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RESEARCH ARTICLE

A nose-to-nose comparison of the physiological and molecular responses of rainbow trout to high environmental ammonia in seawater *versus* freshwater

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SUMMARY

Steelhead rainbow trout acclimated to either freshwater (FW) or seawater (SW) were exposed to high environmental ammonia (HEA, 1000 μmol l^{-1} NH_4HCO_3, pH 7.8–8.0) for 24 h. SW trout restored ammonia excretion more rapidly (3–6 h *versus* 9–12 h in FW), despite higher production rates and lower plasma pH. Plasma total ammonia levels stabilized at comparable levels below the external HEA concentration, and blood acid–base disturbances were small at both salinities. The electrochemical gradients for NH_4^+ entry ($F_{NH_4^+}$) were the same in the two salinities, but only because FW trout allowed their transepithelial potential to rise by ~15 mV during HEA exposure. Elevation of plasma [cortisol] during HEA exposure was more prolonged in SW fish. Plasma [glucose] increased in SW, but decreased in FW trout. Plasma [urea-N] also decreased in FW, in concert with elevated urea transporter (*UT*) mRNA expression in the gills. Of 13 branchial transporters, baseline mRNA expression levels were higher for *Rhcg1*, *NHE2*, *NKCC1a* and *UT*, and lower for *NBC1* and *NKA-α1a* in SW trout, whereas *NKA-α1b*, *NHE3*, *CA2*, *H⁺-ATPase*, *Rhag*, *Rhbg* and *Rhcg2* did not differ. Of the Rh glycoprotein mRNAs responding to HEA, *Rhcg2* was greatly upregulated in both FW and SW, *Rhag* decreased only in SW and *Rhcg1* decreased only in FW. *H⁺-ATPase* mRNA increased in FW whereas *NHE2* mRNA increased in SW; *NHE3* did not respond, and V-type H^+-ATPase activity declined in SW during HEA exposure. Branchial Na^+,K^+-ATPase activity was much higher in SW gills, but could not be activated by NH_4^+. Overall, the more effective response of SW trout was explained by differences in physical chemistry between SW and FW, which greatly reduced the plasma NH_3 tension gradient for NH_3 entry, as well as by the higher [Na^+] in SW, which favoured Na^+-coupled excretion mechanisms. At a molecular level, responses in SW trout showed subtle differences from those in FW trout, but were very different than in the SW pufferfish. Upregulation of *Rhcg2* appears to play a key role in the response to HEA in both FW and SW trout, and NH_4^+ does not appear to move through Na^+,K^+-ATPase.

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Key words: Rhesus glycoprotein, *Oncorhynchus mykiss*, gene expression, cortisol, transepithelial potential, PNH_3, ammonia transport, Na^+,K^+-ATPase.

INTRODUCTION

Ammonia excretion in fish has been studied since the time of Homer Smith (Smith, 1929) and August Krogh (Krogh, 1939), but the exact mechanisms remained highly controversial throughout the last century (Wood, 1993; Wilkie, 1997). However, a paradigm shift started 7 years ago with the proposal of Weihrauch and co-workers (Weihrauch et al., 2004) that specific channels, the Rhesus (Rh) proteins, were involved in ammonia excretion in crabs. Only in the last 4 years has it been realized that these Rh proteins play a crucial role in facilitating ammonia efflux across the gills of fish (Nakada et al., 2007a; Nakada et al., 2007b), especially in the face of high environmental ammonia (HEA) (Hung et al., 2007; Nawata et al., 2007). A rapid generation of new information has quickly reinforced these initial findings; Wright and Wood (Wright and Wood, 2009) and Weihrauch et al. (Weihrauch et al., 2009) provide critical reviews.

When expressed *in vitro*, these Rh proteins bind NH_4^+ but transport NH_3 (Nawata et al., 2010a). Therefore, transport of H^+ ions across the epithelium by a separate mechanism can create an acidic environment in the external boundary layer (Wilson et al., 1994), which converts NH_3 to NH_4^+, thereby sustaining the gradient for facilitated diffusion of NH_3 *via* the Rh channel. H^+ transport mechanisms may include V-type H^+-ATPases (which energize Na^+ channels), direct Na^+/H^+ exchangers, as well as CO_2 diffusion/hydration catalyzed by carbonic anhydrase (Wilson et al., 1994; Tsui et al., 2009; Wu et al., 2010). The excretion mechanism manifests as a coupling of ammonia excretion to Na^+ uptake, the scheme first proposed by Krogh (Krogh, 1939). However, as the coupling is indirect and loose, involving several interacting transporters, Wright and Wood (Wright and Wood, 2009) have described it as a 'Na^+/NH_4^+ exchange metabolon'. *In vitro*, this system is upregulated at the mRNA level by cortisol in the presence of HEA, and ammonia transport capacity is also augmented by this combined stimulus (Tsui et al., 2009). *In vivo*, circulating cortisol levels are known to rise markedly during HEA exposures (Ortega et al., 2005; Tsui et al., 2009), so this hormone is now suspected to contribute to the increased ammonia excretion capacity that occurs at this time.

Recent studies suggest that the mechanisms for dealing with HEA may differ between seawater (SW) and freshwater (FW) teleosts. The SW pufferfish *Takifugu rubripes* (Nawata et al., 2010b) regulated plasma ammonia levels and re-established ammonia excretion far more quickly than the FW trout *Oncorhynchus mykiss* (Nawata et al., 2007) in response to HEA. In FW trout, mRNA

transcript levels of *Rhcg2* and *Rhbg* were upregulated in the gills, whereas in the SW pufferfish, *Rhcg1* was upregulated and *Rhbg* and *Rhag* were downregulated, suggesting a barrier function for the latter response. There were several other molecular differences, including increased mRNA and activity of Na^+,K^+-ATPase in the pufferfish but not in the trout, though V-type H^+-ATPase expression and activities were elevated during HEA exposure in both species.

Long before it was known that Rh proteins are regulated during HEA exposure, Wilson and Taylor performed a classic nose-to-nose comparison study of the responses of rainbow trout to HEA in FW *versus* SW (Wilson and Taylor, 1992). Surprisingly, they concluded that trout faced greater difficulties in SW as indicated by a larger rise in plasma total ammonia (T_{Amm}) despite less severe plasma NH_3 tension (PNH_3) diffusion gradients and NH_4^+ concentration gradients during the same HEA exposure. They attributed this difference to a greater cation (i.e. NH_4^+) permeability through the tight junctions of the gills in SW teleosts (Evans et al., 1989; Evans et al., 2005). However, Wilson and Taylor (Wilson and Taylor, 1992) did not have the benefit of modern molecular tools, and did not measure actual ammonia fluxes, plasma cortisol, or gill Na^+,K^+-ATPase and V-type H^+-ATPase activities. They also did not record the true electrochemical potential for NH_4^+ (Kirschner, 1970) but rather just the NH_4^+ concentration gradient.

In the current investigation, we have performed a nose-to-nose comparison of rainbow trout exposed to the same HEA level in FW *versus* SW. The study was similar in concept to that of Wilson and Taylor (Wilson and Taylor, 1992), but included all of the abovementioned additional measurements and had a particular focus on the molecular responses in the gills. The mRNA expressions of 13 genes thought to be involved in ammonia and Na^+ transport were measured by qPCR. Our overall hypothesis was that there would be marked differences between the responses at the two salinities, with different mechanisms akin to those seen in earlier non-parallel studies on FW trout (Nawata et al., 2007) and SW pufferfish (Nawata et al., 2010b).

MATERIALS AND METHODS

Experimental animals

Rainbow trout (*Oncorhynchus mykiss*, Walbaum 1792) of the coastal steelhead strain [subspecies *irideus* (Gibbons 1855)], typical mass approximately 40 g, were obtained on 1 May 2010 from the Robertson Creek Hatchery (Fisheries and Oceans Canada), Port Alberni, BC, Canada, where they had been raised in FW. At Bamfield Marine Sciences Centre (BMSC) they were either held in Bamfield FW (μmol l^{-1}: Na^+ 300, Cl^- 233, K^+ 5, Ca^{2+} 144, Mg^{2+} 48; titratable alkalinity 43 μmol l^{-1}, pH 7.0–7.2) or gradually acclimated to 100% Bamfield SW (mmol l^{-1}: Na^+ 452, Cl^- 515, K^+ 9.8, Ca^{2+} 9.5, Mg^{2+} 52; titratable alkalinity 2.2 mmol l^{-1}, pH 7.9–8.1) over 2 weeks. Conditions included natural photoperiod, flow-through FW or SW, and daily satiation feeding with commercial pellets, with 7 d fasting prior to experiments. Experiments were performed in June on approximately 50 trout from each salinity. All procedures were approved by McMaster and BMSC Animal Care Committees, and conformed to Canadian Council of Animal Care guidelines. Apart from cannulation failures, only one trout died during the experiments (at 12–24 h of HEA exposure in SW), so the exposures were essentially sublethal.

Series 1: ammonia and urea fluxes during HEA exposure

FW (*N*=6) or SW trout (*N*=6) were transferred to individual flux chambers (darkened 2.5 l polyethylene containers) served with flowing FW or SW, and allowed to settle for 8–12 h. The chambers were then closed, the volume was set to 2.0 l, and the water pH was set to 7.8–8.0 using 1 mol l^{-1} KOH or HCl. This constituted a rise in pH for FW trout, and essentially no change for SW trout. For the control flux, an initial water sample was taken for ammonia and urea analysis, followed by a final sample 10–12 h later. The chamber was then flushed, and HEA exposure was started by adding 1000 μmol l^{-1} of NH_4HCO_3, the volume was reset to 2.0 l, and the pH was reset to 7.8–8.0. Water samples were taken at 0, 3, 6, 9 and 12 h, and then the chambers were flushed several times with HEA water at the correct pH and a final flux measurement was performed in the same manner over 12–24 h. Throughout all of the flux periods, water pH was periodically monitored and adjusted as required.

Series 2: blood acid–base status, transepithelial potential, plasma ammonia and trans-branchial gradients during HEA exposure

The largest trout (51–97 g) were selected for these experiments (*N*=9–11 at each salinity); nevertheless, these were still relatively small for cannulation. In preliminary trials, we found that caudal artery catheters (Wood et al., 1988) were better tolerated than traditional dorsal aortic catheters, so the former were used throughout. Surgery was performed under MS-222 anaesthesia (Syndel Laboratories, Vancouver, BC, Canada; 0.15 g l^{-1}, neutralized with 1 mol l^{-1} KOH). The catheter was filled with Cortland salmonid saline containing 50 i.u. ml^{-1} of lithium heparin (Sigma-Aldrich, St Louis, MO, USA). The fish were then placed in the same individual chambers as used in Series 1 for 24 h recovery in FW or SW (pH 7.8–8.0 in both), with water exchanges at 12 and 24 h.

Control measurements were taken 2–3 h later, and then the water was changed to HEA (1000 μmol l^{-1} NH_4HCO_3, pH 7.8–8.0). Experimental measurements were taken at 4, 12, and 24 h, though to minimize blood loss, experimental samples were not taken at all times in most fish. Water was changed after 12 h. At each sampling time, transepithelial potential (TEP) was measured first, and then blood (300 μl) was sampled via the catheter for acid–base status. Red blood cells were resuspended in non-heparinized saline and re-infused to maintain hematocrit. Plasma was immediately separated by centrifugation (5000 ***g***, 30 s), frozen in liquid N_2, and stored at –80°C for later analysis of plasma total ammonia ([T_{Amm}]). A water sample was taken for [T_{Amm}] and pH measurements to allow precise calculation of trans-branchial PNH_3 and electrochemical NH_4^+ gradients.

Series 3: molecular responses, enzymatic activities and additional plasma parameters during HEA exposure

FW and SW trout were treated exactly as in Series 1. However, at each sampling time (control, 4, 12 and 24 h HEA), fish (*N*=5–9) were killed for additional plasma measurements and collection of gill tissue for molecular analyses. A water sample was taken for measurement of pH and [T_{Amm}], then a slurry of pH-adjusted MS-222 (to yield a final concentration of 0.15 g l^{-1}) was added to each chamber. The fish (unconscious but still ventilating) was then irrigated on an operating table with FW or SW, as appropriate, containing HEA and 0.15 g l^{-1} MS-222, so as to maintain exposure conditions right up until death. A blood sample was drawn by caudal puncture, and then 0.2 ml of 1000 i.u. ml^{-1} lithium heparin in saline was injected. After 5 min, the ventral aorta was exposed and cannulated with PE60. Ten millilitres of ice-chilled saline containing 10 i.u. ml^{-1} of lithium heparin were then perfused through the gills to wash out the red blood cells. The cleared gills were removed and blotted. One portion was added to five volumes of RNA*later*™ (Ambion Inc., Austin, TX, USA) and stored at 4°C for later

molecular analyses, while the other was flash-frozen in liquid N_2 and stored at –80°C for later enzymatic assays. Plasma was immediately separated by centrifugation (5000 ***g***, 30 s), aliquotted into several vials, frozen in liquid N_2, and stored at –80°C for later analysis of plasma [T_{Amm}], [urea-N], [cortisol], [glucose], [lactate], [Na^+] and [K^+].

Physiological analyses and calculations

TEP was measured by means of 3 mol l^{-1} KCl-agar bridges connected *via* Ag/AgCl electrodes to a high impedance electrometer (Radiometer pHM 84 meter, Copenhagen, Denmark). The measurement bridge was connected to the blood catheter, and the reference bridge was placed in the water in the fish chamber. TEP values (mV) were expressed relative to the water side as 0 mV after correction for junction potential.

Blood samples for acid–base status were drawn into gas-tight Hamilton syringes. Plasma and water pH were measured using Radiometer GK401C electrodes calibrated with precision buffers (Radiometer). The blood electrode was seated in a custom-made chamber at 12°C. True plasma total CO_2 was measured using a Corning model 965 analyzer (Lowell, MA, USA). Arterial plasma carbon dioxide tensions ($PaCO_2$) and bicarbonate concentrations ([HCO_3^-]a) were calculated using the Henderson–Hasselbalch equation with α_{CO_2} (solubility) and pK_{app} (apparent pK) values at 12°C according to Severinghaus (Severinghaus, 1956) and Boutilier et al. (Boutilier et al., 1984).

Water [T_{Amm}] was determined by the salicylate hypochlorite method (Verdouw et al., 1978) with standards made up in FW or SW. Plasma [T_{Amm}] was measured using a kit (Raichem, San Diego, CA, USA) based on the glutamate dehydrogenase/NAD method. The two methods were cross-validated in both FW and SW. Plasma and water [NH_3], [NH_4^+] and PNH_3 were calculated from [T_{Amm}] and pH measurements using the Henderson–Hasselbalch equation with appropriate pK' (operational pK) and α_{NH_3} (solubility) values for trout plasma, FW and SW at 12°C from Cameron and Heisler (Cameron and Heisler, 1983). [Urea-N] in water and plasma was determined by the diacetyl monoxime method (Rahmatullah and Boyde, 1980).

Flux rates of total ammonia (J_{Amm}) and urea ($J_{Urea\text{-}N}$) were calculated from changes of concentration in the closed chambers, factored by fish mass, volume and time. A negative J_{Amm} indicates a net excretion and a positive J_{Amm} indicates a net uptake of ammonia into the fish.

The PNH_3 gradient across the gills (ΔPNH_3) was calculated as:

$$\Delta PNH_3 = P_{out}NH_3 - P_{in}NH_3, \quad (1)$$

where $P_{in}NH_3$ is the PNH_3 in the plasma and $P_{out}NH_3$ is the PNH_3 in the outside water. A positive value of ΔPNH_3 will tend to drive NH_3 into the fish whereas a negative value will drive NH_3 out of the fish.

The Nernst potential for NH_4^+ ($E_{NH_4^+}$) was calculated as:

$$E_{NH_4^+} = \frac{RT}{zF} \ln \frac{[NH_4^+]_{out}}{[NH_4^+]_{in}}, \quad (2)$$

where z is the valence, R is the gas constant, T is the absolute temperature, F is Faraday's constant, and $[NH_4^+]_{in}$ and $[NH_4^+]_{out}$ are the concentrations of NH_4^+ in the blood plasma and the outside water, respectively (Kirschner, 1970).

The true electrochemical potential or net driving force ($F_{NH_4^+}$) for NH_4^+ across the gills was calculated as:

$$F_{NH_4^+} = E_{NH_4^+} - TEP. \quad (3)$$

A positive value of $F_{NH_4^+}$ will tend to drive NH_4^+ into the fish whereas a negative value will drive NH_4^+ out of the fish.

Plasma [cortisol] was measured using a commercial ^{125}I radioimmunoassay kit (CA-1529 GammaCoat™, DiaSorin Inc., Stillwater, MN, USA). Plasma [Na^+] and [K^+] were measured by flame atomic absorption spectroscopy (Varian SpectrAA-220FS, Mulgrave, Australia). Commercial clinical micrometers, calibrated with standards made up in Cortland saline, were used for assay of plasma [glucose] (Accu-Chek GT Compact Plus™, Roche Diagnostics, Mannheim, Germany) and [lactate] (Lactate Pro™ LT-1710, Arkray Inc., Kyoto, Japan).

Molecular analyses

Total RNA was extracted from the gills of Series 3 fish (uncannulated) with Trizol (Invitrogen, Burlington, ON, Canada) and quantified on a Nanodrop spectrophotometer (ND-1000; Nanodrop Technologies, Wilmington, DE, USA). 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). Using the above-described cDNA, the mRNA expressions of carbonic anhydrase 2 (*CA2*), V-type proton ATPase, B subunit (*H^+-ATPase*), sodium bicarbonate co-transporter 1 (*NBC1*), sodium hydrogen exchanger 2 (*NHE2*), sodium hydrogen exchanger 3 (*NHE3*), sodium potassium two chloride co-transporter 1a (*NKCC1a*), sodium potassium ATPase isoform α1a (*NKA α1a*), sodium potassium ATPase isoform α1b (*NKA α1b*), four Rhesus glycoproteins (*Rhag*, *Rhbg*, *Rhcg1* and *Rhcg2*) and the urea transporter (*UT*) were quantified (see Table 1 for gene accession numbers and primers, all of which are for rainbow trout). Real-time PCR reactions were performed using Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen) and products were sequenced to verify primer specificity. Analyses were performed on an Mx3000P QPCR System (Stratagene, Cedar Creek, TX, USA). Melt-curve analysis further confirmed production of a single product and no-template controls were run in parallel. Values were extrapolated from standard curves generated by serial dilution of one random sample. For normalization, elongation factor-1α (*EF-1α*) expression was measured in all samples, and did not vary significantly with salinity or experimental treatment.

Enzymatic analyses

Na^+,K^+-ATPase and V-type H^+-ATPase activities were measured in perfused gills from the control and 24 h HEA samples of Series 3 using methods from McCormick (McCormick, 1993) and Lin and Randall (Lin and Randall, 1993), respectively, as modified by Nawata et al. (Nawata et al., 2007). Protein concentrations were measured with Bradford Reagent and BSA standards (Sigma-Aldrich). In order to test whether NH_4^+ could activate trout branchial Na^+,K^+-ATPase, various concentrations of NH_4^+ (as NH_4Cl) were added to the assay medium to either compete with or replace the native concentrations of K^+ (as KCl) in the buffers (see Results).

Statistical analyses

Data are expressed as means ± 1 s.e.m. (N), where N is the number of fish. In Series 1 and 2, a repeated-measures ANOVA was followed by a Dunnett's paired multiple comparison test to detect specific differences within a treatment group (FW or SW) relative to the pre-exposure control value. In Series 3, groups at each sample time were independent, so regular ANOVA followed by the Bonferroni test was used to detect values that were significantly different from the pre-exposure control. qPCR, V-type H^+-ATPase and Na^+,K^+-ATPase activity data were analyzed using ANOVA

Table 1. Primer list and accession numbers for mRNA transcripts assayed by qPCR

Primer name	Forward/reverse sequence (5′–3′)	Accession no.
CA2[1]	gccagtctcccattgacatc/cctgtacgtccctgaaatgg	AY514870
EF-1α[1]	ggaaagtcaaccaccacag/gataccacgctccctctcag	AF498320
HATP[1]	tcagccttggttgtgagatg/caacattggtgggaaacagg	AF14002
NBC1	ttggaggttaggttccgatg/tcactgacagccagatcgag	AF434166
NHE2[1]	tatggccattgtgacctgtg/caggcctctccacactaagg	EF446605
NHE3[2]	agagcagccgtgacagaact/aaccagcacaaccacctctc	EF446606
NKAα1a[1]	ttgacctggatgaccacaag/ggatctccttagcccgaac	AY319391
NKAα1b[1]	tataagctggtggcgacctc/ggtcatccaggtcaacttcc	AY319390
NKCC1a	tatcagcttgtccccccagag/aactttgtggatccgagtgg	DQ864492
Rhag[1]	ctggcggccaatgatgttg/atggcgaagaggtcagagtg	EF667352
Rhbg[1]	cgacaacgacttttactaccgc/gacgaagccctgcatgagag	EF051113
Rhcg1[1]	catcctcagcctcatacatgc/tgaatgacagacggagccaatc	DQ431244
Rhcg2[1]	cctcttcggagtcttcatc/ctatgtcgctggtgatgttg	AY619986
UT[3]	gtataggccaggtgtatggg/gatcgcctcaaatggagctg	EF688013

Primers previously published by: [1]Nawata et al. (Nawata et al., 2007), [2]Ivanis et al. (Ivanis et al., 2008) and [3]Hung et al. (Hung et al., 2008).

followed by a Fisher's least significant difference *post hoc* test. In all series, specific differences between treatments at the same sampling times were evaluated using a Student's unpaired *t*-test. Paired or unpaired *t*-tests, as appropriate, were also used to evaluate some of the enzyme activity data. All tests were two-tailed and a significance level of 0.05 was used throughout.

RESULTS

Series 1

The control ammonia excretion rate (J_{Amm}) was 80% greater in SW than in FW trout. Upon exposure to HEA, there was an immediate significant reversal of J_{Amm} to positive values (i.e. net ammonia uptake) in both groups (Fig. 1A,B). This reversal was approximately twice as large in SW trout, but was more quickly corrected, such that rates not significantly different from control were re-established after 3 h (Fig. 1B). In contrast, net ammonia uptake (i.e. positive J_{Amm}) was maintained until 9 h in FW trout (Fig. 1A). By 12–24 h, J_{Amm} was again significantly greater in SW than in FW. Despite their higher baseline rates of J_{Amm}, cumulative ammonia loading over 24 h tended to be less in the SW than in FW trout [7670±2352 μmol kg^{-1} (6) *versus* 10,093±2692 μmol kg^{-1} (6), n.s.].

Urea-N fluxes were low (30% of J_{Amm}) and could only be resolved over 12 h periods (Table 2). As with J_{Amm}, control $J_{Urea\text{-}N}$ was significantly higher in SW than in FW. $J_{Urea\text{-}N}$ fell significantly during the first 12 h of HEA exposure in the SW trout; there were no other significant differences.

Series 2

Control plasma pH (pHa) was significantly higher in FW than in SW trout by approximately 0.1 unit, and this difference was maintained during HEA exposure (Fig. 2A). Both groups responded with a rise in pHa of approximately 0.1 unit, significant only in FW trout. By 24 h, pHa fell below control levels in SW fish, but this did not occur in FW animals.

The higher pHa in FW was explained by slightly lower $PaCO_2$ (Fig. 2B) and slightly higher [HCO_3^-]a (Fig. 2C), neither of which was significant by itself at most time points. The rise in pHa at 4 h (Fig. 2A) was associated with a rise in [HCO_3^-]a, indicative of slight

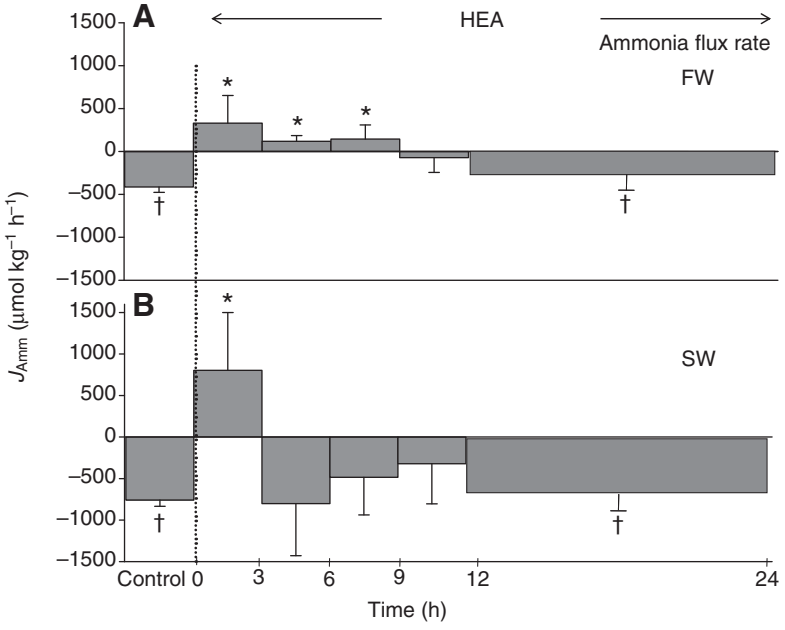

Fig. 1. Net ammonia flux rates (J_{Amm}) in (A) freshwater (FW) trout and (B) seawater (SW) trout prior to and during 24 h high environmental ammonia (HEA) exposure in Series 1. Data are means ± 1 s.e.m. (N=6 in both groups). Asterisk indicates a significant difference (P<0.05) from the pre-exposure control rate in the same group; dagger indicates a significant difference (P<0.05) between the two groups at the same time period.

Table 2. Net urea-N flux rates ($J_{Urea\text{-}N}$; μmol N kg^{-1} h^{-1}) in freshwater (FW) and seawater (SW) trout prior to and during 24 h high environmental ammonia (HEA) exposure in Series 1

	Control	0–12 h HEA	12–24 h HEA
FW	–149±13	–146±21	–203±50
SW	–209±13†	–155±15*	–211±22

Data are means ± 1 s.e.m. (*N*=6).
*Significant difference (*P*<0.05) from the pre-exposure control rate in the same group; †significant difference (*P*<0.05) between the two groups at the same time period.

metabolic alkalosis, significant only in the FW treatment (Fig. 2C). The fall in pHa at 24 h in SW trout (Fig. 2A) was correlated with a significant increase in $PaCO_2$ (Fig. 2B) and further increase in [HCO_3^-]a (Fig. 2C), indicative of respiratory acidosis.

Under control conditions, TEP was negative in FW but slightly positive in SW trout, a highly significant difference [–16.3±1.7 mV (11) *versus* +2.5±0.4 mV (9)]. Upon exposure to HEA, TEP did not change in SW, but FW trout allowed their TEP to rise substantially, reaching values not significantly different from either 0 mV or the mean TEP in SW by 24 h (Fig. 3A).

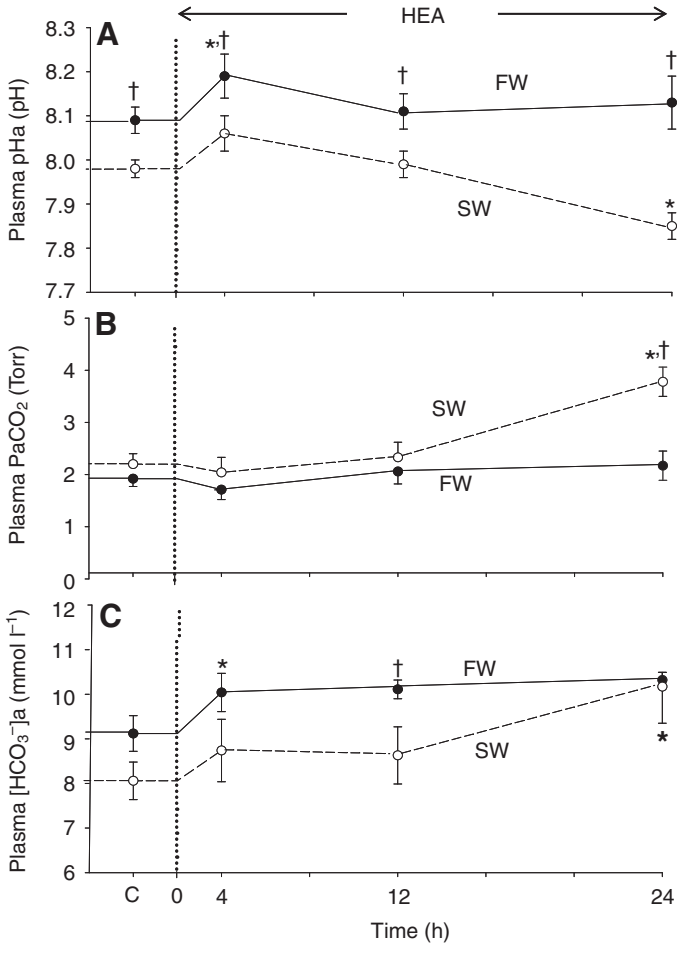

Fig. 2. Arterial blood plasma acid–base status in FW and SW trout prior to and during 24 h HEA exposure in Series 2. (A) Arterial pH (pHa). (B) Arterial CO_2 tension ($PaCO_2$). (C) Arterial plasma bicarbonate concentration ([HCO_3^-]a). Data are means ± 1 s.e.m. (for both groups, *N*=9–11 under control conditions and *N*=5–9 under HEA exposure). C, control. Other details as in legend of Fig. 1.

Control plasma T_{Amm} concentrations were similar (~160 μmol l^{-1}) in the two groups. Upon HEA exposure, they increased sharply at 4 h, then more slowly thereafter. The increases were greater in the FW trout, a difference that was significant at 12 h (Fig. 3B). By 24 h, plasma [T_{Amm}] reached approximately 725 and 820 μmol l^{-1} in SW and FW trout, respectively, still significantly below simultaneously measured water [T_{Amm}] (~1040 μmol l^{-1}, paired *t*-test). These measurements allowed calculation of water ($P_{out}NH_3$) and blood plasma ($P_{in}NH_3$) NH_3 tensions (Fig. 4) and $E_{NH_4^+}$ (Fig. 5).

Prior to HEA exposure, $P_{in}NH_3$ (~60 μTorr; 1 μTorr=133×10^{-6} Pa) was significantly greater than $P_{out}NH_3$ (~15 μTorr), such that there was a negative ΔPNH_3 (Eqn 1) of approximately –45 μTorr driving NH_3 out of the fish in both groups. During HEA exposure, $P_{out}NH_3$ rose to approximately 460 μTorr in FW (Fig. 4A), but to only 170 μTorr in SW (Fig. 4B), a highly significant difference.

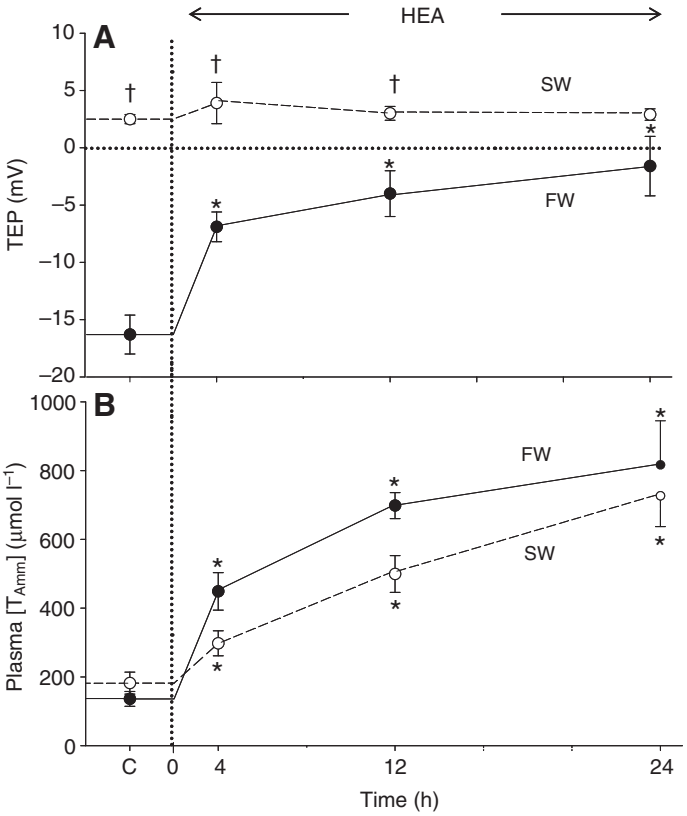

Fig. 3. (A) Transepithelial potential (TEP) across the gills (relative to the outside water as 0 mV) and (B) total arterial plasma ammonia concentration (T_{Amm}) in FW and SW trout prior to and during 24 h HEA exposure in Series 2. Data are means ± 1 s.e.m. (*N* as in legend of Fig. 2). C, control. Other details as in legend of Fig. 1.

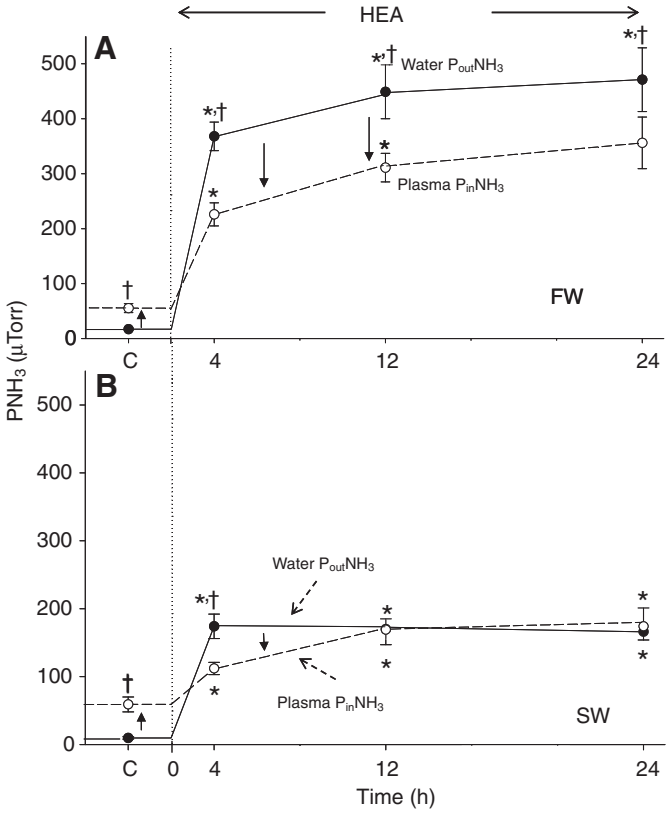

Fig. 4. Partial pressures of NH_3 in the external water ($P_{out}NH_3$) and the arterial blood plasma ($P_{in}NH_3$) in (A) FW and (B) SW trout prior to and during 24 h HEA exposure in Series 2. The direction of the ΔPNH_3 gradient ($P_{out}NH_3$–$P_{in}NH_3$) is indicated by arrows; a positive value of ΔPNH_3 tends to drive the influx of NH_3 into the fish whereas a negative value tends to drive the efflux of NH_3 out of the fish. Data are means ± 1 s.e.m. (*N* as in legend of Fig. 2). Asterisk indicates a mean significantly different ($P<0.05$) from the pre-exposure control value for the same parameter; dagger indicates a significant difference ($P<0.05$) between $P_{out}NH_3$ and $P_{in}NH_3$ at the same time point. C, control.

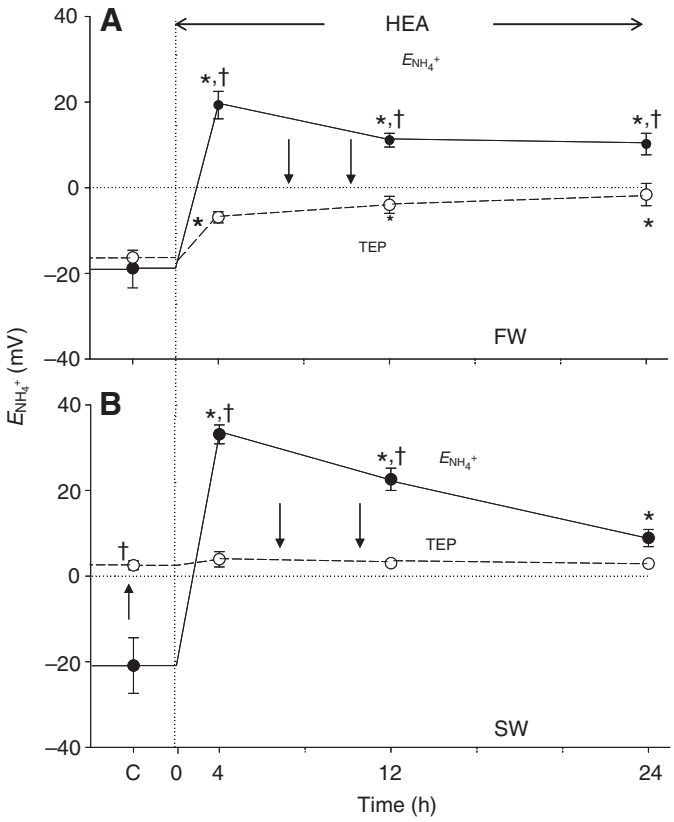

Fig. 5. Nernst potentials for NH_4^+ ($E_{NH_4^+}$) between the arterial blood plasma and the external water in (A) FW trout and (B) SW trout prior to and during 24 h HEA exposure in Series 2. The TEP is also shown. The direction of the $F_{NH_4^+}$ gradient ($E_{NH_4^+}$–TEP) is indicated by arrows; a positive value of $F_{NH_4^+}$ tend to drive the influx of NH_4^+ into the fish whereas a negative value tends to drive the efflux of NH_4^+ out of the fish. Data are means ± 1 s.e.m. (*N* as in legend of Fig. 2). Asterisk indicates a mean significantly different ($P<0.05$) from the pre-exposure control value for the same parameter; dagger indicates a significant difference ($P<0.05$) between $E_{NH_4^+}$ and TEP at the same time point. C, control.

In FW fish, plasma $P_{in}NH_3$ rose in parallel to $P_{out}NH_3$, but stabilized at approximately 350 μTorr, significantly below the simultaneous $P_{out}NH_3$. Therefore, ΔPNH_3 stabilized at approximately +110 μTorr, which would tend to drive NH_3 into the FW fish (Fig. 4A). The responses were very different in SW (Fig. 4B). Plasma $P_{in}NH_3$ increased significantly but stabilized at a much lower value, the same as $P_{out}NH_3$, at 12 and 24 h. Thus there was only a small, inwardly directed ΔPNH_3 (+55 μTorr) at 4 h, which decreased to 0 μTorr at 12 h and 24 h.

In Fig. 5, the Nernst potentials ($E_{NH_4^+}$) are plotted together with TEP values to illustrate the two components of the net driving force on NH_4^+ ($F_{NH_4^+}$). Control $E_{NH_4^+}$ was approximately –20 mV in both groups, almost identical to the TEP (~–16 mV) in FW fish (Fig. 5A), but far below that (~+3 mV) in SW animals (Fig. 5B). Therefore, there was a negligible $F_{NH_4^+}$ in FW trout, but a highly negative $F_{NH_4^+}$ (~–29 mV) favouring NH_4^+ efflux in SW trout. Upon exposure to HEA, $E_{NH_4^+}$ rose greatly to positive values, though to a greater initial extent in SW (~+33 mV) than in FW trout (~+19 mV), reflecting the slower rise in plasma [T_{Amm}] in these fish (Fig. 4). As these values were far above TEP, there were strong positive $F_{NH_4^+}$ gradients tending to drive NH_4^+ into the fish at both salinities at 4 h (+26 to +29 mV). Thereafter, $E_{NH_4^+}$ stabilized in FW (Fig. 5A) but fell in SW (Fig. 5B). Note that by allowing their TEP to rise, FW trout were able to attenuate the increase in $F_{NH_4^+}$ by approximately 15 mV, bringing it to a level similar to that in SW fish.

Series 3

Plasma parameters

Here the trout were not cannulated, and samples were taken by caudal puncture after rapid anaesthetization. Control plasma [T_{Amm}] was virtually identical (~70 μmol l^{-1}) in FW and SW trout. Again the initial increase during HEA was greater in FW than in SW trout, significant at 4 h (Fig. 6A). However, thereafter, values tended to stabilize (SW) or fell significantly from 12 to 24 h (FW) rather than continuing to rise, in contrast to the cannulated trout of Series 2 (cf. Fig. 3B). At 24 h, there was no significant difference between FW (~235 μmol l^{-1}) and SW values (~335 μmol l^{-1}) of plasma [T_{Amm}] (Fig. 6A) and these remained far below simultaneously measured water [T_{Amm}] (~1000 μmol l^{-1}).

In light of the unexpected fall in plasma [T_{Amm}] at 24 h only in the FW group (Fig. 6A), which was not observed in the cannulation series (Fig. 3B), we performed another 24 h HEA exposure, again sampling by caudal puncture. A difference in [T_{Amm}] was not seen

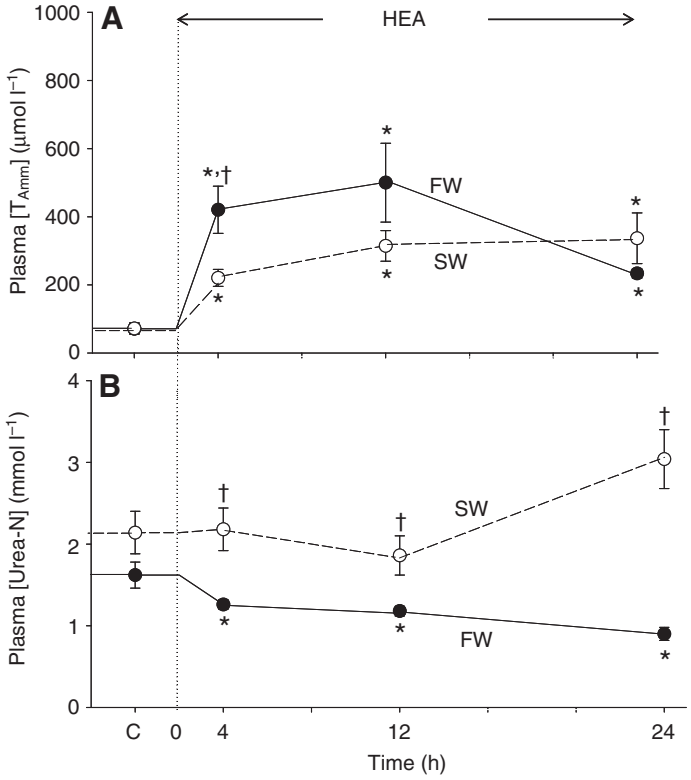

Fig. 6. (A) Total plasma ammonia concentrations (T_{Amm}) and (B) plasma urea-N concentrations in FW and SW trout prior to and during 24 h HEA exposure in Series 3. Samples were taken by caudal puncture. Data are means ± 1 s.e.m. (*N*=6). C, control. Other details as in legend of Fig. 1.

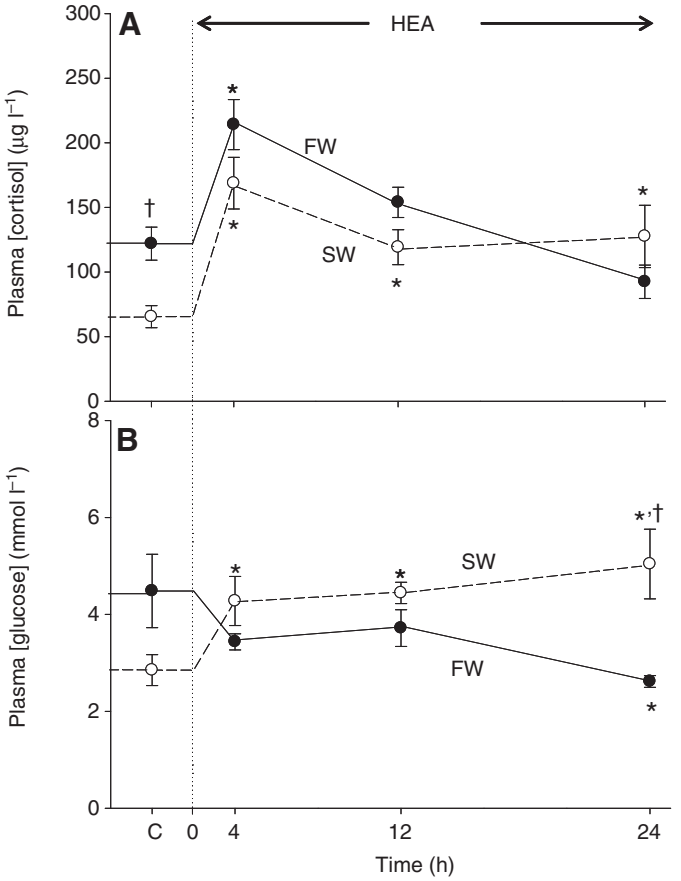

Fig. 7. (A) Plasma cortisol and (B) glucose concentrations in FW and SW trout prior to and during 24 h HEA exposure in Series 3. Samples were taken by caudal puncture. Data are means ± 1 s.e.m. (*N*=6). Other details as in legend of Fig. 1.

in this test [FW 406±66 μmol l^{-1} (6) *versus* SW 483±47 μmol l^{-1} (6), n.s.], so the conservative conclusion is that plasma [T_{Amm}] stabilizes after 24 h of HEA exposure at similar levels in FW and SW trout.

Control plasma [urea-N] (~2 mmol l^{-1}) was about 30-fold higher than plasma [T_{Amm}], and did not differ significantly between FW and SW trout. However, upon exposure to HEA, [urea-N] fell progressively in FW fish, reaching 55% of control values by 24 h (Fig. 6B). Plasma [urea-N] did not change significantly during HEA in SW, but tended to rise to values threefold greater than in FW animals by 24 h. These differences were confirmed by the second 24 h HEA experiment [FW 1.06±0.04 mmol N l^{-1} (6) *versus* SW 2.32±0.25 mmol N l^{-1} (6), $P<0.001$].

The ΔPNH_3 and $F_{NH_4^+}$ gradients in this series were also calculated using measured water pH, water [T_{Amm}] and plasma [T_{Amm}] in combination with plasma pH and TEP data from Series 2; the results are shown in supplementary material Fig. S1. Overall trends were qualitatively similar to those of Series 2 (cf. Figs 4, 5). ΔPNH_3 values were negative under control conditions, favouring NH_3 efflux, but became highly positive during HEA exposure in FW fish, fluctuating between +100 and +250 μTorr, thereby favouring NH_3 influx (supplementary material Fig. S1A). In contrast, in SW fish, ΔPNH_3 reached only +60 μTorr at 4 h, and thereafter was not significantly different from 0 μTorr at 12 and 24 h. Under control conditions, $F_{NH_4^+}$ values were below 0 mV in both treatments, with significantly more negative values in SW trout favouring NH_4^+ efflux (supplementary material Fig. S1B). During HEA exposure, $F_{NH_4^+}$ switched over to positive values (+22 to +35 mV) that were virtually identical at all times in FW and SW animals, thereby tending to drive NH_4^+ influx.

Control plasma [cortisol] was twofold higher in FW than in SW trout, but during HEA exposure, immediately increased to similar concentrations (~200 μg l^{-1}) in the two groups, thereafter gradually declining (Fig. 7A). By 24 h, [cortisol] had returned to control levels in FW fish, but remained significantly elevated in SW trout.

Control plasma [glucose] did not differ significantly between FW and SW trout (Fig. 7B). However, during HEA exposure, levels increased in SW but decreased in FW fish, resulting in a highly significant difference by 24 h. There were no significant differences in plasma [lactate], but plasma [Na^+] and [K^+] were consistently higher by 5–20% in SW trout (Table 3). During HEA, [Na^+] increased at 4 h in SW, but fell significantly at this time in FW animals; there were no other differences. In contrast, [K^+] increased at both 4 and 24 h in both treatments but to a much greater extent in SW trout.

Molecular responses

Branchial mRNA expression of *Rhag* was approximately two orders of magnitude lower than the other three Rh glycoproteins *(Rhbg*, *Rhcg1* and *Rhcg2*; Fig. 8). *Rhag* expressions were comparable at the two salinities under control conditions and did not change during HEA exposure in FW trout (Fig. 8A). However, *Rhag* mRNA declined progressively in SW trout, a response (55% fall) that was significant by 24 h. *Rhbg* expressions were also comparable under control conditions, but during HEA exposure tended to

Table 3. Mean concentrations of lactate, Na^+ and K^+ ($mmol\,l^{-1}$) in the plasma of FW and SW trout prior to and during 24 h HEA exposure in Series 3

	Control	4 h HEA	12 h HEA	24 h HEA
Lactate FW	0.82±0.02	1.17±0.17	1.08±0.18	1.00±0.16
Lactate SW	0.80±0.01	0.92±0.09	0.97±0.10	0.80±0.01
Na^+ FW	158±2	151±1*	153±3	161±2
Na^+ SW	167±6	186±6*,†	171±5†	173±6
K^+ FW	2.58±0.05	3.16±0.18*	2.59±0.09	2.85±0.10*
K^+ SW	2.82±0.09†	3.74±0.21*	3.08±0.13†	3.84±0.30*,†

Data are means ± 1 s.e.m. (N=5–9).
*Significant difference (P<0.05) from the pre-exposure control mean in the same group; †significant difference (P<0.05) between the two groups at the same time period.

increase in FW but decrease in SW trout, resulting in significant twofold differences at 12 and 24 h (Fig. 8B). *Rhcg1* expression was initially threefold higher in FW trout, but quickly declined during HEA exposure to levels that were not significantly different from those in SW animals from 4 h onwards; there were no changes in the latter (Fig. 8C). *Rhcg2* levels tended to be higher in SW animals, a difference that became significant at 12 and 24 h of HEA as expression more than doubled in both salinities (Fig. 8D).

For nine other branchial transport proteins, baseline mRNA expression levels varied by less than one order of magnitude (Fig. 9A,E). Under control conditions, mRNA expression levels were higher in SW trout for *NHE2* (Fig. 9D), *NKCC1a* (Fig. 9H) and *UT* (Fig. 9I), but higher in FW trout for *NBC1* (Fig. 9C) and *NKA-α1a* (Fig. 9F).

During HEA exposure, *CA2* expression remained unchanged in FW but fell slightly in SW fish, resulting in a significant difference at 12 h (Fig. 9A). *V-type H^+-ATPase* expression gradually increased in FW trout, a difference that became significant at 24 h, whereas there was a small decline in SW trout only at 4 h (Fig. 9B). There were no changes in *NBC1* mRNA levels during HEA exposure, but expression remained threefold to fivefold higher in SW animals throughout (Fig. 9C). *NHE2* mRNA exhibited a small increase only at 4 h in FW trout, but in SW, there was a progressive increase, with levels more than twofold higher than in FW at 24 h (Fig. 9D). In contrast, *NHE3* exhibited no significant changes (Fig. 9E). The twofold to fivefold higher *NKA-α1a* expression in FW fish tended to decline during HEA exposure, and was significant at 12 h; levels remained unchanged in SW trout (Fig. 9F). *NKA-α1b* mRNA also fell significantly at 12 h in FW, but tended to decline in SW trout as well, a change which became significant at 24 h; expression was twofold higher in FW animals at 4 and 24 h of HEA exposure (Fig. 9G). *NKCC1a* expression was unaltered during HEA exposure at either salinity, but remained 5-fold to 10-fold higher in SW trout at all times (Fig. 9H). *UT* mRNA in FW fish exhibited one of the most dramatic responses to HEA exposure (Fig. 9I). Initially less than 20% of SW values, *UT* expression increased progressively (significant at all time points) reaching levels comparable to those of SW trout by 24 h. *UT* expression did not change in response to HEA exposure in SW animals.

Enzymatic responses

V-type H^+-ATPase activity was identical in FW and SW trout gills under control conditions (Fig. 10A). However, after 24 h HEA exposure, activity declined by 40% in SW, but remained unchanged in FW animals. Depending upon assay conditions, branchial Na^+,K^+-ATPase activity was 3-fold to 10-fold higher in SW trout than in FW trout (Fig. 10B). There were no significant changes following 24 h HEA exposure in any of the assays.

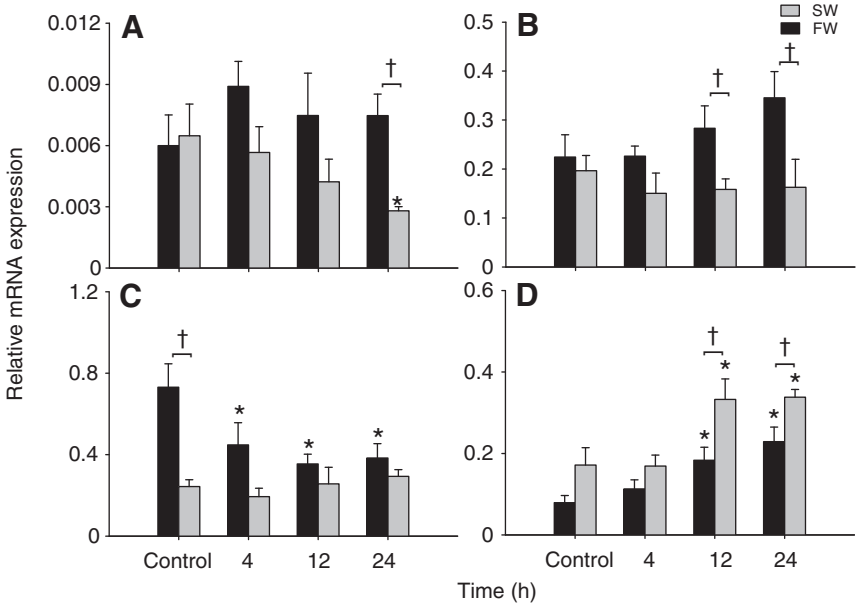

Fig. 8. Branchial mRNA expression levels of four Rh glycoproteins in FW and SW trout prior to and during 24 h HEA exposure in Series 3. (A) *Rhag*, (B) *Rhbg*, (C) *Rhcg1* and (D) *Rhcg2*. All data were normalized to the expression of elongation factor 1α (*EF-1α*), which did not vary with salinity or HEA exposure. Data are means ± 1 s.e.m. (N=6). Asterisk indicates a mean significantly different (P<0.05) from the pre-exposure control value in the same group; dagger indicates a significant difference (P<0.05) between the two groups at the same time point.

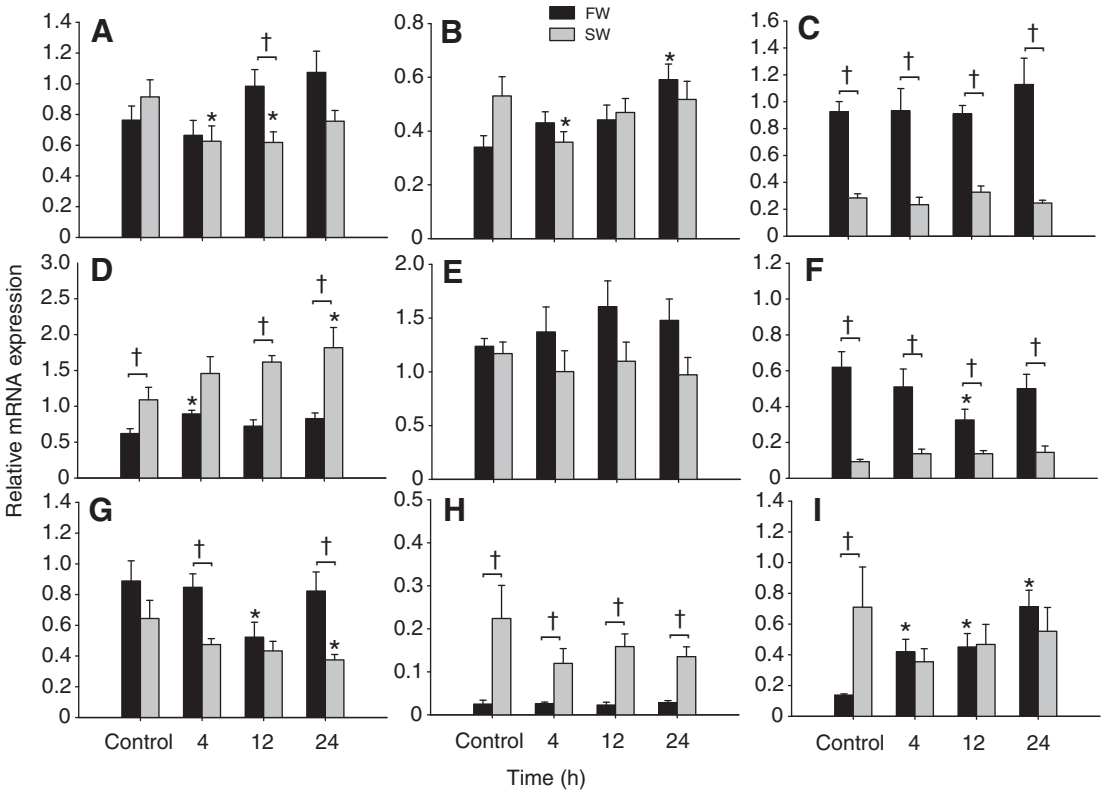

Fig. 9. Branchial mRNA expression levels of nine transport proteins in FW and SW trout prior to and during 24 h HEA exposure in Series 3. (A) Carbonic anhydrase 2 (*CA2*), (B) V-type proton ATPase (*H⁺-ATPase*), (C) sodium bicarbonate co-transporter 1(*NBC1*), (D) sodium hydrogen exchanger 2 (*NHE2*), (E) sodium hydrogen exchanger 3 (*NHE3*), (F) sodium potassium ATPase isoform α1a (*NKA-α1a*), (G) sodium potassium ATPase isoform α1b (*NKA-α1b*), (H) sodium potassium two chloride co-transporter isoform 1a (*NKCC1a*) and (I) urea transporter (*UT*). Other details as in legend of Fig. 8.

The ability of NH_4^+ to activate Na^+,K^+-ATPase activity in trout gills was examined in light of the finding by Nawata et al. (Nawata et al., 2010b) that substitution of 10 mmol l^{-1} NH_4Cl for 10 mmol l^{-1} KCl in the assay medium maintained activity unchanged in the gills of the SW pufferfish. In the course of these trials, we found that when typical plasma levels of K^+ (3 mmol l^{-1}, as KCl) were employed, in contrast to the normally used 10 mmol l^{-1}, Na^+,K^+-ATPase activity was depressed by 60% in preparations from FW trout gills (Fig. 10B). However, the opposite occurred in preparations from SW gills, where lowering K^+ from 10 to 3 mmol l^{-1} resulted in a paradoxical doubling of activity (Fig. 10B). When 1 mmol l^{-1} NH_4^+ (as NH_4Cl) was added in the presence of 3 mmol l^{-1} K^+, representing a physiological plasma level of T_{Amm} (Fig. 3B) as well as K^+ (Table 3) during HEA exposure, there was no change in activity in preparations from control or HEA gills, regardless of whether these were from FW or SW trout (Fig. 10B). A range of lower NH_4^+ concentrations (0.1–1.0 mmol l^{-1}) also had no effect (data not shown). We also examined whether NH_4^+ alone could activate the enzyme in preparations from SW gills. NH_4^+ concentrations of 1 or 3 mmol l^{-1} provided less than 10% of the activity with 3 mmol l^{-1} K^+, and even raising NH_4^+ to 10 mmol l^{-1} provided only 30% of the activity with 10 mmol l^{-1} K^+ (Table 4).

DISCUSSION

Faster recovery of ammonia excretion in SW *versus* FW trout

In contrast to Wilson and Taylor (Wilson and Taylor, 1992), we found that SW trout cope more easily with HEA exposure, re-establishing ammonia excretion more quickly than FW trout despite higher baseline rates of J_{Amm} (Fig. 1), and suffering no greater net ammonia load or rise in plasma [T_{Amm}] (Fig. 3B, Fig. 6A). The results concur with our earlier work indicating that SW pufferfish (Nawata et al., 2010b) compensate more quickly than do FW trout during HEA exposure (Nawata et al., 2007). However, our hypothesis that the mechanisms involved would be akin to those seen in these studies with different species was not supported, as outlined below.

There are several possible reasons for the lack of agreement with Wilson and Taylor (Wilson and Taylor, 1992). Their conclusion was based only on plasma [T_{Amm}] measurements with speculation as to fluxes, whereas we directly measured J_{Amm} as well as [T_{Amm}]. They compared SW trout at 12°C with FW trout at 15°C; the relatively lower pK and solubility at higher temperature would tend to increase PNH_3 values, and metabolic ammonia production would also be higher. In contrast, our comparisons were made at 12°C for both salinities. They used $(NH_4)_2SO_4$ whereas we used NH_4HCO_3, which was chosen because it has negligible effects on water pH at pH 7.9, it avoids adding a potentially toxic anion (SO_4^{2-}), and it allowed direct comparison with previous studies from our laboratory (Nawata et al., 2007; Nawata et al., 2010a; Nawata et al., 2010b). Their fish were also much larger (300–800 g *versus* 30–100 g), and their FW had twofold higher [Na^+] and [Cl^-] and fivefold higher [Ca^{2+}] than our FW. We used rainbow trout of the coastal steelhead strain (subspecies *irideus*), which naturally go to sea, whereas the strain used by Wilson and Taylor (Wilson and Taylor, 1992) was not stated.

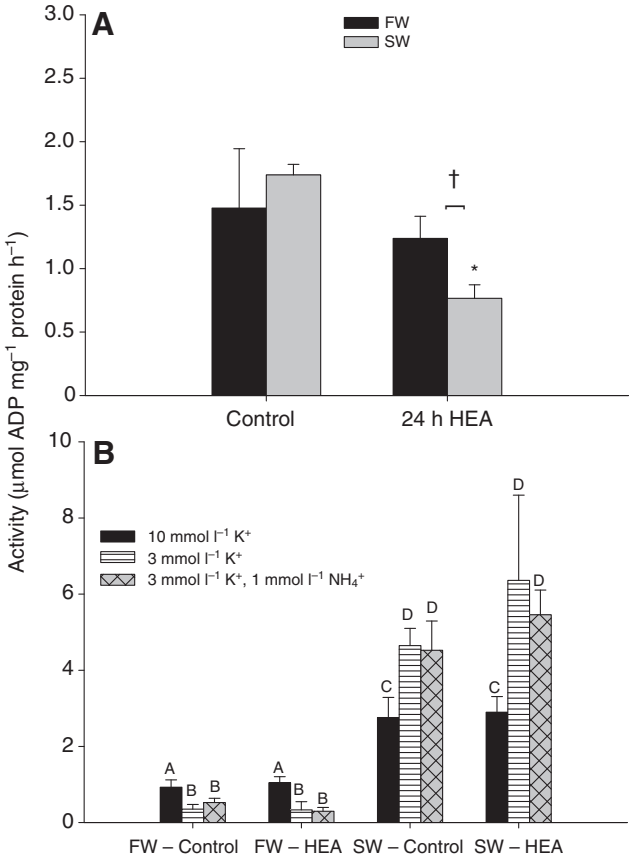

Fig. 10. (A) Branchial V-type H^+-ATPase activity in FW and SW trout prior to and following 24 h HEA exposure in Series 3. Data are means ± 1 s.e.m. (*N*=6). Other details as in legend of Fig. 1. (B) Branchial Na^+,K^+-ATPase activity in FW and SW trout prior to and following 24 h HEA exposure in Series 3, under differing concentrations of K^+ (as KCl) and NH_4^+ (as NH_4Cl) in the assay medium. Data are means ± 1 s.e.m. (*N*=6). Means not sharing the same letter are significantly different (*P*<0.05) from one another.

Table 4. Na^+,K^+-ATPase activities (μmol ADP mg^{-1} protein h^{-1}) in gill extracts of control SW trout from Series 3, measured with different levels of NH_4^+ (as NH_4Cl) and K^+ (as KCl) in the assay medium

	Na^+,K^+-ATPase activity
1 mmol l^{-1} NH_4^+ only	0.39±0.06^A
3 mmol l^{-1} NH_4^+ only	0.31±0.04^A
10 mmol l^{-1} NH_4^+ only	0.83±0.12^B
3 mmol l^{-1} K^+ only	4.65±0.45^C
10 mmol l^{-1} K^+ only	2.76±0.53^C

Data are means ± 1 s.e.m. (*N*=6).
Means not sharing the same letter are significantly different (*P*<0.05) from one another.

The important role of physical chemistry

One notable point of agreement between the two studies is the important role of physical chemistry. Because of the much greater ionic strength in SW, the pK′ values and NH_3 solubility coefficients were both higher in SW than in FW (Cameron and Heisler, 1983), resulting in substantially lower values of $P_{out}NH_3$ for the same level of water [T_{Amm}] and, therefore, much smaller ΔPNH_3 gradients driving the entry of NH_3 across the gills (Fig. 4, supplementary material Fig. S1A). Wilson and Taylor (Wilson and Taylor, 1992) reached the same conclusion, but also suggested that the NH_4^+ gradient was lower in SW trout during HEA exposure (because plasma $[NH_4^+]_{in}$ was greater). However, the true gradient driving NH_4^+ across the gills is not the simple difference in concentration ($\Delta[NH_4^+]=[NH_4^+]_{out}-[NH_4^+]_{in}$), but rather the difference between the Nernst potential and TEP ($F_{NH_4^+}=E_{NH_4^+}-TEP$). When this was measured in the present study, $F_{NH_4^+}$ values driving NH_4^+ entry during HEA exposure were very similar at the two salinities (Fig. 5, supplementary material Fig. S1B). However, in order for this to occur, the FW trout allowed their TEP to rise by approximately 15 mV (Fig. 3A), thereby attenuating the $F_{NH_4^+}$ by 30–50% and bringing it to a similar level to that in SW fish where TEP did not change. This phenomenon was also seen *in vitro* when HEA was imposed on cultured trout epithelia exposed to apical FW (Tsui et al., 2009). The negative TEP in FW teleosts is usually interpreted as a diffusion potential reflecting the differential permeability of the gills to Na^+ *versus* Cl^- (i.e. $P_{Na^+}/P_{Cl^-}>1.0$) (Potts, 1984). HEA exposure has been reported to increase the diffusive efflux of Na^+ across the gills and lower plasma [Na^+] in FW trout (Twitchen and Eddy, 1994), as seen at 4 h (Table 3), but whether this also brings the P_{Na^+}/P_{Cl^-} ratio closer to 1.0 is unknown. The phenomenon is clearly worthy of further study as an important adaptive mechanism in FW trout exposed to HEA.

Physiological responses to HEA in SW *versus* FW

The slightly lower pHa (~0.1 unit) in SW relative to FW fish (Fig. 2A) was also seen by Wilson and Taylor (Wilson and Taylor, 1992) and is a common observation probably associated with the strong ion difference phenomenon (Stewart, 1981). Alternatively or additionally, it might be explained by the excretion of a portion of the metabolically derived HCO_3^- *via* the intestine rather than the gills in SW fish, thus lowering the pH and HCO_3^- levels of plasma relative to those of FW fish (Wilson and Grosell, 2003). This difference actually puts the SW trout at a disadvantage during HEA exposure, because it reduces $P_{in}NH_3$ and thus tends to raise the ΔPNH_3 gradient for NH_3 entry while having negligible effect on $F_{NH_4^+}$. Nevertheless, ΔPNH_3 was still far less in SW animals because of the physico-chemical reasons discussed earlier. Alterations in acid–base status during HEA exposure were relatively small (Fig. 2). We did not see the large changes reported by Wilson and Taylor (Wilson and Taylor, 1992), i.e. respiratory alkalosis throughout HEA exposure superimposed on metabolic acidosis in FW and on metabolic alkalosis in SW. Instead, a slight metabolic alkalosis occurred at both salinities, coherent with NH_3 uptake, with a compensating late respiratory acidosis (rise in $PaCO_2$) at 24 h only in SW (Fig. 2). There were no changes in plasma [lactate] (Table 3), which has been reported to rise in trout exposed to high environmental pH, another treatment known to cause ammonia loading (Wilkie and Wood, 1991; Wilkie et al., 1996). However, it is possible that longer-term exposure might have resulted in lactate generation in muscle (Wilkie et al., 1996).

Plasma [cortisol] increased during HEA exposure at both salinities (Fig. 7A), a commonly observed response in salmonids exposed to HEA (e.g. Ortega et al., 2005; Tsui et al., 2009). The ‘normal’ response accompanying elevation of plasma [cortisol] in fish is usually a sustained increase in plasma [glucose] (Wendelaar Bonga, 1997; Mommsen et al., 1999). This was seen clearly in SW trout, but curiously not in FW trout where [glucose] fell during HEA exposure (Fig. 7B). It is possible that the energy-demanding processes needed to restore ammonia excretion outstripped the rate of supply of glucose by gluconeogenesis.

The progressive fall in plasma [urea-N] that occurred only in FW trout during HEA exposure (Fig. 6B) was an unexpected finding. Elevated plasma [cortisol] increases urea-N excretion rates in FW trout (McDonald and Wood, 2004b). Inhibition of ammonia excretion by high environmental pH also increases urea-N excretion rates (Wilkie and Wood, 1991). Notably, we found a marked increase in branchial mRNA expression of *UT* occurring in FW trout only during HEA exposure (Fig. 9I). *UT* appears to promote urea-N excretion by facilitating its diffusion across the basolateral membranes of trout gill cells (McDonald and Wood, 2004a), and elevated cortisol (Fig. 7A) promotes urea production through a glucocorticoid-mediated pathway (McDonald and Wood, 2004b). We speculate that urea-N production was upregulated during HEA exposure as an alternative route for waste-N excretion, and that excretion exceeded production, resulting in the observed fall in plasma concentrations. However, this could not be confirmed by the measured urea-N flux rates ($J_{Urea\text{-}N}$), which exhibited only a non-significant increase at 12–24 h in FW fish (Table 2). However, precision on $J_{Urea\text{-}N}$ was much lower than on J_{Amm} measurements, so this topic is worthy of future investigation.

Differences in baseline gene expression between FW and SW trout

Several of the differences in mRNA expression between FW and SW trout under control conditions were predictable based on other recent studies. These include the higher expression of two basolateral Na^+ transporters, the 'freshwater' isoform *NKA-α1a* of Na^+,K^+-ATPase (Fig. 9F) (Richards et al., 2003; Shrimpton et al., 2005; Bystriansky et al., 2006) as well as *NBC1* in FW (Fig. 9C). The latter appears to play a major role in Na^+ uptake in FW fish (Perry et al., 2003; Parks et al., 2007). Surprisingly, baseline expression of the 'seawater' isoform *NKA-α1b* of Na^+,K^+-ATPase (Richards et al., 2003; Bystriansky et al., 2006) did not differ between the two salinities (Fig. 9G), but there are two other reports where this was also the case (Shrimpton et al., 2005; Kiilerich et al., 2007). The higher expression of *NKCC1a* in SW trout (Fig. 9H) is consistent with the SW-adaptive role of this basolateral transporter in net NaCl extrusion (McCormick, 2001; Tipsmark et al., 2002; Scott et al., 2005; Scott et al., 2008; Kiilerich et al., 2007). Control *NHE2* expression was also greater in SW trout (Fig. 9D); there have been many studies of this apical transporter in various teleosts (reviewed by Ivanis et al., 2008), but the only previous salinity study suggested that expression of *NHE2* transiently increased after transfer from brackish water to FW in the killifish (Scott et al., 2005). The lower expression of *Rhcg1* in SW (Fig. 8C) was consistent with zebrafish larvae exposed to brackish water exhibiting a downregulation of *Rhcg1* (Nakada et al., 2007a). To our knowledge, *UT* expression has not been measured previously in the gills of salmonids subjected to salinity differences, but its greater baseline expression in SW trout (Fig. 9I) fits with the general belief that urea-N excretion is more important in marine teleosts (Wood, 1993).

Gene expression responses to HEA in FW trout

In these FW steelhead trout, key mRNA responses were generally similar to those we have reported previously in an inland aquacultural strain challenged with HEA (Nawata et al., 2007; Tsui et al., 2009; Zimmer et al., 2010). These included upregulation of the putative basolateral (*Rhbg*; Fig. 8B) and apical (*Rhcg2*; Fig. 8D) ammonia transporters, as well as apical *H⁺-ATPase* (Fig. 9B), thought to be a key mechanism for acidifying the boundary layer and energizing Na^+ uptake in FW trout (Parks et al., 2007; Parks et al., 2008). As part of the Na^+/NH_4^+ exchange metabolon (Wright and Wood, 2009), these mechanisms act to facilitate increased NH_3 efflux across both cell membranes and its diffusion trapping as NH_4^+ in the acidified boundary layer (Wilson et al., 1994), as well as help restore plasma [Na^+] (Table 3). Based on gill cell fractionation, these responses (upregulation of *Rh* proteins and *H⁺-ATPase*) occur mainly in the pavement cells (PVCs), which constitute the bulk of the branchial epithelium, rather than in the mitochondria-rich cells (MRCs) (Nawata et al., 2007). Another component, apical *NHE2,* was only slightly increased at 4 h HEA (Fig. 9D) as seen in some HEA studies in FW trout (Tsui et al., 2009; Zimmer et al., 2010).

However, a new observation was the progressive downregulation of *Rhcg1* (Fig. 8C) during HEA exposure in FW. *Rhcg1* is thought to be an apical ammonia transporter whose expression, at least in FW zebrafish and SW pufferfish, is restricted to MRCs (Nakada et al., 2007a; Nakada et al., 2007b; Braun et al., 2009a). In previous studies with FW trout, weatherloach and zebrafish, *Rhcg1* expression did not change during HEA exposure (Nawata et al., 2007; Nakada et al., 2007a; Braun et al., 2009a; Moreira-Silva et al., 2010). However, in our earlier study on FW trout, there was a 60% fall in *Rhcg1* in the MRC fraction only; this was not significant because of variability (Nawata et al., 2007). Very probably, the trout in the present study would have had more MRCs because of the much more dilute FW to which they were acclimated (Greco et al., 1996), and thus decreases in *Rhcg1* expression were detected more precisely. Like other Rh glycoproteins, *Rhcg1* is a bidirectional transporter (Nawata et al., 2010a), so its downregulation could serve as a barrier to prevent backflux of NH_3 through the MRCs during HEA exposure.

Another new observation was the progressive increase in *UT* expression occurring only in FW fish during HEA exposure (Fig. 9I). As noted earlier, urea-N excretion may have been upregulated as an alternative route of N excretion so as to deal with the more difficult ammonia loading situation in FW trout, though this speculation is not supported by the $J_{Urea\text{-}N}$ data (Table 2). *UT* expression also increased in the gills of FW zebrafish during HEA exposure, together with a transient rise in $J_{Urea\text{-}N}$ (Braun et al., 2009).

Gene expression responses to HEA in SW trout

Our hypothesis that the molecular responses of SW trout to HEA would be similar to those of the marine pufferfish (Nawata et al., 2010b) was not supported. In contrast to the pufferfish, there were no marked increases in branchial expression of *Rhcg1*, *NHE3*, *NKCC1a*, *H⁺-ATPase* or *NKA-α*, or downregulation of *Rhbg* (Figs 8, 9), and there was no increase in the activities of either V-type H^+-ATPase (Fig. 10A) or Na^+,K^+-ATPase (Fig. 10B). The only point of agreement was in the downregulation of *Rhag* (Fig. 8A). Based on the original immunohistochemical localization of transporters by Nakada et al. (Nakada et al., 2007b), the pufferfish responses were interpreted as a co-ordinated mechanism to increase active NH_3 excretion through the MRCs, while decreasing passive backflux of NH_3 through the PVCs. Instead, the SW trout seems to rely on similar mechanisms to those in FW, with again an upregulation of *Rhcg2* (Fig. 8D) and a much larger increase in expression of *NHE2* (Fig. 9D). Notably, *H⁺-ATPase* expression did not change (Fig. 9B) and V-type H^+-ATPase activity fell in response to HEA in SW animals (Fig. 10A). The approximately 1500-fold greater availability of Na^+ in SW than in FW may explain the different responses in *H⁺-ATPase* and *NHE2*, because of electrochemical and energetic considerations (Parks et al., 2008). The initial elevation of plasma [Na^+] at 4 h HEA exposure (Table 3) may represent a transient consequence of increased Na^+ importation by *NHE2* at a time when the fish is activating the system to enhance ammonia excretion. It

is unclear whether SW trout continue to route the bulk of their NH_3 excretion through the PVCs. On the one hand, this seems probable in light of the lack of increase in Na^+,K^+-ATPase expression (Fig. 9F,G) or activity (Fig. 10B) during HEA exposure. On the other hand, *NHE2* expression has been localized mainly to the MRCs in FW trout (Ivanis et al., 2008), though the situation in SW trout remains unknown. It also remains unknown whether the *NHE2* protein could perform direct Na^+ *versus* NH_4^+ exchange in SW.

There were two notable differences in the molecular responses of SW *versus* FW trout to HEA. First, downregulation of *CA2* mRNA was seen only in SW fish (Fig. 9A). Curiously, this has been reported previously in FW trout (Nawata et al., 2007; Tsui et al., 2010), so it is unclear why it occurred only in SW trout in the present study. Wright and Wood interpreted carbonic anhydrase downregulation as a homeostatic compensation to minimize disturbances in intracellular pH at times of elevated ammonia extrusion (Wright and Wood, 2009). Perhaps this explains why SW trout in the present study excreted ammonia at a much faster rate than FW trout (Fig. 1). Second, *Rhag* expression declined during HEA exposure (Fig. 8A), but only in SW fish. There is general agreement that *Rhag* expression is confined to the pillar cells in the gills when the tissue is properly cleared of erythrocytes (Nakada et al., 2007b; Braun et al., 2009), as in the present study; this is probably the reason for its much lower expression level than the other three Rh glycoproteins (Fig. 9). The pillar cells underlie the epithelium and separate it from the blood space in the secondary lamellae. Downregulated *Rhag* has been interpreted as another barrier mechanism to help prevent NH_3 backflux from water to blood during HEA exposure in SW pufferfish (Nawata et al., 2010b); the same explanation may apply in SW trout.

Na^+,K^+-ATPase activity

As expected, Na^+,K^+-ATPase activity was substantially higher in the gills of SW than FW trout (Fig. 10B), a difference that has been observed in many studies on salmonids (reviewed by McCormick, 1995; Richards et al., 2003). The potential ability of NH_4^+ to substitute for K^+ and/or to augment the activity of branchial Na^+,K^+-ATPase proved negligible in both FW and SW trout, regardless of whether the fish had been exposed to HEA for 24 h (Fig. 10B). Indeed, NH_4^+ was a very poor substrate for the enzyme in trout (Table 4). These results confirm an earlier report on FW trout gill preparations, where only lower NH_4^+ concentrations were used (Salama et al., 1999). In this respect, trout clearly differ from marine toadfish (Mallery, 1983), mudskippers (Randall et al., 1999) and pufferfish (Nawata et al., 2010b), where active NH_4^+ movement across the gill basolateral membranes on the 'K^+ site' of the Na^+,K^+-ATPase molecule may be very important during HEA exposure. These results, together with the absence of increased *NKA-α1a* or *NKA-α1b* transcription (Fig. 9F,G), argue against a role for the MRCs in active ammonia efflux in SW trout.

The finding that the SW enzyme exhibited a higher activity with 3 mmol l^{-1} K^+, and the FW enzyme with 10 mmol l^{-1} K^+ (Fig. 10B), was unexpected, and did not correlate with any marked differences in plasma [K^+] in SW *versus* FW trout (Table 3). Potentially, this difference may relate to the different balance of *NKA-α1a* and *NKA-α1b* isoforms at the two salinities (Fig. 9F,G) and/or to the differential incorporation of the molecules into lipid rafts in SW *versus* FW gill cell membranes (Lingwood et al., 2005).

A role for cortisol in the responses to HEA?

Elevated plasma [cortisol] (Fig. 7A) is a commonly observed response to HEA (Ortega et al., 2005; Tsui et al., 2009). At a gene expression level, many of the components of the Na^+/NH_4^+ exchange metabolon (Wright and Wood, 2009) in the trout appear to be sensitive to cortisol, at least in the presence of elevated ammonia. These include *Rhcg2*, *Rhbg*, *H⁺-ATPase* and *NHE2* (Tsui et al., 2009). *H⁺-ATPase* (Lin and Randall, 1993) and *NHE2* (Ivanis et al., 2008) are also sensitive to cortisol alone. We speculate that the observed elevations in plasma [cortisol], which were more prolonged in SW trout (Fig. 7A), may have contributed to the gene expression responses (Figs 8, 9) and thereby helped to augment ammonia excretion during HEA exposure. In future, it will be of interest to determine whether these are indirect or direct effects of cortisol, and whether they operate through mineralocorticoid, glucocorticoid or non-genomic receptors.

Conclusions

Integrated responses to HEA in FW and SW trout

SW rainbow trout deal with the same level of HEA more rapidly and effectively than FW trout, contrary to a previous report (Wilson and Taylor, 1992). Their response is favoured by differences in physical chemistry, which greatly reduce the ΔPNH_3 gradient for NH_3 entry, and the much higher Na^+ concentration in SW, which favours Na^+-coupled excretion mechanisms. The electrochemical gradients for NH_4^+ entry ($F_{NH_4^+}$) are the same at the two salinities, but only because FW trout allow their TEP to rise by approximately 15 mV, thereby reducing the $F_{NH_4^+}$ gradient by 30–50%. At a molecular level, the gill Rh glycoprotein and associated transporter responses are not fundamentally different between FW and SW trout, though FW trout appear to rely more on V-type *H⁺-ATPase* and SW trout more on *NHE2* for boundary layer acidification and ammonia trapping. In the trout, upregulation of *Rhcg2* appears to play a key role in the response to HEA in both FW and SW animals, and NH_4^+ does not appear to move through Na^+,K^+-ATPase. The molecular responses in the SW trout are very different than in the pufferfish, the only other marine fish that has been examined in detail (Nawata et al., 2010b). Nevertheless, this is consistent with other recent studies showing that the importance of *Rhcg1 versus Rhcg2* and *H⁺-ATPase versus NHE2* or *NHE3* as ammonia trapping mechanisms varies even amongst FW teleosts (for reviews, see Wright and Wood, 2009; Weihrauch et al., 2009). Indeed, the currently available data, which indicate somewhat different mechanisms both among and between FW and SW teleosts, suggest that the mechanisms of coping with HEA may be both diverse and flexible.

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