

Physiological and molecular analysis of the interactive effects of feeding and high environmental ammonia on branchial ammonia excretion and Na^+ uptake in freshwater rainbow trout

Alex M. Zimmer · C. Michele Nawata ·
Chris M. Wood

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Abstract Recently, a “ $\text{Na}^+/\text{NH}_4^+$ exchange complex” model has been proposed for ammonia excretion in freshwater fish. The model suggests that ammonia transport occurs via Rhesus (Rh) glycoproteins and is facilitated by gill boundary layer acidification attributable to the hydration of CO_2 and H^+ efflux by Na^+/H^+ exchanger (NHE-2) and H^+ -ATPase. The latter two mechanisms of boundary layer acidification would occur in conjunction with Na^+ influx (through a Na^+ channel energized by H^+ -ATPase and directly via NHE-2). Here, we show that natural ammonia loading via feeding increases branchial mRNA expression of Rh genes, NHE-2, and H^+ -ATPase, as well as H^+ -ATPase activity in juvenile trout, similar to previous findings with ammonium salt infusions and high environmental ammonia (HEA) exposure. The associated increase in ammonia excretion occurs in conjunction with a fourfold increase in Na^+ influx after a meal. When exposed to HEA (1.5 mmol/l NH_4HCO_3 at pH 8.0), both unfed and fed trout showed differential increases in mRNA expression of Rhcg2, NHE-2, and H^+ -ATPase, but H^+ -ATPase activity remained at control levels. Unfed fish exposed to HEA displayed a characteristic reversal of ammonia excretion, initially uptaking ammonia, whereas fed fish (4 h after the meal) did not show this reversal, being able to immediately excrete ammonia against the gradient imposed by HEA. Exposure to HEA also led to a depression of Na^+ influx, demonstrating that ammonia excretion can be uncoupled from Na^+ influx. We suggest that the efflux of H^+ , rather

than Na^+ influx itself, is critical to the facilitation of ammonia excretion.

Keywords Rhesus glycoproteins · Ammonia excretion · High environmental ammonia (HEA) · Rainbow trout · Feeding

Abbreviations

HEA	High environmental ammonia
J_{amm}	Ammonia flux
$J_{\text{in}}^{\text{Na}}$	Sodium influx
$J_{\text{out}}^{\text{Na}}$	Sodium efflux
$J_{\text{net}}^{\text{Na}}$	Sodium net flux
mRNA	Messenger RNA
NHE-2	Na^+/H^+ exchanger-2
P_{NH_3}	Partial pressure of NH_3
Rh	Rhesus glycoprotein
T_{amm}	Total ammonia concentration

Introduction

In rainbow trout (*Oncorhynchus mykiss*), as well as in a majority of other freshwater teleost fish, a large proportion of nitrogenous waste is excreted as ammonia via the gills with a relatively small amount being excreted renally (Smith 1929; Fromm 1963; Wood 1993). Ammonia excretion increases after ammonia loading via direct infusion (Maetz 1972; Wilson et al. 1994; Salama et al. 1999; Nawata and Wood 2009) and is inhibited by exposure to high environmental ammonia (HEA), but with later recovery (Fromm and Gillette 1968; Payan 1978; Wilson et al. 1994; Nawata et al. 2007). These treatments are

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A. M. Zimmer (✉) · C. M. Nawata · C. M. Wood
Department of Biology, McMaster University,
Hamilton, ON L8S 4K1, Canada
e-mail: zimmea@mcmaster.ca

associated with increases in Na^+ influx from the water during ammonia infusion (Wilson et al. 1994; Salama et al. 1999) and decreases in Na^+ influx and later recovery during HEA (Twitchen and Eddy 1994; Wilson et al. 1994). A relatively new idea is the potential involvement of Rh proteins in these processes (Weihrauch et al. 2009; Wright and Wood 2009).

Rhesus (Rh) proteins are thought to be implicated in ammonia transport due to their similarity to the methylammonium/ammonium permease (MEP)/ammonia transporter (Amt) protein families (Marini et al. 1997). It remains controversial whether they transport NH_3 or NH_4^+ (Khademi et al. 2004; Bakouh et al. 2004; Li et al. 2007). Recently, several Rh cDNA sequences were identified in the gills of rainbow trout (Nawata et al. 2007), mangrove killifish (Hung et al. 2007), pufferfish (Nakada et al. 2007b), and zebrafish (Nakada et al. 2007a). When expressed in *Xenopus* oocytes, trout Rh proteins significantly increased the flux of ammonia, with experimental results favoring the transport of NH_3 rather than NH_4^+ (Nawata et al. 2010). Furthermore, fish exposed to HEA or infused with ammonia displayed increases in branchial mRNA levels of Rhcg2 as well as Na^+/H^+ exchanger (NHE-2) and/or V- type H^+ -ATPase (Hung et al. 2007; Nawata and Wood 2009; Nawata et al. 2007). Tsui et al. (2009) demonstrated that, in vitro, gill cells of rainbow trout pre-exposed to HEA and cortisol show increases in Rhcg2, H^+ -ATPase, and NHE-2 gene expression.

The partial pressure gradient for NH_3 (P_{NH_3}), which is thought to drive ammonia excretion, appears to be maintained by the acidification of the gill boundary layer (Avella and Bornancin 1989; Randall and Wright 1987; Wilson et al. 1994; Salama et al. 1999). This causes protonation of NH_3 after it diffuses across the gills, transforming it to NH_4^+ and thus maintaining a positive P_{NH_3} gradient from blood to water under normal conditions (see Wood 1993). Acidification of the gill boundary layer is thought to be due in part to an apically located V- type H^+ -ATPase (Lin et al. 1994; Sullivan et al. 1995), an apical H^+/Na^+ exchanger (NHE; Wilson et al. 2000; Ivanis et al. 2008), and also the hydration of CO_2 (see Wilkie 2002). In freshwater fish, active pumping of H^+ out of the gills by the apical H^+ -ATPase is believed to create an electrochemical gradient, which drives Na^+ into the gills (Avella and Bornancin 1989; Lin and Randall 1991, 1993; Wilson et al. 2000). Apical NHEs may also function in Na^+ uptake in conjunction with a basolaterally located Na^+/K^+ -ATPase and an electrogenic $\text{Na}^+\text{HCO}_3^-$ cotransporter (NBC-1) (Wilson et al. 2000; Perry et al. 2003; Scott et al. 2005; Parks et al. 2007). Thus, the gill boundary layer acidification that facilitates the transport of ammonia would be linked to Na^+ influx in this scheme.

Recently, Wright and Wood (2009) proposed an extension of this model for ammonia excretion in freshwater

fish, a “ $\text{Na}^+/\text{NH}_4^+$ exchange complex”, which was based mainly on data of Tsui et al. (2009) obtained from cultured gill epithelia in vitro. The model suggests that ammonia moves passively through Rhcg (presumably Rhcg2 in rainbow trout) as NH_3 and is coupled to the efflux of H^+ through H^+ -ATPase and NHE-2. These mechanisms may provide the driving force necessary for Na^+ uptake (through NHE-2 or through a Na^+ channel energized by H^+ -ATPase), but may not necessarily do so in a 1:1 ratio. Very recently, partial support for this model was provided by Wu et al. (2010) from in situ studies of mitochondrial-rich cells (MRCs) in the larvae of Japanese medaka. Here ammonia excretion through Rhcg (Rhcg1) was dependent on H^+ excretion by NHE-3 and appeared to be tightly linked to Na^+ uptake. However, in this case, H^+ flux was shown to be insensitive to bafilomycin A1, demonstrating that H^+ -ATPase does not necessarily play a role (Wu et al. 2010). This work contrasts with the findings of Nakada et al. (2007a) that Rhcg1 co-localizes with V- H^+ -ATPase and Rhcg1 in MRCs in the skin of larval zebrafish. These studies demonstrate the complexity and variability of the relationship among different transport proteins and different species. However, to date, what has been missing is any examination in intact animals of the potential linkages of ammonia flux via Rh proteins to Na^+ influx via NHE or H^+ -ATPase.

Also overlooked to date is the potential role of feeding, as mechanistic in vivo studies are traditionally done on fasted fish to standardize N status. Fromm (1963) showed that during unfed periods, total nitrogenous waste excretion of trout falls substantially to a baseline level. It is now well established that plasma total ammonia concentration (T_{amm}) and ammonia excretion rate increase markedly after feeding in fish (Kaushik and Teles 1985; Wicks and Randall 2002a, b; Wood 1993; Gelineau et al. 1998; Bucking and Wood 2008). Based on findings with HEA and ammonia infusion discussed above, we hypothesized that this more natural pathway of internal ammonia loading in trout would cause upregulation of Rhcg2, NHE-2, and H^+ -ATPase mRNA in the gills and increased Na^+ influx after feeding.

This study aims to determine the effects of feeding, HEA exposure, and a combination of both feeding and HEA on ammonia excretion, plasma T_{amm} , Na^+ uptake, and gill mRNA levels of Rhcg2, NHE-2, and H^+ -ATPase in juvenile rainbow trout, as well as branchial H^+ -ATPase activity. Specifically, we hypothesized that fed fish under normal conditions (no HEA) would demonstrate increases in ammonia excretion, plasma T_{amm} , Na^+ uptake, and mRNA expression of associated gill transport proteins. Secondly, we hypothesized that this prior upregulation would allow fed fish to excrete ammonia against HEA better than unfed fish. Thirdly, we predicted that HEA would inhibit Na^+ influx in both fed and unfed fish, but

recovery of both Na^+ influx and ammonia excretion would be more rapid in fed fish since they would already have increased levels of Rhcg2, NHE2, and H^+ ATPase.

Methods and materials

Animals

Juvenile rainbow trout (*Oncorhynchus mykiss*) weighing 3–9 g were obtained from Humber Springs Trout Hatchery, Ontario, Canada. The fish were kept in dechlorinated Hamilton tap water (moderately hard: $[\text{Na}^+] = 0.6$ mequiv/l, $[\text{Cl}^-] = 0.8$ mequiv/l, $[\text{Ca}^{2+}] = 1.8$ mequiv/l, $[\text{Mg}^{2+}] = 0.3$ mequiv/l, $[\text{K}^+] = 0.05$ mequiv/l; titration alkalinity 2.1 mequiv/l; pH ~ 8.0 ; hardness ~ 140 mg/l as CaCO_3 equivalents; temperature 12.5–15°C), and fed $\sim 1\%$ of their weight per day with commercial trout pellets. The measured Na^+ and Cl^- contents of the pellets were approximately 200 mequiv/kg each. Fish were fasted for 48 h prior to the start of experiments.

Experimental series

The study consisted of a variety of experiments, all performed at a pH of 8.0, with each fish placed in an individual 80-ml chamber containing aerated water at approximately 15°C and limited light exposure to reduce stress. The treatments included exposure to high environmental ammonia (HEA; 1.5 mM NH_4HCO_3 at pH 8.0), feeding to satiation, feeding to satiation followed by HEA exposure, and no feeding and no HEA exposure. The pH was monitored throughout using a Radiometer-Copenhagen combination glass electrode (GK2401C) and maintained by the addition of 0.1 M KOH or 0.1 M HCl to the chambers as necessary.

Ammonia excretion experiments

A variety of experiments designed to evaluate ammonia excretion were performed. Exposures had durations of 6, 12, or 24 h. Those lasting longer than 6 h were flushed with new water every 6 h to limit ammonia buildup in the chambers. For measurements of ammonia excretion rates, water samples (2 ml) were taken at 2-h intervals (in one experiment at 3-h intervals). For fed treatments, trout were fed to satiation in their communal holding tanks (they would not feed in the individual chambers) and then transferred immediately to the chambers. For an initial 12-h post-prandial evaluation of ammonia excretion, flux measurements were started immediately after transfer. Based on this initial test (see Fig. 1), as well as the study of Gelineau et al. (1998), in subsequent experiments, fed fish began the HEA or corresponding

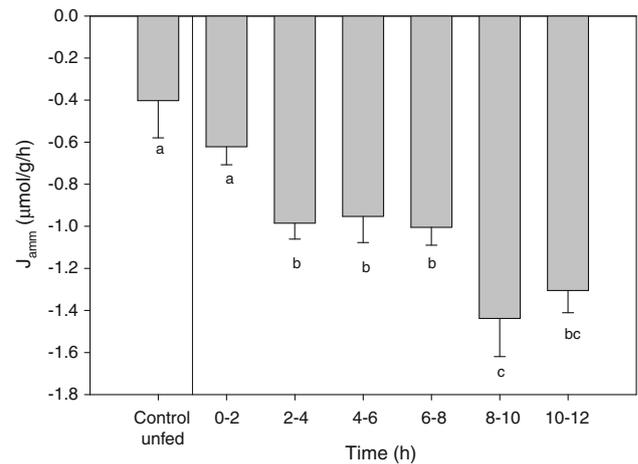


Fig. 1 Effects of feeding on ammonia excretion rates (J_{amm}) over 12 h including rates from a 6-h control using an unfed group of fish. Feeding occurred at 0 h. Means sharing the same letter are not significantly different from one another. Comparisons were made using one-way ANOVA statistical analysis followed by a Fisher LSD post hoc multiple comparison test ($P < 0.05$, $N = 8$)

control exposures at 4 h after feeding (i.e., 3+ h after transfer) as this seemed to be the time at which elevated ammonia excretion and, potentially, transporter upregulation, would be well established in trout. Therefore, for these HEA exposures and corresponding control exposures (no HEA), both fed and unfed fish were acclimated to the chambers for at least 3 h prior to the start of the exposures.

Plasma ammonia and tissue collection experiments

Fish were exposed to the same conditions as mentioned above. Fed and unfed fish were euthanized at times corresponding to 2, 4, 6, 8, and 10 h post-feeding. Trout were killed using an MS-222 solution (0.1 g/l), which had been set to the same pH and ammonia concentration as the experimental conditions. Blood samples were collected into sodium-heparinized plastic microhematocrit capillary tubes from the caudal vessels after severing the tail, and plasma was separated from red cells by centrifugation (10,000g for 2 min). Though this method of collection had the possibility of raising plasma T_{amm} (see “Discussion”), the effect would likely be uniform across treatments. Plasma samples were subsequently flash-frozen in liquid nitrogen and stored at -70°C . Gill (entire gill basket) samples were dissected from the fish following blood sampling and were immediately flash-frozen in liquid nitrogen and stored at -70°C until analysis.

Sodium flux experiments

Experiments to determine sodium flux were performed separately from all other experiments. Fish were placed

into identical chambers (at appropriate times post-feeding) as described above and 1 $\mu\text{Ci/l}$ of ^{22}Na was added to each chamber. When chambers were flushed at 6-h time periods, radioisotope was re-administered to each chamber. All other procedures were performed identically to the ammonia excretion experiments described above. However, terminal plasma collection was done using ammonium-heparinized glass microhematocrit capillary tubes. These samples were required for measurement of final plasma ^{22}Na and total Na^+ levels, for backflux correction (see below).

Determination of mRNA expression

Total RNA was extracted from gill samples using TRIzol (Invitrogen, Burlington, ON), quantified via spectrophotometry (Nanodrop ND-1000, Nanodrop Technologies, Wilmington, DE, USA) and electrophoresed on 1% agarose gels stained with ethidium bromide to verify integrity. cDNA was synthesized from 1 μg total RNA (DNaseI-treated, Invitrogen) with an oligo(dT₁₇) primer and Superscript II reverse transcriptase (Invitrogen). mRNA expression levels of Rhcg2, H⁺-ATPase (v-type, B-subunit), and NHE2 in the gill were determined by quantitative real-time PCR (qPCR) using the above synthesized cDNA and transporter-specific primers published previously (Nawata et al. 2007). Analyses were performed on an Mx3000P QPCR System (Stratagene, Cedar Creek, TX). Reactions (20 μl) containing 4 μl of 5 \times diluted cDNA described above, 4 pmol each of forward and reverse primers, 0.8 μl ROX dye (1:10 dilution), and 10 μl Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen), were performed at 50°C (2 min) and 95°C (2 min), followed by 40 cycles of 95°C (15 s) and 60°C (30 s). No-template controls and non-reversed-transcribed controls were run in parallel while melt-curve analysis verified the presence of a single product. Values were extrapolated from standard curves generated by the serial dilution of one randomly selected control sample. Because the mRNA expression levels for three housekeeping genes, beta-actin, elongation factor-1 α , and 18S, were unstable across treatments, data were normalized to ng total RNA, an established method of normalization (Bustin 2000, 2002), which we have used previously for these genes in trout gills (Nawata and Wood 2008, 2009).

Determination of branchial H⁺-ATPase activity

Gill H⁺-ATPase activity measurements were obtained from the same unperfused tissue samples (control, fed no HEA, fed and unfed HEA) used for mRNA expression measurements. A protocol for measuring H⁺-ATPase activity was modified from Lin and Randall (1993) and adapted to a Na⁺/K⁺-ATPase activity assay (McCormick

1993) using sodium azide and NEM (N-ethylmaleimide) and ouabain as inhibitors to measure H⁺-ATPase activity levels in control and experimental fish gills. This was the same protocol used by Nawata et al. (2007). Activities are reported as activity (no inhibitor) minus inhibitor-treated activity (sodium azide with N-ethylmaleimide (NEM) for V-type H⁺-ATPase), measured at 340 nm and at room temperature in a kinetic microplate reader (SpectraMAX Plus; Molecular Devices, Menlo Park, CA) at 15-s intervals for 30 min. Protein concentrations were measured using Bradford Reagent (Sigma) and BSA standards.

Analytical techniques

For plasma total ammonia (T_{amm}) analyses, freshly thawed plasma samples were assayed enzymatically using a Raichem kit (Cliniqa Corporation, San Diego, CA). For water total ammonia analyses, the collected water samples were refrigerated at -4°C for no more than 24 h or frozen (-20°C) until assay. Water ammonia concentration was determined (in triplicate) via spectrophotometry using a modified protocol based on indophenol formation with sodium salicylate (Verdouw et al. 1978). The two different ammonia assays were cross-validated. Gamma-radioactivity of ^{22}Na (in counts per minute, cpm) was measured by gamma counting (Perkin Elmer Wizard 1480 3" Auto Gamma Counter) and sodium concentration was measured by atomic absorption spectroscopy (SpectrAA 220FS Atomic Absorption Spectrometer).

Calculations

Ammonia flux rates ($\mu\text{mol/g/h}$) were calculated as:

$$J_{\text{Amm}} = ([\text{Amm}]_i - [\text{Amm}]_f) \times V / (t \times M), \quad (1)$$

where $[\text{Amm}]_i$ and $[\text{Amm}]_f$ are the initial and final concentrations of ammonia in the water (in $\mu\text{moles/l}$) obtained from comparison to a standard curve. V indicates volume (l), t time (h), and M mass (g). Sodium influx ($\mu\text{mol/g/h}$) was measured using values obtained from gamma counting (cpm/ml), and water and plasma concentrations ($\mu\text{mol/ml}$) obtained from atomic absorption spectroscopy. The following equation described by Maetz (1956), which incorporates backflux corrections, was used:

$$J_{\text{in}}^{\text{Na}} = [(R_i - R_f)V_{\text{ext}} - SA_{\text{int}}([\text{Na}]_i - [\text{Na}]_f)] / [(SA_{\text{ext}} - SA_{\text{int}}) \times M \times t], \quad (2)$$

where R_i and R_f are the initial and final radioactivity levels (cpm/ml) in the water for each flux period, V_{ext} indicates final volume (ml) of water, SA_{int} and SA_{ext} are the mean internal and external specific activities over the flux period, and $[\text{Na}]_i$ and $[\text{Na}]_f$ are the initial and final concentrations

of sodium (in $\mu\text{mol/ml}$) measured by atomic absorption. Net sodium flux ($\mu\text{mol/g/h}$) was obtained using the following equation:

$$J_{\text{net}}^{\text{Na}} = ([\text{Na}]_i - [\text{Na}]_f) \times V / (M \times t). \quad (3)$$

Finally, sodium efflux ($\mu\text{mol/g/h}$) was obtained using the following equation:

$$J_{\text{out}}^{\text{Na}} = J_{\text{net}}^{\text{Na}} - J_{\text{in}}^{\text{Na}}. \quad (4)$$

To calculate values for water and plasma ammonia chemistry ($[\text{NH}_4^+]$, $[\text{NH}_3]$, and P_{NH_3}), pK and αNH_3 constants for the appropriate experimental pH and temperature were obtained from Cameron and Heisler (1983). Using pK and the experimental T_{amm} values, $[\text{NH}_4^+]$ was derived from a modified version of the Henderson–Hasselbalch equation:

$$[\text{NH}_4^+] = T_{\text{amm}} / [1 + \text{antilog}(\text{pH} - pK)] \quad (5)$$

From this, $[\text{NH}_3]$ was calculated from the following equation:

$$[\text{NH}_3] = T_{\text{Amm}} - [\text{NH}_4^+] \quad (6)$$

Then P_{NH_3} (in μTorr) was calculated using the appropriate αNH_3 values from Cameron and Heisler (1983) using the following equation:

$$P_{\text{NH}_3} = \text{NH}_3 / \alpha\text{NH}_3 \quad (7)$$

$\Delta^{P_{\text{NH}_3}}$ values were calculated by subtracting the corresponding P_{NH_3} value calculated for water from that calculated for blood plasma at the same time for each individual fish. For these calculations, a blood plasma pH of 7.9 was assumed (see “Discussion”).

Statistical analyses

Data have been expressed as means ± 1 SEM (N = number of fish). In the case of the 6-h ammonia flux (J_{amm}) experiments, controls are representative of an average of a 6-h flux made over the corresponding time period for trout subjected to no HEA, no feeding, or feeding only, as appropriate (see “Results”). Comparisons between these controls (significant differences represented by asterisks) were made using a Mann–Whitney rank sum test. Comparisons made between experimental data points and a given control were analyzed using a one-way ANOVA followed by a Dunnett’s method post hoc test. In the case of a failed normality test, an ANOVA on ranks analysis was performed using a Dunn’s post hoc test with multiple comparisons versus a control. All comparisons made among multiple treatments within time points (significance represented using the lettering system, whereby means sharing the same letter are not significantly different) were analyzed using a one-way ANOVA analysis with

a Fisher LSD post hoc test. In the case of a failed normality test, an ANOVA on ranks analysis was performed using a Dunn’s post hoc test with multiple pair-wise comparisons. In all cases, statistical significance was accepted at the $P = 0.05$ level. Figure legends specify the statistical test employed to determine significance within the given data.

Results

Control experiments (6 h) with unfed fish were performed in all experimental series as a check on handling disturbance. As ammonia excretion rates and Na^+ flux rates do not vary significantly over time, average values are shown as mean control values in figures.

Ammonia excretion

Prior to feeding, ammonia excretion (J_{amm}) was typically around $-0.4 \mu\text{mol/g/h}$ in juvenile trout, which had been fasted for 48 h. Following feeding, J_{amm} increased 2.4-fold by 2–4 h, and 3.6-fold by 8–10 h (Fig. 1).

When exposed to HEA, unfed fish displayed a reversal of ammonia flux from a control (no HEA) value of $-0.40 \mu\text{mol/g/h}$ to $+0.62 \mu\text{mol/g/h}$ (i.e., net ammonia uptake) over the first 3 h of exposure (Fig. 2). However, after 3 h of HEA, these fish were able to re-establish ammonia excretion to a value not significantly different from the control, a situation which persisted through 12–24 h despite the continued presence of HEA (Fig. 2). Therefore, in all subsequent experiments, fed fish were

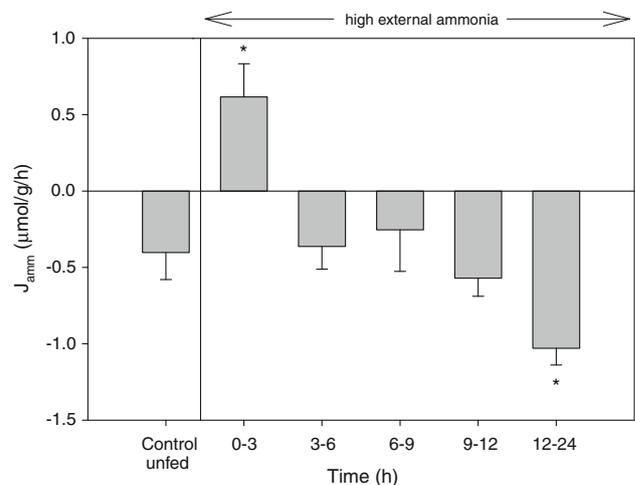


Fig. 2 Ammonia excretion rates (J_{amm}) in unfed fish during HEA exposure including rates from a 6-h control using an unfed group of fish. HEA exposure began at 0 h. Asterisks denote values that are significantly different from the control value based on a one-way ANOVA statistical analysis followed by a Dunnett’s post hoc test against the control value ($P < 0.05$, $N = 6-8$)

exposed to HEA at 4–10 h post-feeding, a time by which the elevated J_{amm} response was well established, and measurements were taken of J_{amm} at 2-h intervals commencing with the start of HEA exposure, to capture the time period over which J_{amm} would be reversed and ammonia loading would occur in unfed fish.

A subsequent experiment compared the J_{amm} responses of unfed and fed trout to HEA exposure at a time corresponding to 4–10 h post-feeding (Fig. 3). The control values in Fig. 3 represent averages of 6-h control fluxes (no HEA) over the appropriate period for fed and unfed fish, respectively. Note that the fed control value ($-0.88 \mu\text{mol/g/h}$) was significantly greater than the unfed control value ($-0.40 \mu\text{mol/g/h}$), reinforcing the observation that feeding elevates J_{amm} (Fig. 3). As in the earlier series (Fig. 2), unfed fish again experienced a reversal of J_{amm} over the first 2 h of HEA, with a subsequent return to control rates thereafter. In contrast, fed fish exposed to HEA (4 h post-feeding) displayed no significant changes from the control over 6 h of exposure (Fig. 3), thereby maintaining J_{amm} in the face of HEA.

Plasma total ammonia levels

Feeding alone with no HEA induced a marked, but delayed increase of plasma total ammonia (T_{amm}) from $555 \mu\text{mol/l}$ in the control unfed group to $1,060 \mu\text{mol/l}$ at 8 h post-feeding;

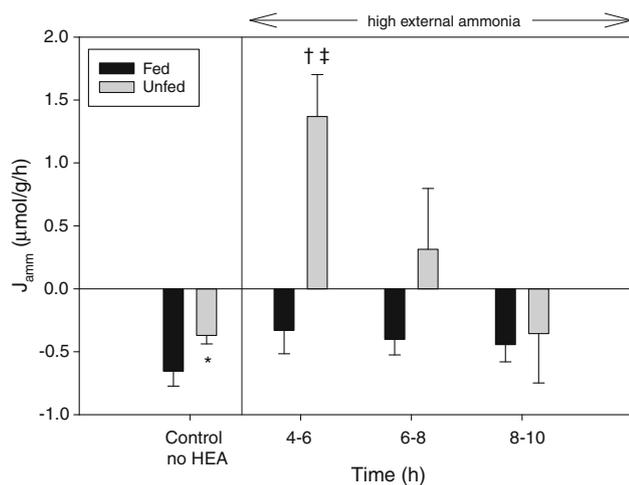


Fig. 3 Effects of a 6-h HEA exposure on ammonia excretion rates (J_{amm}) in both fed and unfed fish. Feeding occurred at 0 h; HEA exposure began at 4 h. Control groups represent the average of 6-h unfed and fed, no HEA experiments. *Crosses* represent values that are significantly different from the unfed control; *double crosses* represent those that differ from the fed control. *Asterisk* indicates a significant difference between the two controls. Comparisons between the controls were made using a Mann–Whitney rank sum test; comparisons between controls and other data points were made using an ANOVA on ranks statistical analysis followed by a Dunn's post hoc test against the control values (as comparisons failed a normality test) ($P < 0.05$, $N = 6-8$)

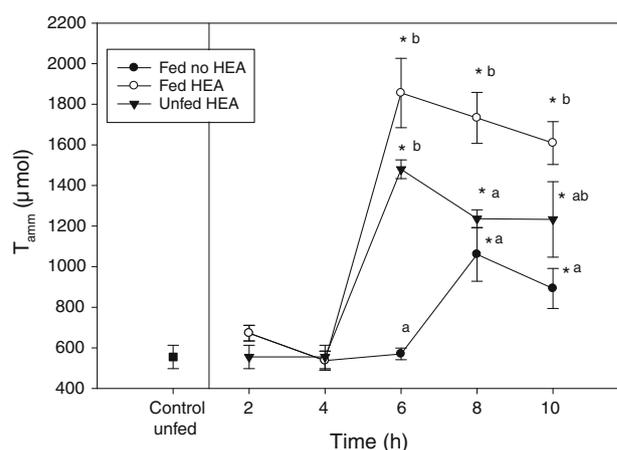


Fig. 4 Plasma total ammonia (T_{amm}) values after feeding and during HEA exposure including a control value from unfed fish. Feeding occurred at 0 h; HEA exposure began at 4 h. *Asterisks* represent values that are significantly greater than the control based on an ANOVA on ranks statistical analysis followed by a Dunn's post hoc test (as comparisons failed a normality test). Means sharing the same letter are not significantly different from one another within a given time point. Comparisons among treatments at the same time points were made using a one-way ANOVA statistical analysis followed by a Fisher LSD post hoc test ($P < 0.05$, $N = 5-14$)

this elevation persisted at 10 h (Fig. 4). When exposed to HEA, unfed fish displayed an immediate threefold increase in plasma T_{amm} (after 2 h, a time corresponding to 6 h in the fed groups). This increase was followed by a subsequent small decline to a value not significantly different from the plasma T_{amm} of the fish in the fed (no HEA) group at later time points. When fed fish were exposed to HEA, they showed a similar but somewhat greater response (fourfold increase) in plasma T_{amm} in comparison with the unfed HEA group, and much higher T_{amm} than in the unfed HEA treatment. Indeed, the T_{amm} responses of these latter treatments were approximately additive, accounting for the large increases seen in the fed HEA group, at least at 8 and 10 h post-feeding (Fig. 4).

Sodium uptake and exchange

In fasted trout, control sodium influx rates ($J_{\text{in}}^{\text{Na}}$) were approximately $1.0-1.5 \mu\text{mol/g/h}$. Control efflux rates ($J_{\text{out}}^{\text{Na}}$) were slightly greater, so net balance ($J_{\text{net}}^{\text{Na}}$) was slightly negative. Feeding induced a progressive rise in $J_{\text{in}}^{\text{Na}}$, which became significant at 6–8 h post-feeding (fourfold increase relative to the control value) such that ($J_{\text{net}}^{\text{Na}}$) became positive; $J_{\text{out}}^{\text{Na}}$ was also significantly elevated at this time (threefold increase; Fig. 5a). However, overall, $J_{\text{net}}^{\text{Na}}$ did not vary significantly throughout the entire experimental period in this fed, no HEA exposure treatment (Fig. 5). Exposure to HEA (at a time corresponding to 4–10 h post-feeding in the fed group) led to an immediate depression in $J_{\text{in}}^{\text{Na}}$ in both fed and

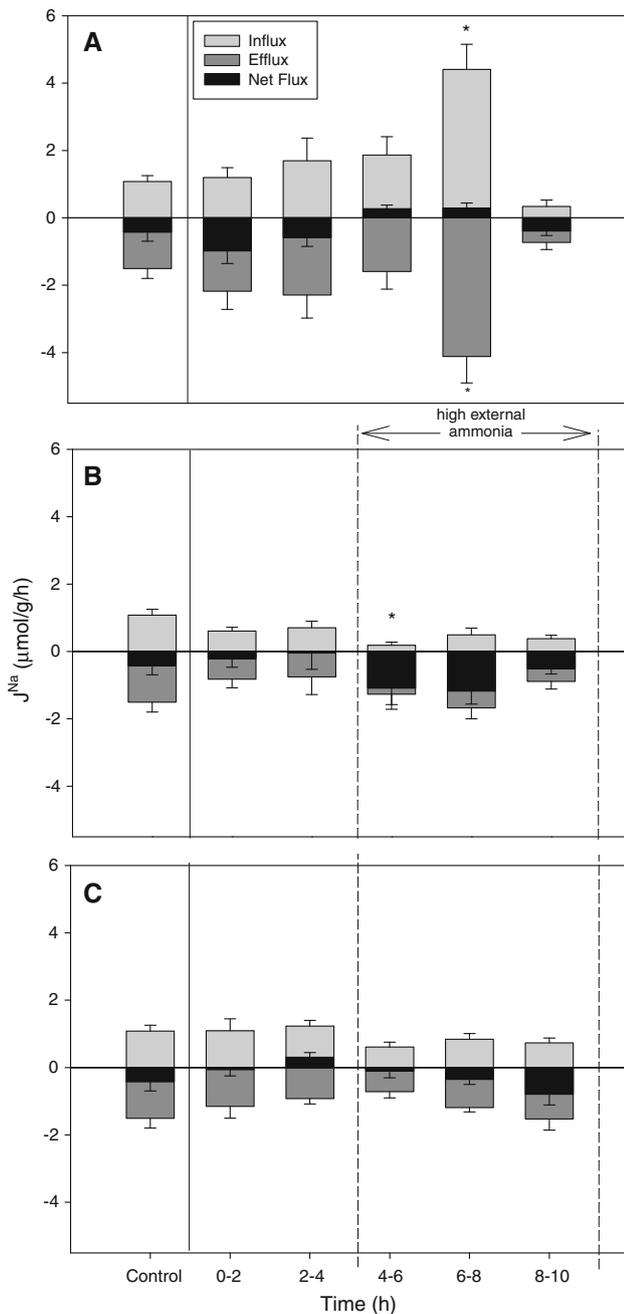


Fig. 5 Effects of **a** feeding, **b** feeding combined with a 6-h HEA exposure, and **c** exposure to 6 h of HEA alone on unidirectional ($J^{\text{Na}}_{\text{in}}$, upward light gray bars; $J^{\text{Na}}_{\text{out}}$, downward dark gray bars) and net rates ($J^{\text{Na}}_{\text{net}}$, black bars) of sodium flux over a 10-h time period. The rates from a 6-h control using an unfed group of fish are included. Feeding occurred at time 0; HEA began at 4 h. Asterisks denote values that are significantly different from their respective control values based on a one-way ANOVA statistical analysis followed by a Dunnett's post hoc test against the control value. In the case of a failed normality test, an ANOVA on ranks statistical analysis was used followed by a Dunn's method post hoc test ($P < 0.05$, $n = 8$)

unfed fish (at 4–6 h), though this decrease was not significant ($P = 0.230$) in the unfed group (Fig. 5b, c). Thereafter, $J^{\text{Na}}_{\text{in}}$ tended to recover in both the fed and unfed HEA groups despite the persistence of HEA. $J^{\text{Na}}_{\text{out}}$ values and $J^{\text{Na}}_{\text{net}}$ tended to be more negative during HEA in both these groups, but there was high variability and no significant overall differences from the control (Fig. 5b, c).

Gill mRNA expression

In all three treatment groups, significant increases in the mRNA expression levels of transporters of interest in the gills were not seen until times corresponding to 6 and 10 h post-feeding (i.e., 2 and 6 h of HEA; Fig. 6a, b, c). There were no significant changes at 2 or 4 h.

Elevations in Rhcg2 expression levels were significant in all three treatments at times corresponding to 6 and 10 h post-feeding (i.e., 2 and 6 h of HEA; Fig. 6a). The HEA-exposed groups exhibited generally greater upregulation (fed HEA = 23- and 93-fold increases; unfed HEA = 111- and 75-fold increases over the control at 6 and 10 h, respectively) than in the fed, no HEA control group (32- and 30-fold increases over the control at 6 and 10 h, respectively; Fig. 6a).

There was also a sixfold increase in NHE-2 mRNA expression at 6 h in fed fish not exposed to HEA; the mean value at 10 h remained about the same but was no longer significantly different from the control ($P = 0.08$) because of increased variability (Fig. 6b). In unfed fish, exposure to HEA caused a significant increase in the expression of NHE-2 at 6 and 10 h (18- and 8-fold, respectively), whereas fed fish exposed to HEA displayed a tenfold increase only after 10 h (Fig. 6b).

The unfed HEA treatment was the only group to display a significant increase (sevenfold) in H^+ATPase mRNA expression at 6 h; however, the increase in the fed only treatment was close to being significant ($P = 0.07$; Fig. 6c). After 10 h, all treatment groups showed significant increases in H^+ATPase expression (10-, 11-, and 10-fold for fed no HEA, fed HEA, and unfed HEA groups, respectively; Fig. 6c).

Branchial $\text{H}^+\text{-ATPase}$ activity

Branchial $\text{H}^+\text{-ATPase}$ activity increased significantly by about 1.7-fold at 4 h after the meal in the fed fish (Fig. 7). Thereafter, activity tended to decline, with a tendency for greater decreases in the two HEA treatments. However at 6 and 10 h, none of these values were significantly different from the control or from one another.

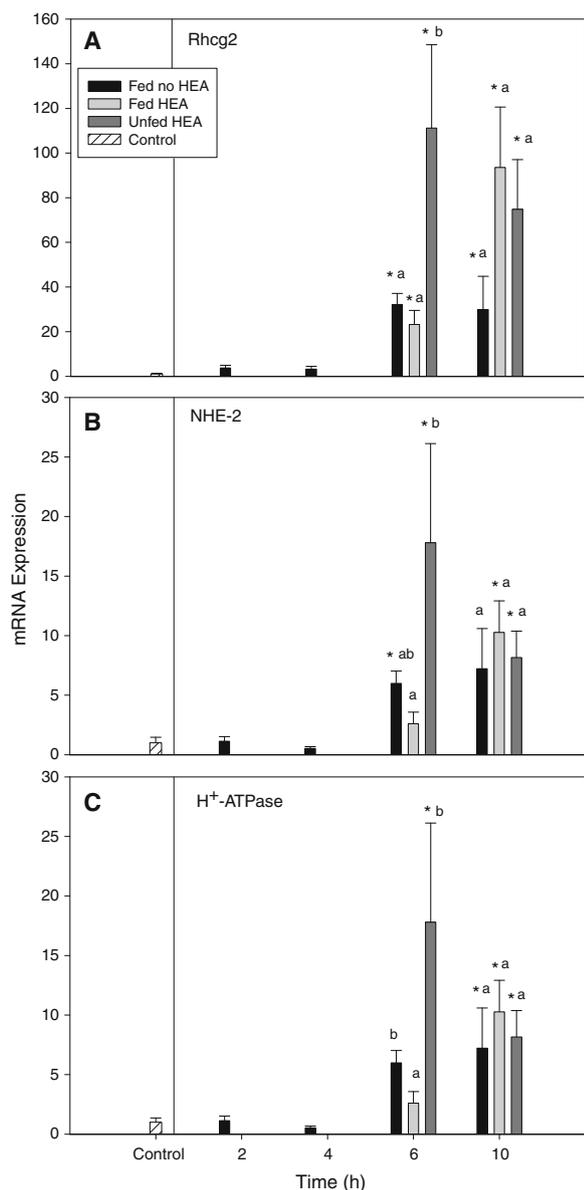


Fig. 6 mRNA expression of **a** Rhcg2, **b** NHE-2, and **c** H⁺-ATPase relative to total RNA in the gills of unexposed (no HEA) fed fish as well as fed and unfed fish exposed to HEA. The control value represents gene expression in the gills of unfed, no HEA fish. Sampling for both fed groups (HEA and non-HEA) took place at 2, 4, 6, 8, and 10 h. The 2 and 4 h time points (where fish are yet to be exposed to HEA) are the same for each of these groups, as the treatment up to these times was identical, and so only a single value is shown at each of these time points. Sampling for the unfed HEA group took place only at 6, 8, and 10 h, as 2 and 4 h points for this treatment would represent the unfed, no HEA control. Feeding occurred at 0 h; HEA exposure began at 4 h. Asterisks denote values that are significantly different from the control levels based on an ANOVA on ranks statistical analysis followed by a Dunn's post hoc test (as all comparisons failed a normality test). Means sharing the same letter are not significantly different from one another within a given time point. Since all comparisons among treatments at a given time point failed a normality test, all comparisons were made using an ANOVA on ranks statistical analysis followed by a Dunn's post hoc test ($P < 0.05$, $N = 5-6$)

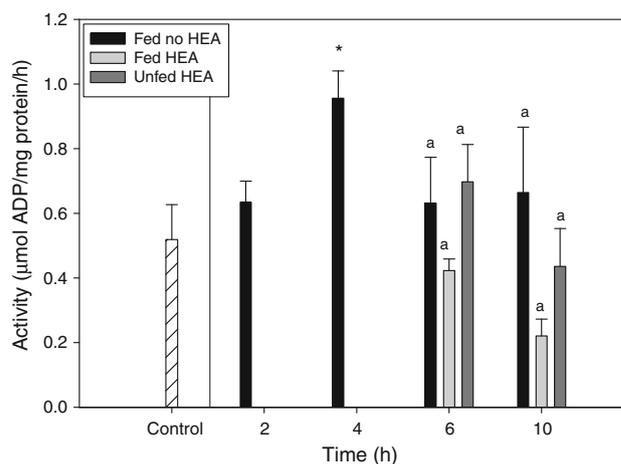


Fig. 7 H⁺-ATPase activity in the gills of unexposed (no HEA) fed fish as well as fed and unfed fish exposed to HEA. The control value represents gene expression in the gills of unfed, no HEA fish. Sampling for both fed groups (HEA and non-HEA) took place at 2, 4, 6, 8, and 10 h. The 2 and 4 h time points (where fish are yet to be exposed to HEA) are the same for each of these groups, as the treatment up to these times was identical, and so only a single value is shown at each of these time points. Sampling for the unfed HEA group took place only at 6, 8, and 10 h, as 2 and 4 h points for this treatment would represent the unfed, no HEA control. Feeding occurred at 0 h; HEA exposure began at 4 h. Asterisks denote values that are significantly different from the control levels based on an ANOVA on ranks statistical analysis followed by a Dunn's post hoc test (as all comparisons failed a normality test). Means sharing the same letter are not significantly different from one another within a given time point ($P < 0.05$, $N = 5-6$)

Discussion

Overview

Two of our original three hypotheses were confirmed. Firstly, feeding did cause upregulation of Rhcg2, NHE-2, and H⁺-ATPase mRNA expression in the gills, a transient increase in branchial H⁺-ATPase activity, and a transient rise in Na⁺ influx. Though the mRNA expression data do not explicitly demonstrate that the expression or activity of the associated proteins increased, they certainly suggest a role for these proteins during feeding and in response to HEA as demonstrated in other treatments from this study. Notably, for one gene (H⁺-ATPase), we recorded both mRNA expression and functional activity and found a marked temporal disconnect between the two. Secondly, after feeding, trout were able to excrete ammonia against HEA more effectively than unfed fish. With respect to our third hypothesis, the results are complex. HEA did inhibit Na⁺ influx as predicted, though the effect was significant only in the fed fish. Ammonia excretion was not inhibited at all by HEA in fed fish, so in a sense “recovery” of ammonia excretion in the face of HEA was more rapid in fed fish. However, contrary to prediction, there was no

difference in the rates at which Na^+ influx recovered in the face of continued HEA between fed and unfed fish. As outlined below, we believe the interpretation of these results may be more complicated than the original ideas behind our hypotheses, involving important roles for simple diffusion, for changing P_{NH_3} gradients, for non-genomic modification of transporter activity, and for uncoupling of Na^+ uptake from H^+ and ammonia efflux during HEA.

To explore the role of P_{NH_3} gradients from blood plasma to water, we have estimated them based on individual measurements of bulk water T_{amm} , plasma T_{amm} , water pH, and an assumed constant blood plasma pH of 7.9 (Fig. 7). Because of the small size of the fish, it was not possible to obtain accurate measurements of plasma pH by cannulation, and measurements of plasma T_{amm} may have been elevated by the disturbance of caudal severance sampling (discussed by Wood 1993). Furthermore, without knowledge in these small fish of the existence or time course of the postprandial alkaline tide, which occurs in adult trout (Cooper and Wilson 2008; Bucking and Wood 2008), this factor, which would potentially elevate plasma P_{NH_3} , could also not be taken into account. Bulk water pH measurements overlook possible acidification of the micro-environment in the gill boundary layer. Therefore, the observed P_{NH_3} gradients should be taken as indicative of general trends only (Fig. 8). Also important to note is that T_{amm} gradients (which may also be indicative of ammonia excretion gradients as Rh ammonia transport processes have not yet been fully resolved) showed very similar trends and thus have not been shown. Nevertheless, these data are sufficient to indicate that the bulk P_{NH_3} gradient was positive (i.e., in favor of excretion) throughout most of the post-feeding period in fed fish, which were not exposed to HEA. In contrast, the gradient was highly negative throughout the period of HEA in both unfed and fed treatments, indeed somewhat more so in the latter, so that these fish were achieving ammonia excretion against bulk P_{NH_3} gradients.

Effects of feeding alone

In previous studies on freshwater trout, feeding has been demonstrated to cause increases in both J_{amm} and plasma T_{amm} (Kaushik and Teles 1985; Wicks and Randall 2002a, b; Wood 1993; Gelineau et al. 1998; Bucking and Wood 2008). In the present study, J_{amm} increased significantly as early as 2–4 h post-feeding (Fig. 1), but significant increases in plasma T_{amm} did not occur until 8 h (Fig. 4). These observations differ from those of Bucking and Wood (2008) on adult trout (300–400 g) held in water of identical chemistry, where increases in plasma T_{amm} occurred within 2–4 h post-feeding and preceded increases in J_{amm} . Bucking and Wood (2008) also showed the basal level of plasma T_{amm} to be approximately 100 μM , whereas levels in this study were

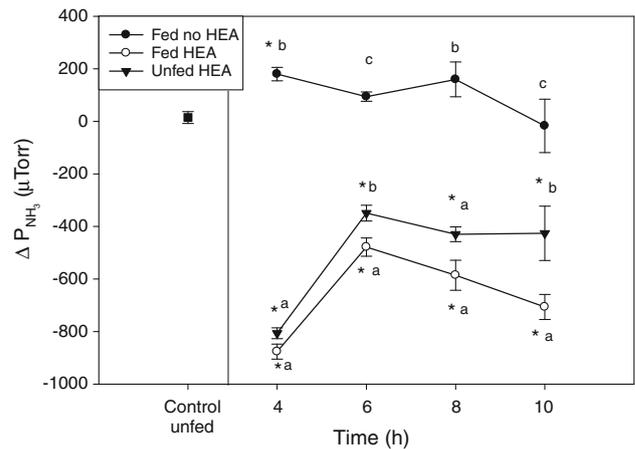


Fig. 8 Effects of feeding, HEA exposure or both feeding and HEA exposure on the estimated partial pressure gradient of NH_3 from blood to water. Feeding occurred at 0 h; HEA began at 4 h. Asterisks represent values that are significantly different from their respective control values based on a one-way ANOVA statistical analysis followed by a Dunnett's post hoc test (or, in the case of a failed normality test, an ANOVA on ranks statistical analysis followed by a Dunn's method post hoc test was used). Means sharing the same letter are not significantly different from one another within a given time point. Comparisons among treatments at a given time point were made using a one-way ANOVA statistical analysis followed by a Fisher LSD post hoc test. In the case of a failed normality test, an ANOVA on ranks statistical analysis was used followed by a Dunn's method post hoc test ($P < 0.05$, $N = 5\text{--}14$)

approximately five times that (Fig. 4). It is possible that this difference was due to sampling techniques (caudal puncture vs. tail severing method), stress effects, or differing metabolisms of the varying sizes of fish. However, for the purposes of this study, we are more concerned in the qualitative and temporal trends, than in the absolute values. The smaller fish (3–9 g) used in the present study therefore appear to upregulate excretion in response to feeding more quickly than do larger trout. We interpret the lack of detectable change in plasma T_{amm} over the first 6 h post-feeding to mean that J_{amm} is increased sufficiently by 2–4 h to maintain normal plasma T_{amm} levels for some time.

To our knowledge, the present study is the first to demonstrate that gill *Rhcg2*, *NHE-2*, H^+ -ATPase expression levels, as well as branchial H^+ -ATPase activity levels, are increased by feeding. The latter response was significant at 4 h post-feeding, while the *Rhcg2* and *NHE-2* mRNA expression increases were significant at 6 h, whereas the H^+ -ATPase mRNA expression increase did not become significant until 10 h. These results provide a cautionary note as to the importance of strict control of the feeding regime in any future studies on gill transporter expression and activity. Interestingly, *Rhcg2* expression also increased after a meal in adult rainbow trout (C.M. Nawata and C.M. Wood, unpublished results), but not until 12 h post-feeding, re-enforcing the idea that small trout respond more quickly.

We interpret these as responses to “natural” internal ammonia loading associated with de-amination of absorbed amino acids and proteins, comparable to those seen when trout were infused with exogenous ammonium salts (Nawata and Wood 2009), though it is possible that the complex hormonal events associated with feeding (Buddington and Krogdahl 2004) may have played a modulating role.

The elevation of J_{amm} by 2–4 h post-feeding (Fig. 1) and the maintenance of normal levels of plasma T_{amm} through 6 h (Fig. 4) suggest that the branchial ammonia excretion mechanism(s) were quickly upregulated after feeding. This is not obvious from the mRNA expression of the putative transport proteins, since at 2 and 4 h post-feeding there were no significant increases in Rhcg2, NHE-2, or H^+ -ATPase (Fig. 6). However, these data represent only the messages for the proteins, and it was instructive to find that branchial H^+ -ATPase activity had increased significantly by 4 h post-feeding, long before the significant increase in mRNA expression at 10 h. Indeed, it is very possible that non-genomic upregulation of the activity of existing proteins was sufficient for early elevation of J_{amm} , prior to later, larger increases in J_{amm} , which may have been due to the significantly increased plasma T_{amm} levels occurring at 8 h (Figs. 1, 4) associated with slow processing of the dietary N-load. In fact, Seshadri et al. (2006a) demonstrated that when acidosis is induced in rats, a condition that is known to cause an increase in urinary ammonia excretion, Rhcg protein expression increased in several areas of the kidney though mRNA expression of Rhcg remained unchanged. In a follow-up study, it was demonstrated that acidosis induced an increase in apical protein expression of Rhcg and a decrease in cytosolic protein expression, suggesting that these proteins are present in vesicles and are inserted during acidosis (Seshadri et al. 2006b). This, together with the early transient increase in H^+ -ATPase activity at 4 h, is a potential explanation for the increase in ammonia excretion capacity, which occurs in the absence of an increase in mRNA expression demonstrated here. The delayed and larger increase in J_{amm} seen 8 h after a meal (Fig. 1) was presumably associated with increases in protein synthesis associated with increases in Rhcg2 and NHE-2 mRNA levels at 6 h post-feeding (Fig. 6a, b). It was not until 10 h post-feeding that a significant increase in H^+ -ATPase mRNA also occurred (Fig. 6c), perhaps a response to the increase in plasma T_{amm} that occurred at 8 h (Fig. 4). A generally higher bulk P_{NH_3} gradient favoring excretion was seen throughout most of the post-feeding period (Fig. 8) and may also have contributed to the sustained elevation in J_{amm} , by either Rh-mediated transport (Nawata et al. 2010) or simple diffusion (Kelly and Wood 2001; Tsui et al. 2009). If a postprandial alkaline tide (Bucking and Wood 2008; Cooper and Wilson 2008) occurs in these juvenile trout, then the contribution of this component would be increased.

Also critical to the induction of elevated J_{amm} was the observed transient increase in $J_{\text{in}}^{\text{Na}}$, which became significant at 6 h after the meal (Fig. 6a). This transient increase, which may be related to a sustained increase in J_{amm} , could be an indication of the rather loose coupling between these processes. In situ studies of MRCs in the larvae of Japanese medaka indicated that ammonia excretion through Rhcg (Rhcg1) was dependent on H^+ excretion by NHE-3, which appeared to be tightly linked to Na^+ uptake (Wu et al. 2010). These were similar, but not identical to the findings of Tsui et al. (2009) where in vitro studies on cultured trout gill epithelia indicated that Rhcg-2, NHE-2, and H^+ -ATPase all played a role in the linkage of a portion of ammonia excretion to Na^+ uptake, whereas a portion occurred by simple diffusion along P_{NH_3} gradients. In vivo, this increase in $J_{\text{in}}^{\text{Na}}$ probably occurred in response to internal ammonia loading associated with feeding, since the infusion of exogenous ammonium salts is also known to cause an increase in $J_{\text{in}}^{\text{Na}}$ in intact fish (Kerstetter and Keeler 1976; Maetz and Garcia-Romeu 1964; Maetz 1973; Kerstetter and Keeler 1976; Wilson et al. 1994; Salama et al. 1999). The increase in branchial H^+ -ATPase activity seen at 4 h post-feeding may also have played a role here. To our knowledge, this is the first demonstration that feeding a normal meal causes an increase in $J_{\text{in}}^{\text{Na}}$ in freshwater fish; notably, $J_{\text{out}}^{\text{Na}}$ also increased, so Na^+ homeostasis was preserved (Fig. 6a). Previous studies have instead focused on the inhibition of $J_{\text{in}}^{\text{Na}}$ and large elevations of $J_{\text{out}}^{\text{Na}}$, which are associated with ingestion of experimental high-NaCl meals (Smith et al. 1995; Pyle et al. 2003).

Overall, these observations and the increases in mRNA expression of NHE-2 (Fig. 6b) and H^+ -ATPase (Fig. 6c) demonstrate that the handling of ammonia post-feeding is likely to be, at least in part, coupled to Na^+ uptake. Thus, on an overall basis, feeding causes similar responses in gill mRNA expression patterns, $J_{\text{in}}^{\text{Na}}$, and J_{amm} to ammonium salt infusion (Wilson et al. 1994; Salama et al. 1999; Nawata and Wood 2009). These in vivo results support the “ $\text{Na}^+/\text{NH}_4^+$ exchange complex” model of Rhcg2, NHE-2, H^+ -ATPase function proposed by Wright and Wood (2009), which loosely links Rhcg2-mediated NH_3 excretion to active Na^+ uptake through boundary layer acidification. As originally proposed by Heisler (1990), there may be a plasma T_{amm} threshold above which this “active” mechanism cuts in.

Effects of HEA exposure in unfed fish

Exposure to HEA led to an initial reversal of ammonia excretion (Figs. 2, 3) as earlier demonstrated by Wilson et al. (1994) and Nawata et al. (2007) in unfed adult trout. In response to HEA exposure, there was a dramatic increase in plasma T_{amm} , which was rapid (within 2 h), compared to the delayed and less prominent increase seen

in response to feeding (within 8 h; Fig. 4). This increase occurred in conjunction with the reversal of ammonia excretion during HEA (Figs. 2, 3). A trend of decreasing plasma T_{amm} ensued (Fig. 4) as unfed fish were able to re-establish ammonia excretion by 4–6 h of exposure to HEA (Fig. 3). This ability to overcome a large inwardly directed P_{NH_3} gradient (Fig. 7) may be attributed to the induction of branchial ammonia excretion mechanisms, which are potentially active. Exposure to HEA led to an increase in the mRNA expressions of Rhcg2, NHE-2, and H^+ -ATPase mRNA after only 2 h of exposure (corresponding to the 6 h time point in Fig. 6), similar to responses to ammonium salt infusion (Nawata and Wood 2009). These increases were more immediate than the increases seen in response to feeding, which did not occur until 6–10 h after the meal (Fig. 6). Notably, these responses to HEA occurred more rapidly than in adult trout faced with the same HEA challenge in identical water chemistry (Nawata et al. 2007). In the adult trout, similar increases in Rhcg2 and H^+ -ATPase mRNA expression were not seen until 12–48 h, and re-establishment of positive J_{amm} did not occur until 12–24 h (Nawata et al. 2007), again highlighting the more rapid responses of these juvenile trout.

High environmental ammonia exposure tended to depress $J_{\text{in}}^{\text{Na}}$, though not by a significant margin (Fig. 5c). This response contrasts with the response to feeding alone, where $J_{\text{in}}^{\text{Na}}$ (likely coupled to boundary layer acidification) was clearly increased (Fig. 5a) as a putative mechanism enhancing J_{amm} (Fig. 1). This inhibition of $J_{\text{in}}^{\text{Na}}$ by HEA is similar to results reported by Twitchen and Eddy (1994) and Wilson et al. (1994) and may be attributed to competition by NH_4^+ with Na^+ for external binding sites on Na^+ transport proteins (Na^+ channels and/or NHE-2). Whether this simply slows down Na^+ uptake, or whether it results in greater NH_4^+ uptake (i.e., increased ammonia loading) in substitution for Na^+ uptake through these pathways is unknown. However, the latter would clearly be maladaptive, otherwise why then upregulate NHE-2 and H^+ -ATPase mRNA during HEA exposure (Fig. 6b, c)? A more likely scenario is that Na^+ influx is slowed, but H^+ efflux is maintained or increased. This scenario envisages that Na^+ uptake may be uncoupled from H^+ flux and ammonia excretion. Using low doses of amiloride (10^{-5} M) in the brown trout, *Salmo trutta*, Nelson et al. (1997) demonstrated that $J_{\text{in}}^{\text{Na}}$ could be reduced by 75% while H^+ efflux and J_{amm} continued at control rates. We speculate that during HEA exposure, NH_4^+ acts like low dose amiloride, such that H^+ efflux and boundary layer acidification for trapping of Rhcg2-mediated NH_3 efflux can still occur. By this scenario, upregulation of NHE-2 would serve to maintain some degree of Na^+ uptake and linked H^+ excretion, while H^+ -ATPase (now uncoupled from Na^+ uptake), together with CO_2 hydration, would provide the bulk of the H^+ efflux and

acid trapping mechanism. H^+ -ATPase mRNA was upregulated (Fig. 6c), whereas H^+ -ATPase activity remained at control levels though with a non-significant tendency to decline in HEA (Fig. 7). Presumably, H^+ -ATPase activity would increase over the longer term if HEA were maintained, as reported by Nawata et al. (2007) in adult trout.

Protective effects of feeding against HEA exposure

Unlike unfed fish exposed to HEA, fed fish were able to immediately maintain normal ammonia excretion rates when exposed to HEA, demonstrating that feeding has a protective effect against HEA exposure (Fig. 3). There was no initial reversal of J_{amm} to negative values. These fed trout also displayed increases in plasma T_{amm} during HEA similar to or greater than those seen in the unfed, HEA group, reflecting the additive effect of feeding (Fig. 4). However, on an individual fish basis, the bulk P_{NH_3} gradient against which they were excreting was in fact even more negative than in the unfed HEA group (Fig. 7), owing to the fact that they were actively excreting ammonia into the water from the beginning of the exposure, effectively increasing the P_{NH_3} gradient by increasing water T_{amm} .

Contrary to our original ideas (see “Introduction”), this increased ability to excrete ammonia against the gradient cannot be attributed to prior upregulation of Rhcg2, NHE-2, and H^+ -ATPase mRNA expression associated with feeding, for two reasons. Firstly, after 2 h of HEA (6 h time point in Fig. 6), upregulation of message in these fed fish exposed to HEA was quantitatively equal to or less than in fed fish, which had not been exposed to HEA at the same time. Secondly, compared to unfed fish exposed to HEA, fed fish exposed to HEA demonstrated a delayed upregulation of mRNA expression of NHE-2 and H^+ -ATPase, which was never greater in magnitude (Fig. 6b, c). Though the upregulation of Rhcg2 mRNA expression was not delayed, it also was not greater in magnitude than that seen in unfed fish exposed to HEA (Fig. 6a).

We speculate that the explanation for this apparent paradox lies in the upregulated ammonia excretion capacity seen early on in response to feeding alone (within 2–4 h after the meal), without mRNA recruitment, as discussed earlier. It is likely that by the time of HEA exposure (4 h post-feeding), fed fish already possessed increased ammonia transport activity levels in their gill cells due to non-genomic modification. Note for example the significant increase in branchial H^+ -ATPase activity at 4 h post-feeding (Fig. 7), prior to any significant increase in H^+ -ATPase message (Fig. 6c). Any later protein synthesis associated with mRNA increases would likely add to this activity. This may be the explanation for the finding of Wicks and Randall (2002a) that feeding protects rainbow trout from ammonia toxicity, elevating the LC_{50} during the first 24 h of HEA exposure.

Unlike the unfed HEA group, fed fish exposed to HEA displayed a depression of J_{in}^{Na} , which was significant (Fig. 5b), and recovery of J_{in}^{Na} was certainly no faster even though J_{amm} was well maintained (Fig. 3). It is unclear why the inhibitory effect of high NH_4^+ should be greater in fed fish; perhaps, these trout were relying more on NHE-2 at this time compared to unfed fish. Their branchial H^+ -ATPase activity levels also tended to be lower (Fig. 7). Regardless, these data demonstrate that upregulation of Na^+ influx is not a requirement for “active” ammonia excretion against the P_{NH_3} gradient during HEA exposure. The depression of J_{in}^{Na} may seem counter-intuitive given that expressions of both NHE-2 (Fig. 6b) and H^+ -ATPase mRNA (Fig. 6c) were increased significantly at 6 h after exposure to HEA (10 h after the meal). However, as in the case of the unfed HEA group, it is likely that NH_4^+ was competing for Na^+ binding sites, such that H^+ efflux and ammonia excretion were then uncoupled from Na^+ uptake at that time. Perhaps with longer HEA exposure, a more complete recovery or increase of J_{in}^{Na} would have been seen in both HEA treatments.

Conclusions

Rhcg2, NHE-2, and H^+ -ATPase all appear to play significant roles in ammonia excretion in trout in vivo, in accordance with the “ Na^+/NH_4^+ exchange complex” model of Wright and Wood (2009), but simple diffusive flux along the P_{NH_3} gradient is also important. Juvenile trout can elevate ammonia excretion more rapidly than adult trout in response to feeding or HEA. After feeding, the ammonia excretion capacity of the gills increases before elevated Rhcg2, NHE-2, and H^+ -ATPase mRNA levels are seen, suggesting non-genomic activation of existing transport protein function. This confers a greater capacity for fed fish to excrete ammonia against a bulk P_{NH_3} gradient in the face of HEA. Later after feeding, elevated Na^+ influx appears to play an important role in augmenting ammonia excretion, but this is not the case during HEA exposure. The influx of Na^+ is loosely tied to an efflux of H^+ (either by NHE-2 or H^+ -ATPase) and it is this efflux of H^+ , not the influx of Na^+ , which is responsible for facilitating ammonia excretion. This becomes evident during HEA where Na^+ influx is depressed even though Rhcg2, NHE-2, and H^+ -ATPase are all upregulated and ammonia excretion is restored: i.e., Na^+ influx is uncoupled from H^+ and ammonia efflux. In future studies, it would be interesting to examine ammonia and net H^+ excretion during HEA when Na^+ is not present in the water. This would quantify whether or not ammonia excretion during HEA (in fed or unfed fish) can, in fact, be completely uncoupled from Na^+ influx. Studies examining

the post-transcriptional regulation of the implicated transporters will also be essential to realizing the complete model for ammonia excretion during feeding and HEA exposure. The temporal disconnect between recorded changes in H^+ -ATPase mRNA expression and functional activity observed in the present study underscores the need for such studies. Finally, the use of pharmacological blockers to inhibit H^+ excretion (bafilomycin, EIPA, and acetazolamide) during HEA would be useful in determining the relative contribution to ammonia excretion of H^+ -ATPase, NHE-2, and carbonic anhydrase, respectively.

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References

- Avella M, Bornancin M (1989) A new analysis of ammonia and sodium transport through the gills of the freshwater rainbow trout (*Salmo gairdneri*). J Exp Biol 142:155–175
- Bakouh N, Benjelloun F, Hulin P, Brouillard F, Edelman A, Cherif-Zahar B, Planelles G (2004) NH_3 is involved in the NH_4^+ transport induced by the functional expression of the human Rh C Glycoprotein. J Biol Chem 279:15975–15983
- Bucking C, Wood CM (2008) The alkaline tide and ammonia excretion after voluntary feeding in freshwater rainbow trout. J Exp Biol 211:2533–2541
- Buddington RK, Krogdahl A (2004) Hormonal regulation of the fish gastrointestinal tract. Comp Biochem Physiol A 139:261–271
- Bustin SA (2000) Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. J Mol Endocrinol 25:169–193
- Bustin SA (2002) Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): trends and problems. J Mol Endocrinol 29:23–39
- Cameron JN, Heisler N (1983) Studies of ammonia in the rainbow trout: physico-chemical parameters, acid–base behaviour, and respiratory clearance. J Exp Biol 105:107–125
- Cooper CA, Wilson RW (2008) Post-prandial alkaline tide in freshwater rainbow trout: effects of meal anticipation on recovery from acid–base and ion regulatory disturbances. J Exp Biol 211:2542–2550
- Fromm PO (1963) Studies on renal and extra-renal excretion in a freshwater teleost, *Salmo gairdneri*. Comp Biochem Physiol 10:121–128
- Fromm PO, Gillette JR (1968) Effect of ambient ammonia on blood ammonia and nitrogen excretion of rainbow trout (*Salmo gairdneri*). Comp Biochem Physiol 26:887–896
- Gelineau A, Medale F, Boujard T (1998) Effect of feeding time on postprandial nitrogen excretion and energy expenditure in rainbow trout. J Fish Biol 52:655–664
- Heisler N (1990) Mechanisms of ammonia elimination in fishes. In: Truchot JP, Lahlou B (eds) Animal nutrition and transport processes. Transport, respiration and excretion: comparative and environmental aspects. Comp Physiol, vol 2, Karger, Basel, pp 137–151
- Hung CYC, Tsui KNT, Wilson JM, Nawata CM, Wood CM, Wright PA (2007) Rhesus glycoprotein gene expression in the mangrove killfish *Kryptolebias marmoratus* exposed to elevated environmental ammonia levels and air. J Exp Biol 210:2419–2429

- Ivanis G, Esbaugh AJ, Perry SF (2008) Branchial expression and localization of SLC9A2 and SLC9A3 sodium/hydrogen exchangers and their possible role in acid-base regulation in freshwater rainbow trout (*Oncorhynchus mykiss*). *J Exp Biol* 211:2467–2477
- Kaushik SJ, Teles AD (1985) Effect of digestible energy on nitrogen and energy balance in rainbow trout. *Aquaculture* 50:89–101
- Kelly SP, Wood CM (2001) The cultured branchial epithelium of the rainbow trout as a model for diffusive fluxes of ammonia across the fish gill. *J Exp Biol* 204:4115–4124
- Kerstetter TH, Keeler M (1976) On the interaction of NH_4^+ and Na^+ fluxes in the isolated trout gill. *J Exp Biol* 64:517–527
- Khademi S, O'Connell J, Remis J, Robles-Colmenares Y, Miercke LJW, Stroud RM (2004) Mechanism of ammonia transport by Amt/MEP/Rh: Structure of AmtB at 1.35 Å. *Science* 305:1587–1596
- Li X, Jayachandran S, Nguyen HHT, Chan MK (2007) Structure of the *Nitrosomonas europaea* Rh protein. *Proc Natl Acad Sci USA* 104:19279–19284
- Lin H, Randall D (1991) Evidence for the presence of an electrogenic proton pump on the trout gill epithelium. *J Exp Biol* 161:119–134
- Lin H, Randall D (1993) H^+ -ATPase in crude homogenates of fish gill tissue: inhibitor sensitivity and environmental and hormonal regulation. *J Exp Biol* 180:163–174
- Lin H, Pfeiffer DC, Vogl AW, Pan J, Randall DJ (1994) Immunolocalization of H^+ -ATPase in the gill epithelia of rainbow trout. *J Exp Biol* 195:169–183
- Maetz J (1956) Les échanges de sodium chez le poisson *Carassius auratus* L. Action d'un inhibiteur de l'anhydrase carbonique. *J Physiol Paris* 48:1085–1099
- Maetz J (1972) Branchial sodium exchange and ammonia excretion in the goldfish *Carassius auratus*. Effects of ammonia loading and temperature changes. *J Exp Biol* 56:601–620
- Maetz J (1973) $\text{Na}^+/\text{NH}_4^+$, Na^+/H^+ exchanges and NH_3 movement across the gill of *Carassius auratus*. *J Exp Biol* 58:255–275
- Maetz J, Garcia-Romeu F (1964) The mechanism of sodium and chloride uptake by the gills of fresh-water fish, *Carassius auratus*: II. Evidence for $\text{NH}_4^+/\text{Na}^+$ and $\text{HCO}_3^-/\text{Cl}^-$ exchanges. *J Gen Physiol* 47:1209–1227
- Marini AM, Urrestarazu A, Beauwens R, Andre B (1997) The Rh (Rhesus) blood group polypeptides are related to NH_4^+ transporters. *Trends Biochem Sci* 22:460–461
- Nakada T, Hoshijima K, Esaki M, Nagayoshi S, Kawakami K, Hirose S (2007a) Localization of ammonia transporter Rhcg1 in mitochondrion-rich cells of yolk sac, gill, and kidney of zebrafish and its ionic strength-dependent expression. *Am J Physiol Regul Integr Comp Physiol* 293:R1743–R1753
- Nakada T, Westhoff CM, Kato A, Hirose S (2007b) Ammonia secretion from fish gills depends on a set of Rh glycoproteins. *FASEB J* 21:1067–1074
- Nawata CM, Wood CM (2008) The effects of CO_2 and external buffering on ammonia excretion and Rhesus glycoprotein mRNA expression in rainbow trout. *J Exp Biol* 211:3226–3236
- Nawata CM, Wood CM (2009) mRNA expression analysis of the physiological responses to ammonia infusion in rainbow trout. *J Comp Physiol B* 179:799–810
- Nawata CM, Hung CYC, Tsui TKN, Wilson JM, Wright PA, Wood CM (2007) Ammonia excretion in rainbow trout (*Oncorhynchus mykiss*): evidence for Rh glycoprotein and H^+ -ATPase involvement. *Physiol Genomics* 31:463–474
- Nawata CM, Wood CM, O'Donnell MJ (2010) Functional characterization of Rhesus glycoproteins from an ammoniotelic teleost, the rainbow trout, using oocyte expression and SIET analysis. *J Exp Biol* 213:1049–1059
- Nelson ID, Potts WTW, Huddart H (1997) The use of amiloride to uncouple branchial sodium and proton fluxes in the brown trout, *Salmo trutta*. *J Comp Physiol B* 167:123–128
- Parks SK, Tresguerres M, Goss GG (2007) Interactions between Na^+ channels and $\text{Na}^+/\text{HCO}_3^-$ cotransporters in the freshwater fish gill MR cell: a model for transepithelial Na^+ uptake. *Am J Physiol Cell Physiol* 292:935–944
- Payan P (1978) A study of the $\text{Na}^+/\text{NH}_4^+$ exchange across the gill of the perfused head of the trout (*Salmo gairdneri*). *J Comp Physiol* 124:181–188
- Perry SF, Furimsky M, Bayaa M, Georgalis T, Shahsavarani A, Nickerson JG, Moon TW (2003) Integrated responses of $\text{Na}^+/\text{HCO}_3^-$ cotransporters and V-type H^+ -ATPases in the fish gill and kidney during respiratory acidosis. *Biochim Biophys Acta* 30:175–184
- Pyle GG, Kamunde CN, McDonald DG, Wood CM (2003) Dietary sodium inhibits aqueous copper uptake in rainbow trout (*Oncorhynchus mykiss*). *J Exp Biol* 206:609–618
- Randall DJ, Wright PA (1987) Ammonia distribution and excretion in fish. *Fish Physiol Biochem* 3:107–120
- Salama A, Morgan IJ, Wood CM (1999) The linkage between Na^+ uptake and ammonia excretion in rainbow trout: kinetic analysis, the effects of $(\text{NH}_4)_2\text{SO}_4$ and NH_4HCO_3 infusion and the influence of gill boundary layer pH. *J Exp Biol* 202:697–709
- Scott GR, Claiborne JB, Edwards SL, Schulte PM, Wood CM (2005) Gene expression after freshwater transfer in gills and opercular epithelia of killifish: insight into divergent mechanisms of ion transport. *J Exp Biol* 208:2719–2729
- Seshadri RM, Klein JD, Kozlowski S, Sands JM, Kim YH, Han KH, Handlogten ME, Verlander JW, Weiner ID (2006a) Renal expression of the ammonia transporters, Rhbg and Rhcg, in response to chronic acidosis. *Am J Physiol Renal Physiol* 290:397–408
- Seshadri RM, Klein JD, Smith K, Sands JM, Handlogten ME, Verlander JW, Weiner ID (2006b) Changes in subcellular distribution of the ammonia transporter, Rhcg, in response to chronic metabolic acidosis. *Am J Physiol Renal Physiol* 290:1443–1452
- Smith HW (1929) The excretion of ammonia and urea by the gills of fish. *J Biol Chem* 81:727–742
- Smith NF, Eddy FB, Talbot C (1995) Effect of dietary salt load on transepithelial Na^+ exchange in freshwater rainbow trout (*Oncorhynchus mykiss*). *J Exp Biol* 198:2359–2364
- Sullivan GV, Fryer JN, Perry SF (1995) Immunolocalization of proton pumps (H^+ -ATPase) in pavement cells of rainbow trout gill. *J Exp Biol* 198:2619–2629
- Tsui TKN, Hung CYC, Nawata CM, Wilson JM, Wright PA, Wood CM (2009) Ammonia transport in cultured gill epithelium of freshwater rainbow trout: the importance of Rhesus glycoproteins and the presence of an apical $\text{Na}^+/\text{NH}_4^+$ exchange complex. *J Exp Biol* 212:878–892
- Twitche ID, Eddy FB (1994) Effects of ammonia on sodium balance in juvenile rainbow trout *Oncorhynchus mykiss* Walbaum. *Aquat Toxicol* 30:27–45
- Verdouw H, van Echteld CJA, Dekkers EMJ (1978) Ammonia determination based on indophenols formation with sodium salicylate. *Water Res* 12:399–402
- Weihrauch D, Wilkie MP, Walsh PJ (2009) Ammonia and urea transporters in gills of fish and aquatic crustaceans. *J Exp Biol* 212:1716–1730
- Wicks BJ, Randall DJ (2002a) The effect of feeding and fasting on ammonia toxicity in juvenile rainbow trout, *Oncorhynchus mykiss*. *Aquat Toxicol* 59:71–82
- Wicks BJ, Randall DJ (2002b) The effect of sub-lethal ammonia exposure on fed and unfed rainbow trout: the role of glutamine in regulation of ammonia. *Comp Biochem Physiol A* 132:275–285
- Wilkie MP (2002) Ammonia excretion and urea handling by fish gills: present understanding and future research challenges. *J Exp Zool* 293:284–301

- Wilson RW, Wright PM, Munger S, Wood CM (1994) Ammonia excretion in freshwater rainbow trout (*Oncorhynchus mykiss*) and the importance of gill boundary layer acidification: lack of evidence for $\text{Na}^+/\text{NH}_4^+$ exchange. *J Exp Biol* 191:37–58
- Wilson JM, Laurent P, Tufts BL, Benos DJ, Donowitz M, Vogl AW, Randall DJ (2000) NaCl uptake by the branchial epithelium in freshwater teleost fish: an immunological approach to ion-transport protein localization. *J Exp Biol* 203:2279–2296
- Wood CM (1993) Ammonia and urea metabolism and excretion. In: Evans DH (ed) *The physiology of fishes*. CRC Press, Boca Raton, pp 379–425
- Wright PA, Wood CM (2009) A new paradigm for ammonia excretion in aquatic animals: role of Rhesus (Rh) glycoproteins. *J Exp Biol* 212:2303–2312
- Wu SC, Horng JL, Liu ST, Hwang PP, Wen ZH, Lin CS, Lin LY (2010) Ammonium-dependent sodium uptake in mitochondrion-rich cells of medaka (*Oryzias latipes*) larvae. *Am J Physiol* 298:C237–C250