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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

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Summary

This study was designed to elucidate the strategies adopted by mudskippers to handle endogenous ammonia during aerial exposure in constant darkness. Under these conditions, specimens exhibited minimal locomotory activity, and the ammonia and urea excretion rates in both *Periophthalmodon schlosseri* and *Boleophthalmus boddaerti* decreased significantly. As a consequence, ammonia accumulation occurred in the tissues of both species of mudskipper. A significant increase in urea levels was found in the liver of *P. schlosseri* after 24 h of aerial exposure, but no similar increase was seen in the tissues of *B. boddaerti*. It is unlikely that these two species of mudskipper detoxified ammonia to urea during aerial exposure since *B. boddaerti* does not possess a complete ornithine–urea cycle (OUC) and, although all the OUC enzymes were present in *P. schlosseri*, the activity of carbamoyl phosphate synthetase present in the liver mitochondria was too low to render the OUC functional for ammonia detoxification. Peritoneal injection of $^{15}NH_4Cl$ into *P. schlosseri* showed that this mudskipper was capable of incorporating some of the labelled ammonia into urea in its liver. However, aerial exposure did not affect this capability and did not induce detoxification of the accumulated ammonia to urea. Mudskippers exposed to terrestrial conditions and constant darkness did, however, show significant decreases in the total free amino acid content in the liver and blood, in the case of *P. schlosseri* and in the muscle of *B. boddaerti*. No changes in the alanine or glutamine content of the muscle were found in either species. Analyses of the balance between the reduction in nitrogenous excretion and the increase in nitrogenous accumulation further revealed that these two species of mudskipper were capable of reducing their protein and amino acid catabolic rates. Such adaptations constitute the most efficient way to avoid the build-up of internal ammonia, and would render unnecessary the detoxification of ammonia through energetically expensive pathways. This finding may be the first report of a teleost fish showing a reduction in proteolysis and amino acid catabolism in response to aerial exposure.

Key words: aerial exposure, amino acids, ammonia, *Boleophthalmus boddaerti*, mudskipper, *Periophthalmodon schlosseri*, proteolysis.

Introduction

Fish have a remarkable capacity to utilize amino acids both as metabolic fuel and as precursors for protein, lipid and carbohydrate synthesis (Wood, 1993; Moon and Johnston, 1981; Mommsen and Walsh, 1992). Dietary carbohydrate does not appear to be an important aerobic fuel, although fish have a high capacity for gluconeogenesis from exogenous amino acids (Wood, 1993). In contrast, dietary lipid is important, especially in carnivores. If the diet is deficient in lipid, then a greater proportion of dietary protein is metabolised for energy or deaminated for conversion to fat and carbohydrates, more ammonia is excreted and a lower percentage of dietary nitrogen is retained for growth (Wood, 1993).

Fish exposed on land would have difficulty in maintaining ammonia excretion, leading to its accumulation in the body. Ammonia toxicity can be ameliorated by preventing this accumulation by decreasing production, maintaining or enhancing excretion and/or converting ammonia to less toxic compounds for storage or excretion. Whatever the circumstances, if the rate of proteolysis remains the same but the rate of amino acid catabolism decreases, ammonia

production will decrease (even though ammonia may accumulate in the tissues if its excretion is impeded), but concentrations of free amino acids will increase. Hence, increases in the concentration of free amino acids under experimental conditions in which fishes have difficulty in excreting ammonia may not indicate the detoxication of ammonia to amino acids as suggested by Iwata et al. (Iwata et al., 1981; Iwata, 1988) for the mudskipper *Periophthalmus cantonensis*. Instead, it may simply suggest a decrease in the rate of amino acid catabolism or a change in the catabolic pathways involved.

It was suggested that mudskippers reduce the rate of nitrogen metabolism in response to aerial exposure (Gordon et al., 1969; Gordon et al., 1970: Gordon et al., 1978), although Gordon et al. did not provide any data to support this proposition. In contrast, Ip et al. (Ip et al., 1993) observed increases in total free amino acid (TFAA) levels and branched-chain free amino acid (FAA) levels in the tissues of *Periophthalmodon schlosseri* after 24 h of aerial exposure but not in those of *Boleophthalmus boddaerti*. They attributed this difference between the two species of mudskipper to the greater activity of *P. schlosseri* on land. In addition, they speculated that *P. schlosseri* increased the rate of amino acid catabolism to sustain this activity on land. However, their proposition did not take into consideration a probable decrease in nitrogenous excretion when specimens were exposed to terrestrial conditions.

In this study, we have examined whether mudskippers are able to reduce their rate of amino acid catabolism in response to aerial exposure. The eyes of mudskippers are adapted for aerial vision (Clayton, 1993), so these fish can be stimulated visually to exhibit mild to violent locomotory activities during aerial exposure. Through observations in the laboratory, it was discovered that mudskippers remain relatively quiescent in total darkness (Y. K. Ip and S. F. Chew, unpublished results). Experiments were, therefore, designed to study mudskippers in constant darkness, eliminating visually stimulated physical activity. Mudskippers are diurnal. Under a 12 h:12 h light:dark regime in the laboratory, they show two activity peaks in the light period (Clayton, 1993). Under continuous light or dark, a similar periodicity of activity was maintained but at a much lower level in the dark (Clayton, 1993).

The species chosen for this study were *P. schlosseri* and *B. boddaerti*. These are two species of mudskipper that inhabit the intertidal zone of mudflats in Singapore and Malaysia. Although they share the same habitat, they show very different patterns of behavior. *B. boddaerti* appears to be less well-adapted to land than *P. schlosseri*, and their burrows are found on the lower regions of the mudflats. Individuals are usually seen on the mudflats at low tide and, as the tide rises, they retreat into their burrows and remain submerged until the tide ebbs. In comparison, the burrows of *P. schlosseri* are found higher on the mudflats. At high tide, they are usually found swimming along the edge of the water or moving on land above the water level.

One aim of this study was to quantify the reduction in rates of ammonia and urea excretion by these species of mudskipper during aerial exposure. Decreases in ammonia and urea excretion rates in *P. schlosseri* and *B. boddaerti* during aerial exposure could lead to (1) an accumulation of ammonia in tissues and organs, (2) the detoxification of ammonia to urea and storage in the tissues, (3) a reduction in the catabolic rates of protein and/or amino acids or (4) various combinations of the above. Therefore, we also measured the accumulation of ammonia, urea and free amino acids in various tissues and organs to construct a balance sheet of nitrogenous excretion and accumulation. If the rates of amino acid catabolism and/or proteolysis remain relatively constant during aerial exposure, the increase in nitrogenous accumulation should balance the reduction in nitrogenous excretion. The hypothesis to be tested was that mudskippers were capable of reducing their rate of ammonia production under terrestrial conditions. During the course of the study, an attempt was also made to determine whether these two species of mudskipper were capable of detoxifying accumulated ammonia by conversion to urea.

Materials and methods

Collection and maintenance of mudskippers

Periophthalmodon schlosseri (Pallas, 1770) (90–100 g body mass) and *Boleophthalmus boddaerti* (Pallas, 1770) (7–19 g body mass) were captured at Pasir Ris, Singapore. They were maintained in plastic aquaria in 50 % sea water (15‰ salinity) at 25 °C in the laboratory, and the sea water was changed daily. No attempt was made to separate the sexes. The fishes were acclimated to laboratory conditions for 1 week. During the adaptation period, *P. schlosseri* were fed small guppies and *B. boddaerti* an artificial diet. Food was withdrawn 24 h prior to experiments, which gave sufficient time for the gut to be emptied of all food and waste.

Exposure of mudskippers to experimental conditions

24 h before the experiment, specimens from a group of animals were individually totally submerged in 50 % sea water. At the end of the 24 h period, some specimens were killed as 0 h controls. They were anaesthetized by the introduction of 3-aminobenzoic acid ethyl ester (MS222) at a final concentration of 0.02 %. Others were exposed to terrestrial conditions in plastic aquaria (25 cm×14 cm×12 cm; length×width×height) containing 20 ml of 50 % sea water. These aquaria were kept in a temperature-controlled (25 °C) cabinet in total darkness. After 24 h, the fish were anaesthetized for 10 min in an atmosphere saturated with diethyl ether. Another batch of fish was resubmerged for 3 h in 50 % sea water following 24 h of aerial exposure. After 3 h in sea water, the fish were anaesthetized as described above for the controls.

Anaesthetized fish were killed immediately by pithing. The lateral muscle and the liver were quickly excised. No attempt was made to separate the red and white muscle. The excised tissues and organs were immediately freeze-clamped in liquid nitrogen with precooled tongs. Frozen samples were kept at –80 °C until analysed. A separate group of fish exposed to

similar conditions was used for the collection of blood samples. The caudal peduncle of the anaesthetized fish was severed, and blood exuding from the caudal artery was collected in heparinized capillary tubes. The collected blood was centrifuged at 4000 ***g*** at 4 °C for 10 min to obtain the plasma. The plasma was deproteinized in 2 volumes (v/v) of ice-cold 6 % $HClO_4$ and centrifuged at 10,000 ***g*** at 4 °C for 15 min. The resulting supernatant was kept at −80 °C until analysis.

Analyses of ammonia and urea

The frozen samples were weighed, ground to a powder in liquid nitrogen, and homogenized in 5 volumes (w/v) of 6 % $HClO_4$ for ammonia and urea analyses. For ammonia analysis, the pH of the deproteinized sample was adjusted to between 5.5 and 6.0 with 2 mol l^{-1} K_2CO_3. The ammonia content was determined as described previously (Bergmeyer and Beutler, 1985). Freshly prepared NH_4Cl solution was used as the standard for comparison.

Urea was determined colorimetrically (Jow et al., 1999). The pH of the sample was neutralized with 2 mol l^{-1} K_2CO_3. The difference in absorbance obtained from the samples with and without urease treatment was used for estimation of the urea concentration in the sample, using a urea standard processed through the same procedure for comparison. Results were expressed as μmol g^{-1} wet mass tissue or μmol ml^{-1} plasma.

Analysis of free amino acids

Samples for FAA analysis were homogenized three times in 5 volumes (w/v) of 6 % trichloroacetic acid (TCA) at 24,000 revs min^{-1} for 20 s each with intervals of 10 s between each homogenization. The homogenate was centrifuged at 10,000 ***g*** at 4 °C for 15 min, and the supernatant was retained for FAA analysis. The plasma was deproteinized in 2 volumes (v/v) of ice-cold 6 % TCA and centrifuged at 10,000 ***g*** at 4 °C for 15 min. The supernatant obtained was adjusted to pH 2.2 with 4 mol l^{-1} lithium hydroxide and diluted appropriately with 0.2 mol l^{-1} lithium citrate buffer (pH 2.2). The level of FAAs was analyzed using a Shimadzu LC-6A amino acid analysis system (Kyoto, Japan) with a Shim-pack ISC-07/S1504 Li-type column. Results of FAA analyses were expressed as μmol FAA g^{-1} wet mass or μmol FAA ml^{-1} plasma.

Ammonia and urea excretion rate

Specimens were submerged individually in plastic aquaria tanks containing 3.5 l of aerated 50 % sea water at 25 °C. Preliminary experiments on the ammonia and urea analysis of water sampled at 6 h and 24 h showed that the ammonia and urea excretion rates were linear up to at least 24 h. Thus, subsequently, water was sampled for ammonia and urea analysis after 24 h of exposure. The same individuals were exposed to terrestrial conditions in plastic tanks containing 20 ml of 50 % sea water. After 24 h, the fish were sprayed thoroughly with 50 % sea water. The water collected was used for ammonia and urea analysis. After aerial exposure, the fish were submerged again in 50 % sea water to study the rates of ammonia and urea excretion upon recovery. Ammonia and urea were determined as described above. Preliminary results indicated that less than 3 % of the ammonia excreted during aerial exposure was volatilized as ammonia gas.

Preparation of samples for the determination of ornithine-urea cycle enzymes

The livers of experimental fishes were excised quickly for the isolation of mitochondrial and cytosolic fractions (using a modification of the procedure of Jow et al., 1999). In the case of *B. boddaerti*, livers from several individuals were pooled in a single sample.

The excised liver was minced and suspended in 10 volumes (w/v) of ice-cold mitochondria extraction buffer (285 mmol l^{-1} sucrose, 3 mmol l^{-1} EDTA and 3 mmol l^{-1} Tris-HCl, pH 7.2), and homogenized using three strokes of a Teflon-glass homogenizer. The homogenized sample was centrifuged at 600 ***g*** for 15 min at 4 °C to remove any unbroken cells and nuclei. The supernatant obtained was further centrifuged for 15 min at 10,000 ***g*** to obtain a mitochondrial pellet. The mitochondrial pellet was washed twice with the extraction buffer, and centrifuged for 15 min at 10,000 ***g***. The final mitochondrial pellet was suspended in 1 ml of suspension buffer (100 mmol l^{-1} Hepes, pH 7.6, 100 mmol l^{-1} KCl, 1 mmol l^{-1} EDTA and 1 mmol l^{-1} dithiothreitol). It was then sonicated three times for 20 s with a 10 s break between each sonication. The sonicated sample was centrifuged at 4,000 ***g*** at 4 °C for 3 min. After centrifugation, the supernatant was passed through either a 10 ml (for *P. schlosseri* liver samples) or a 5 ml (for *B. boddaerti* liver samples) Bio-Rad P-6DG column (Bio-Rad Laboratories; CA, USA) equilibrated with suspension buffer.

To obtain the cytosolic fraction, the freshly excised liver was homogenized with 5 volumes (w/v) of mitochondria extraction buffer and centrifuged at 10,000 ***g*** for 15 min. The resulting supernatant was collected and passed through a 10 ml Bio-Rad column equilibrated with the suspension buffer. The collected filtrates of the mitochondrial and cytosolic fractions were used for the subsequent enzyme analyses.

Enzyme assays

Carbamoyl phosphate synthetase (CPS; E.C. 2.7.2.5) activity was determined according to the method of Anderson and Walsh (Anderson and Walsh, 1995). Radioactivity was measured using a Wallac 1414 liquid scintillation counter (Bio Laboratories, USA). The CPS activity was expressed as μmol [^{14}C]carbamoyl phosphate min^{-1} g^{-1} wet mass.

Ornithine transcarbamoylase (OTCase; E.C. 2.1.3.3) activity was determined by combining the methods of Anderson and Walsh (Anderson and Walsh, 1995) and Xiong and Anderson (Xiong and Anderson, 1989). Absorbance was measured at 466 nm using a Shimadzu UV 160 UV VIS recording spectrophotometer. The OTCase activity was expressed as μmol citrulline min^{-1} g^{-1} wet mass.

Argininosuccinate synthetase (E.C. 6.3.4.5) and lyase (E.C. 4.3.2.1) activities were determined together, assuming that

both were present, by measuring the formation of [^{14}C]fumarate from [^{14}C]aspartate (using the method of Cao et al., 1991). Radioactivity was measured using a Wallac 1414 liquid scintillation counter. Argininosuccinate synthetase and lyase activity were expressed as μmol [^{14}C]fumarate min^{-1} g^{-1} wet mass.

Arginase (E.C. 3.5.3.1) was assayed as described by Felskie et al. (Felskie et al., 1998). Urea was determined as described above. Arginase activity was expressed as μmol urea min^{-1} g^{-1} wet mass.

Glutamine synthetase (GS; E.C. 6.3.1.2) activity was measured by the method of Shankar and Anderson (Shankar and Anderson, 1985). The formation of γ-glutamylhydroxymate was determined at 500 nm using a Shimadzu UV 160 UV VIS recording spectrophotometer. Glutamine synthetase activity was expressed as μmol γ-glutamylhydroxymate min^{-1} g^{-1} wet mass.

$^{15}NH_4Cl$ studies

Eight *P. schlosseri* were submerged in aerated 50 % sea water. After 24 h, they were injected peritoneally with $^{15}NH_4Cl$ at a concentration of 7 μmol g^{-1} fish. Four fish were then exposed to terrestrial conditions for 48 h as described above. The others were returned to submerged conditions in aerated 50 % sea water for 48 h. Another two groups of fish were exposed to 48 h of terrestrial or submerged conditions without $^{15}NH_4Cl$. At the end of the experimental period, the fish were killed, and liver samples were excised and stored immediately at −80 °C. Samples were analyzed for ammonia and urea as stated above.

Ammonia and urea were isolated in glass vials (22 ml) with a rubber stopper from which a slip of glass microfibre filter (Whatman GF/C) moistened with 1 % H_2SO_4 was suspended. The glass microfibre filter was baked at 450 °C for 24 h before using it to trap ammonia. To obtain the ammonia fraction, 0.5 ml of the supernatant fluid was added to the glass vial, followed by 0.1 ml of 0.1 mol l^{-1} borate buffer (pH 10.4). Immediately after the addition of the buffer, the vial was tightly capped with the rubber stopper and left for 20–24 h at 25 °C. Liberated ammonia was absorbed on the slip of glass microfibre filter, and then dried in a desiccator at 25 °C.

After the removal of ammonia-N, the sample in the vial was neutralized with 0.5 mol l^{-1} HCl, and 0.2 ml of 0.2 mol l^{-1} phosphate buffer (pH 7.2) and 7.5 units ml^{-1} urease (Sigma Chemical Co., MO, USA) were added. The vials were incubated at 37 °C for 1 h, and the liberated ammonia was trapped on the glass microfibre filter. Samples of glass microfibre filter were analyzed using a Shimadzu QP-2000 quadrupole mass spectrometer. Known concentrations of $^{14}NH_4Cl$ or $^{15}NH_4Cl$ (50–100 μg) were used to test the recovery efficiency of the method used above.

Statistical analyses

Results are presented as means ± S.E.M. Analysis of variance (ANOVA) followed by multiple comparisons of means by Duncan's procedure were used to evaluate differences between means where applicable. Arcsine transformation was performed on the 'percentage' data before statistical analyses. Differences were regarded as statistically significant at $P<0.05$.

Results

Aerial exposure in constant darkness significantly decreased the rates of ammonia excretion by *P. schlosseri* (Fig. 1A) and *B. boddaerti* (Fig. 1B) in comparison with the submerged control. Upon resubmergence after aerial exposure, the rates returned to the initial control values. Similar observations were made on the rates of urea excretion (Fig. 1A,B).

There were significant increases in concentrations of ammonia in the muscle, liver and plasma of *P. schlosseri* (Fig. 2A), and in the muscle of *B. boddaerti* exposed to terrestrial conditions in constant darkness (Fig. 2B). For *P. schlosseri*, the hepatic ammonia content was about 1.8 times that of the control after 24 h of aerial exposure (Fig. 2A). The ammonia level in the liver of *B. boddaerti* exposed to terrestrial conditions, however, remained relatively constant (Fig. 2B).

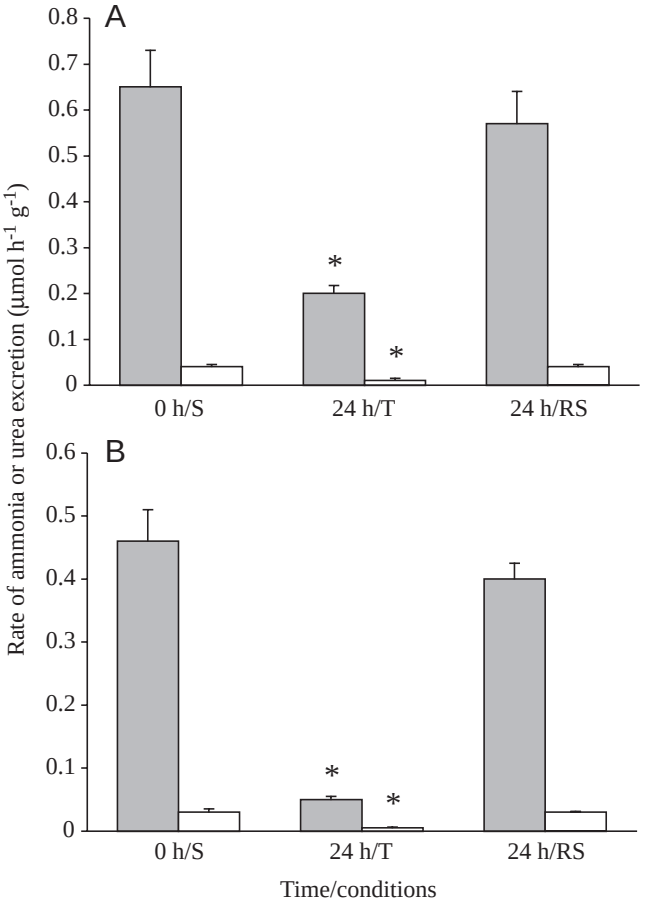

Fig. 1. Effects of 24 h of aerial exposure (24 h/T) or 24 h of resubmergence after 24 h of aerial exposure (24 h/RS), in constant darkness, on the rate of ammonia (shaded bars) and urea (open bars) excretion by *Periophthalmodon schlosseri* (A) and *Boleophthalmus boddaerti* (B). Values are means ± S.E.M. (N=4). *Significantly different from the 0 h submerged (0 h/S) condition, $P<0.05$.

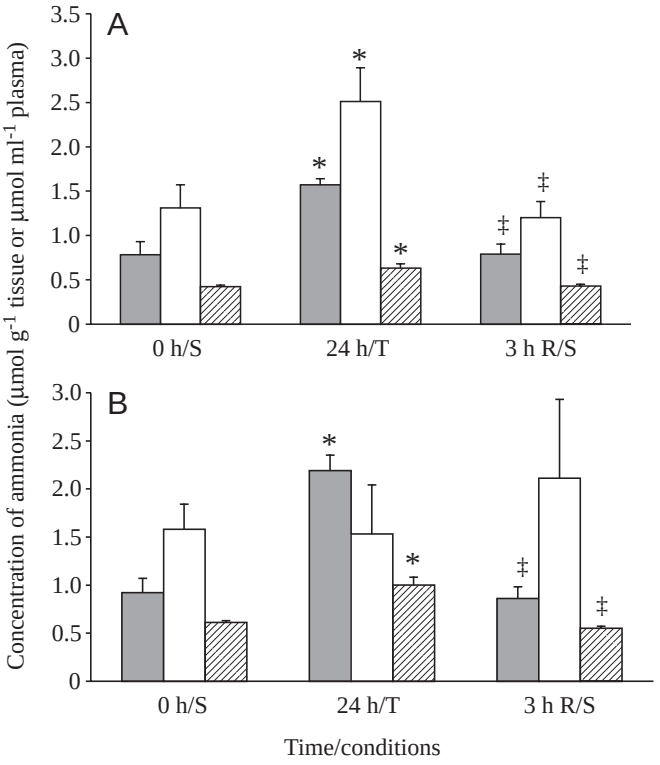

Fig. 2. Effects of 24 h of aerial exposure (24 h/T) or 3 h of resubmergence after 24 h of aerial exposure (3 h/RS), in constant darkness, on the concentrations of ammonia in the muscle (shaded bars), liver (open bars) and plasma (hatched bars) of *Periophthalmodon schlosseri* (A) and *Boleophthalmus boddaerti* (B). Values are means ± S.E.M. (*N*=4). *Significantly different from the 0 h submerged (0 h/S) condition, $P<0.05$; ‡significantly different from the 24 h/T condition, $P<0.05$.

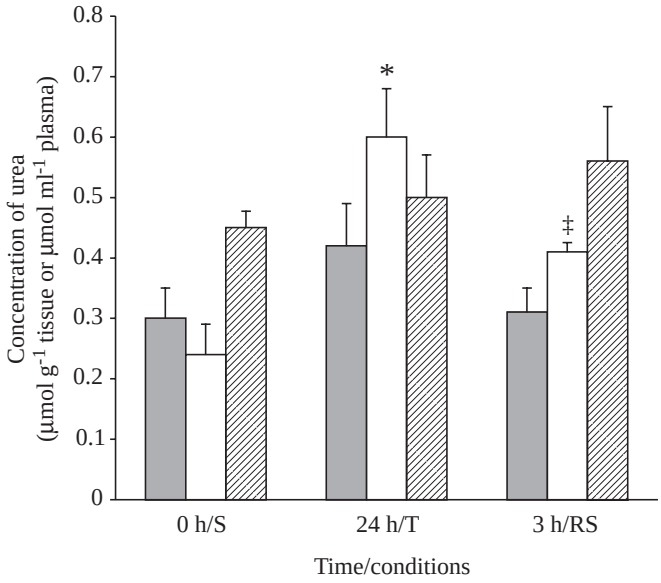

Fig. 3. Effects of 24 h of aerial exposure (24 h/T) or 3 h of resubmergence after 24 h of aerial exposure (3 h/RS), in constant darkness, on the concentrations of urea in the muscle (shaded bars), liver (open bars) and plasma (hatched bars) of *Periophthalmodon schlosseri*. Values are means ± S.E.M. (*N*=4). *Significantly different from the 0 h submerged (0 h/S) condition, $P<0.05$; ‡significantly different from the 24 h/T condition, $P<0.05$.

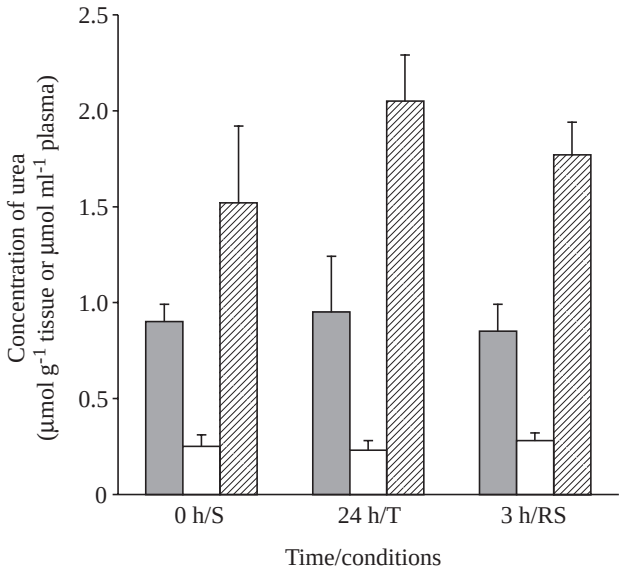

Fig. 4. Effects of 24 h of aerial exposure (24 h/T) or 3 h of resubmergence after 24 h of aerial exposure (3 h/RS), in constant darkness, on the concentrations of urea in the muscle (shaded bars), liver (open bars) and plasma (hatched bars) of *Boleophthalmus boddaerti*. Values are means ± S.E.M. (*N*=4).

The level of urea increased significantly in the liver of *P. schlosseri* after exposure to terrestrial conditions in constant darkness for 24 h (Fig. 3). In contrast, aerial exposure had no significant effects on the urea levels in the tissues and organs of *B. boddaerti* (Fig. 4).

Carbamoyl phosphate synthetase (CPS) activity was detected in the mitochondrial fraction from the liver of *P. schlosseri* (Table 1). Ammonium ions are apparently a more effective substrate than glutamine for this CPS. In addition, the enzyme could not be activated by *N*-acetyl-L-glutamate (NAG). In contrast to the CPS activity in the cytosol, it was not inhibited by UTP. Ornithine transcarbamoylase and arginase activities were detected largely in the liver mitochondrial fraction of the *P. schlosseri* (Table 2). In contrast, argininosuccinate synthetase + lyase (ASS+L) activities were found in the liver cytosolic fraction of this mudskipper (Table 2). Exposure of *P. schlosseri* to terrestrial conditions for 48 h did not result in an increase in the activities of the OUC enzymes.

No CPS activity was detected in the mitochondrial fraction from the liver of *B. boddaerti* exposed to the submerged or terrestrial conditions. In the cytosolic fraction, a CPS activity (0.43 ± 0.15 nmol min^{-1} g^{-1}) that utilized glutamine as a substrate, and was inhibited by UTP, was detected (Table 1). In addition, ASS+L activities were undetectable in both the mitochondrial and cytosolic liver fractions. Ornithine transcarbamoylase and arginase were detected in the cytosolic

Table 1. *Effects of 48 h of aerial exposure on the specific activity of carbamoyl phosphate synthetase in the mitochondrial and cytosolic fractions from the liver of* Periophthalmodon schlosseri

Condition	Substrate and/or effector present	Carbamoyl phosphate synthetase (CPS) activity: Mitochondria (μmol min^{-1} g^{-1})	Carbamoyl phosphate synthetase (CPS) activity: Cytosol (nmol min^{-1} g^{-1})
Submerged	Ammonia	0.055±0.016	ND
	Ammonia+NAG	0.056±0.010	ND
	Ammonia+NAG +UTP	0.075±0.040	ND
	Glutamine	0.030±0.010	2.40±0.01
	Glutamine+NAG	0.029±0.001	2.30±0.01
	Glutamine+NAG +UTP	0.030±0.002	0.24±0.01
Terrestrial	Ammonia	0.099±0.009	ND
	Ammonia+NAG	0.086±0.060	ND
	Ammonia+NAG +UTP	0.117±0.090	ND
	Glutamine	0.035±0.010	2.16±0.01
	Glutamine+NAG	0.032±0.010	2.00±0.01
	Glutamine+NAG +UTP	0.029±0.002	0.22±0.01

CPS activity was measured in mitochondria as μmol carbamoyl phosphate min^{-1} g^{-1} wet mass and in cytosol as nmol carbamoyl phosphate min^{-1} g^{-1} wet mass.

Values are means ± S.E.M. (N=5).

NAG, *N*-acetyl-L-glutamate; UTP, uridine triphosphate; ND, not detectable.

fraction as well as in the mitochondria of the liver of this mudskipper (Table 2).

Studies using $^{15}NH_4Cl$ revealed that *P. schlosseri* was capable of incorporating ammonia into urea in its liver. However, there was no induction of detoxification of ammonia to urea when *P. schlosseri* was exposed to terrestrial conditions (Table 3).

The TFAA content in the muscle of *P. schlosseri* was little affected by aerial exposure in total darkness (Table 4). In contrast, the TFAA content of both the liver and the plasma of these experimental fish decreased significantly after 24 h aerial exposure in constant darkness (P<0.05) (Table 4). In the liver, decreases in TFAA content were attributable to several amino acids, including alanine, asparagine, aspartate, glutamate, glycine, isoleucine, arginine, threonine, proline, tyrosine and lysine (Table 4). Significant decreases in alanine, glutamine, glycine, valine, isoleucine, and threonine were also observed in the plasma after 24 h of aerial exposure (Table 4).

In *B. boddaerti*, the TFAA content in the muscle was approximately 50 % of the submerged values after 24 h of aerial exposure in constant darkness (Table 5). This was attributable to significant decreases in asparagine, glutamine and serine in the muscle from animals exposed to the terrestrial condition for 24 h. The TFAA content in the liver of *B. boddaerti* was not significantly affected by aerial exposure in constant darkness (Table 5), although there was a significant increase in aspartate and glutamate for this experimental condition. Again, there was no significant change in the TFAA concentration in the plasma of *B. boddaerti* after 24 h of aerial exposure under constant darkness (Table 5).

Table 2. *Effects of 48 h of aerial exposure on the specific activities of ornithine transcarbamoylase, argininosuccinate synthetase + lyase, arginase and glutamine synthetase in mitochondrial or cytosolic fractions from the liver of* Periophthalmodon schlosseri *or* Boleophthalmus boddaerti

Condition	Enzyme	Enzyme specific activity (μmol min^{-1} g^{-1} wet mass): *P. schlosseri* Mitochondria	*P. schlosseri* Cytosol	*B. boddaerti* Mitochondria	*B. boddaerti* Cytosol
Submerged	OTCase	7.2±1.6‡	0.20±0.01‡	46.5±10.8	20.3±0.1
Terrestrial		7.6±2.3‡	0.24±0.02‡	49.1±9.0	22.5±1.2
Submerged	ASS+L	ND	1.6±0.4	ND	ND
Terrestrial		ND	1.6±0.4	ND	ND
Submerged	Arginase	58.5±6.4‡	2.4±0.4‡	20.5±2.8	6.7±0.8
Terrestrial		60.5±9.0‡	2.6±0.5	20.0±4.3	3.8±0.6*
Submerged	GS	0.36±0.09	0.21±0.03	0.28±0.02	0.30±0.04
Terrestrial		0.44±0.11	0.24±0.01	0.16±0.07	0.30±0.09

OTCase, ornithine transcarbamoylase; ASS+L, arginosuccinate synthetase + lyase; GS, glutamine synthetase.

Values are means ± S.E.M. (N=5).

*Significantly different from the submerged condition, P<0.05; ‡significantly different from the corresponding value of *B. boddaerti*, P<0.05.

ND, not detectable (detection limit=0.01 μmol min^{-1} g^{-1} wet mass).

Table 3. *Effects of aerial exposure on the incorporation of $^{15}NH_4^+$ (as $^{15}NH_4Cl$) into urea in the liver of* Periophthalmodon schlosseri

		[Ammonia]		[Urea]	
Condition	$^{15}NH_4Cl$	(μmol g^{-1})	% atom excess	(μmol g^{-1})	% atom excess
Submerged	–	1.9±0.1	0	0.13±0.04	0
Submerged	+	2.4±0.3	27.3±2.4	0.15±0.02	18.5±2.7
Terrestrial	–	1.9±0.2	0	0.26±0.02	0
Terrestrial	+	2.9±0.1	22.6±4.3	0.32±0.03	19.1±3.2

Values are means ± S.E.M. (*N*=4).

Discussion

The most important excretory products of nitrogen metabolism in teleost fishes are ammonia and urea, with ammonia usually being the largest component (Mommsen and Walsh, 1992). The contribution of urea is variable, but is usually 20–40 % of the total nitrogen excreted in air-breathing teleosts. In freshwater fishes, over 99 % of the ammonia is excreted via the gills, with the kidney and skin contributing the remainder (Mommsen and Walsh, 1992). However, amphibious fishes with limited gill ventilation during aerial exposure may face problems in excreting the toxic ammonia. Indeed, under terrestrial conditions the ammonia excretion rate of *P. schlosseri* was 1/4 and that of *B. boddaerti* 1/14 of that of the submerged controls. Similarly, urea excretion rates decreased significantly after 24 h of aerial exposure.

Urea synthesis serves as a means for long-term ammonia detoxification and is usually found in fishes that may encounter problems with water availability. Rates of urea excretion in the Chilean clingfish, *Sicyases sanguineus*, rose from 0.76 mmol N kg^{-1} h^{-1} in water to 2.62 mmol N kg^{-1} h^{-1} when the fish was exposed to terrestrial conditions for 20 h (Gordon et al., 1970). Urea levels in the plasma, liver and muscle of *B. boddaerti* were unaffected by aerial exposure, indicating that the accumulated ammonia was not channelled into urea production in this mudskipper. In contrast, there were significant increases in the levels of urea in the liver of *P. schlosseri* after 48 h of aerial exposure, suggesting, superficially at least, that conversion of ammonia to urea might have occurred.

Urea can be synthesized via three pathways: (1) the ornithine–urea cycle (OUC), (2) routine turnover of arginine by argininolysis and (3) the conversion of uric acid to urea by uricolysis. Although arginase and OTCase activities were detected in the mitochondrial fraction of the liver of *B. boddaerti*, no CPS or ASS+L activities were detected. Hence, in agreement with the above observation, *B. boddaerti* does not possess a complete OUC and is incapable of detoxifying

Table 4. *Effects of various times of aerial exposure (in constant darkness) on the concentrations of various free amino acids (FAA) and total FAA (TFAA) in the muscle, liver and plasma of* Periophthalmodon schlosseri

	FAA concentration					
	Muscle (μmol g^{-1})		Liver (μmol g^{-1})		Plasma (μmol ml^{-1})	
FAA	Submerged (0 h)	Terrestrial (24 h)	Submerged (0 h)	Terrestrial (24 h)	Submerged (0 h)	Terrestrial (24 h)
Alanine	2.5±1.4	0.30±0.19	2.9±1.0	0.29±0.05*	0.15±0.01	0.08±0.01*
Asparagine	0.19±0.03	0.20±0.03	0.61±0.22	0.06±0.02*	0.02±0.01	0.01±0.01
Aspartate	0.22±0.10	0.13±0.02	0.24±0.02	0.13±0.04*	0.05±0.01	0.02±0.01
Arginine	0.15±0.02	0.11±0.02	0.21±0.06	0.05±0.01*	0.05±0.01	0.03±0.01
Glutamine	0.46±0.04	0.34±0.03	1.3±0.2	2.7±0.4	0.12±0.01	0.05±0.01*
Glutamate	0.22±0.06	0.35±0.05	5.1±0.7	0.26±0.05*	0.01±0.01	0.01±0.01
Glycine	1.8±0.9	1.2±0.2	1.0±0.2	0.48±0.14*	0.11±0.01	0.07±0.01*
Histidine	0.30±0.06	0.56±0.18	0.40±0.04	0.16±0.04	0.02±0.01	0.03±0.01
Isoleucine	0.23±0.05	0.07±0.02*	0.15±0.03	0.05±0.01*	0.06±0.01	0.03±0.01*
Leucine	0.42±0.10	0.13±0.02*	0.32±0.06	0.10±0.01	0.12±0.02	0.06±0.01
Lysine	1.2±0.2	1.2±0.3	0.91±0.13	0.24±0.03*	0.11±0.03	0.07±0.02
Phenylalanine	0.19±0.09	0.06±0.01	0.06±0.02	0.05±0.01	0.03±0.01	0.02±0.01
Proline	0.19±0.04	0.08±0.02	0.68±0.05	0.19±0.02*	0.03±0.01	0.01±0.01
Serine	0.25±0.04	0.23±0.03	0.14±0.02	0.06±0.01	0.04±0.01	0.03±0.01
Threonine	0.27±0.04	0.24±0.04	1.3±0.2	0.19±0.07*	0.07±0.01	0.03±0.01*
Tyrosine	0.03±0.01	0.05±0.01	0.08±0.02	0.02±0.01*	0.02±0.01	0.01±0.01
Valine	0.36±0.09	0.21±0.03	0.24±0.04	0.09±0.01	0.10±0.02	0.05±0.01*
TFAA (–Taurine)	7.2±2.0	5.4±0.8	15±2	5.6±0.4*	1.1±0.1	0.58±0.09*
Taurine	9.4±2.4	11±1	23±4	14±2*	0.21±0.03	0.21±0.02

Values are means ± S.E.M. (*N*=4).
*Significantly different from the corresponding 0 h submerged condition, $P<0.05$.

Table 5. *Effects of various times of aerial exposure (in constant darkness) on the concentrations of various free amino acids (FAA) and total FAA (TFAA) in the muscle, liver and plasma of* Boleophthalmus boddaerti

	FAA concentration					
	Muscle (μmol g^{-1})		Liver (μmol g^{-1})		Plasma (μmol ml^{-1})	
FAA	Submerged (0 h)	Terrestrial (24 h)	Submerged (0 h)	Terrestrial (24 h)	Submerged (0 h)	Terrestrial (24 h)
Alanine	1.9±0.4	1.2±0.2	0.25±0.08	0.37±0.20	0.07±0.01	0.06±0.01
Asparagine	0.99±0.37	0.17±0.08*	0.04±0.01	0.03±0.01	0.01±0.01	0.01±0.01
Aspartate	0.45±0.24	0.09±0.03	0.05±0.01	0.12±0.01*	0.02±0.01	0.02±0.01
Arginine	0.48±0.20	0.19±0.08	0.18±0.01	0.23±0.06	0.02±0.01	0.04±0.01
Glutamine	1.6±0.9	0.47±0.16*	0.23±0.09	0.43±0.14	0.03±0.01	0.03±0.01
Glutamate	1.3±0.1	0.77±0.29	0.64±0.12	2.3±0.6*	0.02±0.01	0.03±0.01
Glycine	14±3	15±2	0.32±0.05	0.47±0.08	0.34±0.06	0.18±0.03*
Histidine	0.68±0.17	0.88±0.15	0.07±0.03	0.10±0.02	0.02±0.01	0.02±0.01
Isoleucine	0.19±0.05	0.16±0.03	0.04±0.01	0.05±0.02	0.04±0.01	0.03±0.01*
Leucine	0.33±0.08	0.25±0.04	0.03±0.01	0.12±0.05	0.07±0.01	0.10±0.01
Lysine	1.8±0.8	0.78±0.32	0.10±0.02	0.10±0.04	0.04±0.01	0.06±0.01
Phenylalanine	0.08±0.02	0.12±0.06	0.01±0.01	0.02±0.01	0.02±0.01	0.02±0.01
Proline	0.18±0.04	0.14±0.02	0.32±0.10	0.28±0.08	0.01±0.01	0.02±0.01
Serine	0.59±0.09	0.21±0.10*	0.09±0.03	0.09±0.04	0.02±0.01	0.02±0.01
Threonine	0.34±0.08	0.23±0.05	0.05±0.01	0.08±0.04	0.02±0.01	0.02±0.01
Tyrosine	0.28±0.10	0.11±0.02	0.05±0.01	0.06±0.01	0.01±0.01	0.01±0.01
Valine	0.21±0.06	0.21±0.03	0.09±0.01	0.09±0.03	0.07±0.01	0.07±0.01
TFAA (–Taurine)	24±6	12±4*	2.5±0.4	4.9±1.4	0.84±0.03	0.73±0.04
Taurine	13±3	11±1	6.2±1.3	6.5±1.3	0.21±0.03	0.17±0.01

Values are means ± S.E.M. (N=4).
*Significantly different from the corresponding 0 h submerged condition, P<0.05.

ammonia to urea through this cycle. The ability to synthesize low levels of urea is not necessarily linked to a functional OUC. The activities of arginase in the liver of these two mudskipper species were sufficient to account for the amount of urea produced during the 24 h experimental period. For *P. schlosseri*, a very low CPS activity was detected in the mitochondrial fraction of the liver. Ammonia was required as the substrate, and UTP was not inhibitory. At present, the relationship between this CPS and CPS-I of mammals or CPS-III of elasmobranchs is uncertain. Some similarities exist between the CPS activity from liver mitochondria of *P. schlosseri* and that from lake-adapted tilapia (*Oreochromis alcalicus grahami*); V_{max} obtained using ammonia as a substrate is greater than that obtained with glutamine, and *N*-acetylglutamate has little effect (Lindley et al., 1999). However, judging from the level of activity detected, it can be concluded that the OUC, if present in the liver of *P. schlosseri*, is not an effective ammonia detoxification mechanism. Indeed, results from the $^{15}NH_4Cl$ studies indicate that the accumulated ammonia in the tissues was not detoxified into urea in the liver of *P. schlosseri* during aerial exposure.

Mudskippers remain quiescent in total darkness (Y. K. Ip, unpublished observation). When exposed to terrestrial conditions under constant darkness, TFAA levels in the liver and plasma of *P. schlosseri* decreased. In *B. boddaerti*, the decrease was observed in the muscle instead. To slow down the build-up of ammonia internally, it would be necessary to decrease the rate of ammonia production through amino acid

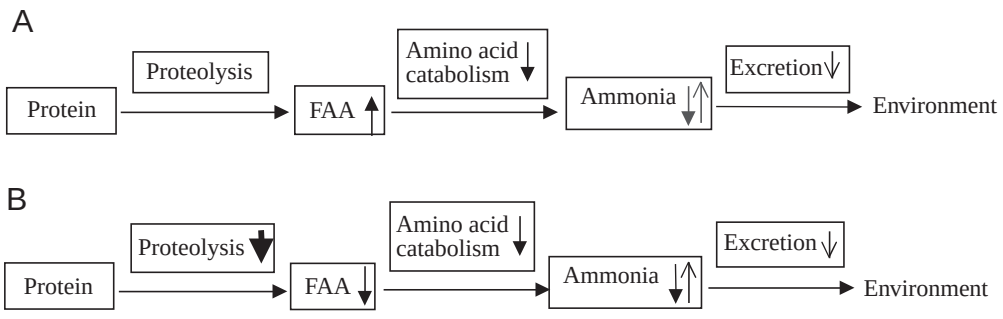

Fig. 5. Proposed changes in the steady state pool of protein, free amino acids (FAAs) and ammonia in mudskippers due to (A) a decrease in the rate of amino acid catabolism and a decrease in the rate of ammonia excretion, or (B) a decrease in the rate of proteolysis, a decrease in the rate of amino acid catabolism and a decrease in the rate of ammonia excretion.

Table 6. *A balance sheet of nitrogenous accumulation and excretion in a 70 g* Periophthalmodon schlosseri *exposed to submerged or terrestrial conditions*

	Nitrogen excretion/accumulation (μmol)		
	Submerged	Terrestrial	Difference
Excreted from *P. schlosseri* (70 g)			
Ammonia	1008	252	−756
Urea	130	25.3	−105
Reduction in nitrogenous excretion			−861
Retained in muscle (42 g)			
Ammonia	31.9	51.2	+19.3
Urea	NS	NS	NS
Amino acids	NS	NS	NS
Retained in liver (2 g)			
Ammonia	2.68	4.14	+1.46
Urea	NS	NS	NS
Amino acids	33.1	16.9	−16.2
Increase in nitrogenous accumulation			+5

NS, no significant difference between the values of the submerged and terrestrial conditions.

Table 7. *A balance sheet of nitrogenous accumulation and excretion in a 7 g* Boleophthalmus boddaerti *exposed to the submerged or terrestrial conditions*

	Nitrogen excretion/accumulation (μmol)		
	Submerged	Terrestrial	Difference
Excreted from *B. boddaerti (7 g)*			
Ammonia	70.6	5.04	−66.6
Urea	8.21	1.21	−7.00
Reduction in nitrogen excretion			−73
Retained in muscle (4.2 g)			
Ammonia	3.78	7.60	+3.82
Urea	NS	NS	NS
Amino acids	111	60.2	−50.8
Retained in liver (0.2 g)	None		
Reduction in nitrogenous accumulation			−48

NS, no significant difference between the values of the submerged and terrestrial conditions.

catabolism. The steady-state concentration of amino acids in the tissues depends on the rates of their degradation and production. In the case of the species in this experiment, amino acids would be produced mainly through proteolysis, as food was withdrawn for 24 h before, and during, experiments. Under such conditions, it is logical to assume that the rate of protein degradation is higher than the rate of protein synthesis, leading to a net proteolysis. If the rate of proteolysis remains relatively constant and is unaffected by aerial exposure, there would be accumulations of FAAs, leading to an increase in the internal TFAA content (Fig. 5A).

Therefore, the decrease in TFAA content seen in some of the tissues of these two mudskipper species indicates that simultaneous decreases in the rates of proteolysis and amino acid catabolism had occurred. In addition, the decrease in proteolytic rate might be greater than the decrease in the rate of amino acid catabolism, leading to decreases in the steady-state concentrations of various FAAs and, consequently lowering the TFAA concentrations (Fig. 5B).

An analysis of the balance between nitrogenous excretion and nitrogenous accumulation in a 70 g *P. schlosseri* (Table 6) and a 7 g *B. boddaerti* (Table 7) supported the above conclusion. The deficits for these two mudskippers were (for *P. schlosseri*) 12.2 [(–861+5)/70] and (for *B. boddaerti*) 17.2 [(−73–48)/7] μmol ammonia-equivalent g^{-1} 24 h^{-1} aerial exposure. However, it is obvious that the decrease in the rate of nitrogenous excretion was much greater than the reduction in the rate of ammonia production, leading to the accumulation of ammonia in the tissues and organs of these two mudskippers.

Given this accumulation of ammonia in the tissues, why do we not observe an increase in ammonia excretion by the mudskippers upon resubmergence? From Fig. 2A, it can be seen that 32.76 μmol ammonia would have accumulated in the muscle of a 70 g *P. schlosseri* after 24 h aerial exposure. If this amount was excreted during the next 24 h of resubmergence in 50 % sea water, the increase in the rate of ammonia excretion would be 0.02 μmol h^{-1} g^{-1} (32.76 μmol 24 h^{-1} 70 g^{-1} fish), which is clearly negligible. This would explain why there was no observable increase in the ammonia excretory rate when *P. schlosseri* was resubmerged in 50 % sea water. A similar argument applies in the case of *B. boddaerti*. In contrast, Gordon et al. (Gordon et al., 1969) observed that when *Periophthalmus sobrinus* was resubmerged after a 12 h period of aerial exposure, the rate of ammonia production was twice that normally seen in water. Hence, they concluded that *Periophthalmus sobrinus* was excreting ammonia that had accumulated in the body. In contrast, the present study exposed *P. schlosseri* and *B. boddaerti* to terrestrial conditions in constant darkness, which minimized the possibility of locomotory activities. We suspect that the observations made by Gordon et al. (Gordon et al., 1969) could be a result of increased protein catabolism during periods of ‘exercise’ or increased activity on land.

The ability to reduce rates of amino acid catabolism during aerial exposure may not be a common phenomenon among fishes. For example, when the marble goby *Oxyeleotris marmoratus*, which remains inactive on land, is exposed to terrestrial conditions for 72 h, it does not undergo a reduction in amino acid catabolism. Instead, it appears that protein and/or amino acid catabolism may increase during aerial exposure because glutamine accumulates to levels far in excess of those needed to detoxify ammonia produced during that period (Jow et al., 1999).

Taken together, our results suggest that mudskippers respond to aerial exposure by reducing their rates of protein and amino acid catabolism. This is an effective strategy for slowing down the internal build-up of ammonia. However, it also prevents the utilization of amino acids as an energy source. Operating by itself, it may not be a good mechanism for fishes such as *P. schlosseri*, which are active during aerial exposure (Kok et al., 1998). It was recently reported that *P. schlosseri* is capable of actively transporting NH_4^+ against a concentration gradient (Randall et al., 1999). Hence, build-up of internal ammonia in this mudskipper could be further reduced by pumping ammonia into the spaces between intrafilamentous interlamellar fusions (Low et al., 1988; Wilson et al., 1999) in adverse conditions. In addition, in the accompanying study (Ip et al., 2001) it is reported that alanine and TFAAs accumulated in the tissues of *P. schlosseri* during aerial exposure (in a dark:light regime). It is possible, therefore, that *P. schlosseri* uses protein and amino acids as energy sources to support activity on land. Future examination of the metabolism of this interesting mudskipper may yet reveal another mechanism that allows the catabolism of amino acids without releasing ammonia (Ip et al., 2001).

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