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ORIGINAL PAPER

Ammonia transport across the skin of adult rainbow trout (*Oncorhynchus mykiss*) exposed to high environmental ammonia (HEA)

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Abstract Recent molecular evidence points towards a capacity for ammonia transport across the skin of adult rainbow trout. A series of in vivo and in vitro experiments were conducted to understand the role of cutaneous ammonia excretion (J_{amm}) under control conditions and after 12-h pre-exposure to high environmental ammonia (HEA; 2 mmol/l NH_4HCO_3). Divided chamber experiments with bladder-catheterized, rectally ligated fish under light anesthesia were performed to separate cutaneous J_{amm} from branchial, renal, and intestinal J_{amm}. Under control conditions, cutaneous J_{amm} accounted for 4.5 % of total J_{amm} in vivo. In fish pre-exposed to HEA, plasma total ammonia concentration increased 20-fold to approximately 1,000 μmol/l, branchial J_{amm} increased 1.5- to 2.7-fold, and urinary J_{amm} increased about 7-fold. Urinary J_{amm} still accounted for less than 2 % of total J_{amm}. Cutaneous J_{amm} increased 4-fold yet amounted to only 5.7 % of total J_{amm} in these fish. Genes (Rhcg1, Rhcg2, Rhbg, NHE-2, v-type H^+-ATPase) known to be involved in ammonia excretion at the gills of trout were all expressed at the mRNA level in the skin, but their expression did not increase with HEA pre-exposure. In vitro analyses using [^{14}C] methylamine (MA), an ammonia analog which is transported by Rh proteins, demonstrated that MA permeability in isolated skin sections was higher in HEA pre-exposed fish than in control fish. The addition of basolateral ammonia (1,000 μmol/l) to this system abolished this increase in permeability, suggesting ammonia competition with MA for Rh-mediated transport across the skin of HEA pre-exposed trout; this did not occur in skin sections from control trout. Moreover, in vitro J_{amm} by the skin of fish which had been pre-exposed to HEA was also higher than in control fish in the absence of basolateral ammonia, pointing towards a possible cutaneous ammonia loading in response to HEA. In vitro MA permeability was reduced upon the addition of amiloride (10^{-4} mol/l), but not phenamil (10^{-5} mol/l) suggesting a role for a Na/H-exchanger (NHE) in cutaneous ammonia transport, as has been previously described in the skin of larval fish. Overall, it appears that under control conditions and in response to HEA pre-exposure, the skin makes only a very minor contribution to total J_{amm}, but the observed increases in cutaneous J_{amm} in vivo and in cutaneous J_{amm} and MA permeability in vitro demonstrate the capacity for ammonia transport in the skin of adult trout. It remains unclear if this capacity may become significant under certain environmental challenges or if it is merely a remnant of cutaneous transport capacity from early life stages in these fish.

Keywords Rainbow trout · Ammonia excretion · Cutaneous excretion · Rhesus (Rh) proteins · Fish skin · High environmental ammonia (HEA)

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Introduction

The majority of freshwater teleost fish excrete ammonia to eliminate the nitrogenous wastes produced by protein

metabolism. In an early pinnacle study, Homer Smith (1929) first determined that the majority (80–90 %) of ammonia excreted by freshwater fish (goldfish; *Carassius auratus* and carp; *Carassius carassius*) occurred via the gills and that the remaining portion (10–20 %) occurred renally. Since this study, a mechanistic model describing ammonia transport across the gills of fish has been developed. The model has incorporated Rhesus (Rh) glycoproteins as the putative ammonia transport channels in gill epithelial cells, with basally and apically expressed Rhbg and Rhcg isoforms (see Weihrauch et al. 2009; Wright and Wood 2009). Furthermore, the branchial excretion model for freshwater fish has been coined a "Na^+/NH_4^+-exchange complex" (Tsui et al. 2009; Wright and Wood 2009) as the excretion of ammonia across the gill is coupled to Na^+ uptake, through an as yet unidentified epithelial Na^+ channel and/or a Na/H-exchanger (NHE). Also key to this model is the presence of an apical H^+-ATPase which facilitates ammonia efflux via acid-trapping and also facilitates Na^+ uptake electrogenically through the Na^+ channels (see Weihrauch et al. 2009; Wright and Wood 2009).

To date, much of our current understanding of the mechanisms underlying ammonia transport in fish has been gained by studying physiological and molecular responses to ammonia challenges. One such challenge, the exposure to high environmental ammonia (HEA), generally leads to an initial reversal of ammonia excretion relative to control values, indicative of ammonia uptake from the environment, and occurs in conjunction with a subsequent rise in plasma total ammonia (T_{amm}) levels (Wilson et al. 1994; Nawata et al. 2007; Zimmer et al. 2010). The rainbow trout (*Oncorhynchus mykiss*) used in these studies were eventually able to re-establish ammonia efflux against the inwardly directed gradient of HEA and this increase in excretion capacity was coupled to increased branchial mRNA expression of Rh proteins and of those transporters involved in Na^+ uptake (Nawata et al. 2007; Zimmer et al. 2010; Wood and Nawata 2011; Sinha et al. 2013). Interestingly, Nawata et al. (2007) demonstrated that Rh genes in the skin of trout were also responsive to HEA exposure, as observed in pufferfish (Nawata et al. 2010a), though the timing and isoform-specificity of this response were different than that seen in the gills. Regardless, this observation suggests the capacity for ammonia transport across an epithelium which is generally believed to be impermeable to both water and ions (Fromm 1968; Kirsch 1972) and which is not believed to contribute to whole-body ammonia excretion in adult freshwater fish (Smith 1929). To date, in vivo cutaneous ammonia excretion in adult fish appears to only occur as a specialized adaptation in a few fish species (e.g., mangrove killifish, *Kryptolebias marmoratus*, Frick and Wright 2002; dab, *Limanda limanda*, Sayer and Davenport 1987). Cutaneous ammonia transport in a typical teleost fish, however, has yet to be demonstrated despite increasing evidence that the skin of these fish appears capable of physiological exchange with the surrounding environment (Glover et al. 2013).

In larval fish, immediately following hatch when the gills are still undeveloped and branchial surface area is low, cutaneous surfaces (body and yolk sac skin) comprise the majority of total fish surface area (Rombough 1999). As such, at this stage the skin is considered to be the major site for exchange with the surrounding environment. It has been demonstrated, to date, that both oxygen and Na^+ uptake are predominantly cutaneous following hatch and eventually progress to become primarily branchial as the gills develop (Wells and Pinder 1996; Fu et al. 2010). Furthermore, several studies have demonstrated that the skin of larval zebrafish (*Danio rerio*) and Japanese medaka (*Oryzias latipes*) is also capable of ammonia excretion which appears to be coupled to Na^+ uptake, in accordance with the current model for branchial ammonia transport (Shih et al. 2008, 2012; Wu et al. 2010; Ito et al. 2013). Similarly, in larval rainbow trout, ammonia excretion and Na^+ uptake both occur cutaneously following hatch and, with development, both processes eventually shift simultaneously to become predominantly branchial, suggesting both cutaneous and branchial coupling of these exchange processes at this life stage (Zimmer AM, Wright PA, Wood CM unpublished results). Thus, it is clear that a capacity for ammonia transport by the skin of larval fish exists, but it is unknown to what degree this capacity might be retained in adult fish. Presently, the general view of the skin in adult fish is that it is an impermeable barrier to water and ion movement which does not contribute to physiological exchange; however, this is currently being contested (see Glover et al. 2013).

The overall aim of the present study was to assess the potential for cutaneous ammonia excretion (J_{amm}) in adult rainbow trout, a model teleost fish. Based on molecular evidence in adult fish (Nawata et al. 2007), we hypothesized that cutaneous J_{amm} in rainbow trout under control conditions would be low but that it would increase and contribute significantly to whole-body J_{amm} in fish exposed to HEA. Thus, the first objective of this study was to directly measure in vivo cutaneous J_{amm} using a divided chamber setup wherein the contribution of the skin to total J_{amm} could be isolated from branchial, renal, and gastrointestinal routes of excretion. Changes in in vivo J_{amm} could then be correlated with possible changes in skin Rh gene expression or Rh protein function. We further hypothesized that the skin of HEA-exposed fish would display an intrinsic increase in ammonia permeability, indicative of increased transport capacity of this tissue. Thus, our second objective was to assess ammonia flux and

permeability to [^{14}C]methylamine (MA) in isolated skin sections in vitro using a modified Ussing chamber design, similar to that of Glover et al. (2011). MA is an ammonia analog previously demonstrated to travel through trout Rh proteins (Nawata et al. 2010b). We also measured mRNA expression in the skin of genes (Rhcg1, Rhcg2, Rhbg, NHE-2, v-type H^+-ATPase) known to be involved in the linkage of Na^+ uptake to ammonia excretion in the gills of trout (Nawata et al. 2007; Tsui et al. 2009; Zimmer et al. 2010; Wood and Nawata 2011; Sinha et al. 2013). The final objective of the present study was to determine if ammonia transport across the skin of adult rainbow trout is linked to Na^+ uptake, as appears to be the case in larval fish (Wu et al. 2010; Shih et al. 2012; Ito et al. 2013), including larval trout (Zimmer AM, Wright PA, Wood CM unpublished results), and in adult mangrove killifish (Cooper et al. 2013) by assessing the effects of pharmacological blockers of Na^+ uptake pathways (amiloride: general NHE and Na^+ channel blocker, and phenamil: specific Na^+ channel blocker) on in vitro MA permeability and J_{amm}. We hypothesized that, similar to the case of larval zebrafish (Shih et al. 2012) and adult mangrove killifish (Cooper et al. 2013), cutaneous ammonia transport would be linked to Rh and NHE function.

Materials and methods

Fish

Adult rainbow trout obtained from Spring Valley Trout Farm (Langley, British Columbia, Canada), weighing 200–450 g, were held in large outdoor tanks at the University of British Columbia in Vancouver, BC, supplied with flow-through dechlorinated Metro Vancouver tap water (in mM: Na^+, 0.06; Cl^-, 0.05; Ca^{2+}, 0.03; Mg^{2+}, 0.007; K^+, 0.004; and in mg/l $CaCO_3$, alkalinity, 3.0; hardness 3.3; pH 7.0) at 10 °C. Fish were fed commercial trout pellets (Skretting, Orient 4–0) at a ration of 1 % per day three times a week. Prior to experiments, fish were fasted for at least 1 week. All experiments were performed at a constant temperature (10.0 ± 0.5 °C) on a natural photoperiod and were approved by the animal care committees of the University of British Columbia and McMaster University.

Series 1: range-finding experiments

To determine the concentration of HEA to be used in experiments, fish were exposed to control conditions and to HEA of 1, 2, and 3 mmol/l NH_4HCO_3 for 12 h. Fish were exposed in a 150-l static system (maximum of 8 per exposure) where pH for all treatments was kept at 7.0 via the addition of HCl. At the end of the 12 h of exposure, half the fish were euthanized and blood was sampled via caudal puncture using a heparinized 1-ml syringe. Blood samples were immediately spun down, and plasma was collected and stored briefly on ice until further analysis. The remaining half of the fish were then removed and placed individually into 4-l flux boxes containing control (i.e., no ammonia added), aerated water. Following a 15-min period to allow fish to settle down, an initial 10-ml water sample was taken from each box and another 10-ml water sample every 2 h for 6 h to determine whole-body ammonia excretion rates (J_{amm}). Following this flux period, fish were lightly anesthetized with a 0.05 g/l neutralized MS-222 solution and weighed. All water samples were kept at −20 °C until further analysis.

Series 2: anesthetic control experiments

Based on these initial results, the 2 mmol/l HEA pre-exposure was deemed appropriate for subsequent experiments as this was the lowest concentration where both plasma ammonia levels and ammonia excretion rates increased significantly following HEA exposure (see "Results"). A second experimental series was then designed to determine the effect of light anesthesia (using MS-222) on whole-body J_{amm} (stage 1; McFarland 1959) which was required to calm fish in the divided chamber system described below to avoid both struggling during handling and escape from the dam. Fish were subjected to the same 12-h protocol described above in control or 2 mmol/l HEA and then their whole-body J_{amm} was measured in 4-l flux boxes in the presence and absence of 0.03 g/l of neutralized MS-222 (pH 7). All water samples were stored at −20 °C until further analysis.

Series 3: divided chamber experiments

In this series, the aim was to determine the contribution of the skin, separate from those of the gills, kidney, and intestinal tract, to total J_{amm} under control conditions and in response to pre-exposure to HEA (2 mmol/l NH_4HCO_3, pH 7.0). First, fish were anesthetized (0.1 g/l neutralized MS-222) and placed onto a V-shaped operating table, with constant anesthetic irrigation of the gills via a recirculating pump, to fit fish with internal urinary bladder catheters (Wood and Patrick 1994). The catheters were made from a length of PE50 tubing with a small flare at one end. The catheter was filled with distilled water and the flared end inserted into the urinary papilla and advanced to the middle of the urinary bladder. The papilla was then tied snugly around the catheter using 2.0 silk thread, and the catheter was held in place using a PE160 sheath (flared at both ends) which was glued to the catheter and held in place close to the papilla using two ligatures on the underside of

the fish. A pin was used to occlude the other end of the distilled water-filled catheter. Following catheterization, the anus of the fish was sewn shut via a ligation made through the underside of the fish, anterior to the rectum. Fish were then transferred to 4-l flux boxes supplied with re-circulating control water.

After a 2-h settling period, individual 4-l flux boxes were supplied with either re-circulating control or HEA water (2 mmol/l NH_4HCO_3, pH 7.0) (~35 l/fish) for 12 h overnight. During this time, pins were removed from the end of the catheters and urine was collected by siphon (approximately 3 cm H_2O head pressure) into pre-weighed, 25-ml Erlenmeyer flasks. After the 12-h flux period, pins were re-inserted into the end of the catheters, urine vials were weighed, and urine was collected and stored at −20 °C until further analysis. Flow to the flux boxes was then stopped and fish were lightly anesthetized via the addition of neutralized MS-222 to a concentration of 0.03 g/l. Next, a latex sheet with a hole cut into its center (approximately 2–3 cm in diameter) was fitted around the anesthetized fish, immediately posterior to the pectoral fins. The latex sheet was tight enough around the fish such that it was not necessary to physically glue the dam to the body of the fish. The fish, with its latex collar, was then loaded tail first into a length of PVC pipe (7-cm diameter, 30-cm length), sealed at the back end with a latex sheet, through which the urinary catheter was pulled, to collect urine during the divided chamber fluxes. The latex collar around the fish was then secured to the front end of the PVC pipe using an elastic band. The PVC pipe, with the head of the fish protruding from the front latex sheet and the urinary catheter exiting through the back latex sheet, was submerged into a 4-l flux box containing 0.03 g/l neutralized MS-222 in control water. The top of each PVC pipe was fitted with a sampling port (a modified 10-ml pipette tip) through which an airline was also inserted for aeration and mixing of the water. A second airline was placed directly into the 4-l box. Fish were allowed to adjust to this setup for 15 min prior to water sampling (10-ml) from the 4-l box (anterior chamber) and the PVC pipe (posterior chamber). Anterior and posterior water samples were taken every 2 h thereafter for a total of 6 h and all water samples were stored at −20 °C until further analysis. Water pH tended to rise slightly over this period, but stayed in the range of 7.0–7.4. Over this 6-h period, urine flow rate was generally depressed and sometimes zero, likely due to the effects of the anesthetic (see "Results"). Following the 6-h flux, a green dye was loaded into the posterior chamber and left to equilibrate for 1 h, after which 1-ml samples were taken from the anterior and posterior chambers to assess potential leakage in the system, determined colorimetrically (spectrophotometry at 590 nm). The sensitivity of this test was such that a leakage corresponding to as low as 1 % of the volume of the posterior chamber could be reliably detected. Fish were then removed from the chambers, weighed, and anterior and posterior final volumes were recorded.

In vitro experiments

In vitro experiments were performed to measure the cutaneous flux rate of ammonia and the permeability of the skin to the ammonia analog [^{14}C]methylamine (MA) (NEC-061, specific activity 2.26 Gbq/mmol, NEN-Dupont, Boston, MA, USA), and to assess regional variability. Trout were euthanized (0.5 g/l neutralized MS-222) either under control conditions or after 12-h exposure to HEA water (2 mmol/l NH_4HCO_3, pH 7.0). Fish were thoroughly rinsed in control water and kept on ice during dissection. Strips of skin were cut by making parallel longitudinal incisions: (1) just above the lateral line and just below the dorsal fin (for epaxial skin), and (2) just below the lateral line and at the point of juncture to the lighter-colored belly (for hypaxial skin). In addition, samples were excised from (3) the ventral midline (belly), (4) the top of the head, and (5) from the caudal peduncle (tail). The samples were taken in the above order. For each strip or sample, the skin was grasped firmly with forceps and peeled off slowly. Bits of adhering muscle tissue were removed, but no attempt was made to scrape the basolateral surface clean so as to avoid damage, so in occasional samples there may have been small remnants of adherent muscle. The scales were left intact for both the in vitro flux experiments and the gene expression studies, but for the latter, any underlying muscle and connective tissues were removed by thorough scraping.

The first samples taken were epaxial, and for some fish, a subsection (approximately 5 cm^2) was immediately immersed in 5 volumes of RNA*later*® (Ambion Inc., Austin, TX, USA) and stored overnight at 4 °C, and then frozen at −20 °C for later analysis of gene expression. All other skin samples were used in flux experiments employing modified Using chambers similar to the design of Glover et al. (2011). These were manufactured from 7-ml plastic scintillation vials with snap-on lids in which the center of the lid had been removed, revealing an aperture of 1.13 cm^2. The vial was filled with 2 ml of Cortland salmonid saline containing either no ammonia or 1 mmol/l NH_4HCO_3 to duplicate blood plasma T_{amm} values measured in fish which had been exposed to HEA (2 mmol/l NH_4HCO_3, referred to as HEA water from this point forward) for 12 h (see Results). The saline was also labeled with [^{14}C]methylamine so as to achieve a radioactivity of approximately 100,000 cpm/ml. The saline had been pre-equilibrated with 99.7 % O_2/0.3 % CO_2 to achieve normal in vivo PCO_2 levels, and the final pH was set to 7.7–7.8 with NaOH. A subsection of skin was placed over the mouth of the scintillation vial such that the basolateral side faced the saline, and was sealed in placed with the snap-on lid, revealing an exposed surface area of 1.13 cm^2. The vial was then blotted

dry and weighed to 1 mg accuracy. The flux period (2 h) was initiated by inverting the vial into a larger plastic container containing 5 ml of dechlorinated Vancouver tap water (pH = 7.0, composition as above) that was continually gassed with water-saturated air (to prevent evaporation). Thus, the basolateral surface of the skin was bathed in saline, and the apical surface in water. Drugs were present in the water in some trials, as outlined below. After 2 h, the flux measurement was terminated by removing the vial from the larger container, blotting dry, and re-weighing. Any preparations that leaked could be detected (and their data rejected) by large weight gains as there was a differential hydrostatic pressure of about 0.3 cm H_2O from the outer water bath to the inner saline bath. Final basolateral saline and apical water samples (1 ml each) were taken and added to 4 ml of Ultima Gold AB fluor (Perkin-Elmer, Waltham, MA, USA) for later scintillation counting. Tests showed that quench was constant, therefore no corrections were made. Additional apical samples were frozen for later analysis of water T_{amm}.

The following experimental series were performed. (1) A survey of regional variation in ammonia flux rates and MA permeability using different skin regions (epaxial, hypaxial, belly, head, tail) from HEA-exposed fish in the presence of 1 mmol/l basolateral NH_4HCO_3 saline; (2) a comparison of ammonia flux rates and MA permeability among epaxial skin preparations from control and HEA-exposed fish, with basolateral control saline or 1 mmol/l NH_4HCO_3 saline, and vice versa; and (3) an assessment of the responses of ammonia flux rates and MA permeability in the hypaxial skin of HEA-exposed fish (in the presence of 1 mmol/l basolateral NH_4HCO_3) to pharmacological blockers (amiloride, phenamil) present in the apical water. In these experiments, amiloride (amiloride hydrochloride hydrate; Sigma-Aldrich, St. Louis, MO, USA) was used at 10^{-4} M, and phenamil (phenamil methanesulfonate, Sigma-Aldrich, St. Louis, MO, USA) was used at 10^{-5} M, and both drugs were first dissolved in dimethyl sulfoxide (DMSO), yielding a final DMSO concentration in the water of 0.1 %. For these experiments, the non-drug control water also contained 0.1 % DMSO. For all series, each treatment was performed in duplicate or triplicate on one fish, and the results averaged, representing $N = 1$. Total N numbers were 4–8 for each series.

Determination of skin gene expression

Total RNA was extracted from minced and homogenized epaxial skin samples using Trizol (Invitrogen, Burlington, ON). Total RNA was then quantified spectrophotometrically (Nanodrop ND-1000, Nanodrop Technologies, Wilmington, DE, USA) and RNA integrity was assessed by running samples on a 1 % agarose gel stained with Redsafe (Froggabio, Toronto, ON). cDNA was synthesized from 1 μg of DNAse1-treated (Fermentas, Fisher Scientific, Pittsburgh, PA) RNA using an oligo(dT_{17}) primer and Superscript II reverse transcriptase (Invitrogen). This synthesized cDNA was then used to determine the mRNA expression levels of EF1α, Rhcg1, Rhcg2, Rhbg, and NHE2, and v-type H^+-ATPase (GenBank accession numbers: AF498320, DQ431244, AY619986, EF051113, EF446605, AF140002, respectively) using quantitative polymerase chain reaction (qPCR). 20 μl reactions consisted of 4 μl of 5 × diluted cDNA, 4 pmol of both forward and reverse primers, previously determined for each gene (Nawata et al. 2007), 0.8 μl of 10 × diluted ROX dye, and 10 μl of Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen) and were performed using a Mx3000P qPCR System (Stratagene, Cedar Creek, TX). Reactions were conducted at 50 °C (2 min) and 95 °C (2 min), followed by 40 cycles of 95 °C (15 s) then 60 °C (30 s). No template controls and non-reverse transcribed controls were run in parallel, and the formation of a single PCR product was verified by melt curve analysis. Relative gene expression values were obtained from a standard curve, generated by the serial dilution of a randomly selected sample, and were then normalized to elongation factor 1α (EF1α) expression which was equal in control and HEA pre-exposed samples.

Estimation of skin surface area

To estimate skin surface area of adult rainbow trout, a separate group of fasted fish were sacrificed with a lethal dose of MS-222 and were weighed. Following this, pectoral, pelvic, dorsal, and anal fins were surgically removed from the fish. Photos were then taken of all of the separated fins, and of lateral, dorsal, and ventral aspects of the whole fish. In each photo, a ruler was included for determination of scale. Photos were then analyzed using ImageJ software (Wayne Rasband, National Institutes of Health, USA) to measure fin surface area (SA) and whole-body skin SA (lateral × 2 + ventral + dorsal). To estimate posterior SA, the same photos were analyzed though only the portion of the fish posterior to the pectoral fins was included in the SA measurement. By doing this, a skin SA/weight (cm^2/g) value for anterior, posterior, and total skin SA was obtained and could be applied to those fish used in the divided chamber experiments for the determination of in vivo cutaneous J_{amm}.

Analytical techniques and calculations

Total ammonia concentrations in water, plasma, and saline samples were measured using the assay described by Verdouw et al. (1978). In the in vitro experiments, the presence of 0.1 % DMSO in apical water samples markedly reduced the sensitivity of the assay, but did not affect

its linearity, therefore standard curves were made up in 0.1 % DMSO for the relevant treatments.

Whole-body ammonia flux rates (J_{amm}; μmol/kg/h) were calculated using the following equation:

$$J_{amm} = (T_{amm}f - T_{amm}i) \times V/(wt \times t), \quad (1)$$

where $T_{amm}f$ and $T_{amm}i$ are the final and initial concentrations of ammonia (μmol/l), respectively, V is volume (l), wt is weight of the fish (kg), and t is time of the flux (h).

Urine flow rate (UFR; ml/kg/h) was calculated using the following equation:

$$UFR = V/(wt \times t), \quad (2)$$

where V is the urine volume (ml), wt is the weight of the fish, and t is flux time. Urinary J_{amm} (μmol/kg/h) was calculated using the following equation:

$$J_{amm} = T_{amm} \times V/(wt \times t), \quad (3)$$

where T_{amm} is the concentration of ammonia in the urine (μmol/l) V is urine volume (l) collected over the measurement period, wt is the weight of the fish (kg), and t is flux time (h).

For in vitro ammonia fluxes in isolated skin preparations, J_{amm} (μmol/cm^2/h) was expressed per unit surface area and calculated as:

$$J_{amm} = T_{amm}f \times V_{ap}/SA/t, \quad (4)$$

where $T_{amm}f$ is the final concentration of ammonia (μmol/l) in the apical water (tests verified that $T_{amm}i$ was zero), V_{ap} is the volume of the apical water (l), SA is the surface area of the skin (cm^2), and t is time of the flux (h).

In vitro MA permeability (P_{MA}, in cm/sec) of isolated skin preparations was calculated using the standard permeability equation (e.g., Wood et al. 1998) as:

$$P_{MA} = \frac{[MA_{ap}]f \times V_{ap}}{0.5([MA_{bl}]i) + ([MA_{bl}]f) \times t \times 3,600 \times SA}, \quad (5)$$

where $[MA_{ap}]f$ is final [^{14}C]MA radioactivity in the apical water (cpm cm^{-3}), $[MA_{bl}]i$ is initial [^{14}C]MA radioactivity in the basolateral saline (cpm cm^{-3}), and $[MA_{bl}]f$ is final [^{14}C]MA radioactivity in the basolateral saline (cpm cm^{-3}), V_{ap} is the volume of the apical water (cm^3), t is time (h), SA is surface area of the skin (cm^2), and 3,600 converts hours to seconds.

Plasma T_{amm} values were measured using the Raichem commercial kit (Cliniqa Corporation, San Marcos, CA) which quantifies the concentration of ammonia in a sample via the disappearance of NADPH (measured at 340 nm) in a reaction catalyzed by glutamate dehydrogenase.

Branchial J_{amm} (μmol/kg/h) was calculated using anterior and posterior J_{amm} values from divided chambers and skin SA values obtained from fasted trout, as:

$$\text{Branchial } J_{amm} = \text{anterior } J_{amm} - [\text{posterior } J_{amm} \text{ per unit SA}] \times [\text{anterior SA/wt}], \quad (6)$$

where anterior J_{amm} is ammonia excretion in the anterior chamber (μmol/kg/h), posterior J_{amm} per unit SA is posterior ammonia excretion per estimated posterior SA (μmol/cm^2/h), anterior SA is the skin surface area isolated to the anterior chamber (cm^2), and wt is the weight of the fish (kg). This assumes that the ammonia excretion through anterior skin per unit surface area is the same as through posterior skin per unit surface area. Cutaneous J_{amm} (μmol/kg/h) was then calculated as:

$$\text{Cutaneous } J_{amm} = \text{total } J_{amm} - \text{branchial } J_{amm}, \quad (7)$$

where total J_{amm} is the sum of posterior and anterior J_{amm} (μmol/kg/h).

Statistical analyses

All data are presented as means ± 1 SEM where N represents sample size, and significance was accepted at the $P < 0.05$ level. All statistical analyses were conducted using SigmaStat version 3.5 (Systat Software, Inc.) and descriptions of the specific analyses used in individual comparisons are included in Figure captions.

Results

In vivo experiments

In Series 1, under control conditions, plasma T_{amm} levels were 55.5 ± 19.6 μmol/l ($N = 4$) and increased by approximately 5-, 20-, and 25-fold in fish exposed to 12 h of 1, 2, and 3 mmol/l HEA, respectively (Fig. 1a). Notably, in all three levels of HEA, plasma T_{amm} remained substantially below water T_{amm}. Pre-exposure to HEA also led to increases in J_{amm}, after transfer to clean water, relative to control fish (J_{amm} = 409.5 ± 57.3 μmol/kg/h; $N = 7$), though this was significant only in 2 and 3 mmol/l HEA-exposed fish where J_{amm} was 1.5- and 3.5-fold higher, respectively, than control J_{amm} (Fig. 1b). Based on these results, a pre-exposure treatment of 2 mmol/l NH_4HCO_3 was selected for all subsequent tests.

Overnight urinary J_{amm} in Series 3, prior to divided chamber experiments, also responded with a 7-fold increase over 12 h of 2 mmol/l HEA exposure relative to control excretion rates (Fig. 2). It is important to note that these differences in urinary J_{amm} were a result of changes in urine T_{amm} as urine flow rate (UFR) was not significantly different between these treatments (Table 1). Based on whole-body J_{amm} from control fish and fish pre-exposed to 2 mmol/l HEA (Fig. 1a), it can be estimated that 0.4 % of

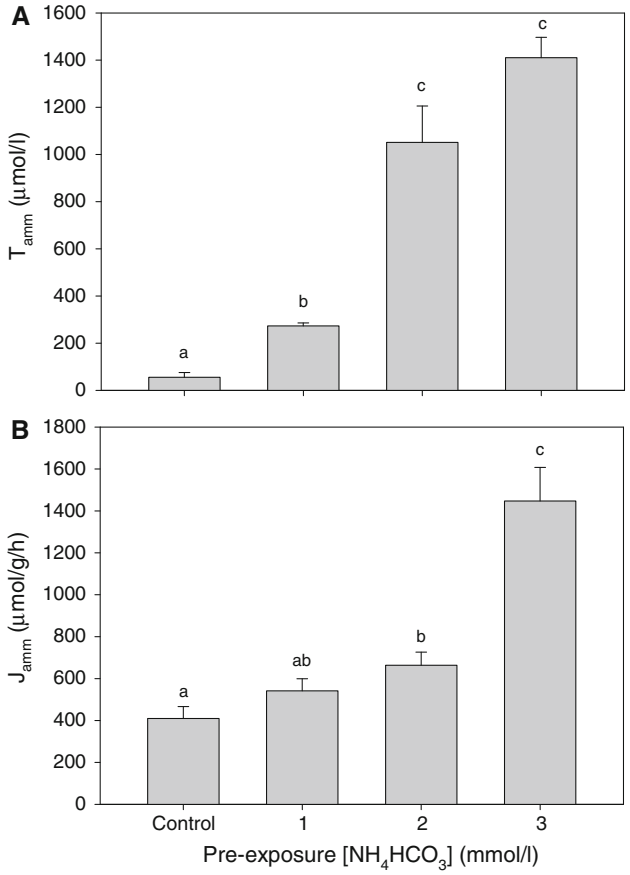

Fig. 1 Plasma total ammonia levels (**a**; T_{amm}) and whole-body ammonia excretion rates (**b**; J_{amm}) after transfer to control water following 12 h of pre-exposure to control conditions or to 1, 2, and 3 mmol/l high environmental ammonia (HEA) as NH_4HCO_3, in Series 1. Means that possess letters that differ indicate statistically significant difference as determined by a one-way ANOVA with a Holm–Sidak post hoc test. Plasma T_{amm} values were normalized using a square root transformation. ($N = 4$, 4, 4, and 2 for control and 1, 2, and 3 mmol/l HEA, respectively, for T_{amm}; $N = 7$, 5, 7, and 2 for control and 1, 2, and 3 mmol/l HEA, respectively, for J_{amm})

total J_{amm} occurred renally under control conditions and that under HEA conditions, this value increased to 1.9 %.

In Series 2, MS-222-induced anesthesia led to a depression of J_{amm} relative to non-anesthetized fish. This amounted to a 56 % decrease in control fish, and a 44 % decrease in fish pre-exposed to HEA and transferred to clean water (Fig. 3). Whole-body J_{amm} of fish in divided chambers was also significantly reduced relative to non-anesthetized fish, but these values did not differ significantly relative to the respective MS-222 anesthetic controls (Fig. 3).

In Series 3, posterior J_{amm} in control fish accounted for 3.6 % of total excretion (Fig. 4a). In HEA pre-exposed fish, posterior J_{amm} after transfer to clean water was 4-fold higher than that seen in control fish but despite this, posterior J_{amm} still accounted for only 4.9 % of total J_{amm} due to a simultaneous 2.4-fold increase in anterior J_{amm} that

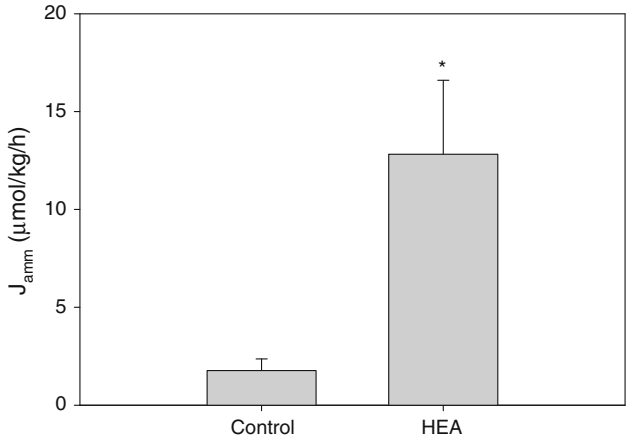

Fig. 2 Urinary ammonia flux rates (J_{amm}) during 12 h of control or HEA (2 mmol/l NH_4HCO_3) pre-exposure, in Series 3. *Asterisks* represent means from HEA-exposed fish, which differed significantly from control means as determined by a two-tailed *t* test. Means were normalized using a log transformation. ($N = 5$–7)

Table 1 Urine flow rates (UFR; ml/kg/h) in rainbow trout during 12 h of overnight control or HEA (2 mM NH_4HCO_3) treatments. ($N = 5$–7)

	Control	HEA
UFR	2.3 ± 0.5	2.3 ± 0.3

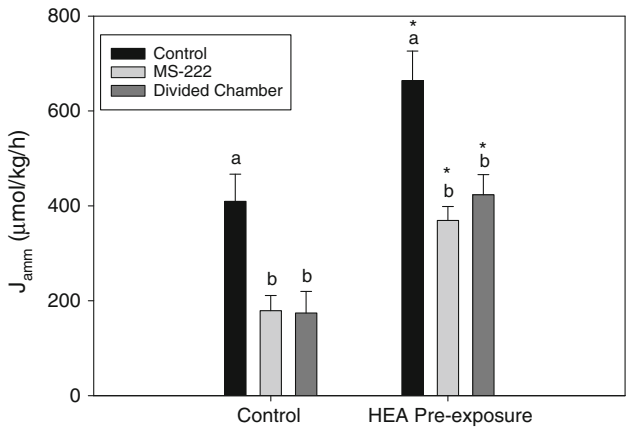

Fig. 3 Total ammonia excretion rates (J_{amm}) after transfer to control water following 12 h of control or HEA (2 mmol/l NH_4HCO_3) pre-exposure under control conditions, MS-222 anesthesia alone, or in divided chambers with MS-222 anesthesia, in Series 2. Means within control or HEA that possess letters that differ indicate statistically significant difference as determined by a one-way ANOVA with a Holm–Sidak post hoc test. *Asterisks* represent means from HEA-exposed fish from control, MS-222 or divided chamber series which differed significantly from respective control means as determined by a two-tailed *t* test. ($N = 4$–7)

was seen at this time (Fig. 4a). Urinary J_{amm} values at this time have not been reported, as urine flow rate was generally depressed and sometimes ceased altogether when the

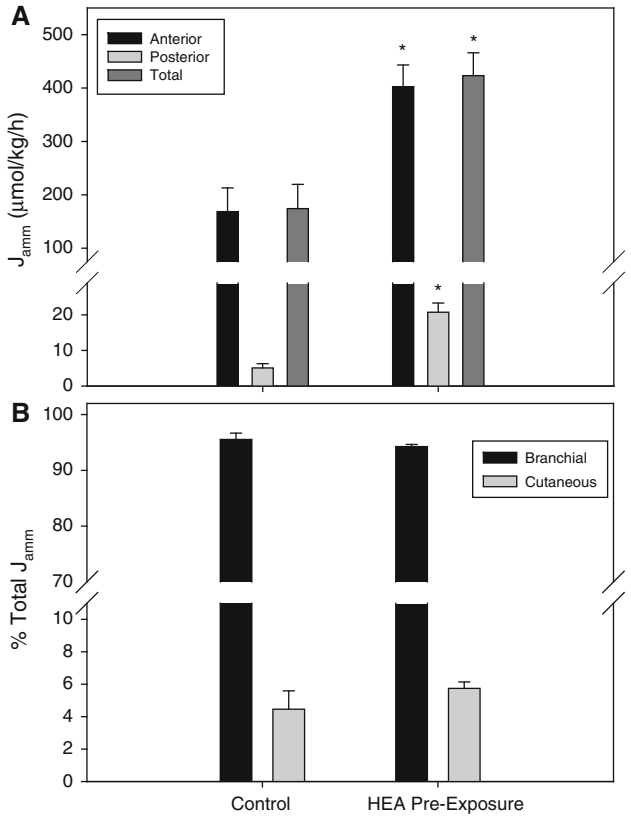

Fig. 4 Anterior, posterior, and total ammonia excretion rates (**a**; J_{amm}) and % of total branchial and cutaneous ammonia excretion (**b**) after transfer to control water following 12 h of control or HEA (2 mmol/l NH_4HCO_3) pre-exposure, in Series 3. *Asterisks* represent anterior, posterior, or total J_{amm} means from HEA-exposed fish, which differed significantly from respective control means as determined by a two-tailed *t* test. ($N = 4$–7)

fish were placed in the divided chambers, despite these fish having normal urine output overnight, prior to experimentation (see "Discussion"). The post-experimental 1-h dye flux period revealed that the seal between anterior and posterior compartments in our setup was effective as no dye was detected (i.e., absorbance equal to or lesser than background) in the anterior chamber following 1 h of equilibration (data not shown). A leak as small as 1 % of the volume of the posterior chamber could have been reliably detected; sensitivity calculations indicate that at most this would have resulted a 1.05-fold overestimation of posterior J_{amm} (because anterior T_{amm} was routinely higher than posterior T_{amm}).

In vitro experiments

Skin samples were taken from different regions of HEA-exposed fish (epaxial, hypaxial, belly, head, and tail) to assess in vitro J_{amm}. In the presence of 1 mmol/l basolateral NH_4HCO_3, a concentration chosen to mimic plasma T_{amm} following 12 h of 2 mmol/l HEA exposure (Fig. 1a), in vitro J_{amm} did not differ between any of the sections and had an overall mean value of 0.36 μmol/cm^2/h (Fig. 5a). In the presence of control basolateral saline, epaxial skin from HEA-exposed fish had J_{amm} values which were significantly greater than those seen in the epaxial skin of control fish. The same trend was observed when preparations from control and HEA fish were incubated in the presence of 1 mmol/l NH_4HCO_3 in the basolateral saline; however, it was not statistically significant ($P = 0.160$; Fig. 5b). The addition of DMSO, a vehicle needed to dissolve the blockers, to the apical water in hypaxial skin preparations from HEA-exposed fish, incubated with 1 mmol/l NH_4HCO_3 in the basolateral saline, resulted in a significant increase in J_{amm} but the further addition of the blockers amiloride (10^{-4} mol/l) or phenamil (10^{-5} mol/l) did not result in a significant difference relative to this value (Fig. 5c).

Unlike the case for in vitro J_{amm}, MA permeability in skin of HEA-exposed fish in the presence of 1 mmol/l basolateral NH_4HCO_3 was generally higher in tail and head sections relative to epaxial, hypaxial, and belly sections, which were not significantly different from one another (Fig. 6a). In the absence of basolateral NH_4HCO_3, MA permeability across epaxial skin taken from HEA-exposed fish was 2.9-fold greater than in epaxial skin from control fish (Fig. 6b). However, in the presence of 1 mmol/l basolateral NH_4HCO_3, this difference was abolished and MA permeability was unchanged regardless of pre-exposure condition (Fig. 6b). Similar to J_{amm}, 0.1 % DMSO significantly increased MA permeability across the hypaxial skin of HEA-exposed fish (Fig. 6a). Unlike J_{amm}, the further addition of amiloride significantly reduced permeability relative to the DMSO only value, whereas phenamil had no such effect, similar to the case for J_{amm} (Fig. 6c).

Surface area estimates and excretion rates

Total skin surface area (SA) per unit weight in fasted rainbow trout was determined to be 1.56 ± 0.07 cm^2/g ($N = 3$). Posterior skin SA/weight was approximately 80 % of total skin SA/weight with a value of 1.27 ± 0.07 cm^2/g ($N = 3$). From these values, in vivo cutaneous J_{amm} was determined using Eqs. (6) and (7). In vivo, fish pre-exposed to HEA displayed cutaneous J_{amm} approximately 3-fold greater than control fish (Table 2). When compared to in vitro cutaneous J_{amm} (from epaxial skin; Fig. 5b), in vivo cutaneous J_{amm} was 91 % lower than in vitro rates in control fish and approximately 83 % lower in HEA-exposed fish (Table 2). Using the surface area values obtained from fasted rainbow trout and J_{amm} values from divided chamber experiments, it was determined that, under control conditions, cutaneous J_{amm} was 6.2 ± 1.5 μmol/kg/h ($N = 4$), accounting for 4.5 % (Fig. 4b) of

Table 2 In vivo and in vitro cutaneous ammonia excretion rates (μmol/cm^2/h; $N = 4$–7 for in vivo fluxes, $N = 4$–5 for in vitro fluxes)

	Control	HEA
In vivo	0.016 ± 0.004a	0.051 ± 0.008b
In vitro	0.18 ± 0.01x*	0.30 ± 0.04y*

Means that possess letters that differ indicate statistically significant difference within in vivo or in vitro data sets. Significance was determined using a *t* test

* Represent in vitro means which differed significantly from respective in vivo means

total J_{amm} measured in the divided chamber experiment. In fish pre-exposed to HEA, this value increased 4-fold to 25.5 ± 3.2 μmol/kg/h ($N = 7$) but still only accounted for 5.7 % (Fig. 4b), similar to the trends seen in posterior J_{amm}.

Gene expression

Rhcg1, Rhcg2, Rhbg, NHE-2, v-type H^+-ATPase were all expressed in the epaxial skin of adult rainbow trout (Fig. 7). In the skin of HEA-exposed fish, relative gene expression (normalized to the expression of elongation factor 1α; EF1α) of all genes measured did not differ significantly from that of control fish, despite a slight increase ($P = 0.106$) in Rhcg2 gene expression in HEA-exposed fish (Fig. 7).

Discussion

Overview

The overall goal of the present study was to use both in vivo and in vitro approaches to assess the potential for cutaneous ammonia excretion in adult rainbow trout in light of previous reports of Rh gene expression in the skin of this species (Nawata et al. 2007; Nawata and Wood 2008, 2009). The range-finding experiments determined that 2 mmol/l was an appropriate HEA exposure for in vivo experiments as this was the lowest exposure level where significant increases in both plasma T_{amm} and J_{amm} were observed (Fig. 1). Notably, the trout were able to keep plasma T_{amm} well below water T_{amm} concentrations, consistent with previous studies where HEA exposure led to increases in ammonia excretion capacity against a prevailing inward ammonia concentration gradient (e.g., Wilson et al. 1994; Nawata et al. 2007; Tsui et al. 2009; Zimmer et al. 2010; Wood and Nawata 2011; Liew et al. 2013; Sinha et al. 2013). Also important to note is the relatively high exposure concentrations used in the present study (up to 3 mM NH_4HCO_3) which were necessary to achieve sufficient ammonia loading and subsequent increases in J_{amm} (Fig. 1) in the Vancouver tap water to which the fish were acclimated, which has a relatively low pH (7.0). The low buffer capacity of this very soft water may also have facilitated elimination of ammonia loads following HEA exposure.

In the divided chamber experiments, fish had to be anesthetized to avoid struggling during handling and to avoid escape from the chamber. As such, a set of controls was designed to assess the effects of anesthesia alone on J_{amm}. In agreement with a similar study on goldfish (Smith et al. 2012), anesthesia led to a significant reduction of J_{amm} (Fig. 3), presumably the result of a reduction in metabolic rate as MS-222 exposure is known to reduce oxygen consumption in rainbow trout (McKim et al. 1987). This reduced excretion rate, however, was not significantly different than the whole-body J_{amm} (anterior + posterior) of fish in the divided chamber setup. Furthermore, the inhibitory effect was approximately equal on a percentage basis, regardless of pre-exposure condition (Fig. 3), suggesting that our anesthetic treatment was effective in reducing experimental stress. Furthermore, the use of divided chamber experiments requires the assessment of dam integrity by dye leakage, an approach used in previous studies (e.g., Frick and Wright 2002). The seal in our system was effective, as there was no detectable dye leakage, whereas we could have reliably detected a 1 % leakage, and sensitivity calculations indicated that such a leakage would have raised posterior J_{amm} by less than 5 %, with negligible impact on the estimation of cutaneous J_{amm}.

Increased urinary J_{amm} occurs in response to an acidosis in fish (e.g., McDonald and Wood 1981; Perry et al. 1987; Wood et al. 1999). In the present study, we have demonstrated that trout also utilize renal routes for the clearance of plasma ammonia loads resulting from HEA exposure (Fig. 2) and that this occurs solely via an increase in urine T_{amm} as UFR was unchanged by HEA exposure (Table 1). Furthermore, the UFR values observed in the present study are quite similar to those reported previously for rainbow trout (Swift and Lloyd 1974; Wood 1988), demonstrating that the catheters were functioning well overnight prior to divided chamber experiments where UFR was inhibited such that the calculation of urinary J_{amm} was not meaningful. Nawata et al. (2007) demonstrated mRNA expression of Rhbg in the kidney of trout, but it is unclear whether this gene would be responsive to HEA exposure. Despite the HEA-induced increase in urinary J_{amm}, however, this route would appear to account for less than 2 % of total J_{amm} in rainbow trout, a much lower contribution than that observed by Smith (1929) in carp and goldfish.

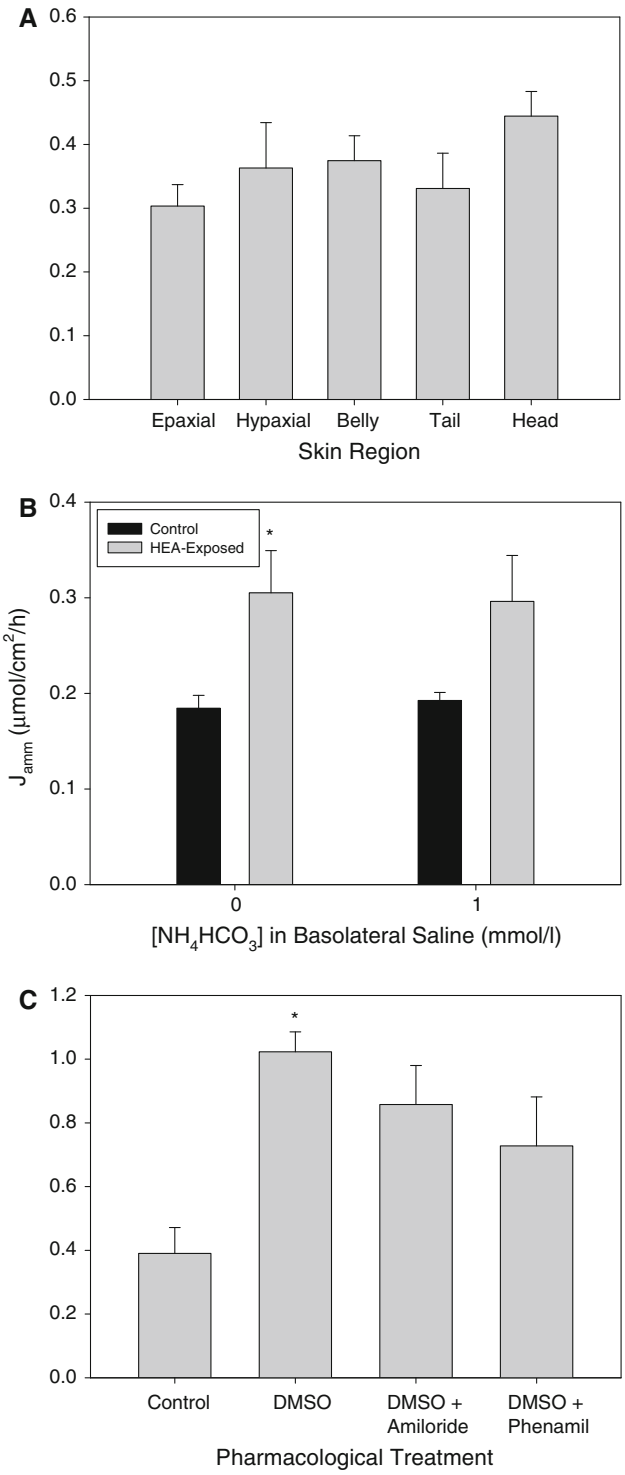

Fig. 5 In vitro ammonia flux rates (J_{amm}) in different skin regions from fish pre-exposed to HEA (2 mmol/l NH_4HCO_3) in the presence of 1 mmol/l basolateral NH_4HCO_3 (**a**), in the epaxial skin of control and HEA-exposed fish treated with either 0 or 1 mmol/l basolateral NH_4HCO_3 (**b**), and in the hypaxial skin of HEA-exposed fish in response to pharmacological blockers (10^{-4} mol/l amiloride, 10^{-5} mol/l phenamil, all in the presence of 0.1 % DMSO) in the presence of 1 mmol/l basolateral NH_4HCO_3 (**c**). **a** No significant differences existed between skin regions. ($N = 4$–7) **b** *Asterisks* represent means from skin of HEA-exposed fish which differed significantly from respective control means as determined by a one-tailed t test. ($N = 4$–5) **c** *Asterisks* represent means from DMSO treatment which differed significantly from control means as determined by a one-tailed paired t test. ($N = 4$–5)

Importance of the skin in ammonia excretion

We hypothesized, based on previous molecular evidence, that the skin of adult trout would become an important site for J_{amm} in response to HEA pre-exposure. In adult trout under control conditions, cutaneous J_{amm} contributed less than 5 % to total J_{amm} (Fig. 4), suggesting that this route of excretion is of little importance to overall J_{amm}. Following HEA exposure; however, cutaneous J_{amm} increased 4-fold but yet only accounted for less than 6 % of total J_{amm} (Fig. 4) because there was a simultaneous 2.5-fold increase in branchial J_{amm}, demonstrating that the gills are indeed by far the most important site for ammonia clearance. Thus our hypothesis could not be confirmed, yet it is clear that this tissue is responsive to HEA exposure. It is not clear, however, if the increase in posterior J_{amm} is a result of greater clearance from the plasma or simply the unloading of a cutaneous tissue ammonia burden accumulated during the 12-h HEA pre-exposure.

In many teleost species, the presence of a secondary circulatory system (SCS), which vascularizes the skin, has been observed (e.g., Chopin et al. 1998; Satchell 1991; Skov and Bennett 2003; Skov and Bennett 2004; Steffensen et al. 1986). The SCS, previously believed to be a lymphatic system due to its lack of red blood cells (Vogel and Claviez 1981), originates from the primary circulation and eventually drains back into the primary circulation via the lateral cutaneous vein (LCV) (see Olson 1996; Satchell 1991 for review). In the longfin eel (*Anguilla reinhardtii*), SCS capillaries periodically emerge from capillary beds, forming hairpin loops in close proximity to mitochondria-rich cells (MRCs) in the epidermis (Skov and Bennett 2004). Indeed, Ishimatsu et al. (1992) observed that plasma [HCO_3^-] and [Cl^-] differed between the primary circulation and the LCV during hypercapnic exposure, suggesting transcutaneous exchange and demonstrating that plasma acid–base corrections during hypercapnic exposure may occur via the skin. Therefore, such a role for the skin may exist in excreting ammonia from plasma following HEA exposure. On the other hand, however, the in vitro results, showing greater cutaneous J_{amm} in preparations from HEA-exposed fish compared to control fish regardless of the presence or absence of basolateral (i.e., plasma) NH_4HCO_3 (Fig. 5b), may indicate a release of ammonia from the skin tissue itself, rather than the transcutaneous clearance of plasma ammonia.

Recent molecular evidence has pointed towards a role for the skin in whole-body J_{amm} in rainbow trout (Nawata

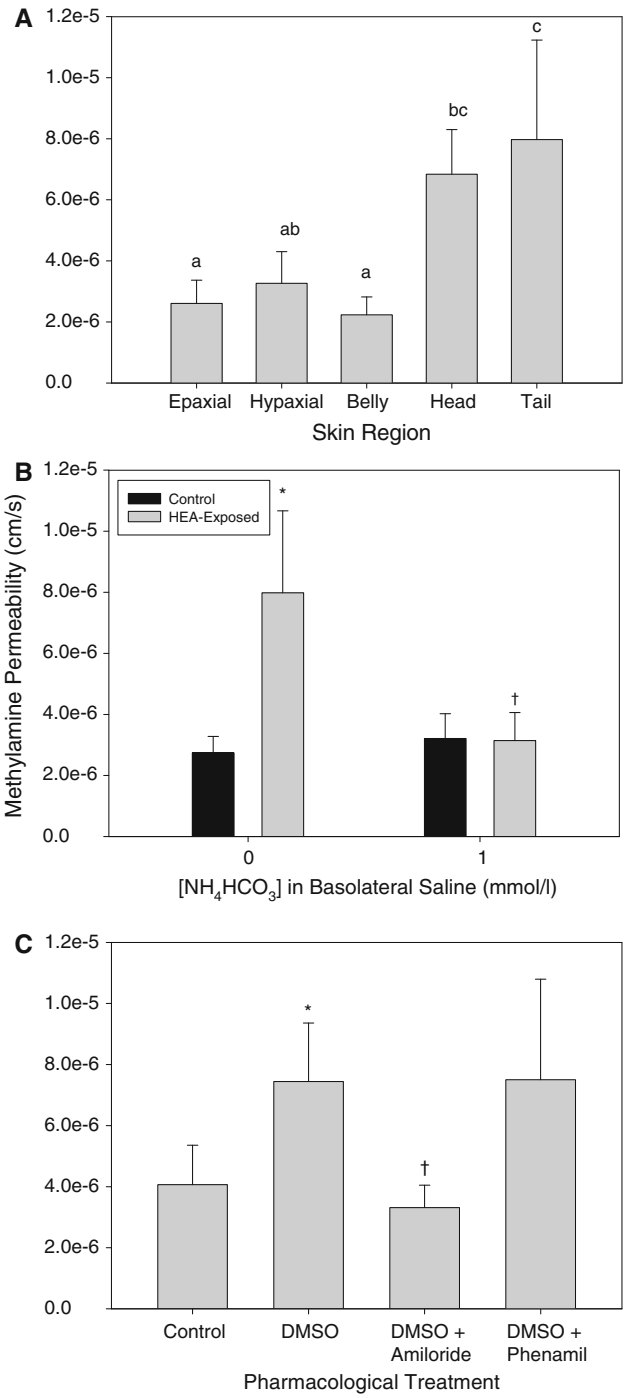

Fig. 6 In vitro methylamine permeability (cm/s) in different skin regions from fish pre-exposed to HEA (2 mmol/l NH_4HCO_3) in the presence of 1 mmol/l basolateral NH_4HCO_3 (**a**), in the epaxial skin of control and HEA-exposed fish treated with either 0 or 1 mmol/l basolateral NH_4HCO_3 (**b**), and in the hypaxial skin of HEA-exposed fish in response to pharmacological blockers (10^{-4} mol/l amiloride, 10^{-5} mol/l phenamil, all in the presence of 0.1 % DMSO) in the presence of 1 mmol/l basolateral NH_4HCO_3 (**c**). **a** Means that possess letters that differ indicate statistically significant difference as determined by a one-way repeated measures ANOVA with a Fisher LSD post hoc test. Permeabilities were normalized using a square root transformation. ($N = 5$–8) **b** *Asterisks* represent means from skin of HEA-exposed fish which differed significantly from respective control means as determined by a one-tailed *t* test using log transformation-normalized data. *Daggers* represent means from 1 mmol/l basolateral NH_4HCO_3 treatments which differed significantly from respective control means as determined by a paired *t* test. ($N = 6$) **c** *Asterisks* represent means from DMSO treatment which differed significantly from control means as determined by a one-tailed paired *t* test. *Daggers* represent DMSO + blocker means which differed significantly from DMSO means as determined by a paired *t* test. ($N = 4$–5)

et al. 2007; Nawata and Wood 2008, 2009). Indeed, in the skin of mangrove killifish, which is known to contribute significantly to overall J_{amm} during air exposure (Frick and Wright 2002; Cooper et al. 2013), HEA exposure led to the induction of Rhcg1 and Rhcg2 (Hung et al. 2007). In the pufferfish, HEA exposure caused an upregulation of Rhbg and Rhcg2 expression (Nawata et al. 2010a). In larval fish, the skin is an important site for J_{amm} (Shih et al. 2008, 2012; Wu et al. 2010; Zimmer AM, Wright PA, Wood CM unpublished results) though it is not known if this capacity is retained into adulthood. Moreover, it is unclear whether the skin of larval trout has an ability to handle HEA challenges in the same way as the gill of adult fish. In rainbow trout embryos, prior to hatch, no significant changes in Rhcg2 gene expression were observed following 4 or 48 h of HEA exposure (Sashaw et al. 2010).

Based on studies with other adult fish, it appears that when gill function is limited, the skin may become an important site for J_{amm} (Glover et al. 2013). For example, the mangrove killifish volatilizes ammonia across the body skin, presumably via Rh proteins, during periods of emersion where gill ventilation may be impeded (Frick and Wright 2002; Hung et al. 2007; Cooper et al. 2013). Similarly, up to 50 % of ammonia excretion occurs over the general body surface of the dab, a flatfish that often lives buried in sand where gill ventilation is limited (Sayer and Davenport 1987). In goldfish, when gill surface area is low due to the presence of an interlamellar cell mass (ILCM), J_{amm} via extra-branchial routes is increased (Smith et al. 2012). Interestingly, in rainbow trout exposed to HEPES buffer, where gill J_{amm} was reduced via an inhibition of branchial acid-trapping mechanisms, Rhcg1 and Rhcg2 mRNA expressions increased in the skin while the expression of these genes was unchanged or reduced in the gills (Nawata and Wood 2008). In future studies, it may be interesting to utilize the in vivo and in vitro systems developed in this study to assess the contribution of the skin to total J_{amm} in adult trout when HEA exposure is continued during the test period, and also when HEPES buffering limits branchial J_{amm}.

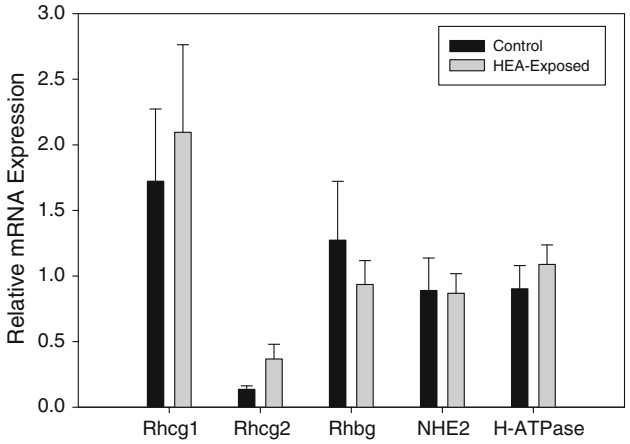

Fig. 7 Relative gene expression (normalized to EF1α gene expression) of Rhcg1, Rhcg2, Rhbg, NHE2, and H^+-ATPase in epaxial skin samples taken from trout following 12 h of control or HEA pre-exposure. ($N = 4$–5)

Mechanisms of cutaneous ammonia excretion

To date, the studies which have addressed the mechanisms of cutaneous J_{amm} in fish have focussed mainly on cutaneous excretion in larval fish. In larval zebrafish, Rhcg1 protein is expressed in MRCs on the yolk sac skin and the increase in expression of this protein over developmental time is consistent with an increase in whole-body J_{amm} (Nakada et al. 2007). Furthermore, J_{amm} in larval fish is coupled to Na^+ uptake via a Rhcg1–NHE metabolon (Kumai and Perry 2011; Ito et al. 2013), though it is unclear if this mechanism is purely cutaneous at this life stage. Using SIET, Shih et al. (2012) confirmed the existence of such a metabolon in the yolk sac skin of zebrafish and it is clear that cutaneous J_{amm} in larval fish is dependent upon acid-trapping (Shih et al. 2008; Wu et al. 2010), in concordance with the branchial model for ammonia transport (Wright and Wood 2009). In adult mangrove killifish, in vitro J_{amm} across isolated skin of freshwater-acclimated fish was inhibited significantly by amiloride, a blocker of NHEs, and also by phenamil and bafilomycin, suggesting that the H^+-ATPase/Na^+ channel system is also important to cutaneous J_{amm} in these fish (Cooper et al. 2013). Thus, the skin is an important site for J_{amm} in the larval stages of several fish species and in the adult stages of at least two highly specialized fish species (*Kryptolebias marmoratus* and *Limanda limanda,* see "Introduction"), but it is not known whether the skin might contribute to J_{amm} generally in adult fish or if this capacity is lost completely over development.

In the present study, pre-exposure to HEA, resulting in a significant increase in cutaneous J_{amm} in vivo (Fig. 4; Table 2), also resulted in an increase in J_{amm} and MA permeability across the skin in vitro in the absence of basolateral NH_4HCO_3 (Figs. 5b, 6b). Interestingly, 1 mmol/l basolateral NH_4HCO_3 abolished the observed HEA-dependent increase in MA permeability, consistent with the *Xenopus* oocyte expression study by Nawata et al. (2010b) which demonstrated that MA flux is facilitated by Rh proteins and that increases in [NH_4Cl] reduce MA uptake, suggesting competition for binding sites on the protein channels. Thus, we interpret our in vitro results as an increase in cutaneous Rh protein function following HEA, though our gene expression data do not directly support this interpretation (Fig. 7). It is possible, however, that non-genomic changes in expression had occurred such as post-translational modification, as indicated by a change in Rh protein molecular size seen in HEA-exposed pufferfish (Nawata et al. 2010a), or increases in protein expression in the absence of changes in mRNA expression or increased plasma membrane insertion, both of which have been demonstrated to occur for Rh proteins (Seshadri et al. 2006a, b). Furthermore, the addition of amiloride (with DMSO as a vehicle) in the present in vitro preparations decreased MA permeability relative to DMSO controls, which may indicate a role for NHE in flux via Rh. This is consistent with the model for cutaneous J_{amm} in larval fish (Kumai and Perry 2011; Shih et al. 2012; Ito et al. 2013), though further pharmacological analyses (bafilomycin, EIPA), flux studies (radioisotopic ^{22}Na fluxes), and immunohistochemical analyses would be necessary to better understand the mechanisms of ammonia transport in the skin of adult trout. Also noteworthy was that phenamil, a blocker of epithelial Na^+ channels, had no effect on MA permeability in trout skin (Fig. 6c), in contrast to skin from freshwater-acclimated mangrove killifish (Cooper et al. 2013).

Important to note is that the overall patterns seen for J_{amm} in vitro (Fig. 5c) do not reflect those for MA permeability (Fig. 6c). However, it is not clear in this preparation if observed ammonia fluxes originate from the release of stored ammonia loads, from metabolic ammonia production, or from degradation of tissue proteins over experimental time. Indeed, skin in vitro J_{amm} was significantly greater than in vivo J_{amm} (Table 2), suggesting that some degradation may have occurred in vitro. Additionally, it is possible that the skin becomes more permeable to ammonia after handling. Moreover, it is possible that some ammonia originates from the small amount of underlying muscle tissue which could not always be removed in the preparation, or even from the scales. Regardless however, in vitro skin J_{amm} did depend on pre-treatment condition (Fig. 5b), with HEA-exposed fish demonstrating a greater J_{amm}, arguing that the J_{amm} in vitro may originate from the release of skin tissue ammonia stores. Moreover, it is likely that any small changes in transcutaneous ammonia fluxes, as indicated by changes in the permeability of the radiolabelled and easily detected ammonia analog MA, are masked by the unloading of tissue ammonia from these various sources.

Finally, in adult trout, a spatial pattern for MA permeability along the body of the fish was observed, with the head and tail demonstrating the highest permeabilities (Fig. 6a). This may be a reflection of a number of different factors including skin thickness, vascularization, mitochondria-rich cell (MRC) density, or Rh protein expression. In larval zebrafish, a gradient for J_{amm} was also seen across the body, though curiously, the head and tail of these fish were the sites of lowest flux rates (Shih et al. 2008).

Concluding remarks

Overall, cutaneous J_{amm} plays a negligible role in whole-body J_{amm} in adult rainbow trout under control conditions and, contrary to our initial hypothesis, its role remains minimal following HEA pre-exposure. Nevertheless, the intrinsic ammonia excretion capacity of this tissue appears to increase under these conditions, as evidenced by increases in in vitro MA permeability and J_{amm} of isolated skin sections, despite its minimal contribution to total J_{amm}. It is not clear if the apparent responsiveness of the skin to ammonia challenges (e.g., Nawata et al. 2007; Nawata and Wood 2008, 2009) is a vestigial trait retained from larval stages where the skin is the dominant site for ammonia transport (Zimmer AM, Wright PA, Wood CM unpublished results) or if these adult trout may, in fact, rely on this site for ammonia excretion under certain conditions. It is also possible that ammonia excretion by the skin occurs largely as a result of its own metabolic activity which can account for up to 15 % of total MO_2 in trout (Kirsch and Nonnotte 1977). If this is the case, this tissue may spare further plasma loading of ammonia during HEA by excreting its waste apically into the surrounding environment as opposed to basally, into the plasma. Overall, it appears that the skin of rainbow trout has some capacity for ammonia transport that warrants further research. In a recent review, it has been suggested that the skin may act as an important surface for physiological exchange in many fish species (Glover et al. 2013). This is a promising area for future research.

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