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mRNA expression analysis of the physiological responses to ammonia infusion in rainbow trout

C. Michele Nawata · Chris M. Wood

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Abstract We recently reported that tissue levels of Rhesus (Rh) mRNA in rainbow trout changed in response to high-external ammonia (HEA). To investigate whether or not these changes could be due to elevated plasma ammonia levels, we infused rainbow trout for 12 h with 140 mmol L^{-1} NH_4HCO_3, or with 140 mmol L^{-1} NaCl as a control for the effects of infusion. We also analyzed the effects of dorsal aortic catheterization alone, without infusion. Catheterization alone resulted in an elevated ammonia excretion rate, a downregulation of Rhbg mRNA in the brain, and mRNA upregulations of Rhbg, Rhcg1, and Rhcg2 in the gill, Rhbg and Rhcg1 in the skin, and Rhag in the erythrocytes. In NH_4HCO_3-infused fish, plasma cortisol peaked at 6 h, erythrocyte Rhag mRNA was downregulated, gill Rhbg, Rhcg1, and Rhcg2 mRNA were upregulated, and skin Rhbg mRNA was also upregulated. NaCl infusion resulted in elevated plasma ammonia and ammonia excretion rates as well as gill mRNA upregulations of Rhbg, carbonic anhydrase, NHE2, H^+-ATPase, Na^+/K^+-ATPase. Taken together, the results indicated that infusion of NH_4HCO_3 induced a similar pattern of Rh transcript changes as that seen when fish were exposed to HEA. Second, catheterization alone, as well as isotonic NaCl infusion, significantly altered mRNA levels, highlighting the necessity for careful data interpretation and inclusion of appropriate controls for gene expression studies in fish that have undergone anaesthesia/surgery and infusion procedures. Finally, elevated plasma ammonia and cortisol may both be involved in the signaling mechanism for Rh gene regulation.

Keywords Rhesus glycoproteins · Ammonia transport · Cortisol · NH_4HCO_3

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C. M. Nawata (✉) · C. M. Wood
Department of Biology, McMaster University,
1280 Main St. West, Hamilton, ON L8S 4K1, Canada
e-mail: nawatacm@mcmaster.ca

Introduction

Like most freshwater teleost fish, rainbow trout are ammoniotelic, excreting the majority of their nitrogenous waste in the form of ammonia across the gills (Wood 1993). Elevated levels of environmental ammonia due to pollution in natural waters and those associated with aquacultural systems can lead to elevated internal levels of ammonia, which can have detrimental effects on the health of fish (Eddy 2005; Mommsen and Walsh 1992; Randall and Tsui 2002). Therefore, the understanding of ammonia handling at the gill has been the subject of much research over the years and the general consensus is that ammonia gas (NH_3) is excreted across the gills by simple diffusion aided by an acidified gill–water boundary layer that traps NH_3 to NH_4^+, in freshwater fish (Wilkie 2002). This model of ammonia excretion, however, is now being modified by the identification of the newest members of the ammonia transporter superfamily, the Rhesus (Rh) proteins. In mammals, Rhag is erythroid-specific whereas Rhbg and Rhcg are non-erythroid proteins present in renal and extrarenal tissues (Huang and Liu 2001). These Rh proteins appear to function as ammonia channels (likely for NH_3, but possibly for NH_4^+) with the additional, albeit controversial function of Rhag as a CO_2 channel (see Cartron 2008).

Rh proteins have also been identified in several fish species (Huang and Peng 2005; Hung et al. 2007; Nakada

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et al. 2007a, b; Nawata et al. 2007). In two species, the mangrove killifish and the rainbow trout, exposures to high-external ammonia (HEA) concentrations have been associated with changes in tissue levels of Rh mRNA, notably the upregulation of Rhcg2 mRNA in the gill (Hung et al. 2007; Nawata et al. 2007). In the rainbow trout, this upregulation of Rhcg2 coincides with the resumption and enhancement of ammonia excretion that was initially blocked by the reversed plasma to water ammonia gradient created by the HEA treatment (Nawata et al. 2007).

In mammals, an increase in ammonia metabolism induced by chronic metabolic acidosis or reduced renal mass is associated with an increase in Rhcg protein expression (Kim et al. 2007; Seshadri et al. 2006) and Rhcg knockdown mice have impaired ammonia excretion (Biver et al. 2008). Therefore, a clear link has been established between ammonia and Rh proteins in both fish and mammals. Presently, however, little is known about the signaling pathway regulating Rh gene expression. Studies with rainbow trout suggest that the observed changes in tissue Rh mRNA levels are directly linked to elevated plasma ammonia levels after exposure to HEA, or when ammonia excretion is blocked at the gill after exposure to Hepes buffer (Nawata et al. 2007; Nawata and Wood 2008). We wanted to further examine this relationship between plasma ammonia levels and Rh mRNA expression, this time by elevating the plasma ammonia concentration in rainbow trout via infusion of 140 mmol L^{-1} NH_4HCO_3 (approximately isotonic to the blood plasma). NH_4HCO_3 infusion avoids the well-known acidosis-inducing effects of ammonium salts containing strong anions [e.g. NH_4Cl or $(NH_4)_2SO_4$—Cameron and Heisler 1983; McDonald and Prior 1988; Salama et al. 1999; Wilson et al. 1994]. Comparable infusion with 140 mmol L^{-1} NaCl served as a control for the effects of isotonic volume loading. These procedures, however, require prior anaesthesia and the surgical placement of a dorsal aortic catheter. Currently nothing is known about the potential impact that catheterization or infusion may have on gene expression.

The use of chronic indwelling dorsal aortic catheters has greatly facilitated physiological studies in fish, allowing for repeated blood sampling and infusion of substances with minimal disturbance to the fish (e.g. Goss and Wood 1990; McGeer and Eddy 1998; Salama et al. 1999; Wilson et al. 1994; Yesaki and Iwama 1992). Although it has been well recognized that anaesthesia and surgical procedures impose stress on fish (Houston 1990; Soivio and Nynolm 1975), the fact that this stress may bias the results in physiological studies has been largely overlooked. Indeed it is commonly assumed that once the plasma cortisol in surgically manipulated fish has reached basal levels after a period of recuperation, the fish are physiologically stable (Tashjian and Hung 2005). At the molecular level, however, the picture may be very different. In fact, it has been shown in mammalian studies, that surgical manipulations can have an impact on gene expression (e.g. Gerloff et al. 1999; Lin et al. 2006; Zhao et al. 2004) and therefore incorporation of appropriate controls is necessary in gene expression studies involving surgical procedures.

The objectives of this study were twofold. The first was to determine whether or not elevated internal plasma ammonia concentrations achieved by ammonia infusion could elicit a similar pattern of mRNA expression in trout tissues as that when trout were exposed to HEA as previously reported (Nawata et al. 2007). The second goal was to determine whether or not surgical procedures or infusion alone could influence mRNA expression levels. An uncatheterized (no-surgery) control and a catheterized control were included to determine the effects of the surgical procedures, and a NaCl-infusion control was employed to examine the effects of infusion itself. The tissue mRNA expression levels of Rhag, Rhbg, Rhcg1, and Rhcg2, as well as carbonic anhydrase (CA2), H^+-ATPase, Na^+/H^+ exchanger 2 (NHE2), and Na^+/K^+-ATPase (NKA) were analyzed. Ammonia excretion rates, and levels of plasma ammonia and cortisol were also evaluated.

Materials and methods

Experimental animals

Rainbow trout, *Oncorhynchus mykiss* (Walbaum), weighing 176–265 g, were acquired from Humber Springs Trout Hatchery, Ontario, Canada and held in dechlorinated Hamilton tapwater ($[Na^+] = 0.6$ mequiv L^{-1}, $[Cl^-] = 1.8$ mequiv L^{-1}, $[Ca^{++}] = 0.8$ mequiv L^{-1}, $[Mg^{++}] = 0.3$ mequiv L^{-1}, $[K^+] = 0.05$ mequiv L^{-1}; titration alkalinity 2.1 mequiv L^{-1}; pH ~8.0; hardness ~140 mg L^{-1} as $CaCO_3$ equivalents; temperature 12–16°C). Feeding was suspended 1 week prior to experimentation. Fish were fitted with dorsal aortic catheters under anaesthesia (0.07 g L^{-1} neutralized MS222, Sigma; St. Louis, MO, USA), transferred to individual darkened boxes with aerated, flowing tap water (15 ± 0.5°C) and allowed to recover for a minimum of 48 h before experimentation.

Infusions and analyses

Fish were infused over a period of 12 h with 140 mmol L^{-1} NH_4HCO_3 (pH 7.8) or as an infusion control, a separate set of fish was infused with 140 mmol L^{-1} NaCl (pH 7.8). The infusion rate was set at the lowest allowable with a polystaltic pump (Buchler Instruments, Inc., Kansas, MO, USA), resulting in an

average rate of 6.18 ± 0.34 mL kg^{-1} h^{-1}. A set of catheterized fish were not infused and served as a catheterized control and an additional set of fish that did not undergo surgery served as an additional (no-surgery) control.

Ammonia excretion rates were measured in both sets of control fish as well as in the infused fish after 3, 6, 9, and 12 h of infusion. During this time, the boxes were closed off to water flow for 1 h and the box water volume was set to 4 L. A 10-mL water sample was removed at the beginning and end of each flux period, frozen at −20°C and later assayed for total ammonia (T_{Amm}) in triplicate, using a modified salicylate-hypochlorite method (Verdouw et al. 1978). Net flux rates of total ammonia (J_{Amm}, μmol kg^{-1} h^{-1}) were calculated as:

$$J_{Amm} = (T_{Ammi} - T_{Ammf}) \times V/(t \times M)$$

where *i* and *f,* respectively, are initial and final concentration (μmol L^{-1}), *V* is box water volume (L), *t* is the total flux time (h) and *M* is fish mass (kg). A negative J_{Amm} indicates a net excretion of ammonia to the water.

Blood samples (400 μL) were collected via the dorsal aortic catheter at 3, 6, 9, and 12 h. The infusion line was disconnected, cleared with Cortland saline (Wolf 1963) and blood was drawn into the catheter three times prior to withdrawing the sample in order to minimize contamination with infusate. In the case of uncatheterized fish, blood samples were removed via caudal puncture after terminal, rapid anaesthetization with neutralized MS222 (0.1 g L^{-1}). The durations of these caudal collections were always under 30 s in order to minimize blood chemistry alterations due to anaesthetization and handling stress. Samples were immediately centrifuged to separate plasma from erythrocytes (2 min, 20,000*g*). Plasma samples were frozen in liquid nitrogen and stored at −70°C and later analyzed in duplicate for T_{Amm} by enzyme assay (Raichem, Hemagen Diagnostics, Inc., San Diego, CA, USA) and for cortisol by radioimmunoassay (Cortisol ^{125}I RIA Kit, MP Biomedicals, Orangeburg, NY, USA).

Tissue sampling

Tissues were extracted from fish at the end of 3, 6, and 12 h of NH_4HCO_3 infusion, and at the end of 3 and 12 h of NaCl infusion. After blood samples were removed, fish were terminally anaesthetized with neutralized MS222. Although the contribution of erythroid Rhbg mRNA to the overall Rhbg mRNA expression in trout tissues is extremely low (Nawata et al. 2007), the same protocol that was used in this previous study was maintained; tissues were perfused with ice-cold, heparinized Cortland saline via the *bulbus arteriosus* in order to minimize erythrocyte contamination. Brain, gill and skin were quickly excised and flash frozen together with the erythrocytes (separated from plasma as described above), in liquid nitrogen and stored at −70°C. The same procedure was carried out for both sets of control fish. For the two infused groups, gill samples were analyzed at all sample times while erythrocytes, brain, and skin samples were analyzed at the 12-h time point. Similarly, only 12-h samples were analyzed in both control groups.

RNA extraction and cDNA synthesis

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

Real-time quantitative PCR (qPCR)

Rh mRNA expression was assessed in the brain, gill, skin, and erythrocytes and carbonic anhydrase (CA2; cytoplasmic), H^+-ATPase (v-type, B-subunit), NHE2, and Na^+/K^+-ATPase (NKA; α1a-subunit) mRNA expression was analyzed in the gill using the cDNA described above and primers previously published (Nawata et al. 2007). Reactions (20 μL) containing 4 μL of cDNA, 8 pmoles of each primer, and 10 μL of RT^2 Real-Time SYBR Green/ROX PCR Master Mix (SuperArray, Bioscience Corp, Frederick, MD, USA) were performed at 95°C for 2 min, followed by 40 cycles of 95°C for 30 s and 60°C for 1 min. Production of a single product was verified by melt-curve analysis and gel electrophoresis. No-template controls and non-reversed-transcribed controls were run in parallel. Values were extrapolated from standard curves generated by serial dilution of one randomly selected control sample. The expression of three reference genes, beta-actin, elongation factor (EF1-α) and 18S proved unstable across treatments, therefore data were normalized against ng total RNA, an acceptable normalization method (Bustin 2000, 2002) as described previously (Nawata and Wood 2008).

Statistical analysis

Data are presented as means ± SEM (*n*, number of fish). The effects of NaCl and NH_4HCO_3 infusions were analyzed by one-way analysis of variance (ANOVA), followed by Fisher's Least Significant Difference post hoc test. A paired *t* test was used to compare the two control sets. Significance was set at $\alpha = 0.05$.

Results

Ammonia and cortisol responses

Catheterization (which involved anaesthesia and surgery) caused a significant 2.5-fold elevation in ammonia excretion relative to the no-surgery control (Fig. 1). Infusion of 140 mmoL L^{-1} NH_4HCO_3 resulted in an average ammonia-loading rate of 918 μmol kg^{-1} h^{-1} and the ammonia excretion rate of NH_4HCO_3-infused fish was elevated at all time points relative to both controls. By 12 h the ammonia excretion rate exceeded the ammonia loading rate and was elevated sevenfold over the no-surgery control rate and 3-fold over that of the catheterized control (Fig. 1). The ammonia excretion rate in NaCl-infused fish was elevated by an average of 3.5-fold above the no-surgery control level at 3, 6 and 12 h, but the rates were not significantly different from those of the catheterized control (Fig. 1).

Plasma ammonia levels were not significantly different in the two control groups of fish, but were significantly elevated in the NH_4HCO_3-infused fish at all time points, reaching a level 29-fold above that of the controls by 12 h (Fig. 2). The NaCl-infused fish also had increased plasma ammonia levels after 6 h and were 1.7-fold higher than the controls by 12 h (Fig. 2). Plasma cortisol levels were not significantly different between the two control groups but were elevated 6.8-fold over both control levels after 6 h of NH_4HCO_3 infusion (Fig. 3). However, this effect disappeared by 12 h of infusion. Plasma cortisol concentrations were not significantly altered by NaCl infusion.

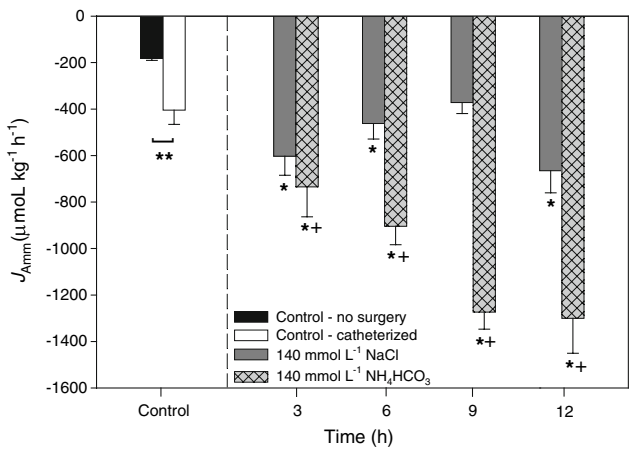

Fig. 1 The ammonia excretion rates (J_{Amm}) in rainbow trout infused with 140 mmol L^{-1} NaCl or 140 mmol L^{-1} NH_4HCO_3 over a course of 12 h. The no-surgery control fish did not undergo experimental manipulation. The catheterized control fish underwent anaesthesia and surgery for catheter placement, but were not infused. The negative values indicate excretion into the water. *Single asterisks* represent values significantly different from the no-surgery control, *crosses* indicate significant difference from the catheterized control, and *double asterisks* indicate a significant difference between the two control values ($P < 0.05$). Data are means ± SEM ($n = 6$–9)

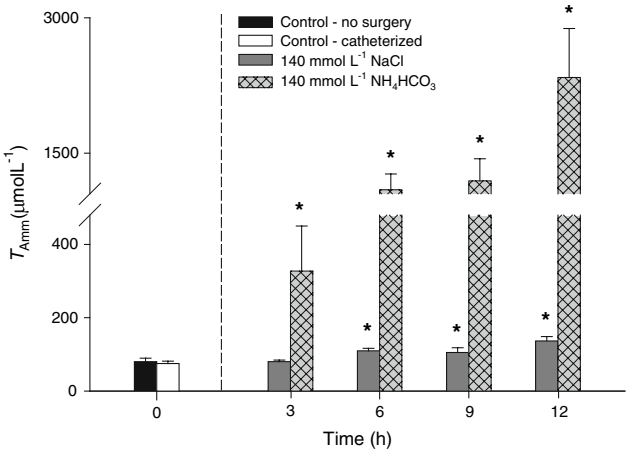

Fig. 2 Plasma total ammonia (T_{Amm}) in rainbow trout infused with 140 mmol L^{-1} NaCl or 140 mmol L^{-1} NH_4HCO_3 over a course of 12 h. The no-surgery control fish did not undergo experimental manipulation. The catheterized control fish underwent anaesthesia and surgery for catheter placement, but were not infused. *Asterisks* represent plasma values significantly different from both control values. Controls were not significantly different from each other ($P < 0.05$). Data are means ± SEM ($n = 6$)

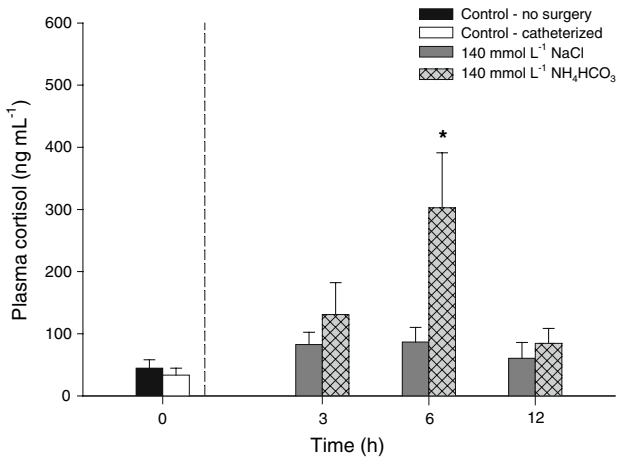

Fig. 3 Plasma cortisol levels during 12 h of infusion with 140 mmol L^{-1} NaCl or 140 mmol L^{-1} NH_4HCO_3. The no-surgery control fish did not undergo experimental manipulation. The catheterized control fish underwent anaesthesia and surgery for catheter placement, but were not infused. *Asterisk* indicates significant difference from both control values. Controls were not significantly different from each other ($P < 0.05$). Data are means ± SEM ($n = 6$)

Expression of Rh genes

In the gills, the mRNA expression levels of Rhbg, Rhcg1 and Rhcg2 were significantly increased in the catheterized

control when compared to the no-surgery control by 4-, 2-, and 4.7-fold, respectively. Similarly, gill mRNA levels of Rhbg, Rhcg1, and Rhcg2 in the NH_4HCO_3-infused fish were all elevated at 6 h and by 12 h, Rhbg was still 7-fold higher while Rhcg2 was 12-fold higher than the levels in the no-surgery controls. At 3 h, gill Rhbg mRNA in the NaCl-infused fish was 10.5-fold higher than that in the no-surgery controls (Fig. 4). There were no significant differences between the catheterized control fish and either set of infused fish.

Erythrocyte Rhag mRNA was significantly upregulated in the catheterized controls by twofold relative to the no-surgery control (Fig. 5). After 12 h of NH_4HCO_3 infusion, Rhag was significantly down-regulated by sevenfold when compared to levels in the catheterized control fish, but there was no significant difference when compared to the no-surgery control levels. Similarly, no significant change was seen in Rhag expression in the erythrocytes after 12 h of NaCl infusion when compared to that in both controls (Fig. 5). Rhcg1 and Rhcg2 are not expressed in trout erythrocytes and Rhbg is very weakly expressed (Nawata et al. 2007) therefore, these mRNAs were not analyzed.

Brain levels of Rhbg mRNA were about 50% lower in the catheterized control fish when compared to no-surgery controls, but there were no significant changes after 12 h of either NaCl or NH_4HCO_3 infusion when compared to either of the control groups (Fig. 6a). In the skin, both Rhbg and Rhcg1 mRNA were significantly elevated in the catheterized control when compared to the no-surgery control by three and twofold, respectively (Fig. 6b). Rhbg mRNA was elevated fivefold in the skin of NH_4HCO_3-infused fish at 12 h compared to the no-surgery controls. However, there were no significant differences between the catheterized control fish and either group of infused fish (Fig. 6b).

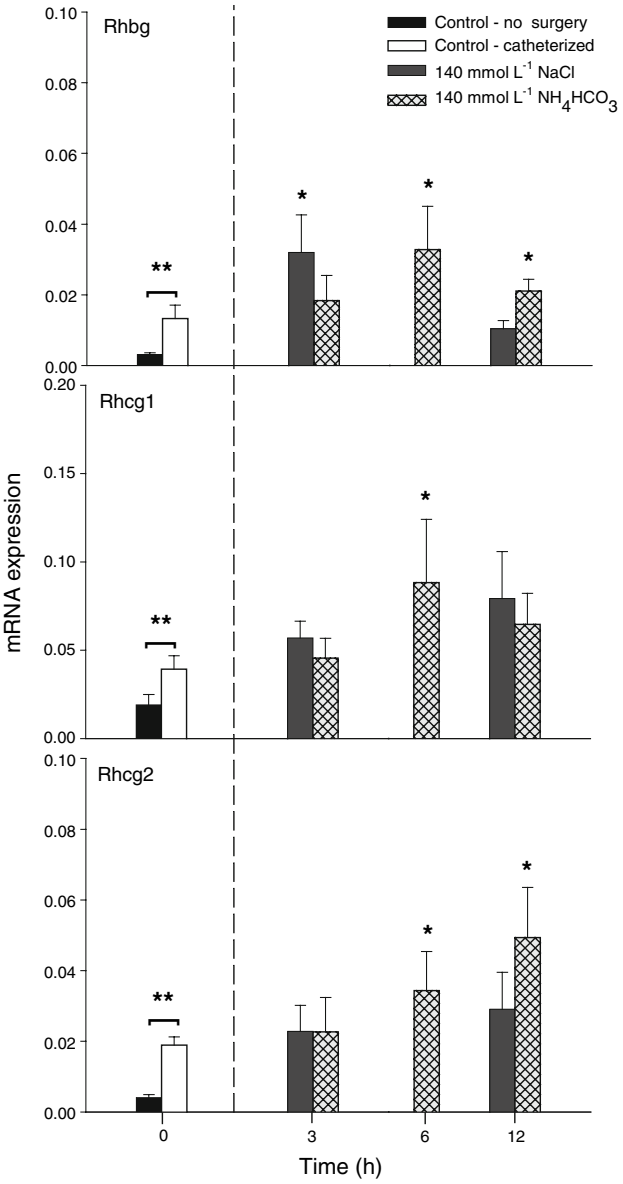

Fig. 4 Gill Rh mRNA expression in rainbow trout during 12 h of infusion with 140 mmol L^{-1} NaCl or 140 mmol L^{-1} NH_4HCO_3. The no-surgery control fish did not undergo experimental manipulation. The catheterized control fish underwent anaesthesia and surgery for catheter placement, but were not infused. Expression data were normalized to ng total RNA concentration. *Single asterisks* indicate significant difference from the no-surgery control. *Double asterisks* indicate significant difference between the two control values. No significant differences were found between the catheterized control fish and both sets of infused fish ($P < 0.05$). Data are means ± SEM ($n = 6$)

Expression of other transport genes in the gills

Catheterization alone resulted in a ten-fold elevation of gill CA2 mRNA and 3 h of NaCl infusion elevated the level to almost 28-fold over the no-surgery control (Fig. 7). Catheterization also caused a fourfold increase in gill NHE2 mRNA expression when compared to the no-surgery control (Fig. 7). At 3 h, however, NHE2 mRNA expression in the gills of the NaCl-infused fish was significantly higher than both controls while in the NH_4HCO_3-infused fish, the expression was elevated fivefold over the no-surgery control at 6 and 12 h. Gill H^+-ATPase mRNA expression was similar in both controls but 3 h of NaCl infusion resulted in a threefold increase in H^+-ATPase mRNA over that in the controls (Fig. 7). NH_4HCO_3 infusion also resulted in 3.5-fold higher levels of H^+-ATPase mRNA in the gills compared to that in the no-surgery controls at 3 and 12 h. NKA mRNA levels in the gill were similar in the no-surgery and catheterized controls (Fig. 7). However, 3 h of NaCl-infusion resulted in a 6-fold increase and 12 h of NH_4HCO_3 infusion caused a 4.5-fold increase in NKA mRNA when compared to that in both control sets of fish.

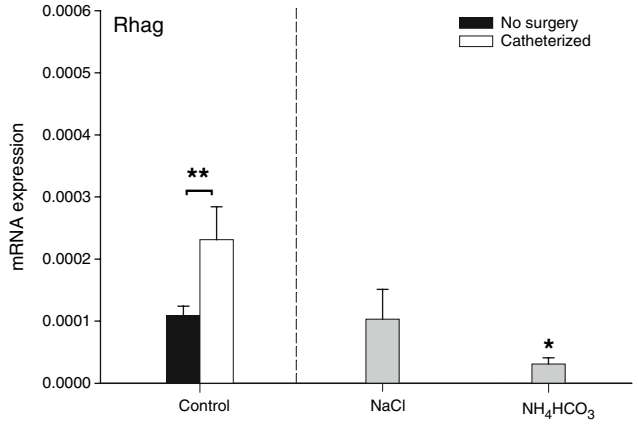

Fig. 5 Erythrocyte Rhag mRNA expression in rainbow trout after 12 h of infusion with 140 mmol L^{-1} NaCl or 140 mmol L^{-1} NH_4HCO_3. The no-surgery control fish did not undergo experimental manipulation. The catheterized control fish underwent anaesthesia and surgery for catheter placement, but were not infused. Expression data were normalized to ng total RNA concentration. The *single asterisk* indicates a significant difference from the catheterized control. *Double asterisks* indicate significant difference between the control values. There were no significant differences between the no-surgery control fish and both sets of infused fish ($P < 0.05$). Data are means ± SEM ($n = 6$)

Discussion

Overview

The recent identification of the Rh glycoprotein ammonia transporters has fueled several studies related to ammonia transport in fish (Hung et al. 2007, 2008; Nakada et al. 2007a, b; Nawata et al. 2007; Nawata and Wood 2008; Shih et al. 2008; Tsui et al. 2009). In two of those studies (Nawata et al. 2007; Nawata and Wood 2008) an association was made between high-plasma ammonia levels and changes in Rh mRNA expression in rainbow trout tissues. These high-plasma ammonia levels were achieved after trout were exposed to high-external ammonia (HEA) or after the acidified gill boundary layer was eliminated with Hepes treatment. In the present study, plasma ammonia levels were elevated in rainbow trout via infusion with NH_4HCO_3. This ammonium salt was chosen to avoid acidosis, although we have previously shown that acidosis produced under hypercapnic conditions had no effect on Rh mRNA expression (Nawata and Wood 2008). This allowed us to correlate changes in Rh mRNA levels to

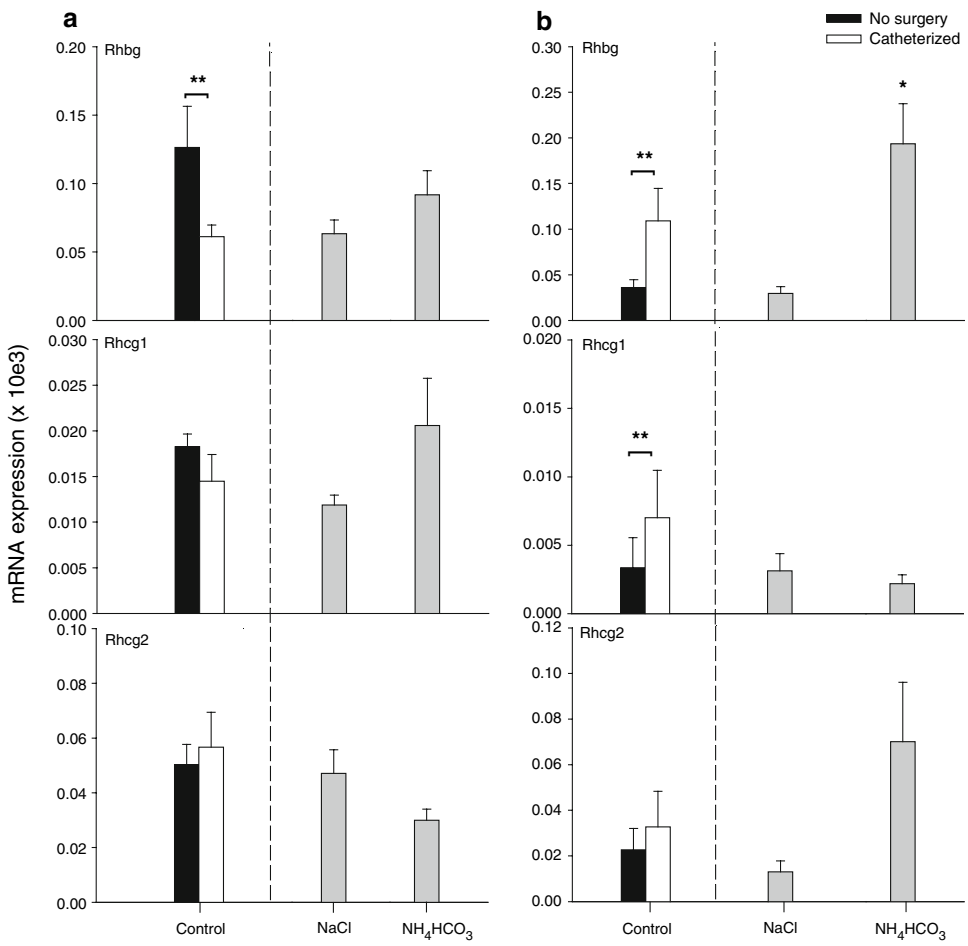

Fig. 6 Rh mRNA expression in the (**a**) brain and (**b**) skin of rainbow trout after 12 h of infusion with 140 mmol L^{-1} NaCl or 140 mmol L^{-1} NH_4HCO_3. The no-surgery control fish did not undergo experimental manipulation. The catheterized control fish underwent anaesthesia and surgery for catheter placement, but were not infused. Expression data were normalized to ng total RNA concentration. The *single asterisk* indicates a significant difference from the no-surgery control value. *Double asterisks* indicate significant difference between the control values. No significant differences were noted between the catheterized control fish and both sets of infused fish ($P < 0.05$). Data are means ± SEM ($n = 6$)

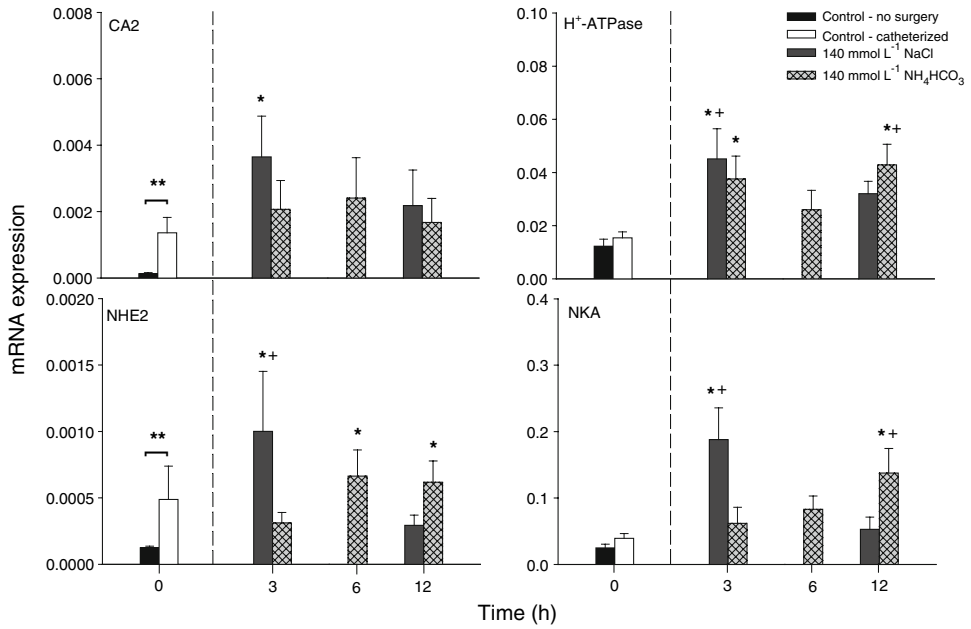

Fig. 7 The effect of 12 h of infusion with 140 mmol L^{-1} NaCl or 140 mmol L^{-1} NH_4HCO_3 on the mRNA expression of carbonic anhydrase (CA2), H^+-ATPase, NHE2, and Na^+/K^+-ATPase α-1a (NKA) in the gills of rainbow trout. The no-surgery control fish did not undergo experimental manipulation. The catheterized control fish underwent anaesthesia and surgery for catheter placement, but were not infused. Expression data were normalized to ng total RNA concentration. *Single asterisks* indicate significant differences from the no-surgery control and *crosses* indicate a significant difference from the catheterized control value. *Double asterisks* indicate a significant difference between the control values ($P < 0.05$). Data are means ± SEM ($n = 6$)

changes in plasma ammonia levels and at the same time, assess the relative impact of surgical procedures (includes anaesthesia) and infusion on the results. The results clearly show that elevated plasma ammonia levels as well as surgical procedures and infusion have marked effects on physiology and on the mRNA expression levels of Rh and other genes. However, in general, internal ammonia loading induced a similar pattern of Rh transcript changes as that seen when fish were exposed to HEA.

The influence of surgical procedures and infusion

Catheterization of the dorsal aorta under anaesthesia, followed by recovery, so as facilitate serial blood sampling and infusion of substances for physiological studies in fish has a long history (see Tashjian and Hung 2005), but the possible influence of this surgical procedure on gene expression has not been explored. From the present study, it was clear that catheterization alone (followed by 48 h recovery), without infusion, resulted in many changes in mRNA expression levels when compared to the no-surgery control. Dramatic differences were seen in Rh mRNA levels in the gill; the mRNA expressions of all three Rh genes were significantly elevated (Fig. 4). These catheterized control fish also had an elevated ammonia excretion rate (Fig. 1), suggesting that endogenous ammonia production was high. This increased ammonia excretion, possibly facilitated by an upregulation of Rh ammonia transporters, likely explains the unaltered control levels of plasma ammonia (Fig. 2).

What is not clear, however, is which component of the surgical procedure may have affected mRNA levels. Anaesthetics are known to alter the blood chemistry of fish (Iwama et al. 1989), so it is plausible that they could also elicit changes in mRNA levels. Although it is possible to study the effects of anaesthesia alone on mRNA expression, it is not possible to isolate the influence of the surgery itself since anaesthesia is a requisite part of the surgical procedure. Therefore, the effects of the surgical procedures could have been due to the anaesthesia, the surgery, or a combination of both. If indeed anaesthesia has an effect on mRNA expression levels, then timing could make a difference. A 48-h recovery period was used in the present study. In future studies, it will be worthwhile to evaluate the effects of anaesthesia alone on mRNA expression levels and plasma parameters, and also to investigate whether or not there are any correlations between the time elapsed following anaesthesia/surgery and changes in these parameters.

Studies that utilize infusion to examine physiological parameters typically incorporate isotonic NaCl infusion as a control. This causes expansion of the extracellular fluid volume and diuresis, but generally has little effect on plasma Na and Cl levels or acid-base status (e.g. Curtis and

Wood 1992; Goss and Wood 1990; Vermette and Perry 1987a, b, c). The infusion rate employed in the present study was 1.5–2-fold higher than that used in these earlier studies and it was apparent from our data that this NaCl infusion induced changes in ammonia metabolism and gene expression. Both plasma T_{Amm} and the ammonia excretion rates were elevated at most time points (Figs. 1, 2). Like the catheterized control, this suggests that endogenous ammonia production was high, but the elevated plasma ammonia levels during NaCl infusion additionally indicate that the rate of ammonia excretion was insufficient to keep up with the production of endogenous ammonia. Furthermore, the elevation in plasma ammonia was apparently not of sufficient magnitude to elicit the same changes in Rh mRNA expression as seen in the NH_4HCO_3-infused fish (Fig. 4). The only change noted was the upregulation of Rhbg mRNA at 3 h and this mRNA was also upregulated in the catheterized controls. This upregulation appears to be unrelated to plasma ammonia since a similar upregulation did not occur in the NH_4HCO_3-infused fish (Fig. 2). As with catheterization alone, NaCl infusion did not significantly elevate cortisol levels (Fig. 3).

Previous studies on trout which used lower rates of isotonic NaCl infusion, showed negligible disturbances of blood acid-base status or plasma NaCl levels (Curtis and Wood 1992; Goss and Wood 1990; Vermette and Perry 1987a, b). However, in humans at least, metabolic acidosis can result from high rates of NaCl infusion because the anion gap is reduced, i.e., HCO_3^- concentration is reduced (Constable 2003; Scheingraber et al. 1999). In fact, when we examined other gill proteins, the upregulations at 3 h of NKA and H^+-ATPase (Fig. 7) were clearly related to the NaCl-infusion itself and not to the surgical procedure since there were no differences between the two control levels of these mRNAs. Indeed Perry et al. (2006) reported that NKA mRNA and protein expression were upregulated in trout that were subjected to high-NaCl loading through the diet. Similarly, although NHE2 mRNA was upregulated in the catheterized control, it was further upregulated over this control at 3 h after NaCl-infusion, suggesting that NaCl-infusion itself stimulated the upregulation of NHE2 mRNA (Fig. 7). There was also an upregulation of CA2 at 3 h but this mRNA was also upregulated in the catheterized control, suggesting that the change may be due in part to the surgical procedures (including anaesthesia). CA2, NHE2, and H^+-ATPase have all been implicated in the branchial acid secretion mechanism of fish at the transcript level (Edwards et al. 2005; Georgalis et al. 2006; Perry et al. 2000). Therefore, the upregulations of CA2, NHE2, and H^+-ATPase mRNA in the NaCl-infused fish may be indicative of an acidosis resulting from the high rate of infusion and reduced anion gap, and/or a response to extracellular volume expansion. Overall, we conclude that despite the elevated plasma ammonia level and ammonia excretion rate, isotonic NaCl infusion resulted in changes in mRNA levels that reflected responses to the side-effects of infusion itself, rather than specific responses to ammonia.

Responses to internal ammonia loading

In trout, natural elevations in plasma ammonia that occur post-prandially are approximately threefold higher than the control level (Bucking and Wood 2008; Wicks and Randall 2002a, b). Similar elevations occur after strenuous exercise (Mommsen and Hochachka 1988; Wood 1988). Our present study was designed to elevate plasma ammonia to levels tenfold or greater above the control value, comparable to levels attained previously with HEA exposure (Nawata et al. 2007; Nawata and Wood 2008). This was done in order to determine whether or not the changes in Rh mRNA expression were in response to high-plasma ammonia, rather than to externally elevated ammonia. Although these values were unnaturally elevated and approaching levels thought to be toxic (Wilkie 2002), no mortalities occurred. Indeed it has been reported that trout survived a 7-day external exposure to 2,000 μmol L^{-1} NH_4Cl with circulating plasma ammonia levels elevated tenfold above that of the control (Tsui et al. 2009).

NH_4HCO_3 infusion resulted in the upregulations of Rhbg and Rhcg2 mRNA in the gill (Fig. 4) similar to HEA-exposure which resulted in mRNA upregulations of gill Rhcg2 after 12 h, and Rhbg in the gill pavement cells after 48 h as demonstrated previously (Nawata et al. 2007). As with the HEA-exposed fish, these upregulations appeared in conjunction with enhanced ammonia excretion (Fig. 1). Unlike the HEA-exposed fish, however, expression of Rhcg1 mRNA was also elevated in the gill (Fig. 4). It is possible that an early peak in Rhcg1 mRNA also occurred under HEA conditions but was missed during the time course of the study, or it could be a response that was specific to NH_4HCO_3 infusion. Since it only occurs after infusion of NH_4HCO_3 and not after infusion of NaCl, this suggests that it was a response related to high-plasma ammonia and not the infusion itself.

In HEA-exposed trout, the mRNA expression and activity of CA2 were decreased while the NHE2 mRNA expression remained unchanged (Nawata et al. 2007). In the current study, the mRNA expression levels of CA2 in NH_4HCO_3-infused fish remained unchanged and the NHE2 mRNA levels were significantly elevated at 6 and 12 h, relative to the no-surgery control (Fig. 7). Both of these mRNAs were elevated in the catheterized controls and therefore true levels of CA2 mRNA could have been masked by the upregulation caused by the surgical

procedures (including anaesthesia). Similarly, elevations of NHE2 may also be related to these surgical procedures and not to the infusion. It is possible, however, that at very high concentrations of plasma ammonia, NHE2 is recruited into the ammonia excretion mechanism. A recent in vitro study showed that NHE2 mRNA levels increased after cultured rainbow trout gill epithelia were pre-exposed to 2 mM NH_4Cl and 1000 ng mL^{-1} cortisol (Tsui et al. 2009). In the present study, the plasma ammonia was very close to 2 mM by 12 h of NH_4HCO_3 infusion, but in the HEA study, plasma ammonia levels only reached about half of this value (Nawata et al. 2007). Additionally, Ivanis et al. (2008) reported that NHE2 mRNA levels in rainbow trout increased in the presence of cortisol. Elevated cortisol levels were observed at 6 h of NH_4HCO_3 infusion in the present study (Fig. 3) and therefore could have also contributed to the upregulation of NHE2 mRNA.

The early elevation of H^+-ATPase mRNA at 3 h, which was also seen in the NaCl-infused fish, is likely a consequence of the infusion and not the surgical procedures, since levels in the two control sets were not significantly different from each other (Fig. 7). The later upregulation of H^+-ATPase mRNA at 12 h, however, does correspond with the elevation of H^+-ATPase activity and mRNA level seen after 12 and 48 h of HEA exposure (Nawata et al. 2007), thus reinforcing the importance of this transporter in the acid-trapping model of ammonia excretion. Finally, the upregulation of NKA mRNA at 12 h (Fig. 7) corresponds with the upregulation seen in the gill pavement cells of HEA-exposed trout after 48 h of exposure (Nawata et al. 2007).

Previously, it was shown that both Rhbg and Rhcg1 were downregulated in the brain after 48 h of HEA (Nawata et al. 2007). In the present study, the surgical procedures (including anaesthesia) resulted in a significant downregulation of Rhbg in the brain, but infusions of either NaCl or NH_4HCO_3 did not lead to significant changes (Fig. 6a). The fact that a downregulation of Rhbg occurred in the catheterized controls suggests that this change may be related to other factors, rather than high-ammonia levels. A finer time-line analysis would clarify this.

A recent report by Shih et al. (2008) confirmed the involvement of skin Rh proteins in ammonia excretion using Rhcg1 knockdown zebrafish larvae. In the present study there was an upregulation of Rhbg at 12 h in the skin of NH_4HCO_3-infused fish, and an upregulation of both Rhbg and Rhcg1 in the skin of the catheterized controls (Fig. 6b). These skin Rh expression changes were less pronounced than those during HEA exposure (Nawata et al. 2007) or Hepes exposure (Nawata and Wood 2008) in trout, or HEA exposure in the mangrove killifish (Hung et al. 2007) where Rhcg1 and/or Rhcg2 were also upregulated. This suggests that Rh gene responses in the skin may differ with treatment.

The decrease in Rhag mRNA in the erythrocytes of NH_4HCO_3-infused fish (Fig. 5) was also observed previously in erythrocytes of HEA- and Hepes-exposed fish (Nawata et al. 2007; Nawata and Wood 2008). Since the Rhag mRNA levels were actually upregulated in the catheterized controls, the downregulation that occurred after NH_4HCO_3 infusion was likely in direct response to the elevated plasma ammonia and not related to either the surgical procedures or infusion alone. We speculate that this may help protect erythrocyte respiratory functions against deleterious effects of high-plasma ammonia, an area worthy of future study. The significance of the downregulation of Rhag when plasma ammonia levels are high is not clear but the fact that it was upregulated after the surgical procedure along with the other Rh mRNAs in the gill suggests that is responding to plasma ammonia and that there may be a negative feedback response when ammonia levels reach a certain threshold.

Does cortisol play a role in Rh gene expression and ammonia excretion?

Cortisol treatment has been shown to upregulate the transcript levels of a number of genes in fish including the aforementioned NHE2 (Ivanis et al. 2008) and several genes involved in osmoregulation (Kiilerich et al. 2007; McCormick et al. 2008) but a clear relationship between cortisol levels and Rh mRNA expression was not established in earlier in vivo experiments on rainbow trout (Nawata and Wood 2008). However, in in vitro experiments with cultured trout gill epithelia pre-exposed to a combination of ammonia and cortisol, there was an upregulation of both Rhbg and Rhcg2 mRNA, while treatment with ammonia or cortisol alone failed to produce a similar effect (Tsui et al. 2009). This suggests that cortisol and elevated ammonia act synergistically, and indeed, there were synergistic effects on the ammonia permeability of the cultured epithelium. Whether or not cortisol or ammonia can independently induce changes in Rh transcript levels in vivo remains to be determined.

The time course of NH_4HCO_3 infusion used in the current study revealed a transient increase of cortisol at 6 h (Fig. 3) and this cortisol surge may have contributed to the subsequent increase in Rh mRNA seen at 6 and 12 h (Fig. 4). In the gulf toadfish, a similar transient surge in cortisol was found to be responsible for an increase in hepatic glutamine synthetase activity, mRNA, and protein (Hopkins et al. 1995; Kong et al. 2000). The transitory nature of cortisol increases could explain why a clear correlation between elevated levels of plasma ammonia (or Rh mRNA) and cortisol were not detected earlier

(Nawata and Wood 2008) despite the apparent linear relationship between plasma ammonia and cortisol levels observed by Ortega et al. (2005). Also, the fact that chronically elevated levels of cortisol do not increase plasma ammonia or ammonia excretion rates (DeBoeck et al. 2001; Hopkins et al.1995; McDonald and Wood 2004), whereas acute exposure by injection does (Chan and Woo 1978), reinforces the idea that pulses in cortisol are stimulatory in fish. Indeed, chronically elevated cortisol levels may have an autoregulatory effect, decreasing the affinity and number of glucocorticoid receptors (Shrimpton and Randall 1994).

Taken together, these observations suggest that both pulses of plasma cortisol and elevated internal ammonia levels are involved in the regulation of Rh genes at the transcript level. Future studies should also investigate Rh changes in trout following feeding or exercise events since both cortisol (Bry 1982; Milligan 1996) and plasma ammonia and ammonia excretion rates (Bucking and Wood 2008; Mommsen and Hochachka 1988; Wicks and Randall 2002a, b; Wood 1988) are naturally elevated for short periods in these situations.

Conclusions

In summary, infusion of NH_4HCO_3 resulted in elevated plasma ammonia and increased ammonia excretion rates with patterns of Rh mRNA expression in trout tissues (erythrocytes, gill, and skin) similar to that seen when plasma ammonia levels were elevated after HEA exposure. Inclusion of a no-surgery control revealed that surgical placement of the dorsal aortic catheter (and associated anaesthesia) resulted in an increase in ammonia excretion rate accompanied by the upregulation of all three Rh mRNAs in the gill. Whether or not the changes resulted from the anaesthesia itself, the surgery alone, or a combination of both these factors remains unknown. Similarly, results from NaCl infusion reflected changes from these surgical procedures as well as effects associated with the high rate of NaCl infusion alone. What is clear, however, it is that any changes associated with NH_4HCO_3 infusion would have gone undetected if either the catheterized control or the NaCl infusion were used alone as a control. Finally, the results suggest that a transient surge in cortisol along with elevated plasma ammonia may be key components in the regulation of Rh genes and the subsequent control of ammonia transport in the rainbow trout.

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