The Journal of Experimental Biology 212, 3846-3856
Published by The Company of Biologists 2009
doi:10.1242/jeb.034157

The responses of zebrafish (*Danio rerio*) to high external ammonia and urea transporter inhibition: nitrogen excretion and expression of rhesus glycoproteins and urea transporter proteins

Marvin H. Braun[1,*], Shelby L. Steele[2] and Steve F. Perry[2]

[1]Hotchkiss Brain Institute, University of Calgary, 3330 Hospital Drive NW, Calgary, Alberta, Canada T2N 4N1 and
[2]University of Ottawa, 30 Marie Curie, Ottawa, ON, Canada K1N 6N5

*Author for correspondence (mhbraun@ucalgary.ca)

Accepted 18 August 2009

SUMMARY

While adult zebrafish, *Danio rerio*, possess ammonia and urea transporters (Rh and UT proteins, respectively) in a number of tissues, they are most heavily concentrated within the gills. UT has a diffuse expression pattern within Na^+–K^+-ATPase (NKA)-type mitochondrion-rich cells and Rh proteins form a network similar to the arrangement seen in pufferfish gills (Nakada et al., 2007b). Rhag expression appeared to be limited to the pillar cells lining the blood spaces of the lamellae while Rhbg was localized to the outer layer of both the lamellae and the filament, upon the pavement cells. Exposure to high external ammonia (HEA) or phloretin increased tissue levels of ammonia and urea, respectively, in adult and juvenile zebrafish; however, the responses to these stressors were age dependent. HEA increased mRNA levels for a number of Rh proteins in embryos and larvae but did not elicit similar effects in adult gills, which appear to compensate for the unfavourable ammonia excretory gradient by increasing expression of V-type H^+-ATPase. Phloretin exposure increased UT mRNA levels in embryos and larvae but was without effect in adult gill tissue. Surprisingly, in both adults and juveniles, HEA increased the mRNA expression of UT and phloretin increased the mRNA expression of Rh proteins. These results imply that, in zebrafish, there may be a tighter link between ammonia and urea excretion than is thought to occur in most teleosts.

Key words: Rh glycoproteins, urea transporter, ammonia transport, gills, gene expression.

INTRODUCTION

How animals excrete the toxic products of amino acid catabolism (specifically ammonia and urea) has been a topic of study for nearly a century (Smith, 1929). While it was originally thought that ammonia and urea moved passively through tissues along partial pressure or concentration gradients, it is now known that they require transporters [Rh proteins for ammonia (Marini et al., 1997), UT proteins for urea (Levine et al., 1973; You et al., 1993)] to efficiently cross plasma membranes. While much of the original work was done on mammalian models and cell lines, in recent years there has also been a great deal of interest regarding the functional arrangement of these transporters in teleost fish (Braun et al., 2009; Hung et al., 2008; Hung et al., 2007; Nakada et al., 2007a; Nakada et al., 2007b; Nawata et al., 2007; Shih et al., 2008; Tsui et al., 2009).

While most adult teleosts are ammonotelic (producing ammonia as the dominant nitrogenous excretory product), urea excretion plays a vital role during development, as the young of several fish species exhibit ureotely (Barimo et al., 2004; Chadwick and Wright, 1999; Essex-Fraser et al., 2005; Steele et al., 2001; Wright et al., 1995). However, urea excretion also appears to be important in adults because UT proteins are present not only in the ureotelic toadfish (*Opsanus beta*) (Walsh et al., 2000) and Lake Magadi tilapia (*Alcolapia grahami*) (Walsh et al., 2001) but also in ammonotelic teleosts such as the rainbow trout (*Oncorhynchus mykiss*) (Pilley and Wright, 2000), Japanese eel (*Anguilla japonica*) (Mistry et al., 2001) and zebrafish (Braun et al., 2009). Recent data from toadfish suggest that within the gill, UT mRNA is only expressed by mitochondrion-rich cells (McDonald et al., 2009), a finding which supports the work on seawater-adapted Japanese eels demonstrating UT protein expression on the basolateral surface of chloride cells (Mistry et al., 2001).

Information regarding the location and function of Rh proteins in teleosts began with the discovery that pufferfish (*Takifugu rugripes*) possess a number of ammonia-transporting Rh proteins (Nakada et al., 2007b). Within the gills, specific members of the Rh family are expressed in discrete cell layers, as ammonia moving from the blood to the water passes from Rhag to Rhbg to Rhcg1/Rhcg2, a pattern similar to that within the mammalian kidney (Eladari et al., 2002; Quentin et al., 2003; Verlander et al., 2003). However, while recent publications have revealed the existence of one or more Rh proteins in rainbow trout (Hung et al., 2008; Nawata et al., 2007; Tsui et al., 2009), mangrove killifish *Krytobelias marmoratus* (Hung et al., 2007) and zebrafish (Braun et al., 2009; Nakada et al., 2007a; Shih et al., 2008), our knowledge of the specific expression pattern of Rh proteins in adult gills remains limited to the initial work on pufferfish.

Excretory stress in fish [high external ammonia (HEA) to limit ammonia efflux or phloretin to limit urea efflux] often results in short-term inhibition, followed by the resumption of normal nitrogenous excretion (Cameron, 1986; Claiborne and Evans, 1988; Steele et al., 2001; Wilson et al., 1994). This pattern may reflect, in part, a re-establishment or an increase of the outwardly directed gradients for ammonia and urea, but it may also be due to the ability of fish to increase transcription of Rh proteins in response to excretory stressors (Hung et al., 2007; Nawata et al., 2007; Nawata and Wood, 2008), potentially increasing excretory flux. Among the reasons why a complete understanding of this response is

confounded is the fact that different species regulate different Rh proteins in different tissues (Hung et al., 2007; Nawata et al., 2007; Nawata et al., 2008) and because there is little known regarding the effect of excretory stress on UT.

In the present study, we tested the hypothesis that part of the zebrafish response to the excretory stressors HEA and phloretin is an increased expression of Rh proteins and UT. However, embryonic and larval zebrafish excrete ammonia and urea in a fundamentally different manner from adults, due in part to a variable expression of transporter proteins with age (Braun et al., 2009). Therefore, while we hypothesized that they both possess the ability to regulate the expression of Rh and UT proteins when exposed to high levels of ammonia and urea, we predicted that the responses to both HEA and phloretin would be different between adults and juvenile fish.

MATERIALS AND METHODS

Animals

Adult zebrafish (*Danio rerio* Hamilton-Buchanan 1822) were kept in the University of Ottawa Aquatic Care Facility where they were maintained in plastic tanks (10 l) supplied with aerated, dechlorinated City of Ottawa tap water at 28°C. Fish were subjected to a constant 10 h L:14 h D photoperiod and were fed daily with No.1 crumble-Zeigler™ (Aquatic Habitats, Apopka, FL, USA).

Embryos were obtained using standard techniques for zebrafish breeding (Westerfield, 1995) and newly spawned eggs were collected from random groups of adult breeders and kept in rearing tanks at 28°C until needed. All procedures for animal use were carried out according to institutional guidelines and in accordance with those of the Canadian Council on Animal Care (CCAC).

Antibody production

Affinity purified polyclonal rabbit antibodies against zebrafish Rhbg (accession no. NM_200071.2) and UT (accession no. AY788989.1) were generated by 21st Century Biochemicals (Marlboro, MA, USA). The peptide sequences used as antigens were:

UT: Ac-TDEKKQQGLEKINSGQRFKANLC-amide (amino acid nos 48–69)

Rhbg: CLNEVSTQNEVEKLNS-OH (amino acid nos 445–459).

Immunohistochemistry

Adult zebrafish were killed with a blow to the head and gills were dissected out and incubated for 20 min at 4°C in a solution of 4% paraformaldehyde (prepared in PBS, pH 7.4) before being sectioned using a cryostat. The sections were rinsed again with PBS (3×5 min) and subsequently blocked with 3% bovine serum albumin (BSA) in PBST (PBS with 0.5% Triton X-100) for 3×5 min. Sections were incubated for 2 h at room temperature with PBS-diluted primary antibodies. Rhag (generously supplied by S. Hirose, Tokyo Institute of Technology), a polyclonal rabbit antibody developed against the Rhag protein of pufferfish (*Takifugu rugripes*) (Nakada et al., 2007b), was used at a concentration of 1:50, Rhbg and UT were used at a concentration of 1:40 and α5, a mouse anti-chicken antibody for Na^+–K^+-ATPase (University of Iowa, Hybridoma Bank), was used at a concentration of 1:25. Concanavalin A (Con A, 2 μg ml^{-1}) was used to highlight basal lamina (Kudo et al., 2007) on some sections.

Sections were washed (3×5 min) with PBS before incubation for 1 h with 1:400 Alexa-546 anti-rabbit and 1:200 Alexa-488 goat anti-mouse (Molecular Probes, Burlington, ON, Canada). The sections were then washed (3×5 min) in PBS before being examined with a confocal scanning system (Olympus BX50WI, Melville, NY, USA) equipped with an argon laser. Images were collected using Fluoview 2.1.39 graphics software (Fluoview, Melville, NY, USA).

Western blots

Proteins were prepared from fresh tissues by homogenization on ice in 1:5 w/v of extraction buffer containing 50 mmol l^{-1} Tris HCl, 150 mmol l^{-1} NaCl, 1% NP-40, 0.5% sodium deoxycholate, 2 mmol l^{-1} sodium fluoride, 2 mmol l^{-1} EDTA, 0.1% SDS and protease inhibitor cocktail (Roche, Laval, Quebec, Canada). The samples were incubated on ice for 10 min and briefly sonicated to break up any DNA that might have been extracted. The samples were centrifuged at 16,000 ***g*** for 20 min at 4°C, and the supernatants were stored at –20°C until use. Protein concentrations were determined using a bicinchoninic acid protein assay (Pierce, Rockford, IL, USA) with BSA as standard. Samples (100 μg) were size fractionated by reducing SDS-PAGE using 10% separating and 4% stacking polyacrylamide gels and transferred to nitrocellulose membranes (Bio-Rad, Mississanga, ON, Canada). After protein transfer, each membrane was blocked for 2 h in 5% milk powder/TBS-T (1×TBS, 0.1% Tween 20) before probing.

To test the efficacy of the new antibodies (Rhbg and UT), three lanes of a gel were loaded with extracted gill proteins and each lane was incubated for 3 h at room temperature with pre-immune serum (1:2000), one of the two primary antibodies (Rhbg, 1:2000; UT, 1:2000), or 1:2000 of preabsorbed antibody (antibodies were incubated with 10× the respective antigenic peptide overnight at 4°C). The membranes were washed (4×5 min) in PBS and incubated for 1 h at room temperature with peroxidase-conjugated secondary anti-rabbit Ig (Rhbg, 1:50,000; UT, 1:20,000). The specific bands were detected by enhanced chemiluminescence (SuperSignal West Pico Chemiluminescent Substrate, Pierce). The protein size marker used was obtained from Fermentas Life Sciences (Burlington, Ontario, Canada).

For semi-quantitative assessments of protein changes, gill proteins were extracted from adult zebrafish exposed to one of three treatment groups for 5 days (for treatment procedures see below). Western blots were performed on the proteins using antibodies for Rhag, Rhbg, Rhcg1, UT and α-tubulin (H-300, Santa Cruz Biotechnology, Santa Cruz, CA, USA) at the following concentrations: Rhag 1:500, Rhbg 1:2000, Rhcg1 1:500, UT 1:2000 and tubulin 1:1000. The same membranes were probed with all five antibodies and were stripped in between different antibodies using Re-Blot Plus (Chemicon International Inc., Millipore, Billerica, MA, USA) following the manufacturer's directions. However, the same stripping protocol does not always bring about the same results as all antibodies do not bind their antigens with the same strength. Therefore, on some blots, bands from earlier antibodies can still be seen. When this happened, the bands were not re-stripped to completely remove all traces of previous antibodies as we tried to minimize the protein loss caused by the stripping process.

The size and density of the protein bands were quantified using ImageJ software (http://rsb.info.nih.gov/ij/). To control for variations in protein loading and stripping, the sizes of the four transporter bands were normalized to the size of the tubulin band.

Phloretin and ammonia exposure

Groups of 6–8 adult zebrafish were placed in 4 l plastic containers containing regular water, water with elevated ammonia (0.5 mmol l^{-1} NH_4Cl) or water with phloretin (0.05 mmol l^{-1}). Ethanol was used as the vehicle for dissolving the phloretin; 100 μl of ethanol for 4 l of water, a concentration which had no noticeable effect on fish in pilot studies. Water flow to the containers was stopped and air was

bubbled to maintain oxygenation. Every 24 h, the containers were refreshed by starting water flow at a rate of 3 l min^{-1} for 15 min, after which the experimental concentrations of ammonia or phloretin were restored. The fish used in these experiments were fed once a day during refreshing of the water. Food was added to the tanks for 3 min after which any remaining particles were removed.

Groups of embryos (30–50) were raised in Petri dishes containing the same concentrations of ammonia and phloretin as used for the adults. While the water-refreshing protocol was the same, the larvae were not fed as the yolk sac was still present. Experimental concentrations of ammonia were selected by pilot studies to ascertain a level of ammonia that was not toxic but which provided a significant change in tissue ammonia levels. Phloretin levels were chosen based on those used previously (Pilley et al., 2000).

Ammonia and urea assays

Adult zebrafish were placed in a modified syringe holding 10 ml of aerated water. To measure nitrogen excretion rates, water samples (1 ml) were collected at 0 and 1 h and immediately frozen (–20°C) for later determination of ammonia and urea content. Embryonic zebrafish were pooled so that between 30 and 50 embryos were placed in one of six modified 3 ml syringes containing 1 ml of aerated water (an air supply was fed through the rubber stopper). Water samples (0.1 ml) were taken at 0 and 3 h, and immediately frozen (–20°C) for later determination of ammonia and urea levels. For all experiments, a seventh chamber was left empty to serve as a blank and to control for background production of ammonia and urea. Following each experiment, the animals were weighed.

Water samples were analysed for ammonia and urea levels using the colorimetric assays of Verdouw and colleagues (Verdouw et al., 1978) and Rahmatullah and Boyde (Rahmatullah and Boyde, 1980), respectively. Excretion rates (μmol N g^{-1} h^{-1}) were calculated as the difference in concentration in μmol l^{-1} multiplied by the volume of the chamber, divided by the mass of the tissue and the time period. Urea excretion rates were multiplied by 2 to account for the two nitrogen atoms removed with every molecule of urea.

For measuring whole-animal tissue levels of ammonia and urea, adults (killed by a blow to the head) and embryos or larvae [killed with an anaesthetic overdose (benzocaine)] were frozen with liquid nitrogen and stored at –80°C. While the embryos and larvae were sampled over a range of ages, adult tissue levels were measured after 5 days in either HEA or phloretin. Samples were ground to a fine powder in liquid nitrogen, deproteinized in 4 volumes of ice-cold 6% perchloric acid and centrifuged at 16,000 ***g*** for 15 min at 4°C. The supernatant was neutralized with ice-cold 2 mol l^{-1} K_2CO_3 and spun again at 16,000 ***g*** for 10 min (4°C). The final supernatant was directly analysed for ammonia levels using a tissue ammonia kit (Sigma, AA0100; St Louis, MO, USA) and for urea levels using the colorimetric assay (see above). Values were corrected for the various dilutions and expressed as μmol N g^{-1} of tissue.

Real-time PCR

For tissue distributions, adult zebrafish were killed with an overdose of benzocaine, and all tissues were dissected and flash frozen in RNase-free 1.5 ml centrifuge tubes. Gills of adults exposed to HEA or phloretin as described above were collected in the same manner. For HEA- and phloretin-exposed larvae, pooled samples of 30–50 larvae were used. Total RNA was extracted from frozen tissue samples in 1 ml of TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Tissues were homogenized by repeatedly drawing the larvae and TRIzol through a 20 gauge needle attached to a 5 ml syringe. Total RNA was re-suspended in 10 μl of nuclease-free water and quantified using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific), and stored at –80°C until use. Prior to synthesis of cDNA, 2 μg of total RNA per sample was treated with DNase I, amplification grade (Invitrogen) according to the manufacturer's protocol to minimize possible genomic DNA contamination. This RNA was used to synthesize cDNA using RevertAid^{TM} M-MuLV reverse transcriptase (Fermentas, Burlington, Ontario, Canada) using 200 ng of random hexamers according to the manufacturer's protocol. Non-reverse transcribed samples, used as controls for DNA contamination, were created by selecting random RNA samples and completing the cDNA reaction without the M-MuLV enzyme. Final cDNA products were diluted 1 in 5 for target genes and 1 in 1000 for 18S in sterile water. An undiluted aliquot of each cDNA sample was taken and pooled to create a dilution series for determination of real-time PCR efficiency by construction of a standard curve. PCR efficiency was deemed satisfactory between 85% and 115% with $R^2 \geq 0.98$.

Real-time PCR was performed on an MX3000P QPCR system using MXPro software version 4.0 (Stratagene, Cedar Creek, TX, USA). PCR reactions were carried out with 4.56 μl sterile water, 6.25 μl Brilliant SYBR Green Master Mix (Stratagene), 0.5 μl of each forward and reverse primer [primers used and efficiencies are given in Braun et al. (Braun et al., 2009); to a final concentration of 0.1 μmol l^{-1}], 0.19 μl of diluted reference dye (Stratagene) and 0.5 μl of cDNA for a final volume of 12.5 μl. Reactions were run using the MXPro software default SYBR Green program with an annealing temperature of 58°C. The program included a dissociation curve performed at the end of each PCR run to ensure the purity of the reactions. All data were analysed using the modified $\Delta\Delta Ct$ method (Pfaffl, 2001). Expression of all Rh and UT genes was normalized to the expression of 18S in each sample. For the developmental distribution, all values were expressed relative to the 1 d.p.f. (day post-fertilization) values. For the adult tissue distributions, all mRNA levels were expressed relative to those found in the gut except for Rhcg1, which is given relative to the eye.

Data presentation and statistical analysis

Data are presented as means ± 1 s.e.m. Statistical analysis was performed using Sigma Stat (version 3.0, SPSS Inc., Chicago, IL, USA). Except for real-time RT-PCR results (analysed using one-sample *t*-tests), the data were assessed for statistical significance using a one-way ANOVA followed by a Tukey *post-hoc* test for pairwise comparisons. In all cases, significance was set at $P<0.05$.

RESULTS

Tissue distribution of ammonia and urea transporters

mRNA coding for Rhag, Rhbg, Rhcg1 or UT was found predominantly in the gills of adult zebrafish (Fig. 1) with mRNA for the Rh proteins also being abundant in the kidney. Rhag was also expressed in large quantities in the heart and to a lesser extent in the muscle, eye and brain (Fig. 1A); Rhbg was also expressed in the brain and eye (Fig. 1B); Rhcg1 was also found in the brain (Fig. 1C). Conversely, UT was almost exclusively expressed in the gills, with only a relatively small amount of mRNA found in muscle, eye and heart (Fig. 1D). Because of the small size of the fish, non-perfused tissues were used, invariably resulting in contamination with red blood cells, a tissue known to express Rhag in mammals (Westhoff et al., 2002) and teleosts (Nawata and Wood, 2008). Therefore, the Rhag tissue distribution may be skewed toward highly vascular tissues.

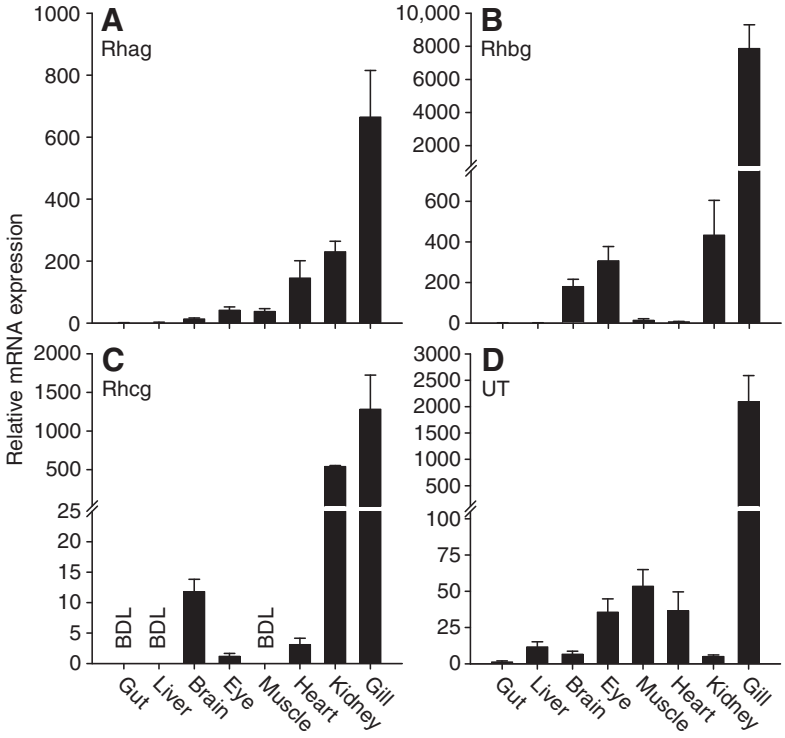

Fig. 1. Tissue distribution of (A) Rhag, (B) Rhbg, (C) Rhcg1 and (D) UT mRNA in adult zebrafish (*Danio rerio*). All tissue mRNA levels are expressed relative to the level in the gut except for Rhcg, which is expressed relative to mRNA levels in the eye. BDL, below detection limit.

Location of transporters in the gill

Western blotting of gill protein with the homologous Rhbg antibody revealed a single immunoreactive band at ~50 kDa; no immunoreactivity was detected in white muscle (Fig. 2A, inset). The immunoreactive band was not observed when the antibody was pre-absorbed with 10-fold excess antigen or when pre-immune serum was used (data not shown). Rhbg immunofluorescence was seen on both the filaments and lamellae (Fig. 2A) and higher magnification images showed that Rhbg appeared to be limited to the outer cell layer (Fig. 2Bi) and did not co-localize with Con A (Fig. 2Bii, Fig. 2Biii). Rhag immunofluorescence was restricted to the lamellae (Fig. 3A), where it had a similar staining pattern to Con A, encircling the intra-lamellar blood spaces (Fig. 3Bi–Biii). A western blot performed using the homologous UT antibody yielded a single immunoreactive band at ~47 kDa in gill and muscle (Fig. 4A, inset). As with the Rhbg antibody, the immunoreactive band was not observed when the antibody was pre-absorbed with 10-fold excess antigen or when pre-immune serum was used (data not shown). UT was observed on specific cells at the base of the lamellae where it was co-localized with Na^+–K^+-ATPase (NKA; Fig. 4Ai, arrowheads). Analysis of sequential optical sections from the apical to the basal surface of an immunoreactive cell demonstrated diffuse UT staining throughout this mitochondrion-rich cell (Fig. 4B–P).

Nitrogenous excretion in embryos and larvae

Inhibition of urea excretion using phloretin resulted in a significant increase in the rate of ammonia excretion at 0 and 1 d.p.f. (Fig. 5A, inset). However, at 2 and 4 d.p.f., chronic phloretin exposure produced the opposite effect, causing a significant fall in the ammonia excretion rate. As expected, chronic phloretin exposure caused a significant decrease in the rate of urea excretion in larvae older than 0 d.p.f. (Fig. 5B).

When exposed to chronically elevated ammonia levels, ammonia excretion rates were significantly decreased at 4 and 5 d.p.f. (Fig. 5A). Chronic exposure to HEA had no effect on the urea excretion rate for any juveniles younger than 5 d.p.f. (Fig. 5B). Larvae at 5 d.p.f. showed a large increase in urea excretion during HEA exposure, with the rate of urea excretion nearly tripling.

Nitrogenous excretion in adults

Phloretin caused a significant decrease in ammonia excretion during the first 2 days of exposure (Fig. 6A); urea excretion only fell significantly on days 1, 4 and 5 of exposure (Fig. 6B). HEA significantly decreased ammonia excretion during days 2–5 of exposure, with ammonia excretion appearing to be completely disrupted after 4 days of exposure as influx of ammonia into the fish was greater than efflux (Fig. 6A). After 2 days of exposure, HEA exposure caused a significant increase in urea excretion (Fig. 6B), but this elevated level of excretion returned to control levels for the following 3 days of exposure.

Tissue concentrations of ammonia and urea

In larval zebrafish, phloretin exposure caused tissue ammonia levels to increase; however, this trend was only statistically significant at 3 d.p.f. (Fig. 7A). Adults exposed to chronic phloretin for 5 days also showed a significant increase in tissue ammonia. Tissue ammonia concentrations significantly increased in larvae (2–5 d.p.f.) and adults (Fig. 7A) during exposure to HEA.

Phloretin treatment resulted in a large rise in tissue urea levels at all ages except 3 d.p.f. (Fig. 7B). However, the lack of significance at 3 d.p.f. appeared to be due to the unusually high levels of urea in the control larvae rather than a deviation in the trend towards increased tissue urea levels with phloretin exposure. Regardless of age, HEA did not raise tissue urea levels in any of the exposed zebrafish; conversely, HEA appeared to result in a significantly decreased total tissue urea concentration at 3 d.p.f.; however, this significant result also appears to be due to the unusually high control values at 3 d.p.f. (Fig. 7B).

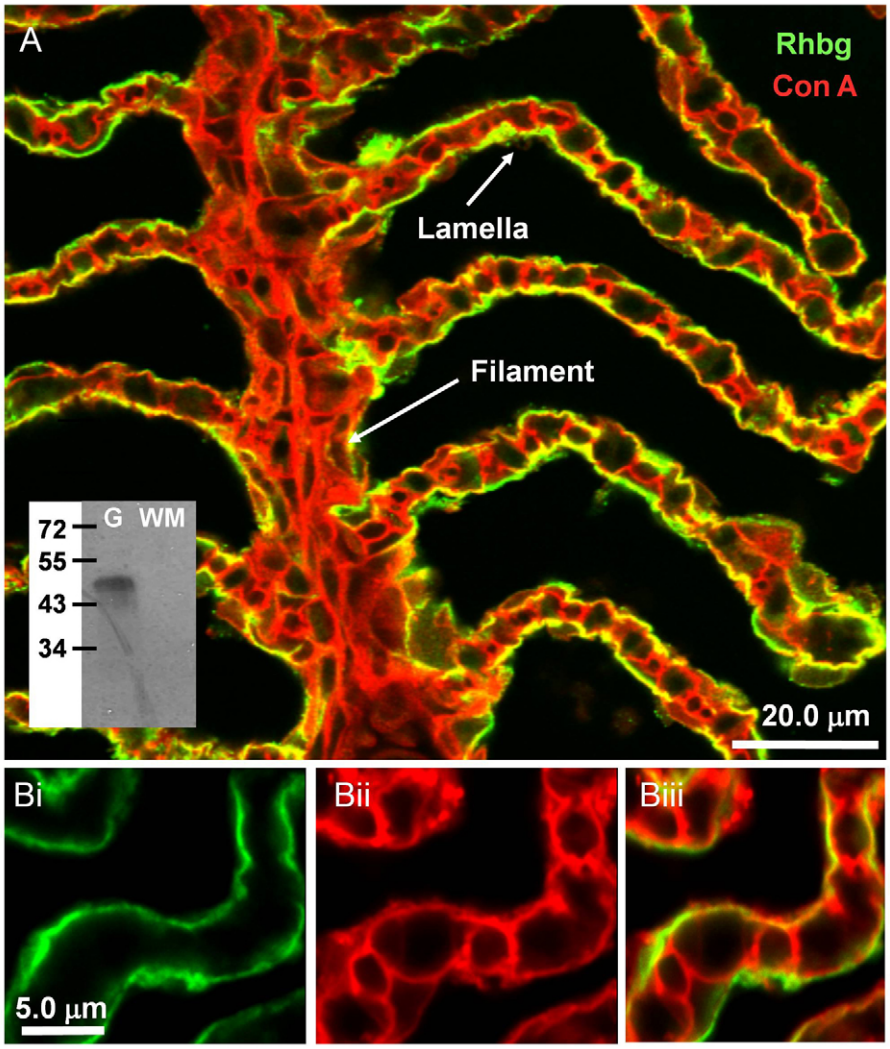

Fig. 2. Immunolocalization of Rhbg in zebrafish gill. (A) Low magnification micrograph showing the expression pattern of Rhbg (green) on gill filaments and lamellae; basal lamina were stained with concanavalin A (Con A, red). The inset is a representative western blot illustrating the specificity of the Rhbg antibody against gill (G) but not white muscle (WM) proteins separated by SDS-PAGE. Size markers are in kDa. (B) Higher magnification images of a single lamella showing the separate distributions of Rhbg (Bi) and Con A (Bii), and a merged image (Biii).

Real-time PCR

The effects of chronic phloretin exposure on the expression of Rh and UT mRNA were variable (Fig. 8A). At 1 d.p.f., only the expression of Rhag was significantly increased; at 2 d.p.f., only UT was significantly increased while at 3 d.p.f., Rhag, Rhcg1 and UT exhibited increased expression. In adults, Rhag, Rhbg and V-type H^+-ATPase mRNA levels were significantly increased during chronic phloretin exposure.

HEA was without effect on the expression patterns of 1 d.p.f. embryos; however, 2 d.p.f. embryos possessed significantly elevated mRNA levels of Rhbg, UT and V-ATPase, while 3 d.p.f. embryos exhibited increased levels of Rhag, Rhcg1, UT and V-type H^+-ATPase (Fig. 8B). In adults, only V-type H^+-ATPase mRNA expression was significantly increased during exposure to HEA.

Semi-quantitative protein measurements in adults

Five days of chronic exposure to either HEA or phloretin resulted in only a single significant change in protein levels; fish exposed to phloretin showed a significant increase in Rhcg1 levels in their gills (Fig. 9).

DISCUSSION

Location of Rh and UT proteins within the adult gill

Rhcg1 is localized to the H^+-ATPase-rich cells of the zebrafish gill (Nakada et al., 2007a), mitochondrion-rich cells akin to the chloride cells where Rhcg1 is found in the pufferfish (Nakada et al., 2007b). In this study, we have found that the locations of Rhbg and Rhag in zebrafish also mirror their locations in the pufferfish gill. Rhbg appeared to be located along the outer surface of both the filament and the lamellae, implying that it is located upon or within the pavement cells (Fig. 2). Rhag, however, was found surrounding the lamellar blood spaces and thus, as in pufferfish (Nakada et al., 2007b), may be expressed on the pillar cells (Fig. 3). The diffuse expression pattern of Rh proteins in zebrafish larvae becomes increasingly centred on the gills as development progresses (Braun et al., 2009), and the current study reveals that the end result of this process is similar to the situation in pufferfish whereby a network of Rh proteins forms within the adult gill which presumably allows ammonia movement across the various cell layers.

In both zebrafish (Fig. 4) and eel (Mistry et al., 2001) gills, diffuse UT expression occurs in mitochondrion-rich cells (NKA cells in zebrafish *versus* chloride cells in eel). Mistry and colleagues (Mistry et al., 2001) attributed the diffuse staining pattern to the localization of UT to basolateral membranes which, in chloride cells, are infolded and extend throughout the cytoplasm (Kessel and Beams, 1962; Laurent and Dunel, 1980; Perry, 1997). While a basolateral membrane localization for UT in zebrafish is also a possibility, we believe that immunodetection using high magnification transmission electron micrographs is required to determine whether UT is truly

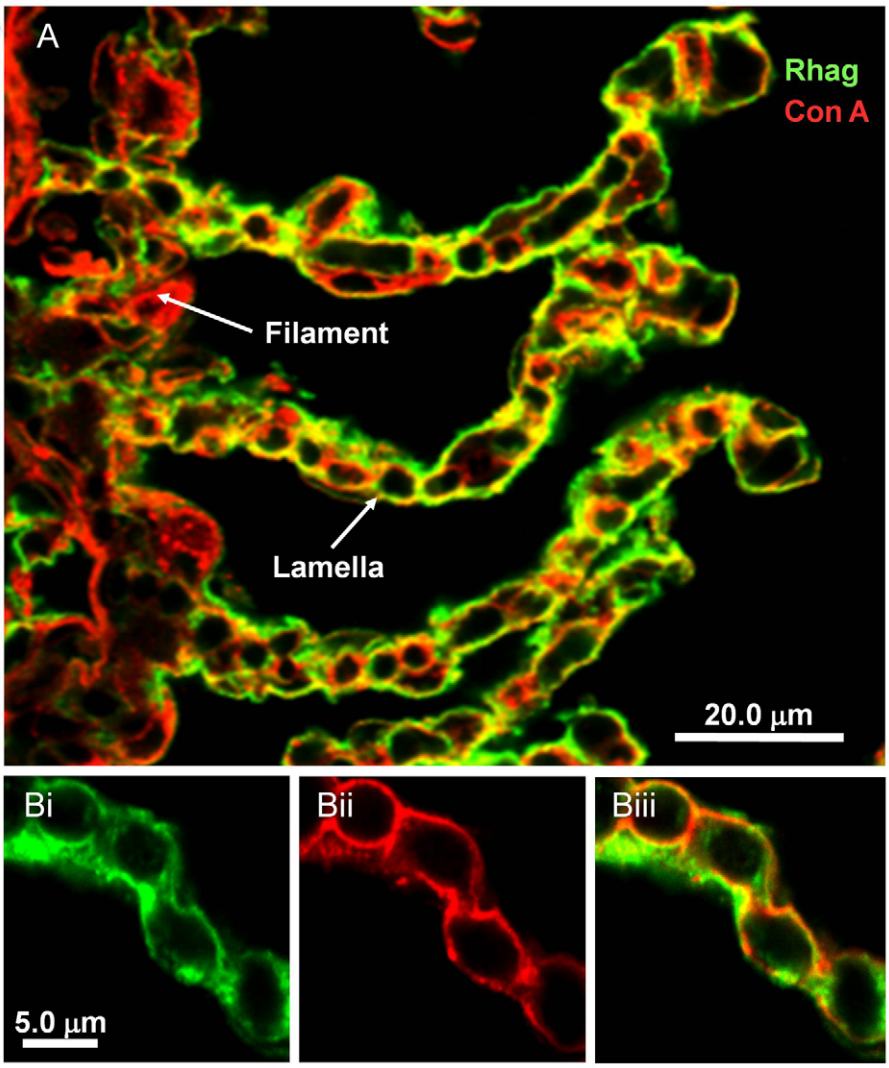

Fig. 3. Immunolocalization of Rhag in zebrafish gill. (A) Low magnification micrograph showing the expression pattern of Rhag (green) on gill filaments and lamellae; basal lamina were stained with Con A (red). (B) Higher magnification images of a single lamella showing the separate distributions of Rhag (Bi) and Con A (Bii), and a merged image (Biii).

confined to basolateral membranes or alternatively distributed within vesicles throughout the cytoplasm.

Tissue expression of Rh and UT proteins

The lipid phase of plasma membranes is not as permeable to ammonia as was previously thought (Hill et al., 2004; Kelly and Wood, 2001), and it is now accepted that Rh proteins are necessary to move ammonia throughout, as well as out of, the body. However, the particular Rh proteins used by fish and where they occur appear to be highly variable. For example, while the tissue distribution of Rhbg is broad in rainbow trout (Nawata et al., 2007), basal expression in killifish (Hung et al., 2007) and pufferfish (Nakada et al., 2007b) appears to be limited to the gills and skin. Furthermore, Nakada and colleagues (Nakada et al., 2007a) suggest that Rhbg expression in zebrafish is specific to the gills, while we detected Rhbg mRNA in eight tissues (Fig. 1B). Similarly, while we detected Rhcg1 mRNA in five different tissues (Fig. 1C), other studies report a much narrower tissue distribution, with the gill being the predominant site of expression in rainbow trout (Nawata et al., 2007), pufferfish (Nakada et al., 2007b), zebrafish (Nakada et al., 2007a) and the mangrove killifish (Hung et al., 2007). While the inconsistencies may reflect differences in the sensitivities of the PCR performed in the various studies, they may also be a consequence of differing environmental conditions. Excretory stress appears to induce diversification of Rh expression as mangrove killifish (*Kryptolebias marmoratus*) exposed to HEA express Rhbg in seven additional tissues (Hung et al., 2007), while in both rainbow trout (Nawata et al., 2007) and mangrove killifish (Hung et al., 2007) the number of tissues expressing Rhcg1 increases during HEA exposure. Development may also play a role as Rhcg1 is highly expressed in the adult zebrafish kidney (Fig. 1C) but not in the larval kidney (Nakada et al., 2007a). Similarly, during the development of zebrafish, overall Rh protein expression changes from a predominantly epidermal pattern to become localized around the gills (Braun et al., 2009). These data suggest that the tissues expressing Rh proteins might change as a result of stress, development and growth, resulting in the variability seen both within and between species.

Development also appears to play a large role in the expression of UT as it is expressed in a broader range of embryonic and larval tissues (Braun et al., 2009) than in adults (Fig. 1D). The fact that UT was largely localized to the gills in adult zebrafish (Fig. 1D) suggests that additional isoforms may be required to move urea between different tissues, a hypothesis supported by the expression pattern of eels, which possess both a gill and a kidney isoform of UT (Mistry et al., 2001; Mistry et al., 2005).

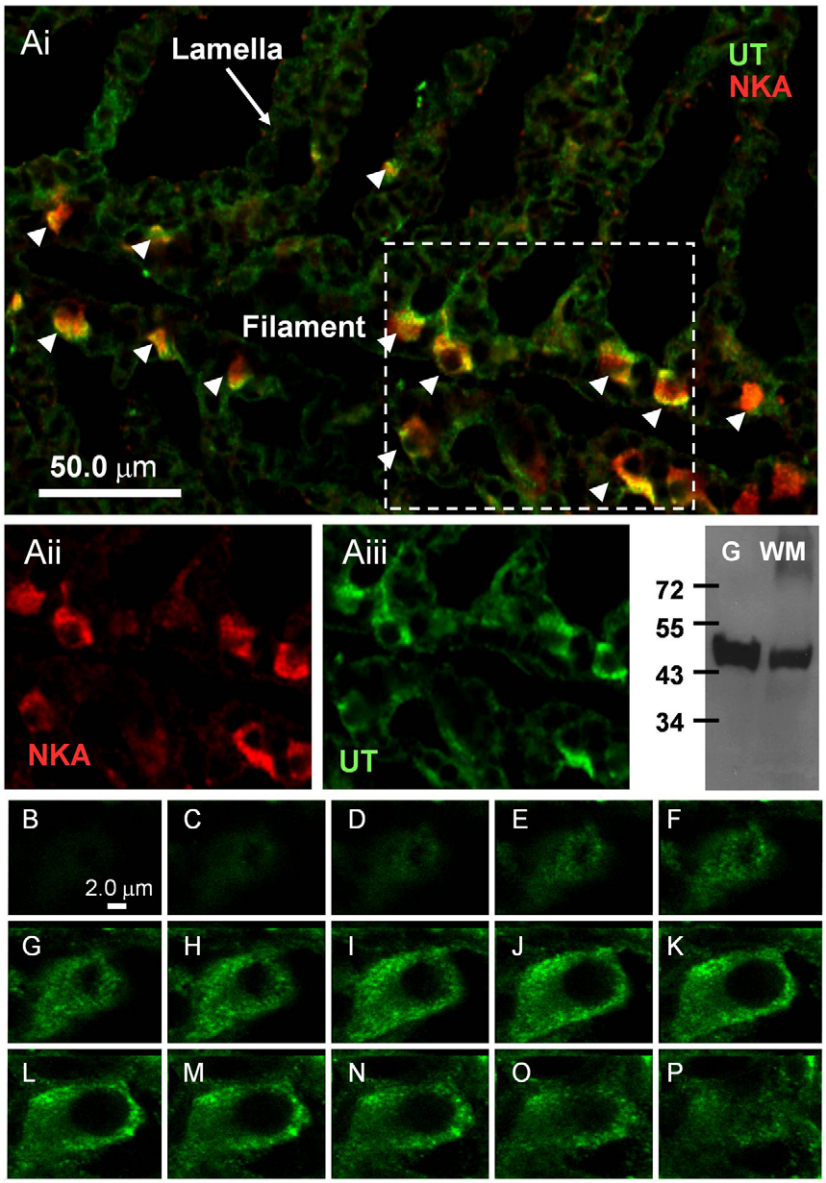

Fig. 4. Immunolocalization of UT and Na^+–K^+-ATPase (NKA) in zebrafish gill. (Ai) UT (green) was co-localized with NKA (red) in mitochondrion-rich cells (arrowheads). The area within the dashed box was used in Aii and Aiii to show the separate distributions of NKA (Aii) and UT (Aiii). The inset is a representative western blot illustrating the specificity of the UT antibody against gill (G) and white muscle (WM) proteins separated by SDS-PAGE. Size markers are in kDa. (B–P) Sequential optical slices through a mitochondrion-rich cell from apical to basal poles reveal the diffuse nature of UT expression (green).

High external ammonia (HEA)

Efficient ammonia excretion in teleosts relies on the movement of both NH_3 and H^+ out of the body, NH_3 *via* the Rh proteins (Braun et al., 2009; Shih et al., 2008) and H^+ *via* the V-type H^+-ATPase (Nawata et al., 2007; Shih et al., 2008) or from the hydration of carbon dioxide. Through the process of 'acid trapping' (Wright et al., 1986), the secreted H^+ combines with NH_3 to create NH_4^+ and maintain a gradient for NH_3 diffusion. Therefore, it may be possible to increase excretion by increasing flow through the two pathways, even against an unfavourable gradient. In fact, a proton gradient established by proton pumps is used by bacteria and fungi to sequester high concentrations of ammonia against a gradient within acid vacuoles (Soupene et al., 2001).

In rainbow trout gills, Rhcg2 and the V-type H^+-ATPase appear to act together as an ammonium pump (Tsui et al., 2009), while in zebrafish, Rhcg1 is found on V-type H^+-ATPase-rich cells (Shih et al., 2008), which have the capacity to increase proton pumping for ionoregulatory functions (Horng et al., 2007). Therefore, any increase in NH_3 flux through Rhcg1 could be matched by an increase in the activity of the V-type H^+-ATPase. Conversely, in larval zebrafish, knockdown of either the Rh proteins (Braun et al., 2009) or the V-type H^+-ATPase (Shih et al., 2008) significantly reduces ammonia excretion.

Under normal conditions, ammonia excretion may be restricted in young zebrafish owing to underdeveloped gills (Kimmel et al., 1995; Rombough, 2002) and the limited expression of Rh proteins, which peaks between 3 and 4 d.p.f. (Braun et al., 2009). However, despite a potentially restricted excretory capacity, HEA did not impede ammonia excretion in zebrafish younger than 4 d.p.f. (Fig. 5A). Transcription of V-type H^+-ATPase and Rh proteins was significantly induced during this period, with Rhag and Rhcg1 mRNA levels increasing 4- and 8-fold, respectively (Fig. 8B). Thus, the increased flux of NH_3 and H^+ through these proteins may account for ammonia excretion being unchanged. After 4 d.p.f., however, ammonia excretion was significantly inhibited by HEA, while tissue concentrations of ammonia were more than double the control values (Fig. 7A).

Despite the absence of any increase in tissue urea levels (Fig. 7B), HEA resulted in increased urea excretion rates at 5 d.p.f. (Fig. 5B), implying that some of the excess ammonia was being metabolized

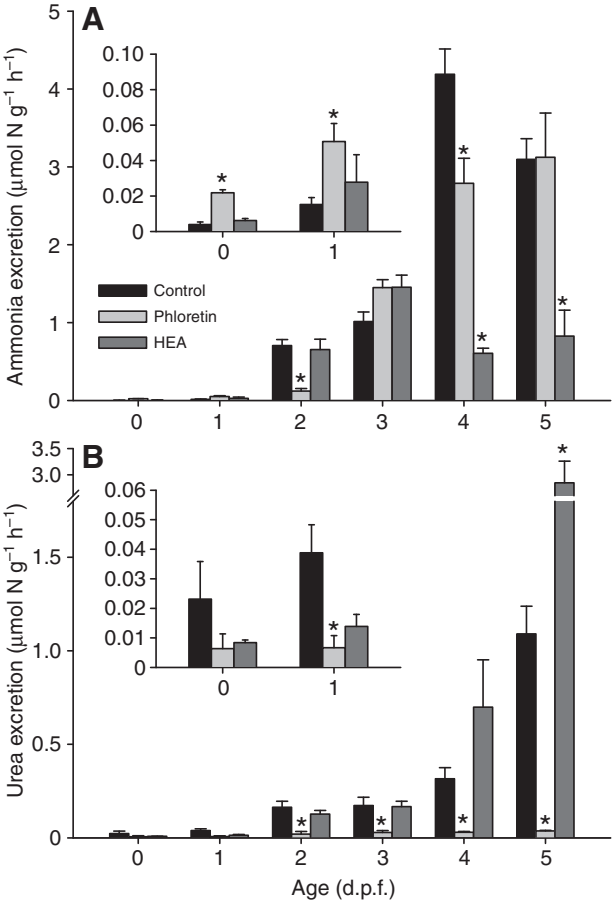

Fig. 5. Excretion rates of (A) ammonia or (B) urea in zebrafish during the first 5 days post-fertilization (d.p.f.) upon exposure to high external ammonia (HEA, 0.5 mmol l^{-1} NH_4Cl) or phloretin (0.05 mmol l^{-1}). The inset in each panel is an expanded view of 0 and 1 d.p.f. *N*=6; values are +1 s.e.m.; *significant difference from the control value at each time point.

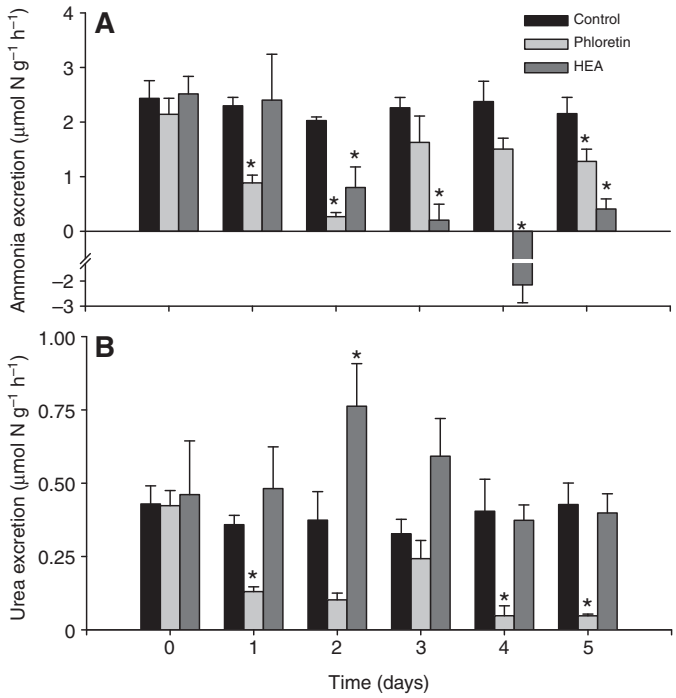

Fig. 6. Excretion rates of (A) ammonia or (B) urea in adult zebrafish during 5 days exposure to either HEA (0.5 mmol l^{-1} NH_4Cl) or phloretin (0.05 mmol l^{-1}). *N*=6; values are +1 s.e.m.; *significant difference from the control value at each time point.

into urea, possibly *via* the ornithine urea cycle (OUC). An active OUC would result in a markedly increased production of urea if tissue ammonia increased, which may have triggered the increased expression of UT (Fig. 8B). The increased excretion of urea may prevent the fish from accumulating excessive nitrogenous waste.

Like the larval zebrafish (Fig. 8B), adult mangrove killifish respond to HEA with a widespread and large (>7-fold) increase in Rh expression (Hung et al., 2007). However, adult zebrafish increased neither the mRNA nor the protein levels of the Rh proteins during HEA. Instead, V-type H^+-ATPase transcription increased, suggesting that the adults either were making use of an increased pH gradient to remove excess ammonia *via* acid trapping or were unable to increase Rh transcription. The latter situation may be the case as HEA significantly decreased ammonia excretion in adults, to the point where it was completely inhibited after 4 days of exposure. At 2 days of exposure, HEA also significantly increased urea excretion (Fig. 6B), even though adult zebrafish, like most teleosts, are unlikely to possess a functional OUC. However, extremely high levels of internal ammonia may result in an increased production of urea synthesized *de novo* through CPSase III when pathways of ammonia excretion are blocked by HEA (Mistry et al., 2001).

Phloretin

While phloretin effectively decreased urea flux, it also had the unexpected effect of decreasing ammonia excretion in both adults (after 1, 2 and 5 days exposure) and juveniles (at 2 and 4 d.p.f.), possibly a stress-induced effect related to the high tissue urea levels. Phloretin had an additional puzzling effect on embryos; it caused an increase in ammonia excretion at 0 and 1 d.p.f. (Fig. 5A) and increased tissue ammonia levels (Fig. 7A). While ammonia can be converted into urea *via* the enzymes of the OUC, converting tissue urea into ammonia excretion is not trivial. Presumably, as tissue urea levels increase, the biochemical pathways for urea production are inhibited, resulting in the shunting of various precursors and intermediate species into alternative ammonia-producing pathways; however, the specifics are unclear.

Transcription of UT significantly increased at 2 and 3 d.p.f. in fish exhibiting phloretin-mediated inhibition of urea excretion, seemingly in response to increased tissue urea levels. In adults, however, rather than increasing UT mRNA levels, 5 days of phloretin exposure caused a large increase in the levels of V-type H^+-ATPase, Rhag and Rhbg mRNA and a significant increase in Rhcg1 protein expression (Fig. 9). The different results obtained for mRNA and protein are likely to reflect differences in the time courses of transcription and translation; thus, the increases in Rhag and Rhbg mRNA levels had not yet been manifested in protein changes. However, an increase in the amount of Rhcg1 mRNA during compromised urea excretion was previously seen in zebrafish embryos experiencing diminished urea excretion following UT knockdown (Braun et al., 2009). Whether the Rhcg1 increase is in response to increased tissue urea levels or to a subsequent increase in tissue ammonia levels is unknown; however, Rhcg has also been

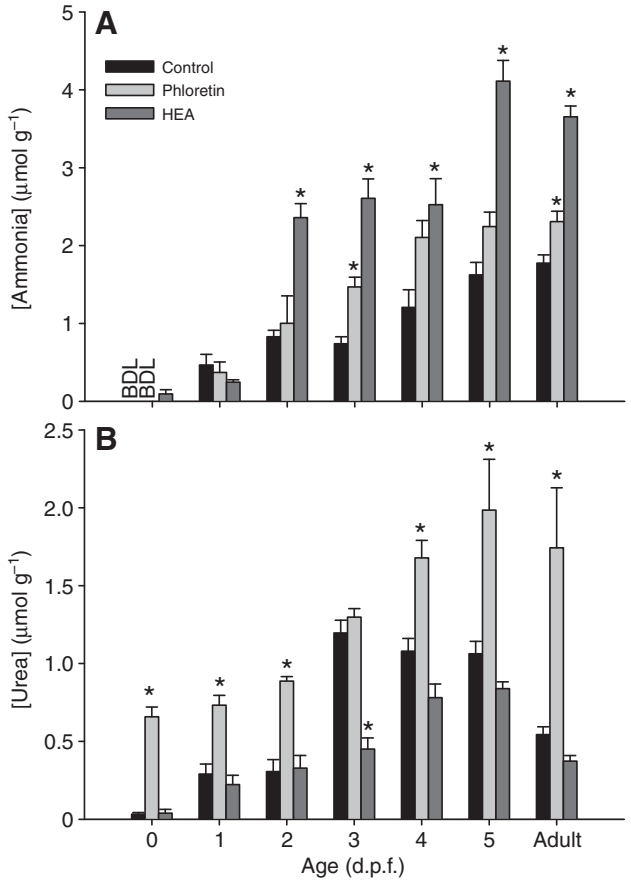

Fig. 7. Whole-body concentrations of (A) ammonia or (B) urea in zebrafish embryos and larvae (0–5 d.p.f.) and adults exposed for 5 days to HEA (0.5 mmol l^{-1} NH_4Cl) or phloretin (0.05 mmol l^{-1}). *N*=6; values are +1 s.e.m.; *significant difference from the control value at each time point. BDL, below detection limit.

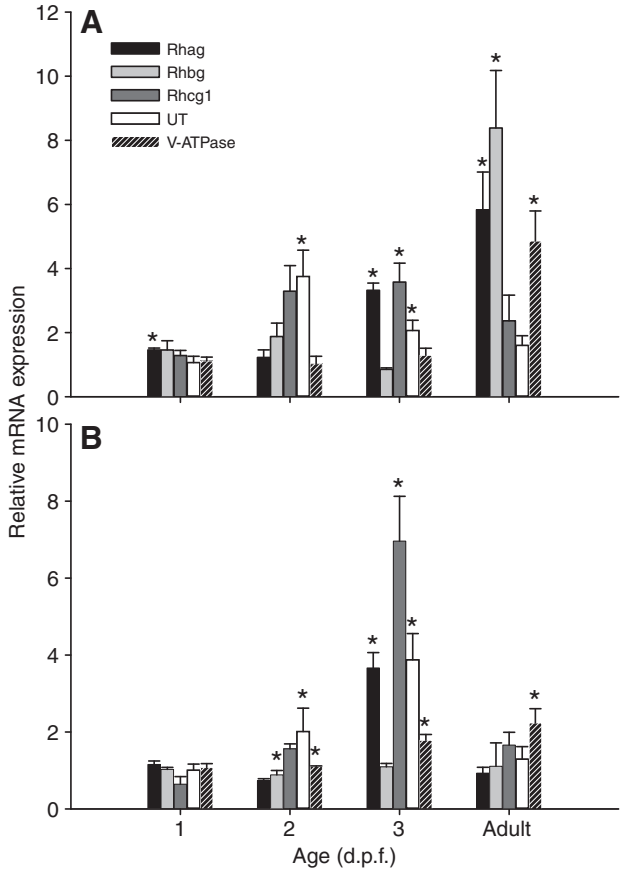

Fig. 8. mRNA expression levels of Rhag, Rhbg, Rhcg1, UT and V-type H^+-ATPase in whole-body larvae and adult gills. mRNA levels are relative to the expression of each respective gene in control fish at the same time point. (A) mRNA levels in fish exposed to 0.05 mmol l^{-1} phloretin; adults were exposed for 5 days. (B) mRNA levels in fish exposed to 0.5 mmol l^{-1} NH_4Cl; adults were exposed for 5 days. All values are presented as +1 s.e.m.; *significant difference from the control value at each time point.

implicated in the excretory stress response in other fish, as killifish increase the levels of Rhcg1 mRNA during periods of air exposure or HEA (Hung et al., 2007).

Interestingly, the increased transcription of Rh proteins in adults was 6- to 8-fold higher in phloretin-treated fish than the changes which occurred during HEA (Fig. 8A,B), implying that high urea levels are more effective than high ammonia levels at inducing ammonia transporters. However, phloretin also caused a significant increase in tissue ammonia levels and thus it may be that the combination of significantly elevated tissue ammonia and urea is what induced expression of Rh proteins.

Adult *versus* juvenile responses to HEA

The results of the present study suggest that adult and larval zebrafish exploit fundamentally different mechanisms for dealing with excretory stress. During exposure to HEA, embryos and larvae appear to favour a large increase in the expression of Rh proteins while adult zebrafish respond by increasing the expression of V-type H^+-ATPase (Fig. 8B). Therefore, the rate of ammonia movement through the adult gill appears to be set by proton pumping rather than by NH_3 flux through Rh proteins. Furthermore, if the rate of ammonia excretion is set by the movement of protons, there must be excess capacity to move NH_3 through the Rh network. In larvae, however, the Rh proteins are not arranged in series, but are scattered across the skin, yolk sac and developing gills (Braun et al., 2009), possibly limiting the efficiency of NH_3 removal and requiring increased Rh protein expression during HEA exposure. Interestingly, Rhbg, which has the highest level of expression during development (Braun et al., 2009), did not increase expression during HEA – suggesting that it may already be expressed in excess in embryos and larvae.

That Rh expression did not increase in adult gills does not preclude an increase in Rh expression in other tissues, as HEA often causes expression of Rh proteins in additional tissues (Hung et al., 2007; Nawata et al., 2007). It may be that under excretory stress, the limitation on excretion is not movement out of the body from the gills, but movement through the body to the gills. Therefore, the increased Rh expression levels in the embryos may be a by-product of whole-body mRNA measurements.

Alternatively, the ability of embryos to increase expression of Rh proteins may be a function of developmental plasticity, an ability lost in adulthood. Embryos and larvae have traditionally had an increased tolerance to high levels of ammonia (Daniels et al., 1987; McCormick et al., 1984; Rice and Stokes, 1975) and while this may be due to their ability to utilize the OUC to detoxify ammonia, it may also be due to their capacity to increase expression of Rh proteins. However, while adult zebrafish appear to be unable to increase transcription of Rh proteins, adults of other species have

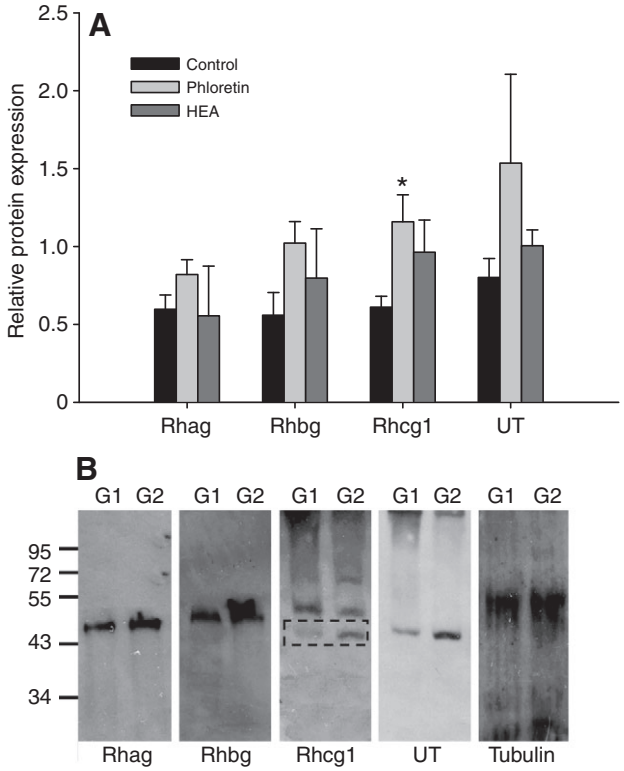

Fig. 9. (A) Semi-quantitative measurements by western blotting of protein changes in gills of adult zebrafish exposed to 0.05 mmol l^{-1} phloretin or 0.5 mmol l^{-1} NH_4Cl. The size and darkness of the bands were normalized to those of the tubulin bands. All values are presented as +1 s.e.m. (N=6); *significant difference from the control. (B) Representative western blot showing two examples of gill proteins (G1, G2) after sequential probing with the five antibodies. The membranes were probed and stripped in the order: Rhag–Rhbg–UT–Rhcg1–tubulin. As mentioned in Materials and methods, the stripping was not always complete and the UT bands can still be seen on the Rhcg1 blot (dashed box).

certainly demonstrated this capacity and the responses to excretory stress in adult fish may thus be species specific.

Future directions

While this study has certainly not disproved the notion that most teleosts are ammonotelic, it has provided intriguing data implying that urea and ammonia excretion in both larval and adult fish are more closely linked than previously thought; high levels of ammonia result in an increase in urea excretion and *vice versa* (Figs 5 and 6); Rh proteins, putative ammonia channels, are affected by increases in urea (Fig. 8A, Fig. 9); and UT, a urea transporter, is affected by increases in ammonia (Fig. 8B). In young fish, the links between urea and ammonia excretion may be due to the fact that embryonic and larval fish are thought to possess a functional OUC (Barimo et al., 2004; Chadwick et al., 1999; Essex-Fraser et al., 2005; Steele et al., 2001; Wright et al., 1995). With an OUC, increased tissue ammonia will result in an increased production of urea, which may trigger an increase in expression of UT. The links between urea and ammonia excretion in adults are not as clear and questions remain regarding the interplay between ammonia, urea and their respective transporters in teleosts.

Perspectives

With the discovery of the locations of ammonia-transporting Rh proteins in the gills of pufferfish (Nakada et al., 2007b), there has been a large increase in the study of Rh proteins in teleosts. Much of this work has focused on the transcriptional changes of Rh proteins under various conditions and during development, and in the same vein the present study has shown that zebrafish also possess the ability to alter Rh proteins during excretory stress. In addition, we have revealed that the methods used by the fish to deal with excretory stress change during development, possibly as a consequence of transporter location. While larval zebrafish possess a diffuse pattern of Rh and UT expression, occurring on the yolk sac, gill and head (Braun et al., 2009), adult zebrafish have an expression pattern which is very similar to that first shown to occur in pufferfish, with specific Rh proteins located in specific cell layers within the gill. The fact that two very different fish, one marine and one freshwater, possess such a similar arrangement of Rh proteins in the gills, suggests that a related pattern may be common to most teleosts.

This study was supported by NSERC of Canada Discovery and RTI grants to S.F.P. S.L.S. was the recipient of NSERC and Government of Ontario graduate scholarships. We'd like to thank Pat Walsh for helpful comments on an early draft and S. Hirose for the generous donation of the Rhag antibody which was used in this study.

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