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The giant mudskipper *Periophthalmodon schlosseri* facilitates active NH_4^+ excretion by increasing acid excretion and decreasing NH_3 permeability in the skin

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Summary

Periophthalmodon schlosseri **is an amphibious and obligatory air-breathing teleost, which is extremely tolerant to environmental ammonia. It actively excretes NH_4^+ in ammonia loading conditions. For such a mechanism to operate efficaciously the fish must be able to prevent back flux of NH_3. *P. schlosseri* could lower the pH of 50 volumes (w/v) of 50% seawater in an artificial burrow from pH 8.2 to pH 7.4 in 1 day, and established an ambient ammonia concentration of 10 mmol l^{-1} in 8 days. It could alter the rate of titratable acid efflux in response to ambient pH. The rate of net acid efflux (H^+ excretion) in *P. schlosseri* was pH-dependent, increasing in the order pH 6.0<7.0<8.0<8.5. Net acid flux in neutral or alkaline pH conditions was partially inhibited by bafilomycin, indicating the possible involvement of a V-type H^+-ATPase. *P. schlosseri* could also increase the rate of H^+ excretion in response to the presence of ammonia in a neutral (pH 7.0) external medium. Increased H^+ excretion in *P. schlosseri* occurred in the head region where active excretion of NH_4^+ took place. This would result in high concentrations of H^+ in the boundary water layer and prevent the dissociation of NH_4^+, thus preventing a back flux of NH_3 through the branchial epithelia. *P. schlosseri* probably developed such an 'environmental ammonia detoxification' capability because of its unique behavior of burrow building in the mudflats and living therein in a limited volume of water. In addition, the skin of *P. schlosseri* had low permeability to NH_3. Using an Ussing-type apparatus with 10 mmol l^{-1} NH_4Cl and a 1 unit pH gradient (pH 8.0 to 7.0), the skin supported only a very small flux of NH_3 (0.0095 μmol cm^{-2} min^{-1}). Cholesterol content (4.5 μmol g^{-1}) in the skin was high, which suggests low membrane fluidity. Phosphatidylcholine, which has a stabilizing effect on membranes, constituted almost 50% of the skin phospholipids, with phosphatidyleserine and phsophatidylethanolamine contributing only 13% and 15%, respectively. More importantly, *P. schlosseri* increased the cholesterol level (to 5.5 μmol g^{-1}) and altered the fatty acid composition (increased total saturated fatty acid content) in its skin lipid after exposure to ammonia (30 mmol l^{-1} at pH 7.0) for 6 days. These changes might lead to an even lower permeability to NH_3 in the skin, and reduced back diffusion of the actively excreted NH_4^+ as NH_3 or the net influx of exogenous NH_3, under such conditions.**

Key words: ammonia, excretion, permeability, cholesterol, fatty acid, gill, H^+-ATPase, lipid, membrane, membrane fluidity, mudskipper, *Periophthalmodon schlosseri*, phospholipid, proton pump, skin.

Introduction

The giant mudskipper *Periophthalmodon schlosseri* is an amphibious gobioid teleost, living in the mangrove swamps of Singapore and peninsular Malaysia. It builds burrows on high grounds in the estuarine mud, which are used as a refuge upon disturbance, or to lay eggs and protect the hatching larvae during the breeding season.

P. schlosseri has a very high tolerance of environmental ammonia. It can survive for more than 1 week in 100 mmol l^{-1} NH_4Cl, an ammonia concentration that most other fish species would not survive for a few hours (Peng et al., 1998). It does not produce urea, or store ammonia in its body when exposed to high concentrations of environmental ammonia. Under such

experimental conditions, the plasma ammonia concentration remains low (Peng et al., 1998; Randall et al., 1999). Randall et al. (1999) demonstrated that *P. schlosseri* could actively excrete NH_4^+ even when the blood-to-water NH_3 partial pressure gradient (ΔP_{NH3}) was reversed. Using a pharmacological approach, it was shown that the branchial Na^+/K^+ (NH_4^+)-ATPase and the Na^+/H^+ (NH_4^+) exchanger were involved in the process. Wilson et al. (2000) confirmed the presence of these transporters using immunolocalization techniques.

Active pumping of NH_4^+ is energetically more efficient than turning ammonia into urea or glutamine (Ip et al., 2001; Jow et al., 1999), because only one mole of ATP is required for every two moles of NH_4^+ eliminated (substituting K^+ transport *via* Na^+,K^+-ATPase). However, for such a system to function effectively there must be mechanisms to prevent the back diffusion of NH_3 when the concentration of total ammonia in the environment reaches a level that would impose an inwardly directed ΔP_{NH3}. There are two possible solutions to such a problem. Firstly, *P. schlosseri* may be capable of acidifying the external medium, as suggested by Chew et al. (2003); the excreted NH_4^+ stays in the ionized form and is prevented from penetrating back into the body as NH_3. Secondly, the skin of *P. schlosseri* may have a low permeability to NH_3, despite the fact that cutaneous respiration accounts for 50% of aerial or aquatic respiration (Clayton, 1993), a percentage that is greater than in fishes that respire in water. Indeed, membranes with low NH_3 permeability have been suggested to be present in the apical membrane of the ascending limb of the loop of Henle in the mammalian kidney (Kikeri et al., 1989).

To test the validity of the first possible solution, i.e. acid excretion, attempts were made to measure the pH of the water within the burrows of *P. schlosseri* in its natural habitat in comparison to open waters in the vicinity. We speculated that the pH of the former was lower than that of the latter. Next, in order to show that the fish is the major cause of the decrease in pH of the external medium, efforts were made to verify the capabilities of *P. schlosseri* to actively excrete NH_4^+ to, and lower the pH of, the bulk water in an artificial burrow. To analyze the environmental signals that prompted the increase in acid excretion, effects of environmental pH or ammonia on the rate of acid excretion in *P. schlosseri* were studied. In order to verify that acid excretion in this fish involves a V-type H^+-ATPase, bafilomycin was tested as an inhibitor. Furthermore, we hypothesized that mechanisms involved in NH_4^+ and acid excretions in *P. schlosseri* occurred in close proximity to each other in the head region of the fish. Therefore, experiments were performed with a specimen positioned in an artificial chamber with the head and tail regions separated by a rubber septum just behind the opercula into two compartments, in which waters at various pH values or concentrations of NH_4Cl were introduced.

To test the validity of the second possible solution, i.e. low NH_3 permeability in the skin, $^{15}NH_4Cl$ was introduced into the head or tail compartments in the artificial chamber, and the recovery of ^{15}N-ammonia in the tail or head compartments were analyzed. We hypothesized that the permeability of NH_3 through the surfaces of the head region was greater than that of the tail region of the fish. In a separate experiment, dissected skin was set up across an Ussing-type apparatus to determine the permeation of NH_3 through the skin directly. We also examined the role of skin lipids in mediating ammonia permeability. While carriers like aquaporins can enhance the permeability of membranes to NH_3 (Wood, 1993; Nakhoul et al., 2001), the lower limits of permeability are set by the lipid properties of membranes. The lipid–water partition coefficient for NH_3 is low (Evans and Cameron, 1989; Wood, 1993) suggesting membrane permeability to NH_3 is generally low. In certain biological situations, however, membrane permeability to NH_3 has to be further reduced. For example, the very low NH_3 permeability of the ascending limb of the loop of Henle in the mammalian kidney has been attributed to its membrane lipid composition (Kikeri et al., 1989). Several mechanisms for reducing the permeability of membranes to NH_3 are apparent from model studies. In particular, the cholesterol and phospholipid fatty acid contents of artificial membranes have been shown to affect the permeability or artificial membranes to NH_3 (Lande et al., 1994, 1995). In the face of very high external levels of total ammonia such as those encountered by *P. schlosseri*, reductions of the permeability of skin would help reduce the influx of NH_3. Hence, we analyzed the lipid composition (phospholipids, phospholipid fatty acids and cholesterol) of the skin of *P. schlosseri* to test the hypothesis that its skin would have a lipid composition that would render low NH_3 permeability. In addition, attempts were made to elucidate if *P. schlosseri* was capable of altering the lipid composition of its skin in response to long-term ammonia exposure.

In this report, NH_3 represents unionized molecular ammonia, NH_4^+ represents ammonium ion, and total ammonia refers to the sum of NH_3 and NH_4^+.

Materials and methods

Animals

Specimens of *Periophthalmodon schlosseri* (Pallas 1770) (60–80 g body mass) were collected from Benut, Malaysia. No attempt was made to separate the sexes. They were maintained in 50% seawater (SW; 15‰) in individual transparent plastic tanks (5 l total volume, containing 1 l of water each), at room temperature (27–32°C). The water was changed every second day, and the fish were fed guppies *ad libitum*. 50% seawater was prepared by dissolving Instant Ocean (Aquarium Systems, France) sea salt in dechlorinated tapwater 24 h before use. Salinity was measured using an YSI Model 33 S-C-T meter (Yellow Springs Instrument Co. Inc., Ohio, USA). The mudskippers were acclimated to this condition for at least 3 days before being exposed to experimental conditions. All fish were fasted 24 h prior to experiments to clear the gut and were not fed during the experimental period.

Preparation of SW for experiments

50%SW was aerated for at least 24 h before the addition

of a biological buffer. Trizma-Base (pK_a=8.1, buffering range=7–9) was used to buffer the pH of the 50%SW at 7.0, 7.6, 8.0 or 8.5. 2-(*N*-morpholino)ethanesulfonic acid (MES; pK_a=6.1, buffering range=5.5–6.7) was used to buffer the 50%SW at pH 6.0. The final concentration of buffer used was 2 mmol l^{-1}. After adding the buffer, the pH of the 50%SW was adjusted to the desired value using concentrated HCl or 2 mol l^{-1} NaOH. The buffered water was strongly aerated over night and the pH adjusted accurately again the next day before usage. The pH was determined with an Orion model 420A pH-meter (Boston, MA, USA) and a Corning G-P Combo w/RJ Tris-electrode (Halstead, Essex, UK). Preliminary results obtained indicated that Tris did not affect the ammonia excretory rate in *P. schlosseri*.

Experiment 1: pH of water from natural and artificial burrows

Water samples were collected from six different burrows of *P. schlosseri* on the mudflat and from the adjacent canal (*N*=6) at Pasir Ris, Singapore, during the non-breeding season between July and September 2001. The identity of the burrow was judged by the characteristic fin marks left around the opening of the burrow as a result of the mudskipper's locomotory activities on land. Sampling was made at the water surface or 30 cm deep inside the burrow. The pH and salinity of the water sample were determined by a hand-held pH meter and an YSI Model 33 S-C-T meter (Yellow Spring, OH, USA), respectively.

Specimens (70–80 g, *N*=4) were placed individually in each artificial burrow containing unbuffered 50%SW (pH 8.2). The artificial burrow was constructed using a translucent plastic hose (5 cm i.d.; 260 cm length; Togawa Super Sun-braid hose, Japan), with a plastic box containing a thin layer of water attached to one end, and a rubber stopper at the other. The hose (5000 ml) was bent to a U-shape and mounted on a wood board. The box served as the 'land-surface', with which the fish could have the choice of entering into the artificial burrow or staying on 'land'. The artificial burrows were kept in a dark cabinet such that the behaviour of the fish could be observed with minimal disturbances. A three-way stopcock valves (Connecta, Helsingborg, Sweden) was installed at 170 cm from the opening end of the hose to facilitate the collection of water samples. Water samples were collected at 0 h, 3 h, 6 h and 24 h during the first day of experiment. Subsequently, water samples were taken every 24 h, and the experiment was continued for 8 days in order to verify the steady state concentration of total ammonia build-up inside the burrow by *P. schlosseri*. The pH of the ambient 50%SW was measured using an Orion model 420A-pH meter and a Corning G-P Combo w/RJ Tris-electrode. Ammonia concentration was determined according to Chew et al. (2003).

Experiment 2: Effects of environmental pH or ammonia on the rate of acid excretion

Specimens were placed individually into darkened 1000 ml conical flasks and acclimatized overnight in ordinary 50%SW. After acclimation, specimens were pre-adapted for 1 h in 5 volumes (w/v) of 50%SW containing 2 mmol l^{-1} Tris adjusted to pH 7.0 at 25°C. During pre-adaptation, experimental and recovery periods, strong aeration (using an air stone fully immersed in the medium) was maintained in the conical flask to drive off any CO_2 produced by the fish, thereby removing the acidification effect resulted from the hydration of the excreted CO_2. Preliminary studies revealed that if this was not done, the rates of H^+ excretion would be lower than those reported herein because excreted CO_2 also contributed to the decrease in environmental pH, which in turn influenced the rate of H^+ excretion.

After the pre-adaptation period, the ordinary 50%SW was drained off and buffered 50%SW of pH 7.0 (control condition) was added. After exactly 1 h, this buffered pH 7.0 50%SW was collected in a plastic vial for determination of the baseline rates of acid and ammonia excretion. The same volume of buffered 50%SW containing 2 mmol l^{-1} MES at pH 6.0 or 2 mmol l^{-1} Tris at pH 7.0, 7.6, 8.0 or 8.5 (experimental condition) was immediately added to the conical flask. This buffered 50%SW was again collected in a plastic vial after exactly 1 h for determination of the effects of pH on the rates of acid and ammonia excretion. The fish was rinsed several times with ordinary 50%SW to get rid of remnants of the test solution, and then left to recover overnight in 50%SW with strong aeration in the conical flask. Recovery studies in buffered pH 7.0 50%SW were conducted on the following day.

Preliminary results showed that strong aeration affected the pH of the buffered 50%SW during the experimental or recovery period. Therefore, a flask containing buffered 50%SW of the same pH but without fish was used as a blank for comparison. The blank was strongly aerated to the same extent as the solutions used in the experiments with fish, and the pH recorded after 1 h.

Titration was done using a burette, and water pH was measured using an Orion model 420A pH-meter and a Corning G-P Combo w/RJ Tris-electrode. The titrant used was 0.01 mol l^{-1} NaOH or 0.01 mol l^{-1} HCl. The pH of the media in the presence of fish was titrated back to those of the corresponding blank values. Preliminary results obtained revealed that the titratable acid flux obtained using this method was similar to that obtained by titration to pH 4.0, as prescribed in the method of McDonald and Wood (1981).

For total ammonia analysis, water samples were acidified to pH 2.0 with 1 mol l^{-1} HCl to keep the ammonia present in its ionized form (NH_4^+). The ammonia concentrations of water samples were analyzed using a Tecator Aquatec System (Hoganas, Sweden) equipped with an Ammonium Cassette. The resolution power of the Aquatec Analyzer was tested by calibrations at three different concentrations (30, 31 and 32 mmol l^{-1}) of standard NH_4Cl solution. The calculated concentrations (*N*=5) from the readings obtained were 30.16±0.26, 30.96±0.34 and 32.12±0.23 mmol l^{-1}, respectively, which were significantly different from each other.

Net acid flux (μmol h^{-1} g^{-1} fish) was obtained by summing the fluxes (μmol h^{-1} g^{-1} fish) of titratable acid and total ammonia, because a portion of the excreted acid (H^+) would react with the ammonia excreted as NH_3 to form NH_4^+.

To verify that a V-type H^+-ATPase was involved, specimens (26–34 g) were exposed to pH 7.0 (*N*=3) or pH 9.0 (*N*=3) in the presence of bafilomycin A1 (Sigma Chemical Co.) for 1 h during the experimental condition (using a 500 ml conical flask). Six fish were consecutively tested in the same 120 ml volume of buffered (10 mmol l^{-1} Tris) medium containing 8 μmol l^{-1} of bafilomycin. Bafilomycin was dissolved in dimethysulphoxide (DMSO) before mixing with the 120 ml of buffered 50%SW. A control medium without bafilomycin, but containing 0.17% DMSO, was used to test whether DMSO has any effect on the rates of H^+ and ammonia excretion by the fish. At the end of the 1 h period, the pH of the bafilomycin-containing medium was recorded and 0.05 ml of the medium was collected for total ammonia assay. The pH of the remaining medium was then adjusted back to the desired value (either pH 7.0 or 9.0) before putting the next fish into it.

To evaluate the effects of environmental ammonia alone on the rate of net acid flux, specimens were pre-adapted for 1 h at pH 7.0 following the acclimation period. For the control condition, the fish was exposed to buffered 50%SW at pH 7.0 or 8.0 for 1 h. For the experimental condition, the fish was exposed to buffered 50%SW at pH 7.0 or 8.0 containing 10, 20 or 30 mmol l^{-1} NH_4Cl. The titratable acid flux and ammonia flux were determined in order to obtain the net acid flux. Again, flasks containing the test media with buffer, but without fish, served as blanks.

Experiment 3: Ammonia and H^+ fluxes in the head or tail portion of specimens in two half chambers separated by a rubber septum

Specimens were anaesthetized with neutralized MS 222 (Sigma Chemical Co., USA) at a final concentration (w/v) of 0.125% for 10 min. The anesthetized specimen was positioned in a special chamber with the rubber septum, creating a partition between the head and the tail of the fish. The apparatus was fabricated locally using transparent plastic. The volumes of the 'head' and the 'tail' compartments were 100 ml and 400 ml, respectively. Preliminary tests using $KMnO_4$ proved that the set-up was leak-proof for a minimum of 24 h. The fish was left to acclimatize in the chambers in ordinary 50%SW for at least 2 h. After that, the 50%SW in the compartments was replaced with 50%SW containing Tris alone (pH 7.0 or pH 8.0) or 50%SW containing Tris (pH 7.0 or 8.0) and 30 mmol l^{-1} NH_4Cl. Water samples were taken at the start of the experiment (0 h) and at 24 h. The pH and total ammonia concentration in the water samples were determined as described above.

To evaluate the effects of ammonia exposure on ammonia and H^+ fluxes in the head and tail regions, specimens were exposed to 30 mmol l^{-1} NH_4Cl in a plastic aquarium for 6 days, with renewal of the external medium every 2 days. They were then thoroughly rinsed with Tris-50%SW, anesthetized and placed individually in the compartment chamber to determine ammonia and H^+ fluxes under various experimental conditions.

Experiment 4: The flux of ^{15}N-ammonia through the head or tail regions of live specimens

Experiments were performed using the compartment chambers and $^{15}NH_4Cl$ (99.7%, Boehringer, Germany). The 50%SW in the compartments was replaced with Tris–50%SW (pH 7.0 or pH 8.0) alone or Tris–50%SW (pH 7.0 or pH 8.0) containing 5, 10 or 20 mmol l^{-1} $^{15}NH_4Cl$. One water sample was taken immediately at 0 h and another after 6 h. Due to the limited amount of $^{15}NH_4Cl$ available at the time of the experiments, results were obtained as average of duplicates for each experimental condition only.

^{15}N- and ^{14}N-NH_3 were released from samples using 0.1 mol l^{-1} borate buffer (pH 10.4) and trapped on slips of glass filter paper (Whatman CF/C) in glass vials (22 ml) according to the method of Iwata and Deguchi (1995). ^{15}N-measurement was performed with a quadrupole type mass spectrometer (QP 2000, Shimadzu, Japan). The ion source temperature was maintained at 250°C under vacuum conditions for sample analysis. The ratio of ^{14}N-ammonia and ^{15}N-ammonia in each water sample was determined and the concentration of ^{15}N-ammonia calculated and expressed as % atom excess. Calculation of % atom excess was as follows (Constantin and Schnell, 1990): $(Ratio_{sample}–Ratio_{standard})\times 100$, where $Ratio=^{15}NH_3/(^{14}NH_3+^{15}NH_3)$.

Known concentrations of $^{14}NH_4Cl$ or $^{15}NH_4Cl$ (50–100 μg) were used to test the recovery efficiency of the method used above. Ammonia liberated on the glass filter paper was introduced directly to the mass spectrometer. The recovery was 98.9%±0.2 (*N*=6).

Experiment 5: Transepithelial ammonia flux across the dissected skin

A portion of the fish skin (about 4 cm^2) was carefully descaled, dissected out from a freshly killed specimen, rinsed with saline solution (0.9% NaCl, with 2 mmol l^{-1} Hepes buffer; this same composition was used throughout the experiment), and immediately mounted in an Ussing-type apparatus. The apparatus was made of two separate pieces of Plexiglass, between which, the skin could by mounted with two rubber O-rings. The chambers had a total volume of 13 cm^3 and a circular opening of 3.14 cm^2 (2 cm diameter).

After mounting the skin, both half chambers were filled with saline solution (pH 7.0), and the skin was allowed to acclimatize to this artificial condition for 10 min. Subsequently, the solution was replaced, with one half-chamber being filled with saline solution at pH 7.0 alone, and the other half with saline solution containing 10 mmol l^{-1} NH_4Cl at pH 8.0. There were two gradients in this setup: (1) a tenfold NH_4Cl gradient and (2) a 1-unit pH gradient. In this way, an ammonia concentration gradient was set up across the skin, and any NH_3 diffusing down the chemical potential

gradient would be trapped as NH_4^+. Mixing of the media in the two half chambers was achieved by two streams of air currents directed at the surface of the media through two Pasteur pipettes, which also ensured oxygenation of the media. A sample (100 μl) was collected every 10 min for a period of 1 h, and immediately acidified with 5 μl of 2 mol l^{-1} HCl. Total ammonia content was determined later colorimetrically according to the method of Chew et al. (2003). By reversing the ammonia gradient across the skin, both NH_3 efflux (serosa–mucosa) and influx (mucosa–serosa) could be determined.

Since *P. schlosseri* is 'amphibious', with the skin highly adapted for cutaneous respiration in water and on land (Clayton, 1993), experiments were also performed on the skin of the frog *Rana catesbiana* (100–120 g), obtainable commercially from markets in Singapore, for comparison. Naturally, the skins of these animals had different thickness (x), which directly affects the concentration (c) gradient ($\Delta c/\Delta x$). The thickness (μm) of the skins was determined using a Leica TCS-SP2 RS Confocal Laser Scanning Microscope (Tokyo, Japan) at 488 nm with a reflection model 10× objective lens. Since flux = $-P(\Delta c/\Delta x)$, where P is the permeability constant, the NH_3 flux across the skins of these animals cannot be compared directly, but because Δc was constant in these experiments, it can be incorporated into P. Hence, in this study, permeability constants, calculated as flux (Δx), i.e. μmol min^{-1} cm^{-2} (10^{-4} cm), or 10^{-4} μmol min^{-1} cm^{-1}, were compared instead.

Experiment 6: The lipid composition of the skin

Specimens were exposed to 50%SW containing 10 mmol l^{-1} Tris (pH 7.0) with (ammonia exposed) or without (control) 30 mmol l^{-1} NH_4Cl for 6 days. The external medium was renewed daily. No food was provided throughout the experimental period. After 6 days, specimens were killed with a strong blow to the head. The lateral skin was dissected immediately, with special care not to sample any muscle, rinsed in saline solution and frozen in liquid nitrogen. Samples were stored at –80°C until analysis.

Lipids were extracted from the skin according to the method of Folch et al. (1957). Phospholipids were isolated from the neutral lipids by acetone precipitation using a modified technique based on the procedure of Hoevet et al. (1968). The neutral lipids (cholesterol is part of this fraction) and phospholipid fractions were dried separately under nitrogen.

For the determination of cholesterol, the neutral lipids were redissolved in 1 ml of isopropanol per gram of skin (w/w). Thereafter, 10 ml of sample were assayed for cholesterol using a Sigma Diagnostics Kit #401 (St Louis, MO, USA).

The dried phospholipids were resuspended in 0.2 ml g^{-1} tissue (w/w) of chloroform:methanol (1:1), and the phospholipids separated on silica-coated chromatography plates (Z29297-4, Merck, Darmstadt, Germany) using a solvent system consisting of chloroform:methanol:acetic acid:water, 100:75:7:4 (v:v:v:v). Developed plates were exposed to iodine vapour for visualization of phospholipid spots. Six phospholipid classes were detected and completely separated. The total amount of phospholipids (Tot PLs) in each class was estimated by summing the different fatty acids recovered in that class. Fatty acid composition of individual phospholipid classes was analyzed according to the methods of Gillis and Ballantyne (1999). Methylated fatty acids were analyzed using a chromatography column (J & W DB-225MS, Folsom, CA, USA; Cat. # 122-2932, dimension – 30 m length × 0.2 mm i.d. × 0.25 μm film thickness) in a gas chromatograph (HP 6890; Hewlett Packard-Agilent, Toronto, ON, Canada) fitted with a flame ionization detector (FID, HP 6890 series) and an automatic injector (HP 6890 series Split/Splitless Inlet). The column oven temperature was programmed with an initial increase from 150°C to 210°C in the first 6 min followed by isothermal separation at 210°C for 30 min. Fatty acids were identified by comparison of retention times of a known standard containing all fatty acids of interest. Chain lengths shorter than C:14 were not resolved under the present conditions, and thus are not reported.

Statistical analysis

Results are presented as means ± standard error (S.E.M.). Differences between means were evaluated by one-way analysis of variance (ANOVA) followed by Student–Neuman–Keul's multiple range test or two-tail Student's *t*-test, where applicable. Any difference where $P<0.05$ was regarded as statistically significant.

Results

pH and salinity of water sampled from the mudskipper's burrow on the mudflat

The pH and salinity of samples collected from the surface water in the burrow (N=6) were 7.05±0.08 and 8±2‰, respectively. The respective values for samples collected 30 cm below the water surface inside the burrow (N=6) were 6.89±0.03 and 11±3‰. Water samples (N=6) collected at the same time in the adjacent canal had a pH of 7.84±0.02 and a salinity of 10±2‰.

The ammonia concentrations in water samples collected from the surface water and 30 cm below the water surface in the burrow were 2.67±0.34 and 2.98±0.27 mmol l^{-1}, respectively. For water collected from the adjacent canal, the ammonia concentration was 0.034±0.003 mmol l^{-1}, which was significantly lower than those in the burrow water.

Capability of P. schlosseri to alter the pH of the medium and actively excrete ammonia into it within an artificial burrow

In the presence of a specimen of *P. schlosseri*, the pH of the 50%SW in the artificial burrow changed significantly from pH 8.2 to pH 7.0 within 24 h. (Fig. 1A). A decrease in pH of about 0.60 units was observed in the burrow during the first 3 h (Fig. 1A). The ammonia concentration in the ambient 50%SW in the artificial burrow reached 1 mmol l^{-1} after 1 day (Fig. 1B). By day 6, the ambient ammonia concentration increased to 10 mmol l^{-1} with no further changes thereafter.

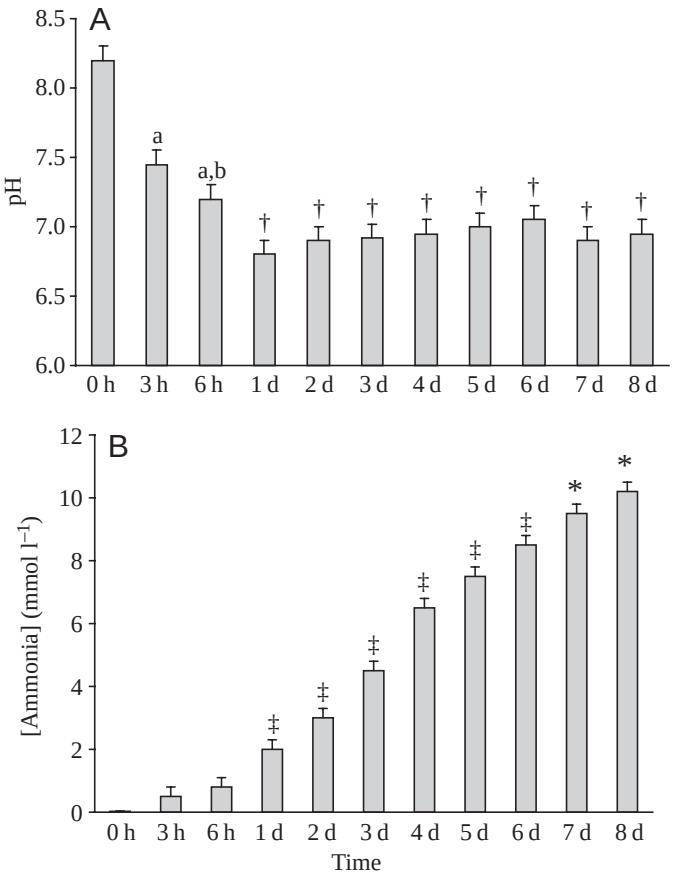

Fig. 1. A time course study on the (A) pH and (B) concentration of ammonia of the ambient seawater at a specific position of the artificial burrow in which *P. schlosseri* was exposed. d, days. Values are means ± S.E.M. [a]Significantly different from the corresponding 0 h values, $P<0.05$; [b]significantly different from the corresponding 3 h values, $P<0.05$; [†]significantly different from the corresponding 0, 3 and 6 h values, $P<0.05$; [‡]significantly different from all the preceding values, $P<0.05$; *significantly different from values 0 h to 5 days, $P<0.05$.

Proton excretion in response to environmental pH or ammonia

There was an apparent influx of titratable acid to the fish when the specimen was exposed to pH 6.0 (Fig. 2). In contrast, at pH 7.0, 7.6, 8.0 or 8.5, there were significant increases in the titratable acid flux to the medium (Fig. 2), increasing in the order pH 7.0<7.6<8.0<8.5. Alkaline pH had no effect on ammonia excretion in this mudskipper; the ammonia excretion rates at pH 8.5 or 8.0 were comparable to that at pH 7.0 (Fig. 2). As a result, the net acid efflux in *P. schlosseri* increased when the mudskipper was exposed to alkaline pH (Fig. 2). Between pH 7.0 and pH 8.5, the net acid flux appeared to vary linearly with the change in the pH in the external medium. Bafilomycin exhibited a large inhibitory effect on the net acid flux of specimens exposed to pH 7.0 or 9.0 (Fig. 3).

When exposed to pH 7.0 in the presence of 20 or 30 mmol l^{-1} NH_4Cl, the net acid fluxes were significantly higher than the respective control values (Fig. 4). Increases in net acid flux were observed in specimens exposed to 10, 20 or 30 mmol l^{-1} NH_4Cl at pH 8.0.

Ammonia and H^+ excretion in the head or tail regions of P. schlosseri

Ambient total ammonia concentration in the head compartment increased when specimens were exposed to increasing NH_3 concentrations (achieved *via* increasing pH) in the body compartment (Table 1). Preliminary experiments showed that specimens exposed to 30 mmol l^{-1} NH_4Cl at pH 9.0 in the tail compartment died after 6 h. The final pH of the media in the head or tail compartments was comparable to the initial pH values. With increasing NH_3 concentration (because of the increasing pH) in the tail compartment, ammonia excretion through the head region still occurred despite the presence of 30 mmol l^{-1} NH_4Cl in the head compartment (Table 2). *P. schlosseri* was able to drastically alter the pH of the medium in the head compartment but not that in the tail compartment (Tables 1, 2).

Table 1. *Ammonia concentration in the external medium in the head or body compartments of the chamber in which* P. schlosseri *was exposed for 24 h to 50%SW at pH 7.0 in the head compartment and 0 or 30 mmol l^{-1} NH_4Cl at pH 7.0 or pH 8.0 in the tail compartment*

		Condition		[Ammonia] (mmol l^{-1}) in the external medium			
Set	Compartment	pH	[NH_4Cl] (mmol l^{-1})	0 h	6 h	24 h	pH after 24 h
1	Head	7	0	0.021±0.006	1.8±0.2[a]	7.6±0.8[a,b]	7.12±0.12
	Tail	7	0	0.024±0.005	0.11±0.03	0.69±0.23[a]	7.02±0.11
2	Head	7	0	0.021±0.005	2.3±0.3[a]	8.2±0.3[a,b]	7.06±0.08
	Tail	7	30	29.7±0.8	29.5±0.7	29.4±0.8	7.03±0.07
3	Head	7	0	0.023±0.004	5.0±1.2[a,1]	19.5±2.5[a,b,1,2]	7.04±0.06
	Tail	8	30	31.2±0.8	30.5±0.9	28.9±1.1	7.82±0.10

Values are means ± S.E.M. (N=5).

[a]Significantly different from the 0 h value; [b]significantly different from the 6 h value; [1]significantly different from the corresponding set 1 value; [2]significantly different from the corresponding set 2 value.

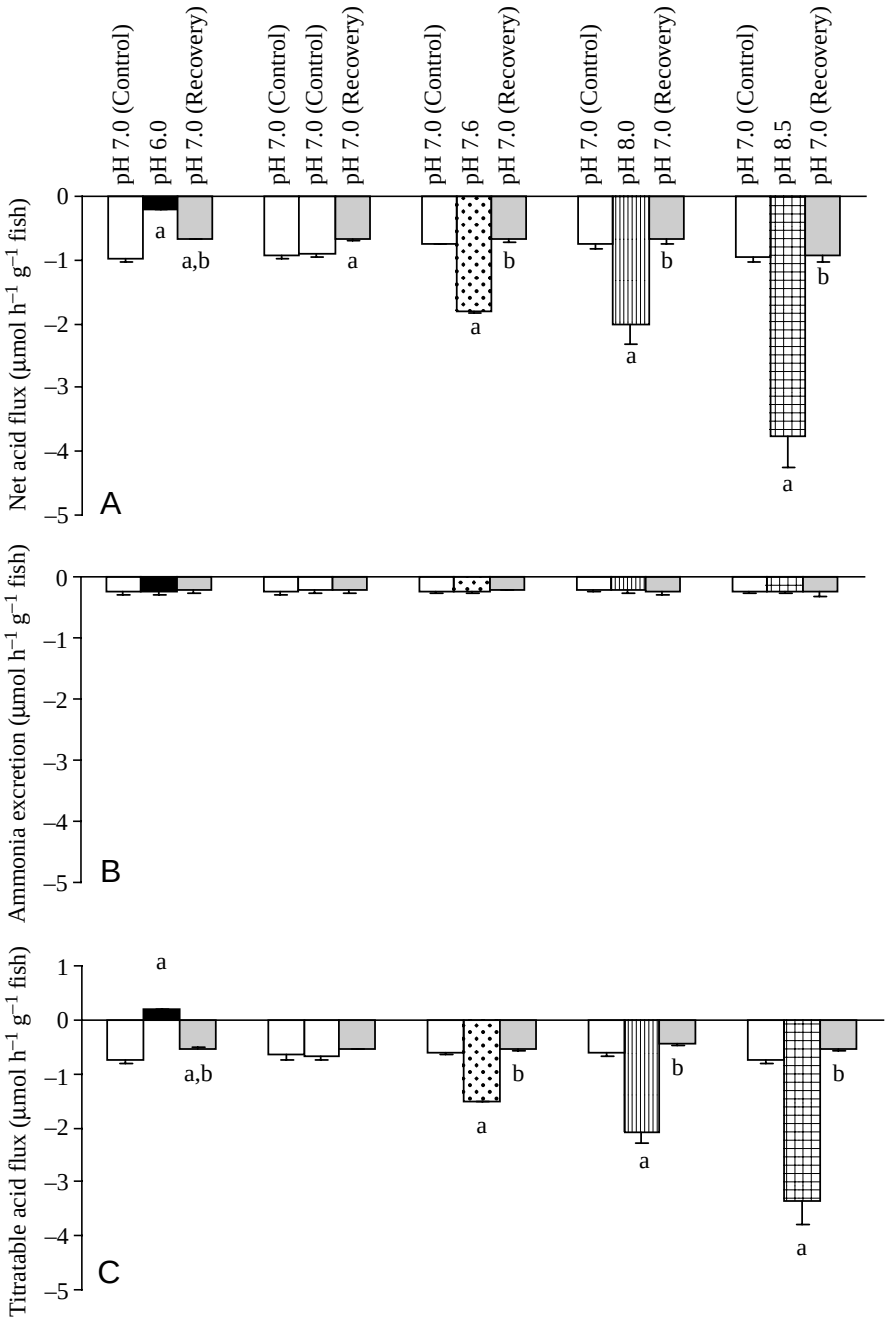

Fig. 2. (A) Net acid flux, (B) ammonia flux and (C) titratable acid flux (μmol h^{-1} g^{-1} fish, N=4) in *P. schlosseri* exposed to pH 7.0 (Control and Recovery) and various other pH values in 50% seawater. Values are means ± S.E.M. [a]Significantly different from the corresponding control (pH 7.0) value, P<0.05; [b]significantly different from the corresponding experimental condition, P<0.05.

The total ammonia concentrations built up in the head compartment containing specimens subjected to prior exposure to 30 mmol l^{-1} NH_4Cl at pH 7.0 for 6 days (Table 3) were lower than in those containing specimens not subjected to prior ammonia exposure (Tables 1, 2).

Permeation of $^{15}NH_4Cl$ through the head or tail regions of P. schlosseri

When specimens were exposed to $^{15}NH_4Cl$ at pH 7.0 or 8.0 in the head compartment, ^{15}N-ammonia enrichment (expressed as % atom excess of ^{15}N) was detected in the external medium in the tail compartment after 6 h (Table 4). In contrast, when $^{15}NH_4Cl$ was added to the tail compartment containing medium at pH 7.0, no ^{15}N-ammonia was detected in the medium in the head compartment, except with 20 mmol l^{-1} NH_4Cl (Table 5). However, with a pH 8.0 medium in the tail compartment, a greater enrichment of ^{15}N-ammonia was observed in the medium in the head compartment (Table 5).

NH_3 flux through dissected skin in an Ussing-type apparatus

The skin of *P. schlosseri* (average thickness 132 μm) was symmetrical with respect to NH_3 permeability. The NH_3 fluxes (N=5) from mucosa to serosa (0.0105±0.0005 mmol min^{-1} cm^{-2}) and from serosa to mucosa (0.0096±0.0007 mmol min^{-1} cm^{-2}) were comparable. The permeability constant was calculated to be 1.33×10^{-4} mmol min^{-1} cm^{-1}.

The NH_3 flux across the skin of *R. catesbiana* (average thickness 440 μm) was 0.0054±0.0011 mmol min^{-1} cm^{-2} (N=3). The permeability constant was 2.38×10^{-4} mmol min^{-1} cm^{-1}, which was 1.8-fold that of *P. schlosseri*.

The lipid compositions in the skin of P. schlosseri

Six classes of phospholipids were detected from the skin of *P. schlosseri*, namely phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidylethanolamine (PE), cardiolipin (CL) and sphingomyelin (SM). PC was the most abundant phospholipid class, comprising almost half of the total phospholipid content in the skin (Table 6). The next highest were PS and PE, contributing about 15% each, followed by SM with 13%. The two minor categories were PI and CL, with 5 and 1%, respectively. The skin of *P. schlosseri* had a high cholesterol content (Table 7). The ratio of cholesterol:phospholipids was 6.2±0.3 (N=5).

In these lipids, the amount of total saturated fatty acids was twice that of the total monounsaturated fatty acids, which in turn were twofold higher than that of the total polyunsaturated fatty acids (Table 7). Among all the phospholipids determined in this study, the most abundant

fatty acid was the saturated fatty acid 18:0 (about 29%), followed by the monounsaturated fatty acid 18:1 (23%), and by another saturated fatty acid, 16:0 (17%) (Table 7). The major saturated fatty acids recovered were 18:0 and 16:0 (52% and 34% of the total saturated fatty acids, respectively). For monounsaturated fatty acids, 18:1 was the major fatty acid (84% of the total monounsaturated fatty acids), followed by 16:1 (12%). All others represented only 1.5% or less of this class. Among the polyunsaturated fatty acids (PUFAs), 20:4(n-6) was the main representative (37% of the total PUFAs), followed by 18:2(n-6) and 22:4(n-6) (22% and 18%, respectively). A few fatty acids were absent in this class, e.g. 20:3(n-3) and 22:2(n-6), or present only in traces (less than 1 nmol g^{-1}), e.g. 18:3(n-3) and 20:2(n-6). Specifically, among the PUFAs, n3 was completely absent from SM and PI in the control specimens. Cardiolipin also lacked n3 PUFAs in the control fish (Table 8), despite its being an exclusively mitochondrial phospholipid.

Effects of ammonia exposure on lipid compositions in the skin

The cholesterol content in the skin increased significantly to 5.5±0.03 μmol g^{-1} after specimens were exposed to 30 mmol l^{-1} NH_4Cl for 6 days (Table 7). Ammonia exposure led to a significant decrease in SM, from 13.3% to 12.7% (Table 6), but had no effect on the content of other phospholipids. Exposure to 30 mmol l^{-1} NH_4Cl for 6 days led to significant increases in the amounts of total fatty acids in the phospholipid fraction, the total saturated fatty acids and the total monounsaturated fatty acids, but not the PUFAs (Table 7). With respect to the percentages of various fatty acids within a certain class of phospholipid in the skin of *P. schlosseri*, changes were detected in the PC and PI fractions (Table 8). In PC, the total PUFAs increased from 11.3 to 13.1%, and total n6 PUFAs from 9.4% to 10.8%. In PI, both total polyunsaturated fatty acids and n6 PUFAs decreased (from 30.4% to 26.4%).

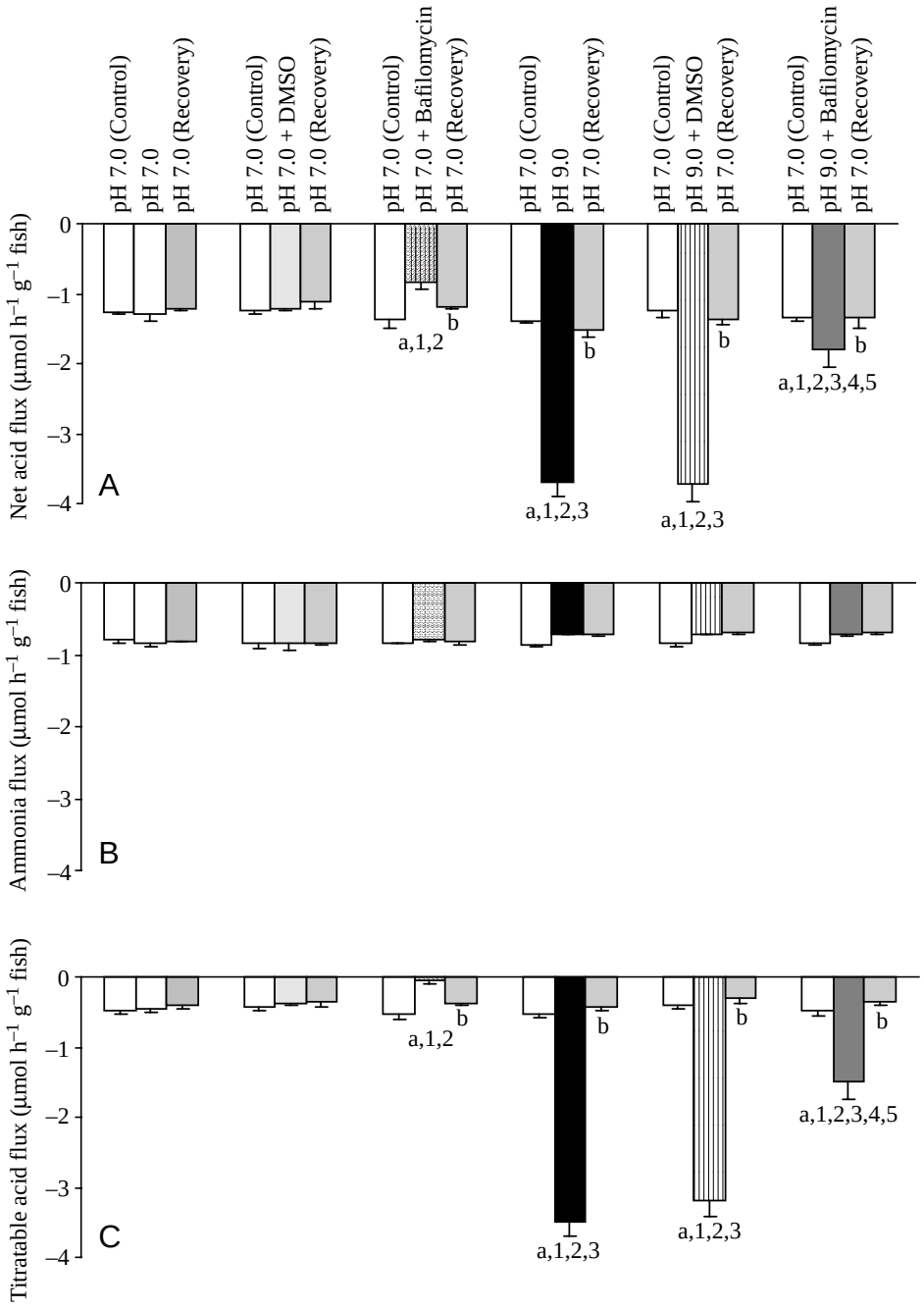

Fig. 3. (A) Net acid flux, (B) ammonia flux and (C) titratable acid flux (μmol h^{-1} g^{-1} fish, N=4) in *P. schlosseri* exposed to 8 μmol l^{-1} bafilomycin in 50% seawater containing 10 mmol l^{-1} Tris at pH 7.0 or pH 9.0, pH 7.0 (Control), pH 7.0+DMSO, pH 7.0+Bafilomycin, pH 9.0, pH 9.0+DMSO, pH 9.0+Bafilomycin, and pH 7.0 (Recovery). Values are means ± S.E.M. [a]Significantly different from the corresponding control (pH 7.0) value, P<0.05; [b]significantly different from the corresponding experimental value, P<0.05; [1]significantly different from the experimental pH 7.0 value, P<0.05; [2]significantly different from the experimental pH 7.0+DMSO value, P<0.05; [3]significantly different from the experimental pH 7.0+Bafilomycin value, P<0.05; [4]significantly different from the experimental pH 9.0 value, P<0.05; [5]significantly different from the experimental pH 9.0+DMSO values, P<0.05.

Discussion

P. schlosseri *could lower ambient pH and increase the ammonia concentration in the artificial burrow*

The significantly higher total ammonia concentration in the burrow water as compared with that in the adjacent estuarine water was likely to result from the excretion of ammonia by *P. schlosseri*. This is confirmed by the results obtained with artificial burrows in the laboratory. During the non-breeding season, *P. schlosseri* only makes brief stays in the burrow during high tides. However, after eggs are laid inside the burrow during the breeding season between April and May, the mudskipper stays therein to take care of the developing embryos. Hence, the ammonia concentrations of the burrow water during the breeding period can be much higher than 2.8 mmol l^{-1}. Ammonia is toxic, and fishes can rarely tolerate >2 mmol l^{-1} total environmental ammonia at pH 8.2 (Ip et al., 2001). The difference in pH between the water in the canal and that inside the mudskipper burrow indicates that *P. schlosseri* was capable of adjusting the pH of a finite volume of external medium, confirming the observations made by Chew et al. (2003) in the laboratory. Taken together, these field results suggest that there could be a relationship between environmental ammonia tolerance in and manipulation of environmental pH by *P. schlosseri*.

In the laboratory, we confirmed that *P. schlosseri* was capable of lowering the pH of the water in the artificial burrow and elevating the total ammonia concentration therein. At the beginning, the concentration of total ammonia in the 50%SW inside the artificial burrow was close to zero, and it can be deduced that the specimen altered the pH of the external medium solely as a response to the alkalinity of the medium (pH 8.2). Ammonia excretion at this stage might be achieved through NH_3 diffusion and 'NH_3 trapping' (Wilkie, 1997). Surprisingly, within 8 days, a single specimen of *P. schlosseri* was able to establish the ambient total ammonia concentration inside the artificial burrow to 10 mmol l^{-1}. In nature, burrows of *P. schlosseri* are constructed on high ground and subjected to tides only 2–3 days a year

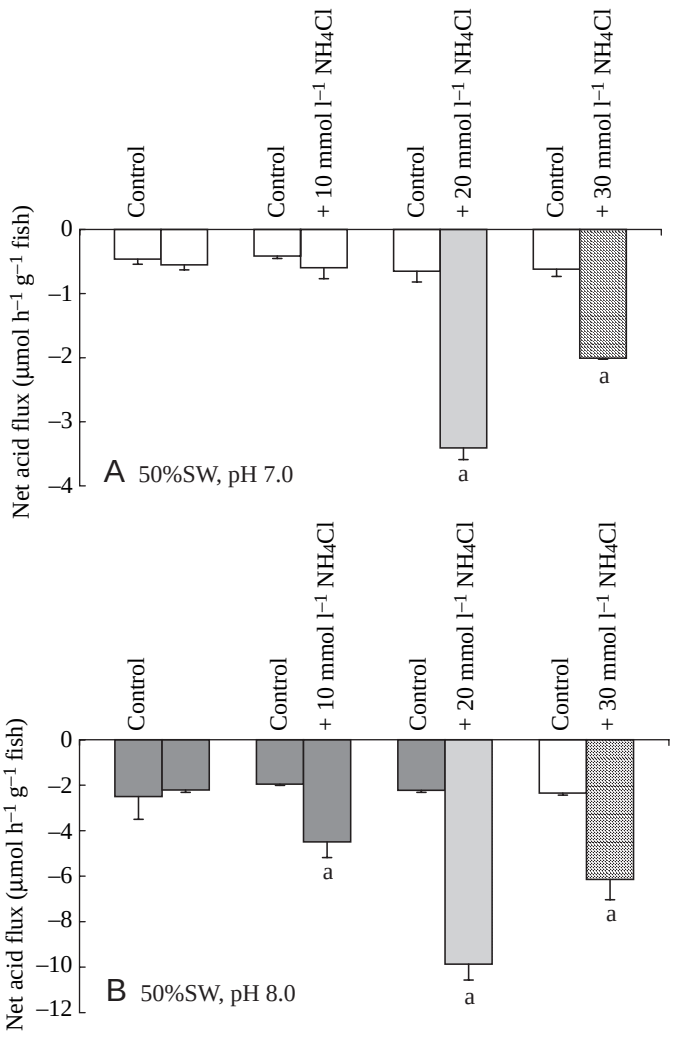

Fig. 4. Net acid flux (μmol h^{-1} g^{-1} fish, *N*=4) in *P. schlosseri* exposed to (A) 50% seawater (SW) pH 7.0 or (B) 50%SW pH 8.0, containing various concentrations (10, 20 or 30 mmol l^{-1}) of NH_4Cl. Values are means ± S.E.M. [a]Significantly different from the corresponding control (pH 7.0) values, $P<0.05$.

Table 2. *Ammonia concentration in the external medium in the head or body compartments of the chamber in which* P. schlosseri *was exposed for 24 h to 50%SW containing 30 mmol l^{-1} NH_4Cl at pH 7 or pH 8 in both the head and tail compartments*

		Condition		[Ammonia] (mmol l^{-1}) in the external medium			
Set	Compartment	pH	[NH_4Cl] (mmol l^{-1})	0 h	6 h	24 h	pH after 24 h
1	Head	7	30	28.7±0.5	29.8±0.4[a]	38.1±3.1[a,b]	6.91±0.08
	Tail	7	30	30.1±0.6	29.9±0.5	29.7±0.6	7.05±0.06
2	Head	7	30	29.3±0.9	33.7±1.8[a]	42.6±2.8[a,b]	6.93±0.07
	Tail	8	30	30.8±0.4	30.1±0.9	29.2±0.9	7.85±0.08
3	Head	8	30	29.8±1.0	31.6±1.2[a]	45.1±1.3[a,b]	7.28±0.06
	Tail	8	30	31.0±1.1	29.8±0.9	28.5±0.8	7.88±0.09

Values are means ± S.E.M. (*N*=5).
[a]Significantly different from the 0 h value; [b]significantly different from the 6 h value.

Table 3. *Ammonia concentration in the external medium in the head or tail compartments of the chamber in which* P. schlosseri *was exposed for 24 h to 0 or 30 mmol l^{-1} NH_4Cl at pH 7.0 in the head compartment and 0 or 30 mmol l^{-1} NH_4Cl at pH 7.0 or pH 8.0 in the tail compartment*

		Condition		[Ammonia] (mmol l^{-1}) in the external medium			
Set	Region	pH	[NH_4Cl] (mmol l^{-1})	0 h	6 h	24 h	pH after 24 h
1	Head	7	0	0.043±0.005	1.1±0.1	3.2±0.3[a,b]	7.04±0.06
	Tail	7	0	0.042±0.006	0.049±0.006	0.53±0.21[a,b]	7.01±0.04
2	Head	7	0	0.038±0.004	1.0±0.2[a]	3.0±0.2[a,b]	7.01±0.05
	Tail	7	30	30.5±0.3	29.8±0.5	29.4±0.2	7.12±0.06
3	Head	7	30	30.1±0.3	30.9±0.4	33.3±0.5[a,b]	6.82±0.03
	Tail	7	30	30.1±0.2	29.8±0.3	29.2±0.2	7.09±0.04
4	Head	7	30	30.1±0.2	33.5±0.3[a]	39.3±0.6[a,b]	6.85±0.04
	Tail	8	30	30.1±0.3	29.0±0.2	28.3±0.3	7.86±0.05

Specimens were exposed to 30 mmol l^{-1} NH_4Cl at pH 7.0 for 6 days prior to the chamber experiment.
Values are means ± S.E.M. (*N*=5).
[a]Significantly different from the 0 h value; [b]significantly different from the 6 h value.

Table 4. *The ambient ammonia concentration and ^{15}N-ammonia in the tail compartment when the tail of* P. schlosseri *was exposed for 6 h to 5, 10 or 20 mmol l^{-1} $^{15}NH_4Cl$ in the head compartment*

		Tail compartment			
Condition	Head compartment	[Ambient ammonia] (mmol l^{-1})		^{15}N-ammonia (% atom excess)	
pH	[NH_4Cl] (mmol l^{-1})	0 h	6 h	0 h	6 h
7.0	5	0.04	0.16	0	73
	10	0.04	0.18	0	83
	20	0.04	0.64	0	69
8.0	5	ND	0.06	0	54
	10	ND	0.09	0	18
	20	ND	1.17	0	45

ND, not detectable.

Table 5. *The ambient ammonia concentration and ^{15}N-ammonia in the head compartment when the tail of* P. schlosseri *was exposed for 6 h to 5, 10 or 20 mmol l^{-1} $^{15}NH_4Cl$ in the tail compartment*

		Head compartment			
Condition	Tail compartment	[Ambient ammonia] (mmol l^{-1})		^{15}N-ammonia (% atom excess)	
pH	[NH_4Cl] (mmol l^{-1})	0 h	6 h	0 h	6 h
7.0	5	0.01	0.53	0	0
	10	0.02	1.99	0	0
	20	0.02	2.34	0	54
8.0	5	ND	0.56	0	26
	10	ND	2.00	0	60
	20	ND	2.32	0	75

ND, not detectable.

(Clayton, 1993); removal of ammonia would be difficult in the absence of tidal inundation. The ability to excrete ammonia against a concentration gradient allows *P. schlosseri* to survive in a 'closed' environment where ammonia could build up to high concentrations, especially during the breeding seasons when eggs are developing therein.

Table 6. *Fatty acid content in each of the six phospholipid classes from the skin of the tail portion of* P. schlosseri *exposed for 6 days to 50%SW with or without (control) 30 mmol l^{-1} NH_4Cl*

	Fatty acid content (% of total)	
	Control	Ammonia exposed
SM	13.3±0.3	12.7±0.3*
PC	49.0±2.0	45.0±1.2
PS	15.7±1.7	20.4±0.3
PI	5.43±0.22	5.61±0.32
PE	15.1±0.4	14.6±1.0
CL	1.48±0.16	1.68±0.32

PC, phosphatidylcholine; PS, phosphatidylserine; PI, phosphatidylinositol; PE, phosphatidylethanolamine; CL, cardiolipin; SM, sphingomyelin.

Values are means ± S.E.M. (*N*=5).

*Significantly different from the corresponding control value.

Table 7. *Different phospholipid parameters in the skin from the tail portion of* P. schlosseri

	Control	Ammonia exposed
[Cholesterol] (μmol g^{-1})	4.50±0.20	5.50±0.03*
Tot PLs (nmol g^{-1})	738±11	833±7*
Tot sat (nmol g^{-1})	393±5	444±4*
Tot mono (nmol g^{-1})	214±1	228±2*
Tot poly (nmol g^{-1})	132±5	161±3
n3 (nmol g^{-1})	20.1±1.5	27.6±0.9
n6 (nmol g^{-1})	111±3	134±3
n3/n6 ratio	0.815±0.033	0.912±0.037
Mono/poly ratio	8.63±0.63	6.06±0.11
Unsat index	844±25	864±14
Chain length	162±2	158±1
PC/PE ratio	3.41±0.08	3.35±0.13

Tot PLs, total fatty acids in the phospholipid fraction; Tot sat, total saturated fatty acids; Tot mono, total monounsaturated fatty acids; Tot poly, or PUFAs, total polyunsaturated fatty acids; n3, n3 PUFAs; n6, n6 PUFAs; mono, monounsaturates; poly, polyunsaturates; Unsat index, unsaturation index; PC/PE ratio, ratio between phosphatidylcholine and phosphatidylethanolamine.

Specimens were exposed to 50%SW with or without (control) 30 mmol l^{-1} NH_4Cl.

Values are means ± S.E.M. (*N*=5, except for cholesterol, *N*=7).

*Significantly different from the corresponding control value.

As the ambient ammonia concentration builds up, it is imperative that the excreted NH_4^+ does not dissociate to NH_3 and H^+ because NH_3 can diffuse back into the fish. If acid excretion were also responsive to environmental ammonia at neutral pH (see below), there would be a continuous excretion of acid. This would maintain a low pH (high concentrations of H^+) in the boundary water layer of the branchial epithelia, preventing the excreted NH_4^+ from dissociating and avoiding a back flux of NH_3. In essence, this is a process of 'NH_4^+

Table 8. *Fatty acid composition of the six phospholipid classes examined in the skin of* P. schlosseri *exposed for 6 days to 50%SW containing 10 mmol l^{-1} Tris buffer, pH 7.2, with or without (control) 30 mmol l^{-1} NH_4Cl*

Phospholipid class	Fatty acid composition (% of total in the class)	
	Control	Ammonia exposed
SM		
Tot sat	78.4±0.5	79.7±1.0
Tot mono	5.64±0.87	3.48±0.90
Tot poly	16.0±0.5	16.9±0.6
n3	–	–
n6	16.0±0.5	16.9±0.6
PC		
Tot sat	44.0±0.7	42.3±0.6
Tot mono	44.7±0.3	44.6±0.4
Tot poly	11.3±0.5	13.1±0.5*
n3	1.90±0.32	2.26±0.03
n6	9.42±0.47	10.8±0.5*
PS		
Tot sat	71.3±2.1	70.6±0.7
Tot mono	12.8±0.6	12.2±0.3
Tot poly	15.8±2.0	17.2±0.8
n3	4.44±0.96	3.86±0.19
n6	11.4±1.2	13.3±0.8
PI		
Tot sat	60.6±1.0	63.7±0.7
Tot mono	9.07±1.09	9.91±0.91
Tot poly	30.4±0.8	26.4±0.9*
n3	–	–
n6	30.4±0.8	26.4±0.9*
PE		
Tot sat	44.7±2.6	42.2±2.0
Tot mono	18.7±1.1	18.9±1.3
Tot poly	36.6±3.5	38.9±3.1
n3	6.7±1.7	10.2±1.4
n6	29.9±3.1	28.6±3.2
CL		
Tot sat	41.5±5.0	30.8±6.7
Tot mono	32.5±3.2	30.5±5.8
Tot poly	26.0±6.7	38.8±3.5
n3	–	0.60±0.60
n6	26.0±6.7	38.1±3.7

The composition of each phopholipid class is expressed as % of the total content in that class.

PC, phosphatidylcholine; PS, phosphatidylserine; PI, phosphatidylinositol; PE, phosphatidylethanolamine; CL, cardiolipin; SM, sphingomyelin.

Tot sat, total saturated fatty acids; Tot mono, total monounsaturated fatty acids; Tot poly, or PUFAs, total polyunsaturated fatty acids; n3, n3 PUFAs; n6, n6 PUFAs.

Values are means ± S.E.M. (*N*=5).

*Significantly different from the corresponding control value.

trapping' in contrast to 'NH_3 trapping' at the beginning of the experiment.

Increased net acid (H^+) excretion in response to environmental pH or ammonia

Instead of studying the expired water from the gills of *P. schlosseri*, we monitored the decrease in pH of the bulk water, which was made up to 10 volumes (w/v) of the fish. The decrease in pH was very large and rapid at pH 8.5 or pH 9.0, even in the presence of 2 mmol l^{-1} of Tris. Hence, large quantities of acid must be excreted to manipulate the external pH of alkaline waters. At pH 6.0, this mudskipper was capable of decreasing the net acid efflux, and achieving an increase in the ambient pH through NH_3 excretion. NH_3 combined with H^+ in the medium, leading to an apparent influx of titratable acid. At alkaline pH, *P. schlosseri* responded by excreting more acid to the external medium. This would explain why the pH of the water within its burrow was close to neutral.

Bafilomycin, a specific inhibitor of V-ATPases (Bowman et al., 1988) had a large inhibitory effect on the net acid flux of specimens exposed to pH 7.0 or 9.0. V-ATPases are H^+-translocating enzymes that occur in the endomembranes of all eukaryotes and in the plasma membranes of many eukaryotes (Merzendorfer et al., 1997). It is possible that the increased rate of proton excretion by the mudskipper was due to the increased rate of fusion of the V-ATPase-containing vesicles to the apical surfaces (Merzendorfer et al., 1997) of the branchial epithelium and opercular membrane, thereby increasing the density of the V-ATPases in these regions.

More importantly, *P. schlosseri* was capable of increasing the rate of net acid excretion in response to the presence of 20 or 30 mmol l^{-1} NH_4Cl in the external medium at pH 7.0 or 8.0, which served as direct evidence linking net acid excretion with defense against environmental ammonia toxicity in these fish.

Active ammonia excretion and acid excretion occurred in the head region of P. schlosseri

In agreement with previous observations made on whole fish (Chew et al., 2003), active NH_4^+ excretion (sets 1, 2 and 3 of Table 2) through the head region of *P. schlosseri* was unaffected by alkaline pH (set 3 of Table 2). More importantly, our results reveal that both active NH_4^+ excretion and H^+ excretion occurred in the head region, where the gills and the opercular membranes are located. Theoretically, it is imperative for these two mechanisms to be located together. The branchial and opercular surfaces have the important functions of allowing passage for gases and other ions. Excretion of acid to trap the actively excreted NH_4^+ is likely to be more effective than modifying the fluidity of these surfaces to change the permeability of NH_3, which would also affect the permeability of other molecules.

The exact pH of the branchial boundary water layer in *P. schlosseri* is unknown. However, with such a large efflux of H^+ (3 μmol min^{-1} g^{-1}) into the bulk of the external medium, it is logical to conclude that the concentration of H^+ in the boundary layer must be high. Hence, the NH_4^+ actively pumped out to the boundary layer was likely to remain in the ionized form. As a result, there would be no back diffusion of NH_3. Active NH_4^+ excretion accompanied by increased acid excretion allows *P. schlosseri* to reserve other ammonia detoxification mechanisms (e.g. glutamine formation; Peng et al., 1998) to function at much higher concentrations of environmental ammonia, rendering it an extremely high ammonia tolerance (96 h LC_{50} of 120 mmol l^{-1} NH_4Cl; Peng et al., 1998).

The skin of P. schlosseri *had a low permeability to NH_3*

Unlike branchial and opercular surfaces in the head region, the skin of the tail portion of *P. schlosseri* apparently adopted other mechanisms to reduce the penetration of exogenous ammonia. Judging from Table 1, the skin of *P. schlosseri* was permeable to NH_3, albeit NH_3 permeability could be low. In animals, membranes with low permeability to NH_3 have been suggested to be present in the ascending limb of the loop of Henle of the mouse (Kikeri et al., 1989), and the luminal surface of colonic crypt cells (Singh et al., 1995) and the apical membranes of bladder cells (Chang et al., 1994) of the rabbit.

In this study, ^{15}N was a qualitative index of NH_3 entry into the specimen, and not an indicator of the actual NH_3 entry rate. With $^{15}NH_4Cl$ added to the head compartment at pH 7.0, high levels of ^{15}N-ammonia enrichment (69–83% atom excess) were detected in the tail compartment, despite only a very small amount of ammonia being excreted through the tail region. This reconfirms that the branchial and opercular epithelial surfaces were indeed permeable to ammonia. For specimens exposed to ammonia but at pH 8.0 in the head compartment, the level of ^{15}N-ammonia enrichment detected in the tail compartment was slightly lower. This was unexpected because the concentration of NH_3 at pH 8.0 was tenfold higher than at pH 7.0. This could be due to the activation of the acid excretion mechanism at pH 8.0 (see above), rendering the boundary water layer acidic which, in effect, reduced the influx of exogenous NH_3 through the branchial surfaces.

In contrast, no ^{15}N-ammonia was detected in the external medium in the head compartment when 5 or 10 mmol l^{-1} $^{15}NH_4Cl$ was added to the tail compartment at pH 7.0. This means that NH_3 entry into the body through the skin was low at neutral pH, and a significant amount of NH_3 entry occurred only at 20 mmol l^{-1} NH_4Cl. These results suggest that the skin of *P. schlosseri*, despite being responsible for 50% of O_2 uptake in air or in water (Clayton, 1993), was much less permeable to NH_3 than the branchial epithelial surface. However, with a higher percentage of NH_3 at pH 8.0 in the tail compartment, the ^{15}N-ammonia recovered from the head compartment increased (from Table 5), confirming that NH_3 could permeate the skin, albeit with relatively low permeability.

Using an Ussing-type apparatus, it could be shown that the flux of NH_3 through the skin of *P. schlosseri* was low (approximately 0.01 μmol min^{-1} cm^{-2}). We could not find equivalent results from other fishes for direct comparison, but the permeability constant for NH_3 calculated for the frog

R. catesbiana, which is also amphibious and dependent on cutaneous respiration, was indeed higher (1.8-fold) than that of *P. schlosseri*. Lohrmann and Feldman (1994) measured the unidirectional ammonia flux through portions of distal colon, using a 20 mmol l^{-1} ammonia solution (with no pH difference) to create a gradient, and reported an ammonia flux of 2.8 μEq h^{-1} cm^{-2}, or equivalent to 0.047 μmol min^{-1} cm^{-2}. While the skin and the colon have different morphologies and functions, the ~80% smaller NH_3 flux under a much greater NH_3 gradient (taking into consideration the pH effect) in the case of *P. schlosseri* suggests that its skin has indeed a low NH_3 permeability.

In order to gauge the usefulness of the decreased NH_3 permeability of the skin in reducing the load on the ammonia excretory capacity of *P. schlosseri*, a theoretical calculation was performed on a hypothetical specimen of 110 g, which had a skin surface area of 9.25 cm^2. For such a specimen, the total ammonia flux in 1 h through the skin was 0.01 μmol min^{-1} cm^{-2}×9.25 cm^2×60 min=5.55 μmol, when exposed to 10 mmol l^{-1} NH_4Cl at pH 8.0. The ammonia excretion rate in *P. schlosseri* at pH 7.0 (or pH 8.0) was 0.65 μmol h^{-1} g^{-1} (Lim et al., 2001), or 71.5 μmol h^{-1} for a 110 g fish. The majority of this NH_3 is excreted through the head region, presumably through the gills. To remove the excess amount of 5.55 μmol (entered through the skin) *via* the gills within the same period (1 h) would mean an extra load of (5.55/71.5)×100=7.76% to the gills in the head region. This is a very small percentage, and the calculated quantity is already an overestimated value because the blood pH was not 7.0 and the plasma ammonia concentration was not zero, which were the conditions in the Ussing-type apparatus *in vitro*.

Lipid compositions of the skin of P. schlosseri *suggested its low fluidity*

The low NH_3 permeability in the skin of *P. schlosseri* was likely to be due to its low membrane fluidity. It has been shown that NH_3 permeability in artificial vesicles decreased with decreasing lipid fluidity (Lande et al., 1995). The fluidity of a membrane is influenced by the cholesterol content, phospholipid composition and the fatty acid composition of the phospholipids.

The rigid steroid ring structure of cholesterol restricts the molecular motion available to adjacent phospholipid hydrocarbon chains, increasing membrane orderness (Robertson and Hazel, 1999). Cellular membranes of fish undergo compensatory lipid compositional changes during thermal acclimation (Hazel and Williams, 1990). For example, gill membranes of trout kept at 5°C have a lower cholesterol content than those kept at 20°C (Robertson and Hazel, 1999). This reflects an increase in structural disorder with acclimation to lower temperatures to compensate for the increasing order due to the lower temperature. The cholesterol content (4.5 μmol g^{-1}) in *P. schlosseri* skin was high compared with those of tissues from other animals (Borchman et al., 1989; Crockett and Hazel, 1995; Cuculeseu et al., 1995; Fines et al., 2001; Molitoris et al., 1985; Smith and Ploch, 1991), indicating the low fluidity and hence low permeability of its skin. Furthermore, ammonia exposure could lead to higher cholesterol content in the skin of *P. schlosseri* (see below).

Compared to fish gills, PE, a destabilizing phospholipid (Gillis and Ballantyne, 1999), is present in a smaller amount in the skin of *P. schlosseri* (<15%). This would also contribute to a more ordered, less permeable membrane. PS is present in a greater amount in the skin of this mudskipper (15–20%) than in fish gill (approximately 8%). Charged lipids such as PS strongly perturb phospholipid head group motion, probably by changing the pattern of hydrogen bonding between water molecules, and thus easily segregate into distinct domains (Aloia and Mlekusch, 1988). According to Hazel and Williams (1990), an increase in PE, using PS as a substrate for its formation, is adaptive at low environmental temperatures as it would enhance membrane fluidity. Hence, these results again suggest that the skin of *P. schlosseri* has relatively low membrane fluidity.

PC was the major phospholipid present in the skin of *P. schlosseri*. It is known to stabilize the bilayer, favoring the formation of a laminar structure (Gillis and Ballantyne, 1999). Stabilizing the lipid bilayer is an important adaptation in warm temperatures (Logue et al., 2000). The PC:PE ratio can be used as an indication of membrane fluidity, and may be altered according to the environmental and physiological conditions encountered by an animal (Hazel and Williams, 1990). The PC:PE ratio of 3.4 in the skin of *P. schlosseri* was much higher than those of tissues (intestinal mucosa or kidney membranes) of other fishes (Acierno et al., 1996; Hazel and Landrey, 1988). Although no information on the lipid compositions of other fish skins is available, together with the results discussed above, it can be concluded that the skin of *P. schlosseri* has a high degree of stability or order, consistent with low membrane fluidity.

When we consider the different types of fatty acids present in various phospholipid classes as percentages of the total fatty acids in *P. schlosseri*, it becomes obvious that the saturated fatty acids (52%) predominate, followed by fatty acids with one double bond (monoenes, 28%) and the PUFAs (20%). These percentages are remarkably different from those of the skin of the trout (Ghioni et al., 1997), in which monoenes predominate (48%), followed by the PUFAs (26%), and the saturated fatty acids (22%). Unsaturated fatty acids adopt a more expanded conformation, occupy greater areas in monomolecular films, pack less compactly, and possess lower melting points than their saturated homologs. Hence, a nearly ubiquitous response to cold temperature is a reduction in the proportion of saturated fatty acids, and a corresponding increase in the proportion of unsaturated fatty acids in the lipids of cellular membranes (Hazel and Williams, 1990). In general, the relatively high content of saturated fatty acids in the skin of *P. schlosseri* supports the above conclusion that its skin is less fluid, and possibly less permeable, to NH_3.

Ammonia exposure affected the lipid composition of the skin of P. schlosseri *and its NH_3 permeability*

After exposure of *P. schlosseri* to ammonia for 6 days, there were detectable changes in the amount of ammonia entering

through the tail region (compare Table 3, sets 2, 3 and 4, with Table 1, sets 2 and 3 and Table 2, set 2). At the same time, in specimens subjected to prior exposure to ammonia, there were changes in lipid compositions in the skin, some of which could be linked to a lower NH_3 permeability. Taken together, these results suggest that the skin in the tail region of *P. schlosseri* decreased its permeability to ammonia after prolonged exposure to NH_4Cl.

More importantly, the cholesterol content in the skin of *P. schlosseri* increased after 6 days of exposure to exogenous ammonia. This result suggests a definite role for cholesterol in an active defense mechanism against environmental ammonia toxicity, because an increase in cholesterol content would imply a decrease in the fluidity of the membrane in the skin. Since the specimens were not fed during the experimental period, it is logical to deduce that cholesterol synthesis must be enhanced in response to ammonia-loading conditions, or that cholesterol was mobilized from other tissues. Lande et al. (1995) studied the NH_3 permeability of artificial unilamellar vesicles, in which cholesterol and SM contents, together with acyl chain saturation, were varied to create a range of fluidities. They concluded that lower fluidity did indeed correspond with lower NH_3 permeability. This would explain why there was an apparent decrease in the influx of NH_3 through the skin of the specimens that had been exposed to NH_4Cl for 6 days, as described above.

An ecological perspective

The tolerance of *P. schlosseri* to environmental ammonia is much higher than those of other fishes, in part because of its capability to actively excrete NH_4^+. For active excretion of NH_4^+ to be efficacious, back diffusion of NH_3 must be prevented. Our results suggest that *P. schlosseri* solves the problem of back diffusion of NH_3 differently through the gills (head region) and through the skin (tail region). Acid excretion occurred in the head region, in response to alkaline pH and/or environmental ammonia. On the other hand, the skin has relatively low permeability to NH_3, and its lipid compositions can be altered after long-term (6 days) exposure to environmental ammonia. *P. schlosseri* is the only species of mudskipper found exclusively in the tropics. Other species of mudskippers (e.g. *Periophthalmus* spp. and *Boleophthalmus* spp.) can be found in both tropical and temperate regions (e.g. Japan and China). It is possible that, by reducing the membrane fluidity of the skin to enhance ammonia tolerance, *P. schlosseri* loses its ability to survive in areas of lower temperature. In temperate regions, the temperature during winter can drop to a level that would cause even lower membrane fluidity, which would limit the permeability of the skin to various other molecules that are essential to life. Therefore, efforts should be made in the future to compare membrane fluidity and permeability to NH_3 in tropical and temperate fish species.

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