result in swelling (Bruce, 2008). Also, the buffering effect of NH_3 could interfere with the Bohr and Haldane effects, and therefore with O_2 and CO_2 transport. In human erythrocyte membranes, RhAG forms a macrocomplex with a number of proteins including the band-3 anion exchanger (AE1) and carbonic anhydrase, and this complex is thought to function in CO_2/O_2 gas exchange (Bruce et al., 2003). Trout erythrocytes also have AE1 present (Michel and Rudloff, 1989), but whether or not it forms a similar complex with Rhag is unknown. However, if Rhag functions as a CO_2 channel, then upregulation along with AE1 could be beneficial during hypercapnia when plasma HCO_3^- is high.

It has also been speculated that the Rh30 proteins could allow CO_2 passage (Callebaut et al., 2006), although Endeward and colleagues found no difference in CO_2 uptake in erythrocytes lacking the RhD (Rh30) protein (Endeward et al., 2007). Data from our study also suggest that the Rh30-like proteins are not involved in CO_2 transport as there were no notable changes in mRNA expression in erythrocytes from either of the two groups exposed to hypercapnia (Fig. 8). *Rh30-like2* mRNA expression, however, was downregulated in the fish exposed to normocapnia and Hepes, like HEA-exposed fish (Nawata et al., 2007), suggesting a response to high ammonia only.

So far, studies have indicated that fish have multiple Rh genes (Huang and Peng, 2005; Hung et al., 2007; Nakada et al., 2007a; Nakada et al., 2007b; Nawata et al., 2007) but the significance of this seeming redundancy is unclear. Nakada and colleagues (Nakada et al., 2007b) proposed that Rhag in the pillar cells of pufferfish may work in conjunction with the basolaterally located Rhbg and apically situated Rhcg2 in the lamellae to keep plasma ammonia levels low, with additional excretion aided by Rhcg1 in the mitochondria-rich cells. Others have shown that some Rh proteins may be dispensable like Rhbg in the mouse (Chambrey et al., 2005) and Rh1 in the slime mould (Benghezal et al., 2001). Likewise, the physiological significance of the multiple variants of *Rhbg2* (Fig. 1) is puzzling, but the presence of uORFs and splicing in the 5′- and 3′-UTRs of the mRNAs suggests complex regulation of this protein. uORFs have the potential to impact gene expression and some may serve as *cis*-acting regulatory elements modulating translation of the main ORF (Meijer and Thomas, 2002; Morris and Geballe, 2000). Similarly, the UTRs are involved in many post-transcriptional pathways that control the localization, stability and translation efficiency of mRNAs (Pesole et al., 2001). The existence of a truncated version of the Rhbg2 protein suggests yet another level of complexity. Truncation alters function and/or intracellular location of some transporters while some alternative-splice products negatively regulate the wild-type protein (Kitayama et al., 1999; Mangravite et al., 2003). Whether the truncated version of Rhbg2 functionally interacts with the full form or whether it has a completely different function has yet to be analysed.

Interestingly, the same 5′-UTR *Rhbg2* variants are present in both the skin and gill, although in the gill they are expressed equally while in the skin they are expressed differentially (Fig. 3). The downregulation of *Rhbg2* mRNA expression that occurred in the skin with Hepes treatment during both hypercapnia and normocapnia appears to be in response to high plasma ammonia rather than high CO_2 as suggested by the unchanged expression in the hypercapnia-treated fish in unbuffered water. To support this idea, we found that HEA-exposed fish exhibited a similar downregulation (Fig. 7). Rhbg was reported to be non-essential during acidosis in knockout mice (Chambrey et al., 2005). An abundance of Rhbg2 in fish skin may similarly be unnecessary during hypercapnia and HEA, and instead Rhbg2 could have a role other than ammonia transport.

Glucocorticoids are known for their role in regulating gene expression in mammals and the same is likely to be true for fish

(Mommsen et al., 1999). Data from this study, however, showed no correlation between Rh mRNA abundance and cortisol levels. Plasma cortisol and gill *Rhcg2* mRNA levels were both elevated during HEA but in the two Hepes-exposed groups *Rhcg2* mRNA was elevated in the skin while the cortisol remained at control levels (Fig. 10). A lack of correlation, however, does not exclude the possibility that cortisol could be involved either directly or indirectly in the control of Rh protein expression. Indeed, cortisol levels need not be elevated in order to have an influence on physiological processes (e.g. Wood et al., 2001) and other factors such as the clearance rate and non-genomic actions of cortisol need to be taken into consideration (Mommsen et al., 1999).

We have evaluated the expression of Rh protein mRNAs in rainbow trout exposed to hypercapnic and normocapnic conditions in the presence and absence of Hepes buffer. Previously, we showed that at the mRNA level, Rh proteins clearly respond to high ammonia conditions in rainbow trout tissues (Nawata et al., 2007). A similar response to high CO_2 would suggest that Rh proteins may have a dual role as CO_2 and NH_3 gas channels in trout tissues. Data from our present study revealed, however, that high CO_2 did not directly elicit changes in Rh mRNA transcription levels in the gill and skin. Instead, the changes that did occur probably reflected responses to high plasma ammonia, and thus reinforce the connection between the Rh proteins and ammonia. As such, a dual role for Rh proteins in the rainbow trout is not apparent from this study. There does remain, however, the possibility that Rhag interacts with both CO_2 and ammonia in the erythrocyte and further work is needed to clarify this.

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