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Rhesus glycoprotein gene expression in the mangrove killifish *Kryptolebias marmoratus* exposed to elevated environmental ammonia levels and air

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Summary

The mechanism(s) of ammonia excretion in the presence of elevated external ammonia are not well understood in fish. Recent studies in other organisms have revealed a new class of ammonia transporters, Rhesus glycoprotein genes (*Rh* genes), which may also play a role in ammonia excretion in fish. The first objective of this study was to clone and characterize *Rh* genes in a fish species with a relatively high tolerance to environmental ammonia, the mangrove killifish *Kryptolebias marmoratus* (formerly *Rivulus marmoratus*). We obtained full-length cDNAs of three *Rh* genes in *K. marmoratus*: *RhBG* (1736 bp), *RhCG1* (1920 bp) and *RhCG2* (2021 bp), which are highly homologous with other known *Rh* gene sequences. Hydropathy analysis revealed that all three *Rh* genes encode membrane proteins with 10–12 predicted transmembrane domains. *RhBG*, *RhCG1* and *RhCG2* are highly expressed in gill tissue, with *RhBG* also present in skin of *K. marmoratus*. Exposure to elevated environmental ammonia (2 mmol l^{-1} NH_4HCO_3) for 5 days resulted in a modest (+37%) increase in whole-body ammonia levels, whereas gill *RhCG2* and skin *RhCG1* mRNA levels were upregulated by 5.8- and 7.7-fold, respectively. *RhBG* mRNA levels were also increased in various tissues, with 3- to 7-fold increases in the liver and skeletal muscle. In a separate group of killifish exposed to air for 24 h, *RhCG1* and *RhCG2* mRNA levels were elevated by 4- to 6-fold in the skin. Thus, the multifold induction of *Rh* mRNA levels in excretory tissues (gills and skin) and internal tissues in response to conditions that perturb normal ammonia excretion suggests that *RhBG*, *RhCG1* and *RhCG2* may be involved in facilitating ammonia transport in this species. Furthermore, the findings support earlier studies demonstrating that the skin is an important site of ammonia excretion in *K. marmoratus*.

Key words: *Kryptolebias marmoratus*, ammonia excretion, gills, skin, ammonia transporter.

Introduction

Most teleost fish excrete ammonia and urea as the primary nitrogenous waste, with ammonia contributing more than 80% of the total (Wood, 1993). The major site of ammonia elimination is the gills, but the kidney and skin may also be involved (for a review, see Wilkie, 2002). Although ammonia is produced endogenously, it is a dangerous neurotoxin at elevated concentrations (Felipo and Butterworth, 2002; Rose, 2006). Normally ammonia is excreted predominantly by passive diffusion down the NH_3 partial pressure gradient (P_{NH_3}) from blood to water across the gills (Wilson et al., 1994). In environments of high external ammonia or elevated pH, the P_{NH_3} gradient across the gills may be diminished or reversed, resulting in ammonia uptake from the environment and/or an inhibition of ammonia excretion (Wright and Wood, 1985; Wilson et al., 1994; Wilkie, 2002).

Fish survive in environments of elevated ammonia by one or more of the following strategies: reducing endogenous ammonia production, converting ammonia to less toxic molecules (e.g. urea, glutamine), or continuing to excrete ammonia against the gradient (for a review, see Chew et al., 2006). Very little is known about the latter possibility because it is very difficult to define the true blood-to-water P_{NH_3} and electrochemical gradients (E_{NH4+}) (Wilson et al., 1994). African sharptooth catfish *Clarias gariepinus* is a species that appears to excrete ammonia actively against high external ammonia, but the exact mechanism of the excretion is currently unknown (Ip et al., 2004). Indeed, only the mudskipper *Periophthalmodon schlosseri* has been reported to actively excrete ammonia against an indisputable gradient (8 and 30 mmol l^{-1} NH_4Cl in the water). The mechanism likely involves basolateral Na^+-K^+(NH_4^+)-ATPase coupled to an apical Na^+/H^+ (NH_4^+) exchanger (Randall et al., 1999).

Another possible mechanism of NH_3/NH_4^+ transport is *via* the Rhesus glycoproteins. The Rhesus glycoproteins (*Rh* genes) represent members from the Amt/MEP/Rh superfamily

found in all three domains of living organisms: Bacteria, Archaea and Eukarya. Amt and Rh proteins are distantly linked (Huang and Liu, 2001). The first ammonia transporter genes were identified from yeast *Saccharomyces cerevisiae* (*ScMep1*) and plant *Arabidopsis thaliana* (*AtAmt1*) (Marini et al., 1994; Ninnemann et al., 1994). In mammals, three Rh genes are identified so far: *RhAG*, *RhBG* and *RhCG*. *RhAG* expression is restricted to the erythrocyte membrane, whereas *RhBG* and *RhCG* are expressed in various tissues in the mammalian systems, including liver, kidney and gastrointestinal tract (Handlogten et al., 2005; Liu et al., 2000; Liu et al., 2001; Weiner et al., 2003; Weiner and Verlander, 2003). Studies in mammalian and plant systems reveal that at least some of those *Rh* genes encode proteins that mediate NH_3/NH_4^+ movement (Khademi et al., 2004; Zheng et al., 2004; Mayer et al., 2006), but the form of ammonia (NH_3 gas or NH_4^+ ion) being transported, and whether the transport is active or passive, are still under much debate (Bakouh et al., 2004; Khademi et al., 2004; Nakhoul et al., 2005). A full-length cDNA of *Rh*-like protein (*Rh-CM*) has been identified recently from the gills of the aquatic crab *Carcinus maenas*, which has a similar predicted transmembrane structure as the mammalian Rh proteins (Weihrauch et al., 2004). However, the precise role of *Rh-CM* in crab ammonia excretion awaits elucidation.

To date, there have been only two reports of *Rh* sequences in fish (Huang and Peng, 2005; Nakada et al., 2007). *RhAG*, *RhBG*, *RhCG1* and *RhCG2* sequences have recently been identified in the pufferfish *Takifugu rubripes*, with unique spatial distribution within the fish gill, and these *Rh* genes mediate movement of the ammonia analogue methylammonia when expressed in *Xenopus* oocytes (Nakada et al., 2007). However, no physiological studies of *Rh* genes have been conducted. This family of Rh transporters may be an important missing link in our understanding of branchial ammonia excretion. The mangrove killifish *Kryptolebias marmoratus* is very tolerant of elevated external ammonia, surviving 48 h of 10 mmol l^{-1} NH_4Cl at pH 8.0 (Frick and Wright, 2002a). Remarkably, there was very little change in tissue ammonia levels in *K. marmoratus* exposed to external ammonia, nor were tissue urea or glutamine concentrations altered (Frick and Wright, 2002a). During prolonged air exposure (11 days), *K. marmoratus* volatilize NH_3 (Frick and Wright, 2002b) by elevating both NH_4^+ concentration and pH on the cutaneous surface (Litwiller et al., 2006). Ammonia excretion during both elevated environmental ammonia and air exposure may require specialized transport mechanisms to move ammonia across cell membranes possibly against the gradient, similar to the mudskipper *P. schlosseri* (Randall et al., 1999).

The first objective of this study was to isolate *Rh* genes from *K. marmoratus* and determine their tissue distribution, with a particular focus on the gill and skin. The second objective was to quantify changes in *Rh* mRNA expression in *K. marmoratus* in response to elevated external ammonia concentrations and aerial exposure. We have cloned three *Rh* genes from *K. marmoratus* and investigated the tissue expression pattern of each *Rh* gene, in control and ammonia-exposed fish. Using quantitative real time-PCR, we have also measured the relative mRNA expression of all three *Rh* genes in response to (i) high external ammonia in the gills and skin and (ii) aerial exposure in the skin. In addition, relative *RhBG* expression levels in brain, liver and muscle of control and ammonia-exposed *K. marmoratus* were also quantified.

Materials and methods

Experimental animals

Fish were obtained from a breeding colony of *Kryptolebias marmoratus* Poey held in the Hagan Aqualab at the University of Guelph, Guelph, ON, Canada (Frick and Wright, 2002a). Adult hermaphrodite fish, at least 1 year of age and weighing approximately 0.06–0.12 g, were used for experiments. Hermaphrodites were identified by the appearance of an overall mottled brown colouration, a characteristic caudal ocellus, and a whitish border on the anal fin. Fish were kept in individual containers under a constant photoperiod (12 h:12 h, L:D), in 15‰ artificial seawater (made with distilled water and Crystal Sea® Marinemix; Marine Enterprises International, Inc., Baltimore, MA, USA), 25°C, pH 8.1. Water changes were performed every 2 weeks and fish were fed *Artemia* five times per week. The experiments in this study were approved by the University of Guelph Animal Care Committee.

Full-length cDNA cloning of the RhBG, RhCG1 and RhCG2 from killifish K. marmoratus

Two fish were used for *Rh* gene cloning. Fish were decapitated, the gills removed and immediately frozen in liquid nitrogen. Total RNA was extracted from killifish gill using Trizol (Invitrogen Canada Inc., Burlington, ON, Canada). First-strand cDNA was synthesized using Superscript reverse transcriptase II (Invitrogen) with an adaptor oligodT primer (T17AP2: GACTCGAGTCGACATCGAT_{17}). A partial sequence (585 bp) was obtained using F primer (GCA-CACTGTTCCTGTGGATG) and R primer (CAGC-AGGATCTCCCCAGA), with the thermal cycle (PTC-200, Peltier Thermal Cycler, MJ Research, Mississauga, ON, Canada): 94°C, 2 min, 45 cycles of 94°C, 30 s; 51°C, 30 s; 68°C, 1 min and a final extension at 68°C, 5 min (Invitrogen Platinum Taq). Sequence of this PCR product was confirmed to be a *Rh* homologue. Thereafter, 3′ RACE was conducted using the F primer and AP2 primer (GACT-CGAGTCGACATCGA) with thermal cycles: 95°C, 5 min, 45 cycles of 94°C, 15 s; 56°C, 1 min; 68°C, 5 min and a final extension at 72°C, 10 min (QIAgen High Fidelity HotStar, Qiagen, Mississauga, ON, Canada). Three PCR products (1.3 kb, 1.6 kb and 1.8 kb) were obtained, gel-purified (QIAquick extraction kit), cloned into pGEM-T easy vectors (Promega, Madison, WI, USA) and transformed into *E. coli* XL-Blue strain. Plasmids from positive clones were extracted (QIAgen miniprep), sequenced (Mobix sequencing facility, McMaster University) and confirmed to be 3′ end sequences of *RhBG*, *RhCG1* and *RhCG2*. 5′ RACE was then carried out

Table 1. *Gene-specific primer sequences for 5′ end cloning*

Cloning primer	Sequence
RhBG 5′GSP	GAGCCGTGAGGGCAGACATGCCATACGTAGAC
RhBG 5′NGSP	GTGCAGGCTGCCAGGGAGTAGTAGGTGTTCA
RhCG1 5′GSP	CGGTGGCAGCCAGAGACAAGTAGGTGTTGATGG
RhCG1 5′NGSP	CGCTCCGTAGGCGATCAAGCATCCGGCCACAC
RhCG2 5′GSP	GTTGTCGTCGGCAGGATCTCCCCAGATGGG
RhCG2 5′NGSP	CCTCCCTGCGTTCCCACACCTCTGTTTGC

with cloning primers (Table 1) (Marathon cDNA Amplification Kit, Clontech, Moutainview, CA, USA).

Full-length sequences submitted to GenBank have the following accession number: DQ995211 (*RhBG*), DQ995210 (*RhCG1*) and DQ423779 (*RhCG2*).

Ammonia exposure

Fish were fasted for 48 h prior to the onset of experiments to eliminate the influence of diet on nitrogen metabolism and excretion. Three groups of animals were studied. Control fish were exposed to 15‰ seawater (pH 8.1) containing no added NH_4HCO_3. Experimental fish were exposed to either 1 or 2 mmol l^{-1} NH_4HCO_3 (15‰, pH 8.1). At the end of 5 days, fish were decapitated. Fish dissections were conducted under a dissecting microscope. Individual tissues (brain, eye, gill, gonad, gut, kidney, liver, skeletal muscle and skin) were excised and immediately frozen in liquid nitrogen and kept at –80°C for later RNA isolation within 2 weeks. Identical groups of fish were treated in the same way, except at the end of exposure, fish were decapitated and whole fish were frozen in liquid nitrogen, and stored at –80°C for whole-body ammonia analysis within 1 week.

We attempted to measure ammonia excretion to the water in fish exposed to 1 and 2 mmol l^{-1} NH_4HCO_3, however, due to the small size of the fish and the high background ammonia, this proved to be difficult. In preliminary experiments we extended the flux period from 4 to 24 h to increase the signal:noise ratio, but this introduced the confounding problem of significant microbial metabolism, which removed ammonia from solution. Hence ammonia excretion data were unreliable and are not presented.

Aerial exposure

Fish exposed to terrestrial conditions were placed in individual plastic containers containing a moist paper of 15‰ seawater at 25°C, as described previously (Ong et al., 2007). After 24 h, fish were decapitated and skins were dissected, frozen immediately in liquid nitrogen and kept at –80°C until analysis within 1 week.

Whole-body ammonia analysis

The frozen fish samples (including heads) were used for whole-body ammonia content measurement. Each sample was powdered using a mortar and pestle under liquid nitrogen, weighed and deproteinized in 10 vols of 10% trichloroacetic acid (TCA) (Fisher Scientific). The homogenate was centrifuged (Sorvall Legend RT, Mandel, ON, Canada) at 12 000 ***g*** at 4°C for 15 min. The deproteinized samples were neutralized to pH 6.5–7.0 with 2 mol l^{-1} $KHCO_3$ (Sigma-Aldrich). Ammonia content was determined by the method of Kun and Kearney (Kun and Kearney, 1974). The change in absorbance at 340 nm (25°C) was monitored using a Spectra Max 90 spectrophotometer (Molecular Devices, Woodbridge, ON, Canada). Freshly prepared ammonium chloride (Sigma-Aldrich) was used as a standard.

Tissue expression

Reverse-transcription PCR (RT-PCR) was used to determine the mRNA expression pattern of *RhBG*, *RhCG1* and *RhCG2* in control and ammonia-exposed tissues. Total RNA extraction and cDNA synthesis were done as described above. A DNase I (Invitrogen) digestion step was used (1 U per 1 μg RNA, 15 min at room temperature) to ensure there was no genomic DNA contamination prior to cDNA synthesis. Three sets of gene-specific primers (Table 2) were used to examine the tissue-specific expression of *RhBG*, *RhCG1* and *RhCG2*; and

Table 2. *Gene-specific primer sequences for tissue expression screening*

Screening primer	Sequence
RhBGF	AGATCCCCAGCATCGCACA
RhBGR	TTCTCCACTCGCCACATCAG
RhCG1F	AGATTTCTGTGTGGCCGGATGC
RhCG1R	ACGGTGGCAGCCAGAGACAA
RhCG2F	GGTCATTCACTGCTTTGGTGGC
RhCG2R	CTTGTGAGCCAAGCTGGAGATG
EF1aF	GAGCGTGGTATCACCATTGACA
EF1aR	CCTTCCATCCCTTGAACCAG

Table 3. *Gene-specific primer sequences for quantitative PCR*

Quantitative PCR primer	Sequence
Q-BGF	GCTTCCTCCATGGCTTGCA
Q-BGR	GCTCCAAATGAGATGAGTACAGA
Q-CG1F	CTGGACTACACTGATGGGAAG
Q-CG1R	CGTGTATGAGATCCAGGATGATA
Q-CG2F	ACCTTTGGCTACCTGTTCATC
Q-CG2R	CTATGAAGCCGCCAAGCATC
Q-18SF	GTGCATGGCCGTTCTTAGTT
Q-18SR	CTCAATCTCGTGTGGCTGAA
Q-EF1aF	GAACTCACCGACACCAGCA
Q-EF1aR	ATCATCGACGCTCCTGGAC

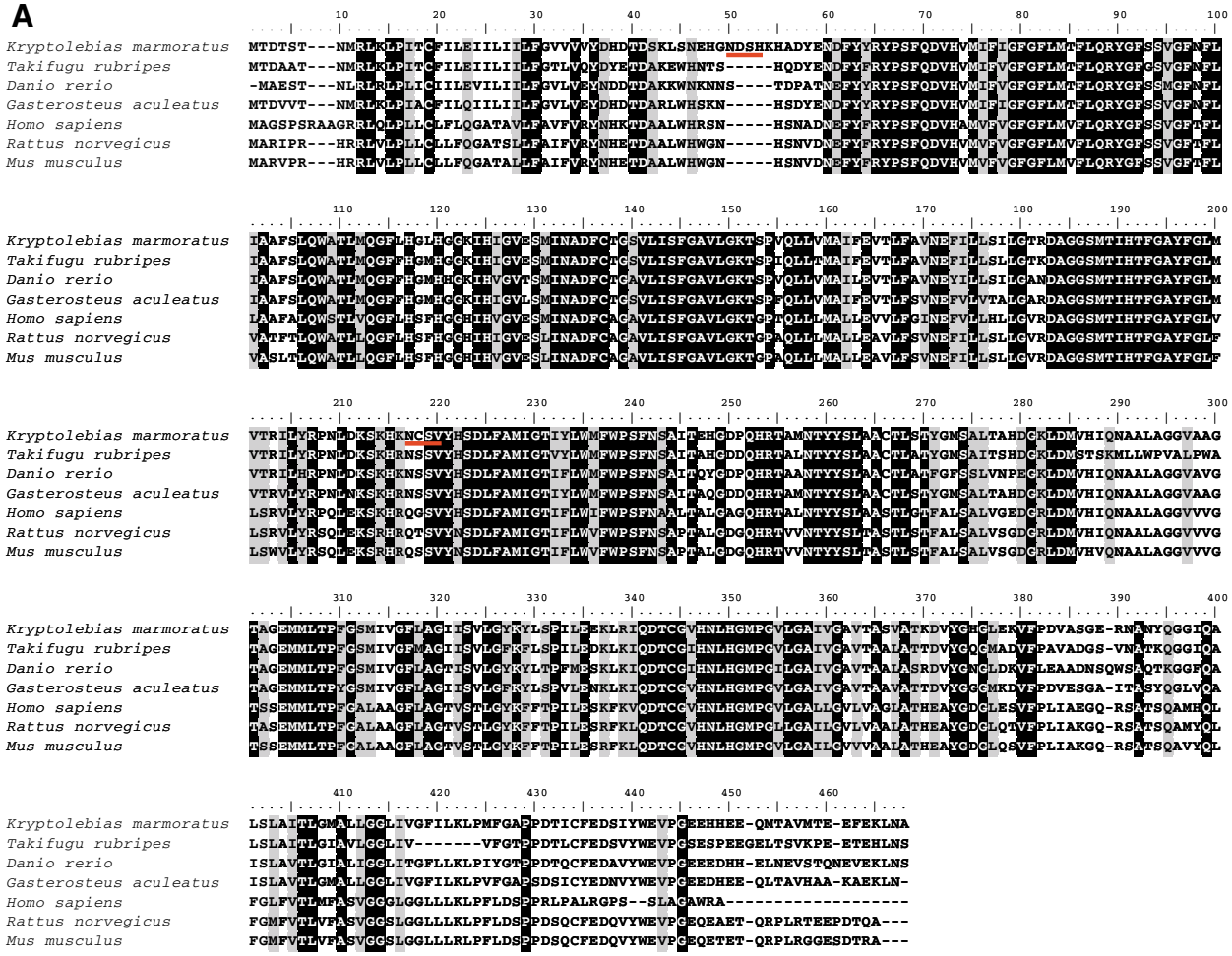

Fig. 1A. See next page for Fig. 1B and legend.

elongation factor 1alpha (*EF1a*) was used as the control gene to ensure that cDNA of individual samples were successfully synthesized.

The thermal cycle used was: 94°C, 3 min, 45 cycles of 94°C, 30 s; 58°C, 30 s (*RhBG* and *RhCG2*) or 55°C, 30 s (*RhCG1* and *EF1a*); 72°C, 1 min and a final extension at 72°C for 5 min (Invitrogen Taq polymerase).

Quantitative PCR analysis

Potential ammonia excretory organs, gill and skin, were used to test for all three *Rh* gene expressions in control and experimental fish by quantitative PCR (Q-PCR). Other internal organs, brain, liver and skeletal muscle, were selected to analyze for the expression of RhBG. In this study, two control genes, *EF1a* and *18S ribosomal RNA* (*rRNA*), were used as normalization factors; and the choice of which normalization factor to use was dependent on which gene exhibited the most consistent expression in control and ammonia-exposed or air-exposed tissues. For each Q-PCR reaction, 5 μl of 5× diluted cDNA sample was used. Q-PCR primers used are listed in Table 3.

Quantification of gene expression was done using Platinum SYBR green qPCR Super-mix-UDG (Invitrogen) with PCR thermal cycle (Mx3000P QPCR System, Stratagene, Cedar Creek, Texas, USA): 50°C for 2 min, 95°C for 2 min, followed by 40 cycles of 95°C for 15 s and 60°C for 15 s. Gel electrophoresis was performed to ensure that only a single PCR product of the desired size was obtained for each reaction. The Q-PCR product was purified (QIAquick PCR purification kit) and sequenced to confirm its identity.

Sequence analysis

Mammalian *Rh* homologues are predicted to be membrane proteins with 12 membrane spanning domains and cytoplasmic N- and C-terminals (Huang and Liu, 2001), but nothing is known about fish *Rh* homologues. To predict the potential localization of killifish Rh proteins, we characterized the primary and secondary structure of the killifish Rh sequences by analyzing their hydropathy profiles (Kyte-Doolittle scale) *via* webserver SDSC Biology WorkBench 3.2 at http://seqtool.sdsc.edu/CGI/BW.cgi. To further confirm that these three *Rh* sequences encode for membrane proteins, which consist of transmembrane (TM), intra- and extracellular motifs, transmembrane domain organizations were predicted at TMHMM server, version 2.0 (http://www.cbs.dtu.dk/services/TMHMM). Furthermore, mammalian Rh homologues have

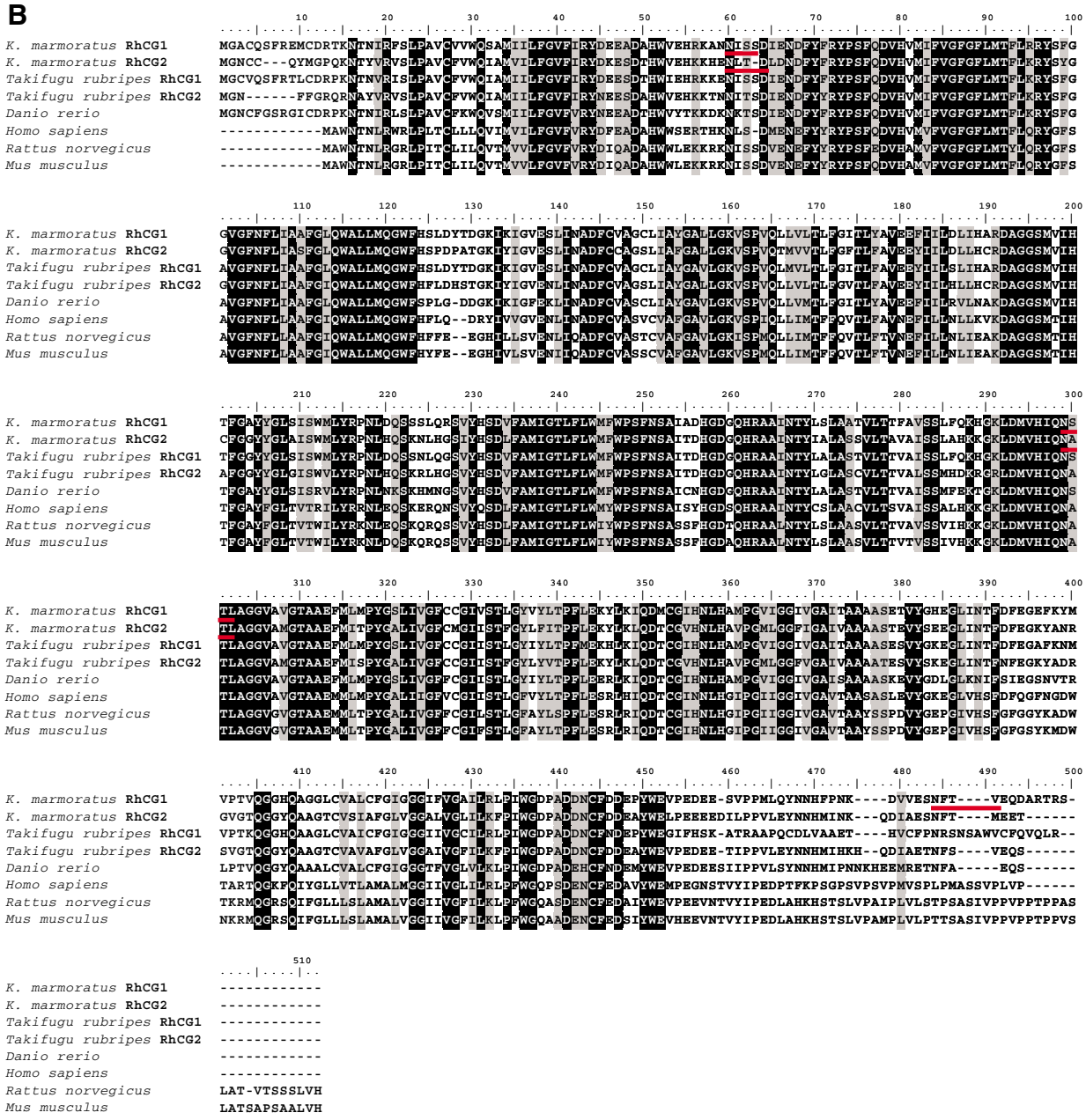

Fig. 1. Amino acid sequence alignment of *Kryptolebias marmoratus* Rh with other Rh sequences. Conserved amino acids are shaded in black and similar amino acids are shaded in grey. Potential N-glycosylation sites of *K. marmoratus* RhCGs are underlined in red. (A) Amino acid sequence alignment of *Kryptolebias marmoratus* RhBG with other RhBG sequences. Accession numbers of sequences: *Takifugu rubripes* AAM48577; *Danio rerio* AAQ09527; *Homo sapiens* NP_065140; *Rattus norvegicus* AAH79365 and *Mus musculus* NP_067350. (B) Amino acid sequence alignment of *K. marmoratus* RhCGs with other RhCG sequences. Accession numbers of sequences: *Takifugu rubripes* RhCG1 AAM48578; *Takifugu rubripes* RhCG2 AAM48579; *Danio rerio* AAM90586; *Homo sapiens* AAH30965; *Rattus norvegicus* NP_898876 and *Mus musculus* NP_062773.

been shown to be glycoproteins (Quentin et al., 2003) and therefore, potential N-glycosylation sites on killifish Rh protein sequences were predicted *via* NetNGlyc 1.0 Server at http://www.cbs.dtu.dk/services/NetNGlyc.

Sequence alignments (Clustal W) were conducted using BioEdit software downloaded at http://www.mbio.ncsu.edu/BioEdit/bioedit.html. The same software was used for molecular mass, isoelectric point (pI), amino acid composition, and percentage sequence identity calculations.

Phylogenetic analysis

Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 3.1 downloaded at http://www.megasoftware.net (Kumar et al., 2004). Sequences

were first aligned by ClustalW and subjected to a Neighbour-joining (NJ) matrix for tree reconstruction and evaluated by means of a Bootstrap of 1000 replicates.

Statistical analysis

All data are expressed as mean ± the standard error of the mean (s.e.m.) (N=6–8). Analysis of variance (ANOVA) was used to compare means of relative *Rh* mRNA expression in the gill with Statistix software (Analytical Software, Tallahassee, FL, USA), followed by the Least Significant Difference (LSD) test to determine where significant differences were present (P<0.05). For *Rh* mRNA expression level between control (immersion) and air-exposed (emersed) fish, an unpaired t-test was conducted to determine if expression was significantly different (P<0.05; control: N=6; air-exposed: N=4).

Results

Nucleotide, protein sequences and structures

We have obtained full-length cDNA sequences of three Rhesus glycoproteins in the killifish *K. marmoratus*: *RhBG*, *RhCG1* and *RhCG2*, and they are 1736, 1920 and 2021 bp long, respectively. *RhCG1* and *RhCG2* are highly homologous in both their nucleotide (72.5% identity) and amino acid (61.1% identity) sequences. *RhCG1* and *RhCG2* are similar to *RhBG*, with 50.6% and 49.5% identity, respectively, for nucleotide sequences and 54.4% and 52.3% identity at the amino acid level. All three *Rh* sequences appear to share high homology with other known *Rh* gene sequences.

RhBG

K. marmoratus RhBG is 76.2–84.8% identical to other known fish RhBGs and 52.2–61.5% identical to known mammalian RhBGs. Sequence alignment revealed that *K. marmoratus* RhBG is highly homologous to other fish RhBGs, with no gap found between ^{55}H and ^{388}E (TM2 to TM9 of *K. marmoratus* RhBG) (Fig. 1A). The open reading frame (ORF) of RhBG encodes a 462-amino acid polypeptide (50.5 kDa), with two potential N-glycosylation sites (^{47}NDSH^{50}, ^{217}NCSV^{220}) (Fig. 1A). When *K. marmoratus* RhBG was aligned with all known RhBG sequences from fish and mammals, it was found that variable regions of fish RhBG sequences are located between amino acids 250–300 and the C-terminal sequences. However, these regions are rather conserved among the mammalian RhBG sequences (data not shown).

Many fragments of the RhBG sequences appear to be class-specific. For example, RhBG amino acid regions ^{200}MVTRIL(Y/H)RPNLD^{211} and ^{312}SMIVGF(L/M)AG(I/T)ISV^{324} are highly conserved among fish sequences. However, the

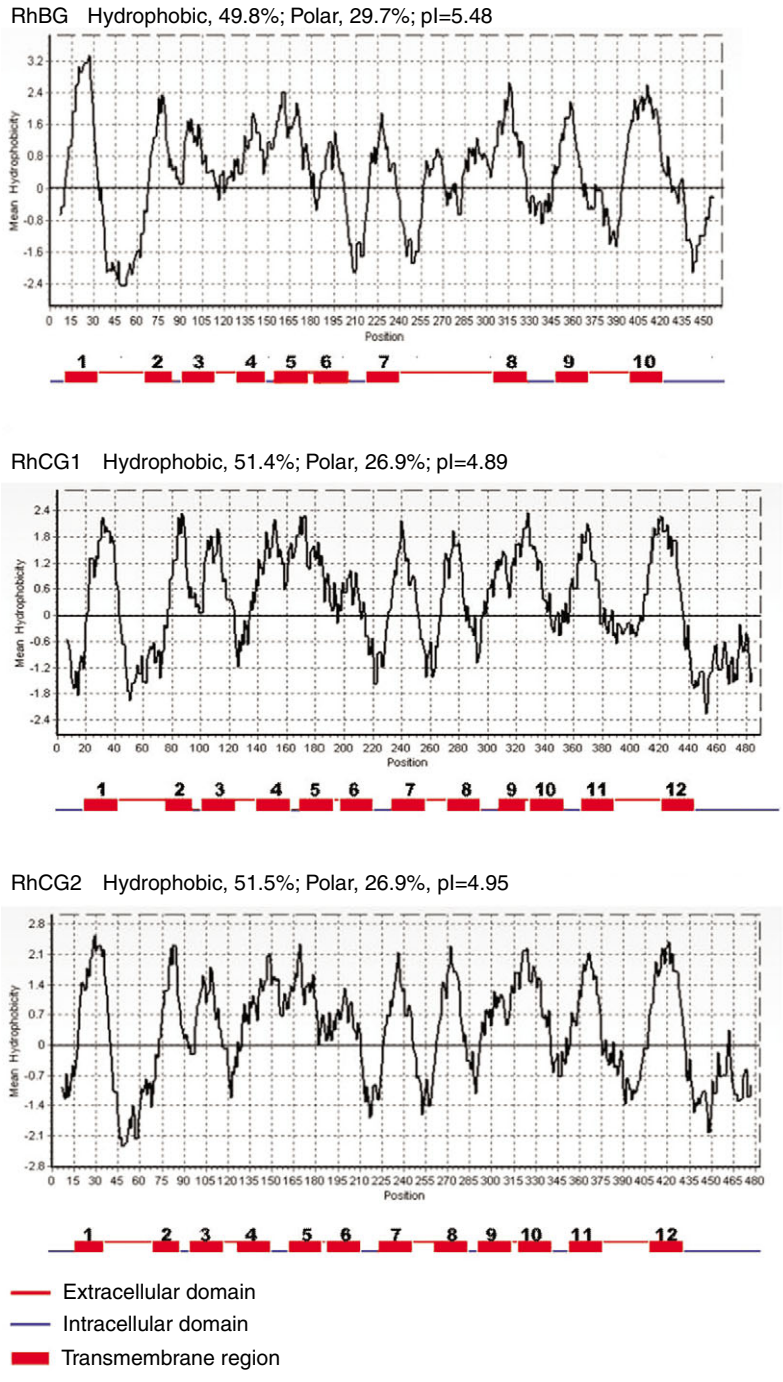

Fig. 2. Protein characteristics of *K. marmoratus* RhBG, RhCG1 and RhCG2. Amino acid compositions indicate that all three Rh proteins consist of hydrophobic and polar amino acids, and all three proteins are negatively charged at physiological pH. Hydropathy profiles (Kyte–Doolittle scale) indicate that high hydrophobicity regions are dispersed along all three sequences and these high hydrophobicity regions correspond to the predicted transmembrane domains (red blocks). All three Rh proteins have intracellular N- and C-terminals.

mammalian RhBG sequences of the same regions are highly conserved among themselves and are encoded as ^{200}(V/F)LS(R/W)VLYR(P/S)QLE^{211} and ^{312}ALAAGFLAGT-VST^{324}, respectively. Such class-specific regions are also located at the N- and C-terminal sequences (data not shown).

RhCG

RhCG1 and *RhCG2* ORFs, encode for polypeptides 490 (54.2 kDa) and 483 (53.3 kDa) amino acids in length, respectively. RhCG1 show 72–84.6% and 53.9–64.6% identity to known fish and mammalian RhCG sequences, respectively. Similarly, the identity between RhCG2 and other fish sequences is 71.2–81.0% and 51–61% with mammalian RhCGs (data not shown). RhCG1 has three potential N-glycosylation sites (^{60}NISS^{63}, ^{299}NSTL^{302}, ^{479}NFTV^{482}), whereas RhCG2 contains two such sites (^{57}NLTD^{60}, ^{295}NATL^{298}) (Fig. 1B). The C-terminal sequences appear to be the least conserved among species and a class-specific conserved region is also found at the C-terminal of RhCG sequences (data not shown).

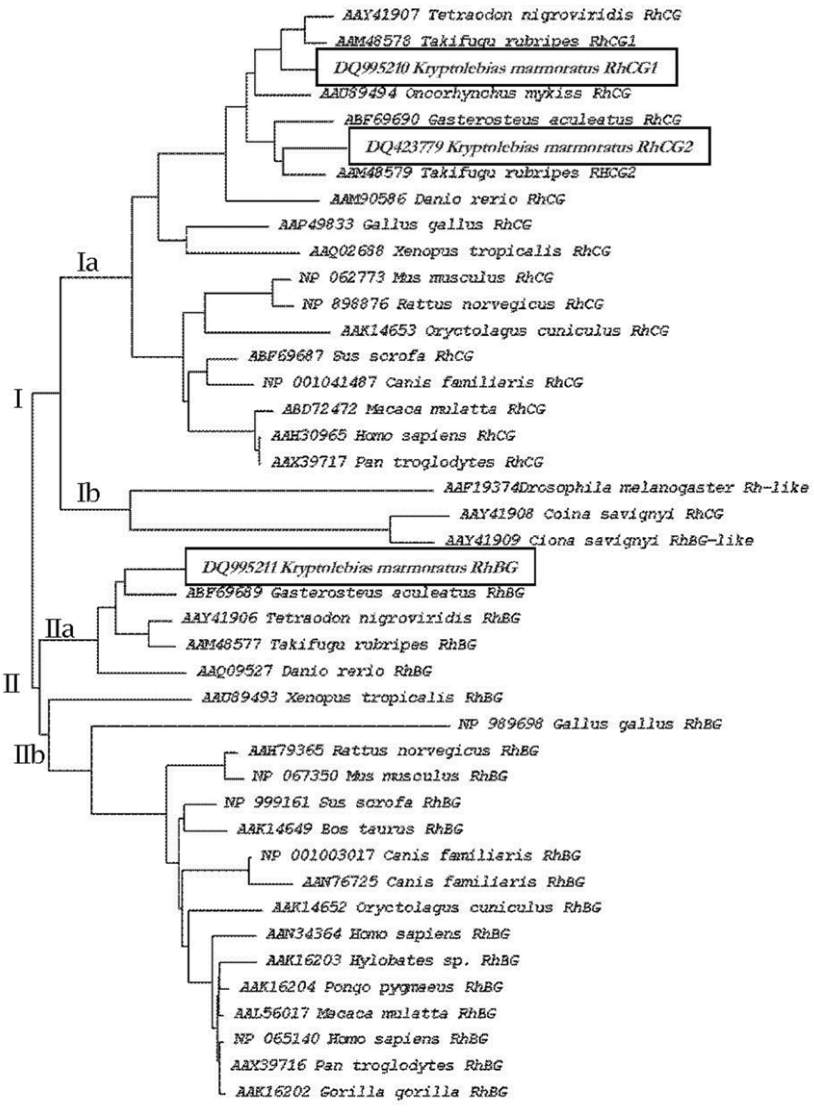

Fig. 3. Phylogenetic relationships of *K. marmoratus RhBG* and *RhCG*s and other homologues. Two major clusters are identified: Cluster I (RhCG), Cluster II (RhBG). Cluster I is subdivided into Ia (fish/amphibian/mammals/aves) and Ib (invertebrates). Cluster II is also further divided into IIa (fish) and IIb (mammals/amphibian/aves). Sequences are obtained from GenBank with accession numbers indicated.

Hydropathy analysis

Hydropathy analysis revealed that all three Rh proteins are highly hydrophobic and negatively charged at physiological pH. They share similar topology that resembles bacterial Amt (Blakey et al., 2002) and mammalian Rh membrane channels (Liu et al., 2000; Liu et al., 2001), with cytoplasmic N- and C-terminals. *K. marmoratus* RhBG is predicted to have ten transmembrane domains, while both RhCG1 and RhCG2 have 12 predicted transmembrane domains (Fig. 2).

Phylogenetic relationships between RhBG and RhCG proteins

The phylogenetic tree reconstruction shows that the Rh proteins form two main clusters: Cluster I (RhCG) and II (RhBG), which are subdivided into smaller clusters (Fig. 3). The invertebrate Rh-like proteins (Ib) are divergent from the RhCG family (Ia). The vertebrate RhCG, on the other hand, diverge into a mammalian RhCG cluster and a cluster including other vertebrate RhCGs, which are then divided later into fish, amphibian and avian RhCG clusters. On the other hand, the RhBG cluster (cluster II), is also subdivided into two groups IIa and IIb. However, it is interesting to note that the fish RhBG (IIa) proteins apparently diverged earlier, forming one distinct cluster, whereas mammalian RhBGs, in this case, have closer relationships with the amphibian and avian homologues and diverged later than the fish RhBGs.

Whole-body ammonia content

The whole-body ammonia level was not altered after 5 days of exposure to 1 mmol l^{-1} NH_4HCO_3. However, after 5 days of 2 mmol l^{-1} NH_4HCO_3 exposure, whole-body ammonia content increased significantly by 37% (Fig. 4).

Tissue expression of Rh genes

Reverse-transcription PCR results show that *RhBG* expression in control *K. marmoratus* is consistently found in gill and skin, whereas *RhCG1* and *RhCG2* are predominantly expressed in gill. After 5 days of exposure to high external ammonia levels (2 mmol l^{-1} NH_4HCO_3), *RhBG* expression is induced in brain, eye, gonad, gut, kidney, liver and skeletal muscle and continues in gill and skin. *RhCG1* expression is also induced in skin of ammonia-exposed fish, but *RhCG2* expression remains restricted to the gill (Fig. 5).

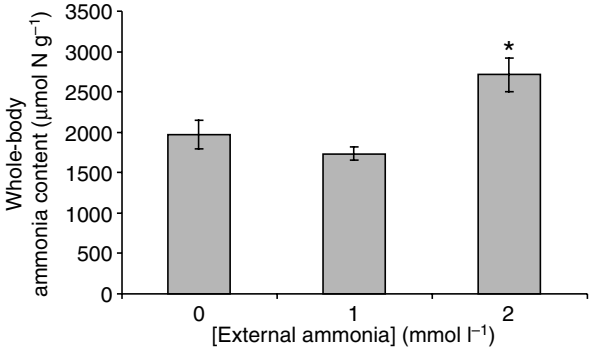

Fig. 4. Whole-body ammonia content of *K. marmoratus* after 5 days of 0 mmol l^{-1}, 1 mmol l^{-1} and 2 mmol l^{-1} ammonia exposure. Asterisk indicates that tissue ammonia was significantly higher at 2 mmol l^{-1} ammonia compared to 0 mmol l^{-1} and 1 mmol l^{-1} ammonia. Values are means ± s.e.m., N=6 (one-way ANOVA, P<0.05).

Quantitative PCR

Response to high external ammonia

Gill *RhCG2* mRNA levels were not altered after 5 days of exposure to 1 mmol l^{-1} NH_4HCO_3, but 2 mmol l^{-1} NH_4HCO_3 exposure resulted in a 5.8-fold increase, relative to the control level. Gill *RhBG* and *RhCG1* mRNA levels did not change in response to either ammonia level (Fig. 6A). In skin, *RhCG1* mRNA levels increased significantly by 2.4-fold and 7.7-fold after 5 days of 1 and 2 mmol l^{-1} of NH_4HCO_3 exposure, respectively, relative to control fish. As well, skin *RhCG2* mRNA levels increased by 3.8-fold after exposure to 1 mmol l^{-1} NH_4HCO_3, but there were no changes when fish were exposed to 2 mmol l^{-1} NH_4HCO_3 relative to control fish (Fig. 6B).

RhBG mRNA levels remained unchanged in the brain but were significantly higher in liver (3.4-fold) and muscle (7.2-fold) tissue in 2 mmol l^{-1} NH_4HCO_3-exposed fish compared to control fish (Fig. 7).

Response to aerial exposure

Significant induction of *RhCG1* (6.2-fold) and *RhCG2* (4.2-fold) mRNA levels were observed in killifish skin after 24 h of air exposure. The *RhBG* mRNA level was not altered (Fig. 8).

Discussion

We have identified three *Rh* genes in killifish *K. marmoratus*: *RhBG*, *RhCG1* and *RhCG2*, which encode transmembrane proteins with cytoplasmic N- and C-terminals. The amino acid sequences of these three *Rh* genes share high homology to known Rh sequences in the GenBank. Since all three Rh proteins contain potential N-glycosylation sites, they are probably expressed as glycoproteins, similar to their mammalian counterparts (Quentin et al., 2003). *K. marmoratus* RhBG shares higher sequence homology to three-spined stickleback (*Gasterosteus aculeatus*) (data not shown), which

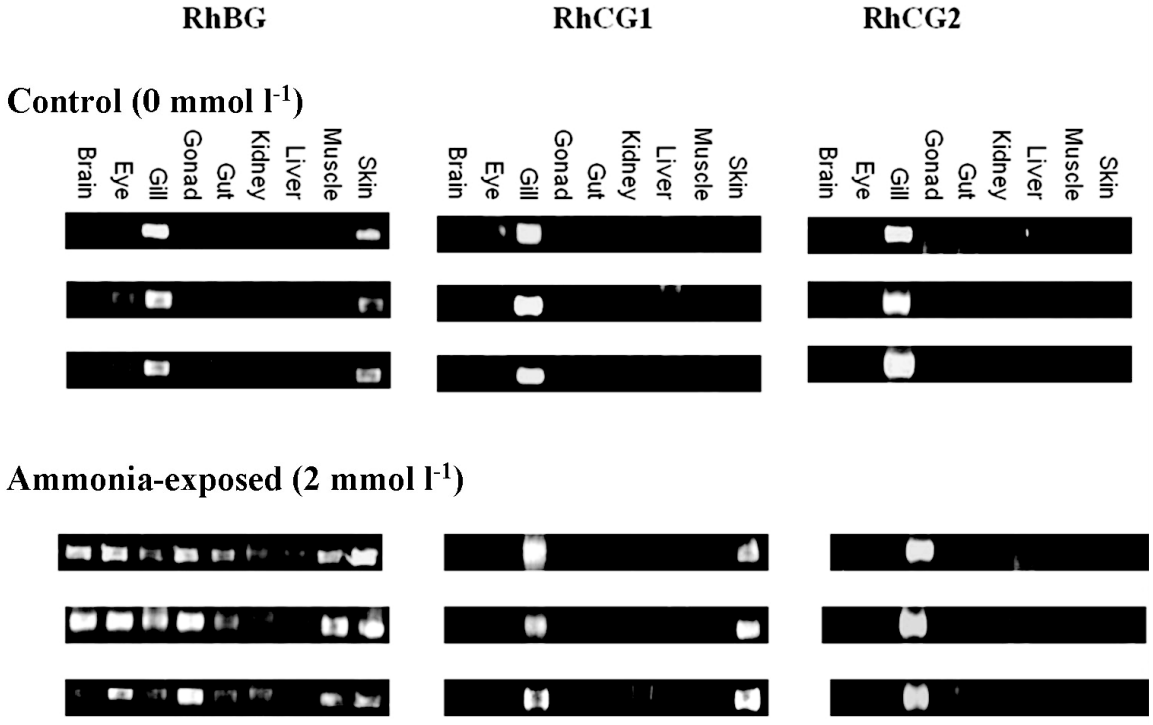

Fig. 5. RT-PCR mRNA expression of *RhBG*, *RhCG1* and *RhCG2* in control (0 mmol l^{-1} ammonia) and ammonia-exposed (2 mmol l^{-1} ammonia) *K. marmaratus*. For each gene, the following tissues are shown from left to right: brain, eye, gill, gonad, gut, kidney, liver, skeletal muscle and skin. Each gel represents one individual (control N=3, ammonia-exposed N=3). Note *RhBG*, *RhCG1* and *RhCG2* are expressed strongly in gill tissue. *RhBG* expression is low in control tissues except in gill and skin, but higher in many tissues in ammonia-exposed fish. *RhCG1* expression in skin is induced with ammonia exposure and *RhCG2* expression remains restricted to gill tissues.

also has ten predicted transmembrane domains but is unlike zebrafish (*Danio rerio*) and pufferfish (*T. rubripes*), both of which have 12 predicted transmembrane domains (data not shown). *K. marmoratus* RhCG1 and RhCG2, on the other hand, have 12 transmembrane domains similar to their mammalian counterparts (Huang and Liu, 2001).

Our study represents the first report of the possible involvement of *Rh* gene/proteins in ammonia excretion in fish during adverse conditions such as high external ammonia and aerial exposure. All three *Rh* mRNAs (*RhBG*, *RhCG1* and *RhCG2*) were expressed under control conditions in the gill, similar to the observation in pufferfish gills (Nakada et al., 2007), and *RhBG* is also expressed in the skin. After exposure to high environmental ammonia (2 mmol l^{-1} NH_4HCO_3, pH 8.1), *RhBG* was widely expressed in many tissues, and *RhCG1* expression extended to the skin. Interestingly, *RhBG* induction is observed in almost all tissues screened (Figs 5 and 7) except gill and skin (Fig. 6). *RhCG1* and *RhCG2* appear to demonstrate tissue specificity during high ammonia exposure (Fig. 6), with *RhCG1* mRNA levels dominating skin *Rh* expression and *RhCG2* dominating gill *Rh* expression.

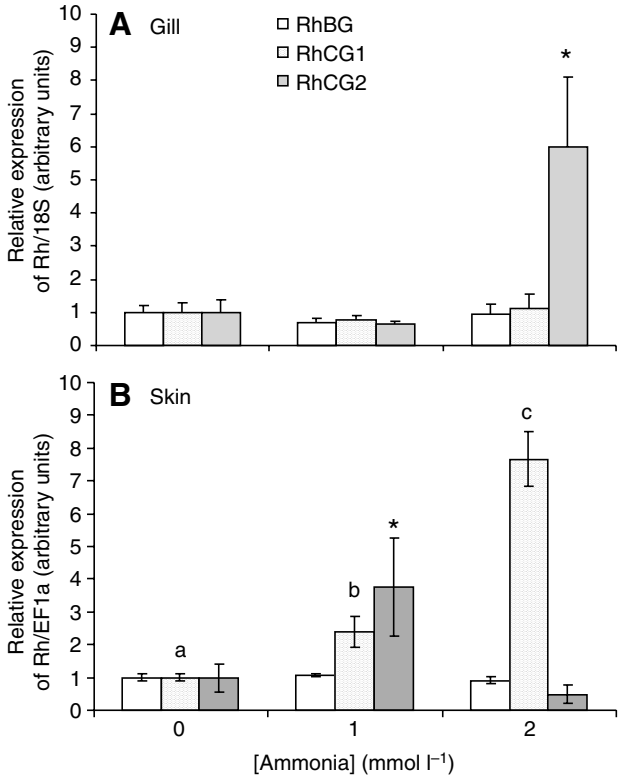

Fig. 6. Relative mRNA expression levels of gill *Rh* genes in excretory organs (gills and skin) of *K. marmaratus* exposed to 0 mmol l^{-1} (control) or 1 and 2 mmol l^{-1} NH_4HCO_3. (A) Gill *RhBG*, *RhCG1* and *RhCG2* relative to 18S rRNA. Asterisk indicates that *RhCG2* mRNA levels were significantly higher than in fish exposed to 2 mmol l^{-1} ammonia compared to 0 or 1 mmol l^{-1} ammonia. *RhBG* and *RhCG1* were not significantly different between control and ammonia-exposed fish. Values are means ± s.e.m.; 0 and 2 mmol l^{-1} ammonia, N=7, 1 mmol l^{-1} ammonia, N=8; one-way ANOVA, P<0.05). (B) Skin *RhBG*, *RhCG1* and *RhCG2* relative to *EF1a*. Letters (a,b,c) indicate that *RhCG1* mRNA levels were significantly higher in fish exposed to 1 mmol l^{-1} relative to 0 mmol l^{-1} ammonia, and significantly higher in 2 mmol l^{-1} relative to 0 and 1 mmol l^{-1} ammonia. Asterisk indicates that *RhCG2* was significantly higher in 1 mmol l^{-1} compared to 0 and 2 mmol l^{-1} ammonia. *RhBG* was not significantly different between control and ammonia-exposed fish. Values are means ± s.e.m.; N=6 (one-way ANOVA, P<0.05).

An earlier study (Frick and Wright, 2002a) indicated that ammonia might be excreted against the gradients when *K. marmoratus* were exposed to high environmental ammonia (see Introduction). Indeed, in the present study, there was no elevation of body tissue ammonia levels after 5 days in 1 mmol l^{-1} NH_4HCO_3 and only a small increase (37%) after the same period of time in 2 mmol l^{-1} NH_4HCO_3 (pH=8.1, $NH_3 \approx 100$ μmol l^{-1}). This is surprising because in other fish, internal ammonia levels typically increase several-fold in response to external ammonia levels (Wilson and Taylor, 1992; Wilson et al., 1994; Knoph and Thorud, 1996; Kong et al., 1998; Steele et al., 2001; Anderson et al., 2002). For example, in the ammonia-tolerant marine toadfish *Opsanus beta*, muscle tissue ammonia concentrations were elevated by threefold after 4 days in water containing 3.5 mmol l^{-1} NH_4Cl (at pH 7.8, $NH_3 \approx 70$ μmol l^{-1}) (Wang and Walsh, 2000), a lower NH_3 level than in this study. In contrast, *K. marmoratus* is capable of preventing excessive accumulation of ammonia at high external ammonia and this appears to be similar to the mudskipper *P. schlosseri* (Randall et al., 1999), a species that is known to excrete ammonia actively during high environmental ammonia exposure.

Although the mode of ammonia excretion against high external ammonia in killifish has not been delineated, we

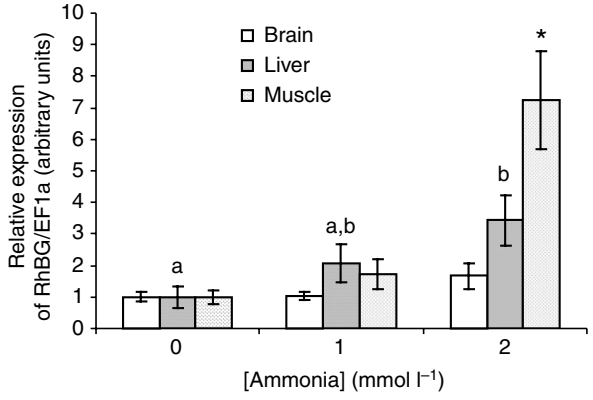

Fig. 7. Relative mRNA expression levels of *RhBG* to *EF1a* in internal organs of *K. marmaratus* exposed to 0 mmol l^{-1} (control) or 1 and 2 mmol l^{-1} NH_4HCO_3. In brain, no significant difference was observed between control and ammonia-exposed fish. In liver, *RhBG* was significantly higher in liver of fish exposed to 2 mmol l^{-1} (b) compared to 0 mmol l^{-1} (a), but there was no significant difference between 0 (a) and 1 mmol l^{-1} ammonia (a,b), as well as between 1 and 2 mmol l^{-1} ammonia-exposed fish. In muscle, *RhBG* in muscle was significantly higher in fish exposed to 2 mmol l^{-1} (asterisk) than 0 and 1 mmol l^{-1} ammonia. Values are means ± s.e.m.; N=6 (one-way ANOVA, P<0.05).

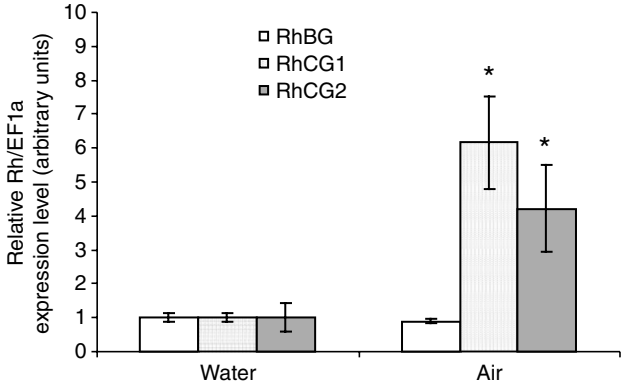

Fig. 8. Relative mRNA expression levels of *RhBG*, *RhCG1* and *RhCG2* to *EF1a* in the skin of control (immersed) or air-exposed *K. marmoratus*. Asterisks indicate both *RhCG1* and *RhCG2* were significantly higher in skin of air-exposed relative to control fish. Values are means ± s.e.m.; *N*=6; air-exposed: *N*=4 (*t*-test, *P*<0.05).

hypothesize that *Rh* genes may be involved. All three Rh proteins are expressed in control pufferfish gills (Nakada et al., 2007). Constitutive expression of Rh proteins is probably required for routine removal of endogenous ammonia from nitrogen metabolism. There was no increase in any of the *Rh* mRNAs in the gills of *K. marmoratus* during 1 mmol l^{-1} exposure, but the upregulation of *RhCG1* and *RhCG2* in the skin (Fig. 6B) and constitutive expression of Rh proteins in the gill, may have been involved in facilitating ammonia excretion and thereby preventing an elevation of whole-body ammonia content (Fig. 4). Likewise, when external ammonia levels were increased to 2 mmol l^{-1}, the upregulation of *RhCG2* mRNA levels in the gill and *RhCG1* mRNA levels in the skin (Fig. 6B) is suggestive of a functional role of *Rh* genes in ammonia transport. It should be noted that transcriptional changes do not necessarily imply a corresponding upregulation of protein content, and verification of these ideas is necessary. Initial experiments using antibodies obtained from mammalian Rh proteins were unsuccessful and future work will require fish-specific antibodies.

In the mammalian kidney, polarized localization of the Rh proteins is observed in epithelial cells of specific regions, including the renal collecting segment and the collecting duct, with RhBG expression in the basolateral membrane and RhCG in the apical membrane (Quentin et al., 2003; Verlander et al., 2003). Such polarized localization is also present in the pufferfish. RhBG is localized basolaterally and RhCG2 proteins apically in the pavement cells of the gills, whereas RhCG1 proteins are found in the apical membrane of the mitochondrial-rich (MR) cells (Nakada et al., 2007). We predict a similar localization of the different Rh proteins in the killifish.

It has been suggested (Weiner, 2006) that RhBG may be playing a role in ammonia-sensing or having roles other than ammonia-transport. This suggestion was based on the basolateral location of RhBG glycoproteins, together with the observation that expressions of *RhBG* mRNA and protein were not increased metabolic acidosis (which results in net excretion of acid and ammonia by the mammalian renal system) (Seshadri et al., 2006). Others have reported that genetic ablation of *RhBG* in the knockout mice did not affect ammonia transport in the kidney and tolerance to chronic acid-loading (Chambrey et al., 2005; Chambrey et al., 2006), although other genes may have been upregulated to compensate for the *Rh* knockdown. *RhBG* expressions are restricted to kidney, liver and gastrointestinal tract in the mammals (human and mouse) (Liu et al., 2001; Handlogten et al., 2005). In contrast, *RhBG* in *K. marmoratus* has a very broad tissue expression (Fig. 5). From the phylogenetic tree (Fig. 3), it is noted that RhBG is relatively more primitive and emerged earlier compared to RhCGs. Induction of *RhBG* was observed in a variety of tissues, whereas the expression of *RhCG*s was restricted to gill and skin during high ammonia exposure (Fig. 4). Fish normally experience much higher extracellular ammonia concentrations [0.1–1.3 mmol l^{-1} (Wood, 1993)] than mammals [0.03–0.1 mmol l^{-1} (Felipo and Butterworth, 2002)]. Taken together, the data suggests that *RhBG* may have important functions in fish to facilitate ammonia transport in various internal organs.

The skin of *K. marmarotus* is an important site for NH_3 volatilization during air exposure (Frick and Wright, 2002b; Litwiller et al., 2006). Following emersion, there was an 18-fold increase in the NH_4^+ concentration on the cutaneous surface, leading to a substantial elevation in the partial pressure of NH_3 (Litwiller et al., 2006). The authors proposed that an active mechanism may be involved in moving ammonia across the skin surface when fish were emersed. Induction of ammonia/ammonium transporter genes (*RhCG1* and *RhCG2*) in the skin during aerial exposure (Fig. 8), therefore, may be involved in the transport of ammonia across the epidermis for subsequent NH_3 volatilization, as well as in aquatic ammonia excretion. According to the model proposed by Khademi and co-workers (Khademi et al., 2004), ammonia approaches the Amt B channel as NH_4^+, then the proton is stripped away and ammonia is transported across the channel as NH_3 gas; it exits the channel as NH_3, then picks up a proton and forms NH_4^+. We propose that fish Rh proteins also conduct ammonia in the gaseous form. During air exposure, ammonia would leave the cutaneous surface of *K. marmoratus* in the form of NH_3, without being protonated and hence volatilized. While in water, RhBG and/or RhCG would facilitate NH_3 excretion across the gill and skin, which would be subsequently trapped by H^+ to form NH_4^+. Acidification of the boundary water layer (Wright et al., 1989) in the cutaneous or gill surface of immersed *K. marmoratus* might be a possible mechanism to accelerate ammonia removal in fish, particularly in the presence of high external ammonia concentration.

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