Ammonia transport in cultured gill epithelium of freshwater rainbow trout: the importance of Rhesus glycoproteins and the presence of an apical Na^+/NH_4^+ exchange complex

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SUMMARY

The mechanisms of ammonia excretion at fish gills have been studied for decades but details remain unclear, with continuing debate on the relative importance of non-ionic NH_3 or ionic NH_4^+ permeation by various mechanisms. The presence of an apical Na^+/NH_4^+ exchanger has also been controversial. The present study utilized an *in vitro* cultured gill epithelium (double seeded insert, DSI) of freshwater rainbow trout as a model to investigate these issues. The relationship between basolateral ammonia concentration and efflux to apical freshwater was curvilinear, indicative of a saturable carrier-mediated component (K_m=66 μmol l^{-1}) superimposed on a large diffusive linear component. Pre-exposure to elevated ammonia (2000 μmol l^{-1}) and cortisol (1000 ng ml^{-1}) had synergistic effects on the ammonia permeability of DSI, with significantly increased Na^+ influx and positive correlations between ammonia efflux and Na^+ uptake. This increase in ammonia permeability was bidirectional. It could not be explained by changes in paracellular permeability as measured by [^{3}H]PEG-4000 flux. The mRNA expressions of Rhbg, Rhcg2, H^+-ATPase and Na^+/H^+ exchanger-2 (NHE-2) were up-regulated in DSI pre-exposed to ammonia and cortisol, CA-2 mRNA was down-regulated, and transepithelial potential became more negative. Bafilomycin (1 μmol l^{-1}), phenamil (10 μmol l^{-1}) and 5-(*N,N*-hexamethylene)amiloride (HMA, 10 μmol l^{-1}) applied to the apical solution significantly inhibited ammonia efflux, indicating that H^+-ATPase, Na^+ channel and NHE-2 pathways on the apical surface were involved in ammonia excretion. Apical amiloride (100 μmol l^{-1}) was similarly effective, while basolateral HMA was ineffective. Pre-treatment with apical freshwater low in [Na^+] caused increases in both Rhcg2 mRNA expression and ammonia efflux without change in paracellular permeability. These data suggest that Rhesus glycoproteins are important for ammonia transport in the freshwater trout gill, and may help to explain *in vivo* data where plasma ammonia stabilized at 50% below water levels during exposure to high environmental ammonia (~2300 μmol l^{-1}). We propose an apical 'Na$^+$/NH$_4^+$ exchange complex' consisting of several membrane transporters, while affirming the importance of non-ionic NH_3 diffusion in ammonia excretion across freshwater fish gills.

Key words: Rhesus glycoproteins, *Oncorhynchus mykiss*, gills, ammonia transport, sodium uptake, cortisol, H^+-ATPase, carbonic anhydrase, transepithelial potential.

INTRODUCTION

Ammonia (throughout this paper, the term 'ammonia' is used to refer to total $NH_3 + NH_4^+$, whereas these chemical symbols are used to refer to the two components) is a toxic end-product of protein catabolism which must be rapidly metabolized or excreted by all animals. Smith (Smith, 1929) and Krogh (Krogh, 1938) established that ammonia excretion and active Na^+ absorption, respectively, take place in gills of animals living in freshwater. Krogh (Krogh, 1939) further suggested that a Na^+/NH_4^+ exchange system exists on the apical membrane of the freshwater fish gill. Since then many studies have been carried out attempting to delineate the mechanisms involved in ammonia excretion and Na^+ uptake. However, these studies often led to conflicting conclusions: some supported the presence of a Na^+/NH_4^+ exchange system (Maetz and Garcia-Romeu, 1964; Maetz, 1973; Payan and Matty, 1975; Payan et al., 1975; Kerstetter and Keeler, 1976; Payan, 1978; Pressley et al., 1981; McDonald and Prior, 1988; McDonald and Milligan, 1988), others suggested that diffusive mechanisms predominated (Avella and Bornancin, 1989; Wilson et al., 1994; Wilkie and Wood, 1994; Wilkie et al., 1996), while still others argued that both mechanisms

were likely to be present (Wright and Wood, 1985; Heisler, 1990; Salama et al., 1999).

Certain observations supported the presence of a Na^+/NH_4^+ exchange system. For example, the amount of ammonia excreted and the amount of Na^+ taken up have been shown to be equivalent in some circumstances (Wright and Wood, 1985; McDonald and Prior, 1988; McDonald and Milligan, 1988; Salama et al., 1999). Furthermore, stimulation of ammonia excretion by ammonia loading resulted in stimulation of Na^+ uptake (Maetz and Garcia-Romeu, 1964; Wilson et al., 1994; Salama et al., 1999). In addition, amiloride, an inhibitor of Na^+ influx, caused a reduction in ammonia excretion (Kerstetter and Keeler, 1976; Payan, 1978; Wright and Wood, 1985; Yesaki and Iwama, 1992).

The controversy, however, continues because of other experimental results that suggested the absence of a Na^+/NH_4^+ exchange system. Using an isolated perfused head preparation (IPHP), Avella and Bornancin (Avella and Bornancin, 1989) observed that when ammonia excretion was increased by increasing the ammonia level in the gill perfusate from 0 to 1 mmol l^{-1}, Na^+ uptake was not affected. They also found that when the pH of the gill perfusate was reduced,

which increased the amount of NH$_4^+$ in the perfusate and should have stimulated ammonia excretion if a Na$^+$/NH$_4^+$ exchanger was present, ammonia excretion was actually decreased (Avella and Bornancin, 1989). Also, at high ambient pH (Wilkie and Wood, 1994) or in strongly buffered water (Wilson et al., 1994), amiloride did not affect ammonia excretion in rainbow trout.

Interpretations of experiments on this topic using live fish or the IPHP are often problematic because of the complexity of the gill architecture and the associated microenvironments. An alternative *in vitro* approach which avoids many of these problems is the gill epithelium of rainbow trout cultured on permeable filter supports ('inserts'); this preparation withstands apical freshwater exposure and allows experimental manipulation of the chemical composition (pH, ammonia level, Na$^+$ concentration, etc.) of solutions on either side of the epithelium (reviewed by Wood et al., 2002). Kelly and Wood (Kelly and Wood, 2001a) used this approach to investigate gill ammonia excretion; their principal conclusion was that ammonia efflux could not be explained by diffusion alone, and that carrier-mediated transport probably also played an important role. Our goal in the present study was to use the same *in vitro* approach to further address this issue of diffusive *versus* carrier-mediated transport, and to identify the latter mechanism(s). Specifically, we employed the double seeded insert (DSI) preparation developed by Fletcher and colleagues (Fletcher et al., 2000), which is a modification of the original preparation of Wood and Pärt (Wood and Pärt, 1997) so as to contain an appropriate amount of mitochondria-rich cells (15%) in addition to pavement cells, thereby better simulating the native epithelium of the trout gill. Cortisol was employed in some experiments because of its demonstrated ability to stimulate active Na$^+$ uptake in DSI preparations without altering Na$^+$,K$^+$-ATPase activity, an effect that may occur through apical channels or transporters (Kelly and Wood, 2001b; Zhou et al., 2003).

In initial experiments where basolateral ammonia concentration was varied, we found evidence of a saturable transport system superimposed on simple diffusion. Our particular focus then became the possible role of Rhesus (Rh) glycoproteins as ammonia carriers. The Rh proteins are now believed to be involved in ammonia transport in diverse organisms (Marini et al., 2000; Liu et al., 2000; Liu et al., 2001; Weihrauch et al., 2004). Our group has recently cloned several Rh proteins from the rainbow trout gill and shown that their mRNA expression responds to ammonia loading (Nawata et al., 2007; Nawata and Wood, 2008) and turns on during embryonic development in parallel with ammonia excretion (Hung et al., 2008). To elucidate the mechanism of ammonia excretion and to further investigate the role of Rh proteins in the rainbow trout gill, we pre-exposed the DSI preparations to cortisol and/or elevated ammonia. We found that mRNA expression of certain Rh proteins, as well as H$^+$-ATPase and Na$^+$/H$^+$ exchanger-2 (NHE-2) were induced, and that the ammonia permeability of the DSI was also increased, while carbonic anhydrase (CA-2) mRNA was down-regulated. With the help of specific inhibitors of the Na$^+$ channel (phenamil), Na$^+$/H$^+$ exchanger [5-(N,N-hexamethylene)amiloride] and H$^+$-ATPase (bafilomycin), we illuminated the mechanism of ammonia transport across DSI epithelia. We also performed an *in vivo* experiment to validate the high level of ammonia exposure used in our *in vitro* experiments, which revealed an interesting finding of reversed blood-to-water ammonia gradients and high plasma cortisol. Overall, our results affirm the importance of non-ionic NH$_3$ diffusion, and suggest that an effective 'Na$^+$/NH$_4^+$ exchange complex' indeed exists on the apical surface of the gill epithelium. However, this complex is probably made up of several membrane transporters, instead of a single Na$^+$/NH$_4^+$ exchanger.

MATERIALS AND METHODS
Preparation of cultured gill epithelia
Rainbow trout (*Oncorhynchus mykiss*; 90–180 g), obtained from Humber Springs Trout Hatchery, Ontario, Canada, were held in dechlorinated running freshwater (Hamilton tapwater from Lake Ontario with composition: [Na$^+$]=0.65, [Cl$^-$]=0.70, [Ca^{2+}]=0.80, [Mg^{2+}]=0.30, [K$^+$]=0.05 mmol l^{-1}, pH 7.8–8.0). Temperature was maintained at 12–14°C and a light–dark cycle of 12 h:12 h was maintained. All procedures used were approved by the McMaster University Animal Research Ethics Board and are in accordance with the Guidelines of the Canadian Council on Animal Care.

Gill cell isolation was conducted in a laminar flow hood using sterile techniques. Procedures used for gill cell isolation were adapted from Kelly et al. (Kelly et al., 2000) and Zhou et al. (Zhou et al., 2003). Briefly, trout were killed by MS-222 anaesthesia (Sigma-Aldrich, St Louis, MO, USA) followed by cephalic concussion. Gill cells were obtained from excised gill filaments by two consecutive cycles of 8 min tryptic digestion [Gibco Life Technologies, Long Island, NY, USA, 0.05% trypsin in phosphate-buffered saline (PBS) with 5.5 mmol l^{-1} EDTA] at room temperature. Gill cells were resuspended in cold culture medium [Leibovitz's L-15, supplemented with 2 mmol l^{-1} glutamine, 5% fetal bovine serum (FBS), 100 IU ml^{-1} penicillin, 100 μg ml^{-1} streptomycin] and seeded on the apical side of Falcon cell inserts (Cyclopore polyethylene terephthalate filters, Becton-Dickinson, Franklin Lakes, NJ, USA; pore density 1.6×10^6 pores cm^{-2}, pore size 0.45 μm, growth surface 0.9 cm^2) at a density of 2.2×10^6 cells cm^{-2}. The total volume of culture medium initially was 0.8 ml on the apical side and 1.0 ml on the basolateral side. At 24 h after seeding, each insert was rinsed with culture medium to remove mucus and unattached cells. Gill cells freshly prepared from a second fish were seeded onto the cell layer of each insert at a density of 2.2×10^6 cells cm^{-2}. After another 24 h, mucus and unattached cells were again removed by rinsing with culture medium, and 1.5 ml and 2.0 ml of culture medium were then added to the apical and basolateral side, respectively. The culture medium was replaced every second day. The cells were kept in an incubator at 18°C. Transepithelial resistance (TER) of each DSI was monitored daily (see below).

Series 1: concentration dependence of ammonia flux across DSI preparations
The objective of these tests was to determine whether ammonia flux showed any indication of saturation kinetics, which would indicate that a transporter or channel was involved. When the TER of DSI stabilized (typically after 6–9 days of culture), asymmetrical preparations were created by replacing the apical culture medium with UV-sterilized freshwater. After 3 h of freshwater treatment, the apical freshwater was replaced with new freshwater and the basolateral solution was replaced with culture medium containing different concentrations of ammonia. In three separate experiments, ranges of 14–237 μmol l^{-1}, 370–2270 μmol l^{-1} and 243–17,768 μmol l^{-1} (measured concentrations) were used. For the lowest ammonia range, PBS with 5% FBS was used instead of L-15 culture medium because L-15 culture medium typically contains 200–300 μmol l^{-1} ammonia. Ammonia was added as NH$_4$Cl, and the pH was maintained at 7.4. Samples of apical freshwater were collected at the beginning and the end of the 3 h flux period for determination of total ammonia concentration, thereby yielding measurements of ammonia flux.

Series 2: cortisol and high [ammonia] pre-exposure
For DSI that received cortisol treatment, cortisol (hydrocortisone 21-hemisuccinate, Sigma Aldrich) was included in the basolateral

solution at $1000\,\mathrm{ng\,ml^{-1}}$ from day 2 of culturing. This dose was chosen based on previous experience with DSI preparations (Kelly and Wood, 2001b). When the TER of DSI stabilized at 6–9 days of culture, asymmetrical DSI were created by changing the apical culture medium to UV-sterilized freshwater (composition identical to acclimation freshwater). After 3 h of freshwater treatment, DSI were exposed to conditions described in Table 1 for 20 h ('pre-exposure'). At the end of the 20 h pre-exposure, all the solutions were removed. The apical side was rinsed with UV-sterilized freshwater and the basolateral side with PBS, in order to remove any residual NH_4Cl. Freshwater was then placed on the apical surface and culture medium containing $650–750\,\mathrm{\mu mol\,l^{-1}}$ total ammonia (see below) and $1000\,\mathrm{ng\,ml^{-1}}$ cortisol (if appropriate) was placed on the basolateral surface. Ammonia flux was then measured over a 3 h period (see below).

For symmetrical DSI experiments, the apical side was never changed to freshwater and the pre-exposure conditions are described in Table 1. At the end of the 20 h pre-exposure, all the solutions were removed and both the apical and basolateral sides were rinsed with PBS in order to remove any residual NH_4Cl. Culture medium containing $650–750\,\mathrm{\mu mol\,l^{-1}}$ total ammonia (see below) and $1000\,\mathrm{ng\,ml^{-1}}$ cortisol (if appropriate) was placed on the basolateral surface, and unamended culture medium was placed on the apical surface. Ammonia flux was then measured over a 3 h period.

Series 3: transporter inhibitor studies

In order to further investigate the mechanism of ammonia transport, $1\,\mathrm{\mu mol\,l^{-1}}$ bafilomycin, $10\,\mathrm{\mu mol\,l^{-1}}$ phenamil and $10\,\mathrm{\mu mol\,l^{-1}}$ HMA were used to specifically inhibit H^+-ATPase, Na^+ channels and Na^+/H^+ exchangers (NHE), respectively. Amiloride ($100\,\mathrm{\mu mol\,l^{-1}}$), a general blocker of both Na^+ channels and Na^+/H^+ exchangers, was also tested because of its common use in previous *in vivo* studies. These drugs were dissolved in dimethyl sulphoxide (DMSO; maximum concentration $\leq 0.05\%$ in the final test solutions) and then added to the apical solution of asymmetrical DSI prior to the standard 3 h ammonia flux test, which was performed exactly as in Series 2. The same concentration of DMSO was used in control experiments. To test whether NHE was involved in basolateral ammonia transport, $10\,\mathrm{\mu mol\,l^{-1}}$ HMA was added to the basolateral solution. In all of these tests, DSI preparations were first pre-exposed to apical freshwater for 3 h followed by a subsequent 20 h high ammonia treatment ($2000\,\mathrm{\mu mol\,l^{-1}}\,NH_4Cl$) on both surfaces (apical freshwater, basolateral culture medium) as in Series 2. Furthermore all preparations in this series had $1000\,\mathrm{ng\,ml^{-1}}$ cortisol in the basolateral solution throughout the experiment. All drugs were from Sigma Aldrich.

Table 1. Pre-exposure conditions for DSI in Series 2

Conditions	Apical solutions	Basolateral solutions
Asymmetrical DSI		
Control	FW	CM
Amm	FW, Amm	CM, Amm
Cort	FW	CM, Cort
Amm+Cort	FW, Amm	CM, Amm+Cort
Symmetrical DSI		
Control	CM	CM
Amm	CM, Amm	CM, Amm
Cort	CM	CM, Cort
Amm+Cort	CM, Amm	CM, Amm+Cort

DSI, double seeded insert; FW, freshwater; CM, culture medium; Amm, $2000\,\mathrm{\mu mol\,l^{-1}}\,NH_4Cl$; Cort, $1000\,\mathrm{ng\,ml^{-1}}$ cortisol.

Series 4: pre-exposure to low [Na⁺] freshwater

Na^+-free water was utilized to investigate whether a prolonged pre-exposure to a low sodium level in the apical freshwater would alter the ammonia permeability of asymmetrical DSI epithelia. The synthetic Na^+-free Hamilton tapwater used was prepared according to the formula in Goss and Wood (Goss and Wood, 1990). Asymmetrical DSI preparations were created as described in Series 2. After the 3 h apical freshwater treatment, the apical freshwater was changed to Na^+-free water. Throughout the subsequent 20 h period, the apical solution was changed every hour in order to keep the apical Na^+ level low. The parallel control asymmetrical DSI received the same treatment, except that the apical solution used was standard freshwater. The measured Na^+ levels in the apical solutions were $660–770\,\mathrm{\mu mol\,l^{-1}}$ and $0–84\,\mathrm{\mu mol\,l^{-1}}$ for control and Na^+-free water, respectively, as determined by atomic absorption spectrophotometry (Varian AA220 FS, Mulgrave, Australia). All DSI epithelia in this series had $1000\,\mathrm{ng\,ml^{-1}}$ cortisol in the basolateral solution throughout the experiment. After the 20 h pre-exposure, ammonia flux was measured for a 3 h period, during which standard freshwater ($[Na^+]=660–770\,\mathrm{\mu mol\,l^{-1}}$) was present on the apical surface and standard total ammonia concentrations of $650–750\,\mathrm{\mu mol\,l^{-1}}$ were present in the basolateral culture medium.

Series 5: evaluation of the effect of pre-exposure to high [ammonia] on active Na⁺ uptake

To test whether ammonia pre-exposure would stimulate active Na^+ uptake, symmetrical DSI preparations were pre-exposed to $2000\,\mathrm{\mu mol\,l^{-1}}\,NH_4Cl$ (both apical and basolateral sides) for 20 h, followed by simultaneous measurement of ammonia efflux and Na^+ influx for 6 h. For the 6 h flux period, the apical solution was changed to freshwater containing $1\,\mathrm{\mu Ci}$ of radioactive ^{22}Na (Perkin Elmer, Boston, MA, USA), and the basolateral solution was changed to culture medium containing $650–770\,\mathrm{\mu mol\,l^{-1}}\,NH_4Cl$. The parallel control DSI epithelia received the same pre-treatment, but without elevated ammonia. All DSI preparations in this series had $1000\,\mathrm{ng\,ml^{-1}}$ cortisol in the basolateral solution throughout the experiment.

Na^+ influx ($J_{in}^{Na^+}$, apical to basolateral flux) was calculated according to:

$$J_{in}^{Na^+} = \Delta[Na*]_{Bl} \times \frac{1}{SA_{Ap}} \times \frac{Volume_{Bl}}{Time \times Area},\tag{1}$$

where $\Delta[Na*]_{Bl}$ is the change in radioactivity due to ^{22}Na on the basolateral side and SA_{Ap} is the mean specific activity on the apical side.

Na^+ net flux ($J_{net}^{Na^+}$) was calculated as:

$$J_{net}^{Na^+} = \Delta[\Sigma Na^+]_{Ap} \times \frac{Volume_{Ap}}{Time \times Area},\tag{2}$$

where $\Delta[\Sigma Na^+]_{Ap}$ is the change in total sodium concentration on the apical side.

Na^+ efflux ($J_{out}^{Na^+}$) was estimated indirectly:

$$J_{out}^{Na^+} = J_{net}^{Na^+} - J_{in}^{Na^+}.\tag{3}$$

The criterion used to detect the presence of active transport was disagreement of the measured flux ratio (J_{in}/J_{out}) with that predicted by the Ussing flux ratio:

$$\frac{J_{in}}{J_{out}} = \frac{A_{Ap}}{A_{Bl}} e^{(zFVIRT)},\tag{4}$$

where A_{Ap} and A_{Bl} are the activities of Na$^+$ on the apical and basolateral solution, z is the valency, V is the measured transepithelial potential (TEP), and F, R and T have their usual thermodynamic meanings. Using a Na$^+$-specific microelectrode, A^{Na} was measured to be 75% of total Na$^+$ concentration in L-15 media. For freshwater, the ion activity was taken as equal to the measured concentrations, in view of the very low concentrations ($<1\,\mathrm{mmol\,l^{-1}}$). ^{22}Na radioactivity measurements were made by gamma-counting using a Wallac 1480 Wizard 3 gamma counter (Perkin-Elmer, Toronto, ON, Canada).

Series 6: *in vivo* responses to high environmental ammonia exposure

To address concerns that the $2000\,\mathrm{\mu mol\,l^{-1}}$ NH$_4$Cl exposure used in the preceding *in vitro* experiments could be toxic, live trout were monitored for 7 days of exposure to this nominal level of NH$_4$Cl in the standard test and acclimation water, dechlorinated Hamilton tapwater (composition as above). The addition of high NH$_4$Cl lowered water pH from 7.8–8.0 to 7.1–7.4, such that NH$_4^+$ constituted about 99.5% of the total, and NH$_3$ constituted 0.5%, yielding a P_{NH_3} of approximately $220\,\mathrm{\mu torr}$ (where $1\,\mathrm{torr} \approx 133\,\mathrm{Pa}$) in the water [based on constants provided by Cameron and Heisler (Cameron and Heisler, 1983)]. This experiment also provided an opportunity to measure *in vivo* plasma levels of total ammonia and cortisol during the exposure. Trout were fasted for 3 days before and throughout the exposure. A control group ($N=10$) were fasted for the same length of time in normal tapwater without added ammonia. For high external ammonia exposure, water flow to the 400 l tank was stopped while aeration was maintained, and sufficient NH$_4$Cl was added to the tank to bring the total ammonia to a nominal concentration of $2000\,\mathrm{\mu mol\,l^{-1}}$. Half of the water was exchanged every day, using fresh pre-dosed water, and water samples were taken before and after the water change to check the water ammonia level. At 20 h ($N=16$) and 7 days ($N=10$), fish were removed individually to a bucket containing $0.5\,\mathrm{mg\,l^{-1}}$ MS-222 in the exposure water neutralized back to the original pH, and a 0.5 ml blood sample was quickly drawn by blind caudal puncture into a sodium-heparinized syringe. A simultaneous water sample was also taken directly from the bucket. Plasma was separated by rapid centrifugation and frozen in liquid N$_2$. Plasma and water samples were stored at –80°C for later analysis of ammonia and cortisol levels.

Electrophysiological measurements

TER was monitored using STX-2 chopstick electrodes connected to a custom-modified EVOM epithelial voltohmmeter (World Precision Instruments, Sarasota, FL, USA). TEP was measured using agar–salt bridges ($3\,\mathrm{mol\,l^{-1}}$ KCl in 3% agar) connected through Ag/AgCl electrodes (World Precision Instruments) to a DVC-3 preamplifier (World Precision Instruments) that was in turn connected to DVC-1000 dual voltage clamp (World Precision Instruments). All TEP measurements were expressed relative to the apical side as 0 mV. Corrections for junction potential and for blank TER of vacant inserts were performed as described by Kelly and Wood (Kelly and Wood, 2001a; Kelly and Wood, 2001b).

Ammonia flux

To create ammonia flux from the basolateral to the apical direction (efflux), $500\,\mathrm{\mu mol\,l^{-1}}$ NH$_4$Cl was added to the basolateral solution (pH 7.4). Together with the ammonia already present in L-15 medium, the total ammonia level in the basolateral solution was 650–$770\,\mathrm{\mu mol\,l^{-1}}$. If the DSI had been pre-treated with cortisol,

$1000\,\mathrm{ng\,ml^{-1}}$ cortisol was also added to the basolateral solution. The apical solution was either freshwater, pH 8.0 (asymmetrical DSI), or culture medium, pH 7.4 (symmetrical DSI). Aliquots of the apical solution were collected at the beginning and at the end of the 3 h flux period. The collected solution was frozen at –20°C until analysis. To create ammonia flux from the apical to the basolateral direction (influx), $500\,\mathrm{\mu mol\,l^{-1}}$ NH$_4$Cl was added to the apical solution. Aliquots of the basolateral solution were collected at the beginning and at the end of the 3 h flux period.

Ammonia assays

The colorimetric method of Ivancic and Degobbis (Ivancic and Degobbis, 1984) was used to measure ammonia levels in freshwater in the *in vitro* experiments. Freshly prepared NH$_4$Cl solutions were used as standards. Because drugs (e.g. phenamil) and DMSO altered colour generation in the assay, we found it essential to use the exact experimental solution (containing DMSO or DMSO+drug) to prepare the NH$_4$Cl standards for each particular experimental treatment. Ammonia levels in the culture medium were measured enzymatically (L-glutamate dehydrogenase; ammonia reagent no. 85446, Raichem, San Diego, CA, USA); tests revealed no drug or DMSO interferences with this assay. In the *in vivo* exposures, the enzymatic assay was used for both water and plasma samples, to ensure direct comparability of measurements for blood-to-water gradients.

Cortisol assays

Plasma cortisol levels were measured on $25\,\mathrm{\mu l}$ samples by radioimmunoassay (cortisol ^{125}I RIA kit, DiaSorin, Stillwater, MN, USA) and values are reported in ng ml^{-1}.

[^{3}H]Polyethylene glycol-4000 permeability

In Series 2 and Series 4, the permeability of DSI epithelia to the paracellular permeability marker, [^{3}H]polyethylene glycol-4000 (PEG-4000; molecular mass 4000 Da; New England Nuclear-Dupont) was measured using methods previously described (Gilmour et al., 1998; Wood et al., 1998). Permeability was determined in the efflux direction (basolateral to apical) after the addition of $1\,\mathrm{\mu Ci}$ [^{3}H]PEG-4000 to the basolateral culture medium. The appearance of [^{3}H]PEG-4000 radioactivity in the apical solution was determined at the end of the 3 h flux period.

[^{3}H]PEG-4000 permeability (P, in cm s^{-1}) was calculated according to:

$$P = \frac{\Delta[PEG^*]_{Ap} \times \mathrm{Volume}_{Ap}}{[PEG^*]_{Bl} \times \mathrm{Time} \times 3600 \times \mathrm{Area}}, \qquad (5)$$

where $\Delta[PEG^*]_{Ap}$ is the change in radioactivity due to [^{3}H]PEG-4000 on the apical side, $[PEG^*]_{Bl}$ is the mean radioactivity on the basolateral side, 3600 converts hours to seconds, and Area defines the area of epithelial growth in the insert (0.9 cm^2). [^{3}H]PEG-4000 radioactivity measurements were made by adding samples to 10 ml of Ultima Gold AB scintillation cocktail (Perkin-Elmer); samples were counted in a liquid scintillation counter (Tri-Carb 2900TR, Perkin-Elmer). Tests demonstrated that quench was constant.

Transporter mRNA expression measurements

At the end of the 3 h ammonia flux period in Series 2 and Series 4, all the solutions were removed and the apical side was rinsed with freshwater and the basolateral side was rinsed with PBS. A 1.0 ml sample of ice-cold Trizol reagent (Invitrogen, Burlington, ON, Canada) was added to the apical side. Mechanical disruption of gill

cells was performed by pipetting repeatedly with a 1 ml pipette. RNA was then extracted from the Trizol samples following the protocol provided by Invitrogen, quantified spectrophotometrically and electrophoresed on 1% agarose gels stained with ethidium bromide to verify integrity. First strand cDNA was synthesized from $2\,\mu g$ total DNase I-treated RNA using an oligo(dT_{17}) primer and Superscript II reverse transcriptase (Invitrogen). Quantitative real-time PCR (qPCR) reactions ($20\,\mu l$) containing $4\,\mu l$ of cDNA (1:4 dilution), 4 pmol of each primer, $10\,\mu l$ of Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen) and $0.8\,\mu l$ of ROX (6-carboxyl-X-Rhodamine dye; 1:10 dilution) were performed at 50°C (2 min), 95°C (2 min), followed by 40 cycles of 95°C (15 s) and 60°C (30 s) using an Mx3000P QPCR System (Stratagene, Cedar Creek, TX, USA). Melt-curve analysis confirmed production of a unique product and gel electrophoresis verified the presence of a single product. Gene-specific primers used were the same as in Nawata et al. (Nawata et al., 2007) for expression of elongation factor-1α (EF-1α; GenBank AF498320), H^{+}-ATPase (V-type, B subunit; GenBank AF14002), carbonic anhydrase-2 (cytoplasmic, CA-2; GenBank AY514870), Na^{+}/K^{+}-ATPase α1a (GenBank: AY319391), NHE2 (GenBank EF446605), Rhbg (GenBank EF051113/EF051114), Rhcg1 (GenBank DQ431244) and Rhcg2 (GenBank AY619986). EF-1α showed stable expression during different experimental conditions and was used as the reference gene to calculate relative mRNA expression by the standard curve method. Standard curves were generated by serial dilution of a random mixture of control samples.

Statistical analysis

In Series 1, iterative curve fitting using SigmaPlot 8.0 was employed to describe the concentration dependence of ammonia flux data. The best fit was obtained using a Michaelis–Menten component, with constants for affinity (K_{m}) and maximum transport capacity (J_{max}), superimposed on a linear component of constant slope. Manual fitting of linear components and Eadie–Hofstee plots were employed as a check on the SigmaPlot outputs.

All data are presented as means ± s.e.m. (N, number of preparations). Values from each condition were analysed using one-way analysis of variance (ANOVA) followed by Fisher's least significant difference *post-hoc* test. Student's unpaired *t*-test (two-tailed) was used when appropriate for simple comparisons of two means. Significance was set at α=0.05.

RESULTS
Series 1: concentration dependence of ammonia efflux

In asymmetrical DSI epithelia, relationships between ammonia efflux rate and basolateral [ammonia] were curvilinear, rather than linear over all ranges tested. The data from the two lower range experiments (i.e. basolateral [ammonia]=14–2270$\,\mu mol\,l^{-1}$) appeared to constitute a single relationship and were therefore combined (Fig. 1A), whereas those from the high range experiment (243–17768$\,\mu mol\,l^{-1}$) followed a different relationship (Fig. 1B). Iterative curve fitting (Sigmaplot 8.0) to the data of Fig. 1A yielded a relationship with a linear component plus a hyperbolic Michaelis–Menten relationship (R^{2}=0.993, P<0.0001) described by the following equation:

$$J_{Amm} = \frac{J_{max}[Amm]}{K_{m}+[Amm]} + C[Amm], \qquad (6)$$

where J_{Amm} is ammonia efflux rate (nmol cm^{-2} h^{-1}), [Amm] is basolateral total ammonia concentration ($\mu mol\,l^{-1}$), J_{max} is maximum
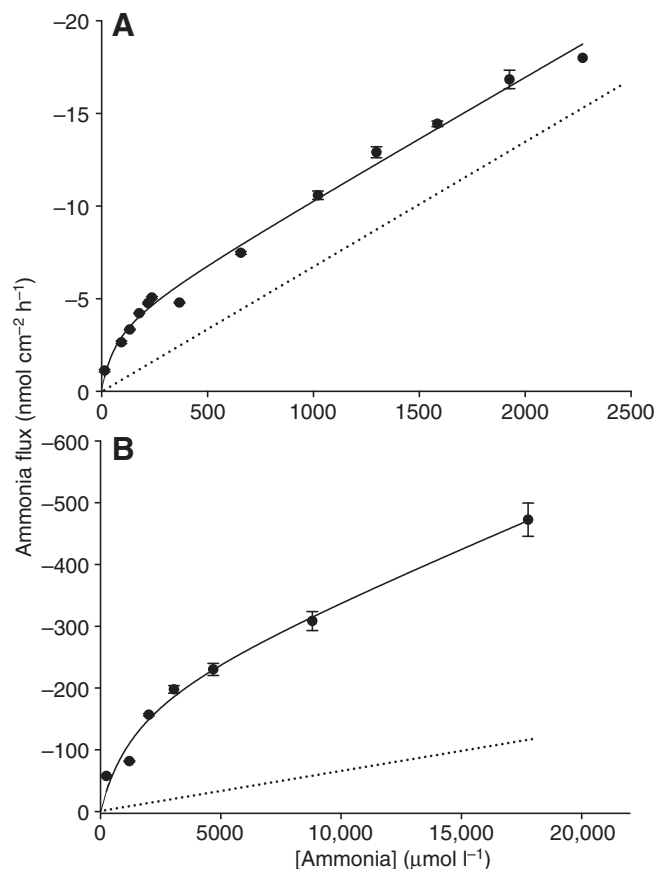

Fig. 1. Relationships between basolateral total ammonia concentration and ammonia efflux rate across asymmetrical DSI in Series 1. Flux rates are portrayed as negative values to represent basolateral-to-apical effluxes. (A) Physiological range of basolateral [ammonia]; data are described by a relationship which combines a Michaelis–Menten saturable component and a linear component (R^{2}=0.993, P<0.0001):

$$J_{Amm} = \frac{J_{max}[Amm]}{K_{m}+[Amm]} + C[Amm],$$

where J_{Amm} is ammonia efflux rate (nmol cm^{-2} h^{-1}), [Amm] is basolateral total ammonia concentration ($\mu mol\,l^{-1}$), J_{max} is maximum ammonia efflux rate (–3.92±0.75 nmol cm^{-2} h^{-1}), K_{m} is the affinity constant (66±44 $\mu mol\,l^{-1}$) equal to the [Amm] which supports 50% of J_{max}, and C is the slope (–0.0066±0.0004 nmol cm^{-2} h^{-1}/$\mu mol\,l^{-1}$) of the linear component. The dotted line indicates the linear component which was subtracted to yield the saturable component. The amount of efflux above this line is due to the saturable component. (B) Supra-physiological range of basolateral [ammonia]; data are described by the same equation as for A (R^{2}=0.953, P<0.0002) with the same linear component but with a much lower affinity (K_{m}=4818±1275 $\mu mol\,l^{-1}$) and higher maximum transport capacity (J_{max}=–429±47 nmol cm^{-2} h^{-1}). The dotted line indicates the same linear component as in A which was subtracted to yield the saturable component. The amount of efflux above this line is again due to the saturable component. Data points are means ± s.e.m. (N=4–6).

ammonia efflux rate (–3.92±0.75 nmol cm^{-2} h^{-1}), K_{m} is the affinity constant (66±44 $\mu mol\,l^{-1}$) equal to the [Amm] which supports 50% of J_{max}, and C is the slope (–0.0066±0.0004 nmol cm^{-2} h^{-1}/$\mu mol\,l^{-1}$) of the linear component. This suggests that a saturable carrier-mediated component with a relatively high affinity (low K_{m}) is superimposed on a large simple diffusion component.

When the same linear component was subtracted from the ammonia flux rates in the high range experiment, an apparent second

system (R^2=0.953, P<0.0002) with much lower affinity (K_m=4818±1275 μmol l^{-1}) but much higher maximum transport capacity (J_{max}=–429±47 nmol cm^{-2} h^{-1}) was revealed. However, in view of the apparently non-physiological range of basolateral [ammonia] in the experiment of Fig. 1B (but see Discussion), further tests concentrated exclusively on the range within Fig. 1A.

Series 2: cortisol and high [ammonia] pre-exposure

In asymmetrical DSI preparations pre-exposed to 2000 μmol l^{-1} NH$_4$Cl or 1000 ng ml^{-1} cortisol or 2000 μmol l^{-1} NH$_4$Cl plus 1000 ng ml^{-1} cortisol for 20 h, the ammonia flux from the basolateral to apical side (efflux) was significantly increased (Fig. 2A). The greatest increase was observed in DSI pre-exposed to both NH$_4$Cl and cortisol. The ammonia flux from the apical to basolateral side (influx), on the other hand, only increased when the inserts were pre-exposed to either NH$_4$Cl alone or NH$_4$Cl plus cortisol, and not to cortisol alone (Fig. 2B).

Results obtained from symmetrical DSI epithelia were slightly different. Significant increases were observed in ammonia efflux in DSI pre-exposed to either NH$_4$Cl or NH$_4$Cl plus cortisol, but not to cortisol alone (Fig. 3A). Exactly the same pattern was observed for ammonia influx (Fig. 3B).

The [^{3}H]PEG-4000 permeability was significantly increased when DSI preparations (both asymmetrical and symmetrical) were exposed to NH$_4$Cl alone (Fig. 4), though the relative changes were much smaller than in ammonia fluxes (Fig. 3). Treatment with cortisol, on the other hand, significantly reduced the [^{3}H]PEG-4000 permeability of DSI (both asymmetrical and symmetrical; Fig. 4). The effects of NH$_4$Cl plus cortisol were therefore intermediate.

For asymmetrical DSI preparations, before the apical culture medium was changed to freshwater, the TER was high (above 30 kΩ cm^2; Fig. 5A). The TER remained high 3 h after the gill culture was exposed to freshwater on the apical side. However, after 20 h of exposure, DSI that did not receive any cortisol treatment had TER values that were significantly lower than those pre-treated with cortisol alone or cortisol plus NH$_4$Cl (Fig. 5A). The TER remained almost the same after the 3 h ammonia flux experiment.

For symmetrical DSI, the apical culture medium was not changed to freshwater and the TER remained unchanged throughout the experiment for all the treatment groups, except in that pre-exposed to NH$_4$Cl alone. Here the TER was found to be significantly lower after the 3 h ammonia flux experiment (Fig. 5B).

In all groups, TEPs were positive (generally +15 to +35 mV with the apical side as zero reference) under symmetrical conditions prior to the start of the experimental pre-treatments (Fig. 6A,B). TEPs were significantly reduced and reversed to negative potentials (generally –5 to –10 mV) when the apical culture medium was changed to freshwater in all asymmetrical DSI experiments (Fig. 6A). After 3 h of apical freshwater treatment, DSI that had received cortisol during culture exhibited significantly more negative TEPs. Their TEPs returned to the same level as those of the control DSI after the 20 h pre-exposure, though a slight difference persisted in the cortisol plus NH$_4$Cl group after the 3 h ammonia flux experiment (Fig. 6A).

For the symmetrical DSI experiments, TEP was significantly reduced in DSI pre-exposed to NH$_4$Cl plus cortisol (Fig. 6B). A significant decrease in TEP was also observed at the end of the 3 h ammonia flux in preparations that had previously been exposed to NH$_4$Cl alone (Fig. 6B).

The mRNA expressions for Rhbg, Rhcg2 as well as NHE-2 were significantly increased by 2.4-, 16.1- and 2.3-fold, respectively, in asymmetrical DSI epithelia that had been pre-exposed to cortisol plus NH$_4$Cl and harvested at the end of the 3 h flux period (Fig. 7).
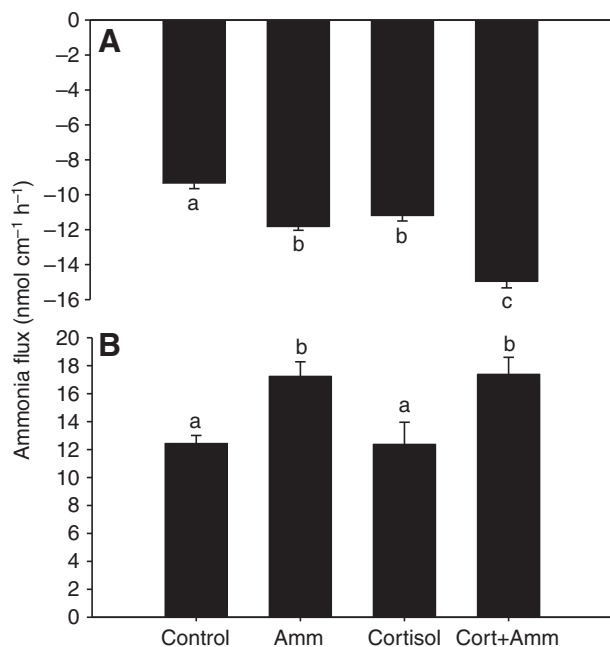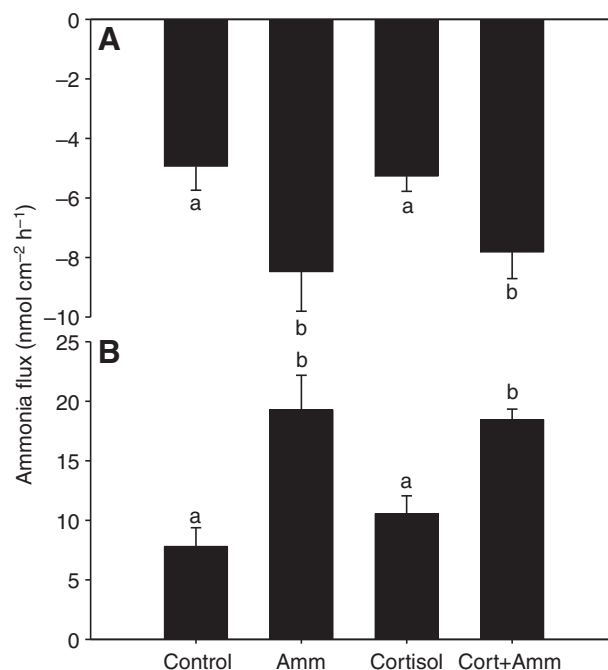

Fig. 2. The effect of 20 h pre-exposure on the ammonia flux across asymmetrical DSI in Series 2. (A) Ammonia efflux (negative); (B) ammonia influx (positive). Amm, 2000 μmol l^{-1} NH$_4$Cl pre-exposure; Cortisol, 1000 ng ml^{-1} cortisol pre-exposure; Cort+Amm, 1000 ng ml^{-1} cortisol and 2000 μmol l^{-1} NH$_4$Cl pre-exposure. Means not sharing the same letter are significantly different from one another (P<0.05). Data are means ± s.e.m. (N=4 or 5).

Fig. 3. The effect of 20 h pre-exposure on the ammonia flux across symmetrical DSI in Series 2. (A) Ammonia efflux (negative); (B) Ammonia influx (positive). Amm, 2000 μmol l^{-1} NH$_4$Cl pre-exposure; Cortisol, 1000 ng ml^{-1} cortisol pre-exposure; Cort+Amm, 1000 ng ml^{-1} cortisol and 2000 μmol l^{-1} NH$_4$Cl pre-exposure. Means not sharing the same letter are significantly different from one another (P<0.05). Data are means ± s.e.m. (N=4 or 5).
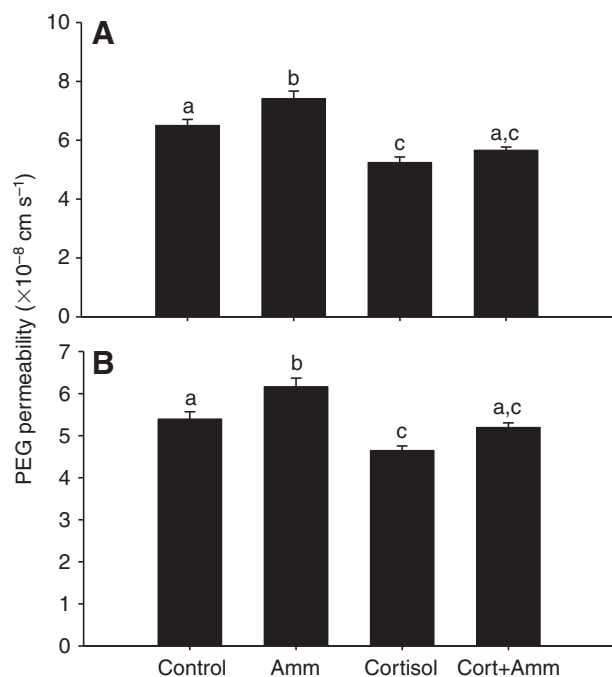
Fig. 4. The effect of 20 h pre-exposure on the [^{3}H]PEG-4000 permeability across (A) asymmetrical DSI and (B) symmetrical DSI in Series 2. Amm, 2000 μmol l^{-1} NH$_4$Cl pre-exposure; Cortisol, 1000 ng ml^{-1} cortisol pre-exposure; Cort+Amm, 1000 ng ml^{-1} cortisol and 2000 μmol l^{-1} NH$_4$Cl pre-exposure. Means not sharing the same letter are significantly different from one another (P<0.05). Data are means ± s.e.m. (N=4 or 5).
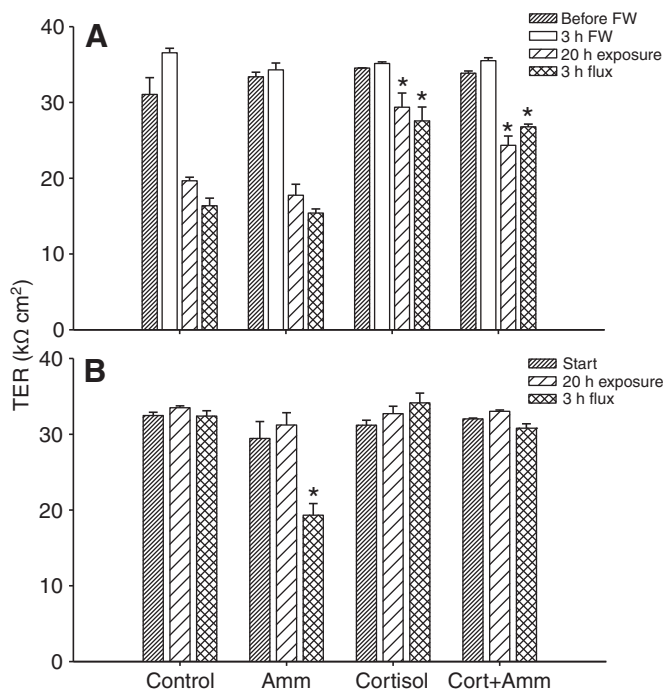

Fig. 5. The effect of 20 h pre-exposure on the transepithelial resistance (TER) across (A) asymmetrical DSI and (B) symmetrical DSI in Series 2. Amm, 2000 μmol l^{-1} NH$_4$Cl pre-exposure; Cortisol, 1000 ng ml^{-1} cortisol pre-exposure; Cort+Amm, 1000 ng ml^{-1} cortisol and 2000 μmol l^{-1} NH$_4$Cl pre-exposure. FW, freshwater. Asterisks represent values significantly different from the corresponding control value (P<0.05). Data are means ± s.e.m. (N=4 or 5).

Pre-exposure to either cortisol or NH$_4$Cl alone did not have these effects. H$^+$-ATPase mRNA expression was significantly increased (1.7-fold) in DSI preparations that were exposed either to NH$_4$Cl alone, or to both NH$_4$Cl and cortisol. Significant decreases in carbonic anhydrase-2 (CA-2) mRNA expression were observed in DSI epithelia exposed to NH$_4$Cl alone (by 50%), cortisol alone (by 70%), as well as both NH$_4$Cl and cortisol (by 75%). There were no significant changes in the mRNA expression of Rhcg1 and Na$^+$/K$^+$-ATPase α1a subunit.

Series 3: effects of transporter inhibitors on ammonia flux

As the most clearcut effects on ammonia efflux and mRNA transporter expression were seen with the combined pre-exposure to NH$_4$Cl and cortisol, this treatment was used to evaluate the effects of inhibitors. Bafilomycin (1 μmol l^{-1}), amiloride (100 μmol l^{-1}), phenamil (10 μmol l^{-1}) and HMA (10 μmol l^{-1}), each applied separately to the apical solution, significantly reduced the ammonia efflux across asymmetrical DSI preparations by 35 to 50% (Fig. 8). HMA (10 μmol l^{-1}) applied to the basolateral solution, on the other hand, had no effect on the ammonia efflux. These results strongly suggest the involvement of H$^+$-ATPase (inhibited by bafilomycin), Na$^+$ channel (inhibited by phenamil and amiloride) and NHE (inhibited by HMA and amiloride) on the apical side in ammonia transport. It should be stressed that alteration of the standard curves in the ammonia assay caused by DMSO and drugs has been corrected (see Materials and methods).

Series 4: low apical [Na$^+$] pre-exposure

Pre-exposure of cortisol-treated DSI preparations to low apical [Na$^+$] for 20 h led to a significant increase in ammonia efflux by 15%

(Fig. 9A). There was no change in [^{3}H]PEG-4000 permeability (Fig. 9B), indicating that the increase in ammonia efflux was not due to an increase in paracellular permeability. In view of the fact that the change in ammonia efflux was small but significant, the experiment was repeated with another batch of DSI epithelia (data not shown). Although baselines fluxes were somewhat higher, the modest significant increase in ammonia efflux but lack of change in [^{3}H]PEG-4000 permeability was highly reproducible.

Analysis of mRNA expression of these DSI epithelia exposed to low apical [Na$^+$] revealed that only Rhcg2 mRNA was significantly induced by about 1.5-fold (Fig. 10C). The mRNA expressions of Rhbg, Rhcg1, H$^+$-ATPase and NHE-2 were not changed (Fig. 10). The importance of Rhcg2 in gill epithelial ammonia transport is thus very apparent.

Series 5: effect of high [ammonia] pre-exposure on active Na$^+$ uptake

Pre-exposure of cortisol-treated DSI epithelia to 2000 μmol l^{-1} NH$_4$Cl for 20 h resulted in significant increases (50–60%) in both Na$^+$ influx (Fig. 11A) and ammonia efflux rates (Fig. 11D). Na$^+$ efflux rate did not change (Fig. 11B). The TEP became slightly more negative (Fig. 11C), as previously seen with this same treatment in the experiments of Series 1 (Fig. 6A). Both Na$^+$ influx (Fig. 12A) and ammonia efflux (Fig. 12B) were negatively correlated with TEP in Series 5. Also, Na$^+$ influx was positively correlated with ammonia efflux with a slope of approximately 4 (i.e. four ammonia effluxed per one sodium taken up; Fig. 12C). Active influx of Na$^+$ was observed in both control and ammonia-exposed DSI preparations as the measured flux ratios were significantly greater than the predicted Ussing ratios for both groups (Table 2). It should be noted
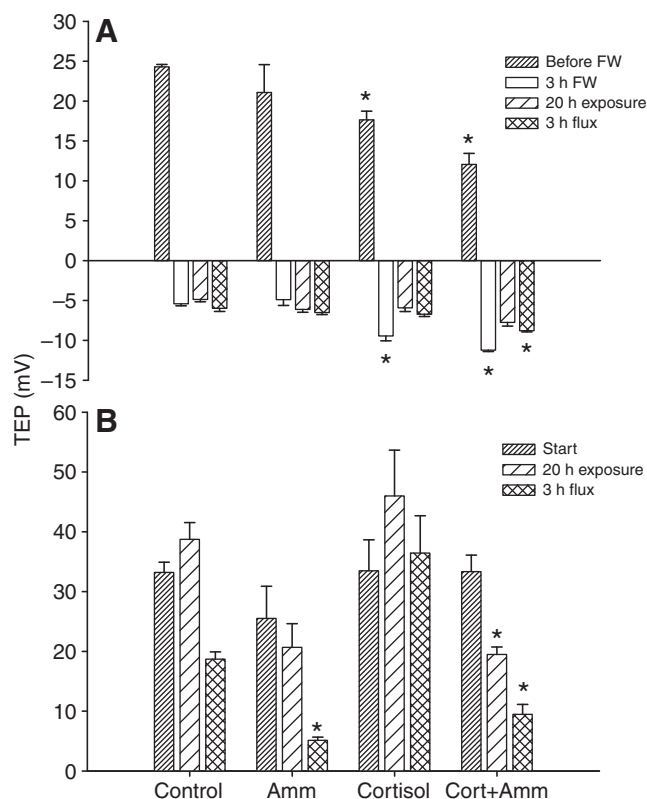
Fig. 6. The effect of 20 h pre-exposure on the transepithelial potential (TEP) across (A) asymmetrical DSI and (B) symmetrical DSI in Series 2. Amm, 2000 μmol l⁻¹ NH₄Cl pre-exposure; Cortisol, 1000 ng ml⁻¹ cortisol pre-exposure; Cort+Amm, 1000 ng ml⁻¹ cortisol and 2000 μmol l⁻¹ NH₄Cl pre-exposure. Asterisks represent values significantly different from the corresponding control value ($P<0.05$). Data are means ± s.e.m. (N=4 or 5).

that cortisol was present in all DSI preparations in this series and active Na⁺ transport from apical freshwater has previously been reported in cortisol-treated DSI epithelia (Zhou et al., 2003).

Series 6: responses of live trout to high environmental ammonia exposure

The high environmental ammonia exposure proved largely sublethal. Of 29 trout exposed to a nominal level of 2000 μmol l⁻¹ NH₄Cl in Hamilton tapwater (measured level=2515±153 μmol l⁻¹; Fig. 13), only three died, all in the first few hours, though about half exhibited erratic swimming behaviour during the first day. By day 7, all appeared healthy though inactive relative to the controls. Blood plasma total ammonia concentrations were elevated more than 10-fold above control levels and did not differ significantly between 20 h and 7 days (Table 3). Surprisingly, these plasma concentrations around 1100 μmol l⁻¹ were only about 50% of the simultaneously measured water levels at the time of sampling (Table 3). Plasma cortisol was elevated 16-fold above control levels to about 160 ng ml⁻¹ at 20 h, and remained close to this level at 7 days (Table 3).

DISCUSSION
Culture conditions

DSI epithelia were cultured as in all of our previous studies (Fletcher et al., 2000; Kelly et al., 2000; Wood et al., 2002; Kelly and Wood, 2001a; Kelly and Wood, 2001b; Zhou et al., 2003) using L-15 media, supplemented with 2 mmol l⁻¹ glutamine and 5% FBS. The epithelia

grow very well in this phosphate-buffered, nominally HCO₃⁻-free medium, but it is designed to be used in an air atmosphere which has very low CO₂ relative to blood. The medium naturally stabilizes at a pH of 7.4, whereas *in vivo* extracellular pH would be 7.7–7.9 at the temperatures used here (e.g. Salama et al., 1999). To maintain the pH in the latter range during the extended culture period would have necessitated the addition of disodium or dipotassium phosphate to the pre-packaged medium, or alternatively adding an organic buffer such as Hepes, or a CO₂/HCO₃⁻ buffer system. We elected to avoid the addition of either phosphate salt, because it would alter the inorganic ion composition and risk precipitation of calcium, or Hepes, because of its well-documented effects in blocking anion channels (Hanrahan and Tabcharani, 1989), whereas the latter approach was impractical as we lacked a low-level CO₂ incubator. We therefore chose to accept this moderately acidotic condition (pH 7.4, nominally CO₂-free and HCO₃⁻-free media) in all our experiments, recognizing that it may alter transport activity. For example, in isolated gill cells, there is evidence that additional pH₁-regulatory pathways are seen when HCO₃⁻ is present in the media (Wood and Pärt, 2000). Nevertheless, one advantage of this approach is that it would probably have ameliorated any toxicity due to the high ammonia pre-exposure (2000 μmol l⁻¹) used in some trials. In future studies, it will be desirable to incorporate a CO₂/HCO₃⁻ buffer system, and in particular to attempt to maintain an outward P_{CO_2} gradient from the basolateral to apical surface, as occurs *in vivo*.

Concentration dependence of ammonia flux

Concentrations of ammonia in arterial blood plasma of teleosts *in vivo* are generally less than 500 μmol l⁻¹, and more normally closer to 100–200 μmol l⁻¹ in fasted fish (e.g. Table 3) but they may be considerably higher in venous plasma (reviewed by Wood, 1993). In salmonids, plasma levels rise after feeding (300–1000 μmol l⁻¹) (Kaushik and Teles, 1985; Wicks and Randall, 2002; Bucking and Wood, 2008), and similarly during sublethal high environmental ammonia (HEA) exposure may reach close to 1000 μmol l⁻¹ (Wilson et al., 1994; Nawata et al., 2007; Nawata and Wood, 2008). In the present study, the HEA exposure (2300–2600 μmol l⁻¹) of Series 6 was right at the upper end of the sublethal range (only 3 of 29 trout succumbed) and plasma ammonia concentrations averaged about 1100 μmol l⁻¹ (Table 3), with one individual value reaching 1982 μmol l⁻¹ in a surviving fish. Experimental variation of basolateral [ammonia] over this physiological range (14–2270 μmol l⁻¹) in asymmetrical DSI preparations yielded evidence of a saturable curvilinear component superimposed on a simple linear diffusive component for ammonia efflux (Fig. 1A). The calculated K_m (66 μmol l⁻¹) appeared reasonable (i.e. within the normal physiological range for blood plasma), providing the impetus to further investigate the nature of this apparent carrier-mediated component. Interestingly, Heisler (Heisler, 1990) presented evidence in rainbow trout *in vivo* that flux *versus* concentration relationships deviated from linearity above arterial plasma total ammonia levels of 200 μmol l⁻¹, and suggested that this represented a threshold for activation of some sort of carrier-mediated process.

Based on the results in Fig. 1A, we chose to work at a 'normal' basolateral total ammonia concentration of about 700 μmol l⁻¹, a concentration at which the carrier-mediated component is close to saturated, and total flux is about 56% diffusive and 44% carrier mediated (see Fig. 1A). Apical and basolateral concentrations of 2000 μmol l⁻¹ were employed for HEA pre-exposures, because it was well above saturation for the normal relationship, and was at the upper end of the physiological range of tolerance, as shown by the *in vivo* HEA exposures of Series 6.
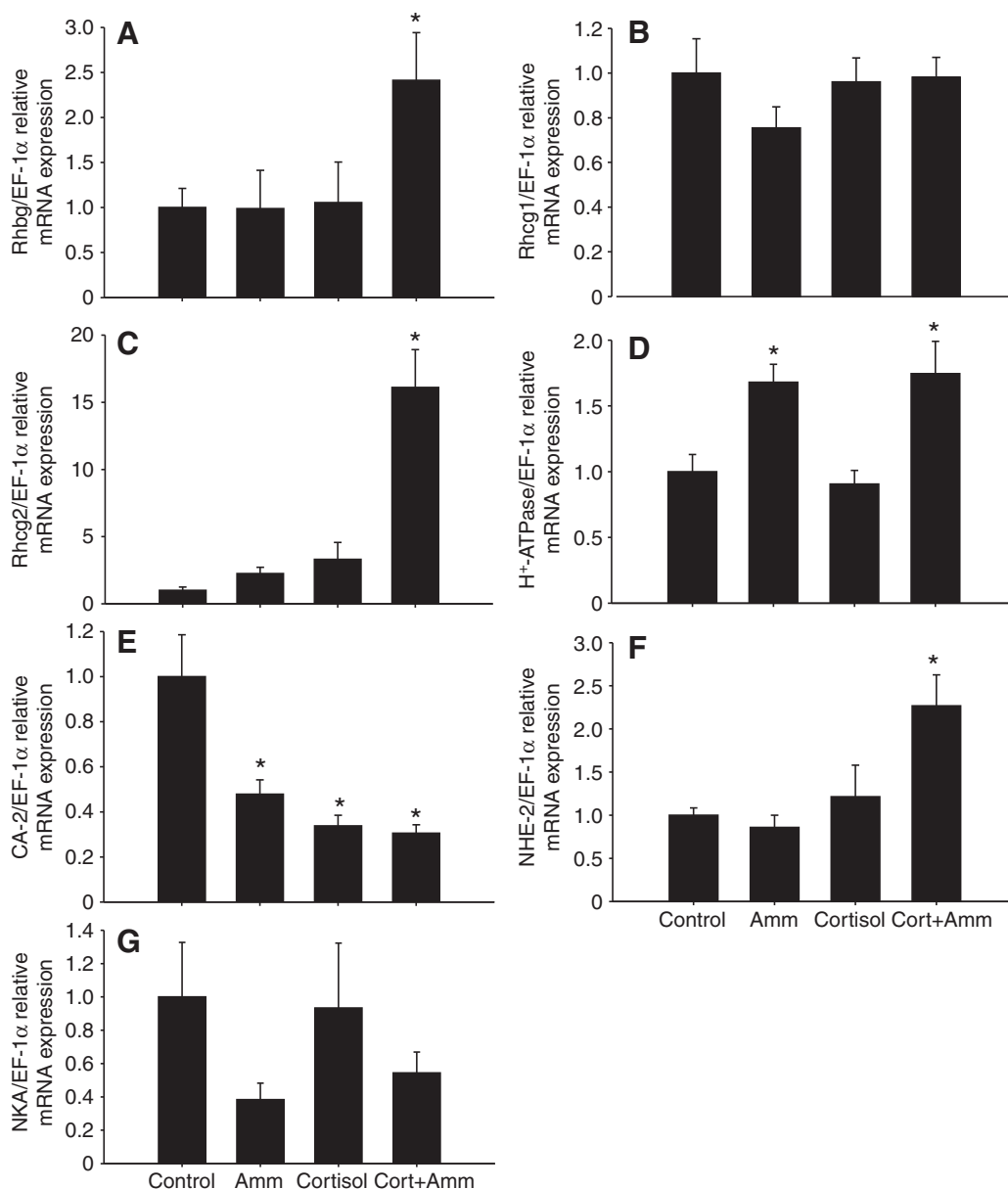
Fig. 7. The effect of 20h pre-exposure on the mRNA expression in asymmetrical DSI, relative to that of elongation factor 1α (EF-1α), of (A) Rhbg, (B) Rhcg1, (C) Rhcg2, (D) H$^+$-ATPase, (E) carbonic anhydrase-2 (CA-2), (F) Na$^+$/H$^+$ exchanger-2 (NHE-2) and (G) Na$^+$/K$^+$-ATPase α1a (NKA) in Series 2. Amm, 2000 µmol l^{-1} NH$_4$Cl pre-exposure; Cortisol, 1000 ng ml^{-1} cortisol pre-exposure; Cort+Amm, 1000 ng ml^{-1} cortisol and 2000 µmol l^{-1} NH$_4$Cl pre-exposure. Asterisks represent values significantly different from control value ($P<0.05$). Data are means ± s.e.m. (N=4 or 5).

However, there was also evidence of a possible low affinity (K_m=4818 µmol l^{-1}), high capacity system present at much higher basolateral concentrations (Fig. 1B). Note that this experiment was performed on a different batch of DSI epithelia with much higher fluxes (5- to 8-fold) in the range of concentration overlap with the epithelia of Fig. 1A. The possible low affinity, high capacity system was not pursued experimentally in the present study because these concentrations are not physiologically relevant relative to normal plasma concentrations. However, in retrospect, there may be physiological relevance. In fish, ammonia distributes across cell membranes according to electrical gradients rather than pH gradients such that intracellular levels of total ammonia are up to 30-fold higher than extracellular levels (reviewed by Wood, 1993). A K_m concentration of 4814 µmol l^{-1} would be a very reasonable intracellular ammonia level. The apparent carrier-mediated components induced by high ammonia and/or cortisol pre-exposure are clearly bidirectional (Figs 2 and 3), so the transport system discussed below must normally encounter high intracellular ammonia concentrations on the inner sides of both cell membranes.

It is possible that Fig. 1B represents the 'intracellular-side' transport behaviour of the system under an artificial, non-steady-state situation where extracellular ammonia concentration is acutely raised to approximate normal intracellular levels.

Rh proteins are important for ammonia transport in DSI

Until recently, ammonia was thought to move across lipoprotein cell membranes mainly by simple diffusion in the form of NH$_3$, without the involvement of protein channels or transporters. While this small molecular weight dissolved gas is commonly considered to be lipid soluble, its solubility in lipid is actually quite low (Evans and Cameron, 1986; Wood, 1993), so passive diffusion alone may not be enough to account for the high ammonia permeability of gill epithelia (see Kelly and Wood, 2001a). The discovery of ammonia transporters in yeast (Marini et al., 1994), plants (Ninnemann et al., 1994) and later in humans (Marini et al., 2000) and other animals (Liu et al., 2000; Weihrauch et al., 2004; Nakada et al., 2007b) may provide an explanation for this discrepancy. The present study, together with other recent work on gill ammonia transport in fish
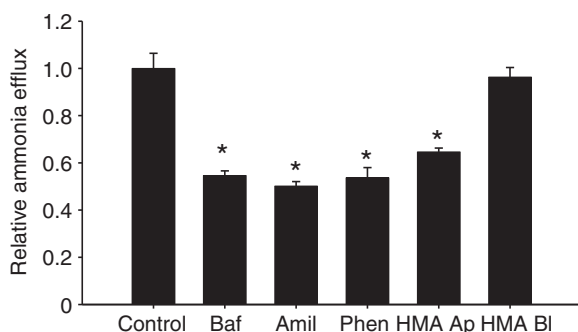
Fig. 8. Effects of different transport inhibitors on ammonia flux across asymmetrical DSI in Series 3. Baf, bafilomycin (1 μmol l^{-1}); Amil, amiloride (100 μmol l^{-1}); Phen, phenamil (10 μmol l^{-1}); HMA, 5-(N,N-hexamethylene)amiloride (10 μmol l^{-1}); Ap, apical solution; Bl, basolateral solution. Asterisks represent values significantly different from control value ($P<0.05$). Data are means ± s.e.m. (N=4 or 5).

(Nakada et al., 2007a; Nakada et al., 2007b; Hung et al., 2007; Hung et al., 2008; Nawata et al., 2007; Nawata and Wood, 2008) strongly indicates the involvement of protein carriers in ammonia excretion.

Nawata and colleagues (Nawata et al., 2007) have demonstrated that the mRNA expression of various Rh proteins was up-regulated in the gills of rainbow trout when exposed to HEA. A similar phenomenon has also been observed in the mangrove killifish (Hung et al., 2007). These up-regulations suggest the involvement of Rh proteins in ammonia transport. When various Rh proteins identified in the puffer fish, *Takifugu rubripes*, were expressed in *Xenopus* oocytes, the uptake of the ammonia analogue methylammonia into these oocytes was up-regulated (Nakada et al., 2007b). It is reasonable to assume that rainbow trout Rh proteins similarly facilitate ammonia transport. At this moment it is not clear whether Rh proteins function as a NH$_3$ gas channel or NH$_4^+$ ion carrier (Javelle et al., 2007). However, there is considerable evidence that NH$_3$ movement plays a major role in gill ammonia excretion, while NH$_4^+$ movement appears to be less important, at least in freshwater fish (Wood, 1993; Wilkie, 1997), so it seems more likely that fish
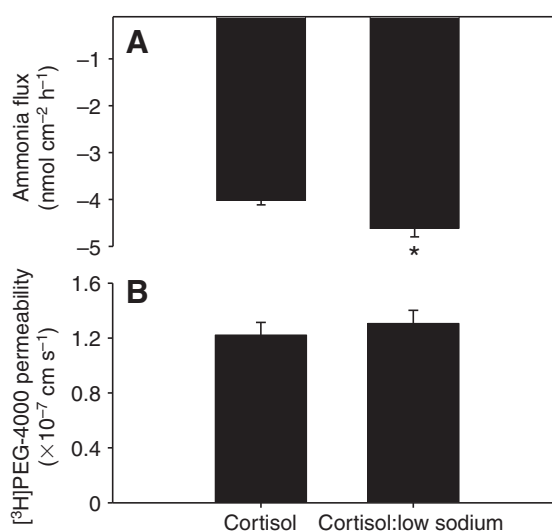

Fig. 9. The effect of 20 h low [sodium] pre-exposure on the apical side on (A) ammonia efflux (negative) and (B) [^{3}H]PEG-4000 permeability across asymmetrical DSI in Series 4. Asterisk represents value significantly different from cortisol value ($P<0.05$). Data are means ± s.e.m. (N=5).

Rh proteins are NH$_3$ gas channels, similar to the ammonia transporter AmtB in *Escherichia coli* (Khademi et al., 2004).

However, the permeability of the gill epithelium to ammonia in the various studies mentioned above could not be directly measured. The present study utilized the cultured gill epithelium where ammonia permeability could be easily measured (see Kelly and Wood, 2001a). We found that up-regulation of mRNA expression of Rh proteins was always associated with increases in ammonia permeability in the DSI preparations. Rhcg2 seems to be of particular importance as its mRNA was up-regulated 16-fold when exposed to cortisol and ammonia (Series 2; Fig. 7C). Moreover, when the epithelia were pre-treated with low apical [Na$^+$], the only gene that showed up-regulation was Rhcg2 (Fig. 10C) and this response was accompanied by an increase in ammonia permeability (Series 4; Fig. 9A). It should also be highlighted that the mRNAs of Rhcg2 in both the rainbow trout and the mangrove killifish gills were up-regulated more than those of other Rh proteins in response to HEA (Nawata et al., 2007; Hung et al., 2007). From these results, we may infer that Rhcg2 was the limiting factor for carrier-mediated ammonia permeability in the DSI.

A general observation in the present study was that various experimental treatments (e.g. pre-treatment with ammonia and/or cortisol, low [Na$^+$] pre-exposure, application of inhibitors) caused changes in ammonia flux which were significant but not massive (generally less than 50% decrease or 100% increase) relative to control values. We interpret this observation to mean that there is a general background level of diffusive permeability to ammonia in the gill epithelia (accounting for about 56% of flux at 700 μmol l^{-1}; Fig. 1A) which is augmented by transport-mediated flux, rather than transport-mediated flux completely dominating. This is in accord with the earlier conclusion of Kelly and Wood (Kelly and Wood, 2001a) who used DSI epithelia which had not been pre-exposed to ammonia, cortisol or low [Na$^+$]. These workers concluded that the basic diffusive NH$_3$ permeability of this preparation was similar to that of many other epithelia, that diffusive NH$_4^+$ flux could augment this permeability under certain asymmetrical conditions, but that neither of these was sufficient to explain total ammonia flux, such that transport-mediated flux must also be important under *in vivo* conditions. Wright and Wood (Wright and Wood, 1985), Heisler (Heisler, 1990), and Salama and colleagues (Salama et al., 1999) reached similar conclusions based on experiments with rainbow trout *in vivo*. Missing in these earlier studies was any realization of the potential role of Rh glycoproteins in the carrier-mediated component.

Various factors up-regulate ammonia permeability

Although Nawata and colleagues (Nawata et al., 2007), Nawata and Wood (Nawata and Wood, 2008), and Hung and colleagues (Hung et al., 2007) have already shown changes in the mRNA expression of transporters potentially involved in ammonia transport in HEA, it was not clear what the signalling molecule(s) for the changes were. Results from the present study shed light on the signalling mechanism for the regulation of gene expression in the gill. Application of cortisol or ammonia alone up-regulated ammonia permeability (Figs 2 and 3). The up-regulation in ammonia permeability was even greater when DSI preparations were pre-treated with both cortisol and ammonia (Figs 2 and 3). The mRNA expression of Rhbg and Rhcg2 was up-regulated 2.4- and 16-fold, respectively, when DSI epithelia were pre-treated with both ammonia and cortisol (Fig. 7A,C). When pre-treated with either cortisol plus ammonia or ammonia alone, there was an up-regulation
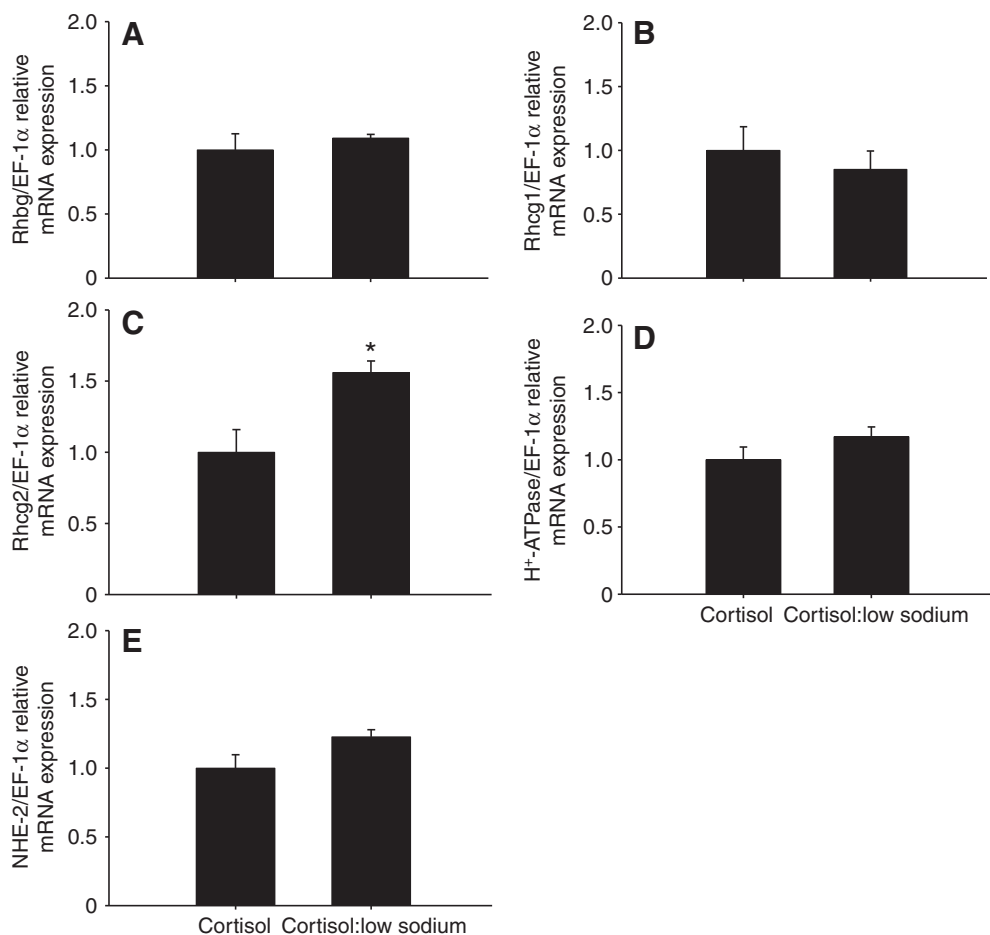
Fig. 10. The effect of 20 h low [sodium] pre-exposure on the apical side on the mRNA expression in asymmetrical DSI relative to EF-1α of (A) Rhbg, (B) Rhcg1, (C) Rhcg2, (D) H⁺-ATPase and (E) NHE-2 in Series 4. Asterisk represents value significantly different from cortisol value ($P<0.05$). Data are means ± s.e.m. ($N=4$ or 5).

of H⁺-ATPase mRNA (Fig. 7D). All of these observations indicated that cortisol and ammonia could affect membrane transporter expression individually as well as synergistically.

TEPs in the cultured epithelia were in the normal range for trout *in vivo* and typical of previous reports using DSI preparations (reviewed by Wood et al., 2002). It is notable that cortisol and ammonia pre-treatment induced more negative TEPs in asymmetrical DSI epithelia (Fig. 6A; Fig. 11C), and that both ammonia efflux and active Na⁺ influx were correlated with the extent of negativity in these preparations (Fig. 12A,B). Traditionally, the TEP across the freshwater gill is interpreted as predominantly a diffusion potential reflecting the differential passive permeability of the whole epithelium to Na⁺ exceeding that to Cl⁻ (e.g. Potts, 1984; Wood and Grosell, 2008). However, an alternative or additional explanation is the electrogenic action of an apical membrane proton pump, extruding positive charge. In accord with this concept, mRNA expression for H⁺-ATPase increased (Fig. 7D) and ammonia flux was inhibited by bafilomycin (Fig. 8) in response to this pre-treatment.

Cortisol is well known to be a stress hormone released whenever the fish face abnormal situations (Wendelaar Bonga, 1997) and HEA is certainly a stressful situation. Indeed, plasma cortisol levels in rainbow trout were positively correlated to plasma ammonia levels during HEA in the study of Ortega and colleagues (Ortega et al., 2005), and increased 4-fold in association with a 10-fold increase in plasma total ammonia concentration in the HEA (1500 μmol l⁻¹) exposure of Nawata and Wood (Nawata and Wood, 2008). In Series 6 of the present study, which used an even higher HEA exposure (2300–2600 μmol l⁻¹), plasma cortisol increased to about the same

concentrations (160 ng ml⁻¹) as measured by Nawata and Wood (Nawata and Wood, 2008), but against a lower baseline. Notably, three of the surviving fish surpassed 300 ng ml⁻¹. Therefore, during HEA episodes, the gill epithelium will encounter both elevated ammonia and elevated cortisol simultaneously. It is probable that, *in vivo*, cortisol rarely if ever reaches the 1000 ng ml⁻¹ used in the *in vitro* experiments of the present study, but it is well established that fish gill cells in culture are much less sensitive to many agents than *in vivo* (Castaño et al., 2003). Cortisol receptors have been discovered and extensively studied (e.g. Alsop and Vijayan, 2008), and full-length sequences are now available for a number of Rh proteins in trout (Nawata et al., 2007; Nawata and Wood, 2008). Cultured trout gill epithelia are very responsive to cortisol (Kelly and Wood, 2001a; Zhou et al., 2003), as also seen in the present study; however, cortisol receptors in the DSI epithelia have not yet been characterized, and very little is known in general about ammonia receptors/sensors.

It was exciting to find that low apical [Na⁺] pre-treatment led to increases in Rhcg2 mRNA expression as well as in ammonia efflux in DSI (Fig. 9A; Fig. 10C). Presumably, during low apical [Na⁺] treatment, adaptive compensation would occur in the gill epithelium to increase Na⁺ uptake in order to compensate for the increased loss of Na⁺ to the apical water. Increased Rhcg2 expression on the apical membrane would allow more ammonia (NH₃ or NH₄⁺) to cross the membrane and emerge on the apical surface. If these ammonia molecules move as NH₃, H⁺ pumped by H⁺-ATPase would then bind to these NH₃ to form NH₄⁺, thereby maintaining an appropriate electrochemical gradient for the H⁺ pump and chemical gradient for the NH₃ transporter. Because there is now increased export of
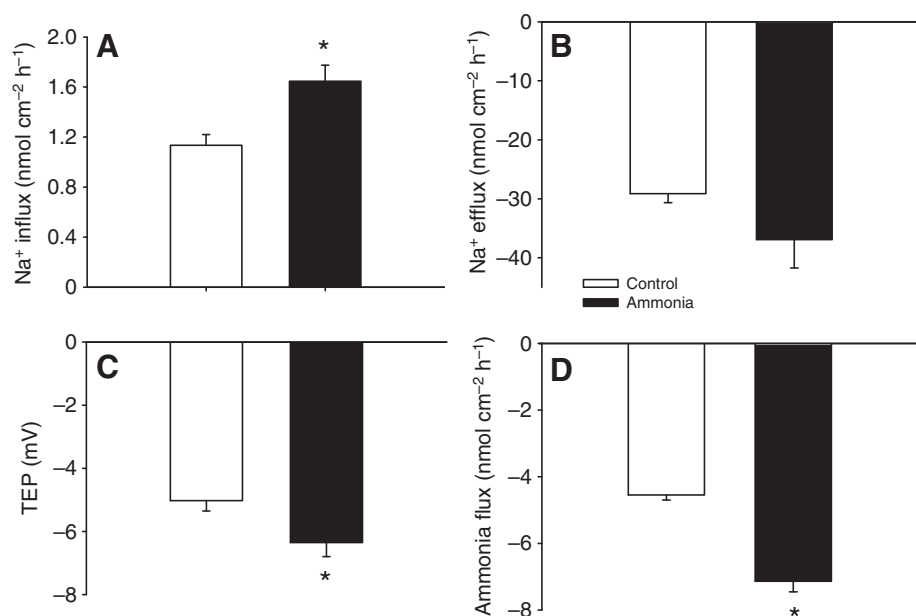
Fig. 11. The effect of 20 h high [ammonia] pre-exposure on (A) Na$^+$ influx (positive), (B) Na$^+$ efflux (negative), (C) TEP and (D) ammonia efflux (negative) of asymmetrical DSI in Series 5. High [ammonia] pre-exposure was performed on symmetrical DSI for 20 h prior to the switch to apical freshwater. Cortisol (1000 ng ml^{-1}) was added to all DSI. Asterisks represent values significantly different from control value (P<0.05). Data are means ± s.e.m. (N=4).

positive charge, increased Na$^+$ uptake *via* the Na$^+$ channel becomes possible. By this scenario, the Rhcg2 and H$^+$-ATPase would act together to function like an ammonium pump, as suggested by Nawata and colleagues (Nawata et al., 2007). A similar phenomenon has been reported in zebrafish larvae, where Rhcg1 was up-regulated when the whole fish was subjected to diluted freshwater (Nakada et al., 2007a). It is interesting that different isoforms of Rhcg were responding to the same stressor in trout (Rhcg2) and in zebrafish (Rhcg1).

An apical Na$^+$/NH$_4^+$ exchange complex may consist of many transporters

From the discussion above, there is a clear link between ammonia excretion and Na$^+$ uptake. Based on the results from mRNA expression analysis of DSI preparations in Series 2 as well as the transport inhibitor studies in Series 3 (Fig. 8) we propose a model of an apical Na$^+$/NH$_4^+$ exchange complex that is made up of several membrane transporters (Fig. 13). In this model, NH$_3$ crosses the apical membrane from cell to the water *via* Rhcg (Rhcg1 or Rhcg2) down a concentration gradient. Upon emergence from the cell, NH$_3$ binds with H$^+$, which is pumped from the cell by H$^+$-ATPase, to form NH$_4^+$. This 'ammonium pump' maintains the transmembrane NH$_3$ gradient and H$^+$ gradients and also provides electrostatic force to drive Na$^+$ uptake through the Na$^+$ channel. In addition, H$^+$ could also exit the cell *via* an NHE-2 exchanger, and thus allow Na$^+$ intake, if thermodynamically feasible.

This model can help reconcile some discrepancies from studies on the Na$^+$/NH$_4^+$ transporter. While ammonia excretion and Na$^+$ uptake are linked, they can also be uncoupled, depending on the experimental approach used. For example, using the IPHP, Avella and Bornancin (Avella and Bornancin, 1989) provided evidence against the presence of a Na$^+$/NH$_4^+$ exchanger after they found that ammonia excretion was reduced when the pH of the gill perfusate was decreased. However, if ammonia leaves the gill epithelium in the form of NH$_3$ *via* Rh proteins, by reducing the perfusate pH, the trans-epithelial NH$_3$ gradient would be lower, resulting in a reduction of ammonia excretion. Avella and Bornancin (Avella and Bornancin, 1989) also found that when ammonia excretion was increased by increasing the ammonia level in the perfusate from 0 to 1 mmol l^{-1},

there was no noticeable change in Na$^+$ uptake. At 0 perfusate ammonia level, H$^+$ excretion was probably maintained and thus Na$^+$ uptake was maintained. At higher perfusate ammonia levels, the Na$^+$ uptake mechanism (Na$^+$ channel and NHE-2) would be saturated but the ammonia excretion mechanism (Rh proteins) would not be saturated. In fact, when the external water [Na$^+$] was reduced, ammonia excretion was significantly reduced, too, though the magnitude of the effect varied among studies (Avella and Bornancin, 1989; Wilson et al., 1994; Salama et al., 1999). The present study also showed that there is a positive correlation between ammonia excretion and Na$^+$ uptake, though not a 1:1 relationship (Fig. 12C).

It is well known that both amiloride (Kerstetter and Keeler, 1976; Payan, 1978; Wright and Wood, 1985; Yesaki and Iwama, 1992) and external buffering separately reduce ammonia excretion *in vivo* (Wright et al., 1989; Wilson et al., 1994; Salama et al., 1999; Nawata and Wood, 2008). Amiloride, which at this concentration (100 μmol l^{-1}) can inhibit Na$^+$-linked H$^+$ excretion by blocking both Na$^+$ channels and Na$^+$ exchangers, was similarly effective *in vitro* in the present study (Fig. 8). However, Wilson and colleagues (Wilson et al., 1994) found that when the external water was buffered, amiloride did not inhibit ammonia excretion. They interpreted this to mean that amiloride inhibited Na$^+$-linked H$^+$ excretion rather than direct Na$^+$/NH$_4^+$ exchange. This explanation actually supports the present model (Fig. 13) because amiloride should not alter NH$_3$ flux when the water is buffered and the diffusion trapping mechanism thereby removed. While a single Na$^+$/NH$_4^+$ exchanger (i.e. with tightly coupled 1:1 stoichiometry) most likely does not exist, a system which is effectively an 'Na$^+$/NH$_4^+$ exchange complex' may consist of several different transporters functioning together (Fig. 13). This would include Rh proteins, H$^+$-ATPase (sensitive to bafilomycin), NHE-2 (sensitive to HMA), and Na$^+$ channels (sensitive to phenamil; Fig. 8).

In the *in vivo* studies of HEA of Series 6, it was intriguing to find that plasma total ammonia concentration stabilized at a level that was only about 50% of that in the external water (Table 3). Bulk water pH and blood pH were probably similar in this situation, so at first glance this result suggests that when the above-described mechanism was induced by elevated ammonia and cortisol (Table 3), trout were able to actively excrete ammonia against the gradient.
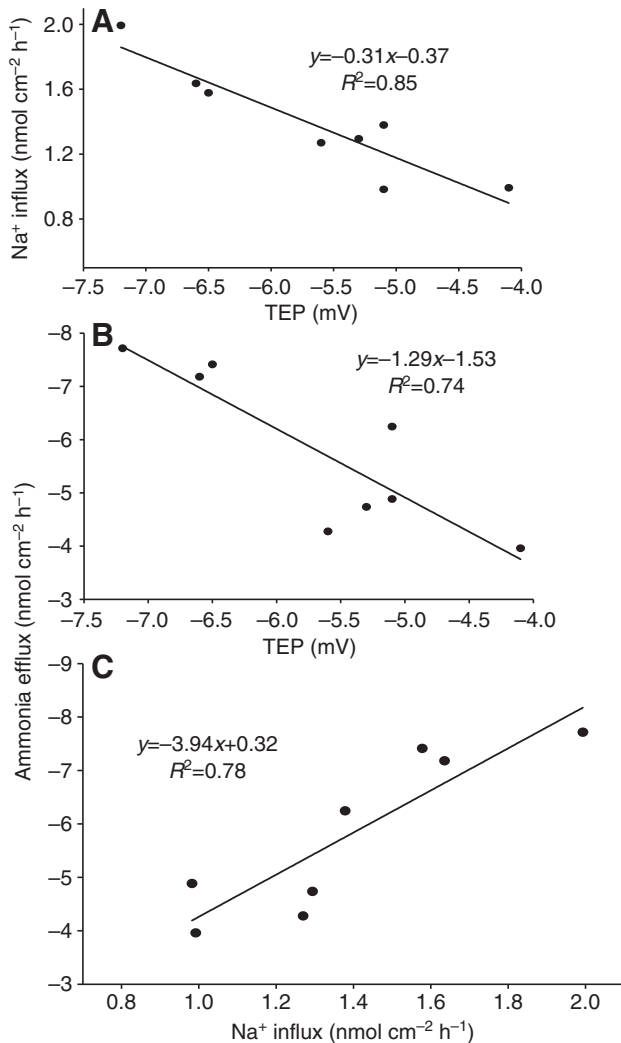
Fig. 12. Correlations between (A) Na$^+$ influx and TEP, (B) ammonia efflux and TEP, and (C) ammonia efflux and Na$^+$ influx in Series 5.

Table 2. Comparison between predicted (Ussing) and measured flux ratio ($\times 10^{-3}$) for Na$^+$ in Series 5

	Predicted	Measured
Control	7.65±0.12	45.1±3.4*
Ammonia	7.79±0.13	46.0±4.5*

Asterisks represent values significantly different from predicted value ($P<0.05$). Data are means ± s.e.m. ($N=4$). Measured flux ratios higher than predicted indicate active apical-to-basolateral uptake.

both Rh proteins and H$^+$-ATPase expressions. Given that several transporters (Rh, H$^+$-ATPase, NHE-2, Na$^+$ channel) are involved in the proposed Na$^+$/NH$_4^+$ exchange complex, and that this carrier-mediated exchange is superimposed on a substantial component which occurs by simple diffusion (Fig. 1A), we suggest that the apparent stoichiometry may depend on the relative expression level of the different membrane transporters, as well as changes in passive permeability.

The situation at the basolateral surface of the epithelium is less clear at this moment (Fig. 13). If Rhbg, which is assumed to be located basolaterally as in other animals (Verlander et al., 2003), functions as a NH$_3$ gas channel, H$^+$ must enter the gill cell directly or as CO$_2$ which is subsequently hydrated to yield HCO$_3^-$ and protons. Although Nakhoul and colleagues (Nakhoul et al., 2006) suggested that mouse Rhbg transported NH$_4^+$, there is still much debate on what form of ammonia is transported by Rh proteins (Javelle et al., 2007). Also, it is not clear how Na$^+$ leaves the gill cell and enters the bloodstream. Although Na$^+$/K$^+$-ATPase appears to be an appropriate candidate for Na$^+$ transport, other NHE isoforms or Na$^+$–HCO$_3^-$ co-transport cannot be ruled out (Wood and Pärt, 2000; Hirata et al., 2003; Perry et al., 2003; Scott et al., 2005).

Randall and Wright (Randall and Wright, 1987; Randall and Wright, 1989) and Wright et al. (Wright et al., 1989) proposed that ammonia excretion was facilitated by acidification of the gill boundary layer. The acidification was suggested to be partially brought about by hydration of CO$_2$ by carbonic anhydrase present
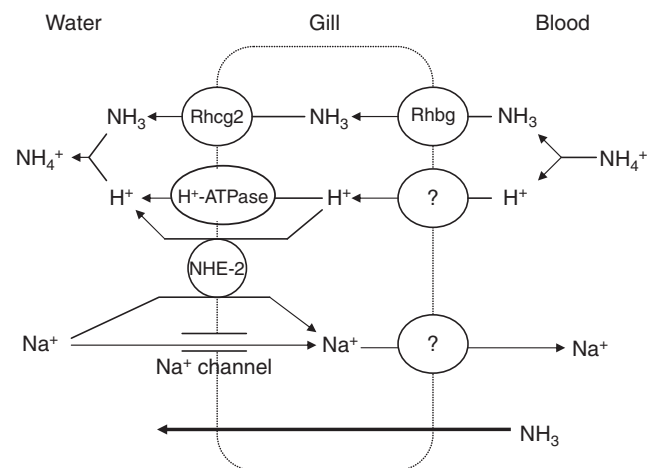
However, in light of knowledge that water pH in the gill boundary layer may be considerably lower than in the bulk water (Wright et al., 1986; Wright et al., 1989; Randall and Wright, 1987; Randall and Wright, 1989; Wilson et al., 1994), this conclusion must remain tentative.

In the present study, the apparent stoichiometry between ammonia excretion and Na$^+$ uptake was 4:1 (Fig. 12A). There are many discrepancies amongst studies on this ratio. Some studies reported a close to 1:1 stoichiometry (Wright and Wood, 1985; McDonald and Prior, 1988), while others noted that the magnitude of change in ammonia excretion was different from that in Na$^+$ uptake (Kirschner et al., 1973; Wilkie and Wood, 1994; Salama et al., 1999) in various experimental scenarios. Different water quality conditions in the wild or during husbandry may affect gill membrane transporter expression. For example, the hardness of water affects H$^+$-ATPase expression (Craig et al., 2007). Both Nakada and colleagues (Nakada et al., 2007b) and the present study have shown that reduced ionic strength of the water affects Rh protein expression, while Nawata and Wood (Nawata and Wood, 2008) reported that water buffering also alters Rh expression. Nawata and colleagues (Nawata et al., 2007) and the present study have shown that ammonia level affects

Fig. 13. Proposed model of a 'Na$^+$/NH$_4^+$ exchange complex' in DSI. The roles of various CA isoforms in ammonia excretion require further investigation; therefore they have not been included in the model. Non-ionic NH$_3$ diffusion, shown by the lower arrow, also plays an important role in ammonia efflux.

Table 3. The influence of exposure to high environmental ammonia (HEA, nominally 2000 μmol l^{-1}, measured=2515±153 μmol l^{-1}) for up to 7 days on plasma total ammonia and cortisol concentrations in live trout in Series 6

	Water [ammonia] (μmol l^{-1})	Plasma [ammonia] (μmol l^{-1})	Plasma [cortisol] (ng ml^{-1})
Control (N=10)	33±5	97±15	10±1
20 h HEA (N=16)	2319±26*	1183±81*,†	163±19*
7 day HEA (N=10)	2260±60*	1079±43*,†	135±17*

The water ammonia concentrations measured at the time of blood sampling are also reported.

*$P<0.05$ relative to corresponding control value; †$P<0.05$ relative to simultaneous water ammonia concentration.

There were no significant differences ($P>0.05$) between 20 h and 7 day values.

in the mucus covering the apical side of the gill (Wright et al., 1986). At first glance, the reduction in carbonic anhydrase mRNA expression in the DSI after ammonia exposure (Fig. 7E) appears to contradict the previous model. However, it should be noted that exactly the same response was seen in rainbow trout gills *in vivo* following HEA (Nawata et al., 2007). The CA-2 investigated in both the present study and that of Nawata and colleagues (Nawata et al., 2007) is the intracellular isoform. It is not known whether the extracellular CA-4 was affected by ammonia exposure. We interpret the present results to indicate that, during ammonia exposure, CA-2 was down-regulated leading to less intracellular H$^+$ production from CO$_2$ hydration, so that H$^+$ from NH$_4^+$ could be preferentially exported by H$^+$-ATPase or NHE-2 (Fig. 13). The roles of various carbonic anhydrase isoforms in ammonia excretion require further investigation; therefore they have not been included in the current model (Fig. 13).

In conclusion, we report that the mRNA expression of different membrane transporters are regulated by ammonia and cortisol, individually as well as synergistically. We have also proposed an apical 'Na$^+$/NH$_4^+$ exchange complex' consisting of several interacting membrane transporters. This model affirms the importance of non-ionic diffusion of NH$_3$ in ammonia excretion. It also explains the coupling phenomenon between ammonia excretion and Na$^+$ uptake. Given the toxicity of ammonia and the constant loss of Na$^+$ in freshwater fish, it is not surprising to find a sophisticated and yet flexible system to deal with ammonia excretion and Na$^+$ uptake.

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