The Journal of Experimental Biology 209, 1737-1745
Published by The Company of Biologists 2006
doi:10.1242/jeb.02197

Rapid increase in the partial pressure of NH_3 on the cutaneous surface of air-exposed mangrove killifish, *Rivulus marmoratus*

S. L. Litwiller[1], M. J. O'Donnell[2] and P. A. Wright[1,*]

[1]*Department of Integrative Biology, University of Guelph, Guelph, Ontario N1G 2W1, Canada and* [2]*Department of Biology, McMaster University, Hamilton, Ontario L8S 4K1, Canada*

*Author for correspondence (e-mail: patwrigh@uoguelph.ca)

Accepted 7 March 2006

Summary

Mangrove killifish, *Rivulus marmoratus*, are tolerant of prolonged periods of air exposure (>30 days). Air-exposed *R. marmoratus* eliminate more than 40% of their total ammonia through NH_3 volatilization; however, the sites and mechanisms are unclear. We hypothesized that the cutaneous surface is an important site of NH_3 volatilization in air-exposed *R. marmoratus*. Ion-selective microelectrodes were used to measure the NH_4^+ concentration and pH in the boundary layer on the cutaneous surface of fish in water or air (acute: 1 h, chronic: 11 days). Following acute and chronic air exposure, there was a ~18-fold increase in the NH_4^+ concentration and a 0.3–0.6 pH unit increase on the cutaneous surface of *R. marmoratus*. In air-exposed fish, the calculated cutaneous partial pressure (P_{NH_3}) was 608–1251 μTorr, representing a 33- to 75-fold increase over control (immersed) fish. The P_{NH_3} on the cutaneous surface water film was more than sufficient to account for the rate of NH_3 volatilization under terrestrial conditions. Together, these data indicate that during air exposure, *R. marmoratus* utilize the cutaneous surface as a key site of NH_3 volatilization.

Key words: NH_3 volatilization, nitrogen excretion, ammonia excretion, water pH, fish skin, boundary layer, amphibious fish, *Rivulus marmoratus*.

Introduction

It has long been known that ammonia is the primary nitrogenous end product of protein catabolism in fishes and that it is mostly excreted across the gill epithelium (e.g. Smith, 1929; Kormanik and Cameron, 1981). Continuous ammonia elimination is necessary because ammonia is toxic if it accumulates in fish tissues. During periods of air exposure, there is no external water available to irrigate the branchial epithelia and thus, gas exchange, ion balance and ammonia elimination are problematic. Nevertheless, some fishes are able to tolerate periods of air exposure and exhibit a variety of strategies to ameliorate elevated ammonia levels in the tissues.

Several strategies to prevent the lethal accumulation of ammonia during air exposure have been documented in the literature. The four-eyed sleeper (*Bostrichthys sinensis*) (Ip et al., 2001), African lungfish (*Protopterus* sp.) (Chew et al., 2004) and the giant mudskipper (*Periophthalmodon schlosseri*) (Lim et al., 2001) suppress proteolysis and amino acid catabolism on land to slow down the accumulation of ammonia. However, air-exposed *P. schlosseri* is also capable of partial amino acid catabolism (Ip et al., 1993), where alanine is formed to support locomotory activities on land, without the release of ammonia. An alternative strategy to prevent ammonia accumulation during air exposure is to store nitrogenous wastes within the tissues in a less toxic form. Both the marble goby (*Oxyeleotris marmoratus*) (Jow et al., 1999) and the sleeper (*B. sinensis*) (Ip et al., 2001) detoxify ammonia to glutamine for storage during air exposure. By contrast, the snakehead (*Channa gachua*) (Ramaswamy and Reddy, 1983), mudskipper (*P. cantonensis*) (Gordon et al., 1978), blenny (*Alticus kirki*) (Rozemeijer and Plaut, 1993) and African lungfish (*Protopterus* sp.) (Janssens and Cohen, 1968; Chew et al., 2004) increase urea retention and/or excretion on land, in order to prevent ammonia accumulation in the tissues.

Some terrestrial invertebrates and fish continue to excrete ammonia *via* NH_3 volatilization while on land. With a pK of ~9–9.5, ammonia exists in solution mostly as NH_4^+ at physiological pH. Alkalinization of the branchial fluid in crabs results in an increase in the non-ionic form of ammonia, NH_3, leading to volatilization if the fluid is in contact with a convective air stream (Weihrauch et al., 2004). The ammonotelic terrestrial isopod, *Ligia beaudiana*, releases gaseous NH_3 after alkalinization of water retained between their pleopods (Wieser, 1972). In two terrestrial crabs, *Geograpsus grayi* and *Ocypode quadrata*, NH_3 volatilization occurs from the alkalinized surface of the branchial chambers

(Greenaway and Nakamura, 1991; De Vries and Wolcott, 1993). However, in the isopod, *Porcellio scaber*, alkalinization is not involved and high ammonia concentrations in the pleon fluid are sufficient to facilitate NH_3 volatilization (Wright and O'Donnell, 1993).

In fishes, volatilization of NH_3 occurs in the leaping blenny (*A. kirki*) (Rozemeijer and Plaut, 1993), amphibious blenny (*Blennius pholis*) (Davenport and Sayer, 1986), giant mudskipper (*P. schlosseri*) (Wilson et al., 1999), weather loach (*Misgurnus anguillicaudatus*) (Tsui et al., 2002), as well as in the mangrove killifish (*Rivulus marmoratus*) (Frick and Wright, 2002b). The weather loach *M. anguillicaudatus* volatilizes NH_3 from an alkaline cutaneous surface and/or digestive tract when exposed to air for 48 h (Tsui et al., 2002). *R. marmoratus* is capable of more than 11 days of air exposure, during which time it continues to excrete both urea (39% of immersed rate) and ammonia (57% of immersed rate) with almost half (approximately 42%) of the total ammonia released through NH_3 volatilization (Frick and Wright, 2002b). Surprisingly, ammonia does not accumulate in the tissues, but after 4 days of air exposure, urea concentrations are elevated modestly (twofold). There appears to be no active ornithine urea cycle pathway, as seen in some amphibious fishes (Frick and Wright, 2002b). Taken together, the information indicates that NH_3 volatilization is a key strategy to avoid ammonia toxicity in *R. marmoratus*.

The purpose of this study was to determine the mechanisms and sites of action involved in NH_3 volatilization in air-exposed *R. marmoratus*. With a decreased reliance on the gill epithelia for nitrogen elimination, amphibious fishes may enhance the use of other routes, such as the kidney, cutaneous surface and digestive tract. *R. marmoratus* has an extensive vascularized epidermis, which may be used for cutaneous respiration (Grizzle and Thiyagarajah, 1987) and ammonia elimination during air exposure. Partitioning of the anterior and posterior regions of *R. marmoratus* revealed nitrogen excretion occurred predominantly in the anterior region (gills; ~57% of total nitrogen excretion) in immersed fish and shifted to the posterior region (kidney and/or skin; ~66% of total nitrogen excretion) in air-exposed fish, however, volatilization was not assessed in these divided chamber experiments (Frick and Wright, 2002b).

In the present study, we tested the hypothesis that the cutaneous surface is an important site of NH_3 volatilization in air-exposed *R. marmoratus*. We predicted that an elevation of the ammonia concentration and/or pH in the boundary layer on the cutaneous surface occurs in air-exposed relative to immersed fish. We further hypothesized that changes in pH and ammonia concentration on the cutaneous surface are progressive over time following air exposure. We predicted that relatively small changes in cutaneous ammonia concentration and pH will be present after 1 h of air exposure with larger changes occurring after 11 days in air. To test these hypotheses, we used ion-selective microelectrodes to measure the NH_4^+ concentration and pH in the boundary layer on the cutaneous surface of immersed and both acutely (1 h) and chronically (11 days) air-exposed *R. marmoratus*.

Materials and methods

Experimental animals

Fish were obtained from a breeding colony of *Rivulus marmoratus* Poey held in the Hagan Aqualab at the University of Guelph, Guelph, ON, Canada (Frick and Wright, 2002a). Adult hermaphrodite fish, at least 1 year of age and weighing approximately 0.07–0.15 g, were used for experiments. Hermaphrodites were identified by the appearance of an overall mottled brown colouration, a characteristic caudal ocellus, and a whitish border on the anal fin. Fish were kept in individual containers under a constant photoperiod (12 h:12 h, L:D), in approximately 16–18‰ artificial seawater (made with distilled water and Crystal Sea® Marinemix; Marine Enterprises International, Inc., Baltimore, MA, USA), 25°C, pH ~8.1. Water changes were performed every 2 weeks and fish were fed *Artemia* three times per week.

Preparation and calibration of ion-selective microelectrodes

Ion-selective and reference microelectrodes were constructed as described previously (Maddrell et al., 1993). The ionophore cocktails (Sigma-Aldrich, Oakville, ON, Canada) used were: H^+ ionophore I, cocktail B; ammonium ionophore I, cocktail A; potassium ionophore I, cocktail B. The pH, NH_4^+ and K^+ microelectrodes were backfilled with solutions of 100 mmol l^{-1} NaCl/100 mmol l^{-1} sodium citrate (pH 6.0), 1 mol l^{-1} NH_4Cl and 500 mmol l^{-1} KCl, respectively. The tip of the reference electrode was filled with 500 mmol l^{-1} sodium acetate and the barrel was then backfilled with 500 mmol l^{-1} KCl. Tips of the microelectrodes were usually broken back to a diameter of ~5 μm to reduce electrode resistance and response time. A chlorided silver wire inserted into the backfilling solution of either the pH, NH_4^+, or K^+ microelectrode was connected to a high-impedance input stage (>10^{15} Ω) of an electrometer, and the electrical ground of the amplifier was connected through a second silver wire to the reference microelectrode.

The pH microelectrodes were calibrated using NaCl solutions that mimicked the ionic strength of the fresh water (FW; ~1 mmol l^{-1}). Two pH calibration solutions were buffered with 20 mmol l^{-1} Hepes (pH ~7.0 and 8.0). Calibration solutions of pH ~7.0 were set at ~0 mV as a reference for pH measurements. The pH microelectrode calibrations were checked throughout the measurements performed on each fish. Published selectivity coefficients (K) for pH microelectrodes are as follows: $K_{H,Na}$ $10^{-10.4}$, $K_{H,K}$ $10^{-9.8}$, $K_{H,Ca}$ $10^{-11.1}$ (Sigma-Aldrich). NH_4^+ microelectrodes were calibrated in solutions containing 1 mmol l^{-1} NaCl and 0.1, 1 or 10 mmol l^{-1} NH_4Cl. Calibration solutions of 0.1 mmol l^{-1} NH_4Cl were set at ~0 mV as a reference for NH_4^+ measurements. The NH_4^+ microelectrode calibrations were checked throughout the measurements performed on each fish. Selectivity coefficients for NH_4^+ microelectrodes were

determined using the separate solution method (Ammann, 1986) and 100 mmol l^{-1} solutions of chloride salts are: $K_{NH4,H}$ $10^{-2.2}$, $K_{NH4,Na}$ $10^{-2.0}$, $K_{NH4,K}$ $10^{-0.6}$. The selectivity coefficient for NH_4^+ relative to K^+, the primary interfering ion, was also determined using the separate solution method under conditions of low ionic strength (1 mmol l^{-1} NaCl) approximating those of the experimental measurements. The value of $K_{NH4,K}$ was $10^{-0.9}$, indicating that the electrode was 7.9 times more selective towards NH_4^+ than for K^+.

To determine if K^+ leakage from the fish was interfering with the NH_4^+ measurements, K^+ concentrations were also measured. K^+ microelectrodes were calibrated in solutions containing 1 mmol l^{-1} NaCl and 0.15 or 1.5 mmol l^{-1} KCl.

Experimental protocol and measurements

To minimize ion interference with the NH_4^+ microelectrode, fish were acclimated to freshwater (FW) over 4 days of daily water changes from 15‰ to 7‰ to 3‰ to ~0‰ FW [chlorine-free wellwater: $[Na^+]$=1.05, $[Cl^-]$=1.47, $[Ca^{2+}]$=5.24, $[Mg^{2+}]$=2.98, $[K^+]$=0.06 mequiv l^{-1}; total alkalinity ($CaCO_3$)=250 mg l^{-1}; total hardness ($CaCO_3$)=411 mg l^{-1}]. FW was adjusted to pH ~8.0 with HCl or NaOH, using an Accumet® AP61 portable pH meter (Fisher Scientific, Ottawa, ON, Canada). Fish were fed *Artemia* every day during the acclimation period and deprived of food during experimentation. Preliminary measurements indicated that there were no significant differences in pH on the cutaneous surface of both immersed and chronically (11 days) air-exposed *R. marmoratus*, between seawater- and freshwater-acclimated fish.

Three series of experiments were conducted post-acclimation. In Series I (immersed), FW-acclimated fish were placed in individual plastic containers and immersed in FW (10 ml) for either 1 h (control I) or 11 days (control II), and NH_4^+ concentration and water pH were measured. Freshwater was replaced every other day during the 11 days of immersion. Water pH remained relatively constant (±0.1 pH unit) over the 11 days. Water oxygen concentration, measured using a DO-166-NP dissolved oxygen needle probe and an Accumet® AB15 pH meter calibrated in mV, dropped 0.76 mg l^{-1} within the first 24 h and then remained constant (5.93 mg l^{-1} ±051) until the freshwater was replaced (48 h). After 1 h (control I) or on the 11th day (control II), the fish were placed in a Petri dish (3.5 cm diameter) in 3 ml of water. Ion-selective microelectrodes and the reference electrode were positioned approximately 5–10 μm from the cutaneous surface (in the boundary layer) of the unanaesthetized fish, and the electrical potential difference was recorded. The measurements were taken on three locations that were dorsoventrally on the mid-section of the fish: an anterior location (near the operculum), a mid-section location (base of the pectoral fin), and a posterior location (base of the caudal fin). Measurements collected from Series I were used as controls for the corresponding air exposure measurements; control I (1 h) was compared to Series II (acute; 1 h) air exposure and control II (11 days) was compared to Series III (chronic; 11 days) air exposure (see below). An external bulk water NH_4^+ concentration or pH value was also obtained under these conditions.

In Series II (acute air exposure), fish were removed from water at the start of the experiment and placed in identical containers as for Series I. Containers were supplied with a moist substratum (layer of cotton batting and filter paper with ~2 ml of 0‰ FW) and relative humidity remained constant at approximately 99%. Cutaneous NH_4^+ concentration and pH were measured after 1 h (acute) of air exposure. The air-exposed fish were placed in a Petri dish with ~10 μl of water. The microelectrodes were positioned either directly onto the moist surface of the fish or in the thin film (~100 μm depth) that collected between the bottom of the fish and the dish. To determine if ammonia accumulation was time dependent, NH_4^+ concentration was measured for an additional 1 h.

In Series III (chronic air exposure), cutaneous NH_4^+ concentration and pH as well as digestive tract pH were measured in fish that had been exposed to air for 11 days. Procedures for air exposure and microelectrode measurements were as described for Series II. To determine if the gut was involved in ammonia volatilization in air-exposed fish, gut pH was measured in anterior and posterior regions of the mucosal surface of the digestive tract. The fish were sacrificed with an overdose of MS-222 and cervical dislocation was performed. The digestive tracts of the fish were immediately removed and a longitudinal section was made in order to measure pH along the mucosal surface at the anterior and posterior end (~3–5 min after sacrifice).

During air exposure, observation of the movement of small pieces of dislodged scales or debris on the cutaneous surface indicated that the fluid was well mixed by the occasional movements of the fish, and measurements at different locations on the cutaneous surface of the fish could not be distinguished. Thus, measurements taken at the locations described above (Series I) were pooled together.

Amphibious fish may experience dehydration during air exposure and thus, wet and dry weights were taken in fish on day 0 and day 11 of immersion and air exposure. By using the formula (wet mass–dry mass)/wet mass, the body water content was estimated to determine if significant water loss had occurred.

Calculations

pH was calculated from the difference in electrical potential recorded between the sample on the cutaneous surface of the fish and a calibration solution with a known value according to the following formula:

$$pH_{skin} = pH_{calibration} + (-\Delta V/\text{slope}) ,$$

where pH_{skin} is the pH of the cutaneous surface of the fish, $pH_{calibration}$ is the pH of the calibration solution, ΔV is the potential difference (mV) measured when the microelectrode is moved between the calibration solution and the unknown, and slope is the change in potential in response to a one unit change in pH.

NH_4^+ concentration was determined as:

$$[NH_4^+]_{skin} = [NH_4^+]_{calibration} \times 10^{(\Delta V/slope)} ,$$

where $[NH_4^+]_{skin}$ is the $[NH_4^+]$ of the sample on the cutaneous surface of the fish, $[NH_4^+]_{calibration}$ is the $[NH_4^+]$ of the calibration solution and slope is the change in potential in response to a tenfold change in $[NH_4^+]$. The NH_4^+ concentrations were corrected for interference of K^+ ions on the NH_4^+ microelectrodes using the following equation [derived from the Nicolsky-Eisenman equation (Ammann, 1986)]:

$$[NH_4^+]_{corrected} = [NH_4^+] - K_{NH_4,K} \times [K^+] .$$

$[NH_3]$ was calculated using the Henderson-Hasselbalch equation, where pK_{amm} was obtained from Cameron and Heisler (Cameron and Heisler, 1983) and pH on the cutaneous surface was taken as an average of individual values [immersed pH=7.28±0.076, N=5 (1 h); pH=7.21±0.040, N=8 (11 days) and air-exposed pH=7.84±0.049, N=9 (1 h); pH=7.49±0.051, N=12 (11 days)]. Average pH was used because the $[NH_4^+]$ measurements were taken on a separate group of fish, since switching microelectrodes during recording was impractical and the standard errors were relatively small. The partial pressure of NH_3 (P_{NH_3} in μTorr; 1 Torr≈133.322 Pa) was calculated using the appropriate solubility coefficients (α_{NH_3}) (Cameron and Heisler, 1983):

$$P_{NH_3} = ([NH_3]/\alpha_{NH_3}) \times 10^6 .$$

Statistical analyses

Data are presented as means ± standard error of the mean (s.e.m.). A two-way analysis of variance (ANOVA) was used to determine significant differences between immersed and air-exposed fish among the two time periods, 1 h and 11 days. A one-way ANOVA was used to determine significant differences within each of the two time periods as well as environment, immersion and air exposure. A two-way ANOVA was used to distinguish significant differences between immersed and air-exposed fish among the anterior and posterior locations along the digestive tract. The Tukey test (SPSS, SPSS Inc., Chicago, Illinois, USA) was used to determine where significant differences were present (P<0.05). Normality tests and data transformations (logarithmic) were performed where appropriate to meet assumptions of the tests above.

Results

Series I – immersed

NH_4^+ concentration and pH on the cutaneous surface of *R. marmoratus* did not differ significantly among the anterior, mid-section and posterior locations on the body of the fish following 1 h (control I) (Fig. 1A,B) and 11 days (control II) (Fig. 2A,B) of immersion. The NH_4^+ concentration on the cutaneous surface in immersed *R. marmoratus* was relatively low at both times (1 h and 11 days) and not significantly different from the bulk water concentration (Fig. 1A, Fig. 2A). The pH on the cutaneous surface of immersed fish was approximately 0.6 (11 days) to 0.8 (1 h) pH units lower than the bulk water pH (Fig 1B, Fig. 2B). Data at different locations for control I (1 h) and control II (11 days) were pooled for comparisons of NH_4^+ concentration and pH with respective Series II (acute; 1 h) and Series III (chronic; 11 days) air exposure (see below).

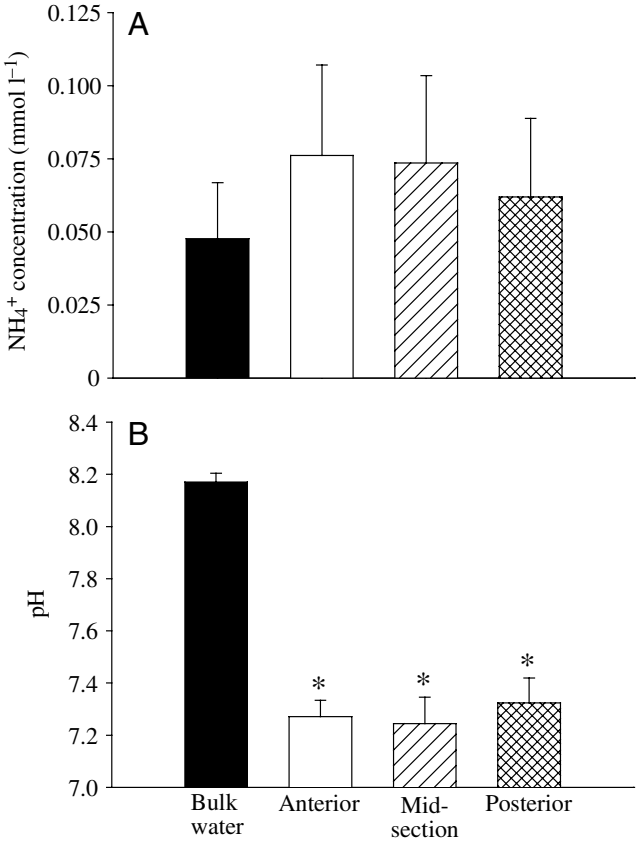

Fig. 1. NH_4^+ concentration (A) and pH (B) of the bulk water (black bars) and cutaneous surface locations on the body of the fish (anterior, white bars; mid-section, hatched bars; posterior, cross-hatched bars) in *R. marmoratus* immersed for 1 h in Series I experiments (control I). Values are means + s.e.m. (N=8). Asterisk indicates significant difference from bulk water (P<0.05). There were no significant differences between locations.

Series II – acute (1 h) air exposure

Following acute air exposure (1 h), there was an ~17-fold increase in the NH_4^+ concentration on the cutaneous surface of the fish when compared to that on immersed fish (Fig. 3A). The pH on the cutaneous surface of air-exposed fish was elevated by ~0.6 pH units compared to that on immersed fish (Fig. 3B). The NH_4^+ concentration and pH measurements were used to calculate the partial pressure of NH_3 (P_{NH_3}). The P_{NH_3} increased significantly (~75-fold) upon acute air exposure, compared to that of immersed fish (Fig. 3C).

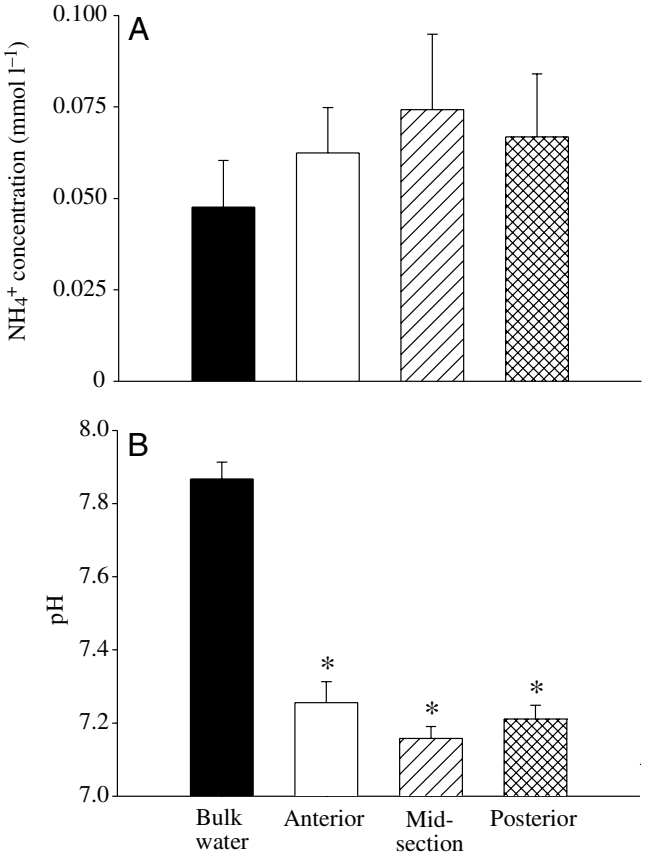

Fig. 2. NH_4^+ concentration (A) and pH (B) of the bulk water (black bars) and cutaneous surface locations on the body of the fish (anterior, white bars; mid-section, hatched bars; posterior, cross-hatched bars) in *R. marmoratus* immersed for 11 days in Series I experiments (control II). Values are means + s.e.m. (N=8). Asterisk indicates significant difference from bulk water (P<0.05). There were no significant differences between locations.

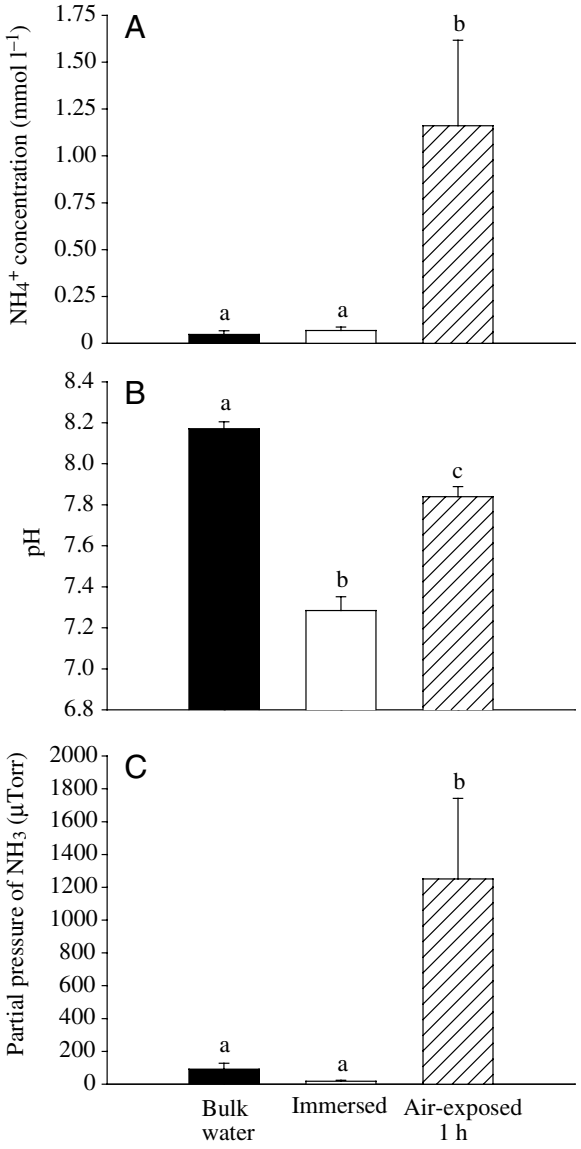

Fig. 3. NH_4^+ concentration (A), pH (B) and the calculated partial pressure of NH_3 (C) of the bulk water (black bars) and cutaneous surface of immersed (white bars) and acutely (1 h) air-exposed (hatched bars) *R. marmoratus* (Series II). Values are means + s.e.m. (N=5 bulk water, N=5 cutaneous immersed, N=9 cutaneous air-exposed). Bars with a different letter are significantly different (P<0.05).

Cutaneous NH_4^+ concentrations did not differ significantly over the time periods of 1 h (0.79±0.14 mmol l^{-1}, N=4), 1.5 h (0.61±0.25 mmol l^{-1}, N=4) and 2 h (1.03±0.33 mmol l^{-1}, N=4) in air-exposed *R. marmoratus*.

Series III – chronic (11 days) air exposure

Following chronic air exposure (11 days), there was an ~18-fold increase in the NH_4^+ concentration on the cutaneous surface of the fish when compared to that on immersed fish (Fig. 4A). The pH on the cutaneous surface of air-exposed fish was elevated by ~0.3 pH units compared to that on immersed fish (Fig. 4B). The calculated P_{NH_3} increased significantly (~33-fold) following chronic air exposure, compared to that on the immersed fish (Fig. 4C).

There were no significant differences in the cutaneous NH_4^+ concentrations and P_{NH_3} between acute and chronic air exposure. As well, the change in cutaneous pH was not significantly different following 1 h and 11 days of air exposure. The bulk water pH of the acute and chronic time periods differed significantly by approximately 0.3 units and, therefore, the cutaneous pH could not be compared directly.

There were no significant differences in gut pH between the anterior (7.25±0.11, N=7) and posterior (7.33±0.17, N=7) regions. After chronic air exposure, there were no significant differences in gut pH (anterior 7.50±0.17, N=6; posterior 7.50±0.17, N=6) relative to that of immersed fish.

To ensure chronic air exposure was not resulting in significant dehydration, percent body water was calculated from wet and dry body mass. *R. marmoratus* had slightly

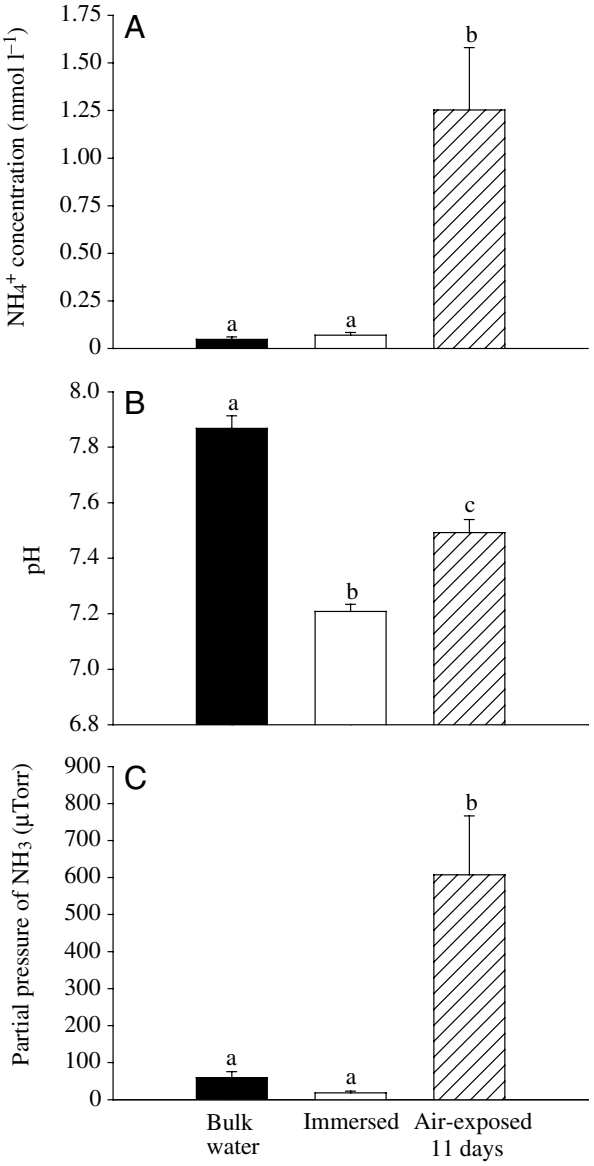

Fig. 4. NH_4^+ concentration (A), pH (B) and the calculated partial pressure of NH_3 (C) of the bulk water (black bars) and cutaneous surface of immersed (white bars) and chronically (11 days) air-exposed (hatched bars) *R. marmoratus* (Series III). Values are means + s.e.m. (*N*=8 bulk water, *N*=8 cutaneous immersed; *N*=6 cutaneous air-exposed NH_4^+ concentration, *N*=12 cutaneous air-exposed pH, *N*=6 cutaneous air-exposed P_{NH_3}). Bars with a different letter are significantly different ($P<0.05$).

elevated (~1%) percent body water content after 11 days in both immersed and air-exposed fish (Fig. 5).

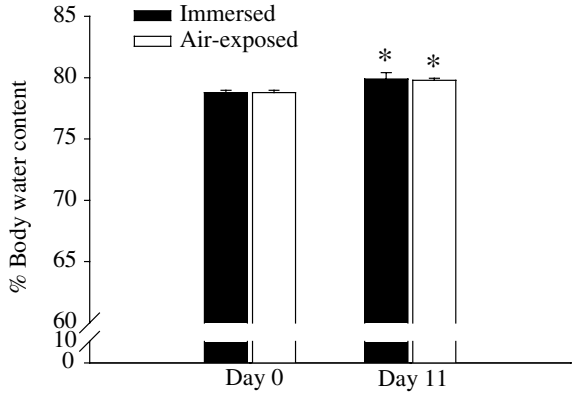

Fig. 5. Percent body water content over 11 days in both immersed (black bars) and air-exposed (white bars) *R. marmoratus*. Values are expressed as mean + s.e.m. (*N*=6 immersed, *N*=8 air-exposed). Asterisk indicates significant difference from day 0.

Discussion

The mangrove killifish, *R. marmoratus*, has adopted a remarkable strategy to survive prolonged air exposure by eliminating excess ammonia *via* NH_3 volatilization (Frick and Wright, 2002b). In the present study, the large increase in the NH_4^+ concentration and accompanying alkalinization results in a greater than 30-fold elevation in the partial pressure of NH_3 (P_{NH_3}) on the cutaneous surface of air-exposed fish. *R. marmoratus* have a thin and highly vascularized epidermis over most of the body (Grizzle and Thiyagarajah, 1987). The dramatic increase in the P_{NH_3} in the fluid surrounding the cutaneous surface of air-exposed *R. marmoratus* suggests that NH_3 volatilization is occurring primarily at this site, but is the P_{NH_3} gradient from the surface to air sufficient to account for the measured rate of volatilization?

The P_{NH_3} on the cutaneous surface required to explain reported rates of NH_3 volatilization in air-exposed *R. marmoratus* can be estimated as described by Wright and O'Donnell (Wright and O'Donnell, 1993). The rates of NH_3 volatilization in air-exposed *R. marmoratus* after 1 day (closest value for 1 h comparison) and 11 days in air were ~0.18 and 0.28 μmol g^{-1} h^{-1} (0.35 and 0.55 μg h^{-1}), respectively (Frick and Wright, 2002b). We made the assumption that most of the NH_3 was volatilized from the cutaneous surface. By using an estimate of the effective permeability of a free water surface in air, we determined the P_{NH_3} required to generate this flux, given the P_{NH_3} for external air is approximately zero. Gravimetric estimates of boundary layer permeabilities for 10–100 μl water droplets in air are approximately 0.0027 μg h^{-1} cm^{-2} $\mu Torr^{-1}$ (Wright and O'Donnell, 1993). Water and ammonia have similar diffusion coefficients in air [~0.221 cm^2 s^{-1} at 25°C and atmospheric pressure (Reid et al., 1977)] and thus, will have similar permeabilities to outward flux. The permeability estimate was then divided into the measured rates of NH_3 volatilization (see above) to generate the P_{NH_3}=0.35 (μg h^{-1})/0.0027 (μg h^{-1} cm^{-2} $\mu Torr^{-1}$)=132.51 μTorr cm^2 for 1 h and=0.55 (μg h^{-1})/0.0027 (μg h^{-1} cm^{-2} $\mu Torr^{-1}$)=206.14 μTorr cm^2 for 11 days. The estimated average cutaneous surface area (Rombough and Ure, 1991) was 3.92 cm^2. Thus, the P_{NH_3} required is 132.51 μTorr cm^2/3.92 cm^2=34 μTorr for 1 h and 206.14 μTorr cm^2/3.92 cm^2=53 μTorr for 11 days. These

values represent the required P_{NH_3} on the cutaneous surface of air-exposed *R. marmoratus* to generate the measured rates of NH_3 volatilization determined by Frick and Wright (Frick and Wright, 2002b). The P_{NH_3} values calculated from our measurements were substantially higher [1251 μTorr (1 h); 608 μTorr (11 days)] than the theoretical estimates above and are more than sufficient to account for the rate of NH_3 volatilization.

In air-exposed *M. anguillicaudatus*, there is a progressive increase of NH_3 volatilization over a 3-day period corresponding to a gradual increase in internal ammonia levels (Tsui et al., 2002). As well, in the isopod *P. scaber*, periodic volatilization depends on the accumulation of high ammonia concentrations in the pleon fluid (Wright and O'Donnell, 1993). NH_3 volatilization in air-exposed *R. marmoratus* did not depend on the gradual build up of ammonia on the cutaneous surface. The cutaneous NH_4^+ concentration was constant over the 1–2 h of air exposure and there were no differences in cutaneous NH_4^+ concentration and pH between acutely (1 h) and chronically (11 days) air-exposed *R. marmoratus*. Furthermore, the increase in P_{NH_3} occurred almost immediately (1 h) upon air exposure. There are two possible explanations for these observations. First, very rapid changes in cutaneous circulation may result in a higher rate of delivery of ammonia to the cutaneous surface (perfusion-limited). For example, it is possible that air exposure triggers a surface vasodilatory response within the first few minutes that facilitates both gas exchange and ammonia excretion across the cutaneous surface. Cutaneous vasodilation has been proposed in *Anguilla vulgaris* (Berg and Steen, 1965) and *Lepidosiren paradoxa* (Johansen and Lenfant, 1967) during air exposure.

The second possibility is that the rate of cutaneous ammonia excretion does not change when they are air-exposed. However, the loss of ammonia from the cutaneous surface of the fish to the air is slower (diffusion-limited) because of the higher solubility of NH_3 in water than in air (e.g. water:air 700:1 mmol l^{-1} $Torr^{-1}$; Dejours, 1988). In this scenario, NH_4^+ concentration increases in the surface fluid within the first few minutes and then stabilizes as the rate of volatilization matches the rate of ammonia transport across the cutaneous surface. The greater diffusion coefficient of NH_3 in air [0.22–0.28 cm^2 s^{-1} (Reid et al., 1977; Incropera and DeWitt, 1990)] relative to water [1.96×10^{-5} cm^2 s^{-1} (Kemper, 1986)], however, suggests that the loss of ammonia from the cutaneous surface of the fish is a greater problem in water than in air. Despite these differences, the time it takes for the fish to eliminate their total ammonia content (turnover time) is longer in air than in water. Taking the values for ammonia tissue concentration and ammonia excretion rates from Frick and Wright (Frick and Wright, 2002b), we calculated turnover time (ammonia tissue concentration/ammonia excretion rate) in air (9.6 h) and water (2.5 h). The turnover time in air is almost fourfold greater than in water. These differences may relate to the factors discussed above, or possibly be influenced by changes in metabolic rate. Metabolic rate has not been directly measured in air-exposed *R. marmoratus*, but based on decreased oxygen consumption upon air exposure in some other air-tolerant fishes such as *Boleophthalmus boddaerti* (Kok et al., 1998), *P. modestus* and *Scartelaos histophorus* (Tamura et al., 1976), it may decrease. The components that regulate the elimination of ammonia in air are complex and require further study.

Immersed *R. marmoratus* had a lower pH in the cutaneous water boundary layer relative to the bulk water. In studies of *Oncorhynchus mykiss*, the gill water boundary layer and mucus on the cutaneous surface have a lower pH relative to the ambient water (Wright et al., 1986). Carbonic anhydrase catalyzes the conversion of excreted CO_2 to HCO_3^- and H^+. Acidification of the gill water boundary layer facilitates NH_3 excretion by converting NH_3 to NH_4^+, thereby maintaining the blood-to-water P_{NH_3} gradient (Wright et al., 1986; Wright et al., 1989). A similar scenario may occur across the branchial (and possibly cutaneous) surface of *R. marmoratus* in water. If we assume an arterial blood pH of ~7.8 (Wilkie, 2002), then cutaneous NH_3 diffusion in immersed fish would be facilitated by the more acidic water at the cutaneous surface (pH ~7.2), relative to the bulk water pH (pH ~7.9–8.2). Hence, in immersed *R. marmoratus* ammonia elimination probably depends on the passive diffusion of NH_3 from the blood to the water, as in other teleost species (Wood, 1993; Wilkie, 2002).

During air exposure, the cutaneous surface pH increased by 0.3–0.6 pH units (pH 7.5–7.8), possibly approaching blood pH values. pH increase on the cutaneous surface was also observed in air-exposed *M. anguillicaudatus* (Tsui et al., 2002). Although alkalinization is advantageous for gaseous NH_3 release from the cutaneous surface to the environment, it would not facilitate NH_3 diffusion from the blood to the boundary layer. It has been demonstrated that *P. schlosseri* actively excrete NH_4^+ across their gills when the blood-to-water P_{NH_3} is reversed (Randall et al., 1999), using the branchial $Na^+/K^+(NH_4^+)$-ATPase and $Na^+/H^+(NH_4^+)$ exchangers (Wilson et al., 2000). A similar mechanism may be involved in moving ammonia across the cutaneous surface in air-exposed *R. marmoratus*. Alkalinization at the cutaneous surface of air-exposed *R. marmoratus* may be the result of changes in the relative rates of cutaneous CO_2, HCO_3^-, H^+ and/or NH_3 excretion. Even very small changes in the composition of the fluid surrounding air-exposed *R. marmoratus* (~100 μm in depth) may markedly influence pH. Overall, very little is known about this microenvironment, and further studies are needed to clarify the role of the cutaneous surface in aerial respiration in *R. marmoratus*.

In immersed *R. marmoratus*, NH_4^+ concentration and pH on the cutaneous surface did not differ among anterior, mid-section and posterior locations along the body of the fish. These results are not consistent with the well-established conclusions of 'divided chamber' experiments, originally developed by Homer Smith (Smith, 1929). Data from a number of studies demonstrate that the majority (~80%) of nitrogen is eliminated *via* the anterior (branchial) region in different species of bony fishes (reviewed by Wood, 1993). Using a divided chamber, Frick and Wright (Frick and Wright, 2002b) reported that

immersed *R. marmoratus* excrete less of their total nitrogen from their anterior region (~57%) compared to many fishes (Wood, 1993). Thus, substantial cutaneous ammonia excretion in water may partly account for the homogenous nature of surface NH_4^+ concentrations in *R. marmoratus*. The discrepancy between previous studies and the present study may also be due to differences in the experimental protocol. In studies using ion-selective microelectrodes on larval rainbow trout, *O. mykiss*, the NH_4^+ concentration was higher and the pH was lower next to the surface of the gills (anterior location) compared to the cutaneous surface at a mid-section location along the body of the fish (Misiaszek, 1996). In previous experiments, measurements were taken on immobilized fish, whereas in the present experiment measurements were taken on unrestrained fish, in which there were occasional small movements of the fish. Any movement of the medium relative to an animal's surface will decrease the thickness of the boundary layer (Feder and Pinder, 1988). In studies on bullfrogs, *Rana catesbeiana*, occasional small movements ($1\ min^{-1}$) of the animal disturbed the boundary layer, decreasing its thickness (Pinder and Feder, 1990). As well, Lighthill has claimed that the lateral movements of the body segments of swimming fish result in a thinner boundary layer than would be expected over the rigid body of an immobilized fish (Lighthill, 1971). In the present study, occasional small movements of the unrestrained fish may have disturbed outer portions of the boundary layer, decreasing its thickness, and causing some mixing, to produce similar ion concentrations along the body of the fish in water.

Occasional small movements of the fish may also have contributed to the observed mixing of the thin film of fluid surrounding air-exposed *R. marmoratus* (personal observation). Thus, it is not known if the NH_4^+ concentration and pH on the cutaneous surface in air-exposed *R. marmoratus* varied at different locations along the body of the fish. Anything that makes a boundary layer thinner should promote the movement of water vapour at an air:water interface, in which water is evaporating into the air (Vogel, 1994). Although boundary layer thickness and flow velocity were not measured, there is a reduced boundary layer in air compared to that in water (Feder and Burggren, 1985) and air movement is usually greater than $10\ cm\ s^{-1}$ even in 'still' air (Nobel, 1974). As mentioned above, any movements of the medium next to an animal, either by environmentally induced currents or by movements of the organism itself, will also decrease the thickness of the boundary layer (Feder and Pinder, 1988). These movements may have led to the observed mixing of debris in the fluid surrounding the fish with the air flow.

Some species of oniscidean isopods use their gut for ammonia elimination by excreting over 90% of ammonia in their faeces, which may then volatilize (O'Donnell and Wright, 1995). The digestive tract of *R. marmoratus* does not seem to be involved in NH_3 volatilization since there were no differences in digestive tract pH in air-exposed relative to immersed fish. These results are not consistent with the findings of Tsui et al. (Tsui et al., 2002), who reported that the anterior region of the digestive tract was significantly more alkaline than the posterior region in air-exposed *M. anguillicaudatus*. Digestive tract NH_3 volatilization has not been directly measured in *M. anguillicaudatus*, but alkalinization of the digestive tract is suggestive of a possible role in NH_3 gaseous release (Tsui et al., 2002). Preliminary attempts were made to measure digestive tract ammonia concentration but insufficient fish were available for pooled samples (30 fish required for n=1). Although we cannot rule out the digestive tract as a site of NH_3 volatilization in *R. marmoratus*, it appears unlikely.

Amphibious fishes may experience dehydration during prolonged air exposure (Gordon et al., 1969; Gordon et al., 1978; Rozemeijer and Plaut, 1993). However, air-exposed *R. marmoratus* remained in a highly humid environment (relative humidity ~99%) and did not lose a significant body water content over time (11 days) compared to immersed fish. In fact, both air-exposed and immersed fish gained body water content over 11 days. Although statistically significant, the ~1% gain in body water content is probably the result of biological variability from using different groups of fish and is, thus, not physiologically relevant. The loss in body mass (~20% both groups) over 11 days is presumably due to fasting.

In conclusion, this study provides evidence that *R. marmoratus* utilizes the cutaneous surface as a primary site of NH_3 volatilization by elevating NH_4^+ concentrations concomitantly with pH during air exposure, thereby increasing P_{NH_3} for volatilization. It is these immediate changes on the cutaneous surface that may allow *R. marmoratus* to initiate and sustain NH_3 volatilization during air exposure. The elimination of ammonia *via* NH_3 volatilization may help to extend the time this species is able to survive out of water.

The authors would like to thank Dr David Noakes for the supply of *R. marmoratus*. This work was supported by a NSERC Discovery Grant to P.A.W.

References

Ammann, D. (1986). *Ion-selective Microelectrodes: Principles, Design and Application*, 346 pp. Berlin: Springer-Verlag.

Berg, T. and Steen, J. B. (1965). Physiological mechanisms for aerial respiration in the eel. *Comp. Biochem. Physiol.* **15**, 469-484.

Cameron, J. N. and Heisler, N. (1983). Studies of ammonia in the rainbow trout: physico-chemical parameters, acid-base behaviour and respiratory clearance. *J. Exp. Biol.* **105**, 107-125.

Chew, S. F., Chan, N. K. Y., Loong, A. M., Hiong, K. C., Tam, W. L. and Ip, Y. K. (2004). Nitrogen metabolism in the African lungfish (*Protopterus dolloi*) aestivating in a mucus cocoon on land. *J. Exp. Biol.* **207**, 777-786.

Davenport, J. and Sayer, M. D. J. (1986). Ammonia and urea excretion in the amphibious teleost *Blennius pholis* (L.) in sea-water and in air. *Comp. Biochem. Physiol.* **84A**, 189-194.

Dejours, P. (1988). *Respiration in Water and Air: Adaptations-Regulation-Evolution*, 179 pp. Amsterdam: Elsevier.

De Vries, M. C. and Wolcott, D. L. (1993). Gaseous ammonia evolution is coupled to reprocessing of urine at the gills of ghost crabs. *J. Exp. Zool.* **267**, 97-103.

Feder, M. E. and Burggren, W. (1985). Cutaneous gas exchange in vertebrates: design, patterns, control and implications. *Biol. Rev.* **60**, 1-45.

Feder, M. E. and Pinder, A. W. (1988). Ventilation and its effect on 'infinite pool' exchangers. *Am. Zool.* **28**, 973-983.

Frick, N. T. and Wright, P. A. (2002a). Nitrogen metabolism and excretion in the mangrove killifish, *Rivulus marmoratus* I. The influence of environmental salinity and external ammonia. *J. Exp. Biol.* **205**, 79-89.

Frick, N. T. and Wright, P. A. (2002b). Nitrogen metabolism and excretion in the mangrove killifish, *Rivulus marmoratus* II. Significant ammonia volatilization in a teleost during air exposure. *J. Exp. Biol.* **205**, 91-100.

Gordon, M. S., Boetius, I., Evans, D. H., McCarthy, R. and Oglesby, L. C. (1969). Aspects of the physiology of terrestrial life in amphibious fishes. I. The mudskipper *Periophthalmus sobrinus*. *J. Exp. Biol.* **50**, 141-149.

Gordon, M. S., Ng, W. W. and Yip, A. Y. (1978). Aspects of the physiology of terrestrial life in amphibious fishes. III. The Chinese mudskipper *Periophthalmus cantonensis*. *J. Exp. Biol.* **72**, 57-75.

Greenaway, P. and Nakamura, T. (1991). Nitrogenous excretion in two terrestrial crabs (*Gecarcoidea natalis* and *Geograpsus grayi*). *Physiol. Zool.* **64**, 767-786.

Grizzle, J. M. and Thiyagarajah, A. (1987). Skin histology of *Rivulus ocellatus marmoratus*: apparent adaptation for aerial respiration. *Copeia* **1987**, 237-240.

Incropera, F. P. and DeWitt, D. P. (1990). *Fundamentals of Heat and Mass Transfer*, 944pp. New York: John Wiley & Sons.

Ip, Y. K., Lee, C. Y., Chew, S. F., Low, W. P. and Peng, K. W. (1993). Differences in the responses of two mudskippers to air exposure. *Zool. Sci.* **10**, 511-519.

Ip, Y. K., Chew, S. F., Leong, I. A. W., Jin, Y., Lim, C. B. and Wu, R. S. S. (2001). The sleeper *Bostrichthys sinensis* (Family Eleotridae) stores glutamine and reduces ammonia production during aerial exposure. *J. Comp. Physiol. B* **171**, 357-367.

Janssens, P. A. and Cohen, P. P. (1968). Biosynthesis of urea in the aestivating African lungfish, *Protopterus aethiopicus*. *Science* **152**, 358-359.

Johansen, K. and Lenfant, C. (1967). Respiratory functions in the South American lungfish, *Lepidosiren paradoxa* (Fitz). *J. Exp. Biol.* **46**, 205-218.

Jow, L. Y., Chew, S. F., Lim, C. B., Anderson, P. M. and Ip, Y. K. (1999). The marble goby *Oxyeleotris marmoratus* activates hepatic glutamine synthetase and detoxifies ammonia to glutamine during air exposure. *J. Exp. Biol.* **202**, 237-245.

Kemper, W. D. (1986). Solute diffusivity. In *Methods of Soil Analysis, Part 1* (2nd edn.) (ed. A. Klute), pp. 1007-1024. Madison: ASA and SSSA.

Kok, W. K., Lim, C. B., Lam, T. J. and Ip, Y. K. (1998). The mudskipper *Periophthalmodon schlosseri* respires more efficiently on land than in water and vice versa for *Boleophthalmus boddaerti*. *J. Exp. Biol.* **280**, 86-90.

Kormanik, G. A. and Cameron, J. N. (1981). Ammonia excretion in animals that breathe water: a review. *Mar. Biol. Lett.* **2**, 11-23.

Lighthill, M. J. (1971). Large-amplitude elongated-body theory of fish locomotion. *Proc. R. Soc. Lond. B Biol. Sci.* **179**, 125-138.

Lim, C. B., Chew, S. F., Anderson, P. M. and Ip, Y. K. (2001). Reduction in the rates of protein and amino acid catabolism to slow down the accumulation of endogenous ammonia: a strategy potentially adopted by mudskippers (*Periophthalmodon schlosseri* and *Boleophthalmus boddaerti*) during aerial exposure in constant darkness. *J. Exp. Biol.* **204**, 1606-1614.

Maddrell, S. H. P., O'Donnell, M. J. and Caffrey, R. (1993). The regulation of haemolymph potassium activity during initiation and maintenance of diuresis in fed *Rhodnius prolixus*. *J. Exp. Biol.* **177**, 273-285.

Misiaszek, C. F. M. (1996). The development of ion regulation in larval rainbow trout, *Oncorhynchus mykiss*. PhD thesis, McMaster University, Hamilton, ON, Canada.

Nobel, P. S. (1974). *Introduction to Biophysical Plant Ecology*, 488 pp. San Francisco: W. H. Freeman.

O'Donnell, M. J. and Wright, J. C. (1995). Nitrogen excretion in terrestrial crustaceans. In *Nitrogen Metabolism and Excretion* (ed. P. J. Walsh and P. A. Wright), pp. 105-118. Boca Raton, FL: CRC Press.

Pinder, A. W. and Feder, M. E. (1990). Effect of boundary layers on cutaneous gas exchange. *J. Exp. Biol.* **143**, 67-80.

Ramaswamy, M. and Reddy, G. T. (1983). Ammonia and urea excretion in three species of air-breathing fish subjected to aerial exposure. *Proc. Indian Acad. Sci. Anim. Sci.* **92**, 293-297.

Randall, D. J., Wilson, J. M., Peng, K. W., Kok, T. W. K., Kuah, S. S. L., Chew, S. F., Lam, T. J. and Ip, Y. K. (1999). The mudskipper, *Periophthalmodon schlosseri,* actively transports NH_4^+ against a concentration gradient. *Am. J. Physiol.* **277**, 1562-1567.

Reid, R. C., Prausnitz, J. M. and Sherwood, T. K. (1977). *Properties of Gases and Liquids* (3rd edn), 688 pp. New York: McGraw-Hill.

Rombough, P. J. and Ure, D. (1991). Partitioning of oxygen uptake between cutaneous and branchial surfaces in larval and young juvenile Chinook salmon *Oncorhychus tshawytscha*. *Physiol. Zool.* **64**, 717-727.

Rozemeijer, M. J. C. and Plaut, I. (1993). Regulation of nitrogen excretion of the amphibious Blenniidae *Alticus kirki* (Guenther, 1868) during emersion and immersion. *Comp. Biochem. Physiol.* **104A**, 57-62.

Smith, H. W. (1929). The excretion of ammonia and urea by the gills of fish. *J. Biol. Chem.* **81**, 727-742.

Tamura, S. O., Morii, H. and Yuzuriha, M. (1976). Respiration of the amphibious fishes *Periophthalmus cantonensis* and *Boleopthalmus chinensis* in water and on land. *J. Exp. Biol.* **65**, 97-107.

Tsui, T. K. N., Randall, D. J., Chew, S. F., Jin, Y., Wilson, J. M. and Ip, Y. K. (2002). Accumulation of ammonia in the body and NH_3 volatilization from alkaline regions of the body surface during ammonia loading and exposure to air in the weather loach *Misgurnus anguillicaudatus*. *J. Exp. Biol.* **205**, 651-659.

Vogel, S. (1994). *Life in Moving Fluids: The Physical Biology of Flow* (2nd edn), 467pp. New Jersey: Princeton University Press.

Weihrauch, D., Morris, S. and Towle, D. W. (2004). Ammonia excretion in aquatic and terrestrial crabs. *J. Exp. Biol.* **207**, 4491-4504.

Wieser, W. (1972). Oxygen consumption and ammonia excretion in *Ligia beaudiana*. *Comp. Biochem. Physiol.* **43A**, 869-876.

Wilkie, M. P. (2002). Ammonia excretion and urea handling by fish gills: present understanding and future research challenges. *J. Exp. Zool.* **293**, 284-301.

Wilson, J. M., Kok, T. W. K., Randall, D. J., Vogl, W. A. and Ip, Y. K. (1999). Fine structure of the gill epithelium of the terrestrial mudskipper, *Periophthalmodon schlosseri. Cell Tissue Res.* **298**, 345-356.

Wilson, J. M., Randall, D. J., Donowitz, M., Vogl, W. A. and Ip, Y. K. (2000). Immunolocalization of ion-transport proteins to branchial epithelium mitochondria-rich cells in the mudskipper (*Periophthalmodon schlosseri*). *J. Exp. Biol.* **203**, 2297-2310.

Wood, C. M. (1993). Ammonia and urea metabolism and excretion. In *The Physiology of Fishes* (ed. D. H. Evans), pp. 379-425. Boca Raton, FL: CRC Press.

Wright, J. C. and O'Donnell, M. J. (1993). Total ammonia concentration and pH of haemolymph, pleon fluid and maxillary urine in *Porcellio scaber* Lattreille (Isopoda, Oniscidea): relationships to ambient humidity and water vapour uptake. *J. Exp. Biol.* **176**, 233-246.

Wright, P. A., Heming, T. and Randall, D. J. (1986). Downstream pH changes in water flowing over the gills of rainbow trout. *J. Exp. Biol.* **126**, 499-512.

Wright, P. A., Randall, D. J. and Perry, S. F. (1989). Fish gill water boundary layer: a site of linkage between carbon dioxide and ammonia excretion. *J. Comp. Physiol. B* **158**, 627-635.