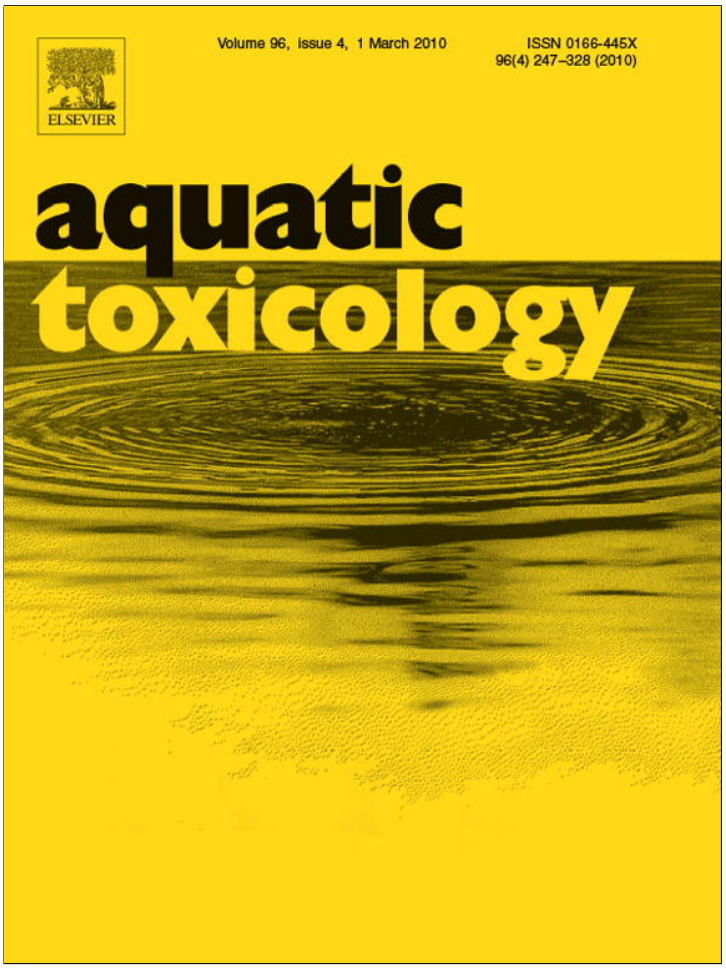

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Rhesus glycoprotein and urea transporter genes in rainbow trout embryos are upregulated in response to alkaline water (pH 9.7) but not elevated water ammonia

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ABSTRACT

Recent studies have shown that genes for the putative ammonia transporter, Rhesus glycoproteins (Rh) and the facilitated urea transporter (UT) are expressed before hatching in rainbow trout (*Oncorhychus mykiss* Walbaum) embryos. We tested the hypothesis that Rh and UT gene expressions are regulated in response to environmental conditions that inhibit ammonia excretion during early life stages. Eyed-up embryos (22 days post-fertilization (dpf)) were exposed to control (pH 8.3), high ammonia (1.70 mmol l^{-1} NH_4HCO_3) and high pH (pH 9.7) conditions for 48 h. With exposure to high water ammonia, ammonia excretion rates were reversed, tissue ammonia concentration was elevated by 9-fold, but there were no significant changes in mRNA expression relative to control embryos. In contrast, exposure to high water pH had a smaller impact on ammonia excretion rates and tissue ammonia concentrations, whereas mRNA levels for the Rhesus glycoprotein Rhcg2 and urea transporter (UT) were elevated by 3.5- and 5.6-fold, respectively. As well, mRNAs of the genes for H^+ATPase and Na^+/H^+ exchanger (NHE2), associated with NH_3 excretion, were also upregulated by 7.2- and 13-fold, respectively, in embryos exposed to alkaline water relative to controls. These results indicate that the Rhcg2, UT and associated transport genes are regulated in rainbow trout embryos, but in contrast to adults, there is no effect of high external ammonia at this stage of development.

1. Introduction

Ammonia is a naturally occurring compound required for protein synthesis, as well as a metabolic waste product in fish. Ammonia is highly toxic to fish (reviewed by Randall and Tsui, 2002) and freshwater rainbow trout are one of the more sensitive species (Thurston et al., 1981). The primary sources of ammonia released to the aquatic environment from human activity are derived from various industries, municipal wastewater effluents and agricultural runoff (CEPA, 2002). As well, intensive fish culture can result in elevated water ammonia levels (Morrison and Piper, 1988). Elevated water pH can also be toxic to fish (Jordan and Lloyd, 1964; Murray and Ziebell, 1984). The pH of freshwater environments are typically between pH 6 and 9, with more alkaline waters found in regions with a bedrock rich in carbonates (Brönmark and Hansson, 2005). One such case is Pyramid Lake, Nevada, where cutthroat trout (*Oncorhynchus clarki henshawi*) are found living in water of pH 9.4. Although the toxicity of ammonia or alkaline water to fish is well established, there is much less information on how the pathways for nitrogen excretion are impacted by these environmental perturbations in early life.

Yolk proteins and amino acids provide the major fuel for developing teleost embryos (reviewed by Wright and Fyhn, 2001) and the removal of nitrogenous waste products is necessary to avoid the accumulation of ammonia to toxic levels. Ammonia and/or urea are excreted prior to hatching and the balance between the two is species-dependent (e.g. Barimo et al., 2004; Braun et al., 2009; Chadwick and Wright, 1999; Korsgaard, 1994; Steele et al., 2001; Terjesen et al., 1997). Urea is synthesized via the ornithine urea cycle (OUC) during early embryonic development in all teleost species studied thus far, including rainbow trout, but the pathway appears to be suppressed in most adult teleosts (Dépêche et al., 1979; Essex-Fraser et al., 2005; Korte et al., 1997; Wright et al., 1995). Griffith (1991) proposed that ureogenesis via the OUC was retained in teleost embryos to prevent ammonia toxicity during the endogenous feeding period.

With the discovery of Rhesus (Rh) glycoproteins in the gills of aquatic animals that transport ammonia (reviewed by Weihrauch et al., 2009; Wright and Wood, 2009), there is renewed interest in

Abbreviations: dpf, days post-fertilization; mRNA, messenger RNA; NHE, sodium hydrogen exchanger; OUC, ornithine urea cycle; PNH_3, partial pressure of NH_3; Rh, Rhesus glycoprotein; UT, urea transporter.

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the mechanisms of ammonia excretion during early life stages in teleost fishes. In zebrafish *Danio rerio* larvae, Rhcg1 was localized to the apical membrane of H^+ATPase-type mitochondrial-rich cells of the yolk sac membrane (Nakada et al., 2007) and there is strong evidence that ammonia and H^+ excretion are linked on the cutaneous surface (Lin et al., 2006; Shih et al., 2008). This scenario is consistent with the model of ammonia excretion proposed for the adult freshwater fish gill where NH_3 diffusion via the Rhcg protein on the apical membrane is linked to H^+ efflux via H^+ATPase and/or NHE (Tsui et al., 2009; Wright and Wood, 2009).

Ammonia excretion in rainbow trout embryos is thought to involve passive diffusion of NH_3 from the embryo to the acidic unstirred boundary layer next to the chorion (Rahaman-Noronha et al., 1996). The skin and yolk sac membranes are the primary site of respiration and ionoregulation, as the gills are poorly developed (Rombough, 1988). mRNAs of Rh genes, Rhbg, Rhcg1 and Rhcg2 were detected at 14 dpf in rainbow trout embryos (10 °C) and Rhcg2 mRNA levels were markedly higher than those of the other two isoforms (Hung et al., 2008). In adult trout, mRNAs of all three of these Rh genes were expressed in the gills, but exposure to elevated external ammonia concentrations (1.5 mmol l^{-1} NH_4HCO_3) that inhibited ammonia excretion rates resulted in the marked upregulation of gill Rhcg2 mRNA levels by 12 h, with no change in Rhcg1 mRNA, and only a small increase in Rhbg mRNA after 48 h. H^+ATPase activity and mRNA levels were also substantially upregulated (Nawata et al., 2007).

Another treatment which is well-known to inhibit ammonia excretion in both embryos (Rahaman-Noronha et al., 1996) and adults (Wilkie and Wood, 1991; Wright and Wood, 1985) of rainbow trout is high environmental pH, an effect which is thought to be due to alkalization of the boundary layer, and therefore an effective decrement of the partial pressure NH_3 (PNH_3) diffusion gradient (Wilkie and Wood, 1996). Trout living chronically in very high pH water tend to excrete less ammonia and more urea (Wilkie et al., 1996; Wright et al., 1993). The Lake Magadi tilapia which thrives in highly buffered water at pH 10 excretes no ammonia at all, but rather entirely urea (Randall et al., 1989; Wood et al., 1989). Whether Rh and associated genes are regulated in embryonic trout in response to either of these environmental challenges (high environmental ammonia, high environmental pH) is unknown.

Urea excretion in trout embryos is dependent on a phloretin-sensitive facilitated urea transporter (Pilley and Wright, 2000), similar to that in the gills of adult trout (McDonald and Wood, 2004). The mRNA of UT gene is expressed in trout (Hung et al., 2008) and zebrafish (Braun et al., 2009) during early life stages. Selective knockdown of the UT gene using morpholinos in zebrafish, inhibited larval urea excretion by ~90% (Braun et al., 2009), indicating that the UT protein plays a critical role in urea efflux in larval fish. In rainbow trout, urea excretion rates and UT mRNA levels remained low until after hatching (~30 dpf at 10 °C; Hung et al., 2008). Exposure to elevated water ammonia (0.2 and 10 mmol l^{-1} NH_4Cl) significantly increased both tissue urea concentrations and urea excretion rates before hatching (Steele et al., 2001). However, there is no information in the literature on UT mRNA levels under these conditions.

Therefore, in this study, we tested the hypothesis that Rh and/or UT gene expression are regulated in response to environmental conditions that inhibit ammonia excretion during early life stages. Pre-hatch embryos (22 dpf) were exposed to control, high ammonia (1.70 mmol l^{-1} NH_4HCO_3) and high pH (pH 9.7) conditions for 48 h. The experimental conditions were selected to correspond to our earlier studies where high ammonia (Nawata et al., 2007; Tsui et al., 2009) and high pH (Rahaman-Noronha et al., 1996; Wright and Land, 1998) inhibited ammonia excretion in rainbow trout. Ammonia and urea excretion rates, ammonia and urea tissue concentrations, and mRNA levels of Rhbg, Rhcg1, Rhcg2, UT, H^+ATPase and NHE2 were determined by quantitative real-time PCR (qPCR) at 4 and 48 h.

2. Methods

2.1. Experimental animals

Rainbow trout embryos were purchased from Rainbow Springs Trout Farm (Thamesford, Ontario, Canada) on the day of fertilization (May 13, 2009). Three males were used to fertilize eggs from three females. Embryos were kept at the Hagen Aqualab (University of Guelph, Guelph, Ontario, Canada) in mesh bottom incubation trays with a continuous flow of local well water (10 °C, pH 8; photoperiod 12 L:12 D; water hardness 411 mg l^{-1} as $CaCO_3$; Ca^{2+} 5.24 mequiv. l^{-1}; Cl^- 1.47 mequiv. l^{-1}; Mg^{2+} 2.98 mequiv. l^{-1}; K^+ 0.06 mequiv. l^{-1}; Na^+ 1.05 mequiv. l^{-1}). Embryos were eyed up at 14 days post-fertilization (dpf) and hatching normally occurred around 30 dpf at 10 °C.

2.2. Experimental protocol

At 22 dpf, embryos were exposed to the three different treatments: control (0 μmol l^{-1} NH_4HCO_3; pH 8.3 ± 0.0), high ammonia (1.70 ± 0.04 mmol l^{-1} NH_4HCO_3; pH 8.3 ± 0.0) and high pH (0 μmol l^{-1} NH_4HCO_3; pH 9.7 ± 0.0) in static, aerated 500 ml plastic containers at 10 °C for 48 h. The age of the embryos was chosen to match our previous study where we found that mRNAs of Rh and UT genes were initially expressed at 21 dpf (Hung et al., 2008). Water pH was maintained over the 4 h flux period and 48 h experimental period by the drop wise addition every 2.6 ± 0.4 h of 0.2 mol l^{-1} H_2SO_4 (control, high ammonia) or 0.1 mol l^{-1} KOH (high pH). For example, over the initial 4 h flux H_2SO_4 was added to control and ammonia treatments to a final concentration of 0.02 mM whereas for the pH 9.7 treatment KOH did not exceed 0.66 mM. Previous experiments have shown that comparable water K^+ concentrations had no apparent physiological effects in rainbow trout (Wilkie and Wood, 1994, 1995). After the first 24 h, water was replaced with new treatment water.

To measure excretion rates, a subset of embryos (42 embryos) were placed in 18 individually aerated 120 ml flux containers and allowed to acclimate for 1 h in control water. Six containers were then assigned to each of the three treatments, with 50 ml of the appropriate treatment water (control; high ammonia; high pH) in each container. Water samples (10 ml) were collected at 0 and 4 or 44 and 48 h, and frozen at −20 °C for later determination of ammonia and urea concentration (within 6 weeks). To ensure there was no loss of ammonia at water pH 9.7 during storage, water samples from the high pH experiment were acidified before freezing (5 μl of 0.2 mol l^{-1} H_2SO_4). To determine if ammonia was lost from the experimental containers over the 4 h flux period, preliminary trial experiments were performed where 6–9 containers (without embryos) of each treatment water with the addition of ammonia (control and high pH: ~35 μmol l^{-1} NH_4HCO_3, high ammonia 2000 μmol l^{-1} NH_4HCO_3) were monitored between 0 and 4 h. In the high pH treatment, on average 23 ± 5% of the initial ammonia was lost over 4 h and therefore, this was taken into account in the calculation of flux rates. In the control and high ammonia treatments, only 2 ± 2% of the initial ammonia was lost and the experimental values were not altered. Flux rates (μmol-N g^{-1} h^{-1}) were calculated as the change in ammonia (or urea) concentration (μmol-N l^{-1}) multiplied by the volume of the chamber (in l) divided by the mass of the tissue (g) and the time period (in h). At the end of the flux experiment, the wet mass of the intact embryos per container was measured. Intact embryos were flash frozen in liquid N_2 and stored at −80 °C for RNA extraction (1 individual/n; n = 4). The

remaining embryos were dechorionated and the embryonic bodies were dissected away from the yolk sac and flash frozen in liquid N_2 and stored at −80 °C for later analysis of tissue ammonia and urea (8 individuals pooled/*n*; *n* = 6, within 7 weeks).

2.3. Analytical techniques

To determine embryonic tissue ammonia, the frozen embryos were ground in liquid N_2 using a mortar and pestle, deproteinized and neutralized using the method described by Wright et al. (1995) with the following differences; 10–15 volumes of ice-cold perchloric acid (8% perchloric acid) was used per tissue sample followed by centrifugation at 12,000 × *g* for 5 min (4 °C). The ammonia concentration in the final supernatant was determined using an enzymatic method (Kun and Kearney, 1974). Water ammonia concentrations were determined using a colorimetric assay (Verdouw et al., 1978).

Tissue preparation for the determination of urea was the same as described above for ammonia. Tissues were deproteinized using 8 volumes of ice-cold perchloric acid (8% perchloric acid) per tissue sample followed by centrifugation at 12,000 × *g* for 5 min (4 °C). The final supernatant was used for analysis. Water and tissue urea concentrations were determined using a colorimetric assay (Rahmatullah and Boyde, 1980).

2.4. Quantitative real-time PCR

Total RNA was extracted from intact rainbow trout embryos using Trizol (Invitrogen Canada Inc., Burlington, Ontario, Canada) as described previously (Hung et al., 2008). cDNA was synthesized and differences in mRNA expression were quantified between the control and experimental treatments (high ammonia, high pH) using quantitative real-time PCR (qPCR) as described by Nawata et al. (2007). Primer sequences for the Rh genes (Rhbg, Rhcg1, Rhcg2) and H^+ATPase (HATP) and the Na^+/H^+ exchanger (NHE2) were provided by Nawata et al. (2007), and the urea transporter (UT) by Hung et al. (2008). Elongation factor-1α (EF-1α, GenBank AF498320) was used as an endogenous standard to calculate relative mRNA expression by the standard curve method. Standard curves were generated by serial dilution of a random mixture of control samples. Melt-curve analysis confirmed production of a unique product, and gel electrophoresis verified the presence of a single band. A non-reversed-transcribed sample controlled for possible genomic DNA contamination.

2.5. Statistical analyses

Statistical analysis was performed using SPSS 14.0. Differences between the control, high ammonia and high pH treatments at each time point (0–4 and 44–48 h) were evaluated using single factor analysis of variance (ANOVA). Equal variance was tested using Levene's Statistic. When unequal variance was detected, Dunnett's T3 test was used to detect significant differences between treatments. For equal variances, Tukey's or Least Squares Difference test was used to determine the differences when significant differences were detected between treatments. Significant significance was accepted when $P \leq 0.05$.

3. Results

Ammonia excretion rates were depressed by almost 90% in embryos exposed to high water pH for 48 h (Fig. 1A). Elevated water ammonia levels had an even more profound impact on ammonia excretion rates with a reversal of the normal pattern, such that the embryos took up considerable amounts of ammonia from the external environment (Fig. 1A). Urea excretion rates were not significantly altered by water conditions (Fig. 1B). However, the % of nitrogen wastes excreted as urea (% urea-N excretion = urea-N excretion [ammonia-N excretion + urea-N excretion]) increased with high water pH and ammonia (% urea-N excretion, 0–4 h: control = 3.0 ± 0.3, high pH = 20.6 ± 1.0, high ammonia = 100 ± 0, 44–48 h: control = 3.9 ± 1.2, high pH = 20.5 ± 6.4, high ammonia = 100 ± 0, *n* = 5–6 for all).

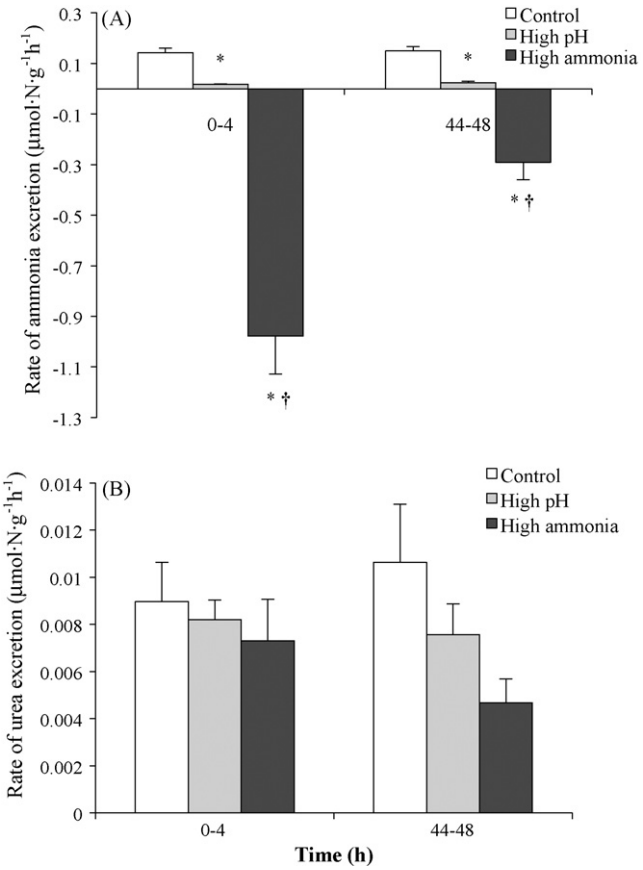

Fig. 1. Rates of ammonia (A) and urea (B) excretion (μmol N g^{-1} h^{-1}) of rainbow trout embryos at time 0–4 h and 44–48 h to one of three treatments: control (0 μmol l^{-1} NH_4HCO_3; pH 8.3 ± 0.0), high ammonia (1.70 ± 0.04 mmol l^{-1} NH_4HCO_3; pH 8.3 ± 0.0) and high pH (0 μmol l^{-1} NH_4HCO_3; pH 9.7 ± 0.0) (means ± SEM, *n* = 6). ANOVA yielded significant differences between the following: asterisk sign indicates significance from control, and dagger indicates significance from high pH group.

Tissue ammonia concentrations were not significantly higher at 4 h but were elevated by 4.3-fold after 48 h of exposure to water of pH 9.7 relative to control embryos (Fig. 2A). Exposure to high water ammonia resulted in larger elevations in tissue ammonia concentrations (2-fold after 4 h, 9.3-fold after 48 h; Fig. 2A). Small but significant changes were observed in tissue urea concentrations in embryos exposed to high ammonia (+24% after 4 h; Fig. 2B).

Changes in mRNA levels were detected after 4 h of treatment (not 48 h) and only in response to high water pH. Relative to control embryos, Rhcg2 (Fig. 3), UT (Fig. 3), H^+ATPase (Fig. 4) and NHE2 (Fig. 4) mRNAs were 3.5, 5.6, 7.2 and 13 times higher after 4 h of exposure to water of pH 9.7. There were no significant changes in Rhbg and Rhcg1 mRNA expression in any treatment (data not shown). EF1α mRNA levels were unchanged between treatments.

4. Discussion

The findings of this study provide evidence to support our hypothesis that both Rh and UT genes are regulated before hatching in rainbow trout embryos in response to environmental perturbations. mRNA expression of Rhcg2, UT, H^+ATPase and NHE2 was upregulated in response to alkaline water, but curiously not in

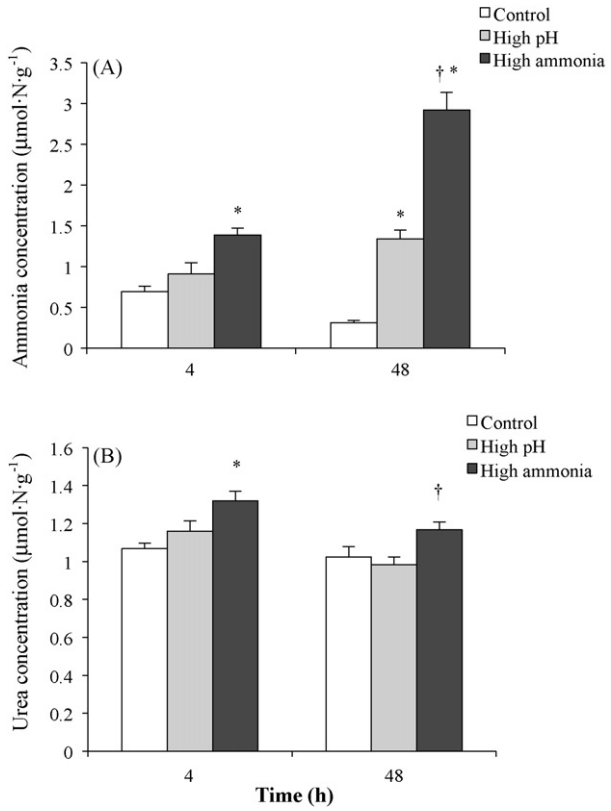

Fig. 2. Ammonia (A) and urea (B) concentration (μmol N g^{-1}) in the embryonic tissue after 4 h and 48 h of exposure to one of three treatments control (0 μmol l^{-1} NH_4HCO_3; pH 8.3 ± 0.0), high ammonia (1.70 ± 0.04 mmol l^{-1} NH_4HCO_3; pH 8.3 ± 0.0) and high pH (0 μmol l^{-1} NH_4HCO_3; pH 9.7 ± 0.0) (means ± SEM, n = 6). ANOVA yielded significant differences between the following: asterisk indicates significance from control, and dagger indicates significance from high pH group.

response to high ammonia as has been reported for adult rainbow trout (Nawata et al., 2007; Tsui et al., 2009). Hence, the mRNA expression of genes involved in ammonia (Rhcg2) and urea (UT) excretion in trout embryos are regulated before hatching, but not under all environmental conditions that perturb nitrogen excretion. Overall, our findings are consistent with the model of ammonia transport across the freshwater trout gill (Tsui et al., 2009; Wright and Wood, 2009) and importance of Rhcg, H^+ATPase and UT proteins in ammonia and urea transport in early life stages (Braun et al., 2009; Nakada et al., 2007; Pilley and Wright, 2000; Shih et al., 2008).

We measured changes in mRNA expression in this study, but were unable to quantify Rh and UT protein expression because trout-specific antibodies were unavailable. In previous trials we determined that zebrafish Rh glycoprotein antibodies (donated by S. Hirose) were incompatible with trout Rh glycoproteins. To our knowledge, there have been no quantitative comparisons of changes in Rh and UT mRNA versus protein expression data for any fish exposed to environmental manipulations. Nawata et al. (2007), however, demonstrated that changes in gill H^+ATPase and carbonic anhydrase mRNA levels were correlated with similar changes in protein activity in trout subjected to elevated environmental ammonia concentrations. Changes in mRNA expression may result in corresponding changes in protein expression. Alternatively, a change in mRNA levels without a change in protein levels may indicate a change in mRNA turnover rates. In either case, if a change in mRNA expression occurred (relative to the control gene), then it must reflect a specific response in the transcriptional regulation of the gene or suite of genes which is one regulatory step in the overall control of the nitrogen excretion pathway.

Nitrogen excretion in early life stages of salmonids is impacted by the immediate water conditions surrounding the chorion. Elevated water pH or ammonia, decrease or reverse the normal tissue-to-water PNH_3 gradients and impede ammonia excretion in rainbow trout embryos (Rahaman-Noronha et al., 1996; Steele et al., 2001; Wright and Land, 1998; present study), as well as in adults (McGeer and Eddy, 1998; Nawata et al., 2007; Wilkie and Wood, 1991, 1996; Wright and Wood, 1985). Typically, plasma/tissue ammonia levels are elevated under these conditions and this was confirmed in the present study. In the process of re-establishing homeostasis in the face of unfavourable NH_3 gradients and elevated tissue ammonia levels, fish may attempt to enhance ammonia excretion by increasing the expression of ammonia transporters (Rh glycoproteins) and accessory proteins (e.g. H^+ATPase, NHE). Indeed, recovery of ammonia excretion rates in adult rainbow trout after 12–24 h of external ammonia (1.5 mmol l^{-1} NH_4HCO_3) was correlated with a more than 6-fold increase in gill Rhcg2 mRNA levels, as well as a significant increase in H^+ATPase mRNA and activity levels (Nawata et al., 2007). However, contrary to the findings in adults, ammonia excretion rates did not recover, the mRNA expression of the genes was not altered and tissue ammonia levels continued to increase over 48 h of elevated external ammonia exposure in trout embryos in the present study.

In contrast, in parallel experiments with elevated water pH (9.7), there was a strong, rapid (4 h) induction of Rhcg2, H^+ATPase, NHE and UT mRNA levels. In these embryos, ammonia continued to be excreted (albeit at a low level), with a small accumulation of tissue ammonia and no accumulation of tissue urea over 48 h. If changes in mRNA expression lead to corresponding changes in protein levels, then higher levels of Rhcg2, UT and associated proteins may have expedited nitrogen excretion in embryos exposed to an unfavourable environment (i.e. high pH) for ammonia excretion.

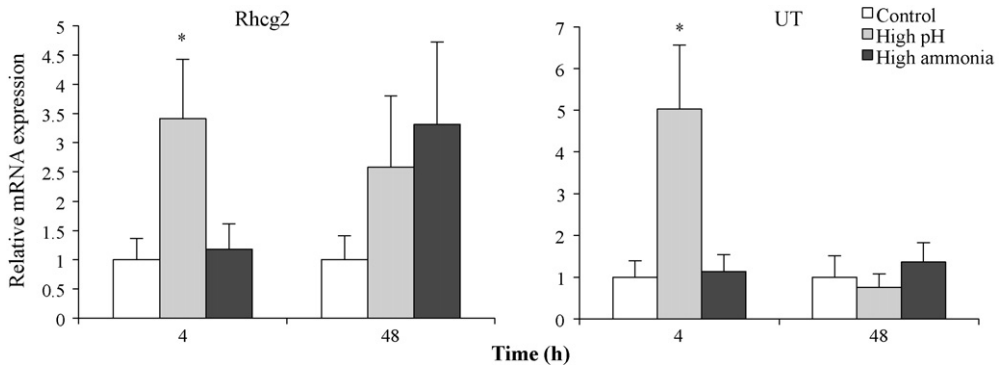

Fig. 3. mRNA expression of Rhcg2 and UT relative to the elongation factor 1 α (EF1α) after 4 h and 48 h of exposure to one of three treatments control (0 μmol l^{-1} NH_4HCO_3; pH 8.3 ± 0.0), high ammonia (1.70 ± 0.04 mmol l^{-1} NH_4HCO_3; pH 8.3 ± 0.0) and high pH (0 μmol l^{-1} NH_4HCO_3; pH 9.7 ± 0.0). *Significant differences from the control (P < 0.05). Data are means ± SE (n = 6).

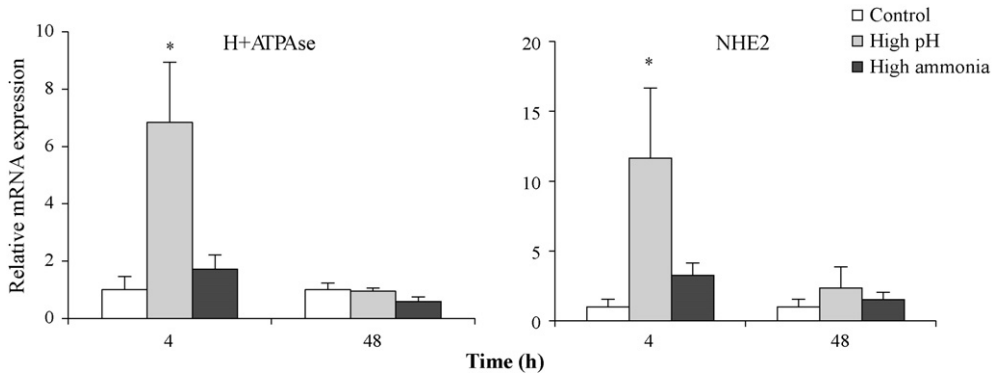

Fig. 4. mRNA expression of H^+ATPase and NHE2 relative to the elongation factor 1α (EF1α) after 4 h and 48 h of exposure to one of three treatments control (0 μmol l^{-1} NH_4HCO_3; pH 8.3 ± 0.0), high ammonia (1.70 ± 0.04 mmol l^{-1} NH_4HCO_3; pH 8.3 ± 0.0) and high pH (0 μmol l^{-1} NH_4HCO_3; pH 9.7 ± 0.0). *Significant differences from the control ($P < 0.05$). Data are means ± SE ($n = 6$).

Acidification of the unstirred layer next to the chorion and perivitelline fluid is thought to be critical in maintaining an outwardly directed NH_3 gradient and facilitating ammonia excretion in trout embryos (Rahaman-Noronha et al., 1996). In zebrafish larvae, Shih et al. (2008) elegantly demonstrated the tight coupling of ammonia and H^+ flux (via H^+ATPase) across the cutaneous surface. In the present study, acidification of the fluid surrounding the cutaneous surface when embryos were exposed to water of pH 9.7 was undoubtedly vital for increasing the conversion of NH_3 to NH_4^+ and enhancing ammonia excretion. Upregulation of H^+ATPase and NHE2 mRNA levels after 4 h in alkaline water is consistent with the model of H^+-linked ammonia transport (Tsui et al., 2009; Wright and Wood, 2009).

It is intriguing that exposure to alkaline water but not high ammonia induced the mRNA expression of genes involved in ammonia and urea transport. Wright and Wood (2009) suggested that cortisol may play a key role in regulating expression of the Rh genes in trout in response to elevated ammonia. Previous studies have shown that sublethal ammonia exposure exerts a stress response in adult fish (Jeney et al., 1992) with a positive correlation between plasma ammonia levels and plasma cortisol levels (Ortega et al., 2005). Using a cultured gill epithelium preparation, Tsui et al. (2009) found that exposure to cortisol and elevated ammonia together upregulated mRNA expression of Rh genes more profoundly than either treatment alone. In addition, the stress associated with cannulation and saline infusion results in elevated plasma ammonia levels and increased expression of gill Rh genes (Nawata and Wood, 2009). However, in rainbow trout embryos, cortisol levels are low and do not change in response to stress (Barry et al., 1995a). Indeed cortisol release in response to stress does not occur until 2 weeks after hatch (Barry et al., 1995a, 1995b). If cortisol is an important component in the regulation of Rh gene expression during exposure to high ammonia, then this may explain why in this study rainbow trout embryos did not alter Rh mRNA levels, despite the accumulation of tissue ammonia.

The fact that gene expression was regulated in response to alkaline water, however suggests that a different signaling pathway may be involved. We suggest that internal alkalosis, in combination with elevated internal ammonia levels, may provide an alternate signal. For example, adult trout exhibit a marked blood alkalosis ("alkaline tide") and increased plasma ammonia levels and ammonia excretion rates following feeding (Bucking and Wood, 2008). Gill Rhcg2 mRNA expression is also upregulated after feeding (C.M. Nawata and C.M. Wood, unpublished results), and there is evidence that systemic alkalosis after feeding can act as a metabolic signaling mechanism in elasmobranchs (Wood et al., 2008).

mRNA expression of Rhcg2 but not Rhcg1 and Rhbg was upregulated in response to high water pH in the present study. In our previous study, we showed that Rhcg2 mRNA levels were 25–88-fold higher compared to Rhcg1 and Rhbg mRNA levels at a similar developmental time in trout (21 dpf; Hung et al., 2008). In adult trout exposed to elevated external ammonia, the pattern of changes in gill Rh mRNA expression parallels that observed with high pH in the current study (Nawata et al., 2007). Although zebrafish larvae (6 dpf) upregulate Rhcg1 mRNA levels in response to water of low ionic strength (Nakada et al., 2007), it appears that Rhcg2 is the more responsive isoform in rainbow trout.

When embryos were exposed to elevated ammonia, far less ammonia accumulated in the tissues than predicted from the large negative ammonia excretion rates at 4 and 48 h (i.e., uptake from the water to the embryo). Steele et al. (2001) found that ammonia accumulated in the yolk, but not the embryonic tissues of trout embryos exposed to 0.2 mmol l^{-1} NH_4Cl for 4 days. Although a small amount of ammonia was converted to urea (as in the present study), there were no increases in tissue individual amino acid concentrations in Steele et al. (2001) study, implying that large scale detoxification of ammonia to glutamine or glutamate did not occur. Thus, we propose in the current study that a portion of the ammonia absorbed from the environment was sequestered in the yolk compartment, where yolk pH is more acidic relative to embryonic tissues and the surrounding water and NH_3 would be trapped as NH_4^+ (Rahaman-Noronha et al., 1996).

After 4 h of exposure to elevated pH, UT mRNA levels were upregulated by >5-fold (this study), even though UT mRNA levels are relatively low prior to hatching in trout (Hung et al., 2008). Pilley and Wright (2000) were the first to provide evidence that urea transport in embryonic rainbow trout is dependent on a phloretin-sensitive facilitated urea transporter. In zebrafish larvae, morpholinos targeted to the UT gene significantly inhibited urea excretion rates (Braun et al., 2009), confirming the importance of the UT protein in urea excretion during early life stages. Exposure to high water pH over 48 h did not alter urea excretion rates in the current study, but in an earlier study, urea excretion rates were significantly increased with acute exposure (4 h) to water pH 9.0 and 9.5 in larval trout (Wright and Land, 1998). Taken together it appears that UT gene expression is sensitive to external environmental pH very early in development and these changes are correlated with the maintenance of urea excretion rates and an increase in the % of nitrogen excreted as urea. In the Magadi tilapia which excretes 100% urea-N at pH 10, this excretion occurs via a UT protein in the gills (Walsh et al., 2001).

In conclusion, alkaline water (pH 9.7) but not high external ammonia (1.70 mmol l^{-1} NH_4 HCO_3) induces mRNA expression of Rhcg2 gene, as well as that of other genes (H^+ATPase, NHE2, UT) involved in nitrogen excretion in rainbow trout embryos. Since both alkaline water and high ammonia inhibited ammonia excre-

tion rates and elevated tissue ammonia levels, the differential effect on mRNA expression suggests that different signaling pathways are involved.

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