RESEARCH ARTICLE

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

Chris M. Wood1,2,* and C. Michele Nawata1,2

1Department of Biology, McMaster University, 1280 Main St. West, Hamilton, ON L8S 4K1, Canada and 2Bamfield Marine Sciences Centre, 100 Pachena Drive, Bamfield, BC V0R 1BO, Canada

*Author for correspondence (woodcm@mcmaster.ca)

Accepted 25 July 2011

SUMMARY

Steelhead rainbow trout acclimated to either freshwater (FW) or seawater (SW) were exposed to high environmental ammonia (HEA, 1000 μmol l−1 NH4HCO3, 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 NH4+ entry (FNH4+) 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 transport capacity. Branchial Na+,K+-ATPase activity was much higher in SW gills, but could not be activated by NH4+. Overall, the more effective response of SW trout was explained by differences in physical chemistry between SW and FW, which greatly reduced the plasma NH3 tension gradient for NH4+ 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 NH4+ does not appear to move through Na+,K+-ATPase.

Supplementary material available online at http://jeb.biologists.org/cgi/content/full/214/21/3557/DC1

Key words: Rhesus glycoprotein, Oncorhynchus mykiss, gene expression, cortisol, transepithelial potential, PNH3, 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., 2006; Nakada et al., 2007b), especially in the face of high environmental ammonia (HEA) (Hunziker 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 NH4+ but transport NH3 (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 NH3 to NH4+, thereby sustaining the gradient for facilitated diffusion of NH3 via the Rh channel. H+ transport mechanisms may include V-type H+-ATPases (which energize Na+ channels), direct Na+/H+ exchangers, as well as CO2 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+/NH4+ 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., 2006b) 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 (TAM) despite less severe plasma NH₃ tension (PNH₃) diffusion gradients and NH₄⁺ concentration gradients during the same HEA exposure. They attributed this difference to a greater cation (i.e. NH₄⁺) 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 NH₄⁺ (Kirschner, 1970) but rather just the NH₄⁺ 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 (Oncorhyncus mykiss, Walbaum 1792) of the coastal steelhead strain [subspecies iridus (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 (µmol1⁻¹: Na⁺ 300, Cl⁻ 233, K⁺ 5, Ca²⁺ 144, Mg²⁺ 48; titratable alkalinity 43µmol1⁻¹, pH 7.0–7.2) or gradually acclimated to 100% Bamfield SW (µmol1⁻¹: Na⁺ 452, Cl⁻ 515, K⁺ 9.8, Ca²⁺ 9.5, Mg²⁺ 52; titratable alkalinity 2.2µmol1⁻¹, 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 2.51 polystyrene 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 mol1⁻¹ 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 1000µmol1⁻¹ of NH₄HCO₃, 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 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 g1⁻¹, neutralized with 1 mol1⁻¹ KOH). The catheter was filled with Cortland salmonid saline containing 501 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 (1000µmol1⁻¹ NH₄HCO₃, 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 (300µl) 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 N₂, and stored at –80°C for later analysis of plasma total ammonia ([TAM]). A water sample was taken for [TAM] and pH measurements to allow precise calculation of trans-branchial PNH₃ and electrochemical NH₄⁺ 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 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 [TAM], then a slurry of pH-adjusted MS-222 (to yield a final concentration of 0.15 g1⁻¹) 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 g1⁻¹ 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 RNXalater™ (Ambion Inc., Austin, TX, USA) and stored at 4°C for later
molecular analyses, while the other was flash-frozen in liquid N2 and stored at –80°C for later enzymatic assays. Plasma was immediately separated by centrifugation (5000g, 30s), aliquoted into several vials, frozen in liquid N2, and stored at –80°C for later analysis of plasma [TAmn], [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 Radiometer GK401C electrodes calibrated with precision buffers (Radiometer). The blood electrode was seated in a custom-made Radiometer GK401C electrodes calibrated with precision buffers (Radiometer). The blood electrode was seated in a custom-made Radiometer GK401C electrodes calibrated with precision buffers (Radiometer). The blood electrode was seated in a custom-made Radiometer GK401C electrodes calibrated with precision buffers (Radiometer). The blood electrode was seated in a custom-made Radiometer GK401C electrodes calibrated with precision buffers (Radiometer). The blood electrode was seated in a custom-made Radiometer GK401C electrodes calibrated with precision buffers (Radiometer).

Flux rates of total ammonia (JAmn) and urea (JUrea,N) were calculated from changes of concentration in the closed chambers, factored by fish mass, volume and time. A negative JAmn indicates a net uptake of ammonia into the fish.

The PNH3 gradient across the gills (ΔPNH3) was calculated as:

$$\Delta PNH3 = P_{inNH3} - P_{outNH3}$$

(1)

where $P_{inNH3}$ is the PNH3 in the plasma and $P_{outNH3}$ is the PNH3 in the outside water. A positive value of $\Delta PNH3$ will tend to drive NH3 into the fish whereas a negative value will drive NH3 out of the fish.

The Nernst potential for NH4⁺ ($E_{NH4^+}$) was calculated as:

$$E_{NH4^+} = \frac{RT}{zF} \ln \left( \frac{[NH4^+]_{out}}{[NH4^+]_{in}} \right)$$

(2)

where z is the valence, R is the gas constant, T is the absolute temperature, F is Faraday’s constant, and $[NH4^+]_{in}$ and $[NH4^+]_{out}$ are the concentrations of NH4⁺ in the blood plasma and the outside water, respectively (Kirschner, 1970).

The true electrochemical potential or net driving force ($F_{NH4^+}$) for NH4⁺ across the gills was calculated as:

$$F_{NH4^+} = E_{NH4^+} - TEP$$

(3)

A positive value of $F_{NH4^+}$ will tend to drive NH4⁺ into the fish whereas a negative value will drive NH4⁺ out of the fish.

Plasma [cortisol] was measured using a commercial 125I radioimmunoassay kit (CA-1529 GammaCoatᵀᴹ, DiaSorin Inc., Stillwater, MN, USA). Plasma [Na+] and [K+] were measured by flame atomic absorption spectroscopy (Varian SpectraAA-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 Plusᵀᴹ, Roche Diagnostics, Mannheim, Germany) and [lactate] (Lactate Proᵀᴹ LT-1710, Arkay Inc., Kyoto, Japan).

Molecular analyses
Total RNA was extracted from the gills of Series 3 fish (unwaxed) 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 (DNase-treated; Invitrogen) with an oligo(dT17) 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 (NH2), sodium hydrogen exchanger 3 (NH3), sodium potassium two chloride co-transporter 1 (NKCC1a), sodium potassium ATPase isofrom α1a (NKA α1a), sodium potassium ATPase isofrom 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 analyses 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 NH4⁺ could activate trout branchial Na⁺,K⁺-ATPase, various concentrations of NH4⁺ (as NH4Cl) 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.
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 ($J_{\text{Amm}}$) was 80% greater in SW than in FW trout. Upon exposure to HEA, there was an immediate significant reversal of $J_{\text{Amm}}$ 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 $J_{\text{Amm}}$) was maintained until 9 h in FW trout (Fig. 1A). By 12–24 h, $J_{\text{Amm}}$ was again significantly greater in SW than in FW. Despite their higher baseline rates of $J_{\text{Amm}}$, 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 $J_{\text{Amm}}$) and could only be resolved over 12 h periods (Table 2). As with $J_{\text{Amm}}$, control $J_{\text{Urea-N}}$ was significantly higher in SW than in FW. $J_{\text{Urea-N}}$ 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 PaCO₂ (Fig. 2B) and slightly higher [HCO₃⁻]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 [HCO₃⁻]a, indicative of slight
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 PaCO2 (Fig. 2B) and further increase in [HCO3–]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).

Control plasma TAmm 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 [TAmm] reached approximately 725 and 820 μmol l–1 in SW and FW trout, respectively, still significantly below simultaneously measured water [TAmm] (~1040 μmol l–1, paired t-test). These measurements allowed calculation of water (PoutNH3) and blood plasma (PinNH3) NH3 tensions (Fig. 4) and $E_{NH4+}$ (Fig. 5).

Prior to HEA exposure, PinNH3 (~60 μTorr; 1 μTorr = 133/1100310–6 Pa) was significantly greater than PoutNH3 (~15 μTorr), such that there was a negative ∆PNH3 (Eqn 1) of approximately –45 μTorr driving NH3 out of the fish in both groups. During HEA exposure, PoutNH3 rose to approximately 460 μTorr in FW (Fig. 4A), but to only 170 μTorr in SW (Fig. 4B), a highly significant difference.
In FW fish, plasma $P_{\text{ar}}$NH$_3$ rose in parallel to $P_{\text{out}}$NH$_3$, but stabilized at approximately 350 µTorr, significantly below the simultaneous $P_{\text{out}}$NH$_3$. Therefore, $\Delta P$NH$_3$ stabilized at approximately +110 µTorr, which would tend to drive NH$_3$ out of the fish.

In SW fish, $P_{\text{ar}}$NH$_3$ increased significantly but stabilized at a much lower value, the same as $P_{\text{out}}$NH$_3$, at 12 and 24 h. Thus there was only a small, inwardly directed $\Delta P$NH$_3$ (+55 µTorr) at 4 h, which decreased to 0 µTorr at 12 h and 24 h.

In Fig. 5, the Nernst potentials ($E_{\text{NH}}^+$) are plotted together with TEP values to illustrate the two components of the net driving force on NH$_4^+$ ($F_{\text{NH}}^+$). Control $E_{\text{NH}}^+$ was approximately $-20$ mV in both groups, almost identical to the TEP ($-16$ mV) in FW fish (Fig. 5A), but far below (−3 mV) in SW animals (Fig. 5B). Therefore, there was a negligible $F_{\text{NH}}^+$ in FW trout, but a highly negative $F_{\text{NH}}^+$ (−29 mV) favouring NH$_4^+$ influx in SW trout. Upon exposure to HEA, $E_{\text{NH}}^+$ 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 $[T_{\text{Amn}}]$ in these fish (Fig. 4). As these values were far above TEP, there were strong positive $F_{\text{NH}}^+$ gradients tending to drive NH$_4^+$ into the fish at both salinities at 4 h (+26 to +29 mV). Therefore, $E_{\text{NH}}^+$ 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 $F_{\text{NH}}^+$ 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 $[T_{\text{Amn}}]$ 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 fall 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 $[T_{\text{Amn}}]$ (Fig. 6A) and these remained far below simultaneously measured water $[T_{\text{Amn}}]$ (−1000 µmol l$^{-1}$).

In light of the unexpected fall in plasma $[T_{\text{Amn}}]$ 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 $[T_{\text{Amn}}]$ was not seen
in this test [FW 406±66µmol l⁻¹ (6) versus SW 483±47µmol 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±0.04mmol Nl⁻¹ (6) versus SW 2.32±0.25mmol Nl⁻¹ (6), P<0.001].

The ΔPNH₃ and F_NH₄⁺ 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). ΔPNH₃ values were negative under control conditions, favouring NH₃ efflux, but became highly positive during HEA exposure in FW fish, fluctuating between +100 and +250µTorr, thereby favouring NH₃ influx (supplementary material Fig.S1A). In contrast, in SW fish, ΔPNH₃ reached only +60µTorr at 4 h, and thereafter was not significantly different from 0µTorr at 12 and 24 h. Under control conditions, F_NH₄⁺ values were below 0mV in both treatments, with significantly more negative values in SW trout favouring NH₄⁺ efflux (supplementary material Fig.S1B). During HEA exposure, F_NH₄⁺ switched over to positive values (+22 to +35mV) that were virtually identical at all times in FW and SW animals, thereby tending to drive NH₄⁺ influx.

Control plasma [cortisol] was twofold higher in FW than in SW trout, but during HEA exposure, immediately increased to similar concentrations (~200µg 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...
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). NKCC1α 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.

---

**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**

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

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.
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$_4$Cl 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$^+$ (3-3.5 mmol l$^{-1}$ K$^+$, and 10 mmol l$^{-1}$ KCl) were employed, in contrast to the normally used SW gills, NH$_4^+$ concentrations of 1 or 3 mmol l$^{-1}$ resulted in a paradoxical doubling of activity (Fig. 10B). A range of lower NH$_4^+$ concentrations (0.1–1.0 mmol l$^{-1}$) 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, establishing ammonia excretion more quickly than FW trout despite higher baseline rates of $J_{\text{Am}}$ (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 $J_{\text{Am}}$ as well as [T Amm]. They compared SW trout at 12°C with FW trout at 15°C; the relatively lower pH 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$_4$)$_2$SO$_4$ whereas we used NH$_4$HCO$_3$, 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 iridus), which naturally go to sea, whereas the strain used by Wilson and Taylor (Wilson and Taylor, 1992) was not stated.
were both higher in SW than in FW (Cameron and Heisler, 1983), activity in FW and SW trout prior to and following 24 h of water [T Amm] and, therefore, much smaller the Nernst potential and TEP (Wilson and Taylor, 1992, supplementary material Fig. 2A), as seen at 4h (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 $P_{Na}$NH$_3$ and thus tends to raise the $\Delta$PNH$_3$ gradient for NH$_3$ entry while having negligible effect on $F_{NH_4^+}$. Nevertheless, $\Delta$PNH$_3$ 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; Mommersen 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 branched mRNA expression of UT occurring in FW trout only during HEA exposure (Fig. 9D). 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 (\( J_{\text{Urea-N}} \)), which exhibited only a non-significant increase at 12–24h in FW fish (Table 2). However, precision on \( J_{\text{Urea-N}} \) was much lower than on \( J_{\text{NH}_4} \) 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-\( \alpha \)-\( \alpha \) 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-\( \alpha \)-\( \beta \) 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; Kielerich 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; Kielerich 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⁺/NH₄⁺ exchange metabolon (Wright and Wood, 2009), these mechanisms act to facilitate increased NH₃ efflux across both cell membranes and its diffusion trapping as NH₄⁺ 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 4h 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 NH₃ 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 \( J_{\text{Urea-N}} \) data (Table 2). UT expression also increased in the gills of FW zebrafish during HEA exposure, together with a transient rise in \( J_{\text{Urea-N}} \) (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-\( \alpha \), 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 NH₃ excretion through the MRCs, while decreasing passive backflux of NH₃ 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 4h HEA exposure (Table 3) may represent a transient consequence of increased Na⁺ import 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 NH₃ 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 NH₄⁺ 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 excretion (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 NH₃ 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 NH₄⁺ to substitute for K⁺ 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 24h (Fig. 10B). Indeed, NH₄⁺ 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 NH₄⁺ 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 NH₃ 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⁺/NH₄⁺ 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 APNH₃ gradient for NH₃ entry, and the much higher Na⁺ concentration in SW, which favours Na⁺-coupled excretion mechanisms. The electrochemical gradients for NH₄⁺ entry (F_NH₄⁺) 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_NH₄⁺ 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 NH₄⁺ 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; Weinbrauch 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.

**ACKNOWLEDGEMENTS**

We thank the Director and staff of Bamfield Marine Sciences Centre, particularly the Research Co-ordinator, Dr Dave Riddell, for their help; Cai Harley of Robertson Creek Hatchery for raising the steelhead; Linda Diao and Sunita Nadella for excellent technical assistance; and two anonymous reviewers for constructive comments.

**FUNDING**

This study was funded by NSERC (Natural Sciences and Engineering Research Council of Canada) Discovery and CFI (Canada Foundation for Innovation) Awards [C.M.W.]. C.M.W. is supported by the Canada Research Chair Program.

**REFERENCES**


**THE JOURNAL OF EXPERIMENTAL BIOLOGY**

C. M. Wood and C. M. Nawata
seawater acclimation of three salmonid fishes that vary in their salinity tolerance. J. Exp. Biol. 209, 1848-1858.


THE JOURNAL OF EXPERIMENTAL BIOLOGY
A.

B.

\[ \Delta \text{PNH}_3 \]

\[ \Delta F_{\text{NH}_4^+} \]