The Journal of Experimental Biology 205, 91–100 (2002)
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JEB3719

Nitrogen metabolism and excretion in the mangrove killifish *Rivulus marmoratus*

II. Significant ammonia volatilization in a teleost during air-exposure

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Accepted 22 October 2001

Summary

The mangrove killifish *Rivulus marmoratus* can tolerate prolonged periods of air-exposure (>1 month). During these periods of emersion, we hypothesized that *R. marmoratus* would convert potentially toxic ammonia into urea and free amino acids (FAAs). In air-exposed fish, both ammonia (J_{Amm}) and urea (J_{Urea}) excretion continued at approximately 57 % and 39 %, respectively, of submerged rates. Remarkably, approximately 42 % of the total ammonia excreted during air-exposure was through NH_3 volatilization. Ammonia did not accumulate in whole-body tissues of air-exposed fish, but levels of both urea and some FAAs (primarily alanine and glutamine) were up to twofold higher after 10 days. The activities of the ornithine–urea cycle enzymes carbamoyl phosphate synthetase III and ornithine transcarbamylase increased (by approximately 30 % and 36 %, respectively) in whole-body tissues of air-exposed fish, while levels of arginase remained unchanged. The activities of enzymes involved in amino acid and oxidative metabolism were not significantly different between control and air-exposed fish. Partitioning of the anterior and posterior ends of immersed fish revealed that just over half (57 %) of the total nitrogen (ammonia+urea) was excreted through the anterior end of the fish, presumably *via* the branchial tissues, while emersed fish increased excretion *via* the posterior end (kidney+skin). *R. marmoratus* do not undergo a shift towards ureotelism during air-exposure. Rather, we propose that *R. marmoratus* are able to survive on land for extended periods without significant ammonia accumulation because they continuously release ammonia, partially by NH_3 volatilization.

Key words: urea, air-tolerant teleost, amino acid, glutamine, glutamate, alanine, urea cycle enzyme, gaseous NH_3 excretion, mangrove killifish, *Rivulus marmoratus*.

Introduction

Rivulus marmoratus, the mangrove killifish, is a small cyprinodont fish that can tolerate prolonged periods of emersion (>1 month), living amongst moist leaf litter, detritus or in hollowed logs. This ability to survive and remain active for extended periods out of water is remarkable because most other species of ‘amphibious’ or ‘air-breathing’ fishes are unable to tolerate more than a few days completely out of water, e.g. *Heteropneustes fossilis*, <4 days (Saha and Ratha, 1989), *Oxyeleotris marmoratus*, approximately 7 days (Jow et al., 1999). Fish that do remain on land for weeks or months often undergo a profound metabolic depression (e.g. African lungfish) (Smith, 1930; Hochachka and Guppy, 1987). *R. marmoratus* do not aestivate when emersed, and it has been suggested that they respire cutaneously *via* a highly vascularized epidermis (Grizzle and Thiyagarajah, 1987).

Many air-tolerant fishes shift from ammoniotelism to ureotelism during air-exposure. For example, the climbing perch (*Anabas scandens*) and the snakehead (*Channa gachua*) (Ramaswamy and Reddy, 1983), the mudskipper (*Periophthalmus cantonensis*) (Gordon et al., 1978), the amphibious blenny (*Alticus kirki*) (Rozemeijer and Plaut, 1993) and the African lungfish (*Protopterus* sp.) (Forster and Goldstein, 1966; Janssens and Cohen, 1968) increase urea tissue levels and/or the percentage of urea excreted [urea-N excretion/(urea-N excretion+ammonia-N excretion)] on land. In some fishes, a shift towards ureotelism is accompanied by increased synthesis of urea *via* the ornithine–urea cycle (OUC). Air-exposure induces the activity of several OUC enzymes, including the key enzyme carbamoyl phosphate synthetase III (CPSase III) in *H. fossilis* (Saha and Ratha, 1998).

Not all air-breathing fish become ureotelic during air-exposure. It appears that some air-tolerant fishes, such as the mudskippers *Periophthalmodon schlosseri* and *Boleophthalmus boddaerti*, reduce proteolysis and amino acid catabolism on land, particularly when held under constant darkness (Lim et al., 2001). When terrestrial conditions are accompanied by a natural photoperiod, however, active *P. schlosseri* have elevated free amino acid (FAA) concentrations in their muscle tissue (Ip et al., 1993, 2001). Ip and coworkers propose that activity on land is fuelled by partial amino acid

catabolism (glutamate+pyruvate→α-ketoglutarate+alanine, catalysed by alanine aminotransferase). α-Ketoglutarate enters the Krebs cycle, undergoes partial oxidation to malate and is subsequently converted to pyruvate, catalysed by malic enzyme (ME). This metabolic scenario was supported by the observation that alanine levels were elevated in the tissues of air-exposed active *P. schlosseri* (Ip et al., 1993, 2001). The key advantage of partial amino acid catabolism is that ATP is produced without the net release of ammonia (Ip et al., 2001).

A few species of fish continue to excrete primarily ammonia when out of water, e.g. *Blennius pholis* (Davenport and Sayer, 1986). In the terrestrial environment, the absence of gill ventilation will presumably result in a decreased reliance on the gills and an increased reliance on the skin or kidneys for nitrogen excretion. Ammonia elimination *via* NH_3 volatilization has been documented in *B. pholis*, but accounts for only a small percentage (approximately 8 %) of the total nitrogen excreted (Davenport and Sayer, 1986).

In the accompanying study, we discovered that mangrove killifish do not elevate tissue ammonia, urea or FAA levels following 4 days of exposure to high external ammonia levels (223 μmol l^{-1} NH_3) (Frick and Wright, 2002). Rather, *R. marmoratus* may continue to excrete ammonia actively. In this study, we propose that, for *R. marmoratus* to survive out of water for extended periods, they must have evolved very efficient strategies for eliminating or detoxifying the nitrogen end-product, ammonia. We tested the following four hypotheses: (i) that prolonged air-exposure will elevate tissue ammonia, urea and FAA levels, with a corresponding induction of the enzymes involved in their synthesis; (ii) that, during emersion, a significant proportion of nitrogen will be eliminated from the fish as gaseous NH_3; (iii) that, following emersion, accumulated ammonia and urea will diffuse out of the fish, resulting in higher rates of nitrogen excretion during the initial part of the recovery period; and (iv) that the site of ammonia excretion during emersion will switch from primarily branchial to primarily renal and/or cutaneous.

To test these hypotheses, the rates of ammonia (J_{Amm}) and urea (J_{Urea}) excretion were measured together with tissue ammonia and urea levels in air-exposed and submerged fish. The activities of three enzymes in the OUC, CPSase III, ornithine transcarbamylase (OTCase) and arginase (ARG), and of an accessory enzyme, glutamine synthetase (GS), were also measured to assess the role of the OUC in *R. marmoratus*. Changes in tissue FAA levels and in the activities of several enzymes involved in amino acid metabolism were also determined. Alanine aminotransferase (Ala-AT), aspartate aminotransferase (Asp-AT) and glutamate dehydrogenase (GDH) catalyze the reactions that synthesize or degrade the amino acids alanine, aspartate and glutamate, respectively. Malic enzyme (ME) replenishes the pyruvate supply by converting malate to pyruvate (see above). In addition, citrate synthase (CS) and cytochrome *c* oxidase (CCO) (enzymes of oxidative metabolism) activities were measured in control and air-exposed fish. These enzymes are indicators of metabolic rate because their activities reflect the rates of oxygen consumption of both individual tissues and whole organisms (Simon and Robin, 1971; Somero and Childress, 1980; Smith and Chong, 1982).

A divided chamber was used to partition the anterior (representing primarily branchial excretion) and posterior (cutaneous, kidney and intestinal excretion) portions of *R. marmoratus*, and J_{Amm} and J_{Urea} were measured in air-exposed and submerged fish. Finally, the pattern of nitrogen excretion in response to air-exposure was compared in laboratory-reared and wild-caught *R. marmoratus*. This experiment was important to establish that the physiological responses of a colony of laboratory-reared fish were essentially the same as those of wild fish (Frick and Wright, 2002).

Materials and methods

Experimental animals

Laboratory-reared *Rivulus marmoratus* Poey were maintained as described previously (Frick and Wright, 2002). Wild-caught fish were collected from Twin Cays and Little Lagoon Cay, near Dangriga, Belize, and held as described previously (Frick and Wright, 2002).

Experimental protocol

Excretion during emersion

Two separate experiments were performed on laboratory-reared fish. (i) Fish were exposed in air for 12 h (N=18) and then returned to water; excretion rates were measured every 2 h following emersion and compared with rates measured 'pre-emersion'. (ii) Fish were exposed in air for 11 days (N=6), and excretion rates were measured during emersion and compared with rates measured in fish under control conditions (immersion).

Under air-exposed conditions, the fish were removed from water and placed onto a moist substratum (filter paper and cotton batting saturated with approximately 2 ml of 16 ‰ sea water). In experiment ii, the amount of urea and ammonia excreted over a 24 h period was measured after 1, 3, 5, 7, 9 and 11 days of air-exposure and under control conditions. Technical control experiments were performed in which a known amount of ammonia and urea were placed on the filter paper and left for 24 h to determine whether there was any loss (or gain) due to bacterial contamination or other factors. The filter paper and cotton were collected after 24 h, and the water was carefully extracted.

Ammonia volatilization

To measure the amount of ammonia excreted through volatilization, an apparatus was constructed similar to that used by Davenport and Sayer (1986). Air was first bubbled through acidified distilled water (ADW) (pH 6) to remove any ammonia in the air supply. The air was then passed into a second chamber containing the fish on filter paper saturated with 16 ‰ sea water at pH 6. The air exiting the second chamber was bubbled through a third chamber containing 0.5 mol l^{-1} KOH, which removed CO_2. Air from the third chamber was bubbled

through five separate acid traps each containing ADW to 'fix' any ammonia present in the air. Preliminary studies showed that five acid traps were necessary to ensure maximal recovery of the volatilized ammonia. Control trials were run in which filter paper was saturated (i) with ammonia-free, pH 6, 16‰ sea water or (ii) with a known amount of ammonia (100 μmol l^{-1}) in 16‰ sea water, pH 6. These control trials established that no ammonia volatilization was measured in the absence of a fish.

Routes of excretion

To determine the sites of J_{Amm} and J_{Urea} in laboratory-reared *R. marmoratus*, a Plexiglas chamber (see Wells and Pinder, 1996) was used to separate the anterior (representing primarily branchial excretion) and posterior (cutaneous, kidney and intestinal excretion) of the fish. Fish were lightly anaesthetized in 2-phenoxy-ethanol (1.2 ml l^{-1}) to reduce stress during the restraining period. The head end of the fish was then positioned through a small hole (approximately 5 mm in diameter) in a 3 cm×3 cm piece of dental dam, forming a tight seal around the fish just posterior to the operculum. Under control conditions (N=7), 4 ml of 16‰ sea water was placed into both the front and back ends of the chamber. In a separate group of fish, moist pieces of cotton were placed in the front and back ends of the chamber during air-exposure (N=7). After 1 h, water or cotton/filter samples were collected for later determination of ammonia and urea concentrations. In a preliminary experiment, to test for leakage across the dental dam barrier, a dye was placed in one half of the chamber and distilled water in the other half. Absorbance at 660 nm was used to quantify leakage. There was negligible (<1 %) leakage between the anterior and posterior portions of the chamber.

Nitrogen metabolism

Laboratory-reared fish held under air-exposed and control conditions were killed after 1, 4 and 10 days. Tissue samples were stored at –80 °C and analysed within 3 weeks. Tissue ammonia, urea and FAA levels and enzyme activities were measured in whole-body samples (N=6) as adequate amounts of individual tissues were not available because of the small size of the fish and the limited number of fish available. Although whole-animal samples include both intracellular and extracellular fluid, values represent mostly tissue intracellular concentrations (approximately 95 % of volume).

Amphibious fish typically experience water loss during emersion, which could concentrate tissue solutes (e.g. ammonia, urea or FAAs). Wet mass measurements were taken in laboratory-reared fish after 1, 3 and 10 days of air-exposure (N=7) and under control conditions (N=7) to test for significant mass loss.

Analytical techniques

Ammonia and urea analyses

The concentrations of ammonia and urea levels in the water were determined as described previously (Frick and Wright, 2002). Ammonia levels in the acid traps of the volatilization experiment were determined using the method of Verdouw et al. (1978).

Tissue ammonia and urea levels of whole fish were determined as described previously (Frick and Wright, 2002) and were expressed as mmol N g^{-1} wet mass. All spectrophotometric measurements were performed using a Perkin Elmer UV/VIS spectrophotometer (Lambda 2) (Perkin Elmer Corp., Norwalk, CT, USA).

Tissue amino acid analysis

Tissue FAA levels were measured using high-performance liquid chromatography (HPLC) (Hewlett-Packard series II 1090 liquid chromatograph), as described previously (Frick and Wright, 2002).

Enzyme analysis

Frozen tissues (whole fish) were ground to a fine powder under liquid nitrogen. The powdered tissue was then diluted 23-fold with extract buffer (50 mmol l^{-1} Hepes buffer, pH 7.5, 50 mmol l^{-1} KCl, 1 mmol l^{-1} dithiothreitol and 0.5 mmol l^{-1} EDTA), homogenized on ice (Euro Turrax T20b homogenizer) for three bursts of 10 s and then sonicated (Vibracell CV18 2368) for three bursts of 10 s. The homogenate was centrifuged (14 000 ***g***, 4 °C) for 10 min, and the resulting supernatant was used to measure the activities of the enzymes Ala-AT, Asp-AT, GDH, ME, CCO and CS. For the OUC enzymes (CPSase, OTCase and ARG) and the accessory enzyme GS, an additional step was carried out. The resulting supernatant was passed through a Sephadex G-25 column (10 ml) equilibrated with extract buffer to remove low-molecular mass (<5000 g mol^{-1}) substrates and effectors (Felskie et al., 1998). The protein concentration of the extract was measured before and after filtration to calculate the dilution factor of the column.

The activities of OUC enzymes were measured as described previously: OTCase by Wright et al. (1995), ARG by Felskie et al. (1998) and GS by Shankar and Anderson (1985). CPSase activity was determined using the reaction mixture described by Chadwick and Wright (1999), and [^{14}C]carbamoyl phosphate production was measured using the technique described by Anderson et al. (1970). OTCase, ARG, GS and CPSase activities were measured at 27 °C for consistency with methods in the literature. The limits of detection for these assays were estimated to be 0.04 nmol g^{-1} min^{-1} for CPSase, 0.01 μmol g^{-1} min^{-1} for OTCase and ARG and 0.08 μmol g^{-1} min^{-1} for GS (Chadwick and Wright, 1999).

The activities of both CPSase II and III were measured. CPSase II requires glutamine as a substrate, does not require N-acetylglutamate, AGA (nor is it affected by the addition of AGA) and is inhibited by UTP. CPSase III requires glutamine and AGA as substrates, and is not inhibited by UTP. Thus, to differentiate between CPSase II and III, activities were measured in the presence of glutamine alone (CPSase II), glutamine+AGA (CPSase II and III) and glutamine+AGA+UTP (CPSase III). To assess the ability of these fish to utilize ammonia as a substrate in the OUC rather than glutamine (as reported in *H. fossilis*) (Saha et al., 1997),

preliminary tests were run using 5 mmol l^{-1} NH_4Cl in place of glutamine. As activities in the presence of ammonia were relatively low compared with activities in the presence of glutamine, only glutamine was used as a substrate in subsequent experiments.

GDH, Ala-AT, Asp-AT, CS and ME were measured at 25 °C, as described by Singer and Ballantyne (1991). CCO activity was measured at 25 °C using the assay of Blier and Guderley (1988). The protein concentrations of extracts were measured using the method of Bradford (1976) with bovine serum albumin as a standard.

Statistical analyses

All data are presented as means ± standard error of the mean (S.E.M.). Single-factor analyses of variance (ANOVAs) were used to examine the differences between control and air-exposed values for tissue analysis (levels of urea and ammonia and enzyme activities) and excretion rates. Amino acid data were analyzed using a General Linear models procedure using the SAS system (version 6.12; SAS Institute Inc., Cary, NC, USA). The Tukey test was used to determine where differences were significant ($P \leqslant 0.05$) between treatment and control fish. Assumptions for normality were verified by generating appropriate residual plots. Data transformations (logarithmic, square root and inverse square root) were used when appropriate to meet the above assumptions.

Results

Ammonia and urea excretion following emersion

Following 12 h of emersion, there were no significant changes in J_{Amm} compared with pre-emersion rates (Fig. 1A). Urea excretion rates were found to vary according to the time of day (Frick and Wright, 2002), and statistical analysis was therefore performed between pre- and post-emersion excretion rates at the appropriate time periods. For example, 2, 4 and 6 h post-emersion sampling occurred between 12:00 and 18:00 h, and these values were therefore compared with pre-emersion excretion rates for the same time period. Using this method of analysis, no significant differences were found between pre- and post-emersion J_{Urea} (Fig. 1B).

Ammonia and urea excretion during emersion

Laboratory-reared fish

During long-term air-exposure (11 days), both J_{Amm} and J_{Urea} were significantly lower in air-exposed fish than in controls, but continued at approximately 57 % and 39 %, respectively, of submerged rates (Fig. 2). In water, ammonia excretion was significantly higher on day 1 but remained unchanged throughout the following 10 days (Fig. 2A). In air, the rate of ammonia excretion did not change with the duration of exposure (Fig. 2A). Approximately 42 % of the total ammonia excreted during air-exposure was *via* NH_3 volatilization (Fig. 2A). In water, urea excretion remained unchanged over the 11 days, but in air urea excretion was significantly lower on day 7 compared with day 1 (Fig. 2B).

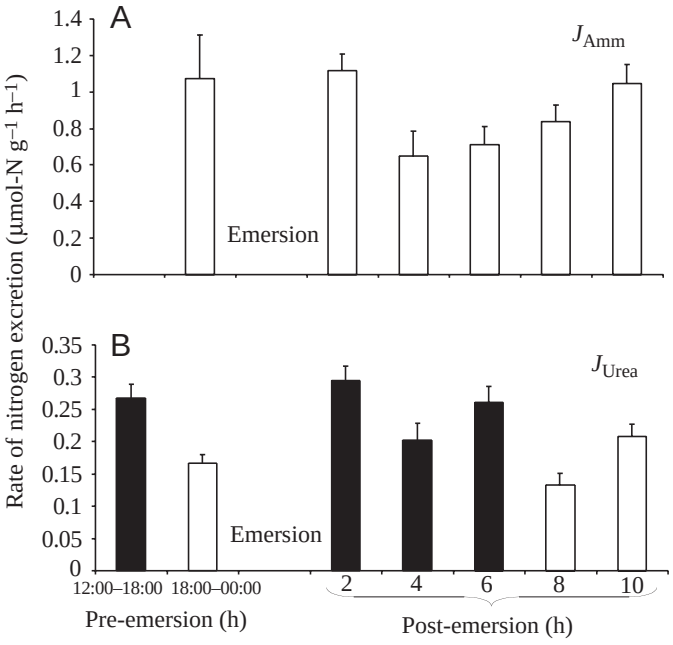

Fig. 1. Ammonia excretion (J_{Amm}) (A) and urea excretion (J_{Urea}) (B) in laboratory-reared *Rivulus marmoratus* before emersion and at 2 h intervals following 12 h of emersion. Statistical comparisons were performed between pre- and post-emersion excretion rates. For J_{Urea} (B), comparisons were made at the appropriate time periods to take into consideration the diurnal pattern of J_{Urea} (i.e. filled columns pre-emersion were compared with filled columns post-emersion). No significant differences were found between pre- and post-emersion J_{Amm} or J_{Urea}. Values are expressed as means + S.E.M. (N=18).

Wild-caught fish

Under conditions of air-exposure, wild-caught *R. marmoratus* altered their nitrogen excretion patterns in a manner similar to that of laboratory-reared fish. There were no significant differences between excretion rates in laboratory-reared (J_{Amm}=0.22±0.03 μmol-N g^{-1} h^{-1}; J_{Urea}=0.06±0.01 μmol-N g^{-1} h^{-1}, N=6) and wild-caught (J_{Amm}=0.28±0.03 μmol-N g^{-1} h^{-1}; J_{Urea}=0.05±0.01μmol-N g^{-1} h^{-1} , N=14) fish following 1 day of air-exposure.

Tissue ammonia and urea levels

Ammonia did not accumulate in the tissue of *R. marmoratus* during air-exposure (Fig. 3A). After 4 and 10 days of air-exposure, tissue urea levels were significantly higher (twofold) than corresponding control values (Fig. 3B).

Tissue amino acid levels

The total individual tissue FAA levels following 1, 4 and 10 days of air-exposure did not change significantly in *R. marmoratus* (Table 1). Of the essential amino acids, phenylalanine and lysine levels were significantly higher after 4 and 10 days of air-exposure, while leucine levels were higher on day 4 only. Of the non-essential amino acids, alanine levels were significantly higher (an approximately 1.5-fold increase) after 1, 4 and 10 days of air-exposure compared with control

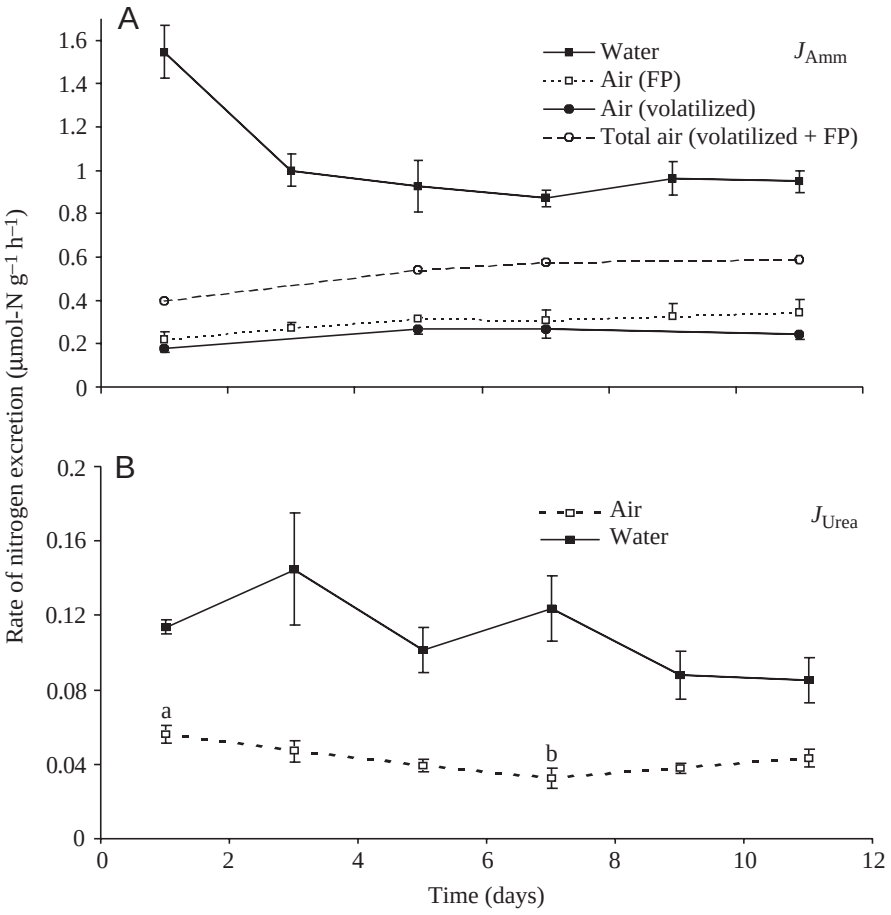

Fig. 2. Ammonia excretion (J_{Amm}) (A) and urea excretion (J_{Urea}) (B) in air-exposed and submerged laboratory-reared *Rivulus marmoratus*. Values are expressed as means ± S.E.M. (N=6). FP, filter paper. For J_{Amm} (A), values presented for total J_{Amm} in air (volatilized+FP) are calculated from the mean of these two rates at each time period. For J_{Urea} (B), excretion rates in air were significantly lower at all time points (P<0.05), and different letters indicate significant differences between time points (P<0.05).

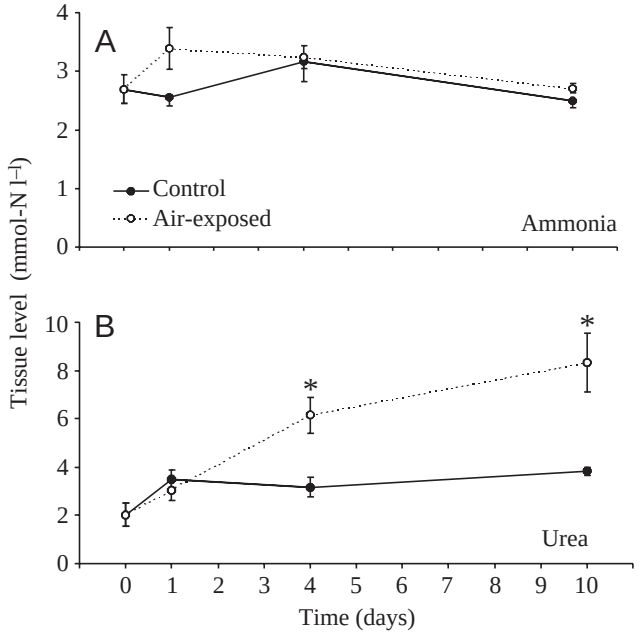

Fig. 3. Whole-body ammonia (A) and urea (B) levels in air-exposed and submerged laboratory-reared *Rivulus marmoratus*. Values are expressed as means ± S.E.M. (N=6). An asterisk denotes a significant difference from the corresponding control value.

fish. Glutamine levels were significantly higher on days 1 (1.8-fold) and 10 (twofold), but not on day 4, whereas glutamate levels were significantly lower on days 1 and 4, but returned to control values by day 10.

Both air-exposed and submerged *R. marmoratus* lost approximately 4, 6.5 and 13 % of their body mass following 1, 3 and 10 days of experimentation, respectively. There were no significant differences in mass loss between air-exposed and submerged fish at any time period.

Enzyme activities

All three enzymes of the OUC (CPSase, OTCase and ARG) and the accessory enzyme GS were detected in *R. mamoratus* (Tables 2, 3). The presence of CPSase III in *R. marmoratus* is indicated by activation with AGA (10–42 %) and little inhibition in the presence of the CPSase II inhibitor UTP (Table 2). After 4 days of air-exposure, significant increases in activity were found under all assay conditions (Table 2). Following 10 days of air-exposure, increased activity was observed with glutamine alone (CPSase II) and with glutamine+AGA+UTP (CPSase III) in the assay mixture (Table 2); however, these increases were relatively modest (approximately 30 %). OTCase activity was significantly higher (approximately 36 %) after 4 days of air-exposure when activity was expressed per gram wet mass (Table 3); however, when expressed per milligram of protein, there was no

Table 1. *Whole-body levels of free amino acids in laboratory-reared mangrove killifish after 1, 4 and 10 days of air-exposure and under control (immersed) conditions*

	Day 1		Day 4		Day 10	
	Control	Air-exposure	Control	Air-exposure	Control	Air-exposure
Essential amino acids						
His	300±59	297±29	234±37	271±36	212±26	283±43
Thr	415±45	411±24	402±39	378±40	373±38	386±50
Val	138±10	147±10	125±10	175±18	142±11	155±18
Met	195±10	220±11	197±16	221±23	176±9	216±13
Trp	506±47	529±39	582±22	537±62	564±22	571±35
Phe	33±7	33±2	31±5	65±11*	25±2	40±6*
Ile	79±11	84±6	70±10	122±21	72±6	83±11
Leu	116±14	124±10	103±13	181±30*	109±11	127±17
Lys	217±22	226±2	230±37	370±68*	267±32	412±59*
Total	1 999±161	2 071±47	1 973±119	2 321±228	1 940±112	2 276±221
Non-essential amino acids						
Asp	1 470±142	1 508±77	1651±240	1 434±250	1 756±140	2 047±251
Glu	1 235±43	938±95*	1 291±58	837±67*	1 079±52	1 099±78
Asn	233±55	252±32	175±19	132±29	158±23	308±51
Ser	240±34	301±25	253±28	324±36	230±31	293±41
Gln	592±39	1039±97*	535±55	692±114	481±42	984±106*
Gly	1 942±232	2 172±118	2 245±324	1 975±90	2 952±201	3 112±206
Ala	621±93	857±46*	571±45	828±73*	565±74	810±53*
Tau	12 561±633	12 500±666	14 022±417	13 653±886	14 097±481	15 737±1 003
Arg	118±9	159±43	160±60	147±16	116±28	147±10
Tyr	108±18	79±13	55±8	73±23	62±12	72 ±9
Hpr	1 731±342	1 450±116	1 722±226	1 456±399	1 875±355	1 570±550
Pro	293±44	184±45	301±64	287±64	295±86	249±75
Total	21 146±1241	21 439±776	22 981±582	21 838±1257	23 665±419	26 429±1315
Total	23 145±1391	23 510±802	24 953±684	24 159±1422	25 604±474	28 704±1475

Values are expressed in nmol g^{-1} wet mass. Values are means ± S.E.M. (N=6).
*A significant difference from the corresponding control value (P<0.05).

Table 2. *Whole-body carbamoyl phosphate synthetase activity in laboratory-reared mangrove killifish after 4 and 10 days of air-exposure and under control (immersed) conditions*

	Day 4		Day 10	
Substrate and/or effector present	Control	Air-exposure	Control	Air-exposed
Glutamine	0.26±0.02	0.36±0.04*	0.32±0.02	0.42±0.05*
AGA	0.26±0.02	0.35±0.03*	0.35±0.02	0.42±0.05
Glutamine+AGA	0.31±0.02	0.51±0.03*	0.38±0.01	0.46±0.06
% Activation	+19	+42	+19	+10
Glutamine+AGA+UTP	0.29±0.02	0.45±0.03*	0.35±0.01	0.46±0.06*
% Inhibition	–6	–11	–8	–0

Values are expressed in nmol g^{-1} wet mass min^{-1}. Values are means ± S.E.M. (N=6).
*A significant difference from the corresponding control value (P<0.05).
AGA, N-acetylglutamate.

significant difference (data not shown). No significant changes were detected in ARG or GS activity (Table 3).

There was a small, but significant, increase (approximately 18 %) in CS activity after 10 days of air-exposure (Table 3) but, when expressed per milligram of protein, there was no significant difference (data not shown). There were no changes in the activities of GDH, Ala-AT, Asp-AT, ME or CCO during air-exposure. It should be noted that expression of enzyme activity per milligram of protein did not change the results, except as noted above.

Table 3. *Whole-body enzyme activities in laboratory-reared mangrove killifish after 4 and 10 days of air-exposure and under control (immersed) conditions*

	Day 4		Day 10	
Enzyme	Control	Air-exposed	Control	Air-exposed
Arginase	0.65±0.09	0.75±0.08	0.57±0.01	0.61±0.03
Ornithine transcarbamylase	0.29±0.04	0.39±0.03*	0.32±0.04	0.34±0.02
Glutamine synthetase	2.95±0.13	3.28±0.35	2.81±0.19	3.15±0.24
Alanine aminotransferase	1.87±0.17	1.71±0.22	1.79±0.12	1.86±0.07
Aspartate aminotransferase	16.62±1.75	18.15±1.42	14.62±1.18	17.24±0.64
Glutamate dehydrogenase	2.68±0.21	3.09±0.19	2.34±0.30	2.49±0.26
Malic enzyme	0.25±0.06	0.28±0.02	0.21±0.03	0.27±0.02
Citrate synthase	2.43±0.15	2.84±0.17	2.34±0.10	2.75±0.16*
Cytochrome *c* oxidase	0.13±0.03	0.10±0.02	0.08±0.02	0.09±0.01

Values are expressed in μmol g^{-1} wet mass min^{-1}. Values are means ± S.E.M. (N=6).
*A significant difference from the corresponding control value (P<0.05).

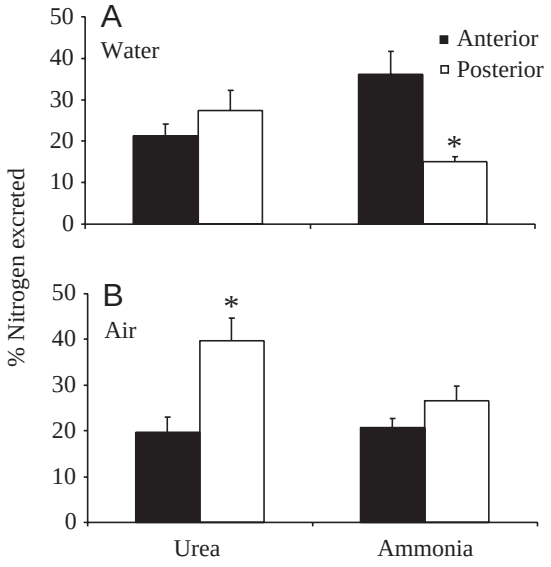

Fig. 4. Percentage of ammonia and urea excretion through the anterior and posterior regions of laboratory-reared *Rivulus marmoratus* in water (A) and in air (B). Values are expressed as means + S.E.M. (N=7). An asterisk denotes a significant difference between the anterior and posterior regions.

Routes of excretion

When *R. marmoratus* were immersed, the majority of nitrogenous wastes (approximately 57 %) were excreted *via* the anterior end of the fish, primarily as ammonia (Fig. 4A). Immersed fish excreted urea relatively equally through the anterior and posterior regions (Fig. 4A); however, in emersed fish, a higher percentage of urea was excreted posteriorly (Fig. 4B). The amount of ammonia excreted onto the filter paper was relatively equal through the front and back ends in air-exposed fish, but it should be noted that these values do not consider NH_3 volatilization. In addition, the high level of stress associated with confinement probably affected the absolute values of J_{Amm} and J_{Urea} and, thus, these results provide a qualitative rather than a quantitative view of the routes of excretion.

Discussion

Nitrogen metabolism and excretion during emersion

R. marmoratus are remarkable in their ability to survive out of water for extended periods. Our findings indicate that they are ammoniotelic both in water and on land and that the physiological responses to emersion are consistent between captively bred and wild fish. To our knowledge, this is the first published report of a teleost fish that releases a substantial proportion (approximately 42 %) of J_{Amm} as gaseous ammonia (approximately 40 % of total nitrogen excretion in air). Furthermore, ammonia volatilization continues unabated for prolonged periods, at least 11 days and presumably longer.

The amphibious blenny (*Blennius pholis*) also remains ammoniotelic following 24 h of air-exposure and is capable of ammonia volatilization, but to a lesser degree (approximately 8 % of ammonia is excreted through volatilization) (Davenport and Sayer, 1986). The weather loach *Misgurnus anguillicaudatus* has been observed to volatilize NH_3 from alkaline (pH 7.9–8.1) surfaces of the body during air-exposure (Tsui et al., 2002). NH_3 volatilization has been documented in terrestrial crustaceans (Greenaway and Nakamura, 1991; Wright et al., 1994; Varley and Greenaway, 1994) and gastropods (Speege and Campbell, 1968).

Mechanisms of ammonia volatilization are poorly understood, but it has been suggested that an elevation of branchial fluid pH is critical in raising the partial pressure of NH_3 (P_{NH_3}) (Wieser and Schweizer, 1970; Wieser, 1984; Varley and Greenaway, 1994). Wright and O'Donnell (1993), however, found no evidence for the involvement of alkalization in NH_3 volatilization in the isopod *Porcellio scaber*. Rather, periodic NH_3 volatilization was correlated with a large rise in ammonia levels in the haemolymph and pleon fluid. In *R. marmoratus*, the rate of NH_3 volatilization (Fig. 2A) was not correlated with a rise in tissue ammonia

Table 4. *Immersed and emersed values of the accumulation and excretion of nitrogen equivalents in laboratory-reared mangrove killifish over 10 days of air-exposure*

	Nitrogen equivalents (μmol-N g^{-1} wet mass)		
	Immersed	Emersed	Difference
Excretion*			
Ammonia	226.20	126.24	–99.96
Urea	26.26	10.22	–16.04
Total			–116.00
Accumulation‡			
Gln	0.96	1.97	+1.01
Glu	–	–	–
Urea	3.83	8.33	+4.50
Ammonia	–	–	–
Total			+5.51
Total	257.25	146.76	–110.49

*Calculated using the average part of ammonia excretion J_{Amm} (Fig. 2A) and urea excretion J_{Urea} (Fig. 2B) (μmol-N g^{-1} h^{-1}) in immersed and emersed fish over the 11 day sampling period, and multiplying this value by 240 h.

‡Amount present in the tissues on day 10 of the experiment.

– No significant change between immersed and emersed fish.

Table 5. *Relative importance of carbamoyl phosphate synthetase III in the production of urea in immersed and emersed laboratory-reared mangrove killifish*

	CPSase III activity (μmol h^{-1})	Rate of urea production (μmol h^{-1})	% of total
Immersed	0.002	0.109*	1.8
Emersed	0.004	0.045†	8.8

*Calculated using the average rate of urea excretion J_{Urea} of immersed fish over the 10 day sampling period.

†Calculated using average J_{Urea} over the 10 day sampling period plus urea accumulated in the tissues of emersed fish.

levels during emersion (Fig. 3A). Further work is necessary to determine whether *R. marmoratus* alkalize and volatilize NH_3 across the mucus covering the external body surface, gills, buccal cavity or possibly other surfaces.

Beyond NH_3 volatilization, we hypothesized that prolonged air-exposure would result in a shift towards ureogenesis (i.e. an increase in tissue urea concentrations, OUC enzyme activities and J_{Urea} following emersion) to combat rising tissue ammonia levels. First, there was no evidence of a 'washout' of urea (or ammonia) following 12 h of emersion (Fig. 1), and this is consistent with the absence of elevation in urea (or ammonia) tissue levels after 24 h of emersion (Fig. 3B). Although this experiment was not conducted after chronic emersion (10 days), one might expect a slightly higher J_{Urea} (but not J_{Amm}) on the basis of the twofold higher tissue urea level at day 10.

Does this increase in tissue urea levels signify a substantial shift to ureogenesis? A nitrogen budget can be estimated by comparing the rates of ammonia and urea excretion in immersed *versus* emersed fish, plus the total accumulation of nitrogenous compounds in the whole body after 10 days of emersion (Table 4). Using this strategy, the relatively large decrease in nitrogen excretion rates in air-exposed fish was not accounted for by the small accumulation of urea in the tissues (Table 4). Furthermore, the OUC does not play a significant role in the synthesis of urea in *R. marmoratus*. Although the four OUC-related enzymes measured were detectable, levels of CPSase III, OTCase and ARG were low compared with those in other ureogenic fish (for a review, see Saha and Ratha, 1998).

CPSase III activity was responsible for approximately 2 % of the urea production in immersed *R. marmoratus* and approximately 9 % in emersed fish (Table 5). However, the absolute levels of CPSase III can be difficult to evaluate because there are often relatively high activities of the pyrimidine-pathway-related CPSase II in most fish tissues (Felskie et al., 1998). This is typically evident by a small activation in the presence of AGA and a large inhibition in the presence of UTP. The data in the present study are somewhat perplexing because neither activation (+AGA) nor inhibition (+UTP) was substantial. Recently, halibut (*Hippoglossus hippoglossus*) larval CPSase III was shown to have significant activity in the absence of AGA (Terjesen et al., 2000). Purification and further characterization of *R. marmoratus* CPSase III is necessary to establish whether the enzyme has similar properties to the halibut larval CPSase III, but it is clear that induction of the OUC is not a major strategy in surviving prolonged air-exposure. These findings are consistent with our accompanying study, in which exposure to elevated levels of external ammonia (223 μmol l^{-1} NH_3) for 7 days did not substantially alter the rates of urea synthesis and excretion (Frick and Wright, 2002).

Ammonia detoxification may also be accomplished through alterations in amino acid metabolism. After 1 and 10 days of air-exposure, whole-body glutamine levels in *R. marmoratus* increased by twofold, similar to the increase found in the muscle of marble goby *Oxyeleotris marmoratus* (Jow et al., 1999) and liver of *P. schlosseri* (Ip et al., 2001). In *R. marmoratus*, glutamate levels were significantly lower following 1 day of emersion, possibly because of increased glutamine synthesis depleting the glutamate pool. Jow et al. (1999) found a positive correlation between glutamine levels and liver GS and GDH activities in air-exposed *O. marmoratus*. Increases in GS and GDH activities in *P. schlosseri* were localized primarily in the brain upon exposure to NH_4Cl (Peng et al., 1998). No increase in either GS or GDH activity was found in whole-body air-exposed *R. marmoratus*, which would mostly reflect enzyme activities of white muscle. In any case, the elevations of glutamine levels during emersion were quantitatively small compared with the total nitrogen budget (Table 4).

The significant increase in tissue alanine levels (1.5-fold) in

air-exposed *R. marmoratus* may reflect metabolic changes aimed at down-regulating ammonia production. Ip et al. (1993, 2001) suggested that partial amino acid catabolism (leading to alanine accumulation) would yield ATP without producing significant amounts of ammonia in air-exposed *P. schlosseri* (see Introduction). However, the increase in tissue alanine levels in *R. marmoratus* can account for only less than 1 % of the reduction in J_{Amm} and, thus, would not contribute substantially to the reduction of ammonia production in this study.

Typically, amphibious fish experience a significant mass loss during air-exposure as a result of dehydration (Gordon et al., 1969, 1978; Rozemeijer and Plaut, 1993). Air-exposed *R. marmoratus* did not lose more mass over time compared with submerged fish, so significant dehydration did not occur. Presumably, the observed approximately 13 % loss of body mass (in both groups) was due to fasting. Unfortunately, not all studies in the literature have considered this, and some of the reported increases in tissue FAA, ammonia and/or urea levels may, in fact, be overestimated as a result of dehydration.

Total nitrogen excretion was significantly lower in air-exposed than in immersed *R. marmoratus*, as has been observed in other air-breathing fish (Morii et al., 1978; Iwata et al., 1981; Davenport and Sayer, 1986; Rozemeijer and Plaut, 1993; Jow et al., 1999; Ip et al., 2001; Lim et al., 2001). The increases in nitrogen stores during air-exposure are of minor importance, cumulatively accounting for less than 2 % of the difference in nitrogen excretion between emersed and immersed fish (Table 4). One possibility is that there was a decrease in standard metabolic rate under terrestrial conditions and, consequently, a decrease in nitrogen production. Although *R. marmoratus* do not aestivate when on land, they are more quiescent, tending to remain motionless unless prompted to move (N. T. Frick, personal observation). Emersed fish were found to consume 24 % less oxygen than immersed fish (Abel et al., 1987), but in the present study there were no corresponding reductions in whole-body CCO or CS activities. Nevertheless, the observed changes in nitrogen excretion in the absence of significant accumulation of nitrogenous end-products suggest a reduction in hepatic nitrogen production.

We proposed that the site of ammonia excretion in emersed fish would switch from primarily branchial to primarily renal and/or cutaneous. Immersed *R. marmoratus* excreted just over half (57 %) of the total nitrogen (ammonia+urea) through the anterior end of the fish, presumably *via* the branchial tissues. Branchial excretion accounts for approximately 80 % of the total nitrogen excreted in teleost fishes (Wood, 1993). Thus, even in water, *R. marmoratus* do not rely heavily on the branchial tissues for nitrogen excretion compared with other teleosts. As predicted, there is an even lower reliance on the gills for nitrogen excretion under terrestrial conditions, with approximately 66 % of nitrogenous wastes excreted through the posterior of the fish (i.e. skin or kidney). Renal nitrogen excretion is considered to be of minor importance in the few species that have been investigated, and data on cutaneous excretion are sparse (for a review, see Wood, 1993). However, in two species of mudskipper, *P. cantonensis* and *B. pectinorostris* (Morii et al., 1978), and in *Limanda limanda* (Sayer and Davenport, 1987), the skin may account for a significant proportion of urea excretion.

J_{Amm} in the divided-chamber experiment was potentially underestimated because NH_3 volatilization was not assessed. If gaseous NH_3 were lost by the emersed fish, then J_{Amm} would have been higher either in the front end (i.e. branchial or buccal excretion) or, more likely, the back end of the chamber (i.e. kidney or cutaneous excretion). If we assume that all NH_3 volatilization occurs *via* the back end of the fish (at the same proportion as previously determined, approximately 42 % of total ammonia) (Fig. 2A), then the percentage of ammonia excreted through the posterior would increase from approximately 26 % (Fig. 4B) to approximately 45 % of total nitrogen excretion, strengthening the importance of the posterior of the fish for waste elimination during air-exposure.

In conclusion, *R. marmoratus* are primarily ammoniotelic in both air and water. Although air-exposed fish accumulate some urea and FAAs in their tissues, quantitatively these increases were of minor importance. Rather, it is the continued excretion of ammonia, particularly the ability to volatilize a significant amount of NH_3, plus the lower rate of ammonia production that allows this species to survive and remain active out of water for extended periods. This study adds to the growing body of literature indicating that amphibious fish species have evolved many different strategies to deal with nitrogenous wastes when on land.

The authors wish to thank Dr David Noakes for supplying *R. marmoratus* and for his advice and interest in this study. We also wish to thank Dr Allan Pinder for his kind donation of the divided chamber and Dr Jim Ballantyne for his helpful comments and suggestions. Funding for this study was provided to N.T.F. from Sigma Xi and a Journal of Experimental Biology Travel fellowship and from NSERC to P.A.W.

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