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RESEARCH ARTICLE

Rh proteins and NH_4^+-activated Na^+-ATPase in the Magadi tilapia (*Alcolapia grahami*), a 100% ureotelic teleost fish

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SUMMARY

The small cichlid fish *Alcolapia grahami* lives in Lake Magadi, Kenya, one of the most extreme aquatic environments on Earth (pH ~10, carbonate alkalinity ~300 mequiv l^{-1}). The Magadi tilapia is the only 100% ureotelic teleost; it normally excretes no ammonia. This is interpreted as an evolutionary adaptation to overcome the near impossibility of sustaining an NH_3 diffusion gradient across the gills against the high external pH. In standard ammoniotelic teleosts, branchial ammonia excretion is facilitated by Rh glycoproteins, and cortisol plays a role in upregulating these carriers, together with other components of a transport metabolon, so as to actively excrete ammonia during high environmental ammonia (HEA) exposure. In Magadi tilapia, we show that at least three Rh proteins (*Rhag*, *Rhbg* and *Rhcg2*) are expressed at the mRNA level in various tissues, and are recognized in the gills by specific antibodies. During HEA exposure, plasma ammonia levels and urea excretion rates increase markedly, and mRNA expression for the branchial urea transporter *mtUT* is elevated. Plasma cortisol increases and branchial mRNAs for *Rhbg*, *Rhcg2* and *Na⁺,K⁺-ATPase* are all upregulated. Enzymatic activity of the latter is activated preferentially by NH_4^+ (*versus* K^+), suggesting it can function as an NH_4^+-transporter. Model calculations suggest that active ammonia excretion against the gradient may become possible through a combination of Rh protein and NH_4^+-activated Na^+-ATPase function.

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Key words: high environmental ammonia, high alkalinity, ammonia transport, urea transport, gills, Na^+,K^+-ATPase, *Alcolapia grahami*.

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INTRODUCTION

Alcolapia grahami, a small cichlid fish living in the extreme environment of Lake Magadi, Kenya [pH ~10, carbonate alkalinity ~300 mequiv l^{-1} (Coe, 1966)], is unique. The Magadi tilapia is the only teleost fish known to be 100% ureotelic; it excretes no ammonia (Randall et al., 1989; Wood et al., 1989). The ornithine-urea cycle (OUC) is present in the muscle tissue as well as in the liver, explaining the exceptionally high rates of production of urea (Randall et al., 1989; Lindley et al., 1999), which is excreted across the gills by a facilitated diffusion type urea transporter (*mtUT*) (Walsh et al., 2001). While this is interpreted as an evolutionary adaptation to overcome the near impossibility of sustaining an ammonia diffusion gradient across the gills against an external pH of 10 (Pörtner et al., 2010), it is noteworthy that the characteristic of 100% ureotelism is obligate. Even when provided with an opportunity to excrete ammonia by acute exposure to neutralized Magadi water or gradual acclimation to freshwater, the Magadi tilapia continues to be 100% ureotelic (Wood et al., 1989; Wood et al., 1994; Wood et al., 2002).

Only recently has it been realized that ammonia excretion across the gills of ammoniotelic fish is facilitated by Rhesus (Rh) glycoproteins. These have been cloned (Nakada et al., 2007; Hung et al., 2007; Nawata et al., 2007) and shown by *in vitro* expression analysis to bind NH_4^+ but facilitate the movement of NH_3 (Nawata et al., 2010a). The transepithelial movement of H^+ ions by other mechanisms appears to play a key role in acid-trapping of NH_3 in the external boundary layer, thereby sustaining the NH_3 movement through the Rh channels [see models in Weihrauch et al. (Weihrauch et al., 2009) and Wright and Wood (Wright and Wood, 2009)]. The system is capable of actively excreting ammonia against gradients, and is activated by elevated external or internal ammonia levels, which cause increased mRNA expression of the Rh proteins and allied transporters such as Na^+,K^+-ATPase (Nawata et al., 2007; Nawata et al., 2010b; Nawata and Wood, 2009). An accompanying elevation of plasma cortisol plays a role in this response (Tsui et al., 2009).

With this background in mind, we questioned whether Rh proteins would be expressed at all at the mRNA or protein levels

in the gills or other tissues in a fish that never apparently excretes ammonia, and/or whether they could be activated by high environmental ammonia (HEA). Magadi tilapia are exceptionally ammonia tolerant (Walsh et al., 1993), and HEA is an environmentally relevant treatment because the bacterial degradation of large amounts of flamingo guano in some areas of the lake can produce high water ammonia concentrations (Wilson et al., 2004). Transport of ammonia by branchial Na^+,K^+-ATPase (i.e. 'NH_4^+-activated Na^+-ATPase') has also been implicated in ammonia tolerance in some species (Mallery, 1983; Balm et al., 1988; Randall et al., 1999; Nawata et al., 2010b), so we examined the response of this enzyme to HEA exposure in terms of gene transcription, enzyme activity and substrate specificity. HEA exposure was previously shown to quickly increase urea excretion in this species (Wood et al., 1989), presumably because ammonia enters across the gills and activates urea production by the OUC in the tissues (Wood et al., 1994; Lindley et al., 1999; Wilson et al., 2004). We therefore also assayed whether the branchial *mtUT* urea transporter was activated at the transcriptional level by HEA. Surprisingly, we show that this 100% ureotelic fish does express Rh proteins; Rh mRNA expression in the gills and related physiological parameters respond to HEA in a manner similar to that seen in 'normal' ammoniotelic fish, *mtUT* is activated, and branchial Na^+-ATPase activity is more responsive to NH_4^+ than to K^+. Model calculations suggest that active ammonia excretion against the gradient may become possible through a combination of Rh protein and NH_4^+-activated Na^+-ATPase function.

MATERIALS AND METHODS

All experiments complied with the laws of Kenya, and were performed under a research permit issued by the National Council for Science and Technology of the Ministry of Higher Education, Science, and Technology of the Republic of Kenya.

Experimental animals

Magadi tilapia [*Alcolapia grahami* (Boulenger 1912), formerly *Oreochromis alcalicus grahami*, formerly *Sarotherodon alcalicus grahami*, formerly *Tilapia grahami*] with a mean mass of 4.11 g (range 2.05–7.57 g) were collected by seine net in July and August 2010 from Fish Springs Lagoon on the edge of Lake Magadi [for maps, see Coe (Coe, 1966) and Narahara et al. (Narahara et al., 1996)]. Collections were made between 06:30 and 08:30 h, and experiments were started within 2–4 h of capture because these highly active fish do not survive well in captivity. All experiments were performed in an outdoor laboratory set up on the balcony of a house kindly provided by the Magadi Soda Company (Tata Chemicals Magadi) in the nearby Magadi township. Although air temperature fluctuated greatly (22–40°C), a water bath ensured that experimental temperature was held at 28–30°C, the same as at the collection site in early morning.

Respirometry series

In previous studies we have found it useful to normalize urea-N production rates to O_2 consumption rates ($\dot{M}_{O_2}$), because of great variations in spontaneous activity levels of these wild fish (Wood et al., 1994; Wood et al., 2002; Wilson et al., 2004). Animals were transferred to individual 0.75 l respirometers filled with freshly collected Lake Magadi water from Fish Springs Lagoon (pH 9.92; Na^+=356, Ca^{2+}=1.30, Mg^{2+}=0.08, Cl^-=112 and titratable carbonate alkalinity=290 mequiv l^{-1}). The chambers were served with individual aeration and could be sealed as respirometers. After a 1–2 h settling period, the experiment was started. Urea-N excretion rates ($\dot{M}_{Urea\text{-}N}$) were measured over a 3 h control period immediately after which HEA (N=21) or continued control exposure (N=12) were instituted (time 0 h), with additional $\dot{M}_{Urea\text{-}N}$ measurements made at 0–3, 3–6, 6–9, 9–18 and 18–24 h. Aeration was stopped and the respirometers sealed for 1 h measurements of $\dot{M}_{O_2}$ in the middle of each period. The nominal ammonia concentration during HEA was 500 μmol l^{-1}, achieved by the addition of NH_4HCO_3, which did not alter water pH. As NH_3 is volatile at pH 9.92, ~90% of the water was renewed and fresh NH_4HCO_3 was added at the beginning of each flux period, taking care to minimize disturbance. In one typical 24 h HEA run (five flux periods on five fish), the mean overall measured ammonia-N concentration was 472±13 μmol l^{-1} (N=25) at the start of flux periods and 443±16 μmol l^{-1} (N=25) at the end; water ammonia concentrations remained less than 5 μmol l^{-1} in the control treatment. Each run included control and HEA blank chambers to correct measurements of $\dot{M}_{Urea\text{-}N}$ and $\dot{M}_{O_2}$ data for microbial activity. This proved negligible for the former, but accounted for up to 25% of $\dot{M}_{O_2}$.

Tissue and blood sampling series

Tilapia were put through identical HEA and control protocols, but without the measurements of $\dot{M}_{Urea\text{-}N}$ and $\dot{M}_{O_2}$. HEA-exposed fish were killed at 4, 12 and 24 h, and control fish at 0 and 24 h (N=10–16 at each time, but not all samples were taken from all fish). For sampling, fish were rapidly anaesthetized (~30 s) by transfer to HEA water or control water (as appropriate) at 1°C, and then killed by cephalic concussion. A blood sample (25–75 μl) was drawn by caudal puncture using a gas-tight Hamilton 100 μl syringe with a bevelled needle customized to sample at the correct depth. The syringe had been pre-rinsed with Cortland saline adjusted to 175 mmol l^{-1} NaCl (Wolf, 1963) and heparinized (1000 i.u. ml^{-1} lithium heparin). After immediate centrifugation (5000 ***g***, 30 s), plasma was decanted and frozen in liquid N_2 for later measurements of plasma total ammonia (T_{Amm}), cortisol, glucose, lactate and ions, whereas the red blood cell pellet was resuspended in approximately five volumes of RNA*later* (Ambion, Austin, TX, USA) and stored at 4°C. The gills and a sample of lateral posterior skin were similarly preserved in RNA*later*. Additional fish were killed in the same manner at 24 h HEA, and at 0 and 24 h control (N=6–8) for plasma metabolites, gill ATPase activity and tissue-specific screening of Rh gene expression. Tissues (gills, brain, liver, intestine, white muscle, skin) were harvested, wrapped in aluminium foil, and immediately frozen in liquid N_2. In addition, some gills were preserved for morphology and immunohistochemistry by immersion fixation in 3% paraformaldehyde in phosphate-buffered saline and then processed for embedding in either paraffin or Tissue-Tek OCT compound (Sakura Finetek, Torrance, CA, USA)

Analytical techniques

Water dissolved O_2 concentration was measured using a portable Accumet polarographic electrode and AB15 meter (Fisher Scientific, Mississauga, ON, Canada) and water urea-N concentration by the diacetyl monoxime method (Rahmatullah and Boyde, 1980). $\dot{M}_{O_2}$ and $\dot{M}_{Urea\text{-}N}$ were calculated from changes in concentration (corrected for the microbial blank), factored by time, chamber volume and fish mass. To adjust for variations in activity levels, $\dot{M}_{Urea\text{-}N}$ was also normalized to O_2 consumption as $\dot{M}_{Urea\text{-}N}/\dot{M}_{O_2}$. Water ammonia-N concentration was checked by the salicylate hypochlorite method (Verdouw et al., 1978). Plasma [T_{Amm}] was measured using a kit (Raichem, San Diego, CA, USA) based on the glutamate dehydrogenase/NAD method. Plasma [cortisol] was measured using a commercial ^{125}I radioimmunoassay kit (CA-1529 GammaCoat, DiaSorin, Stillwater,

Table 1. List of primers (5′→3′) and GenBank accession numbers

Primer	Forward	Reverse	GenBank accession number
Cloning *Rhag*	ccacgcctttggagcttactttggc	ccaggcatgccgtgcagattg	JQ710654[a]
Cloning *Rhbg*	gctgcctatcacctgtttcatcctg	aagctgggccagaacatccacaggta	JQ710655[a]
Cloning *Rhcg2*	cagtgggctcttctcatgcaaggctg	gagttgaaacttggccagaacatcca	JQ710656[a]
RACE *Rhag*	gctatgatcggaaccatctttttgtg	cacaaaaagatggttccgatcatagc	JQ710654[a]
RACE *Rhbg*	ccatgaccattcacacatttggagcc	ggctcccacaatagcgcccataacac	JQ710655[a]
RACE *Rhcg2*	gtccagctgatggttgtcaccttatt	ggtttggtcggtacagcacccagga	JQ710656[a]
Rhcg2 3′UTR	tgttggtttcatcctgaggt	tgaggatataaaggggactgg	XM_003442253.1[b]
qPCR *Rhag*	ctggcccagctttaactcag	gtcagcacatgttcccactg	JQ710654[a]
qPCR *Rhbg*	tatggcttcagcagtgttgg	tccaaacgagatcagcacag	JQ710655[a]
qPCR *Rhcg2*	ctgctgtgctggatctctga	catggagccaccagaatctt	JQ710656[a]
qPCR *GS*	tcgcattcctcgtaatgttg	tcgttcagcaaacaggtgcg	AF503208[b]
qPCR *NKA*	ttggaggccgttgagactct	tgtcaaaccacatgtgagcc	U82549[c]
qPCR *mtUT*	atggcacaccctgacttacc	ccatccattttccaaacacc	AF278537[a]

[a]*Alcolapia grahami*.
[b]*Oreochromis niloticus*.
[c]*Tilapia mossambica*.

MN, USA). Plasma [Na^+], [K^+], [Ca^{2+}] and [Mg^{2+}] were measured by flame atomic absorption spectroscopy (Varian SpectrAA-220FS, Mulgrave, Australia). Clinical micrometers, calibrated with standards made up in Cortland saline, were used for assay of plasma [glucose] (Accu-Chek GT Compact Plus, Roche Diagnostics, Mannheim, Germany) and [lactate] (Lactate Pro LT-1710, Arkray, Kyoto, Japan). Branchial Na^+,K^+-ATPase and V-type H^+-ATPase activities were measured using methods from McCormick (McCormick, 1993) and Lin and Randall (Lin and Randall, 1993) respectively, as modified by Nawata et al. (Nawata et al., 2007). Protein concentrations were measured with Bradford Reagent and BSA standards (Sigma-Aldrich, St Louis, MO, USA). Na^+,K^+-ATPase and V-type H^+-ATPase assays on the same samples were run simultaneously at both pH 7.5 and pH 8.0 to check pH sensitivity. In order to test whether NH_4^+ could activate branchial Na^+,K^+-ATPase, various concentrations of NH_4^+ (as NH_4Cl) and/or K^+ (as KCl) were added to the assay media (see Results).

Molecular and immunohistochemical analyses

Total RNA was extracted from tissues with Trizol (Invitrogen, Burlington, ON, Canada) and concentrations were determined with a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). One microgram of total RNA (DNaseI-treated; Invitrogen) was used to synthesize first strand cDNA with Superscript II reverse transcriptase (Invitrogen). Rh glycoprotein cDNA fragments were first amplified with cloning primers (Table 1). Full-length cDNA sequences of *Rhag*, *Rhbg* and *Rhcg2* were then obtained by 5′- and 3′-rapid amplification of cDNA ends (RACE) (Smart RACE cDNA amplification kit, BD Biosciences Clontech, Mississauga, ON, Canada) using the RACE primers (Table 1) and the protocol described previously (Nawata et al., 2007). However, in the case of *Rhcg2*, 3′ RACE was unsuccessful and the remainder of the 3′ open reading frame was obtained by PCR using a 3′ untranscribed region (UTR) reverse primer designed from *Oreochromis niloticus Rhcg2* and a gene-specific forward primer. Phylogenetic analyses were conducted using ClustalW (Thompson et al., 1994) and MEGA 5.1 software (Tamura et al., 2011) by the neighbour-joining method with support for each node using 1000 bootstrap replicates. Amino acid sequence analyses were performed using BioEdit (Hall, 1999). Hydropathy profile and *N*-glycosylation site predictions were made using TMHMM (Krogh et al., 2001; Sonnhammer et al., 1998) and ScanProsite (de Castro et al., 2006), respectively.

Quantitative real-time PCR (qPCR) was performed on the above synthesized cDNA with the primers for *Rhag*, *Rhbg*, *Rhcg2*, *glutamine synthetase*, *Na^+,K^+-ATPase* (α1-subunit) and urea transporter (*mtUT*) as listed in Table 1, using Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen) on an Mx3000P QPCR System (Stratagene, Cedar Creek, TX, USA). Prior to qPCR analyses, amplification products of all primer pairs were sequenced to verify primer specificity. Expression of the three *Rh* mRNAs was assessed in the gill, skin and red blood cells, and of *glutamine synthetase*, *Na^+,K^+-ATPase* and *mtUT* in the gill. No-template controls were run in parallel and melt-curve analysis verified the production of a single product. Data were extrapolated from standard curves generated by serial dilution of one randomly selected control sample. Beta actin mRNA expression remained stable across treatments and was used for normalization.

Immunohistochemical localization and immunoblotting of Rhcg2, Rhbg and Rhag proteins were detected using rabbit polyclonal antibodies against pufferfish (‘fugu’) Rh proteins (Nakada et al., 2007). Immunohistochemistry was performed as described in Laurent et al. (Laurent et al., 1994) and Wilson et al. (Wilson et al., 2007) for HRP-DAB (horse radish peroxidase, 3,3′-diaminobenzidine) and fluorescence detection, respectively. Using immunofluorescence, some sections were also double-labeled for Rh glycoproteins with Na^+,K^+-ATPase α subunit using the pan-specific α5 monoclonal antibody. Branchial ionocytes can be identified by high Na^+,K^+-ATPase expression (Wilson and Laurent, 2002). Immunoblotting was performed according to Wilson et al. (Wilson et al., 2007) using a Bio-Rad Mini-Protean 3 system and semi-dry transfer apparatus (Bio-Rad Laboratories, Hercules, CA, USA) with enhanced chemiluminescent detection (LAS 4000mini, FujiFilm, Tokyo, Japan). Only control fish were available for immunoblotting.

Statistical analyses

Data have been expressed as means ± 1 s.e.m. (*N*=number of fish). The Michaelis–Menten equation was fitted to enzyme kinetic data using Sigmaplot Windows Version 10.0 (Systat Software, San Jose, CA, USA). $\dot{M}_{O_2}$ and $\dot{M}_{Urea\text{-}N}$ were compared between HEA and simultaneous control treatments at each measurement period by Student’ unpaired *t*-tests. Metabolite, ion, ATPase and mRNA data were analyzed by ANOVA followed by specific *post hoc* multiple comparison tests (Fisher’s least significant difference for mRNA, Bonferroni for all others). The 24 h control means were compared

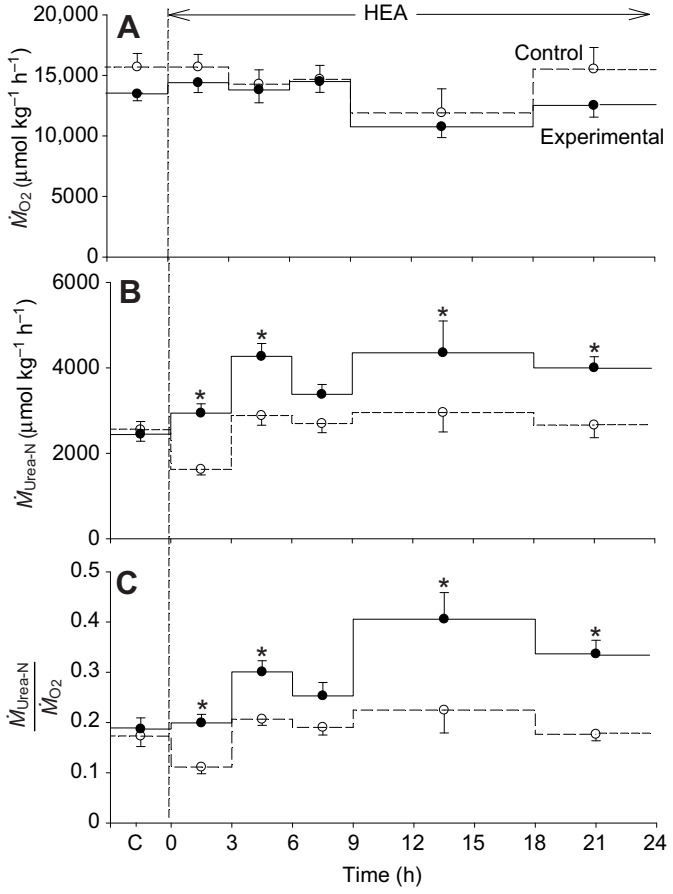

Fig. 1. Influence of 24 h exposure to high environmental ammonia (HEA; 500 µmol l^{-1}; solid symbols, solid lines; *N*=21) or simultaneous exposure to control conditions (open symbols, dashed lines; *N*=12) on (A) oxygen consumption rate ($\dot{M}_{O_2}$), (B) urea-N excretion rate ($\dot{M}_{Urea-N}$) and (C) their ratio ($\dot{M}_{Urea-N}/\dot{M}_{O_2}$) in *Alcolapia grahami*. Data are means ± 1 s.e.m. Initial control values, designated by 'C', were taken prior to HEA exposure. *Significantly different (*P*<0.05) relative to simultaneous control value.

with 0 h control means, and HEA means at each sample time were compared with both the 0 h control mean and the 24 h control mean. For mRNA data, overall effects were evaluated by comparing means from all fish at all three HEA times with means from all fish at the two control times. All tests were two-tailed and a significance level of 0.05 was used throughout.

RESULTS

O_2 consumption and urea-N excretion

HEA had no effect on $\dot{M}_{O_2}$, which remained the same as in the control throughout the 24 h exposure (Fig. 1A). However, $\dot{M}_{Urea-N}$ became greater in the HEA treatment in the first 3 h, and remained elevated relative to the control throughout most periods of the 24 h exposure (Fig. 1B). When normalized to O_2 consumption (i.e. $\dot{M}_{Urea-N}/\dot{M}_{O_2}$), the elevation reached ~85% from 9 through 24 h (Fig. 1C).

Plasma metabolites and ions

In addition to the increased $\dot{M}_{Urea-N}$, plasma T_{Amm} data provided clear evidence of internal ammonia loading during HEA. At 4 h, plasma T_{Amm} increased approximately 2.5-fold from resting levels of ~1.6 mmol l^{-1} and remained significantly elevated at 12 h, relative to both the 0 h and 24 h control means, but was no longer elevated at

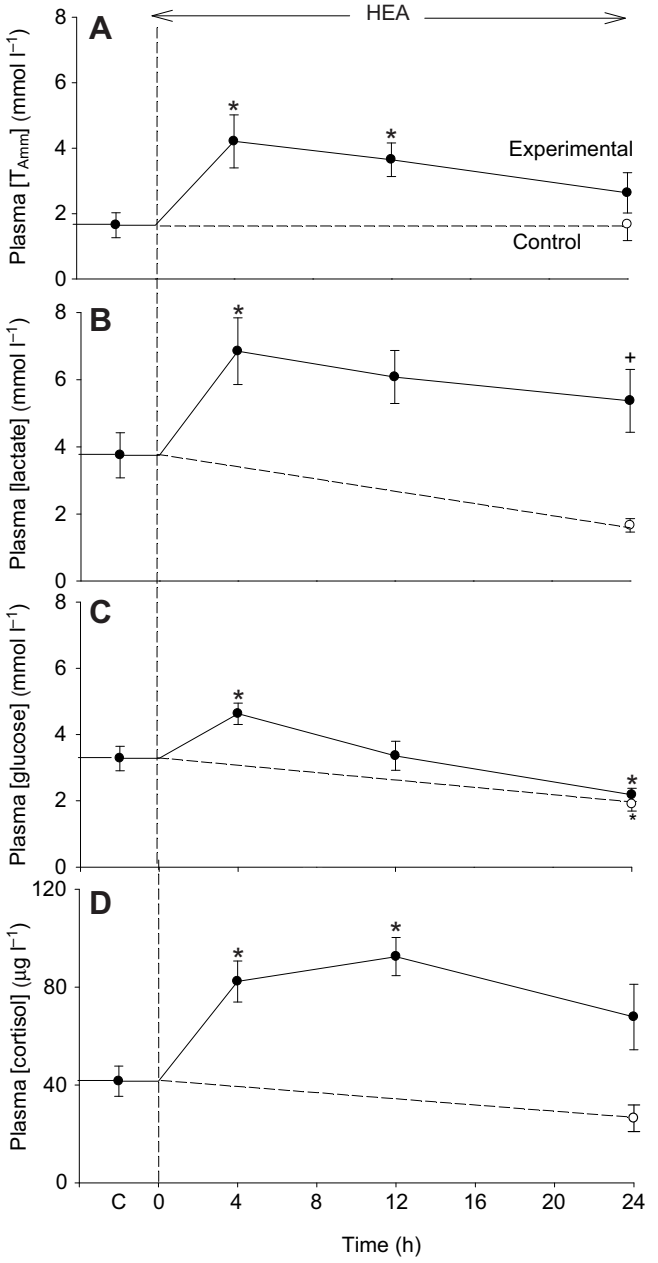

Fig. 2. Influence of exposure to HEA (500 µmol l^{-1}; solid symbols, solid lines) for 4 h (*N*=9–10), 12 h (*N*=10–11) or 24 h (*N*=16–18) on (A) plasma total ammonia (T_{Amm}), (B) lactate, (C) glucose and (D) cortisol concentrations in *Alcolapia grahami*. The 0 h (*N*=13–16) and 24 h control values (*N*=14–16) are also shown (open symbols, dashed lines). Data are means ± 1 s.e.m. *Significantly different (*P*<0.05) relative to 0 h control value; $^{+}$significantly different (*P*<0.05) relative to 24 h control value.

24 h (Fig. 2A). This response in plasma T_{Amm} was accompanied by increases in plasma lactate (Fig. 2B), glucose (Fig. 2C) and especially cortisol concentrations (Fig. 2D), which more than doubled by 12 h HEA. For all these parameters, there was a tendency for 24 h control means to be lower than 0 h controls, but this was significant only for glucose (Fig. 2C). In the HEA treatment, lactate, but not glucose or cortisol, remained significantly elevated at 24 h.

The 0 h control levels of plasma [Na^+] (182±3 mmol l^{-1}, *N*=13), [Cl^-] (171±5 mmol l^{-1}, *N*=13) and [K^+] (4.98±0.70 mmol l^{-1}, *N*=6) did not change significantly during 24 h of HEA exposure, or at the

Table 2. Influence of exposure to high environmental ammonia (HEA; nominally 500 μmol l^{-1}) on plasma [Ca^{2+}] and [Mg^{2+}] concentrations

Sample time	Plasma [Ca^{2+}] (mequiv l^{-1})	Plasma [Mg^{2+}] (mequiv l^{-1})
0 h control	4.56±0.42 (13)	2.04±0.24 (6)
4 h HEA	6.54±0.56 (8)*	2.26±0.28 (7)$^{+}$
12 h HEA	6.86±0.60 (9)*,$^{+}$	2.20±0.12 (5)$^{+}$
24 h HEA	5.70±0.28 (16)*	1.84±0.14 (11)$^{+}$
24 h control	5.16±0.46 (13)	1.50±0.08 (9)*

Data are means ± 1 s.e.m. (*N*). *P<0.05 relative to 0 h control value; ^{+}P<0.05 relative to 24 h control value.

24 h control time (data not shown). However, plasma [Ca^{2+}] and [Mg^{2+}] both increased significantly during HEA exposure (Table 2).

Rh gene expression and molecular responses

Three full-length Rh glycoprotein cDNAs were identified in *A. grahami*: *Rhag* (accession no. JQ710654), 1844 bp, encoding a protein of 488 amino acid residues with 12 predicted transmembrane spanning regions; *Rhbg* (JQ710655), 1691 bp, encoding a protein of 463 amino acid residues with 10 predicted transmembrane regions; and *Rhcg2* (JQ710656), 1578 bp, encoding a protein of 489 amino acid residues with 10 predicted transmembrane regions. However, the complete 3′ UTR was not obtained for Rhcg2. All three proteins have a putative *N*-glycosylation site in the first extracellular domain (Asn-48, -46 and -62 for Rhag, Rhbg and Rhcg2, respectively). The full sequences are shown in supplementary material Fig. S1A. Notably, while Rhag exhibits the standard 12 transmembrane domains, both Rhbg and Rhcg2 have only 10. In the case of Rhbg, this difference appears to be because of a substitution of hydrophobic amino acid residues for hydrophilic ones, rather than deletions; the same phenomenon is seen in the Nile tilapia, *O. niloticus*. However, in the case of Rhcg2, the Magadi sequence is truncated at the 5′ end; this is not the case in *O. niloticus*, which exhibits the full 12 transmembrane domains. Phylogenetic analysis indicates that each paralogue (*Rhag*, *Rhbg* and *Rhcg2*) clearly clusters with its respective orthologue clade (Fig. 3). It was not possible to isolate an *Rhcg1* homologue from gill tissue using the above PCR approach. Attempts using gene-specific primers for *Rhcg1* from the closely related *O. niloticus* were also unsuccessful.

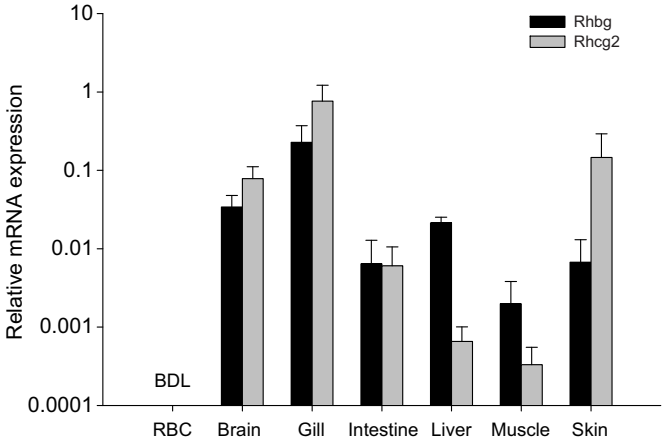

Fig. 4. Relative mRNA expression of *Rhbg* and *Rhcg2* in red blood cells (RBC), brain, gill, intestine, liver, white muscle and skin in *Alcolapia grahami* under control conditions. BDL, below detection limit. Data are means +1 s.e.m. (*N*=4–7). Note that the scale is logarithmic.

Rhbg and *Rhcg2* mRNAs exhibited the highest expression in gill, followed by brain and skin, and were also detected in intestine, liver and muscle (Fig. 4, note the logarithmic scale). However, both were below detectable levels in the red blood cells, so differences in tissue-specific distribution were not due to trapped blood. *Rhag* expression was high in the red blood cells but could not be assessed at other sites as it was not possible to clear the tissues of blood in these very small fish.

Expression of *Rhcg2* in the gills approximately doubled at both 4 and 12 h but had returned to 0 h control levels by 24 h of HEA (Fig. 5A). The elevation at 4 h was significant relative to both controls, whereas that at 12 h was significant only relative to the 24 h control. The overall increase during HEA was significant. *Rhbg* expression in the gills also tended to increase at all three sample times during HEA exposure, which was significant overall, but none of the individual changes were significant (Fig. 5B). *Na^{+},K^{+}-ATPase* expression more than doubled at both 4 and 24 h of HEA exposure, increases that were significant relative to both controls and overall (Fig. 5C). Branchial expression of *mtUT* also increased significantly overall during HEA; the increase at 4 h was significant

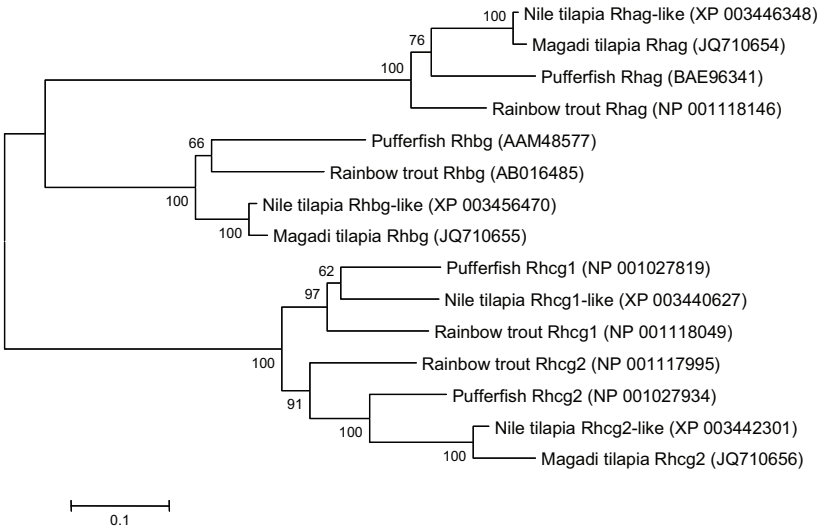

Fig. 3. The evolutionary history of non-erythroid Rhesus glycoprotein was inferred using the neighbour-joining method (Saitou and Nei, 1987) using sequences from Nile tilapia (*Oreochromis niloticus*), Magadi tilapia (*Alcolapia grahami*), pufferfish (*Takifugu rubripes*) and rainbow trout (*Oncorhynchus mykiss*). Accession numbers appear in parentheses. The optimal tree with the sum of branch length=2.34960892 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method (Zuckerkandl and Pauling, 1965) and are in the units of the number of amino acid substitutions per site. The rate variation among sites was modeled with a gamma distribution (shape parameter=1). The analysis involved 15 amino acid sequences. All ambiguous positions were removed for each sequence pair. There was a total of 509 positions in the final data set. Evolutionary analyses were conducted in MEGA5 (Tamura et al., 2011).

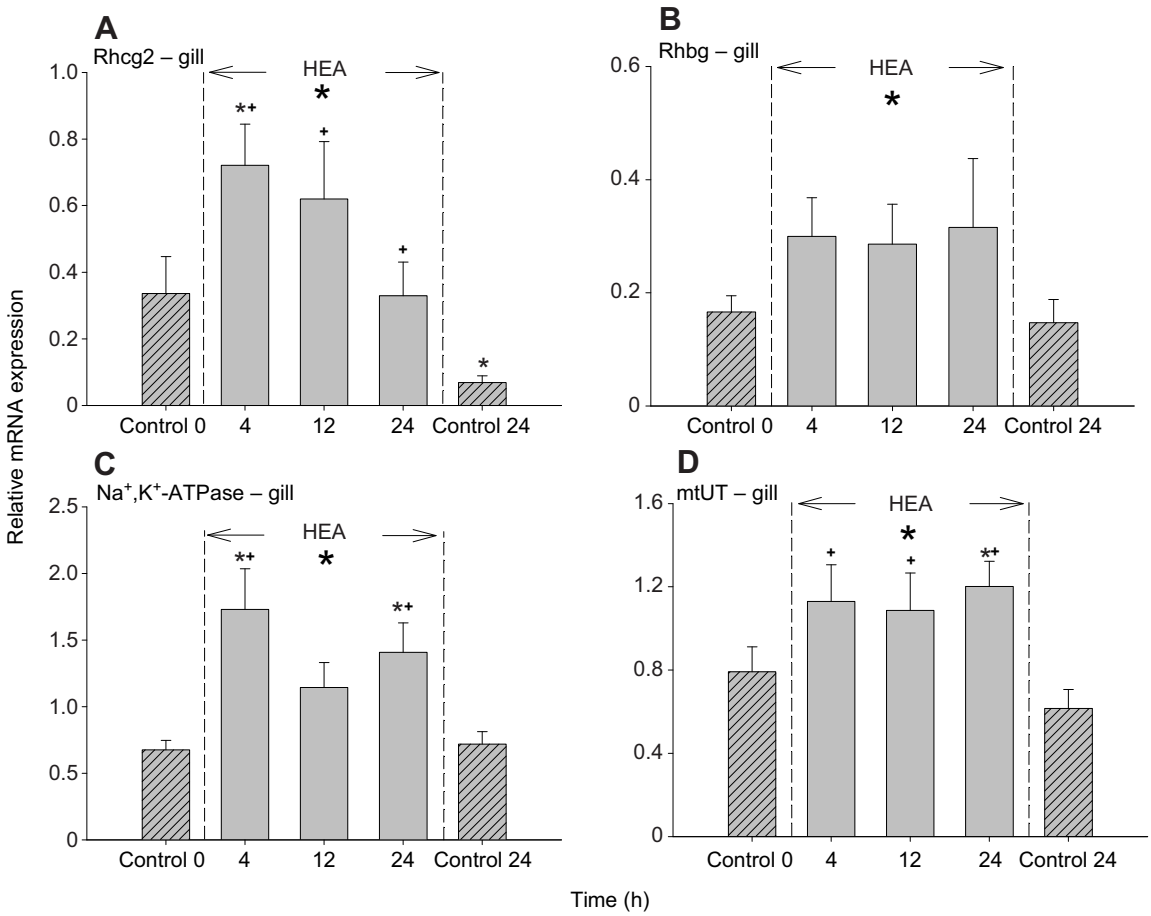

Fig. 5. Relative mRNA expression of (A) *Rhcg2*, (B) *Rhbg*, (C) *Na⁺,K⁺-ATPase* (*α1-subunit*) and (D) *mtUT* (urea transporter) in gills of *Alcolapia grahami* at 4 h (*N*=10), 12 h (*N*=10) and 24 h (*N*=9) of HEA exposure (500 μmol l^{-1}). The 0 h (*N*=9) and 24 h control values (*N*=10) are cross-hatched. Data are means ± 1 s.e.m. *Significantly different (*P*<0.05) relative to 0 h control value; $^{+}$significantly different (*P*<0.05) relative to 24 h control value. A large asterisk indicates an overall significant change (*P*<0.05) during HEA.

relative to the 24 h control, and at 24 h relative to both controls (Fig. 5D).

Expression of *glutamine synthetase* in the gill (shown in supplementary material Fig. S2A) and of *Rhag* in the red blood cells (supplementary material Fig. S2B) did not vary significantly during HEA exposure. *Rhbg* and *Rhcg2* expression in the skin were measured only on 0 h control, 24 h control and 24 h HEA samples. The only difference was a significant decrease in *Rhcg2* expression at 24 h HEA relative to both controls (supplementary material Fig. S2C).

Immunodetection of Rh proteins

Na^+,K^+-ATPase-rich cells, identified by strong whole-cell cytoplasmic immunoreactivity, were found exclusively in the filament epithelium and at the bases of the lamellae on the efferent side of the gill filament of *A. grahami* (Fig. 6A). The whole-cell signal is explained by the invasive nature of the basolateral tubular membrane system in these cells, which is rich in Na^+,K^+-ATPase. The shapes of these cells ranged from cuboidal to ovoid with the apical surface being either flat or forming a crypt (deep invagination). Rhag, Rhbg and Rhcg2-like immunoreactivities were found associated with the lamellae in the downstream (efferent) side of the filament (Fig. 6B,C,D, respectively). Specifically, Rhag and Rhcg2 staining was associated with lamellar pillar cells while Rhbg staining were found in the basal region of the lamellar epithelium (i.e. pavement cells). The HEA exposure did not markedly alter the pattern of *Rhcg2*, *Rhbg* or *Rhag* staining.

Immunoblotting corroborated the specificity of the antibodies used. Na^+,K^+-ATPase α subunit antibody α5 cross-reacted with a band of ~110 kDa, which corresponds to the predicted size range (Fig. 6E). The dominant Rh glycoproteins Rhag, Rhbg and Rhcg2-like immunoreactive bands detected by fugu antibodies were smaller than predicted although within the size range of the unglycosylated peptide (Nawata et al., 2010b). C-terminal peptide sequence alignment homologies for these fugu antibodies against the Magadi tilapia proteins were 74% (Rhag), 53% (Rhbg) and 81% (Rhcg2), respectively.

Branchial ATPase activities during HEA exposure

Na^+-ATPase and V-type H^+-ATPase activities of 0 h control, 24 h control and 24 h HEA gill samples were compared at assay pHs of 7.5 and 8.0, in the presence of either K^+ (10 mmol l^{-1}) or NH_4^+ (10 mmol l^{-1}). Despite the increase in mRNA levels of Na^+,K^+-ATPase during HEA exposure (Fig. 5C), there were no significant differences for the different sample times within each of the four assay conditions (data not shown), so the data were averaged for all sample times (Fig. 7). However, there were clear effects of assay conditions. Na^+-ATPase activity at pH 7.5 was significantly higher by approximately 60% in the presence of 10 mmol l^{-1} NH_4^+ (i.e. Na^+, NH_4^+-ATPase activity) than in the presence of 10 mmol l^{-1} K^+ (i.e. Na^+,K^+-ATPase activity); a similar though non-significant difference was seen at pH 8.0 (Fig. 7A). There were no significant effects of pH on Na^+-ATPase

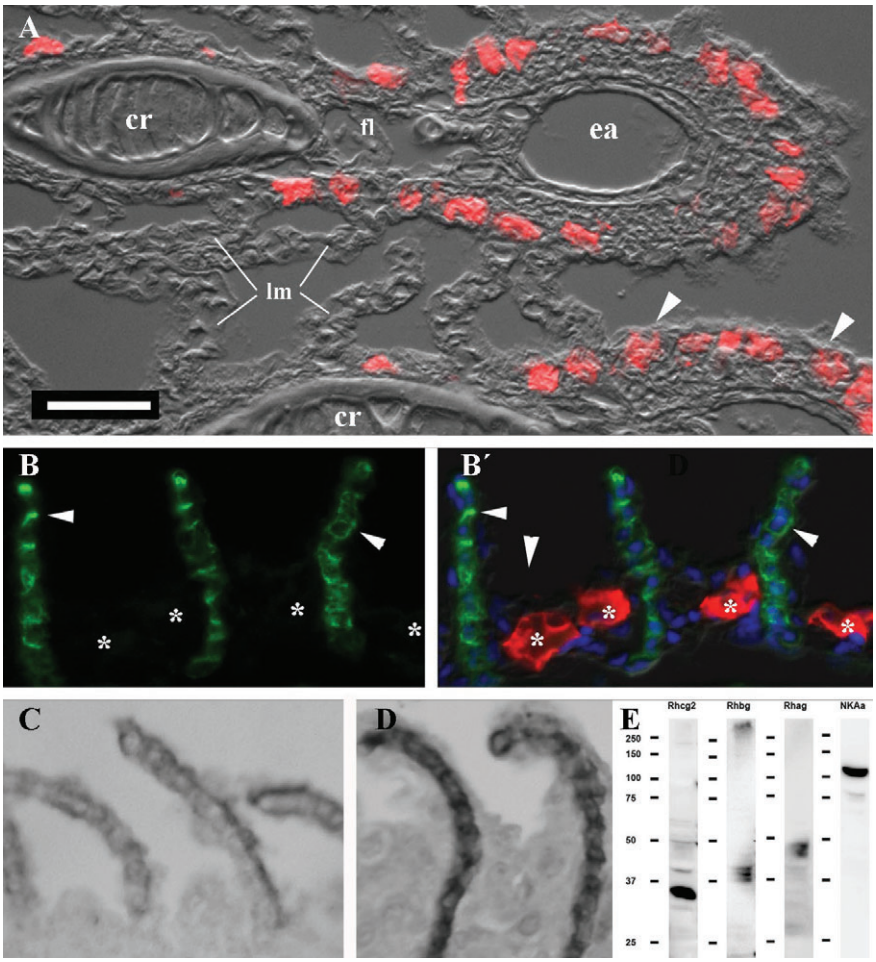

Fig. 6. Immunohistochemical localization of Rh glycoproteins (Rhag, Rhbg and Rhcg2-like) and Na^+,K^+-ATPase (NKA) in the gills of *Alcolapia grahami*. (A) Filament in cross-section showing NKA immunoreactive (IR) ionocytes (red Alexa 568) restricted to the filament epithelium. Arrowheads indicate NKA IR cells with apical crypts. The corresponding differential interference contrast (DIC) image is overlaid. Double labeling of (B) Rhag-like staining (green Alexa 488) associated with lamellar pillar cells (arrowheads) and (B′) *NKA* associated with filament ionocytes (red; indicated by asterisks). In B′ the micrograph is also merged with DAPI (blue) for nuclear staining and overlaid with the DIC image for orientation. (C) Rhbg-like staining associated with basal region of lamellar epithelium and (D) Rhcg2-like staining associated with lamellar pillar cells. Abbreviations: cr, cartilaginous rod; ea, efferent arteriole; lm, lamellae; fl, filament. Immunoreactivity localized by fluorescence (A,B) or HRP-DAB (C,D). Scale bar (applies to A–D): (A) 75 μm, (B–D) 50 μm. (E) Immunoblots of crude gill homogenates for control Magadi tilapia probed with heterologous fugu antibodies (Nakada et al., 2007) for Rhcg2, Rhbg, Rhag and Na^+,K^+-ATPase α subunit (NKAa).

activities. In contrast, increasing the pH of the assay buffer from 7.5 to 8.0 significantly decreased the activity of V-type H^+-ATPase by 23–30% regardless of the substitution of NH_4^+ for K^+, a change that had no significant effect itself (Fig. 7B).

To further explore the ability of NH_4^+ to activate Na^+-ATPase activity and its relevance under *in vivo* conditions, five pools were made from control gill tissues and tested simultaneously under a range of assay conditions, all at pH 7.5. In these pools, 10 mmol l^{-1} NH_4^+ again produced an activity approximately 60% higher than that produced by 10 mmol l^{-1} K^+ (0.811±0.097 *versus* 0.486±0.029 μmol ADP mg^{-1} protein h^{-1}). When 10 mmol l^{-1} NH_4^+ was added to 10 mmol l^{-1} K^+ (the standard concentration in the assay medium) rather than substituted for it as shown in Fig. 7A, activity again increased significantly by approximately 60% (0.776±0.118 μmol ADP mg^{-1} protein h^{-1}). When activity was tested at various K^+ concentrations (1, 3, 10, 20 mmol l^{-1}) in the absence of NH_4^+, there was no concentration dependency whatsoever (Fig. 8). However, when activity was tested over the same range of NH_4^+ concentrations (1, 3, 10, 20 mmol l^{-1}) in the absence of K^+, 3 mmol l^{-1} NH_4^+ provided significantly greater activity, and saturation occurred at 10 and 20 mmol l^{-1} K^+ (Fig. 8). These data were well described by a Michaelis–Menten relationship (r^2=0.90) with K_m=1.18±0.43 mmol l^{-1} NH_4^+ and V_{max}=1.20±0.09 μmol ADP mg^{-1} protein h^{-1}. Thus the measured increase in plasma T_{Amm} to 3–4 mmol l^{-1} (Fig. 2A) would likely have stimulated Na^+-ATPase activity *in vivo*. None of these treatments had any significant effect on V-type H^+-ATPase activity (data not shown).

DISCUSSION

Overview – responses to HEA

The Magadi tilapia expresses at least three Rh proteins (*Rhag*, *Rhcg2* and *Rhbg*) at the mRNA level in the gills, and these are seen at the protein level by immunohistochemistry and immunoblotting using fugu antibodies. The mRNA expression of two of the types (*Rhbg* – usually basolateral; *Rhcg2* – usually apical) increases during HEA. The staining pattern of the various Rh proteins appears typical, as first elucidated in pufferfish (Nakada et al., 2007), but with one exception: *Rhcg2* is associated basolaterally with lamellar pillar cells in Magadi tilapia whereas in pufferfish, it is located apically in lamellar pavement cells. The mass of bands on the immunoblots is smaller than expected, although within the range of the deglycosylated peptides, as has been demonstrated by Nawata et al. (Nawata et al., 2010b) in pufferfish. Otherwise, these responses are comparable to those seen in several ammoniotelic teleosts such as freshwater and marine rainbow trout (Nawata et al., 2007; Tsui et al., 2009; Wood and Nawata, 2011), freshwater zebrafish larvae (Braun et al., 2009), mangrove killifish at intermediate salinity (Hung et al., 2007) and marine pufferfish (Nawata et al., 2010b). This finding is remarkable in that the Magadi tilapia is 100% ureotelic. Ammonia excretion has never been seen in this fish, despite repeated attempts to create favourable conditions for it to occur, such as acute or chronic exposure to lower external pH and/or low external buffering capacity (Wood et al., 1989; Wood et al., 1994; Wood et al., 2002).

Although we were unable to clone *Rhcg1* from Magadi tilapia, we did test the fugu polyclonal Rhcg1 antibody generated by Nakada

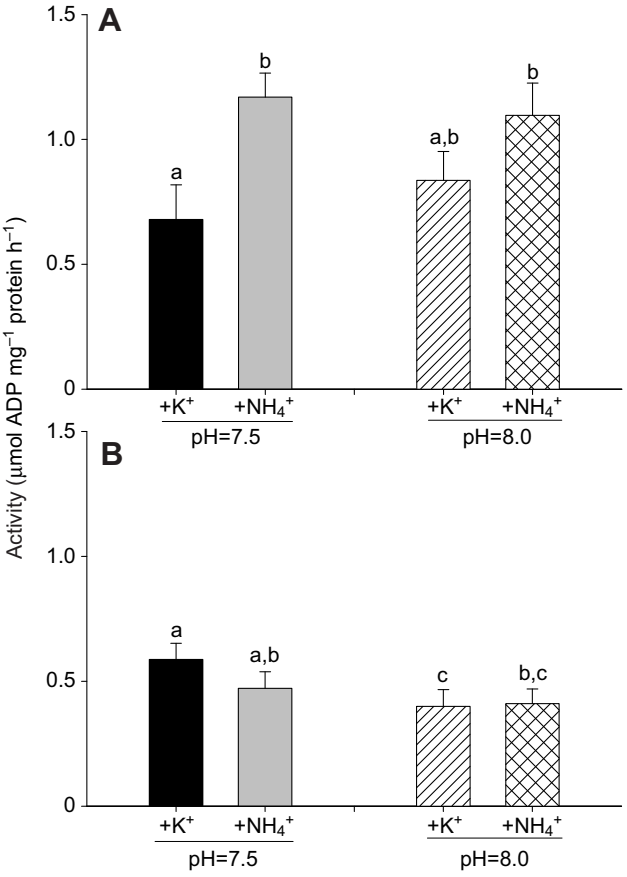

Fig. 7. Influence of assay conditions (the presence of 10 mmol l^{-1} KCl or 10 mmol l^{-1} NH_4Cl; buffer pH 7.5 or 8.0) on the activities of (A) Na^+-ATPase and (B) V-type H^+-ATPase. Data are means ± 1 s.e.m. (*N*=15–17). Data from samples taken at 0 h control, 24 h control and 24 h HEA within an assay condition were not significantly different and have been combined. Within a panel, bars sharing the same letter are not significantly different (*P*>0.05).

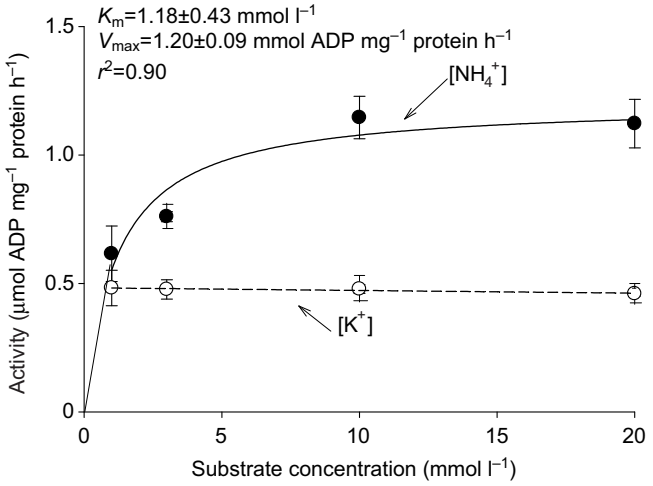

Fig. 8. A comparison of the potency of NH_4^+ (as NH_4Cl) *versus* K^+ (as KCl) in activating branchial Na^+-ATPase. Data are means ± 1 s.e.m. (*N*=5 pools of control gill tissue). *Significantly different (*P*<0.05) relative to the same concentration of K^+. Note the flat relationship for K^+, whereas the NH_4^+ data were well described by a Michaelis–Menten relationship.

et al. (Nakada et al., 2007). In contrast to the other Rh antibodies used (Rhcg2, Rhbg and Rhag), this antibody detected multiple bands on the immunoblot, all of which were larger than the predicted molecular mass range (data not shown). Nevertheless, the Rhg1 antibody did clearly label the apical crypts of some Na^+,K^+-ATPase-rich cells in the filament epithelium (data not shown), in accord with the typical distribution of Rhcg1 in other species (e.g. Nakada et al., 2007; Wright and Wood, 2009). The significance of this observation is unclear at present and more work is needed.

Additional findings that fit with previous HEA studies on some ammoniotelic teleosts include increased plasma cortisol levels [trout (Tsui et al., 2009; Wood and Nawata, 2011)] and Na^+,K^+-ATPase expression [pufferfish (Nawata et al., 2010b)]. Cortisol mobilization may be involved in both the Na^+,K^+-ATPase and the Rh responses (Dang et al., 2000; Tsui et al., 2009). The present results also confirm that HEA exposure results in a rapid increase in urea-N excretion in the Magadi tilapia (Wood et al., 1989), and illuminate this response by showing that plasma [T_{Amm}] is markedly elevated, and that the branchial urea transporter *mtUT* is activated at the transcriptional level. Presumably ammonia enters across the gills and stimulates urea production by the OUC (Wood et al., 1994; Lindley et al., 1999; Wilson et al., 2004). This fits with recent reports (McDonald et al., 2009; Rodela et al., 2011) that cortisol stimulates transcription of the gill urea transporter *tUT* in the toadfish, a facultatively ureotelic teleost. The increases in plasma glucose and lactate observed during HEA are commonly associated with cortisol mobilization in fish (De Boeck et al., 2001), and the increases in plasma [Ca^{2+}] and [Mg^{2+}] may reflect the formation of divalent cation–lactate complexes (Schaer and Bachmann, 1974). Lactic acid production could also serve to combat alkalosis during ammonia loading (Wilkie and Wood, 1991); ammonia is reported to specifically activate phosphofructokinase, a key regulatory enzyme in glycolysis (Kühn et al., 1974).

The role of Rh proteins in Magadi tilapia

Although the Magadi tilapia is an obligate ureotele, its exceptionally high rate of aerobic metabolism (Franklin et al., 1995; Narahara et al., 1996) and N-rich diet [cyanobacteria (Coe, 1966)] likely produces large amounts of ammonia internally. Indeed, both plasma and tissue T_{Amm} levels are comparable to or greater than those in ammoniotelic teleosts (see Wood et al., 1989). Furthermore, the OUC is unusual relative to other ureogenic fish, inasmuch as the key regulatory enzyme carbamoyl-phosphate synthetase III (CPSase III) has kinetic properties that favour ammonia rather than glutamine as its primary substrate (Lindley et al., 1999). Therefore, CPSase III is set up to trap metabolic ammonia directly, and funnel it immediately into urea-N production. The presence of *Rhag* in red blood cells, and of *Rhbg* and *Rhcg2* in OUC tissues such as white muscle and liver, likely facilitates the shuttling of ammonia to sites where it can be trapped by glutamine synthetase and/or detoxified through the OUC by conversion into urea-N. Microbial activity in the faeces also produces ammonia (Wood et al., 1989), which may explain the Rh expression in the intestine (Bucking and Wood, 2012). Rh expression levels are relatively high in brain, which suggests that future studies should check the brain for both OUC activity and other detoxification mechanisms such as glutamine synthesis.

But what about high levels of Rh expression in gills, which cannot be explained by the above argument? One possibility is that gill Rh proteins serve another function, the facilitation of CO_2 rather than NH_3 flux. This idea remains controversial [for background, see Nawata and Wood (Nawata and Wood, 2008), Wright and Wood

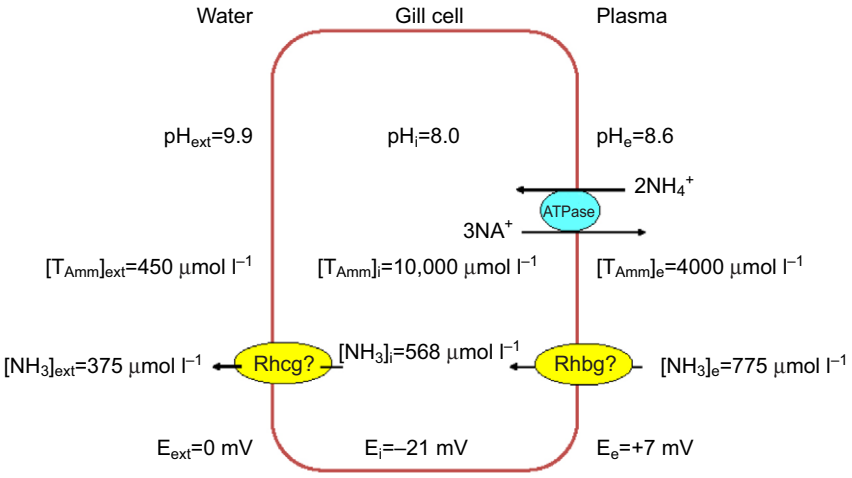

Fig. 9. A hypothetical model of how a basolateral NH_4^+-activated Na^+-ATPase, acting in concert with basolateral (Rhbg?) and apical (Rhcg?) Rh proteins, might achieve active ammonia excretion through gill cells of the Magadi tilapia during HEA exposure. [T_{Amm}], total ammonia concentration; [NH_3], non-ionized ammonia concentration; E, electrical potential; subscripts: ext, external water, i, intracellular; e, extracellular (blood plasma). See Discussion for details.

(Wright and Wood, 2009) and Weihrauch et al. (Weihrauch et al., 2009)], but some preliminary evidence in favour of this idea has recently been presented in zebrafish (Perry et al., 2010). However, because of the high external water pH, Magadi tilapia live in a 'CO_2 vacuum' (Johansen et al., 1975); it is difficult to see why there would be a need to facilitate CO_2 excretion, and why the system would respond to HEA if it is present to manage CO_2 excretion. There may well be other roles for Rh proteins. Notably, the *Rhbg* and *Rhcg2* proteins in Magadi tilapia are unusual, with only 10 transmembrane domains, rather than the 12 that are considered normal (Huang and Ye, 2010). Far simpler changes such as a mere two amino acid substitution can change a mammalian RhAG glycoprotein into a cation-selective channel (Bruce et al., 2009). In mammals, normal and mutated Rh proteins have been implicated in infertility, depression, tumour suppression, migraines and even HCO_3^- transport (Huang and Ye, 2010). The latter is worthy of future investigation, because the Magadi tilapia must actively excrete HCO_3^- across its gills (Wood et al., 2012).

Nevertheless, the facts remain that *Rhbg* and *Rhcg2* responded to HEA at the mRNA level, and HEA is a relevant environmental stressor in Lake Magadi (Wilson et al., 2004). Therefore, at present we favour the idea that the branchial Rh proteins are present in the gills to function as ammonia transporters. Under normal conditions, they might well be inactive. However, during HEA, the branchial Rh proteins would function to facilitate ammonia efflux, by working in concert with a basolateral Na^+-ATPase activated by high plasma [NH_4^+]. While NH_4^+ sensitivity has been reported in several teleosts (Mallery, 1983; Randall et al., 1999; Nawata et al., 2010b), preferential activation by NH_4^+ relative to K^+ has only been reported in one other species, another tilapia (Balm et al., 1988). Acting as an Na^+,NH_4^+-ATPase rather than as an Na^+,K^+-ATPase, this enzyme could potentially transport NH_4^+ across the basolateral membranes, building up very high intracellular concentrations of ammonia in the gill cells.

Model calculations have been performed to see whether this scenario to achieve active ammonia excretion during HEA exposure is feasible. Intracellular pH (pH$_i$) as well as extracellular pH (pH$_e$) appear to be unusually high in Magadi tilapia (Johansen et al., 1975; Wood et al., 1994; Pörtner et al., 2010). In Fig. 9, we have used estimates of pH$_e$=8.6 for blood plasma, and pH$_i$=8.0 for gill ionocytes, measurements of transepithelial potential=+7 mV (blood plasma side) relative to the external environment (Wood et al., 2012), and present data on environmental pH$_{ext}$=9.9, environmental [T_{Amm}]$_{ext}$=0.45 mmol l^{-1}, and plasma [T_{Amm}]$_e$ levels=4 mmol l^{-1} (Fig. 2A) during HEA exposure. We calculate that the NH_4^+-activated Na^+-ATPase would have to maintain a branchial intracellular [T_{Amm}]$_i$ of approximately 10 mmol l^{-1} and a membrane potential of approximately –28 mV relative to the blood plasma in order for there to be a gradient facilitating NH_3 movement through the Rh channels from blood to cell and from cell to water (Fig. 9). These values do not appear unreasonable, so the changes in gene transcription during HEA we have seen may facilitate a functional response to actively excrete ammonia against a gradient. There are several problems with this model, not the least of which is that we have no firm evidence that apical Rh proteins (presumably Rhcg) are expressed in the same epithelial gill cells as basolateral Rh proteins (presumably Rhbg). Furthermore, the source of H^+ supply to sustain apical diffusion trapping in this highly buffered environment remains problematic. More work is needed to determine whether this model is realistic, indeed whether the increased expression of Rh mRNAs has any adaptive significance, or is rather a remnant response of fossil genes and proteins that are no longer functional in this most unusual ureotelic teleost.

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AUTHOR CONTRIBUTIONS

C.M.W. designed the study, performed some of the experiments, and wrote the first draft; C.M.N. performed most of the molecular work; J.M.W. performed additional molecular work and immunohistochemical work; P.L. and C.C. performed histochemical work; H.L.B., A.B., J.N.M., O.E.J., L.F.B., G.D.K., M.B.P. and R.O.O. collected material and performed some of the experiments; R.O.O. organized the field campaign; all authors contributed to the execution and interpretation of the study, and to drafting and revising the article.

COMPETING INTERESTS

No competing interests declared.

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