The Journal of Experimental Biology 205, 79–89 (2002)
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JEB3718

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

I. The influence of environmental salinity and external ammonia

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Accepted 22 October 2001

Summary

At a field site in Belize, mangrove killifish *Rivulus marmoratus* inhabit hypersaline waters (up to 48‰) containing approximately 1 mmol l^{-1} ammonia. We tested the hypotheses that *R. marmoratus* modify their nitrogen metabolism and excretion (i) by accumulating free amino acids (FAAs) and urea in the tissues during hyperosmotic stress and (ii) by shifting to ureotelism and accumulating FAAs during hyperammonia stress. Urea excretion (J_{Urea}) (but not ammonia excretion, J_{Amm}) displayed a diurnal pattern, with significantly less (75 %) urea excreted at night than during the day in both laboratory-reared clones and wild-caught killifish. When fish were exposed to hypersaline conditions (45‰ sea water), J_{Urea} was significantly reduced and tissue urea and FAA levels were elevated compared with those of control fish (15‰ sea water). When *R. marmoratus* were exposed to 0, 1, 2, 5 and 10 mmol l^{-1} NH_4Cl (pH 8) for 48 h, no differences were found in J_{Urea}. Remarkably, prolonged exposure (10 days) to 5 mmol l^{-1} NH_4Cl (pH 8) did not result in an elevation of tissue ammonia levels. In addition, tissue urea and total FAA levels did not differ between control and ammonia-exposed fish after ⩾4 days. We propose that the euryhaline *R. marmoratus* retain urea and FAAs within their tissues in response to extreme osmotic stress. In contrast to many ammonia-tolerant fishes, *R. marmoratus* do not shift to ureotelism during prolonged hyperammonia stress, nor do they convert nitrogenous wastes into FAAs. The data suggest that killifish continue to eliminate ammonia despite an unfavourable blood-to-water gradient, thereby avoiding accumulation of ammonia.

Key words: urea, ammonia, excretion, amino acid, hyperosmotic stress, ureogenesis, ammonia detoxification, osmoregulation, ammonia exposure, mangrove killifish, *Rivulus marmoratus*.

Introduction

The mangrove killifish (*Rivulus marmoratus*) is a small cyprinodontid, hermaphroditic fish that inhabits tropical mangrove forests in Florida, the Caribbean and parts of Central and South America. They inhabit crab burrows or small pools with an ephemeral water supply. Evaporative water loss, wind-blown tides and rainfall routinely alter water salinity to either hypo- or hypersaline conditions (Abel et al., 1987). *R. marmoratus* are incredibly tolerant of environmental extremes, including changes in salinity (0–114‰) (King et al., 1989), temperature (7–45 °C) (Davis et al., 1990), hypoxia (<1 mg l^{-1} O_2) (Dunson and Dunson, 1999), external ammonia (up to approximately 446 μmol l^{-1} NH_3 in 16‰ sea water) (this study), high levels of hydrogen sulphide (Abel et al., 1987) and prolonged air-exposure (>1 month) (Abel et al., 1987).

There is little information in the literature on the effects of external salinity on nitrogen metabolism and excretion in euryhaline fish. Osmoregulating teleosts in sea water (SW) tend to have higher plasma osmolarity and urea levels than those in fresh water (FW) (Wood, 1993), but levels of urea in marine teleosts are 40–70 times lower than those found in ureosmotic regulators (e.g. marine elasmobranchs) (Price and Creaser, 1967; Payan et al., 1973; Forster and Goldstein, 1976). Free amino acids (FAAs) are important intracellular osmolytes in osmoconforming marine invertebrates (Pierce, 1982; Gilles, 1987) and, to a lesser extent, osmoregulating teleost fishes (e.g. Lasserre and Gilles, 1971; Venkatachari, 1974; Assem and Hanke, 1983). FAAs can also serve as an energy source, being directly oxidized to produce ATP for osmoregulatory purposes, such as active ion transport. *R. marmoratus* tolerate a much wider range of salinities (0–114‰) than most euryhaline teleosts (0–34‰). At very high salinities, one might expect an elevation of tissue urea and FAA levels and a compensatory depression of urea excretion (J_{Urea}) and ammonia excretion (J_{Amm}) to aid in the overall osmoregulatory strategy.

In addition to changes in water salinity, *R. marmoratus* may also be exposed to potentially toxic levels of ammonia in their natural environment. Ammonia may accumulate in the burrows from endogenous excretion by the crabs and *R. marmoratus*, especially during dry seasons. Fish living in extreme or variable environments, e.g. mudskippers *Periophthalmodon schlosseri* (Peng et al., 1998), *P. cantonensis* (Iwata, 1988) and

Lake Magadi tilapia *Alcolapia grahami* (Wood et al., 1989), typically have a very high tolerance to ammonia. The conversion of ammonia to the less toxic urea is one mechanism of ammonia detoxification used by several ammonia-tolerant fishes, e.g. singhi catfish *Heteropneustes fossilis* (Saha and Ratha, 1994), walking catfish *Clarias batrachus* (Saha and Das, 1999), Lake Magadi tilapia (Randall et al., 1989), gulf toadfish *Opsanus beta* (Walsh et al., 1990) and abehaze *Mugilogobius abei* (Iwata et al., 2000). Exposure of *H. fossilis* to external ammonia induces enzymes of the ornithine–urea cycle (OUC) (Saha and Ratha, 1994; Saha and Das, 1999). In other species, such as mudskippers *P. schlosseri*, *P. cantonensis* and *Boleophthalmus boddaerti*, excess ammonia is converted to FAAs, particularly glutamate and glutamine, which are stored within the tissues (Iwata, 1988; Peng et al., 1998). Both ureogenesis *via* the OUC and stimulation of glutamine synthetase occur in ammonia-exposed abehaze (Iwata et al., 2000). In addition, active NH_4^+ transport against a blood-to-water concentration gradient was reported in *P. schlosseri* (Randall et al., 1999). In preliminary studies, *R. marmoratus* tolerated relatively high levels of external ammonia (approximately 446 μmol l^{-1} NH_3) similar to that of the ammonia-tolerant species listed above. Hence, *R. marmoratus* may detoxify tissue ammonia by conversion to urea, glutamine and/or glutamate.

The objectives of the present study were to investigate the ability of the mangrove killifish to modify nitrogen metabolism and excretion in order to tolerate wide changes in external salinity and ammonia levels. In the accompanying paper (Frick and Wright, 2002), we discovered the remarkable ability of *R. marmoratus* to release a significant amount of endogenous ammonia by volatilization (42 %) during air-exposure. In the present study, the following two predictions were tested: (i) that, during exposure to high external salinities (up to 60 ‰), FAAs and urea will accumulate in the tissues and, consequently, that rates of ammonia and urea excretion will decrease (as a consequence of lower rates of amino acid catabolism) and (ii) that exposure to relatively high levels of external ammonia (0–10 mmol l^{-1} NH_4Cl) will result in a shift towards ureotelism, evident by the accumulation of urea in the tissues and an increase in the rate of urea excretion. In addition, excess ammonia will be converted into FAAs, particularly glutamate and glutamine, which will be stored in the tissues.

An additional aim of the present study was to compare nitrogen excretion between wild-caught fish and those reared under laboratory conditions. *R. marmoratus* are ideal fish to breed in captivity because of their small size (<2 g) and hardiness. Large numbers of clones can be produced from only a few individuals because they are hermaphroditic. A captive colony was established at the University of Guelph a decade ago, but are the physiological responses of these captive fish the same as those of their wild counterparts? To answer this question, the pattern of nitrogen excretion in laboratory-reared fish was compared with that of wild fish. A further purpose of the study was to assess the water chemistry of the natural habitat of *R. marmoratus* in the mangrove forests. Total ammonia levels, pH, temperature and salinity were measured in water samples collected from crab burrows at Twin Cays, Belize.

Materials and methods

Field water chemistry measurements

Water was collected from burrows (N=10) of the crab *Ucides cordatus* L. where *Rivulus marmoratus* Poey were found in the mangrove forest. All collections were made at Twin Cays, an island adjacent to the Belize barrier reef (approximately 17°N, 88°W). The ammonia concentrations of collected water samples were measured using an HACH ammonia nitrogen reagent set (salicylate method no. 8155) and a HACH DR/2010 portable datalogging spectrophotometer (at 655 nm). All measurements were performed in the field immediately after collection of the water samples because, in preliminary trials, ammonia concentrations changed rapidly, possibly because of bacteria in the water. Salinity, pH and temperature were measured using a refractometer and Accumet AP61 portable pH meter (accurate to 0.01 pH units) with temperature probe (±0.3 °C), respectively. The study area experiences a semidiurnal tidal cycle, and samples presented here were collected at both high (a.m.) and low (p.m.) tide over 2 days (in February) and at high tide following a day of rainfall. The reproductive activity of *R. marmoratus* continues year-round in this study area (D. S. Taylor, personal communication).

Experimental animals

Laboratory-reared fish

A colony of *Rivulus marmoratus* was bred from a single fish captured near Dangriga, Belize, in 1992. Eggs were collected from the parent fish and allowed to hatch and grow to maturity (approximately 0.08–0.15 g), at which time they were used for experimental purposes. Fish were held under the conditions described by Soto and Noakes (1994) (25 °C, 16 ‰, pH 8, 12 h:12 h L:D cycle) and were not fed for 48 h prior to experimentation to eliminate the effects of recent feeding history on nitrogen excretion measurements.

Wild-caught fish

Fish were collected from Twin Cays and Little Lagoon Cay, near Dangriga, Belize. All experiments were conducted within 4 days of capture. Prior to experimentation, fish were kept under their natural photoperiod (approximately 2 h:12 h L:D).

Experimental protocol

An initial experiment was performed under control conditions to determine the pattern of nitrogen excretion in laboratory-reared and wild-caught fish. J_{Amm} and J_{Urea} were measured in laboratory-reared fish (N=18) held in individual chambers containing 15 ‰ artificial SW (prepared from Instant Ocean and distilled water) (25 °C, pH 8, 6 ml) for 60 h. Similar excretion measurements were performed on wild-caught fish (N=14); 15 ‰ SW was made by diluting SW with rain water

(23–26 °C, pH 8). Water samples were collected every 6 h for a period of 36 h for measurement of J_{Amm} and J_{Urea}. It should be noted that, although the pH of the crab burrow water was approximately 7 (see Table 1), the experimental water was at pH 8 to ensure consistency with the rearing conditions of the laboratory-raised fish. At the higher water pH, the ammonia equilibrium will be shifted towards the non-ionic form, thereby reducing the blood-to-water ammonia partial pressure gradient compared with the situation in wild fish (Wright and Wood, 1985).

In addition, laboratory-reared fish were subjected to two experimental series: in series I, they were exposed to a range of salinities (0–60 ‰); in series II, they were exposed to a range of external ammonia concentrations (0–10 mmol l^{-1} NH_4Cl).

Series I

R. marmoratus were exposed to FW ($[Na^+]$≈1.05 mequiv l^{-1}, $[Cl^-]$≈1.47 mequiv l^{-1}, $[Ca^{2+}]$≈5.24 mequiv l^{-1}, $[Mg^{2+}]$≈2.98 mequiv l^{-1}, $[K^+]$≈0.06 mequiv l^{-1}; total alkalinity≈250 mg l^{-1}; total hardness≈411 mg l^{-1}; determined by Xenon Laboratories, Burlington, Ontario, Canada), 15 (control), 30 and 45 ‰ SW, all at pH 8.0. Fish were subjected to a 15 ‰ change in salinity every 2 days. For example, fish in the 45 ‰ group were first transferred from 15 ‰ (control) to 30 ‰ and then held for 2 days before transfer to 45 ‰. Water was changed daily, and water samples were collected for later determinations of ammonia and urea concentrations (on day 1, 3, 5, 7 and 9 at each salinity, *N*=6).

Fish exposed for 7 days to 0, 15 and 45 ‰ SW were killed for analysis of ammonia and urea tissue levels (*N*=5 for the 45 ‰ group and *N*=6 for the 0 and 15 ‰ groups). Whole fish for FAA determination were collected following 4 days of exposure to 0, 15, 30, 45 or 60 ‰ SW (*N*=6). All tissues were stored at –80 °C for no longer than 3 weeks before analysis. It should be noted that, although sampling times in the different experiments were not always identical, the data represent an appropriate time span (i.e. 1–10 days) for comparisons. Tissue ammonia, urea and FAA levels were measured in whole-body samples because it was impossible to collect adequate amounts of individual tissues because of the small size of the fish and the limited number available. As total FAA levels were not significantly different between the 45 and 60 ‰ groups, only the 45 ‰ condition was included for measuring tissue urea and ammonia levels to conserve fish.

Series II

Two separate experiments involving exposure to external NH_4Cl were performed: (i) a short-term exposure to 0, 1, 2, 5 and 10 mmol l^{-1} NH_4Cl during which water samples were collected every 6 h over a 48 h period (*N*=6); and (ii) a long-term exposure to 0 or 5 mmol l^{-1} NH_4Cl during which water samples were collected after 1, 3, 5 and 7 days of exposure (*N*=5).

For both experiments, fish were placed in individual chambers containing 6 ml of 16 ‰ SW at the appropriate concentrations of NH_4Cl (pH 8.0). The concentration of NH_3 present in 1, 2, 5 and 10 mmol l^{-1} NH_4Cl at pH 8 was calculated to be 44, 89, 223 and 446 μmol l^{-1} NH_3, respectively. Because of the high background levels of NH_4Cl in the water samples, only water urea (not ammonia) concentrations were measured.

Fish in 0 or 5 mmol l^{-1} NH_4Cl were killed after 1, 4 and 10 days of exposure for analysis of tissue ammonia and urea concentrations (*N*=6). Both control and ammonia-exposed fish were rinsed with ammonia-free water prior to being killed. Whole fish were collected following 1 and 4 days of exposure to 0 or 5 mmol l^{-1} NH_4Cl (*N*=6), for determination of FAA content, and stored at –80 °C, as described above.

Analytical techniques

Ammonia and urea analysis

Water ammonia levels were quantified using the Indophenol Blue method (Ivancic and Degobbis, 1984), with the following modification. Indophenol was measured at 570 nm instead of 640 nm to increase the linear range of the assay. Water urea levels were measured using the diacetyl-monoxime method (Rahmatullah and Boyde, 1980). Ammonia and urea excretion rates (*J*) were expressed as μmol-N g^{-1} h^{-1}. All spectrophotometric measurements were performed using a Perkin Elmer UV/VIS spectrophotometer (Lambda 2) (Perkin Elmer Corp., Norwalk, CT, USA).

Ammonia and urea tissue levels of whole fish were determined using the method of Chadwick and Wright (1999), except that samples were deproteinized in 10 vols of perchloric acid (8 %). The final supernatant was analyzed for ammonia concentration using an enzymatic Sigma diagnostic kit (171-C), and urea concentration was analyzed using the method of Rahmatullah and Boyde (1980). Ammonia and urea tissue levels were expressed as mmol-N g^{-1} wet mass.

Amino acid analysis

Whole-fish FAA levels were measured using high-performance liquid chromatography (HPLC) [Hewlett-Packard series II 1090 liquid chromatograph equipped with an ultraviolet-visible series II diode array detector (DAD), an automatic injector and a narrow-bore (20 cm×2.1 mm) reversed-phase column (AminoQuant 79916AA-572; Hewlett-Packard)]. Internal and calibration standards were prepared from individual crystalline L-amino acids to a final stock concentration of 2 mmol l^{-1}. Amino acid stock solutions were prepared in 0.1 mol l^{-1} HCl, with the exception of glutamine, asparagine, tryptophan and taurine, which were prepared in 0.1 mmol l^{-1} sodium acetate buffer (pH 7.2). The internal standards for primary and secondary amino acids were norvaline and azetidine 2-carboxylic acid, respectively. Primary and secondary amino acids were derivatized with *o*-phthaldialdehyde (OPA) and 9-fluorenylmethyl chloroformate (FMOC), respectively. Preparation and storage of OPA and FMOC reagents were as described by Barton et al. (1995).

Frozen whole fish were ground to a fine powder under liquid nitrogen and deproteinized in 500 μl of 0.5 % trifluoroacetic acid in methanol and in the presence of a known amount of the internal standards. After centrifugation (16 215 ***g***, 4 °C) for

5 min, 1 mol l^{-1} sodium acetate and 100 mmol l^{-1} NaOH were added, followed by centrifugation (16 215 ***g***, 4 °C) for 25 min. Concentrations of amino acids were expressed as nmol g^{-1} wet mass.

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 treated (salinity- or ammonia-exposed) values for tissue analysis (urea and ammonia levels) 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 whether differences were significant between treatment and control fish ($P \leqslant 0.05$). 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

Field water chemistry

Water in the crab burrows was stagnant, with negligible tidal flushing. The highest concentration of water ammonia was 1.02 mmol l^{-1}, resulting in a calculated NH_3 concentration of 4.7 µmol l^{-1} (Table 1). Water pH was relatively constant (approximately pH 7). Temperature varied between 23 and 28 °C, with slightly lower temperatures after rainfall (approximately 4.8 mm of rain). Salinity ranged between 32 and 48 ‰ and, as expected, was lower after rainfall (Table 1).

Wild versus *laboratory-reared fish*

Laboratory-reared *R. marmoratus* are ammoniotelic, with 70–88 % of the total nitrogen excreted ($J_{Amm}+J_{Urea}$) as ammonia. Ammonia excretion remained constant over time, with no significant changes in excretion rates between time periods after the initial 12 h (Fig. 1A). Urea excretion displayed a diurnal pattern, with significantly less (–75 %) urea excreted at night (00:00 to 06:00 h) than during the day (12:00 to 18:00 h) (Fig. 1B).

Wild fish displayed a similar pattern to that observed in laboratory-reared fish. Fish were ammoniotelic, excreting between 60 and 90 % of nitrogenous waste as ammonia. As for the laboratory-reared fish, wild fish excreted urea (but not ammonia) in a diurnal pattern (00:00 to 06:00 h, –63 % compared with 12:00 to 18:00 h) (Fig. 2A,B).

Changing environmental salinity

Fish exposed to FW (0 ‰) excreted significantly less ammonia than fish in 30 ‰ SW at days 1, 7 and 9 and fish in 45 ‰ SW at day 7 (Fig. 3A). In general, J_{Urea} was significantly lower in fish held in 45 ‰ SW compared with those in 0, 15 and 30 ‰ (Fig. 3B). Total nitrogen excretion ($J_{Amm}+J_{Urea}$) was not significantly different between salinities, with the exception that, on days 1 and 9, FW-acclimated fish excreted less nitrogen than fish in 30 ‰ SW (data not shown). A negative correlation ($r^2=0.92$) between the percentage of urea excreted [$J_{Urea}/(J_{Urea}+J_{Amm})$], and salinity (‰) was evident when values were compared at day 9 (Fig. 4). Following 7 days of exposure to 0 ‰ SW, tissue ammonia levels were significantly higher than those of control fish in 15 ‰ SW (Fig. 5). Tissue urea concentrations were significantly higher (twofold) in fish held in 45 ‰ SW compared with those in both 0 and 15 ‰ SW (Fig. 5).

Total FAA levels in fish held in 60 ‰ SW were approximately 43 % higher than those of control fish (15 ‰) and were significantly higher than those of fish held in 0, 15 and 30 ‰ SW (Table 2). Non-essential FAAs were responsible for approximately 89 % of the increase in total FAA levels between 60 and 15 ‰ SW. Concentrations of tissue threonine, taurine, tyrosine, valine, methionine, phenylalanine, leucine, lysine, glutamine and proline were significantly higher at high salinities (45 and/or 60 ‰) compared with 15 ‰ SW. Most remarkably, proline levels were sevenfold higher in fish at 60 ‰ than in fish in 15 ‰ SW. In addition, glutamine and lysine levels were twofold higher in fish exposed to 0 ‰ SW than in control fish (15 ‰) (Table 2). It should be noted that no mortalities occurred in fish exposed to the range of salinities in this study (0–60 ‰).

Ammonia-exposure

There were no differences in the J_{Urea} of *R. marmoratus* exposed to 0, 1, 2, 5 and 10 mmol l^{-1} NH_4Cl over 48 h (Fig. 6). A diurnal pattern of J_{Urea} was evident at all NH_4Cl concentrations (Fig. 6). After prolonged exposure (7 days) to

Table 1. *Characteristics of water taken from crab burrows in which mangrove killifish are found at Twin Cays, Belize*

	Low tide		High tide		Rainfall	
[Total ammonia] (µmol l^{-1})	390±52	(201–748)	442±49	(220–1018)	306±78	(0–672)
[NH_3] (µmol l^{-1})	1.60±0.29	(0.8–3.7)	1.69±0.27	(0.5–4.7)	1.02±0.25	(0–2.0)
Temperature (°C)	25.9±0.1	(25.5–26.6)	25.5±0.3	(24.2–27.5)	23.6±0.1	(23.2–24.0)*
Salinity (‰)	41.0±0.5	(38–40)	40.9±0.5	(38–48)	38.4±0.8	(32–40)*
pH	7.03±0.03	(6.89–7.14)	6.98±0.02	(6.82–7.15)	7.03±0.04	(6.88–7.18)

Values are expressed as the mean of 10 burrows. Values are means ± S.E.M., with the range shown in parentheses.
An asterisk denotes a significant difference from both low- and high-tide measurements ($P<0.05$).

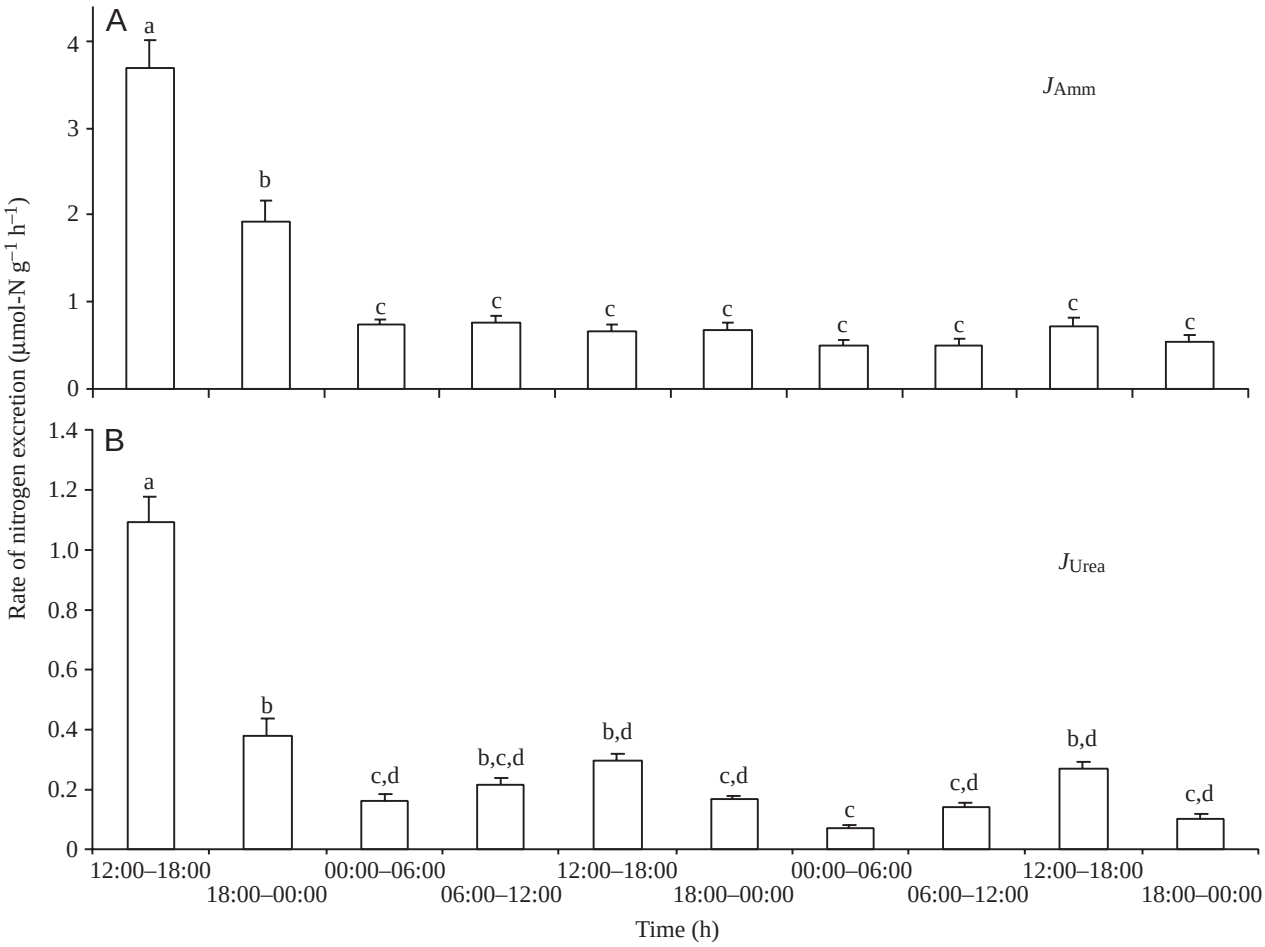

Fig. 1. Diurnal ammonia excretion (J_{Amm}) (A) and urea excretion (J_{Urea}) (B) in laboratory-reared *Rivulus marmoratus*. Values are expressed as means + S.E.M. (N=18). Columns labelled with one or more letters that are the same are not significantly different. Columns labelled with no letters in common are significantly different (P<0.05).

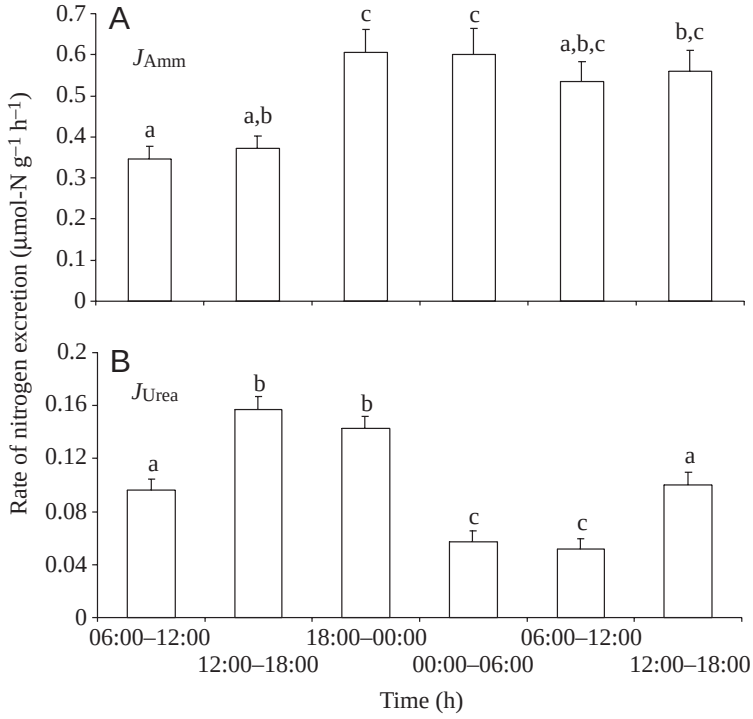

Fig. 2. Diurnal ammonia excretion (J_{Amm}) (A) and urea excretion (J_{Urea}) (B) in wild-caught *Rivulus marmoratus*. Values are expressed as means + S.E.M. (N=14). Columns labelled with one or more letters that are the same are not significantly different. Columns labelled with no letters in common are significantly different (P<0.05).

5 mmol l^{-1} NH_4Cl, urea excretion was significantly higher (by 39 %) at day 5 only (data not shown).

Tissue ammonia levels were significantly higher (1.8-fold) after 1 day of ammonia-exposure compared with controls, but there were no differences on days 4 or 10 (Fig. 7). Tissue urea concentrations were unchanged by ammonia treatment (Fig. 7). Total essential FAA levels were significantly lower after 1 day of ammonia-exposure (1594±87 nmol g^{-1} wet mass) compared with control values (2029±144 nmol g^{-1} wet mass). In particular, the concentrations of three essential FAAs, histidine, valine and isoleucine, were significantly lower (by 1.9-, 1.5- and 1.4-fold, respectively) in ammonia-exposed (5 mmol l^{-1} NH_4Cl) fish after 1 day (data not presented). After 4 days, however, there were no significant differences in tissue FAA levels between control and ammonia-exposed fish (data not presented).

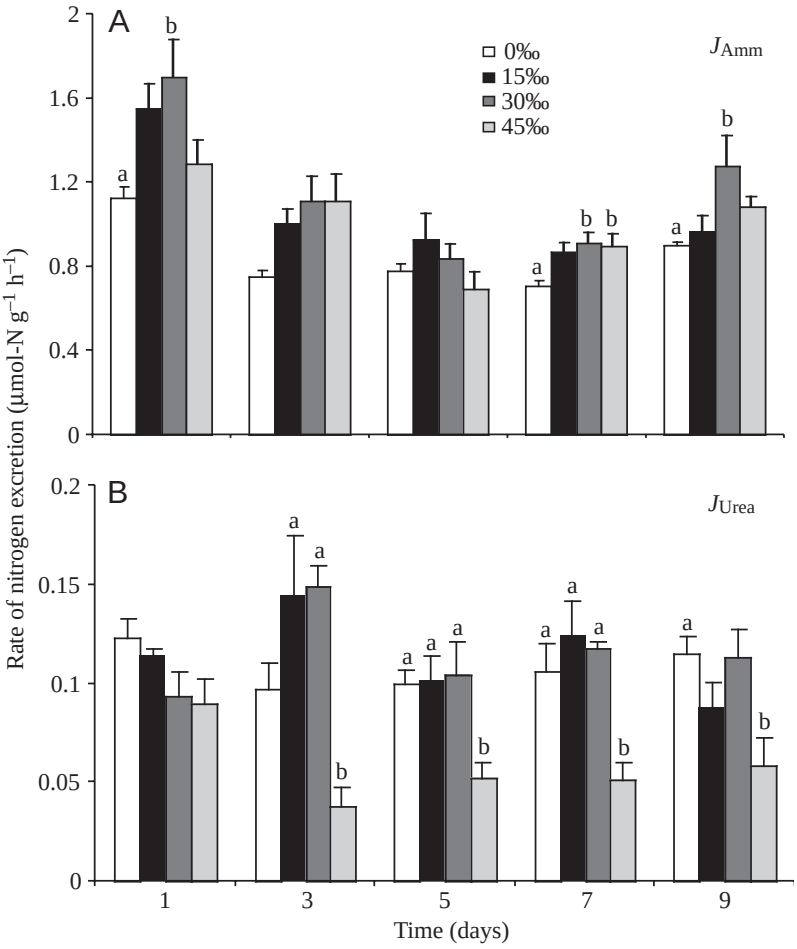

Fig. 3. Ammonia excretion (J_{Amm}) (A) and urea excretion (J_{Urea}) (B) in *Rivulus marmoratus* exposed to 0 (fresh water), 15 (control), 30 and 45 ‰ sea water for 1, 3, 5, 7 and 9 days. Values are expressed as means + S.E.M. (N=6). Columns labelled with one or more letters that are the same on a particular day are not significantly different; the absence of a letter indicates no significant difference. Columns labelled with no letters in common are significantly different (P<0.05).

Discussion

Rivulus marmoratus *in their natural environment*

The water chemistry data presented here were collected over a short period (4 days) and are not representative of the full range of conditions that this species may encounter over the long term. Water characteristics will depend on seasonal effects such as changes in the amount of precipitation, atmospheric temperature, variations in tidal height and bacterial decomposition of substratum organic matter with accompanying release of H_2S (a common occurrence in mangrove forests). Nevertheless, the water conditions in the crab burrows measured even over a short period would not be tolerable for most fish. Water salinity was relatively high (up to 48 ‰), substantially greater than that of normal SW (approximately 33 ‰). Ammonia levels were approximately 1 mmol l^{-1}, considerably higher than in most natural FW or SW habitats, but well below the upper limit of tolerance of *R. marmoratus* (>10 mmol l^{-1}). If burrows are isolated from tidal flushing for significant periods, however, ammonia levels may reach even higher values. Water pH was a full unit lower (approximately pH 7) than that of SW (approximately pH 8.2) and, therefore, the NH_3 level was relatively low (approximately 4.7 μmol l^{-1} NH_3, an order of magnitude lower than at pH 8), considerably lessening the overall potential toxic effect of burrow ammonia. The low pH is possibly due, in part, to the presence of H_2S in the water, suspected to be present at the sampling site from its distinct smell. In addition, CO_2 and H^+ excretion by the crab and fish may also contribute to water acidification.

The pattern of nitrogen excretion in wild-caught *R. marmoratus* was similar to that of laboratory-reared fish. Wild and laboratory-reared fish display a diurnal pattern in urea excretion, but not ammonia excretion. In addition, ammonia

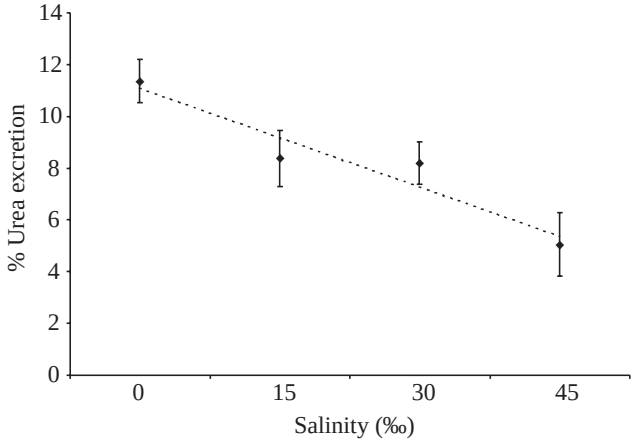

Fig. 4. Relationship between percentage urea excretion [J_{Urea}/(J_{Urea}+J_{Amm})], where J_{Urea} is the rate urea excretion and J_{Amm} is the rate of ammonia excretion, and external salinity in *Rivulus marmoratus* following 9 days of acclimation to each salinity; r^2=0.92, y=–1.91x+13.01. Values are expressed as means ± S.E.M. (N=6).

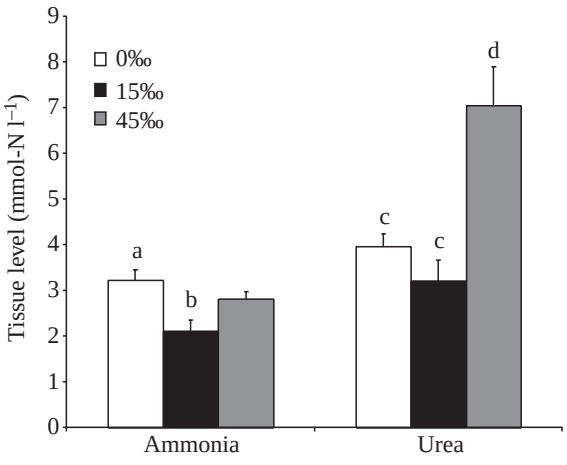

Fig. 5. Whole-body ammonia and urea levels in *Rivulus marmoratus* following 7 days of exposure to 0 (fresh water), 15 (control) and 45‰ sea water. Values are expressed as means + S.E.M., N=5 (45‰ treatment) or N=6 (0 and 15‰ treatment). Columns labelled with one or more letters that are the same are not significantly different; absence of a letter indicates no significant difference. Columns labelled with no letters in common are significantly different (P<0.05).

and urea excretion rates were altered in the same manner in the wild and laboratory-reared fish during air-exposure (Frick and Wright, 2002). Thus, we propose that the use of laboratory-raised clones in these experiments provides an accurate depiction of nitrogen excretion in this species. A diurnal variation in J_{Urea} has also been reported in *H. fossilis* (Saha et al., 1988); however, in *H. fossilis*, J_{Urea} was significantly higher at night than during the day, a situation opposite to that in *R. marmoratus*. Other studies have reported daily variations in J_{Amm} (Tatrai, 1981), but these changes were attributed to variations in food intake. *R. marmoratus* were not fed 2 days prior to or during experimentation, and food intake is therefore not responsible for the observed pattern.

Walsh et al. (1994) reported pulsatile urea excretion in the gulf toadfish (*Opsanus beta*). Within a 24 h period, *O. beta* excrete a single burst of urea (<3 h duration) (Wood et al., 1995), which is correlated with low levels of endogenous cortisol in the plasma (Hopkins et al., 1995) or administration of exogenous arginine vasotocin (Perry et al., 1998). Although *R. marmoratus* (not episodically) excrete urea continuously, cortisol may be implicated in daily cycles. Vijayan et al. (1996)

Table 2. *Whole-body free amino acid levels in mangrove killifish after 4 days of exposure to varying external salinities*

	Free amino acid level (nmol g^{-1} wet mass)				
	0‰	15‰	30‰	45‰	60‰
Essential amino acids					
His	54±15	39±11	44±7	74±14	85±30
Thr	277±51^{a,b}	198±36^{a}	254±18^{a,b}	413±44^{b,d}	542±113^{c,d}
Val	70±8	48±4^{b}	66±5^{b,d}	75±5^{a,d}	88±10^{a,c}
Met	115±18^{a,c}	80±8^{a}	126±17^{a,c}	168±22^{b,c}	206±38^{b}
Trp	376±34	347±29	299±18	360±20	383±58
Phe	18±3	17±2^{a}	23±5	32±6^{b}	36±8^{b}
Ile	42±5	33±4	47±6	56±6	57±12
Leu	56±6^{b,c}	47±4^{a,c}	56±5^{b,c}	75±5^{d}	70±10^{b,d}
Lys	112±25^{d}	53±7^{b}	61±7^{a,b}	90±13^{a,d}	82±6
Total	1120±139^{a,c}	863±97^{a}	976±71^{a,c}	1344±109^{b,c}	1550±265^{b}
Non-essential amino acids					
Asp	1238±176	1172±140	1104±179	1439±1500	1704±282
Glu	580±94	452±81	594±105	737±137	600±105
Asn	96±34	51±9	50±5	147±53	62±6
Ser	116±39	73±11	85±8	149±34	90±9
Gln	333±94^{a,c}	162±20^{b}	189±15^{b,c}	349±75^{a}	299±22^{a}
Gly	875±363	449±73	744±147	1217±342	522±101
Ala	264±81	190±34	237±14	445±112	327±57
Tau	10 348±796^{a}	9987±609^{a}	9572±509^{a}	11 606±600	12 886±1322^{b}
Arg	29±3	24±3	52±16	44±11	47±11
Tyr	41±3^{d}	35±5^{d}	66±8^{a,e}	77±16^{c,e}	124±20^{b,c}
Hpr	1306±154	1109±235	1316±125	1099±210	1376±284
Pro	269±85^{a}	216±60^{a}	279±52^{a}	516±205^{a}	1492±467^{b}
Total	15 494±1497^{a,c}	13 921±1025^{a}	14 243±432^{a}	17 826±1125^{b,c}	19 528±2000^{b}
Total	16 615±1628^{a,c}	14 783±1119^{a}	15 219±482^{a}	19 170±1223^{b,c}	21 077±22334^{b}

Values are expressed as means ± S.E.M. (N=6).

Different letters within a horizontal row represent significant differences (P<0.05) between salinities. The absence of a letter indicates no significant difference.

suggested that cortisol might contribute to the regulation of urea production in the sea raven (*Hemitripterus americanus*) because increased plasma cortisol levels resulted in higher levels of plasma urea (but not ammonia). As cortisol levels vary in a diurnal pattern in some teleosts (Spieler, 1979), cortisol may be involved in the diurnal control of urea metabolism and excretion in *R. marmoratus.*

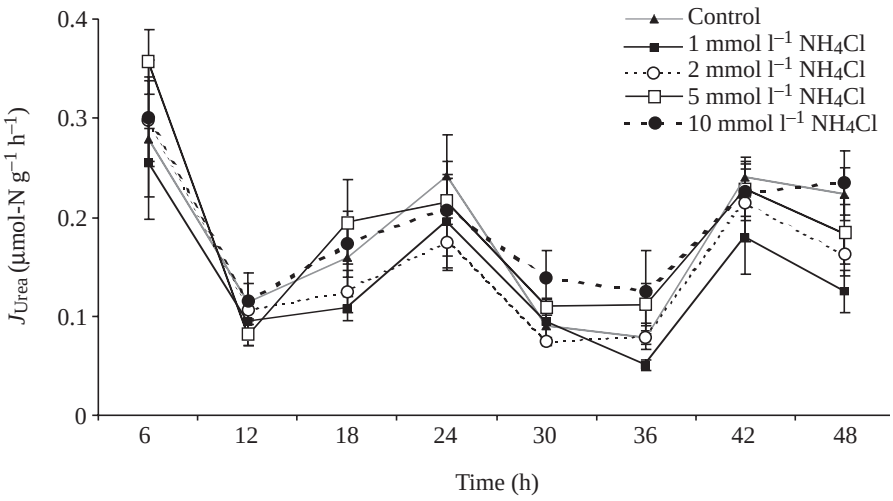

Fig. 6. Urea excretion (J_{Urea}) in *Rivulus marmoratus* exposed to 0–10 mmol l^{-1} NH_4Cl in the external medium. Values are expressed as means ± S.E.M. (N=6). No statistical differences between treatments were found.

Changes in environmental salinity

Water salinity had a pronounced effect on the nitrogen metabolism and excretion of *R. marmoratus*. We predicted that acclimation to high salinities would result in the accumulation of both urea and FAAs in the tissues and a marked reduction in J_{Urea}, and such changes were observed. Are these changes simply a reflection of an overall metabolic shut-down and/or of dehydration of the tissues under hyperosmotic stress? We think not because J_{Amm} and tissue ammonia levels were not depressed at higher salinities and 12 out of 21 individual FAA levels were unchanged by the hypersaline environment. Thus, we propose that the observed changes in urea excretion and tissue urea and FAA concentrations are a coordinated part of a larger osmoregulatory response required in such a high-salt environment.

The twofold elevation of tissue urea levels in osmotically challenged fish (45‰) was accompanied by a significant reduction in J_{Urea} (Fig. 3B). Isaia (1982) found that the permeability of the gill to small non-electrolytes (e.g. urea) was lower in SW- than in FW-acclimated rainbow trout *Oncorhynchus mykiss*, contrary to the increase in NH_4^+ permeability observed in SW-acclimated teleosts (Evans et al., 1989; Wilson and Taylor, 1992). Gill urea permeability is dependent, in part, on urea transport proteins in the marine dogfish and toadfish and in the lake Magadi tilapia (Walsh et al., 2000, 2001; Fines et al., 2001). The lipid composition of cell membranes also influences urea permeability (Lande et al., 1995; Fines et al., 2001) and is affected by changes in environmental salinity (Daikoku et al., 1982). High salinities may therefore facilitate urea retention (Fig. 5) by decreasing the permeability of the gill to urea (Fig. 4), but this hypothesis requires careful testing.

We expected that J_{Amm} would decline with increasing external salinity as a consequence of reduced amino acid catabolism and amino acid retention, assuming no change in gill permeability to ammonia. The higher J_{Amm} at 30‰ compared with 0‰ (Fig. 3A) may relate to greater branchial NH_4^+ diffusion *via* paracellular channels typical of marine fishes (Evans et al., 1989; Wilson and Taylor, 1992). In addition, euryhaline fish in SW typically have a more positive gill transepithelial potential (inside relative to outside) (Potts, 1984), which would promote the outward diffusion of NH_4^+ (Wright et al., 1995). Furthermore, if NH_4^+ excretion is wholly or partially Na^+-dependent, then changes in the availability of Na^+ in the external water will affect J_{Amm} *via* $Na^+/H^+(NH_4^+)$ exchange (Claiborne and Perry, 1991; Claiborne et al., 1999). Yet these potential explanations still do not account for why J_{Amm} was not highest in fish held in 45‰ SW. At salinities above approximately 30‰, a decrease in amino acid catabolism and nitrogen excretion may be connected to amino acid retention (Table 2).

In *R. marmoratus*, approximately 90 % of the rise in tissue FAA levels upon acclimation to 60‰ SW was due to increases in levels of non-essential amino acids (Table 2), a situation similar that reported in other teleosts acclimated to SW (Huggins and Colley, 1971; Lasserre and Gilles, 1971; Colley et al., 1974; Ahokas and Sorg, 1976). The two amino acids that dominated this change were proline and taurine. The sevenfold increase in tissue proline levels has not previously been documented in a teleost. In other teleost fishes in which proline concentration was elevated during SW acclimation, the increases were less than twofold (e.g. Huggins and Colley, 1971; Ahokas and Sorg, 1976). Taurine, a stabilizer of cell membranes, is not found in proteins (Huxtable, 1992) and, thus, protein catabolism would not result in increased tissue taurine levels. This may explain why tissue taurine levels do not usually increase during hyperosmotic stress (Huggins and Colley, 1971; Ahokas and Sorg, 1976; Deaton et al., 1984), but decrease during hypo-osmotic stress (Lasserre and Gilles, 1971; Vislie and Fugelli, 1975; Fugelli and Zachariassen, 1976). The elevation of whole-body taurine levels in *R. marmoratus* can only be explained by an increase in taurine synthesis (i.e. from sulphur amino acids) (King et al., 1980).

Essential FAAs do not typically function as osmolytes because they are not endogenously synthesized and are conserved for various metabolic functions (e.g. protein synthesis) (Ballantyne and Chamberlin, 1994). However, in the present study, increases in levels of several essential FAAs

occurred during acclimation to 45 and 60‰ SW (e.g. threonine, valine, methionine, phenylalanine, leucine and lysine). Of these FAAs, the almost threefold increase in threonine levels in fish held at 60‰ contributed approximately 5% of the increase in total tissue FAA levels. Presumably, the observed changes in essential FAAs at high external salinity were as a result of protein catabolism. Overall, the increase in levels of both essential and non-essential FAAs in tissues of *R. marmoratus* exposed to high salinities (45 and 60‰) may be important in balancing osmolyte concentrations between intra- and extracellular compartments. Moreover, the observation that total FAA levels are not significantly different between 0, 15 and 30‰ suggests that, over a broad range of lower salinities, the fish are not severely osmotically challenged.

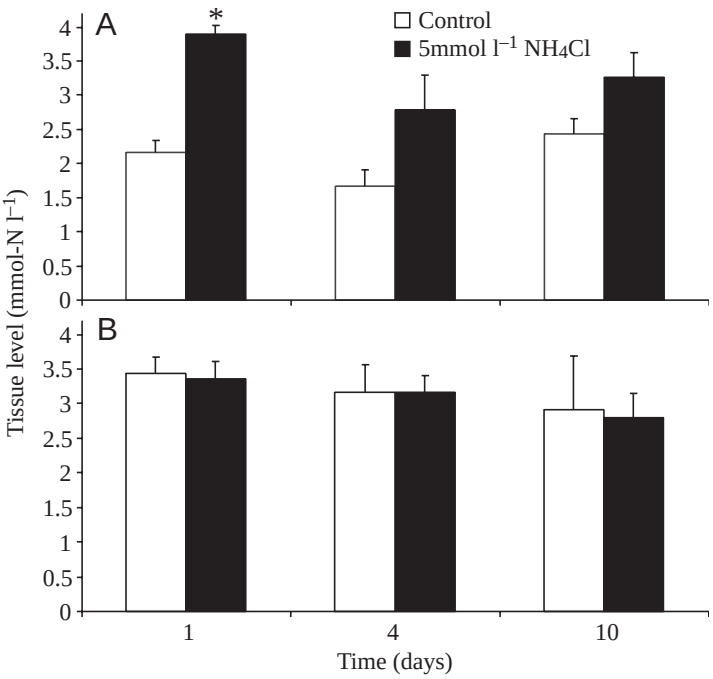

Fig. 7. Whole-body ammonia (A) and urea (B) levels in *Rivulus marmoratus* exposed to 0 or 5 mmol l^{-1} NH_4Cl for 10 days. Values are expressed as means + S.E.M. (*N*=5). An asterisk denotes a significant difference from the corresponding control value.

Ammonia-exposure

Urea production does not play an important role in ammonia detoxification during hyperammonia stress in *R. marmoratus*, contrary to our predictions (Figs 6, 7). In addition, tissue levels of glutamine, glutamate and other FAAs were unchanged after 4 days of ammonia-exposure. The elevation of tissue ammonia levels on day 1 confirms that ammonia was entering the fish and implies that J_{Amm} was initially reversed or reduced, similar to observations in other fish species (e.g. Claiborne and Evans, 1988; Iwata, 1988; Wright, 1993). Quite surprisingly, tissue ammonia levels were not significantly higher after 4 and 10 days of exposure to 5 mmol l^{-1} NH_4Cl (223 µmol l^{-1} NH_3) compared with controls (Fig. 7). Randall et al. (1999) also reported that tissue ammonia levels did not increase in *P. schlosseri* after 6 days of exposure to 8 mmol l^{-1} NH_4Cl; however, at pH 7.2, the NH_3 concentration was only 36 µmol l^{-1}. Thus, *R. marmoratus* are very efficient at maintaining low levels of internal ammonia without invoking ureogenesis or temporary storage of nitrogen in FAAs.

Exposure to relatively high levels of external ammonia would reverse the blood-to-water P_{NH_3} gradient and impair excretion. Randall et al. (1999) have documented active ammonia transport in ammonia-exposed *P. schlosseri*, proposing that both $Na^+/K^+(NH_4^+)$-ATPase and $Na^+/H^+(NH_4^+)$ exchangers are involved in branchial ammonia excretion. It is interesting to note that, when FW-acclimated killifish were exposed to 5 mmol l^{-1} NH_4Cl (well below the tolerance limit for this species), 100% mortality (*N*=6) was observed after less than 24 h (N. T. Frick and P. A. Wright, unpublished observations). In contrast, all fish survived when the same or higher concentrations of NH_4Cl were added to water of 15‰ SW. Hence, we hypothesize that Na^+ is critical for survival in hyperammonia stress, possibly related to active Na^+-dependent ammonia excretion.

Examination of the literature on the nitrogen metabolism and excretion of ammonia-tolerant fishes reveals two emerging patterns. In one group, a tendency towards ureotelism has been documented (see Introduction). However, the results of the present study indicate that *R. marmoratus* fit into the second group that do not undergo a transition to ureogenesis. The synthesis of urea is a metabolically costly process, with at least 2 mol of ATP required to synthesize 1 mol of urea (equivalent to 2 mol of NH_4^+) (Wood, 1993). It may be energetically more favourable to excrete ammonia actively because 1 mol of ATP would potentially eliminate 2 mol of NH_4^+ on the basis of the ATP hydrolysis reaction for Na^+/K^+-ATPase. By inhabiting such a variable and sometimes extreme environment, however, *R. marmoratus* are able to secure their own niche, presumably minimizing predation and competition.

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 Scott Taylor for his assistance and expertise in the field and Dr Bruce Turner and the Smithsonian Institute for the use of research facilities in Belize. 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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