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AMMONIA EXCRETION IN FRESHWATER RAINBOW TROUT (*ONCORHYNCHUS MYKISS*) AND THE IMPORTANCE OF GILL BOUNDARY LAYER ACIDIFICATION: LACK OF EVIDENCE FOR Na^+/NH_4^+ EXCHANGE

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Summary

Net ammonia fluxes (J_{Amm}) were measured in adult freshwater rainbow trout *in vivo* under a variety of conditions designed to inhibit unidirectional sodium uptake (J_{in}^{Na}; low external [NaCl], 10^{-4} mol l^{-1} amiloride), alter transbranchial P_{NH_3} and NH_4^+ gradients [24 h continuous $(NH_4)_2SO_4$ infusion, or exposure to 1 mmol l^{-1} external total ammonia at pH 8] and prevent gill boundary layer acidification (5 mmol l^{-1} Hepes buffer). Inhibition of J_{in}^{Na} with amiloride or low external [NaCl] under normal conditions reduced J_{Amm} by about 20 %, but did not prevent the net excretion of ammonia during exposure to high concentrations of external ammonia. Increasing the buffer capacity of the ventilatory water with Hepes buffer (pH 8) reduced J_{Amm} by 36 % and abolished the effect of amiloride on ammonia excretion.

No evidence could be found to support a directly coupled apical Na^+/NH_4^+ exchange. We suggest that any dependence of ammonia excretion on sodium uptake is caused by alteration of transbranchial P_{NH_3} gradients within the gill microenvironment secondary to changes in net H^+ excretion. Under normal conditions (pH 8, low external ammonia) gill boundary layer acidification facilitates over one-third of the total ammonia excretion. During exposure to high concentrations of external ammonia in poorly buffered water, estimates of transbranchial P_{NH_3} gradients from measurements of bulk water pH and total ammonia concentration (T_{Amm}) may be grossly in error because of boundary layer acidification. Prevention of boundary layer acidification with Hepes buffer during exposure to high cocncentrations of external ammonia revealed that the local transbranchial P_{NH_3} gradient at the gill may in fact be positive (blood to water), negating the need for an active NH_4^+ transport mechanism. In freshwater trout, NH_3 diffusion may account for all ammonia excretion under all experimental conditions used in the present study.

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Introduction

Teleost fish excrete the majority of their nitrogenous waste as ammonia (Smith, 1929). By far the largest proportion of this ammonia output occurs at the gills (Smith, 1929; Sayer and Davenport, 1987) and results from clearance of ammonia from the blood (Payan and Matty, 1975; Cameron and Heisler, 1983; Ogata and Murai, 1988) as it crosses the arterio-arterial circuit through the lamellae (Payan *et al.* 1984). Under normal conditions of low ambient [ammonia], the transbranchial ammonia gradients (ΔP_{NH_3} and ΔNH_4^+) are positive (from blood to water). The bulk of experimental evidence indicates at least three potential mechanisms for the extrusion of ammonia across fish gills: passive diffusion of NH_3 down a partial pressure gradient, passive diffusion of NH_4^+ down an electrochemical gradient, and an apically located electroneutral exchange of intracellular NH_4^+ for either external Na^+ or external H^+ (see reviews by Cameron and Heisler, 1985; Evans and Cameron, 1986; Randall and Wright, 1987; Wood, 1993). Despite numerous attempts to quantify the importance of each of these transport mechanisms, branchial ammonia transfer still remains the subject of much debate.

The passive diffusion of NH_4^+ is probably only of quantitative importance in seawater teleosts, where ionic permeabilities are high (Girard and Payan, 1980; Evans, 1984). In contrast, the gills of freshwater fish are considered to be very 'tight' epithelia (Sardet, 1980; Pisam *et al.* 1987) and transbranchial diffusion of NH_4^+ is almost certainly negligible under normal conditions (Wright and Wood, 1985; McDonald and Prior, 1988). In contrast, diffusion of NH_3 appears to drive a major portion of net ammonia excretion under resting conditions, effected by transbranchial P_{NH_3} gradients in the 50–100 nmHg range (Cameron and Heisler, 1983; Wright and Wood, 1985; Heisler, 1990).

Indirect evidence for the existence of an apically situated Na^+/NH_4^+ exchanger relies predominantly on two observations: (i) correlations between the rate of unidirectional Na^+ influx (J_{in}^{Na}) and net ammonia excretion (J_{Amm}) under certain conditions (Maetz and Garcia-Romeu, 1964; Maetz, 1973; Payan, 1978; Wright and Wood, 1985; Wood, 1988, 1989; McDonald and Prior, 1988) and (ii) the observation that teleosts can apparently maintain negative (inwardly directed) or zero P_{NH_3} and NH_4^+ gradients during prolonged exposure to elevated external total ammonia levels (Cameron and Heisler, 1983; Cameron, 1986; Claiborne and Evans, 1988; Wilson and Taylor, 1992). However, with present techniques it is impossible to distinguish between a directly coupled exchange of NH_4^+ for Na^+ and the diffusion of NH_3 occurring simultaneously alongside a classical Na^+/H^+ exchange (Kinsella and Aronson, 1981) or a primary H^+-ATPase/Na^+ channel arrangement (Avella and Bornancin, 1989; Lin and Randall, 1991). In addition, the accurate measurement of transbranchial P_{NH_3} gradients is severely hampered because water passing over the gills is acidified by CO_2 excretion and/or H^+ and HCO_3^- transport processes (Wright *et al.* 1986; Playle and Wood, 1989; Lin and Randall, 1990).

In the present study we have evaluated the dependence of ammonia excretion on the Na^+ uptake mechanism *in vivo* using treatments that reduce J_{in}^{Na} (low external [Na^+] and the Na^+ transport inhibitor amiloride), under normal and two 'ammonia-loaded' conditions (infusion of ammonium sulphate and exposure to high external ammonia) that

might be expected to stimulate such an exchanger. In addition, we have tested the role of gill boundary layer acidification in branchial ammonia excretion using an artificially buffered medium (5 mmol l^{-1} Hepes) under normal and high external ammonia conditions.

Materials and methods

Animals and surgery

Rainbow trout [*Oncorhynchus mykiss* (Walbaum); 197–463 g] were obtained from Spring Valley Trout Farm, Petersburg, Ontario, Canada, and held in flowing dechlorinated Hamilton tap water ($[Na^+]\approx0.6$ mequiv l^{-1}, $[Cl^-]\approx0.8$ mequiv l^{-1}, $[Ca^{2+}]\approx2.0$ mequiv l^{-1}, $[Mg^{2+}]\approx0.3$ mequiv l^{-1}, $[K^+]\approx0.05$ mequiv l^{-1}, titration alkalinity≈2.1 mequiv l^{-1}, pH≈8.0, temperature, 8–16 °C). At least 1 week prior to experiments animals were transferred to a 15 °C acclimation tank and food was withheld to allow stabilisation of the endogenous fraction of waste nitrogen excretion (Fromm, 1963). For experiments requiring sequential blood sampling, trout were anaesthetised (MS222, 0.05 g l^{-1}; Sigma) and fitted with a dorsal aortic catheter filled with heparinised Cortland saline (Wolf, 1963; 50 i.u. ml^{-1} sodium heparin; Sigma) as described by Soivio *et al.* (1972). All fish, catheterised and uncatheterised, were subsequently transferred to individual, darkened, well-aerated acrylic flux boxes continuously supplied with temperature-controlled (15±0.5 °C), dechlorinated tap water and allowed to recover for 48 h prior to experiments.

Experimental series

The following six experimental treatments were employed to assess the importance of Na^+ uptake and boundary layer acidification in the excretion of ammonia across the gills.

Series 1

In series 1, eight uncatheterised trout (330.6±32.6 g) were exposed for 3 h to low external $[Na^+]$ to determine the dependence of ammonia excretion on the Na^+ uptake mechanism. For the low $[Na^+]$ exposure, boxes were flushed three times with an artificial hard water containing no NaCl but made up to duplicate the $[Ca^{2+}]$, $[Mg^{2+}]$, titratable alkalinity and pH of dechlorinated Hamilton tap water. This artificial hard water was made as described by Goss and Wood (1990), who showed that exposure to this medium caused no changes in transepithelial potential (TEP), in contrast to exposure to distilled water. Series 1 was carried out over a 3 day period with two 3 h control fluxes being performed on days 1 and 3, exactly 24 h prior to, and following, the low NaCl experimental exposure on day 2. Water samples for flux calculations were taken every 15 min during each of the 3 h flux periods to allow high resolution of any rapid changes in J_{Amm}.

Series 2

In series 2, we attempted to artificially stimulate Na^+/NH_4^+ exchange in catheterised trout (230.9±8.2 g, N=7) by ammonium sulphate infusion. We also tested the dependence

of J_{Amm} on J_{in}^{Na} by inhibiting apical sodium uptake pharmacologically using 10^{-4} mol l^{-1} amiloride (Sigma; a 10^{-2} mol l^{-1} stock solution was prepared immediately before use by sonicating amiloride–HCl in distilled water for approximately 10 s). The effects of amiloride were tested both before and after ammonia loading. We predicted that under 'control' (pre-infusion) conditions, amiloride should have a similar effect to the low external [Na^+] treatment in series 1. After testing the effects of amiloride under control conditions, the same animals were then continuously infused with 70 mmol l^{-1} ammonium sulphate over 24 h to allow stabilisation of a new steady state under ammonia-loaded conditions. This protocol differs from previous studies on ammonia excretion, in which bolus injections of ammonium salts have been used to create non-steady-state ammonia loading (e.g. Maetz, 1973; Cameron and Heisler, 1983; McDonald and Prior, 1988). The ammonium sulphate solution [$(NH_4)_2SO_4$; T_{Amm}=140 mmol l^{-1}, adjusted to pH 7.8] was infused through the dorsal aortic catheter using a peristaltic pump (Gilson, Minipuls) at a rate estimated to approximately quadruple the normal J_{Amm}. In practice, the mean infusion rate was 4.014±0.265 ml kg^{-1} h^{-1} (N=7), which provided a total ammonia input of 562.0±37.1 μmol kg^{-1} h^{-1}. In this ammonia-loaded state, J_{Amm} and unidirectional Na^+ fluxes were then measured in the absence and presence of external amiloride. 1 h flux periods were employed both under control conditions (pre-infusion) and following 24 h of ammonium sulphate infusion. In each case a 1 h flux in the absence of amiloride was followed by a 1 h period of flow-through, and then by a second 1 h flux in the presence of amiloride.

Series 3

These experiments consisted of exposing trout to high external ammonia concentrations (HEA; nominal T_{Amm}=1000 μmol l^{-1} as ammonium sulphate, pH≈8), which reverses the normally positive transbranchial ammonia gradients. HEA treatment was continued for 24 h to allow stabilisation of a new steady state. In 16 catheterised trout (284.5±14.3 g), transbranchial ammonia gradients were estimated from blood and water samples taken immediately before, and after 1, 5 and 24 h of HEA. Net ammonia and unidirectional sodium fluxes were measured under identical conditions in 16 uncatheterised trout (242.9±7.7 g). In the latter group, 3 h flux periods were used during the pre-HEA control period and from 0 to 3, 4 to 7 and 21 to 24 h of HEA, with water samples taken at 1 h intervals within each flux period.

Series 4

In Series 4, the HEA protocol of series 3 was repeated in uncatheterised trout (287.1±28.5 g, N=7), but after 24 h of HEA the final flux period was continued for a further 6 h following the addition of 10^{-4} mol l^{-1} amiloride to inhibit any Na^+/NH_4^+ exchange. No blood sampling was carried out in this series.

Series 5

The effect of buffering the water on J_{Amm} under control conditions was examined by exposing eight uncatheterised trout (300.9±21.9 g, N=8) to 5 mmol l^{-1} Hepes buffer at pH 8 for 6 h. A 500 mmol l^{-1} stock solution of Hepes buffer (Sigma; Hepes free acid) was

made up immediately before use and adjusted to pH 8.0 with 1 mol l^{-1} KOH. All flux periods lasted 1 h and were immediately followed by 1 h of flow-through of the appropriate medium (see below). After a pre-experimental control period, fish were exposed to Hepes buffer for 6 h with flux measurements being made at 0–1, 2–3 and 4–5 h of exposure to buffer. Amiloride (10^{-4} mol l^{-1}) was then added to the flux boxes in the continued presence of Hepes buffer to assess whether the effects of amiloride on J_{Amm} could be accounted for by pH changes at the gill surface, rather than by inhibition of Na^+/NH_4^+ exchange. Following 1 h of exposure to Hepes buffer+amiloride, the boxes were returned to flow-through of control dechlorinated tap water for 1 h and followed by a final post-experimental control flux period.

Series 6

In series 6 the HEA protocol of series 3 was repeated using catheterised trout (313.9±12.9 g, N=8), but after 24 h of HEA the final flux period was continued for a further 6 h following the addition of 5 mmol l^{-1} Hepes buffer, to maintain the bulk water pH at approximately 8 but to prevent any localised acidification of inspired water within the gill boundary layers. Blood samples were taken pre-HEA (control), after 1, 5 and 24 h of HEA, and after 2, 4 and 6 h of HEA+buffer. J_{Amm} fluxes were measured in the same animals during the control period, at 0–3, 4–7 and 22–24 h of HEA and at 0–6 h of HEA+buffer.

Experimental protocols

Each experimental series was started with a single control flux (lasting 1–3 h). Following all control and experimental fluxes, each box was flushed three times with a fresh quantity of the appropriate external medium without disturbance to the fish. The box was then returned to a flow-through arrangement for at least 1 h before beginning any subsequent flux period. During a flux period, ammonia obviously accumulates gradually within the boxes and is then rapidly reduced when the boxes are flushed at the end of the flux measurement. The 1 h flow-through recovery period between fluxes was necessary to allow transbranchial ammonia gradients to return to normal by the time the next flux period commences.

Flow-through arrangements during the prolonged experimental exposures when flux measurements were not being made (e.g. high external ammonia for 24 h) consisted of recirculating a 300 l reservoir of the appropriate medium through the flux boxes such that each box received a flow of at least 500 ml min^{-1}. In these cases the recirculated volume was maintained at pH 8.0 using an automatic titration assembly (Radiometer TTT80 titrator, PHM84 pH meter and GK2041C combination electrode).

For measurements of net ammonia and sodium exchanges between the fish and their external medium, the flux chambers were operated as closed, recirculating, low-volume systems (1.9–2.6 l) as previously described (Goss and Wood, 1990). In experiments where unidirectional Na^+ fluxes were measured, ^{22}Na was added to give a final activity of 1 μCi l^{-1} at the beginning of each flux period. In series where amiloride and/or Hepes buffer were also used, all were added within 1 min of each other. In all cases, a 2 min mixing period was allowed before the initial water samples were taken. Periods when the

flux boxes were closed lasted 1–6 h, depending on the experimental series, and water was sampled at 15 min or 1 h intervals for the analysis of pH, T_{Amm}, $[Na^+]$ and ^{22}Na in counts min^{-1}. Water samples for T_{Amm} and $[Na^+]$ analyses (10 ml) were stored frozen until required (−20 °C).

Analytical techniques

In experiments where blood was sampled, 400 μl of blood was drawn through the dorsal aortic catheter into chilled gas-tight 1 ml syringes (Hamilton), and this volume was immediately replaced with saline. In the case of the ammonium sulphate infusion experiment, particular care was taken to avoid contaminating the sampled blood with any infusate still present in the catheter. The infusion line was first disconnected from the catheter and then the catheter was cleared by infusing a small volume of Cortland saline. The catheter was then filled with blood and flushed three times before finally withdrawing the blood sample. Arterial pH, plasma total CO_2 and haematocrit (Hct) were analysed immediately upon collection. Whole-blood pH was measured using a Radiometer G279/G2 glass capillary electrode and K497 calomel reference electrode, connected to a PHM 71 acid–base analyser. Total CO_2 was measured on 50 μl samples of true plasma using a CO_2 analyser (Corning 965). True plasma was obtained anaerobically by centrifuging (9000 ***g*** for 2 min) 80 μl of blood in a sodium-heparinised microhaematocrit tube sealed at the upper end with a drop of paraffin oil. Plasma for measurement of T_{Amm} was obtained by centrifugation (10 000 ***g*** for 2 min) and stored frozen (−70 °C) for later analysis by a micro-modification of a commercial diagnostic kit (GLDH/NADH; Sigma 170-UV). Whole-blood [Hb] was determined using the cyanmethaemoglobin method (Sigma kit no. 525). Whole-blood [lactate] was measured enzymatically (L-lactate dehydrogenase/NADH at 340 nm) using Sigma reagents.

Water pH was measured immediately on samples taken from individual flux boxes using a Radiometer combination pH electrode (GK2401C) and meter (PHM84). Water T_{Amm} was measured in duplicate for 'low external ammonia' experiments (T_{Amm}<150 μmol l^{-1}) using a modified salicylate–hypochlorite method (Verdouw *et al.* 1978). The accuracy and sensitivity of this method for detecting changes in water T_{Amm} during the high external ammonia experiments were improved by (i) bracketing the sample range with 800–1400 μmol l^{-1} standards, (ii) using the 800 μmol l^{-1} standard to set the spectrophotometer absorbance readout to zero, and (iii) measuring all samples and standards in triplicate. For experiments involving low [NaCl] media, amiloride or Hepes buffer, additional standards were prepared within the appropriate background matrix and assayed simultaneously with samples.

Water total $[Na^+]$ was determined by atomic absorption spectrophotometry (Varian AA1275) on undiluted samples. ^{22}Na radioactivity was counted in triplicate on 5 ml water samples in 10 ml of ACS fluor (Amersham) on a liquid scintillation counter (LKB Wallac 1217 Rackbeta).

Calculation of derived variables

P_{NH_3} and $[NH_4^+]$ in plasma and water were calculated from their respective pH and T_{Amm} values using the rearranged Henderson–Hasselbalch equation and using values of

pK′ and NH_3 solubility determined by Cameron and Heisler (1983). Plasma P_{CO_2} and $[HCO_3^-]$ values were similarly calculated from measurements of plasma T_{CO_2} and whole-blood pH, using a rearrangement of the Henderson–Hasselbalch equation and values for CO_2 solubility and pK′ derived from Boutilier *et al.* (1984). The net metabolic (or non-respiratory) acid load (ΔH_m^+) was calculated according to the formula of McDonald *et al.* (1980), using non-bicarbonate buffer values estimated from the blood [Hb] and the regression relationship of Wood *et al.* (1982).

Transbranchial P_{NH_3} and $[NH_4^+]$ gradients (ΔP_{NH_3} and $\Delta[NH_4^+]$) were calculated by simple subtraction:

$$\Delta P_{NH_3} = P_{NH_3} \text{ in plasma} - P_{NH_3} \text{ in water}, \quad (1)$$

where plasma was taken from the dorsal aorta and was therefore postbranchial in origin. In freshwater rainbow trout, the ammonia excreted by the gills is essentially cleared from the blood (Cameron and Heisler, 1983). Ideally, the mean plasma concentration in blood passing through the gill ([arterial]+[venous]/2) should be used to determine the transbranchial gradients. In some previous studies under normal conditions, this has been predicted from the arterial plasma concentration alone, assuming a constant *ratio* for T_{Amm} in pre- and postbranchial blood (from 1.66 to 1.81; Cameron and Heisler, 1983; Wright and Wood, 1985; Ogata and Murai, 1988). However, we deemed such an approach highly problematic for the present study, in which blood T_{Amm} levels changed greatly in response to experimental treatments. Indeed, it is probably more correct to assume a constant *difference* between arterial and venous levels, since the ratio does not remain constant under conditions where the ambient T_{Amm} is raised (see Cameron and Heisler, 1983). In the end, we used a conservative approach whereby transbranchial gradients were simply calculated from dorsal aortic blood plasma measurements. This undoubtedly resulted in an underestimate of ΔP_{NH_3} and ΔNH_4^+ during all conditions.

Net flux rates of total ammonia (J_{Amm}) were calculated from the appearance or disappearance of total ammonia from the water as:

$$J_{Amm} = ([T_{Amm}]_i - [T_{Amm}]_f) \times V/(t \times M), \quad (2)$$

where $T_{Amm,i}$ and $T_{Amm,f}$ are initial and final T_{Amm} concentrations ($\mu mol\,l^{-1}$) in the water, V is the water volume (l) in the box during the flux period, t is the time elapsed (h) during the flux period and M is the mass of the fish (kg). A positive J_{Amm} indicates net excretion and a negative value indicates net uptake of ammonia.

Unidirectional sodium influx rates (J_{in}^{Na}) were calculated as described by Wood (1988):

$$J_{in}^{Na} = (R_i - R_f) \times V/(SA \times t \times M), \quad (3)$$

where R_i and R_f are initial and final radioactivities in water ($cts\,min^{-1}\,ml^{-1}$), SA is the mean specific activity ($cts\,min^{-1}\,\mu equiv^{-1}$) over the flux period in question, and other symbols are as in equation 2. Radioisotope backflux correction (Maetz, 1956) was only required for the final 6 h flux in series 3, when internal specific activity exceeded 5 % of the external activity.

Data have been expressed as mean ± S.E.M. (N=number of animals or number of water samples). Changes in measured variables between the control and the different

experimental treatments were compared using a Student's paired two tailed *t*-test ($P<0.05$) within each series, using each animal as its own control.

Results

Low external [Na^+] (series 1)

For the control fluxes in Fig. 1, external [Na^+] averaged 534.3±3.6 μmol l^{-1} (N=16). This was reduced to 19.5±3.8 μmol l^{-1} (N=8) at the beginning of the low [Na^+] exposure, but had increased to 60.4±6.8 μmol l^{-1} (N=8) by the end of the 3 h experimental flux period. Thus, external [Na^+] remained well below the K_m of 114 μmol l^{-1} and should have maintained J_{in}^{Na} at less than 20 μmol kg^{-1} h^{-1}, based on kinetic uptake curves reported for freshwater rainbow trout *in vivo* under identical conditions (Goss and Wood, 1990).

During the first 15 min of exposure to low external [Na^+], J_{Amm} was significantly reduced by 24 % when compared to the combined average J_{Amm} during the initial 15 min intervals of the two control periods (Fig. 1). For statistical purposes, data from the two control fluxes (days 1 and 3) were pooled to allow for a small decline in J_{Amm} noticed between these two control periods (amounting to a drop in J_{Amm} of about 6 % per day). Over the whole 3 h experimental flux period J_{Amm} was significantly reduced by a similar amount (22.9 %), indicating that there were no rapid changes in ammonia excretion that would be missed by using flux periods of 1 h or more (Fig. 1).

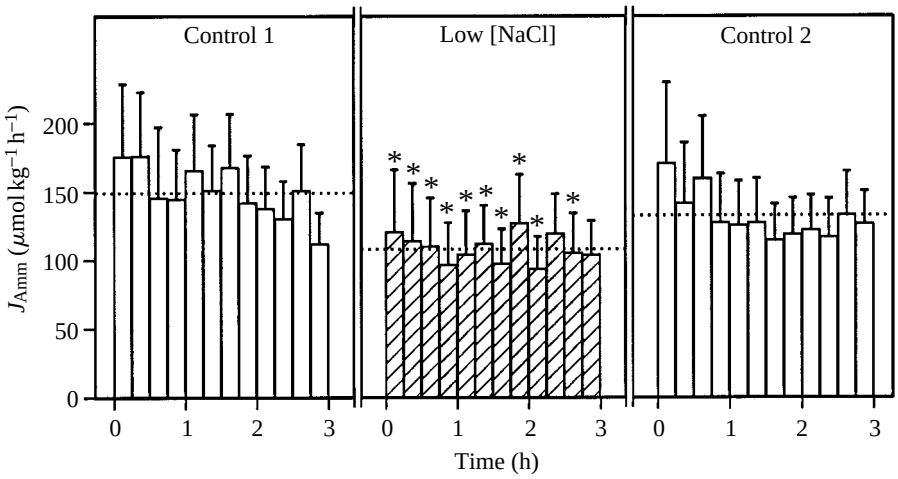

Fig. 1. The effect of a 3 h exposure to low [NaCl] on ammonia excretion (J_{Amm}; measured every 15 min) in rainbow trout. The two control periods (open bars) were performed 24 h before and 24 h after the experimental period (hatched bars). Horizontal dotted lines represent the mean values for each 3 h flux periods (149.6±35.9, 108.5±29.3 and 131.8±32.7 μmol kg^{-1} h^{-1} for control 1, low [NaCl] and control 2 periods, respectively). Asterisks represent significant changes ($P<0.05$) in J_{Amm} for each 15 min flux measurement when compared with the corresponding pooled value from control periods 1 and 2 (see text for details). Means ± S.E.M. (N=8).

Ammonium sulphate infusion and exposure to amiloride (series 2)

Addition of 10^{-4} mol l^{-1} amiloride to the external water prior to ammonium sulphate infusion reduced J_{in}^{Na} from 173±19 to 33±9 μmol kg^{-1} h^{-1}, an inhibition of 81 % (Fig. 2). During the same treatment J_{Amm} was inhibited by only 18 % (Fig. 2). The effect of sodium uptake blockade on J_{Amm} was, therefore, similar to the effect of NaCl removal in series 1. Following 24 h of continuous infusion with 70 mmol l^{-1} ammonium sulphate, J_{Amm} and J_{in}^{Na} were both significantly increased by approximately equivalent amounts (3.7-fold and 3.8-fold, respectively). The measured elevation of J_{Amm} (549±41 μmol kg^{-1} h^{-1}) was not significantly different from the calculated infusion rate (562±37 μmol kg^{-1} h^{-1}), indicating that a new equilibrium state had been achieved. Ammonium sulphate infusion produced a small but significant systemic acidosis: arterial blood pH was reduced from 7.932±0.019 during the control period to 7.851±0.017 (N=7) after 24 h of continuous infusion.

When trout were subsequently exposed to 10^{-4} mol l^{-1} amiloride (following 26 h of

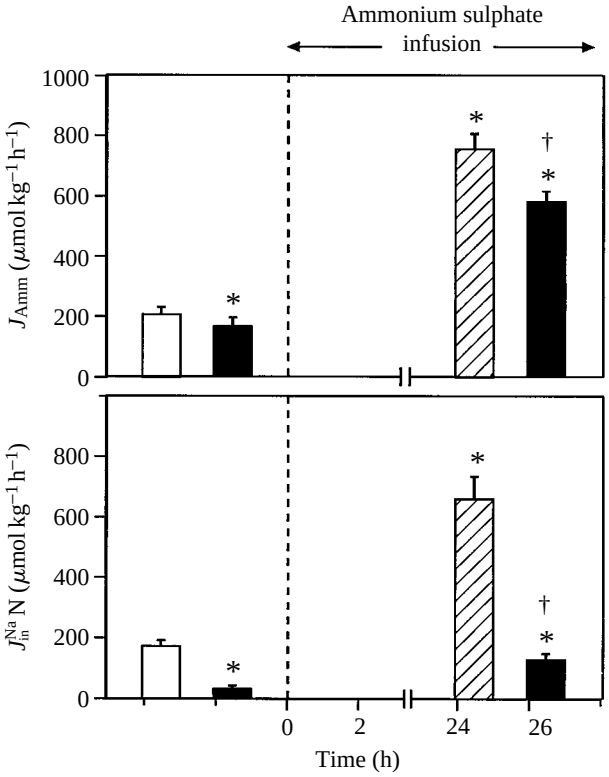

Fig. 2. The effect of externally applied amiloride (10^{-4} mol l^{-1}; filled bars) on ammonia excretion (J_{Amm}) and sodium uptake (J_{in}^{Na}) in rainbow trout before (open bar) and after 24 h of continuous infusion with 70 mmol l^{-1} ammonium sulphate (hatched bar). Asterisks denote values significantly different (P<0.05) from the pre-infusion control value, and daggers represent values significantly different from the flux after 24 h of infusion. Mean ± S.E.M. (N=7).

ammonium sulphate infusion), J_{in}^{Na} was reduced by 80 % and J_{Amm} by 23 %, very similar to the proportional reductions caused by amiloride during control conditions (prior to infusion). However, the absolute reductions in J_{in}^{Na} and J_{Amm} were correspondingly 3.8 and 4.6 times greater than during the pre-infusion amiloride treatment.

High external ammonia (series 3)

Exposure to a high concentration of external ammonia (HEA; T_{Amm}=1106± 12 μmol l^{-1}, pH=8.05±0.01, N=48) resulted in a rapid increase in arterial plasma T_{Amm}, reaching a relatively stable value of 765±38 μmol l^{-1} after 5 h, still 341 μmol l^{-1} below the external T_{Amm} (Fig. 3). Under control conditions the P_{NH_3} and [NH_4^+] gradients were slightly positive, but both were dramatically reversed on exposure to HEA, also reaching new stable values after 5 h but remaining strongly negative throughout the experimental period (Fig. 3).

Initially, exposure to HEA resulted in a period of net ammonia uptake from the water, reversing the normally positive J_{Amm} (i.e. ammonia excretion) seen under control

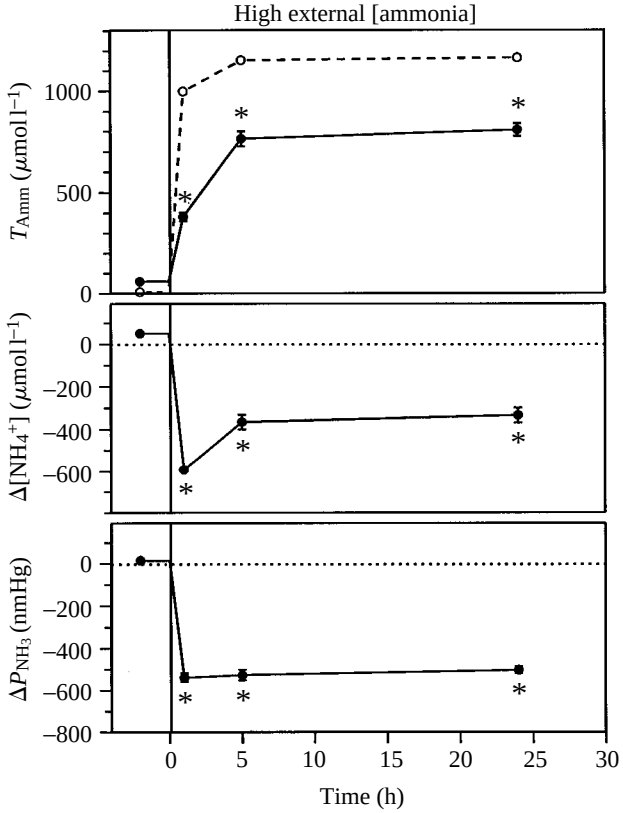

Fig. 3. The effect of 24 h of exposure to high external ammonia (HEA) on plasma (solid line) and water (dashed line) T_{Amm}, and the blood-to-bulk-water [NH_4^+] and P_{NH_3} gradients in rainbow trout. Asterisks indicate values significantly different (P<0.05) from the pre-HEA control value. Mean ± S.E.M. (N=16).

conditions (Fig. 4). In fact, the *influx* of ammonia during the first hour of HEA was almost three times greater in magnitude than the normal *efflux* rate. However, this reversal of the net ammonia flux was short-lived and trout began to excrete ammonia again after about 5 h, coinciding with the stabilisation of plasma T_{Amm} and transbranchial gradients (Figs 3 and 4). After 24 h of HEA, J_{Amm} was not significantly different from the control rate.

Sodium uptake was reduced to half the control J_{in}^{Na} during the second hour of HEA exposure (Fig. 4). However, this inhibitory effect was transitory and, after 24 h, J_{in}^{Na} appeared to have stabilised at a level 32 % greater than the control rate. A mild but persistent blood alkalosis was observed after 5 h of exposure to HEA. This was of a non-respiratory origin (indicated by the negative ΔH_m^+), despite a moderate increase in blood lactate concentration (Fig. 5).

High external ammonia and amiloride (series 4)

In series 4 the changes in J_{Amm} and J_{in}^{Na} over the first 24 h of HEA (Fig. 6) followed patterns similar to those described in series 3 above. This was as expected, since the external T_{Amm} and pH were very similar to those measured in the previous series (T_{Amm}=1113±7 μmol l^{-1}, pH=8.03±0.02, N=32).

On addition of amiloride after 24 h of HEA, water pH remained unchanged during the following 6 h (8.03±0.02, N=16) and J_{in}^{Na} was reduced by 96 % overall. Despite this almost complete blockade of sodium uptake, trout continued to excrete ammonia but at a

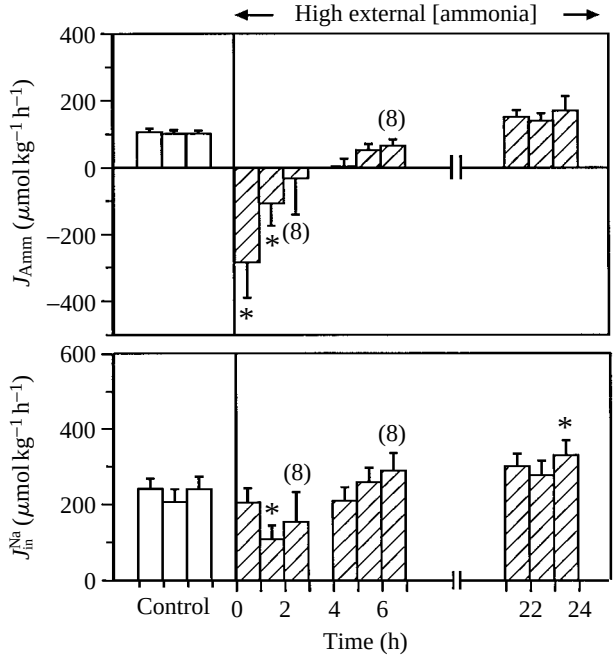

Fig. 4. The effect of 24 h of exposure to high external ammonia (HEA; hatched bars) on net ammonia fluxes (J_{Amm}) and unidirectional sodium uptake (J_{in}^{Na}) in rainbow trout. Asterisks represent values significantly different (P<0.05) from the pre-HEA control value (open bars). Mean ± S.E.M. (N=16, except where indicated in parentheses).

reduced rate (J_{Amm} was inhibited by 27 % during the first hour and 36 % overall), but at no time was J_{Amm} reversed in the presence of amiloride (Fig. 6).

Hepes buffer and amiloride under control conditions (series 5)

Buffering the external medium with 5 mmol l^{-1} Hepes under control conditions (i.e. low external T_{Amm}) immediately reduced J_{Amm} by 36 % (Fig. 7). Bulk water pH (pHw) was unchanged by the addition of Hepes buffer [pHw averaged 8.11±0.03 (N=16) for the two control periods and 8.06±0.02 (N=24) for the three fluxes following the addition of Hepes buffer]. J_{Amm} recovered slightly during the subsequent 4 h in buffered medium but stabilised at a rate that was still below the control J_{Amm}. In contrast to previous experiments using the sodium uptake inhibitor amiloride (series 2 and 4), addition of 10^{-4} mol l^{-1} amiloride had no effect on J_{Amm} when trout were maintained in the buffered medium (Fig. 7). The post-experimental control period was similar to the initial control period, highlighting the rapid reversibility of both Hepes buffer and amiloride treatments.

High external ammonia and Hepes buffer (series 6)

The first 24 h of HEA treatment in series 6 was essentially a repeat of the protocols used

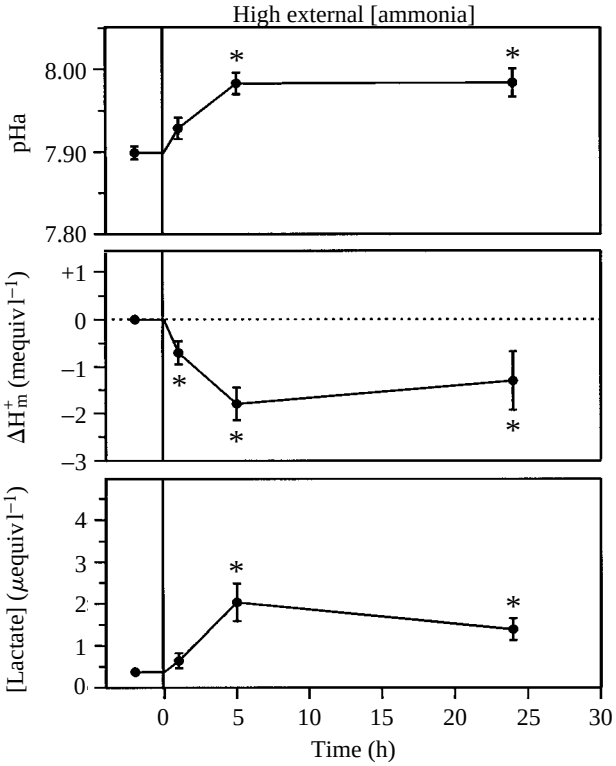

Fig. 5. The effect of 24 h of exposure to high external ammonia (HEA) on whole-blood pH, net non-respiratory acid load (ΔH^+_m) and lactate concentration in rainbow trout. Asterisks indicate values significantly different (P<0.05) from the pre-HEA control value. Mean ± S.E.M. (N=16).

in series 3 and 4. The measured external T_{Amm} and pH in series 6 were slightly lower than in the two previous regimes, averaging 1025±8 μmol l^{-1} (N=80) and 7.95±0.03 (N=40) respectively. As in series 3, elevating the external T_{Amm} caused a rapid increase in plasma T_{Amm} (Fig. 8). After 24 h, plasma T_{Amm} was 471±40 μmol l^{-1} (N=7), still well below the external level, and both P_{NH_3} and NH_4^+ gradients were highly negative (Fig. 8).

During the first 24 h of HEA, J_{Amm} changes were similar to those seen previously in series 3 and 4 (Fig. 9). Matching of the bulk water pH before and after the addition of Hepes buffer was not exact; bulk water in the flux boxes was maintained at the slightly lower pH of 7.81±0.02 (N=32) during the 6 h of HEA+buffer treatment. Addition of Hepes buffer resulted in an immediate reversal of J_{Amm} (Fig. 9). This persisted for the first hour, and by the third hour trout were again excreting ammonia at a rate not significantly different from either the control rate or that after 24 h of HEA. The recovery of net ammonia excretion was accompanied by an increase in the P_{NH_3} gradient such that, after 4 h of HEA+buffer treatment, ΔP_{NH_3} was no longer significantly different from the positive partial pressure gradient observed during the control period (Fig. 8).

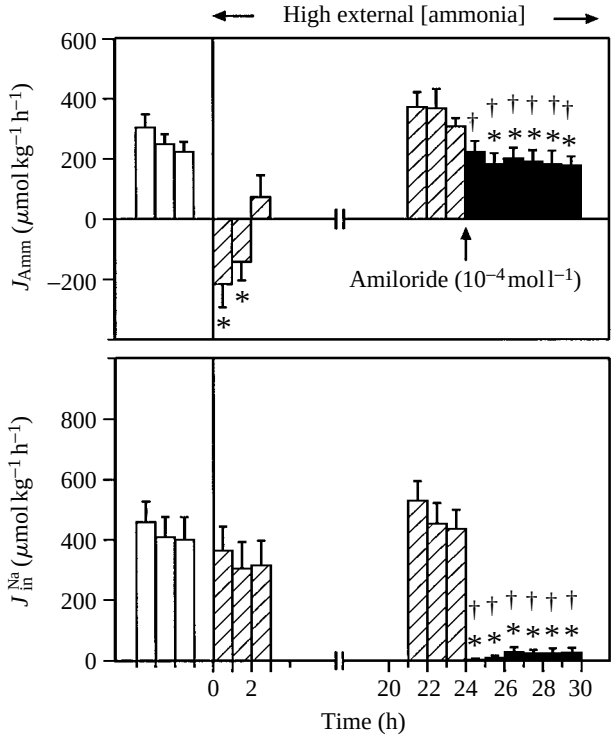

Fig. 6. The effect of amiloride (10^{-4} mol l^{-1}; filled bars) on net ammonia fluxes (J_{Amm}) and unidirectional sodium uptake (J_{in}^{Na}) following 24 h of exposure to high external ammonia (HEA; hatched bars) in rainbow trout. Asterisks represent values significantly different (P<0.05) from the pre-HEA control value (open bars), and daggers represent values significantly different from the final flux at 24 h of HEA. Mean ± S.E.M. (N=7).

Discussion

The link between sodium uptake and ammonia efflux under control conditions

Results from the experiments using low [Na^+] or amiloride indicate that only about 20 % of the net ammonia efflux is dependent upon the active uptake of external Na^+ under control conditions. This is slightly lower than the 23–30 % reductions seen in previous reports of the acute effects of amiloride on freshwater trout preparations *in vivo* (Wright and Wood, 1985) and *in vitro* (Kirschner *et al.* 1973; Payan, 1978; Wright *et al.* 1989). Many people have previously interpreted this as a blockade of the smaller fraction of ammonia excretion that is driven by Na^+/NH_4^+ exchange, with the remaining 70–80 % of ammonia excretion being the result of passive NH_3 diffusion under normal conditions.

Ammonium sulphate infusion

Our attempts to stimulate Na^+/NH_4^+ exchange by infusing ammonium sulphate produced equivalent increases in both J_{in}^{Na} and J_{Amm}, suggesting stimulation of directly coupled Na^+/NH_4^+ exchange. However, if this increased sodium uptake was entirely due to an increase in the rate of Na^+/NH_4^+ exchange, then the subsequent addition of amiloride, which reduced Na^+ influx by 80 %, should have reduced J_{Amm} back to the control level. Instead ammonia excretion continued at a highly elevated rate and was reduced by only about 23 % relative to the rate after 24 h infusion. Thus, the increased rate of ammonia efflux was still largely independent of sodium uptake. Nevertheless, the absolute rate of ammonia excreted that could be inhibited by amiloride was increased from 37 to 169 μmol kg^{-1} h^{-1} following ammonium sulphate infusion. Thus, it could be

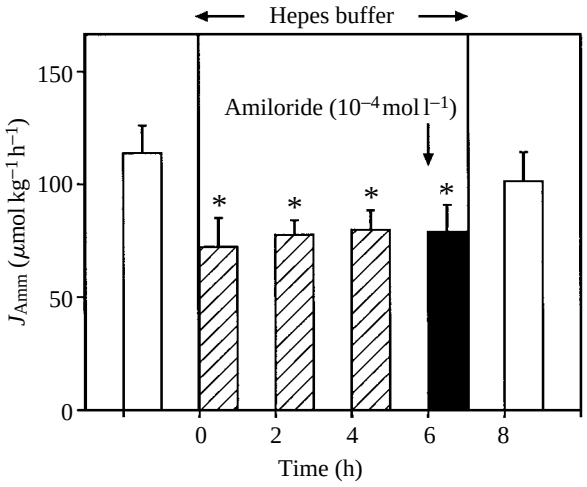

Fig. 7. The effect of buffering ventilatory water (5 mmol l^{-1} Hepes; pH 8.06; hatched bars) on ammonia excretion (J_{Amm}), and the subsequent effect of amiloride (10^{-4} mol l^{-1}; filled bar), on rainbow trout under control conditions. Asterisks represent values significantly different (P<0.05) from the initial value (open bars). Note that the second control period (post-experimental) was not significantly different from the pre-experimental control period, indicating complete reversibility of the Hepes and amiloride treatments. Mean ± S.E.M. (N=8).

argued that a 4.5-fold stimulation of Na^+/NH_4^+ exchange had occurred. Of course, infusion of ammonium sulphate will simultaneously increase both NH_3 and NH_4^+ concentrations in the blood, so one would also expect an elevation of the P_{NH_3} diffusion gradient across the gills. Unfortunately, plasma values for T_{Amm} are not available for this experiment owing to accidental loss of frozen samples. However, it seems logical to assume that some fraction of the increased J_{Amm} during infusion was due to an accelerated diffusive efflux of NH_3. In fact, the more rapid efflux of NH_3 following infusion of an ammonium salt, leaving behind a strong acid (in this case sulphuric acid), is the usual explanation for a post-infusion extracellular acidosis (Cameron and Kormanik, 1982; Cameron and Heisler, 1983; Claiborne and Evans, 1988; McDonald and Prior, 1988). Indeed, it is tempting to attribute the parallel increase in sodium influx following ammonium sulphate infusion to this increased number of protons in the

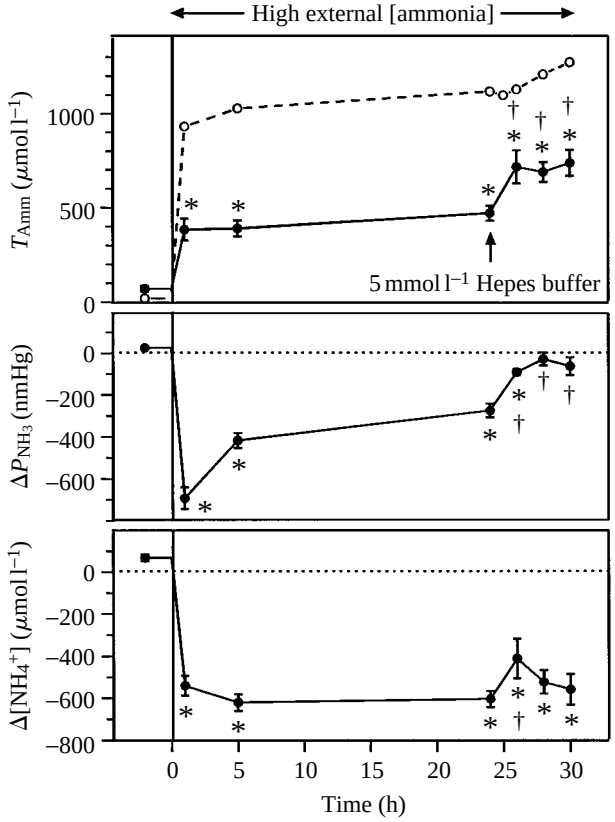

Fig. 8. The effect of adding 5 mmol l^{-1} Hepes buffer to the external water following 24 h of exposure to high external ammonia on T_{Amm} in plasma (solid line) and water (dashed line), blood-to-bulk-water $[NH_4^+]$ and P_{NH_3} gradients in rainbow trout. Asterisks represent values significantly different (P<0.05) from the pre-HEA control value (open bars), and daggers represent values significantly different from the final flux at 24 h of HEA. Note the fall in water T_{Amm} during the first hour of the HEA+buffer flux period, representing a net *uptake* of ammonia, and the subsequent accumulation of T_{Amm} in the water once net excretion has recovered (see Fig. 9 for actual fluxes). Mean ± S.E.M. (N=8).

extracellular fluid becoming available for enhanced Na^+/H^+ exchange or primary H^+ transport linked to Na^+ uptake through an apical channel (Goss and Wood, 1991), rather than to an increased availability of NH_4^+ driving more Na^+/NH_4^+ exchange. The moderate acidosis accompanying the infusion supports this idea. Infusion of ammonium bicarbonate, which produces little or no acid–base disturbances (Claiborne and Evans, 1988), could help to resolve this issue.

High external ammonia concentration

It has been repeatedly observed that teleost fish are able to maintain reversed P_{NH_3} and NH_4^+ gradients when external ammonia levels are elevated (Cameron and Heisler, 1983; Cameron, 1986; Claiborne and Evans, 1988; Wilson and Taylor, 1992). In all these cases it has been concluded that some form of active NH_4^+ extrusion must be operating in order to counteract the inward diffusion of NH_3 (and to some degree NH_4^+ in seawater teleosts) under these extreme conditions. Most commonly, stimulation of apical Na^+/NH_4^+ exchange has been advocated as the counteracting mechanism. Results from series 3 clearly demonstrate that the stabilisation of apparently negative ΔP_{NH_3} and ΔNH_4^+ was accompanied by the return of ammonia fluxes to net excretion after about 5 h. Thereafter, a steady state of net excretion continued for the entire 24 h exposure period. However, there are two lines of evidence that argue strongly against apical Na^+/NH_4^+ exchange as the mechanism of ammonia extrusion during HEA.

First, the addition of amiloride once a new equilibrium had been established in series 4 all but completely inhibited sodium uptake (Fig. 6). If ammonia were being actively extruded by apical Na^+/NH_4^+ exchange at this time, the mechanism should have been abolished upon treatment with amiloride, resulting in a reversal of the net ammonia flux. This was not observed. Although J_{Amm} was reduced following the elimination of Na^+ uptake (similar to the ‘low external ammonia’ amiloride treatments in series 2; see

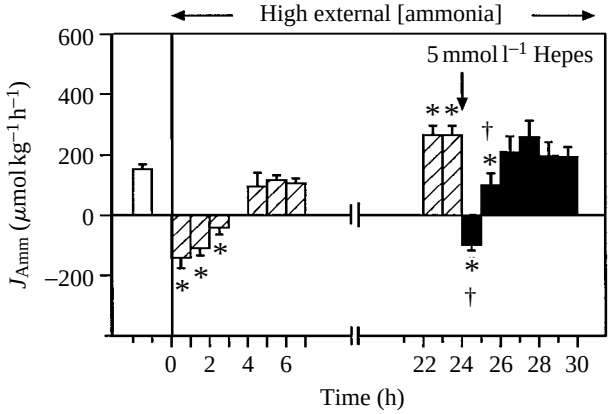

Fig. 9. The effect of adding 5 mmol l^{-1} Hepes buffer (filled bars) to the external water following 24 h of exposure to high external ammonia (hatched bars) on net ammonia fluxes (J_{Amm}) in rainbow trout (N=8). Asterisks represent values significantly different (P<0.05) from the pre-HEA control value (open bars), and daggers represent values significantly different from the final flux at 24 h of HEA. Mean ± S.E.M. (N=8).

Fig. 2), net ammonia excretion continued when amiloride and high external ammonia were combined. Second, one would anticipate an extremely large increase in J_{in}^{Na} if Na^+/NH_4^+ exchange were responsible for the recovery of net ammonia excretion during HEA (Cameron, 1986). If we assume that 80 % of the control net ammonia efflux (i.e. 84 μmol kg^{-1} h^{-1}) was driven by the control ΔP_{NH_3} (15.3 nmHg) then it follows that the new ΔP_{NH_3} after 24 h of HEA (−502 nmHg) should support a passive NH_3 influx of 2756 μmol kg^{-1} h^{-1}. In order to counteract this using a 1:1 Na^+/NH_4^+ exchange, J_{in}^{Na} would have to increase by an even greater amount (to allow for the endogenously produced ammonia). Clearly the small increase in J_{in}^{Na} after 24 h (90 μmol kg^{-1} h^{-1}) was insufficient to explain the recovery of net ammonia excretion. Indeed, to our knowledge, rates of unidirectional Na^+ influx of 2756 μmol kg^{-1} h^{-1} have never been seen in freshwater trout.

It would appear that apical Na^+/NH_4^+ exchange is not a viable explanation for the recovery of net ammonia excretion when the external T_{Amm} is elevated. This was the conclusion of Cameron (1986) following a similar analysis on the freshwater channel catfish (*Ictalurus punctatus*). He proposed that a putative H^+/NH_4^+ exchange was the only plausible mechanism that fitted the accompanying net acid fluxes he measured during 4 h of HEA in the catfish. However, the apparent net H^+ flux data of Cameron (1986) are also

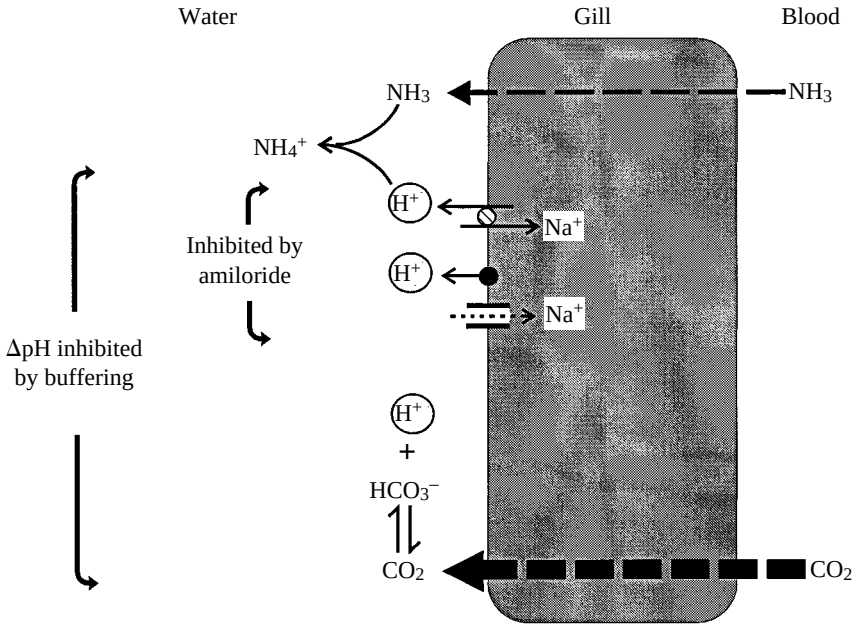

Fig. 10. A schematic diagram of the gill epithelium showing processes thought to be involved in boundary layer acidification and facilitated NH_3 diffusion (protons added to the apical side of the gill epithelium are enclosed in circles for clarity). Boundary layer acidification due to both CO_2 hydration and direct H^+ transport will be inhibited by the addition of buffer to the external water. In contrast, only the fraction of boundary layer acidification due to direct H^+ excretion can be inhibited by external application of amiloride. Solid arrows represent carrier-mediated transport, broken arrows indicate passive diffusion.

compatible with passive net ammonia excretion by NH_3 diffusion if the actual transbranchial P_{NH_3} gradient within the gill micro-environment returns to a positive value during HEA. This hypothesis depends on the strength of gill boundary layer acidification and is discussed below.

The importance of gill boundary layer acidification

In most previous studies it has been assumed that calculation of P_{NH_3} in the bulk water surrounding the animal (well mixed by aeration, and equivalent to the inspired water in this case) is a good estimate of the P_{NH_3} at the gill surface. However, it has been repeatedly demonstrated that inspired water may be acidified by up to 1.5 pH units as it passes over the gills (Wright *et al.* 1986; Playle and Wood, 1989; Lin and Randall, 1990). In these studies acidification was attributed to the hydration of excreted CO_2 and/or direct H^+ transport. However it arises, boundary layer acidification should facilitate branchial ammonia excretion by diffusion-trapping of NH_3 as NH_4^+, i.e. reducing the effective P_{NH_3} at the gill surface (Randall and Wright, 1987; Wright *et al.* 1989; Randall *et al.* 1991). Indeed, this is almost certainly the case in all our experiments that used 'normal' fresh water with limited buffer capacity in series 1–4. By adding buffer to the external water in series 5, our goal was to keep the bulk water pH unchanged but to prevent acidification at the gill surface, in order to quantify the role of boundary layer acidification in facilitating ammonia excretion (Fig. 10). If we assume that buffering with Hepes eliminated acidification of inspired water under control conditions, then the 36 % reduction in J_{Amm} (Fig. 7) suggests that an acidified gill boundary layer normally allows 57 % more ammonia to be excreted than one would have predicted from the blood-to-bulk-water P_{NH_3} gradient. This effect of buffering ventilatory water agrees well with the *in vitro* results of Wright *et al.* (1989) (where J_{Amm} was reduced by approximately 30 % in the presence of 0.4 mmol l^{-1} Tris buffer, pH 8.0), but contrasts markedly with the 50 % increase in J_{Amm} found by Avella and Bornancin (1989) when 50 mmol l^{-1} Tris buffer (pH 8.0) was added to the irrigation solution of the perfused trout head. Avella and Bornancin (1989) explained the latter result as an increased availability of H^+ for diffusion-trapping of NH_3, but it may have been an artefact produced by the high concentration of Tris used. We have found that Tris prevents colour development in the ammonia assay used and, if it is sufficiently permeable to enter the postbranchial perfusate, may have given rise to lower apparent T_{Amm} values, which would be interpreted as increased excretion.

Boundary layer acidification during high external ammonia exposure

Acidification of the gill boundary layer could be even more important during high external ammonia exposure, when transbranchial P_{NH_3} gradients are expected to be strongly reversed. Based on the mean bulk water T_{Amm} at 24 h of HEA in series 3, we calculated that the water layer at the gill surface would only have to be acidified by 0.4 pH units for the actual blood–water P_{NH_3} gradient to be positive, rather than the large negative value (−502 nmHg) that was estimated using the bulk water pH measurements. In other words, if boundary layer acidification were sufficient, then no active NH_4^+

transport (Na^+/NH_4^+ or H^+/NH_4^+ exchange) would be required to explain the recovery of net ammonia excretion under HEA. The experiment performed in series 5 tested this hypothesis by again using Hepes buffer to prevent acidification occurring at the gill surface whilst maintaining bulk water pH at the same value (Fig. 9). In fact the addition of the buffer after 24 h of HEA slightly acidified the bulk water, which would tend to make the blood-to-bulk-water P_{NH_3} gradient more positive. In contrast, J_{Amm} was immediately reversed upon addition of the buffer (Fig. 9), suggesting elimination of a boundary layer acidification that had previously been large enough to create a positive NH_3 diffusion gradient in the presence of negative T_{Amm} and $[NH_4^+]$ gradients. It is worth noting here that, in one natural environment where gill boundary layer acidification is difficult or even impossible (the well-buffered alkaline waters of Pyramid Lake, Nevada, USA), ammonia excretion is chronically depressed in the native Lahontan cutthroat trout (*Oncorhynchus clarki henshawi*; Wright *et al.* 1993).

If the above ideas are correct, the P_{NH_3} gradients measured when J_{Amm} subsequently recovered in the presence of HEA *and* buffer in series 5 should have returned to the slightly positive value measured under control conditions (Fig. 9). Although the final two mean ΔP_{NH_3} values were not significantly different from the control value, neither was made up of consistently positive values. However, the combination of (i) incomplete buffering, (ii) the underestimation of ΔP_{NH_3} by using arterial plasma ammonia measurements (see Materials and methods) and (iii) the inherent problem of measuring small P_{NH_3} gradients (calculated from four independently measured variables) within a background of high T_{Amm}, may all have contributed to this discrepancy.

Overall, it would appear that no definite evidence can be found for a role of Na^+/NH_4^+ in ammonia excretion during HEA. Instead, the role of boundary layer acidification and the creation of a new outwardly directed P_{NH_3} gradient across the gill micro-environment has been highlighted, and appears to be a better explanation for the recovery of net ammonia excretion during HEA. It may even be possible that boundary layer acidification is intensified during HEA. The persistent non-respiratory blood alkalosis during HEA in trout (i.e. net base load or negative ΔH_m^+, despite an increase in blood lactate), coupled with the elevated Na^+ uptake, indicates an activation of H^+ excretion that would amplify the pH changes occurring at the gill surface. Increased anaerobic production of lactate may be a secondary response to compensate for the blood alkalosis (Wilkie and Wood, 1991) even though blood oxygen levels are unaffected by HEA (Wilson and Taylor, 1992). However, the proximate signal(s) for an activation of H^+ excretion (and subsequent activation of glycolysis) remain unexplained. Neither can we explain the opposite acid–base response (i.e. a positive ΔH_m^+) to HEA in freshwater trout found by Wilson and Taylor (1992), although this could be related to the softer water used and considerably lower plasma T_{Amm} achieved in the latter study. Nevertheless, such a response (enhancement of H^+ excretion) would minimise the plasma T_{Amm} and P_{NH_3} levels that could be maintained in the face of HEA and in the absence of an active NH_4^+ transporting mechanism.

The effect of amiloride on boundary layer acidification and J_{Amm}

Amiloride produced a 20–30 % reduction in ammonia excretion during the control,

ammonia infusion and HEA exposures, but had no effect on J_{Amm} when trout were maintained in a buffered medium (Fig. 7). At 10^{-4} mol l^{-1}, amiloride is a potent inhibitor of both the conductive Na^+ entry pathway (a Na^+ channel) and electroneutral Na^+/H^+ exchange (Benos, 1982; Fig. 10). In freshwater trout, the debate continues as to whether apical sodium uptake is *via* the former (linked to an electrochemical gradient set up by a primary proton pump), or is driven by direct exchange for H^+ in the latter. Whichever is the case, inhibition of Na^+ transport by amiloride should simultaneously reduce H^+ excretion to the apical side of the gill, and hence reduce the extent of boundary layer acidification and lower the blood–water P_{NH_3} gradient (Fig. 10). This would explain why amiloride consistently reduced J_{Amm} in normal fresh water, but had no effect when the acidification had already been abolished using Hepes buffer. Interestingly, Wright *et al.* (1993) also found no effect of amiloride on J_{Amm} in the Lahontan cutthroat trout living in the well-buffered alkaline waters of Pyramid Lake mentioned earlier.

It would appear that the effect of amiloride on J_{Amm} in normal fresh water (with minimal buffer capacity) may only be secondary to a reduction in H^+ excretion and boundary layer acidification. If this is the case, then the use of amiloride, and any other form of sodium uptake blockade, cannot be used as evidence for Na^+/NH_4^+ exchange in freshwater fish. Indeed, the existence of 'Na^+/NH_4^+ exchange' as a directly coupled entity is called into question by our results. Instead, we propose that transbranchial ammonia movements occur almost exclusively by passive NH_3 diffusion in freshwater trout under a variety of conditions, and stress the importance of gill boundary layer acidification in determining the true P_{NH_3} gradients driving branchial ammonia excretion.

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