

THE LINKAGE BETWEEN Na^+ UPTAKE AND AMMONIA EXCRETION IN RAINBOW TROUT: KINETIC ANALYSIS, THE EFFECTS OF $(\text{NH}_4)_2\text{SO}_4$ AND NH_4HCO_3 INFUSION AND THE INFLUENCE OF GILL BOUNDARY LAYER pH

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Summary

The nature of the linkage between branchial ammonia excretion (J_{Amm}) and unidirectional Na^+ influx ($J_{\text{in}}^{\text{Na}}$) was studied in the freshwater rainbow trout (*Oncorhynchus mykiss*). Arterial plasma total [ammonia], P_{NH_3} and J_{Amm} were all elevated approximately threefold by intravascular infusion for 24 h with either 70 mmol l^{-1} $(\text{NH}_4)_2\text{SO}_4$ or 140 mmol l^{-1} NH_4HCO_3 at a rate of approximately $400 \mu\text{mol kg}^{-1} \text{ h}^{-1}$. Both treatments markedly stimulated $J_{\text{in}}^{\text{Na}}$. NH_4HCO_3 induced metabolic alkalosis in the blood plasma, whereas $(\text{NH}_4)_2\text{SO}_4$ caused a slight metabolic acidosis. Experiments with Hepes-buffered water (5 mmol l^{-1}) under control conditions demonstrated that increases in gill boundary layer pH were associated with decreases in both $J_{\text{in}}^{\text{Na}}$ and J_{Amm} . Thus, the stimulation of $J_{\text{in}}^{\text{Na}}$ caused by ammonium loading was not simply a consequence of a Na^+ -coupled H^+ extrusion mechanism activated by internal acidosis or by alkalosis in the gill boundary layer. Indeed, there was no stimulation of net acidic equivalent excretion accompanying NH_4HCO_3 infusion. Michaelis–Menten kinetic analysis by acute variation of water $[\text{Na}^+]$ demonstrated that both infusions caused an almost twofold increase in $J_{\text{in}}^{\text{Na}}$ but no significant change in K_m , indicative of an increase in transporter number or internal

counterion availability without an alteration in transporter affinity for external Na^+ . The increase in $J_{\text{in}}^{\text{Na}}$ was larger with $(\text{NH}_4)_2\text{SO}_4$ than with NH_4HCO_3 infusion and in both cases lower than the increase in J_{Amm} . Additional evidence of quantitative uncoupling was seen in the kinetics experiments, in which acute changes in $J_{\text{in}}^{\text{Na}}$ of up to threefold had negligible effects on J_{Amm} under either control or ammonium-loaded conditions. *In vitro* measurements of branchial Na^+/K^+ -ATPase activity demonstrated no effect of NH_4^+ concentration over the concentration range observed *in vivo* in infused fish. Overall, these results are consistent with a dominant role for NH_3 diffusion as the normal mechanism of ammonia excretion, but indicate that ammonium loading directly stimulates $J_{\text{in}}^{\text{Na}}$, perhaps by activation of a non-obligatory $\text{Na}^+/\text{NH}_4^+$ exchange rather than by an indirect effect (e.g. Na^+ -coupled H^+ excretion) mediated by altered internal or external acid–base status.

Key words: *Oncorhynchus mykiss*, rainbow trout, ammonia excretion, Na^+ uptake, gill boundary layer pH, NH_3 diffusion, branchial ion exchange, Na^+/K^+ -ATPase, $(\text{NH}_4)_2\text{SO}_4$ infusion, NH_4HCO_3 infusion.

Introduction

In teleosts, the majority of nitrogenous waste is excreted through the gills as ammonia. In freshwater fish, the excretion takes place either as NH_3 by non-ionic diffusion or as NH_4^+ transport linked in some manner to Na^+ uptake (for a review, see Wilkie, 1997). However, it is still not known which, if either, is the dominant pathway (e.g. Kormanik and Cameron, 1981; Cameron and Heisler, 1983; Wright and Wood, 1985; McDonald and Prior, 1988; Wilson and Taylor, 1992; Wilson et al., 1994). A direct coupling of Na^+ uptake with H^+ excretion by either electroneutral Na^+/H^+ exchange or a primary H^+ pump/ Na^+ channel system is well established (for a review, see

Potts, 1994), but the linkage between Na^+ uptake and NH_4^+ efflux, as first proposed by August Krogh (1939) more than half a century ago, remains uncertain.

On the one hand, several studies have shown a one-to-one relationship between ammonia excretion rates (J_{Amm}) and Na^+ influx rates ($J_{\text{in}}^{\text{Na}}$) under resting physiological conditions in freshwater salmonids (Wright and Wood, 1985; McDonald and Prior, 1988; McDonald and Milligan, 1988) and in the isolated-perfused head preparation (Payan, 1978). Also, when ammonia excretion was stimulated by infusion of $(\text{NH}_4)_2\text{SO}_4$, the increase in J_{Amm} was accompanied by an equivalent increase

in J_{in}^{Na} (Wilson et al., 1994). This tight coupling between J_{Amm} and J_{in}^{Na} suggests that ammonium excretion occurs by Na^+/NH_4^+ exchange.

On the other hand, when the elevated rate of Na^+ uptake accompanying $(NH_4)_2SO_4$ infusion was inhibited by over 80% with amiloride, elevated J_{Amm} continued with only a slight decrement (23%; Wilson et al., 1994). Indeed, under a variety of conditions, 70–95% inhibition of J_{in}^{Na} by amiloride, by low external $[Na^+]$ or by extreme external pH resulted in only a modest reduction (10–30%) in J_{Amm} (Kirschner et al., 1973; Wright and Wood, 1985; Wright et al., 1989; Wilson et al., 1994; Wilkie and Wood, 1994). These results suggest that ammonia excretion and Na^+ uptake are at best only loosely coupled and that non-ionic diffusion of NH_3 may play an important role in ammonia excretion.

The driving force for NH_3 diffusion is the P_{NH_3} gradient. A positive correlation has been demonstrated between J_{Amm} and the P_{NH_3} gradient across the gill epithelium (Cameron and Heisler, 1983; Heisler, 1990; Wright et al., 1993). The gradient is regulated by the pH on either side of the gill membrane, the true external pH being that of the gill boundary layer. The excretion of both CO_2 (forming H^+ and HCO_3^- under carbonic anhydrase catalysis; Wright et al., 1986; Rahim et al., 1988) and H^+ will directly acidify the boundary layer. The pK' of NH_3/NH_4^+ dissociation is approximately 9.5. When NH_3 diffuses out across the epithelium, the availability of protons in the boundary layer water will instantaneously convert NH_3 to NH_4^+ ('diffusion trapping'; Randall and Wright, 1987). An increase in boundary layer pH would be predicted to decrease the P_{NH_3} gradient and therefore to decrease J_{Amm} . Experimentally increasing the buffering capacity of inspired (bulk) water nearly eliminated the pH difference between inspired and expired water (the latter thought to be representative of gill boundary layer water; Wright et al., 1989). At a bulk water pH of 8, this treatment decreased J_{Amm} by approximately 30%, an effect quantitatively similar to that of amiloride (Wright et al., 1989; Wilson et al., 1994). Indeed, once boundary layer pH had been clamped by buffering, amiloride no longer had any effect on J_{Amm} . Wilson et al. (1994) interpreted these results to indicate that the effect of amiloride on J_{Amm} was indirect, acting to block Na^+ -coupled H^+ excretion at the gill epithelium and, thereby, the portion of NH_3 diffusion associated with this portion of boundary layer acidification. Conversely, the stimulation of J_{in}^{Na} and J_{Amm} by $(NH_4)_2SO_4$ infusion was interpreted as the result of increased Na^+ -coupled H^+ excretion (and therefore of increased diffusion trapping), since this treatment leads to an internal acidosis (Cameron and Heisler, 1983; McDonald and Prior, 1988), which in itself can elevate J_{in}^{Na} and coupled H^+ excretion (Tang et al., 1988; Goss and Wood, 1991).

In the present study, we critically evaluated the linkage between ammonia excretion and Na^+ uptake in freshwater rainbow trout in four different ways. First, we separated the effects of NH_4^+ loading from internal acidosis by infusing trout for 24 h with either 70 mmol l^{-1} $(NH_4)_2SO_4$ or 140 mmol l^{-1}

NH_4HCO_3 ; the latter should not create internal acidosis (Claiborne and Evans, 1988). Second, we manipulated the rate of Na^+ uptake by sequentially increasing the external water $[Na^+]$ and observed the effects on rates of ammonia excretion for both infusions. These same experiments furnished a Michaelis–Menten analysis of the kinetics of Na^+ uptake (see Goss and Wood, 1991), thereby determining whether observed changes in J_{in}^{Na} in response to NH_4^+ loading were due to changes in K_m (transporter affinity), in $J_{in}^{Na_{max}}$ (transporter number or the availability of internal counterion) or in both. Third, we performed *in vitro* experiments to determine whether NH_4^+ activation of gill Na^+/K^+ -ATPase activity could explain the stimulation of J_{in}^{Na} accompanying NH_4^+ loading since, at least in the gulf toadfish *Opsanus beta*, NH_4^+ was reported to be more potent than K^+ in dephosphorylating branchial ATPase (Mallery, 1983).

Lastly, by buffering the external water with 5 mmol l^{-1} Hepes, we reduced or eliminated acidification of the gill boundary layer, thereby 'clamping' boundary water pH to bulk water pH. Thus, we were able to measure the effects of relatively small changes in gill boundary layer pH on both J_{Amm} and J_{in}^{Na} . In particular, we employed this approach to test the idea that increased J_{in}^{Na} accompanying NH_4^+ loading could be explained by a control system that normally regulates gill boundary layer pH at a constant level by variations in Na^+ -coupled H^+ excretion. If such a system were present, the alkalization of the boundary layer caused either by increased NH_3 efflux in response to NH_4^+ loading or by raising the boundary layer pH with buffer would both be predicted to activate the system, thereby elevating J_{in}^{Na} and net acidic equivalent efflux.

Materials and methods

Experimental animals

Rainbow trout [*Oncorhynchus mykiss* (Walbaum); 300–500 g; $N=51$] were obtained from Spring Valley Trout Farm, Petersburg, Ontario, Canada, and held in flowing dechlorinated Hamilton tapwater (moderately hard water: $[Na^+]=0.6\text{ mequiv l}^{-1}$, $[Cl^-]=0.8\text{ mequiv l}^{-1}$, $[Ca^{2+}]=2.0\text{ mequiv l}^{-1}$, $[Mg^{2+}]=0.3\text{ mequiv l}^{-1}$, $[K^+]=0.05\text{ mequiv l}^{-1}$, titration alkalinity 2.1 mequiv l^{-1} ; pH 8.0; hardness 140 mg l^{-1} as $CaCO_3$ equivalents; temperature 8–18°C). One week prior to experiments, animals were transferred to an acclimation tank at 15°C, and food was withheld to allow stabilisation of the endogenous fraction of waste nitrogen excretion (Fromm, 1963). For the infusion experiments, trout were anaesthetised (0.05 g l^{-1} MS-222; Sigma) and fitted with a dorsal aortic cannula filled with heparinised Cortland saline (Wolf, 1963; 50 i.u. ml^{-1} sodium heparin; Sigma) as described by Soivio et al. (1972). All fish, cannulated and uncannulated, were subsequently transferred to individual, darkened, well-aerated acrylic flux boxes (McDonald and Rogano, 1986) continuously supplied with flowing (0.51 min^{-1}), temperature-controlled ($15\pm 0.5^\circ\text{C}$), dechlorinated tapwater and allowed to recover for 48 h prior to experiments.

Infusion and kinetic experiments

The experimental protocol for the infusion and kinetic experiments covered 3 days. Day 1 consisted of a control flux measurement (60 min period) followed by the control kinetic series of flux measurements (over approximately 5 h). Day 2 consisted of the start of infusion, followed by flux measurements at 2, 4 and 8 h of infusion (30 min periods). Day 3 consisted of 24 h infusion flux measurement (30 min period), followed by the infusion kinetic series of flux measurements (again over approximately 5 h). J_{Amm} and $J_{\text{net}}^{\text{Na}}$ were measured during all flux periods, whereas unidirectional fluxes of Na⁺ ($J_{\text{in}}^{\text{Na}}$, $J_{\text{out}}^{\text{Na}}$) were measured only during the control and 24 h infusion periods. Titratable alkalinity fluxes were measured only in the NH₄HCO₃ infusion series, at control and 24 h periods.

At the start of each flux period, the boxes were closed and, for the control and 24 h infusion flux period, 0.04 MBq ml⁻¹ ²⁴Na⁺ (1 μCi ml⁻¹) stock solution was added. After a 10 min mixing period, water samples (30 ml) were taken at 0 min (initial) and 30 or 60 min (final) for analysis of water [Na⁺] ([Na⁺]_e), total ammonia concentration (T_{Amm}), titratable alkalinity (T_{Alk}) and ²⁴Na⁺ radioactivity. When operated as a closed system, the volume of each flux box was approximately 2500 ml. Between flux periods, the boxes were returned to the flow-through tapwater system.

At the end of each flux period, a 400 μl blood sample was taken *via* the dorsal aortic cannula into a 1 ml heparinized syringe, and this volume was immediately replaced with saline. In the case of ammonium salt infusions, particular care was taken to avoid contaminating the sampled blood with any infusate still present in the cannula.

The ammonium salt solutions, either 70 mmol l⁻¹ (NH₄)₂SO₄ or 140 mmol l⁻¹ NH₄HCO₃ (both adjusted to pH 7.8), were infused at a nominal rate of 3 ml kg⁻¹ h⁻¹ *via* the dorsal aortic cannula using a peristaltic pump (Gilson, Minipuls). This infusion rate was chosen so as to maximize the ammonia load (without exceeding normal plasma osmolality) while minimizing the volume load to an amount that fish are able to excrete by increasing their urine flow rate (see Goss and Wood, 1990b). The measured infusion rate for (NH₄)₂SO₄ was 2.63 ± 0.12 ml kg⁻¹ h⁻¹ (an NH₄⁺ load of 368.8 ± 17.1 μmol kg⁻¹ h⁻¹, $N=10$) and the infusion rate for NH₄HCO₃ was 3.13 ± 0.09 ml kg⁻¹ h⁻¹ (an NH₄⁺ load of 438.6 ± 12.5 μmol kg⁻¹ h⁻¹, $N=11$) (means ± S.E.M.).

For the kinetic experiments, the boxes were flushed three times with artificial tapwater containing zero NaCl but made up to duplicate the [Ca²⁺], [Mg²⁺], titratable alkalinity, pH and hardness of dechlorinated Hamilton tapwater (for details of synthesis, see Goss and Wood, 1990a). Exposure to this medium causes no change in transepithelial potential in rainbow trout, in contrast to distilled water (Goss and Wood, 1990a). The boxes were then closed and, to achieve the required [Na⁺]_e, a given volume of NaCl was added to the boxes from a common NaCl stock solution (1 mol l⁻¹) containing 0.18 MBq ml⁻¹ (5 μCi ml⁻¹) ²⁴Na⁺. After the stock solution had been added, a 10 min mixing period was allowed,

and water samples were then taken at 0 min (initial) and 30 min (final) for analysis of [Na⁺]_e, T_{Amm} and ²⁴Na⁺ radioactivity. The boxes were then flushed with the NaCl-free artificial tapwater, and the following flux period was started. The flushing between each flux period was performed to prevent ammonia from building up to toxic levels during infusion. Six flux periods of increasing water [NaCl] (nominally 50, 150, 300, 600, 1200 and 2400 μmol l⁻¹) were tested.

Na⁺/K⁺-ATPase activity experiments

Trout were placed in boxes provided with regular dechlorinated tapwater, allowed to settle for 2 days, then killed with an overdose of MS-222 buffered with NaHCO₃. The gills were perfused free of erythrocytes using a modified, phosphate-free Cortland saline (Perry et al., 1984). The filaments were then excised, frozen in liquid N₂ and stored at -70 °C.

Full details of the Na⁺/K⁺-ATPase assay procedure, which followed the method of Holliday (1985), with protein determination by the method of Lowry et al. (1951), are given by Morgan et al. (1997). The activity of gill Na⁺/K⁺-ATPase was calculated from the difference in the amount of organic phosphate liberated by gill homogenates incubated in two media: a 'plus K⁺ medium' containing optimal concentrations of all ions, therefore measuring the activities of all ATPases present, and a 'minus K⁺ plus ouabain medium' which measured the activity of all ATPases except Na⁺/K⁺-ATPase. The only modification was that [K⁺] was set to the normal extracellular (plasma) concentration of 5 mmol l⁻¹ (rather than 30 mmol l⁻¹) in the 'plus K⁺ medium', and various concentrations of ammonium were added to evaluate the potential of NH₄⁺ to stimulate activity under physiological conditions. Na⁺/K⁺-ATPase activity was measured in triplicate at NH₄⁺ concentrations of 0, 50, 150 and 500 μmol l⁻¹ chosen to cover the range of extracellular (plasma) concentrations measured in the control and ammonium-loaded trout of the present study. NH₄⁺ was added as (NH₄)₂SO₄.

Experiments with Hepes-buffered water

The experimental protocol for the Hepes-buffered water experiments was the following: first a control period (C_1) with normal tapwater, then three periods of exposure to Hepes buffer at different pH values, and finally a control period (C_2). For the first set of experiments, a sixth flux period, with a high water [K⁺] ([K⁺]_e) of 4.3 mmol l⁻¹ KCl (but 0 mmol l⁻¹ Hepes) was performed after the second control period as a check on the possible complicating effects of elevated [K⁺]_e during exposures to Hepes. To ensure that the increase or decrease in pH itself did not affect the results, the fish were divided in two groups. One group of fish started with a low-pH Hepes period and the second group of fish with a high-pH Hepes period.

At the start of each flux period, the boxes were closed, and for the Hepes flux periods a given volume of 100 mmol l⁻¹ Hepes (Sigma, Hepes free acid, adjusted to the appropriate pH) stock solution was added to give a final concentration of 5 mmol l⁻¹. After a 10 min mixing period, 0.04 MBq ml⁻¹ ²⁴Na⁺

(1 $\mu\text{Ci ml}^{-1}$) stock solution was added. After a further 10 min mixing period, water samples (30 ml) were taken at 0 (initial) and 60 min (final) for analysis of water $[\text{Na}^+]_e$, $[\text{K}^+]_e$, T_{Amm} , T_{Alk} and $^{24}\text{Na}^+$ radioactivity. Between each flux period, the boxes were returned for 30 min to the flowthrough tapwater system. These water changeovers caused no apparent disturbance to the animals.

For the first set of Hepes experiments, three stock solutions of Hepes buffer (100 mmol l^{-1}) were prepared, and the pH was adjusted to 6.9, 7.5 and 8.1 with KOH. For the second set of Hepes experiments, three stock solutions of Hepes buffer (100 mmol l^{-1}) were made, and the pH was adjusted to 7.7, 8.1 and 8.5 with NaOH. During the mixing period during which Hepes stock solution was added to dechlorinated tapwater at a ratio of 1:20, the water pH stabilized at values slightly different from the nominal pH to which the stock buffer solutions had been calibrated. All results are given as measured water pH. Titratable alkalinity fluxes were measured only in the first series, where buffer pH was adjusted using KOH.

Analytical techniques

Arterial pH (pHa) and plasma total CO_2 concentration (CCO_2) 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 Radiometer PHM 71 acid–base analyser. CCO_2 was measured on 50 μl samples of plasma using a total CO_2 analyser (Corning 965). The plasma was obtained by centrifugation (10000 g, 2 min) and the remainder was frozen in liquid N_2 and stored at -70°C until analysis of plasma T_{Amm} , $[\text{Na}^+]$ and $[\text{Cl}^-]$. Plasma T_{Amm} was measured enzymatically using the GLDH/NAD method (Sigma 170-UV), plasma $[\text{Na}^+]$ was measured by atomic absorption spectrophotometry (Varian AA1275) and plasma $[\text{Cl}^-]$ was measured by coulometric titration (Radiometer CMT10).

Water total $[\text{Na}^+]_e$ was determined by atomic absorption spectrophotometry (Varian AA1275). $^{24}\text{Na}^+$ radioactivity was counted in duplicate on 5 ml water samples in 10 ml of ACS fluor (Amersham) on a liquid scintillation counter (LKB Wallac 1217 Rackbeta). The $^{24}\text{Na}^+$ values (cts min^{-1}) were automatically corrected for radioactive decay by the program of the scintillation counter. Water T_{Amm} was measured in duplicate using a modified salicylate hypochlorite method (Verdouw et al., 1978). Titratable alkalinity (T_{Alk}) was determined by titration of 10 ml water samples to a fixed end point (pH 4.0) with 0.02 mol l^{-1} HCl, according to methods detailed in McDonald and Wood (1981).

Calculations

Plasma P_{CO_2} and $[\text{HCO}_3^-]$ were calculated from pHa and CCO_2 from the Henderson–Hasselbalch equation using α_{CO_2} and pK values from Boutilier et al. (1984).

Plasma P_{NH_3} and $[\text{NH}_4^+]$ were calculated from pHa and T_{Amm} using the Henderson–Hasselbalch equation with α_{NH_3} and pK' values from Cameron and Heisler (1983).

Net flux rates of total ammonia (J_{Amm} , $\mu\text{mol kg}^{-1} \text{h}^{-1}$) were calculated as:

$$J_{\text{Amm}} = (T_{\text{Amm},i} - T_{\text{Amm},f}) \times V / (t \times M), \quad (1)$$

where i and f refer to initial and final concentration ($\mu\text{mol l}^{-1}$), V is the water volume (l) in the box, t is the time elapsed (h) and M is the fish mass (kg).

Net Na^+ flux rates ($J_{\text{net}}^{\text{Na}}$, $\mu\text{mol kg}^{-1} \text{h}^{-1}$) were calculated using an equation analogous to equation 1. Unidirectional Na^+ influx rates ($J_{\text{in}}^{\text{Na}}$, $\mu\text{mol kg}^{-1} \text{h}^{-1}$) were calculated as:

$$J_{\text{in}}^{\text{Na}} = (R_i - R_f) \times V / (SA \times t \times M), \quad (2)$$

where R_i and R_f are initial and final radioactivities in water ($\text{cts min}^{-1} \text{l}^{-1}$), SA is the mean specific activity ($\text{cts min}^{-1} \mu\text{mol}^{-1}$) over the flux period in question, and other symbols are as in equation 1. Unidirectional Na^+ efflux rates ($J_{\text{out}}^{\text{Na}}$, $\mu\text{mol kg}^{-1} \text{h}^{-1}$) were calculated as:

$$J_{\text{out}}^{\text{Na}} = J_{\text{net}}^{\text{Na}} - J_{\text{in}}^{\text{Na}}. \quad (3)$$

Titratable acid flux rates ($\mu\text{mol kg}^{-1} \text{h}^{-1}$) were calculated from titration alkalinity measurements ($T_{\text{Alk},i}$ and $T_{\text{Alk},f}$; and reversing the initial and final values to achieve acid instead of base flux) in an equation analogous to equation 1. Net acidic equivalent flux rates ($\mu\text{mol kg}^{-1} \text{h}^{-1}$) were calculated as the sum, signs considered, of titratable acid flux rate and J_{Amm} (for details, see McDonald and Wood, 1981). For all fluxes, losses by the animal have a negative sign, gains a positive sign.

The effect on $J_{\text{in}}^{\text{Na}}$ of sequentially increasing $[\text{Na}^+]_e$ in the kinetic experiments showed distinctive saturation kinetics, and so transformation of the data to yield K_m and $J_{\text{max}}^{\text{Na}}$ values of the Michaelis–Menten equation was performed by Eadie–Hofstee regression analysis, as outlined in Goss and Wood (1990a).

All results are given as means \pm S.E.M. (where N is the number of animals). Water $[\text{Na}^+]_e$, $[\text{K}^+]_e$ and pH values are the means of the initial and final water samples. Within an infusion, buffer or Na^+/K^+ -ATPase series in which repeated measurements were made on the same fish or homogenate, a paired Student's t -test (two-tailed; $P < 0.05$) was used to determine the significance of changes, with each animal serving as its own control, and with the Bonferroni procedure for multiple comparisons (Nemenyi et al., 1977). For comparisons between different infusions or buffer series, a one-way analysis of variance (ANOVA) was performed followed by the least significant difference (LSD) test. A significance level of $P < 0.05$ was employed throughout.

Results

Responses to ammonium infusion

Infusion of 70 mmol l^{-1} $(\text{NH}_4)_2\text{SO}_4$ led to a threefold increase in arterial plasma T_{Amm} within the first 2 h, from approximately 50 to 150 $\mu\text{mol l}^{-1}$. This elevation persisted throughout the 24 h infusion period (Fig. 1A). Arterial plasma P_{NH_3} also increased threefold (Fig. 1A). Arterial pH decreased slightly, but not significantly (Fig. 1B). However, a small

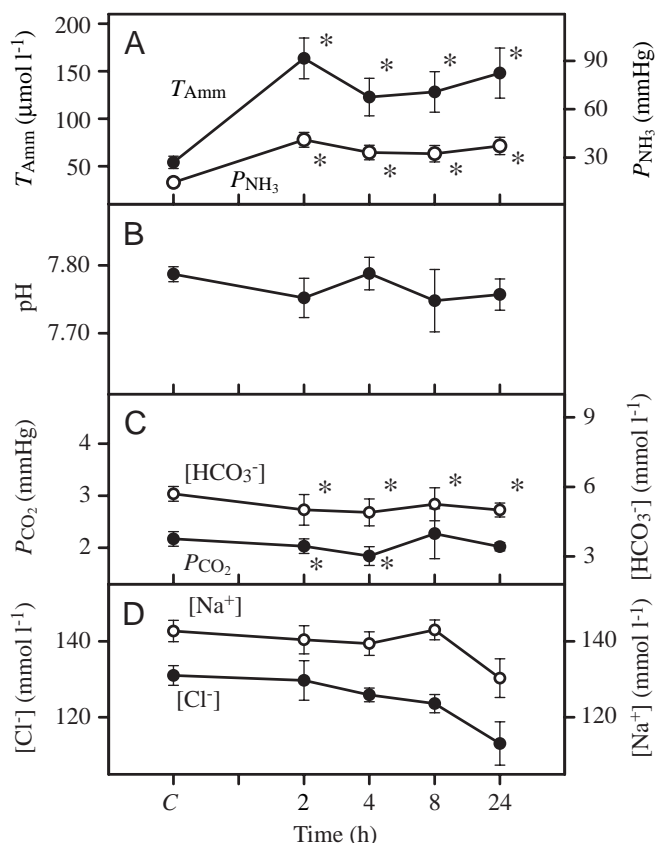


Fig. 1. (A) Plasma total ammonia concentration (T_{Amm}) and P_{NH_3} , (B) blood pH, (C) plasma P_{CO_2} and $[\text{HCO}_3^-]$, and (D) plasma $[\text{Cl}^-]$ and $[\text{Na}^+]$ in the arterial blood during a 24 h infusion of 70 mmol l^{-1} $(\text{NH}_4)_2\text{SO}_4$. *C* is the preinfusion control. Significant differences from the control value ($P < 0.05$) are indicated by an asterisk. Values are means \pm s.e.m. ($N = 7-12$). $1 \text{ mmHg} = 0.133 \text{ kPa}$.

significant decrease in plasma $[\text{HCO}_3^-]$ persisted for 24 h (Fig. 1C), indicating a small metabolic acidosis. There were no significant changes in plasma $[\text{Na}^+]$ and $[\text{Cl}^-]$ (Fig. 1D).

As expected, the $(\text{NH}_4)_2\text{SO}_4$ infusion increased the rate of ammonia excretion (J_{Amm} ; Fig. 2A). The fish reached the new steady-state rate, which was approximately 2.9 times higher than the control rate, within the first 2 h of the infusion, and this rate was maintained throughout the infusion period. This elevation of J_{Amm} was accompanied by a 2.7-fold increase in the rate of Na^+ uptake ($J_{\text{in}}^{\text{Na}}$) measured at 24 h of infusion. The absolute increase in J_{Amm} ($469 \mu\text{mol kg}^{-1} \text{ h}^{-1}$) was actually higher than the rate of NH_4^+ loading ($369 \mu\text{mol kg}^{-1} \text{ h}^{-1}$), while the absolute increase in $J_{\text{in}}^{\text{Na}}$ ($337 \mu\text{mol kg}^{-1} \text{ h}^{-1}$) was approximately 70 % of the absolute increase in J_{Amm} . Net Na^+ flux ($J_{\text{net}}^{\text{Na}}$) was positive (indicating uptake) throughout the infusion period, even though $J_{\text{out}}^{\text{Na}}$ increased significantly at 24 h (Fig. 2B).

Infusion of 140 mmol l^{-1} NH_4HCO_3 also increased arterial plasma T_{Amm} by threefold within the first 2 h, and consequently plasma P_{NH_3} again increased threefold (Fig. 3A). The infusion led to a significant metabolic alkalosis by 4 h reflected in a

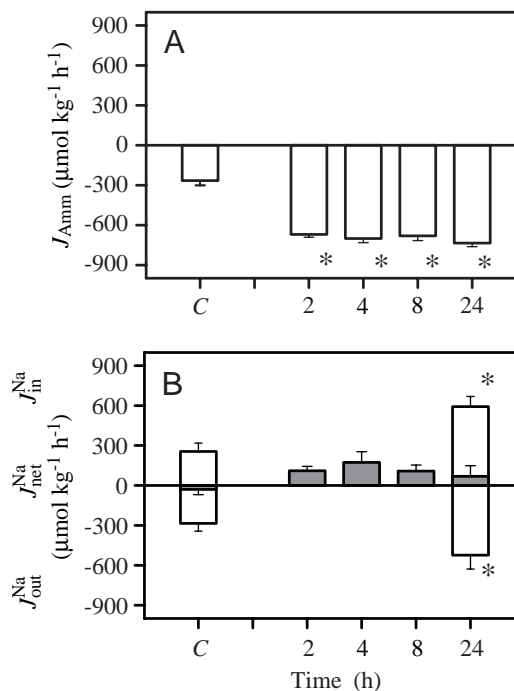


Fig. 2. (A) Total ammonia excretion rate ($\mu\text{mol kg}^{-1} \text{ h}^{-1}$, J_{Amm}) and (B) Na^+ influx ($J_{\text{in}}^{\text{Na}}$), net Na^+ flux ($J_{\text{net}}^{\text{Na}}$, shaded columns) and Na^+ efflux ($J_{\text{out}}^{\text{Na}}$) rates ($\mu\text{mol kg}^{-1} \text{ h}^{-1}$) during a 24 h infusion of 70 mmol l^{-1} $(\text{NH}_4)_2\text{SO}_4$. *C* is the preinfusion control. Significant differences from the control value ($P < 0.05$) are indicated by an asterisk. Values are means \pm s.e.m. ($N = 6$).

significant increase in pHa (Fig. 3B) and plasma $[\text{HCO}_3^-]$ (Fig. 3C). By 24 h, partial compensation had occurred such that the acid-base changes were no longer significant. There were no significant changes in plasma Na^+ or Cl^- concentrations (Fig. 3D).

The infusion of NH_4HCO_3 increased J_{Amm} (Fig. 4A). The pattern was the same as with $(\text{NH}_4)_2\text{SO}_4$ infusion, although the increase in J_{Amm} ($495 \mu\text{mol kg}^{-1} \text{ h}^{-1}$) was slightly higher, 3.3-fold, reflecting the slightly higher rate of NH_4^+ loading ($439 \mu\text{mol kg}^{-1} \text{ h}^{-1}$). In contrast to the $(\text{NH}_4)_2\text{SO}_4$ infusion, $J_{\text{in}}^{\text{Na}}$ increased only 1.7-fold, or approximately 30 % of the increase in J_{Amm} (Fig. 4B). $J_{\text{net}}^{\text{Na}}$ was again positive throughout the infusion period (Fig. 4B), but there was no change in $J_{\text{out}}^{\text{Na}}$. Titratable acid flux rates increased from $+46.1 \pm 55.7 \mu\text{mol kg}^{-1} \text{ h}^{-1}$ (control) to $+510.5 \pm 54.3 \mu\text{mol kg}^{-1} \text{ h}^{-1}$ (infusion, $N = 9$, $P < 0.05$), approximately equal to the increase in J_{Amm} , but there was no change in the net acidic equivalent flux between the control and 24 h infusion flux periods ($-223.1 \pm 63.0 \mu\text{mol kg}^{-1} \text{ h}^{-1}$ and $-234.1 \pm 26.9 \mu\text{mol kg}^{-1} \text{ h}^{-1}$ respectively, $N = 9$).

Na⁺ uptake kinetics: responses to increasing water Na⁺ concentrations

Under control conditions, sequential increases in water $[\text{Na}^+]_e$ from 50 to $2400 \mu\text{mol l}^{-1}$ (nominal concentration) caused a progressive threefold increase in $J_{\text{in}}^{\text{Na}}$ in a pattern that

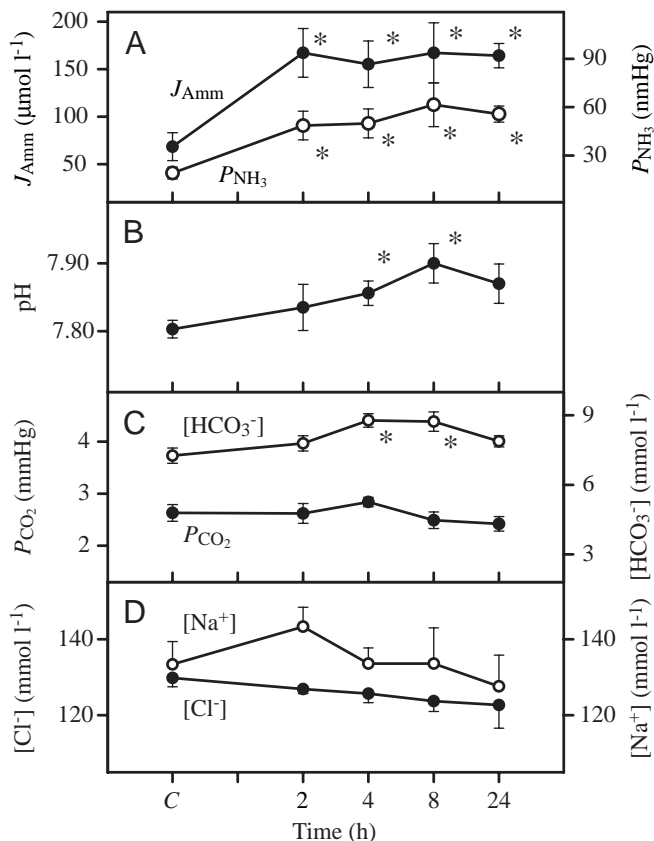


Fig. 3. (A) Plasma total ammonia concentration (T_{Amm}) and P_{NH_3} , (B) blood pH, (C) plasma P_{CO_2} and $[\text{HCO}_3^-]$, and (D) plasma $[\text{Cl}^-]$ and $[\text{Na}^+]$ in the arterial blood during a 24 h infusion of 140 mmol l^{-1} NH_4HCO_3 . *C* is the preinfusion control. Significant differences from the control value ($P < 0.05$) are indicated by an asterisk. Values are means \pm S.E.M. ($N = 7-11$). $1 \text{ mmHg} = 0.133 \text{ kPa}$.

exhibited typical Michaelis–Menten saturation kinetics (Figs 5A, 6A). Infusion of either $(\text{NH}_4)_2\text{SO}_4$ (Fig. 5A) or NH_4HCO_3 (Fig. 6A) for 24 h resulted in significant increases in $J_{\text{in}}^{\text{Na}}$ at virtually all concentrations of $[\text{Na}^+]_e$. In both cases, Eadie–Hofstee analysis revealed that the maximal rate of Na^+ uptake ($J_{\text{max}}^{\text{Na}}$) increased significantly by approximately 1.75-fold relative to control values (Table 1). The $J_{\text{max}}^{\text{Na}}$ of $(\text{NH}_4)_2\text{SO}_4$ infusion was slightly greater than the $J_{\text{max}}^{\text{Na}}$ of NH_4HCO_3 infusion, but the difference was not significant (Table 1). K_m tended to decrease with $(\text{NH}_4)_2\text{SO}_4$ infusion and to increase with NH_4HCO_3 infusion, but again the changes were not significant (Table 1). Nevertheless, the compound effect of these small differences in K_m and $J_{\text{max}}^{\text{Na}}$ between the two treatments quantitatively accounted for the much smaller $J_{\text{in}}^{\text{Na}}$ elevation at ambient water $[\text{Na}^+]_e$ observed with NH_4HCO_3 infusion (Fig. 4B) relative to $(\text{NH}_4)_2\text{SO}_4$ infusion (Fig. 2B).

Under control conditions, the threefold variation in $J_{\text{in}}^{\text{Na}}$ accompanying the acute elevation of $[\text{Na}^+]_e$ in the kinetic protocol had little effect on J_{Amm} (Figs 5B, 6B). Infusion of either ammonium salt greatly elevated J_{Amm} , but again there

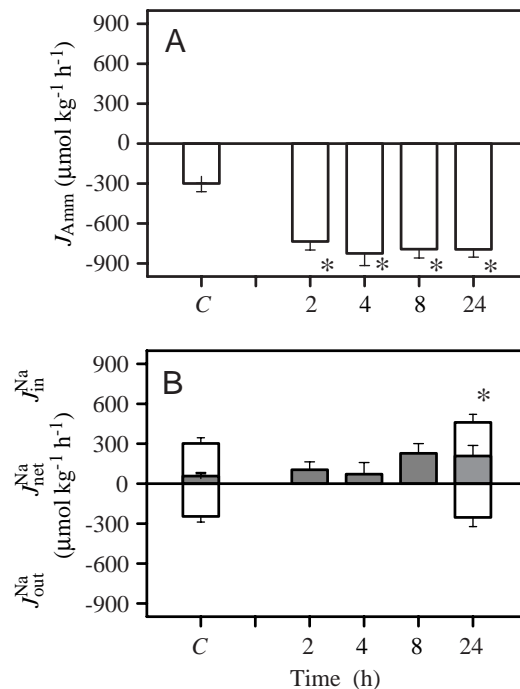


Fig. 4. (A) Total ammonia excretion rate ($\mu\text{mol kg}^{-1} \text{ h}^{-1}$, J_{Amm}) and (B) Na^+ influx ($J_{\text{in}}^{\text{Na}}$), net Na^+ flux ($J_{\text{net}}^{\text{Na}}$, shaded columns) and Na^+ efflux ($J_{\text{out}}^{\text{Na}}$) rates ($\mu\text{mol kg}^{-1} \text{ h}^{-1}$) during a 24 h infusion of 140 mmol l^{-1} NH_4HCO_3 . *C* is the preinfusion control. Significant differences from the control value ($P < 0.05$) are indicated by an asterisk. Values are means \pm S.E.M. ($N = 11$).

were only minor changes in J_{Amm} over the kinetic protocol. With the $(\text{NH}_4)_2\text{SO}_4$ infusion, J_{Amm} at the two lowest levels of $[\text{Na}^+]_e$ (50 and $150 \mu\text{mol l}^{-1}$) was approximately 10% lower than at higher $[\text{Na}^+]_e$ (Fig. 5B). With the NH_4HCO_3 infusion, the same trend was apparent but not statistically significant (Fig. 6B).

Influence of ammonium on Na^+/K^+ -ATPase activity

In the presence of the normal extracellular K^+ concentration (5 mmol l^{-1}), a change in NH_4^+ concentration from nominally

Table 1. Mean estimates of K_m and $J_{\text{max}}^{\text{Na}}$ obtained by one-substrate Michaelis–Menten analysis for the Na^+ transport system during control conditions and after 24 h of infusion of 70 mmol l^{-1} $(\text{NH}_4)_2\text{SO}_4$ or 140 mmol l^{-1} NH_4HCO_3

Treatment	<i>N</i>	K_m ($\mu\text{mol l}^{-1}$)	$J_{\text{max}}^{\text{Na}}$ ($\mu\text{mol kg}^{-1} \text{ h}^{-1}$)
$(\text{NH}_4)_2\text{SO}_4$	Control	10	111.6 ± 24.0
	Infusion	10	67.5 ± 10.4
NH_4HCO_3	Control	11	92.7 ± 21.3
	Infusion	11	107.4 ± 75.1

Values are means \pm S.E.M.

Significant differences from respective control values are indicated by an asterisk.

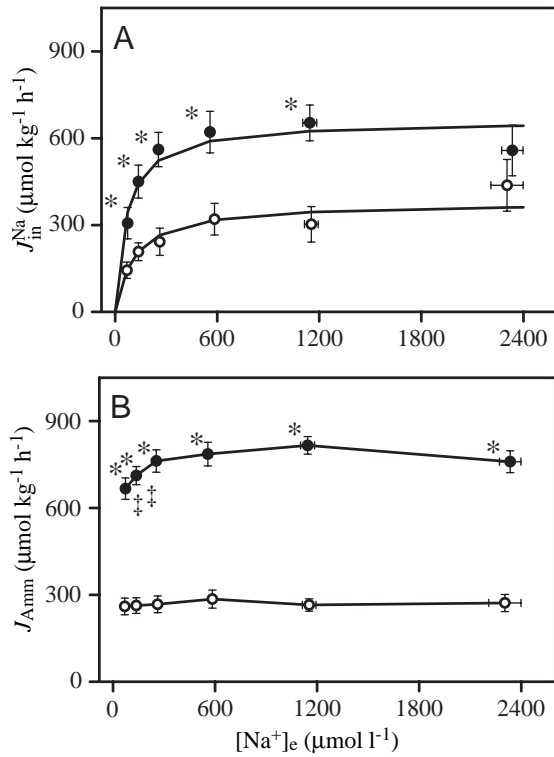


Fig. 5. (A) Na^+ influx rates (J_{in}^{Na}) and (B) total ammonia excretion rates (J_{Amm}) as a function of water $[\text{Na}^+]$ under control conditions (○) and after 24 h of infusion of $70 \text{ mmol l}^{-1} (\text{NH}_4)_2\text{SO}_4$ (●). The curves in A were drawn by Michaelis–Menten analysis from mean estimates of K_m and $J_{in}^{Na_{max}}$ obtained by Eadie–Hofstee regression analysis for all individual fish in the group. Significant differences ($P < 0.05$) between control and infusion values are indicated by an asterisk, and significant differences between successive means within an infusion group (in B only) are indicated by ‡. Values are means \pm S.E.M. ($N=10$).

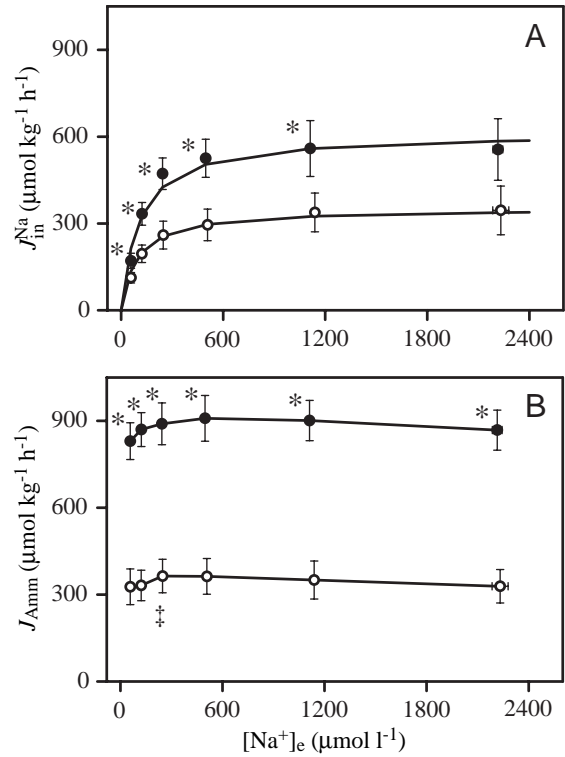


Fig. 6. (A) Na^+ influx rates (J_{in}^{Na}) and (B) total ammonia excretion rates (J_{Amm}) as a function of water $[\text{Na}^+]$ during control conditions (○) and after a 24 h infusion of $140 \text{ mmol l}^{-1} \text{NH}_4\text{HCO}_3$ (●). The curves in A were drawn by Michaelis–Menten analysis from mean estimates of K_m and $J_{in}^{Na_{max}}$ obtained by Eadie–Hofstee regression analysis for all individual fish in the group. Significant differences ($P < 0.05$) between control and infusion values are indicated by an asterisk, and significant differences between successive means within an infusion group (in B only) are indicated by ‡. Values are means \pm S.E.M. ($N=11$).

zero to $500 \mu\text{mol l}^{-1}$ had no significant effect on branchial Na^+/K^+ -ATPase activity measured *in vitro* (Fig. 7). These NH_4^+ levels bracketed the values of T_{Amm} observed *in vivo* in arterial blood plasma during the infusion experiments (cf. Figs 1A, 3A).

Influence of boundary layer buffering

In the experiments with HEPES-buffered water, manipulation of the pH of the 5 mmol l^{-1} HEPES solution necessitated substantial addition of base. In the first set of experiments, KOH was used to adjust the pH of the buffer system, because we wanted to avoid changes in $[\text{Na}^+]_e$ that would probably change J_{in}^{Na} (e.g. Figs 5A, 6A) just by a concentration effect. To control for the possibly disturbing effects of $[\text{K}^+]_e$, a flux test with 4.3 mmol l^{-1} KCl was carried out, slightly above the highest level actually measured in the exposure water during the buffer experiments (Table 2). The high $[\text{K}^+]_e$ had no effects on J_{Amm} [$111.6 \pm 15.5 \mu\text{mol kg}^{-1} \text{h}^{-1}$ (C_1) versus $97.6 \pm 13.1 \mu\text{mol kg}^{-1} \text{h}^{-1}$ (KCl), $N=12$]. However, J_{in}^{Na} decreased significantly [$124.9 \pm 23.4 \mu\text{mol kg}^{-1} \text{h}^{-1}$ (C_1) versus

Table 2. Water $[\text{Na}^+]_e$, $[\text{K}^+]_e$, and pH in the two sets of HEPES-buffering experiments

Treatment	N	$[\text{Na}^+]_e$ ($\mu\text{mol l}^{-1}$)	$[\text{K}^+]_e$ ($\mu\text{mol l}^{-1}$)	pH
KOH				
Control	12	510.5 ± 5.4	42.8 ± 0.4	8.23 ± 0.02
pH 6.9	12	511.8 ± 5.0	867.0 ± 20.7	7.41 ± 0.02
pH 7.5	12	514.2 ± 4.4	1989.0 ± 16.1	7.65 ± 0.02
pH 8.1	12	517.8 ± 5.7	3281.0 ± 28.1	8.03 ± 0.02
High $[\text{K}^+]$	12	529.4 ± 4.4	4301.0 ± 58.0	8.26 ± 0.02
NaOH				
Control	6	508.8 ± 7.2		8.09 ± 0.02
pH 7.7	6	3024.0 ± 25.6		7.75 ± 0.01
pH 8.1	6	3804.0 ± 37.9		7.98 ± 0.02
pH 8.5	6	4368.0 ± 25.9		8.17 ± 0.01

Values are means \pm S.E.M.

$73.7 \pm 26.9 \mu\text{mol kg}^{-1} \text{h}^{-1}$ (KCl), $N=12$, $P < 0.05$). Therefore, the second set of experiments was carried out with HEPES solutions

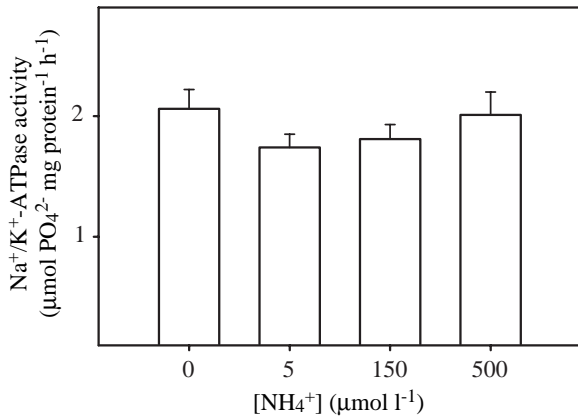


Fig. 7. Branchial Na⁺/K⁺-ATPase activities at four different concentrations of ammonium, added as (NH₄)₂SO₄, in the incubation medium. There were no significant differences. Values are means + s.e.m. (*N*=6).

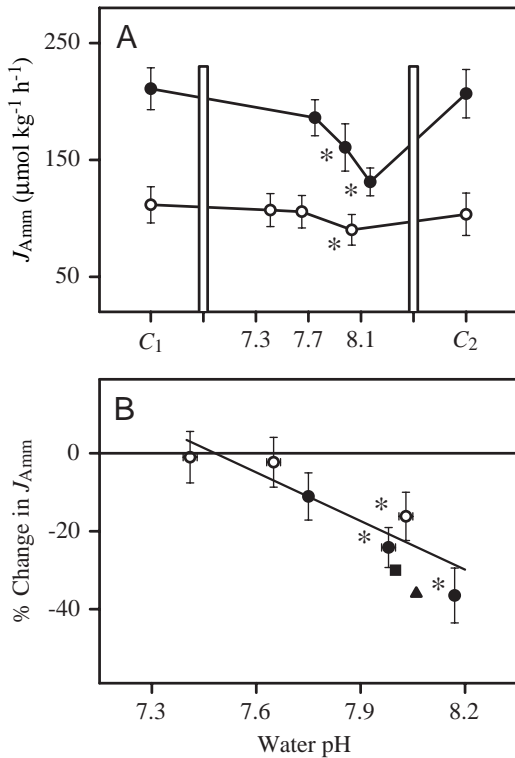


Fig. 8. (A) Total ammonia excretion rates (*J*_{Amm}) and (B) the percentage decrease in total ammonia excretion rates as a function of water pH in HEPES-buffered water; pH was adjusted with NaOH (●) and with KOH (○). C₁ and C₂ are the initial and final control flux periods in tapwater, pH 8.1–8.2. Significant differences from the C₁ value (*P*<0.05) are indicated by an asterisk. Values are means ± s.e.m. (*N*=6 for NaOH, *N*=12 for KOH). ■, value from Wright et al. (1989); ▲, value from Wilson et al. (1994). See text for details of the regression in B.

adjusted with NaOH. The measured increase in [Na⁺]_e in this series (Table 2) would have stimulated *J*_{in}^{Na} by approximately

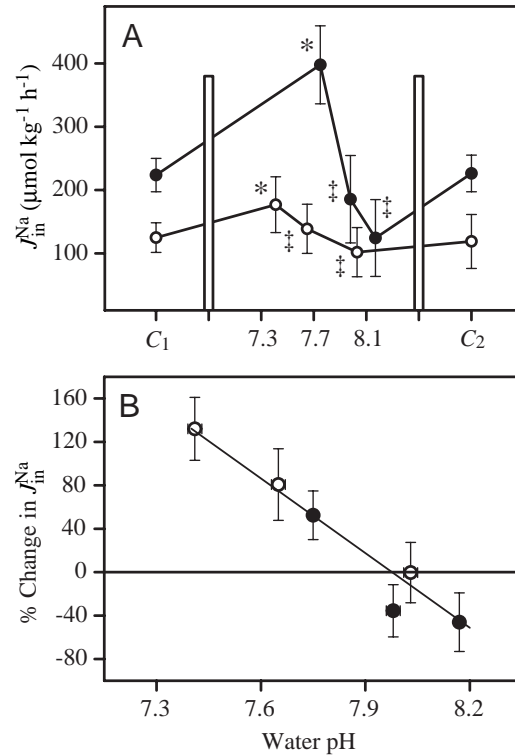


Fig. 9. (A) Na⁺ influx rates (*J*_{in}^{Na}) and (B) the percentage change in Na⁺ influx rates as a function of water pH in HEPES-buffered water, calculated as described in the text; pH was adjusted with NaOH (●) and with KOH (○). C₁ and C₂ are the initial and final control flux periods in tapwater, pH 8.1–8.2. Significant differences (*P*<0.05) from the C₁ value are indicated by an asterisk, and significant differences from the respective lowest pH group are indicated by ‡. Values are means ± s.e.m. (*N*=6 for NaOH, *N*=12 for KOH). See text for details of the regression in B.

15% on the basis of the control relationships in Figs 5A and 6A and Table 1.

The measured pH values in the water achieved and the nominal buffer pH values are reported in Table 2. At a gill boundary layer pH above 7.7, *J*_{Amm} decreased in both experimental series (Fig. 8A). Since control *J*_{Amm} values were different between the two sets of experiments, the *J*_{Amm} flux results were normalized as percentage changes from control values. This analysis revealed a linear correlation between gill boundary layer pH and the percentage decrease in *J*_{Amm} (Fig. 8B): %*J*_{Amm} = -(41.7 ± 9.5)pH + (312.1 ± 73.9) (*r* = -0.52, *N* = 54, *P* < 0.05).

In contrast to the *J*_{Amm} results, there was initially a significant increase in *J*_{in}^{Na} relative to C₁ at each of the lowest gill boundary layer pH values in both series (Fig. 9A). Thereafter, as boundary layer pH increased, *J*_{in}^{Na} decreased back to control levels or below. However, the control *J*_{in}^{Na} values were again different between the two sets of experiments; the first set was complicated by the potential 40% decrease in *J*_{in}^{Na} caused by [K⁺]_e alone, and the second set by the potential 15% increase caused by [Na⁺]_e alone. Therefore,

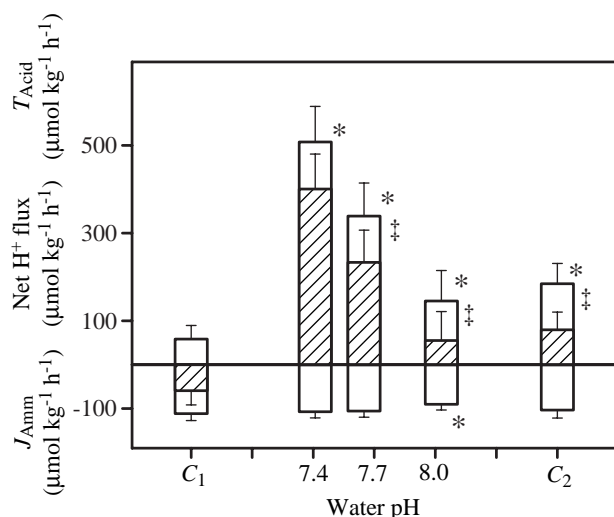


Fig. 10. Titrateable acid flux (T_{Acid}), net acid equivalent flux (shaded columns) and total ammonia excretion (J_{Amm}) rates ($\mu\text{mol kg}^{-1} \text{h}^{-1}$) as a function of water pH in Hepes-buffered water; pH was adjusted with KOH. Significant differences of both T_{Acid} and net H⁺ fluxes ($P < 0.05$) from C₁ value are indicated by an asterisk and from the lowest pH (7.4) group by ‡. Values are means \pm S.E.M. ($N=12$).

the results were normalized as a percentage change from the control values with these correction factors applied. The analysis revealed a linear correlation between gill boundary layer pH and the percentage change in J_{in}^{Na} (Fig. 9B): $\%J_{in}^{Na} = -(230.2 \pm 43.7)\text{pH} + (1836.4 \pm 340.8)$ ($r=0.59$, $N=54$, $P < 0.05$).

The titrateable acid flux rates were measured only in the first (KOH) series. The results demonstrated a strong dependency of titrateable acid flux on boundary layer pH, much greater than could be explained by the much smaller change in J_{Amm} (Fig. 10). The net acidic equivalent flux was very positive (i.e. net H⁺ uptake) at lower pH values and decreased greatly as boundary layer pH was raised from 7.41 to 8.03.

Discussion

(NH₄)₂SO₄ and NH₄HCO₃ infusion produced qualitatively similar branchial flux responses (increased J_{in}^{Na} , increased J_{Amm}), but opposite effects on plasma acid–base status. Whereas (NH₄)₂SO₄ loading produced a small metabolic acidosis, in accord with previous observations in fish (Cameron and Heisler, 1983; McDonald and Prior, 1988; Milligan et al., 1991; Wilson et al., 1994), NH₄HCO₃ loading caused metabolic alkalosis, again in accord with previous reports (Hillaby and Randall, 1979; Claiborne and Evans, 1988). The alkalosis must reflect the fact that at least a portion of the ammonia load from an NH₄HCO₃ infusion is excreted as NH₄⁺. Excretion of NH₃ alone leaves behind H⁺ and HCO₃⁻, forming CO₂, and is therefore neutral in terms of its effect on the acid–base status. In any event, these results provide clear evidence that the stimulation of J_{in}^{Na} accompanying an increase

in J_{Amm} cannot be explained solely as a consequence of an extracellular acidosis causing increased Na⁺-coupled H⁺ excretion (e.g. by an H⁺-ATPase/Na⁺ channel system or a Na⁺/H⁺ antiporter) at the gill epithelium.

Intracellular acid–base status in the gill epithelium was not measured, but it is highly unlikely that the observed responses are a consequence of intracellular acidosis. Exposure of cells to ammonium salts is the basis of the well-known ‘ammonium prepulse’ technique for studying intracellular pH regulation (Roos and Boron, 1981; Part and Wood, 1996). Because NH₃ diffuses into cells faster than NH₄⁺ or H⁺, intracellular alkalization rather than acidification of the gill cells would be predicted in response to continuous infusion of either (NH₄)₂SO₄ or NH₄HCO₃.

We therefore evaluated an alternative explanation – that the stimulation of J_{in}^{Na} by ammonium loading was due to a disturbance of acid–base status in the gill boundary layer water. Increased NH₃ flux across the gill, occurring in response to infusion of either ammonium salt, would be predicted to alkalinize the gill boundary layer. A Na⁺-coupled H⁺ extrusion mechanism present at the apical membrane of the branchial epithelium might be designed to regulate the pH of the boundary layer so as to maintain the diffusion-trapping mechanism for NH₃ excretion. Alkalinization of this layer might therefore stimulate J_{in}^{Na} . However, the results of the buffer experiments argue against this explanation. Experimentally raising the pH of the boundary layer by the use of Hepes-buffered water resulted in inhibition, rather than stimulation, of J_{in}^{Na} (Fig. 9). This finding is in accord with earlier work showing that alkalinization of the bulk water, albeit to more extreme levels, similarly inhibited J_{in}^{Na} (Wright and Wood, 1985; Wilkie and Wood, 1994).

The titrateable acidity flux measurements in the buffer experiments indicate that net acidic equivalent flux across the branchial epithelium is extremely sensitive to boundary layer pH (Fig. 10). However, inasmuch as the net uptake of acid decreased at higher pH as Na⁺ uptake decreased, the mechanism of this flux is unclear, as is the mechanism by which elevation of boundary layer pH inhibits J_{in}^{Na} . *A priori*, one might predict that Na⁺ uptake mechanisms coupled to H⁺ excretion mechanisms might run faster at a higher boundary layer pH, as the proton gradient from gill cell to water becomes more favourable. Clearly, this did not occur.

Since J_{Amm} fell as boundary layer pH increased (Fig. 8), the results of these Hepes experiments are in accord with those of Wright et al. (1989, 1993) and Wilson et al. (1994) and reinforce the importance of simple NH₃ diffusion as the dominant mechanism of ammonia excretion, at least under non-ammonium-loaded conditions. There was a close correlation between the increase in boundary layer pH and the inhibition of J_{Amm} . By interpolation to the buffer pH at which ‘control’ rates of J_{Amm} occurred (Fig. 8B), the normal boundary layer pH would appear to be approximately 7.5 at a bulk (inspired) water pH of 8.1–8.2. Somewhat higher values for boundary layer pH (7.9–8.0) are estimated if interpolation is made to the buffer pH at which J_{in}^{Na} (Fig. 9B) or net acidic

equivalent flux (Fig. 10) were at control levels. The pH values are in broad agreement with those of Wilson et al. (1994), who state that an acidification of the boundary layer by 0.4 pH units (from say pH 8.1 to pH 7.7) in Hamilton tapwater would be sufficient for net excretion of ammonia by NH_3 diffusion. Both our estimates indicate a lesser relative acidification of gill water than previous studies in which expired water pH has been measured (Playle and Wood, 1989; Lin and Randall, 1990). However, these earlier studies were performed in poorly buffered soft water, whereas Hamilton tapwater is quite well buffered (titration alkalinity $2.1 \text{ mequiv l}^{-1}$).

As yet another test of the general hypothesis that increased $J_{\text{in}}^{\text{Na}}$ was in some way linked to a stimulation of Na^+ -coupled H^+ excretion at the gills, we measured net acidic equivalent flux in the NH_4HCO_3 -loaded fish. Obviously, increased net acid excretion into the water would be expected in the acidotic $(\text{NH}_4)_2\text{SO}_4$ -infused trout, and indeed has been seen in other $(\text{NH}_4)_2\text{SO}_4$ loading studies (McDonald and Prior, 1988; Claiborne and Evans, 1988) and an HCl loading study (Goss and Wood, 1991). In the latter, both J_{Amm} and $J_{\text{in}}^{\text{Na}}$ increased markedly in concert with increased net acid excretion. However, *a priori*, an increase in net acid excretion would not be expected in the NH_4HCO_3 -loaded fish, unless a specific H^+ excretion mechanism were activated by the NH_4^+ load. The results revealed no significant change in net acidic equivalent excretion in these fish, again opposing the hypothesis of Na^+ -coupled H^+ excretion.

Taken together, these results therefore suggest that the stimulation of $J_{\text{in}}^{\text{Na}}$ accompanying infusions of ammonium salt is a direct response to NH_3 or NH_4^+ rather than an indirect response mediated through internal or external acid-base status. One possible explanation for this would be a stimulation of Na^+/K^+ -ATPase activity by elevated extracellular NH_4^+ levels acting at the K^+ site on the enzyme, because Mallery (1983) reported that NH_4^+ was, in fact, more effective at this site than K^+ itself. This basolaterally situated enzyme is thought to provide the major source of energy driving Na^+ influx, although not necessarily the only source (Lin and Randall, 1995). However, when tested at normal extracellular $[\text{K}^+]$ across a range of NH_4^+ concentrations spanning the values measured *in vivo* during infusion of the ammonium salts (up to $500 \mu\text{mol l}^{-1}$), $[\text{NH}_4^+]$ had no significant effect on trout gill Na^+/K^+ -ATPase activity *in vitro*. These results appear to be distinctly different from those of Mallery (1983) for the gulf toadfish *Opsanus beta*, in which NH_4^+ was extremely effective in this range, with a K_m of $100 \mu\text{mol l}^{-1}$ at normal extracellular K^+ levels.

A direct $\text{Na}^+/\text{NH}_4^+$ exchange pathway has long been proposed to have a major role in ammonia excretion (Krogh, 1939; Maetz and Garcia-Romeu, 1964; Maetz, 1972, 1973; Payan, 1978; Wright and Wood, 1985; McDonald and Prior, 1988; McDonald and Milligan, 1988), although more recent studies have challenged its very existence (Cameron and Heisler, 1983; Heisler, 1990; Wright et al., 1993; Wilson et al., 1994). If such a mechanism does actually exist, then the explanation for the stimulatory effect of the infusions of

ammonium salt on $J_{\text{in}}^{\text{Na}}$ could be the action of increased internal NH_4^+ levels in driving $\text{Na}^+/\text{NH}_4^+$ exchange. However, several pieces of evidence in the present study, as well as in the literature, argue against this conclusion, at least in terms of an obligatory $\text{Na}^+/\text{NH}_4^+$ mechanism. First, there were clear quantitative discrepancies between the stimulation of $J_{\text{in}}^{\text{Na}}$ and the stimulation of J_{Amm} . With $(\text{NH}_4)_2\text{SO}_4$ infusion, the increase in $J_{\text{in}}^{\text{Na}}$ was approximately 70% of the increase in J_{Amm} , whereas with NH_4HCO_3 infusion, it was only approximately 30% of the increase in J_{Amm} . Interestingly, in both cases, the increases in J_{Amm} were approximately 25% higher than the rate of NH_4^+ loading, perhaps reflecting increased endogenous ammonia production in response to experimental disturbance. Second, when $J_{\text{in}}^{\text{Na}}$ was increased by up to threefold by acutely altering water $[\text{Na}^+]_e$ in the kinetics experiments, negligible changes occurred in J_{Amm} under either the ammonium-loading treatment or the control condition. This finding is in accord with previous kinetic uptake experiments performed only under non-ammonium-loaded conditions (Goss and Wood, 1990a,b, 1991). Lastly, previous studies which have used amiloride, extreme water pH or low $[\text{Na}^+]_e$ to strongly inhibit $J_{\text{in}}^{\text{Na}}$ have shown at most only small effects on J_{Amm} (see Introduction). Clearly, if ammonium loading does stimulate $J_{\text{in}}^{\text{Na}}$ by direct $\text{Na}^+/\text{NH}_4^+$ exchange, the effect can be easily uncoupled, and enhanced J_{Amm} can continue in the absence of $J_{\text{in}}^{\text{Na}}$, presumably by NH_3 diffusion.

Traditionally, supporters of apical $\text{Na}^+/\text{NH}_4^+$ exchange in the gills of freshwater fish have interpreted this phenomenon as a substitution of NH_4^+ for H^+ in a Na^+/H^+ exchange mechanism (Maetz, 1972, 1973; Payan, 1978; Wright and Wood, 1985; McDonald and Prior, 1988). Part and Wood (1996) recently characterized a Na^+/H^+ antiporter responsible for the housekeeping of intracellular pH in freshwater trout gill cells, but interpreted it as a phenomenon that is normally located on the basolateral membrane, as in other cells, rather than on the apical membrane. Indeed, recent theoretical analyses (Avella and Bornacin, 1989; Potts, 1994; Lin and Randall, 1995; Kirschner, 1997) have favoured the H^+ -ATPase/ Na^+ channel system which is now known to be present in the gills of freshwater fish (Laurent et al., 1994; Lin and Randall, 1995; Sullivan et al., 1995, 1996) as the key Na^+ -uptake mechanism on the apical membrane. These same analyses have cast doubt on whether direct Na^+/H^+ exchange could even occur at the apical membrane because of the low values of $[\text{Na}^+]_e$ in most fresh waters and the traditionally low affinity (high K_m) of the antiporter for Na^+ . Thus, if true $\text{Na}^+/\text{NH}_4^+$ exchange does occur at the apical membrane, it is probably a specific mechanism and not *via* substitution at a Na^+/H^+ antiporter. However, a $\text{Na}^+/\text{NH}_4^+$ exchange (or $\text{Na}^+/\text{H}^+/\text{NH}_4^+$ -ATPase) situated on the basolateral membrane, as first proposed by Balm et al. (1988), could be the alternative answer and does not argue against an H^+ -ATPase/ Na^+ channel system located on the apical side.

While several studies have earlier demonstrated that $(\text{NH}_4)_2\text{SO}_4$ (or NH_4Cl) loading stimulates $J_{\text{in}}^{\text{Na}}$ in freshwater fish (Maetz and Garcia-Romeu, 1964; McDonald and Prior,

1988; Wilson et al., 1994), the present investigation is the first to show the same qualitative effect with NH₄HCO₃ infusion. It is also the first to examine the effect of either salt on the kinetics of Na⁺ influx. The smaller stimulation of J_{in}^{Na} by NH₄HCO₃ than by (NH₄)₂SO₄ infusion was probably a reflection of the metabolic alkalosis caused by the bicarbonate salt. Goss and Wood (1990b, 1991) demonstrated that the internal alkalosis caused by NaHCO₃ infusion inhibited J_{in}^{Na} by increasing the K_m (decreasing the affinity) and increasing the J_{max}^{Na} of the uptake mechanism. The tendencies, although non-significant, for both a greater K_m and a smaller increase in J_{max}^{Na} with NH₄HCO₃ infusion [relative to (NH₄)₂SO₄ loading] therefore explain the smaller overall stimulation of J_{in}^{Na} in these fish.

Nevertheless, the overriding effect for both ammonium salt infusions was an increase in J_{in}^{Na} due to an almost doubling of J_{max}^{Na} . This effect is very similar to that seen previously in freshwater trout loaded directly with H⁺ by HCl infusion (Goss and Wood, 1991) and is opposite to that seen in trout infused with NaHCO₃ (i.e. decreased J_{max}^{Na} ; Goss and Wood, 1990b, 1991). Traditionally, an increase in J_{max} has been interpreted as an increase in the number of available transport sites, but more recent 'two-substrate' analyses have demonstrated that it can also result from an increase in the availability of internal substrate ('counterions') for coupled exchangers (Wood and Goss, 1990; Goss and Wood, 1991; Potts, 1994). Goss et al. (1992, 1994b) concluded that both phenomena were important in the case of HCO₃⁻ loading, for increased J_{in}^{Cl} by Cl⁻/HCO₃⁻ exchange through gill chloride cells was partially explained by increased chloride cell fractional area (and therefore presumably transport site numbers) and partially by increased internal HCO₃⁻ availability.

The present results cannot discriminate between these two possibilities in explaining the increase in the J_{max} for Na⁺ uptake that occurs in response to ammonium loading. Indeed, the branchial cell type(s) responsible for Na⁺ uptake remains unidentified at present, although recent studies have pointed to pavement cells rather than chloride cells as the more likely site (Goss et al., 1994a, 1995; Morgan et al., 1994; Morgan and Potts, 1995; Sullivan et al., 1996). Changes in the internal morphology of pavement cells (increased density of mitochondria and apical microvilli) suggestive of increased transport activity have been seen when J_{in}^{Na} has been activated by internal acidosis (Laurent et al., 1994; Goss et al., 1994a, 1995). It would be instructive in future experiments to determine whether similar morphological changes occur during ammonium loading. With respect to internal substrate ('counterion') availability, if apical Na⁺/NH₄⁺ exchange really does occur, then certainly it could be stimulated by increased NH₄⁺ availability. Intracellular NH₄⁺ levels probably increase greatly in gill cells in response to systemic NH₄⁺ infusion because the intracellular pH is lower and the intracellular potential is negative relative to the plasma; both factors magnify intracellular changes in [NH₄⁺] relative to extracellular changes in fish tissues (Wood, 1993).

To conclude, the present experiments are consistent with a

dominant role for NH₃ diffusion as the mechanism of branchial ammonia excretion in freshwater rainbow trout. Nevertheless, they demonstrate that ammonium loading stimulates J_{in}^{Na} and that the effect is not solely a consequence of changes in internal acid-base status, or acid-base status in the external boundary layer water, which might activate a Na⁺-coupled H⁺ excretion mechanism at the gill epithelium. Furthermore, the effect is not due to NH₄⁺-stimulation of Na⁺/K⁺-ATPase activity. Rather, it appears that the stimulation of Na⁺ uptake is *via* a direct action of increased internal NH₃ or NH₄⁺ levels which increase the J_{max} of the Na⁺ transport mechanisms. The elevation of J_{Amm} is quantitatively different from the elevation of J_{in}^{Na} and is uncoupled from it when J_{in}^{Na} is acutely altered by changes in [Na⁺]_e. Activation of a non-obligatory basolateral Na⁺/NH₄⁺ exchange mechanism by ammonium salt infusion is a possible explanation, with the uncoupled portion of J_{Amm} occurring by NH₃ diffusion. In this regard, the proposal of Balm et al. (1988) that a plasma-membrane-bound NH₄⁺-activated Na⁺-ATPase occurs in the gills of freshwater fish, different from the traditional Na⁺/K⁺-ATPase studied in the present investigation, certainly deserves further consideration, especially in the light of previous reports that a Na⁺-dependent, K⁺-independent ATPase exists in the gills of freshwater trout (Pfeiler and Kirschner, 1972; Pfeiler, 1978).

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