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PARTIAL AMINO ACID CATABOLISM LEADING TO THE FORMATION OF ALANINE IN *PERIOPHTHALMODON SCHLOSSERI* (MUDSKIPPER): A STRATEGY THAT FACILITATES THE USE OF AMINO ACIDS AS AN ENERGY SOURCE DURING LOCOMOTORY ACTIVITY ON LAND

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Summary

When the mudskipper *Periophthalmodon schlosseri* was exposed to terrestrial conditions under a 12 h:12 h dark:light regime the fish could be very active, and levels of total free amino acids increased significantly in the muscle and plasma. Alanine levels increased threefold in the muscle, fourfold in the liver and twofold in the plasma. Similar phenomena were not observed in the more aquatic mudskipper, *Boleophthalmus boddaerti*. From these results, we concluded that *P. schlosseri* was capable of partial catabolism of certain amino acids to support activity on land. The amino groups of these amino acids were transferred directly or indirectly to pyruvate to form alanine. The resulting carbon chain was fed into the Krebs cycle and partially oxidized to malate, which could replenish pyruvate through the function of malic enzyme. This favourable ATP yield from partial amino acid catabolism was not accompanied by a net release of ammonia. Such an adaptation would be advantageous to *P. schlosseri* confronted with the problem of ammonia excretion during aerial exposure. Indeed, when *P. schlosseri* were forced to exercise on land after 24 h of aerial exposure, the alanine level in the muscles increased significantly, with no apparent change in glycogen content. In addition, there was no significant change in the ATP level and energy charge of the muscle. In contrast, when *B. boddaerti* were exercised on land, glycogen levels in the muscles decreased significantly and lactate levels increased. In addition, muscle energy charge was not maintained and the ATP level decreased significantly. Hence, it was concluded that when *P. schlosseri* were active on land, they were capable of using certain amino acids as a metabolic fuel, and avoided ammonia toxicity through partial amino acid catabolism. Such a strategy is the most cost-effective way of slowing down internal ammonia build-up without involving energy-expensive ammonia detoxification pathways. Furthermore, an examination of the balance between nitrogenous excretion and accumulation in a 70 g *P. schlosseri* revealed that degradation of amino acids in general was likely to be suppressed to slow down the build-up of ammonia internally. It is possible that such a strategy may be widely adopted, especially by obligatory air-breathing fishes, to avoid ammonia intoxication during aerial exposure.

Key words: aerial exposure, alanine, ammonia, *Boleophthalmus boddaerti*, mudskipper, *Periophthalmodon schlosseri*, locomotory activity, partial amino acid catabolism.

Introduction

The mudskippers *Periophthalmodon schlosseri* and *Boleophthalmus boddaerti* are gobioid teleosts found in the mangrove swamps of Singapore and Southeast Asia. They are amphibious and spend a substantial part of their lives out of water. At low tides, both species of mudskipper move and feed on mudflats. However, *B. boddaerti* stays within its water-filled burrows during high tide, emerging only when the tide ebbs. In contrast, *P. schlosseri* usually swims along the water's edge, with its snout and eyes above water, or can be seen moving on higher ground when the tide is high. Studies on the branchial morphology of *P. schlosseri* reveal that it has many unique adaptations to survive on land (Low et al., 1988; Low et al., 1990; Wilson et al., 1999).

During periods of aerial exposure, branchial ammonia excretion would be inefficient because no external water is available to irrigate the gills. Indeed, there is an accumulation of ammonia in the tissues and organs of *B. boddaerti* and *P. schlosseri* exposed to terrestrial conditions (Ip et al., 1993), and this ammonia is not converted to urea via the ornithine-urea cycle in these two mudskipper species (Lim et al., 2001).

Mudskippers can be very active on land, and urea formation, which is energetically expensive, may not be a very suitable strategy. By exposing these two mudskipper species to terrestrial conditions, in constant darkness to minimize physical activity, Lim et al. (Lim et al., 2001) found that they reduced the rate of proteolysis and amino acid catabolism in response to aerial exposure. In contrast, Ip et al. (Ip et al., 1993) observed increases in the concentrations of alanine, branched-chain free amino acids (FAA) and total free amino acids (TFAA) in the tissues of *P. schlosseri* after a 24 h exposure to terrestrial conditions when the light and dark cycle was not controlled.

The eyes of mudskippers are well adapted for aquatic and terrestrial vision and, according to Smith (Smith, 1945), their vision has a range of at least 10 m in air. Under light conditions mudskippers are easily agitated by visual stimulation but in constant darkness, they remain relatively quiescent. We suspected, therefore, that the discrepancies between these two studies (Ip et al., 1993; Lim et al., 2001) resulted from the activation of a mechanism yet to be discovered in *P. schlosseri* when it is active on land. Kok et al. (Kok et al., 1998) reported that *P. schlosseri* has a higher metabolic rate (greater respiratory rate and heart beat) on land than in water. Reducing the rates of proteolysis and amino acid catabolism in *P. schlosseri* to slow down ammonia build-up during a terrestrial excursion in its natural habitat may not be a good strategy since this would prevent the mudskipper from using protein and amino acids as energy sources. Hence, the objective of this study was to explore whether *P. schlosseri* was capable of catabolizing amino acids without releasing ammonia while on land. Experiments were performed on specimens exposed to terrestrial conditions in a dark:light regime or to short bursts (3 min) of exercise after 3 h or 24 h of aerial exposure.

The gill morphology and morphometry of *B. boddaerti* are similar to those of other aquatic fishes, and this species is less adapted for terrestrial life than *P. schlosseri* (Low et al., 1988; Low et al., 1990). We suspected that the capability to use amino acid as a fuel without polluting the internal environment during aerial exposure was unique to *P. schlosseri* and, to test this, experiments were also performed on *B. boddaerti* for comparison.

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 % (15‰ salinity) sea water at 25 °C in the laboratory, and the sea water was changed daily. No attempt was made to separate the sexes. Fish were acclimated to laboratory conditions for 1 week. During the adaptation period, *P. schlosseri* and *B. boddaerti* were fed small guppies and an artificial diet, respectively. Food was withdrawn 24 h prior to experiments so that the fish had empty guts. All experiments performed in this study were under a 12 h:12 h dark:light regime.

Exposure of mudskippers to experimental conditions and collection of samples

The effects of aerial exposure on specimens

24 h before the experiment, a group of specimens was immersed completely in sea water with continuous aeration. At the end of the 24 h period (0 h control), some of these specimens were anaesthetized by the introduction of 3-aminobenzoic acid ethyl ester (MS-222) at a final concentration of 0.02 % and killed. The rest were exposed for various times to terrestrial conditions in plastic aquaria containing 20 ml of 50 % sea water. These aquaria were kept in a temperature-controlled (25 °C) cabinet in a 12 h:12 h dark:light regime. After 24 h, some of these fish were anaesthetized for 10 min in an atmosphere saturated with diethyl ether. The rest of the fish were resubmerged for 3 h in 50 % sea water before being anaesthetized and killed as described above for the controls.

The effects of locomotory activity on the mudskippers

Specimens were submerged for 24 h in a 12 h:12 h dark:light regime. Some of these specimens were then stimulated by mechanical disturbance to swim in water for 3 min. Others were exposed to terrestrial conditions for 3 h in the light, followed by 3 min of locomotory exercise on land. A separate group of *P. schlosseri* was exposed to terrestrial conditions for 24 h in a 12 h:12 h dark:light regime before being stimulated to move on land for 3 min. No anaesthesia was applied to the exercised fish.

Collection of samples for further analyses

Anaesthetized or exercised 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 pre-cooled 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 4,000 ***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 fluid was kept at –80 °C until analysed.

Analysis of free amino acids (FAAs)

The frozen muscle and liver samples were weighed, ground to a powder in liquid nitrogen, and homogenized in 5 volumes (w/v) of 6 % trichloroacetic acid (TCA) three times (20 s each with 10 s intervals) using an Ultra-Turrax homogenizer at 24,000 revs min^{-1}. The homogenates were centrifuged at 10,000 ***g*** at 4 °C for 15 min to obtain the supernatant for FAA

analyses. 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 to obtain the supernatant.

For analyses of FAA, 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). FAA were analyzed using a Shimadzu LC-6A amino acid analysis system (Kyoto, Japan) with a Shim-pack ISC-07/S1504 Li-type column. The results of FAA analyses are expressed as μmol g^{-1} wet mass tissue or μmol ml^{-1} plasma.

Analyses of ammonia, urea, alanine, lactate, ATP, ADP and AMP

Samples for metabolite analyses were homogenized as stated above except in 5 volumes of 6 % $HClO_4$. After centrifugation at 10,000 ***g*** for 15 min, the supernatant was decanted and the pH adjusted to 5.5–6.0 with 2 mol l^{-1} K_2CO_3. The ammonia content was determined according to the method of Bergmeyer and Beutler (Bergmeyer and Beutler, 1985), urea content according to Felskie et al. (Felskie et al., 1998) and alanine content according to Williamson (Williamson, 1974). Lactate was determined using the method of Gutmann and Wahlefeld (Gutmann and Wahlefeld, 1974) while ATP, ADP and AMP were determined spectrophotometrically (Scheibel et al., 1968). Results were expressed as μmol g^{-1} wet mass tissue or μmol ml^{-1} plasma.

Determination of glycogen content

Frozen samples (0.5–1.0 g) were ground to a powder and digested in 2 ml of 30 % (w/v) KOH in a boiling water bath for 10 min. The glycogen was extracted according to the method of Lim and Ip (Lim and Ip, 1989), and was determined by combining published methods (Bergmeyer et al., 1974; Roehig and Allred, 1974). The glycogen content was expressed as μmol glycosyl units g^{-1} wet mass tissue.

Ammonia and urea excretion rate

Specimens were submerged individually in plastic aquaria (25 cm×14 cm×12 cm, length×width×height) containing 3.5 l of aerated 50 5 sea water at 25 °C. Preliminary experiments on the analysis of ammonia and urea in the water sampled at 6 h and 24 h showed that the ammonia and urea excretion rates were linear up to at least 24 h. Subsequently, water was sampled for ammonia and urea analysis after 24 h of exposure. The same individuals were then 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 analyses. 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.

Statistical analysis

Results are presented as means ± S.E.M. Student's *t*-test or analysis of variance (ANOVA) followed by multiple comparisons of means by Duncan's procedure were used to evaluate differences between means in groups, where appropriate. Differences where $P<0.05$ were regarded as statistically significant.

Results

Effects of aerial exposure in a dark:light regime

Ammonia accumulated in the muscle, liver and plasma of *P. schlosseri* after 24 h of aerial exposure in a dark:light regime (Table 1). The urea content in the liver of these experimental fish also increased significantly (Table 1). After 3 h of resubmergence in water, the ammonia and urea levels returned to near control values (Table 1). In the case of *B. boddaerti*, aerial exposure affected ammonia levels in the muscle and plasma, but not in the liver, and no accumulation of urea was found in any tissues (Table 2).

Levels of alanine, arginine, isoleucine, leucine, lysine, proline, serine and valine in the muscle of *P. schlosseri* increased significantly after 24 h of aerial exposure under a dark:light regime (Table 3). This was accompanied by a significant increase in the levels of TFAA. Increases in the levels of alanine, aspartate, glutamine, glutamate, isoleucine, leucine, lysine, proline and valine and decreases in the levels of glycine and threonine in the liver were also observed (Table 3). Increases in the concentrations of TFAA, alanine,

Table 1. *Effects of aerial exposure for 24 h or a 3 h resubmergence after a 24 h aerial exposure, in a 12 h:12 h dark:light regime, on levels of ammonia and urea in the muscle, liver and plasma of* Periophthalmodon schlosseri

		[Ammonia]			[Urea]		
Condition	Duration (h)	Muscle (μmol g^{-1})	Liver (μmol g^{-1})	Plasma (μmol ml^{-1})	Muscle (μmol g^{-1})	Liver (μmol g^{-1})	Plasma (μmol ml^{-1})
Submerged	0	0.76±0.15	1.34±0.26	0.40±0.01	0.31±0.04	0.23±0.06	0.41±0.02
Terrestrial	24	1.54±0.07*	2.49±0.38*	0.65±0.05*	0.41±0.07	0.59±0.10*	0.51±0.09
Resubmerged	3	0.78±0.11‡	1.18±0.18‡	0.42±0.01‡	0.31±0.05	0.39±0.01‡	0.56±0.08

Values are means ± S.E.M. (N=4).

*Significantly different from the corresponding 0 h submerged condition, $P<0.05$; ‡significantly different from the corresponding 24 h terrestrial condition, $P<0.05$.

Table 2. *Effects of aerial exposure for 24 h or a 3 h resubmergence after a 24 h aerial exposure, in a 12 h:12 h dark:light regime, on levels of ammonia and urea in the muscle, liver and plasma of* Boleophthalmus boddaerti

		[Ammonia]			[Urea]		
Condition	Duration (h)	Muscle (μmol g^{-1})	Liver (μmol g^{-1})	Plasma (μmol ml^{-1})	Muscle (μmol g^{-1})	Liver (μmol g^{-1})	Plasma (μmol ml^{-1})
Submerged	0	0.90±0.11	1.60±0.26	0.59±0.02	0.99±0.12	0.21±0.05	1.48±0.45
Terrestrial	24	2.21±0.16*	1.55±0.51	0.99±0.08*	0.98±0.27	0.18±0.04	1.98±0.17
Resubmerged	3	0.85±0.11‡	2.09±0.82	0.56±0.01‡	0.90±0.11	0.23±0.06	1.81±0.11

Values are means ± S.E.M. (N=4).

*Significantly different from the corresponding 0 h submerged condition, P<0.05; ‡significantly different from the corresponding 24 h terrestrial condition, P<0.05.

Table 3. *Effects of various times of aerial exposure (12 h:12 h dark:light regime) 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	1.07±0.11	3.21±0.39*	1.20±0.12	4.50±0.86*	0.120±0.020	0.260±0.020*
Arginine	0.16±0.02	0.38±0.39*	0.18±0.03	0.19±0.04	0.040±0.020	0.060±0.010
Asparagine	0.08±0.01	0.07±0.01	0.50±0.12	0.35±0.16	ND	ND
Aspartate	0.13±0.01	0.12±0.02	0.32±0.06	0.55±0.04*	0.004±0.001	0.004±0.001
Glutamine	0.15±0.04	0.20±0.05	2.50±0.50	4.50±0.04*	0.006±0.001	0.007±0.001
Glutamate	0.25±0.05	0.22±0.03	4.06±0.25	5.50±0.30*	0.018±0.003	0.015±0.002
Glycine	1.21±0.15	1.30±0.17	1.04±0.12	0.68±0.09*	0.076±0.012	0.083±0.015
Histidine	0.25±0.02	0.32±0.03	0.25±0.02	0.35±0.04	0.024±0.005	0.028±0.003
Isoleucine	0.10±0.01	0.42±0.04*	0.05±0.01	0.18±0.01*	0.056±0.003	0.167±0.004*
Leucine	0.25±0.02	0.65±0.04*	0.20±0.02	0.41±0.02*	0.101±0.009	0.321±0.015*
Lysine	1.01±0.09	1.76±0.09*	0.35±0.02	0.99±0.18*	0.047±0.020	0.152±0.030*
Phenylalanine	0.17±0.03	0.23±0.01	0.21±0.02	0.22±0.03	0.034±0.004	0.049±0.007*
Proline	0.10±0.02	0.26±0.01*	0.36±0.03	0.67±0.05*	0.015±0.001	0.052±0.010*
Serine	0.23±0.01	0.56±0.03*	0.15±0.01	0.16±0.02	0.023±0.002	0.025±0.003
Threonine	0.31±0.02	0.36±0.03	0.92±0.17	0.33±0.04*	0.082±0.010	0.077±0.010
Tyrosine	0.08±0.03	0.15±0.03	0.12±0.02	0.15±0.02	0.018±0.002	0.042±0.003*
Valine	0.20±0.02	0.56±0.03*	0.20±0.02	0.67±0.09*	0.130±0.010	0.150±0.010
TFAA (−Taurine)	5.56±0.30	9.50±0.62*	10.1±0.9	14.8±1.1	0.95±0.08	1.47±0.09*
Taurine	17.8±1.6	18.2±2.3	20.1±3.5	20.2±2.0	11.1±1.8	12.5±1.4

Values are means ± S.E.M. (N=4).

*Significantly different from corresponding 0 h submerged condition, P<0.05.

ND, not detectable.

isoleucine, leucine, lysine, phenyalanine, proline and tyrosine were detected in the plasma (Table 3).

In contrast, levels of arginine and aspartate decreased significantly in the muscle of *B. boddaerti* after 24 h of aerial exposure under a dark:light regime while levels of proline and threonine rose (Table 4). In the liver, alanine levels increased and glycine levels decreased were seen in the plasma (Table 4). The concentrations of TFAAs in all the tissue types in *B. boddaerti* were unaffected by aerial exposure (Table 4).

The rates of ammonia excretion in air-exposed *P. schlosseri* (Fig. 1) and *B. boddaerti* (Fig. 2) were significantly lower than those of the submerged controls. Upon resubmergence, the rates of ammonia excretion returned to the initial control value. Aerial exposure exerted similar effects on the rates of urea excretion in both mudskipper species (Figs 1, 2).

Effects of short burst of locomotory activity

The effects of activity on the levels of various metabolites in the muscle of *P. schlosseri* are shown in Table 5. 3 min of exercise in water after 24 h of submergence led to a significant decrease in glycogen and increase in lactate in the muscle. Increases in the amount of ammonia and alanine in the muscle

Table 4. *Effects of 24 h aerial exposure (12 h:12 h dark:light regime) 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	2.03±0.08	2.56±0.20	0.15±0.02	0.22±0.03*	0.058±0.003	0.068±0.004
Arginine	0.49±0.03	0.25±0.01*	0.020±0.002	0.020±0.002	0.023±0.002	0.025±0.005
Asparagine	0.05±0.01	0.03±0.01	0.05±0.01	0.04±0.01	ND	ND
Aspartate	0.35±0.05	0.11±0.01*	0.25±0.05	0.23±0.05	0.007±0.001	0.007±0.001
Glutamine	0.38±0.05	0.32±0.04	0.36±0.07	0.41±0.09	0.010±0.001	0.005±0.001
Glutamate	0.42±0.06	0.31±0.02	1.85±0.04	2.01±0.13	0.015±0.003	0.014±0.002
Glycine	23.7±0.9	22.5±1.4	1.09±0.08	0.61±0.15*	0.250±0.030	0.210±0.030
Histidine	0.83±0.14	0.98±0.03	0.15±0.02	0.15±0.03	0.200±0.010	0.022±0.130
Isoleucine	0.12±0.01	0.15±0.04	0.04±0.01	0.05±0.01	0.045±0.005	0.047±0.030
Leucine	0.42±0.05	0.41±0.03	0.15±0.02	0.10±0.02	0.120±0.010	0.110±0.010
Lysine	1.58±0.07	1.73±0.11	0.14±0.01	0.14±0.03	0.020±0.003	0.056±0.007
Phenylalanine	0.10±0.01	0.11±0.02	0.04±0.01	0.05±0.01	0.023±0.002	0.024±0.004
Proline	0.20±0.01	0.30±0.01*	0.25±0.05	0.30±0.05	0.010±0.001	0.011±0.001
Serine	0.93±0.06	0.92±0.02	0.15±0.01	0.13±0.02	0.018±0.003	0.017±0.002
Threonine	0.32±0.06	0.54±0.04*	0.04±0.01	0.04±0.01	0.020±0.001	0.025±0.003
Tyrosine	0.23±0.02	0.21±0.01	0.23±0.02	0.21±0.01	0.022±0.003	0.024±0.001
Valine	0.23±0.03	0.25±0.02	0.20±0.05	0.25±0.06	0.086±0.010	0.075±0.008
TFAA (−Taurine)	29.7±1.2	30.8±1.5	4.98±0.52	5.23±0.98	0.75±0.05	0.81±0.05
Taurine	7.31±0.52	8.65±1.31	8.74±0.53	8.96±1.21	8.52±0.90	9.11±0.95

Values are means ± S.E.M. (*N*=4).
*Significantly different from corresponding 0 h submerged condition, $P<0.05$.
ND, not detectable.

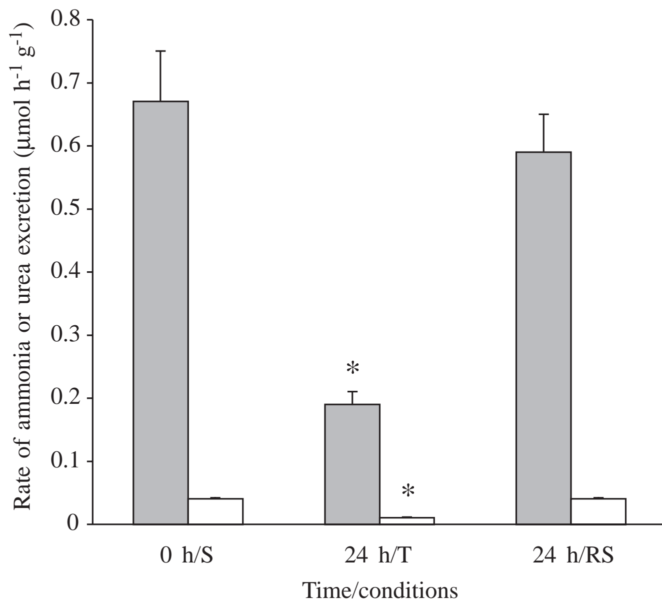

Fig. 1. Effects of a 24 h aerial exposure (24 h/T) or a 24 h resubmergence after a 24 h aerial exposure (24 h/RS), in a 12 h:12 h dark:light regime, on the rate of ammonia (shaded bars) and urea (open bars) excretion by *Periophthalmodon schlosseri.* Values are means ± S.E.M. (*N*=4). *Significantly different from 0 h submerged (0 h/S) condition, $P<0.05$.

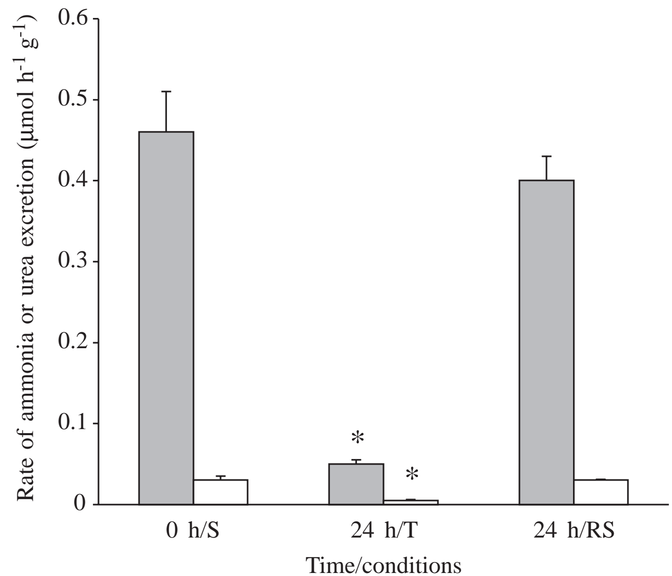

Fig. 2. Effects of a 24 h aerial exposure (24 h/T) or a 24 h resubmergence after a 24 h aerial exposure (24 h/RS), in a 12 h:12 h dark:light regime, on the rate of ammonia (shaded bars) and urea (open bars) excretion by *Boleophthalmus boddaerti.* Values are means ± S.E.M. (*N*=4). *Significantly different from 0 h submerged (0 h/S) condition, $P<0.05$.

Table 5. *Effects of 24 h (12 h:12 h dark:light regime) of submergence followed by exercise (3 min) in 50 % sea water or 3 h of aerial exposure in the light followed by exercise (3 min) on land on the concentrations of various metabolites in the muscle of* Periophthalmodon schlosseri

[Metabolite] (μmol g^{-1} wet mass)	No exercise		Exercise (3 min)	
	Submerged	3 h aerial exposure	Submerged	3 h aerial exposure
Glycogen	38.5±3.4	31.1±0.9	20.1±2.7*	29.6±5.6
Lactate	2.85±0.28	2.81±0.38	4.95±0.37*,‡	4.84±0.73*,‡
Alanine	0.42±0.18	1.33±0.13*	1.22±0.18*	2.16±0.04*,‡,§
Ammonia	0.20±0.03	0.27±0.05	0.97±0.15*,‡	1.66±0.23*,‡,§
Urea	0.30±0.04	0.26±0.04	0.40±0.04‡	0.28±0.02§
ATP	1.95±0.17	2.11±0.06	1.63±0.13	1.66±0.39
ADP	1.81±0.13	1.73±0.08	2.05±0.08‡	2.03±0.05‡
AMP	0.59±0.03	0.63±0.06	0.55±0.01	0.53±0.01
Energy charge	0.65±0.01	0.66±0.01	0.63±0.01	0.60±0.02

The energy charge has no dimensions.

Results are given as means ± S.E.M. (N=4).

*Significantly different from no exercise, submerged values, P<0.05; ‡significantly different from no exercise, 3 h aerial exposure values, P<0.05; §significantly different from exercise, submerged values, P<0.05.

Table 6. *Effects of 24 h (12 h:12 h dark:light regime) of submergence followed by exercise (3 min) in 50 % sea water or 3 h of aerial exposure in the light followed by exercise (3 min) on land on the concentrations of various metabolites in the muscle of* Boleophthalmus boddaerti

[Metabolite] (μmol g^{-1} wet mass)	No exercise		Exercise (3 min)	
	Submerged	3 h aerial exposure	Submerged	3 h aerial exposure
Glycogen	14.3±1.5	15.1±0.6	14.9±2.8	5.67±0.87*,‡,§
Lactate	2.00±0.03	2.90±0.04	2.97±0.53	4.53±0.24*,‡,§
Alanine	2.40±0.05	2.50±0.03	1.22±0.05*,‡	1.37±0.04*,‡,§
Ammonia	0.90±0.19	1.10±0.13	1.63±0.19	2.70±0.33*,‡,§,¶
Urea	0.95±0.05	0.89±0.09	0.66±0.05*,‡	0.79±0.09
ATP	2.00±0.04	2.78±0.08	0.82±0.27*,‡	0.28±0.03*,‡,§
ADP	0.98±0.20	1.10±0.15	2.05±0.24*,‡	1.78±0.09*,‡,§
AMP	0.30±0.05	0.28±0.07	0.46±0.15*,‡	0.64±0.05*,‡,§
Energy charge	0.76±0.01	0.80±0.04	0.55±0.01*,‡	0.43±0.01*,‡,§

The energy charge has no dimensions.

Values are means ± S.E.M. (N=4).

*Significantly different from no exercise, submerged values, P<0.05; ‡significantly different from no exercise, 3 h aerial exposure values, P<0.05; §significantly different from exercise, submerged values, P<0.05; ¶significantly different from the corresponding value of *P. schlosseri*, P<0.05.

were also found. For fish that were exercised on land after 3 h of aerial exposure, the muscle glycogen level remained comparable with that of the control (no exercise), although the lactate content increased. Ammonia and alanine accumulated to levels greater than those of the fish exercised in water after submergence. Exercise on land or in water exerted no effect on the ATP content and energy charge of the muscle but after 3 min of exercise following a 24 h aerial exposure, the alanine level increased to 12 μmol g^{-1} wet mass tissue (Fig. 3).

In contrast, exercise in water after submergence had no effect on the muscle glycogen, lactate or ammonia levels of *B. boddaerti*, but there were significant decreases in the muscle ATP content and energy charge (Table 6). In contrast to the *P. schlosseri* data, muscle alanine content significantly decreased in *B. boddaerti*. When *B. boddaerti* was exercised on land after 3 h, muscle glycogen decreased to one-third of the control (without exercise) value and lactate content increased significantly. Although there was a significant increase in the muscle ammonia content, the alanine content decreased significantly to approximately half the control value. Exercise on land substantially reduced muscle ATP levels in *B. boddaerti* (Table 6).

Discussion

Fish exposed on land have difficulty in maintaining ammonia excretion. Since ammonia is toxic, it is important to avoid ammonia intoxication by slowing down its accumulation

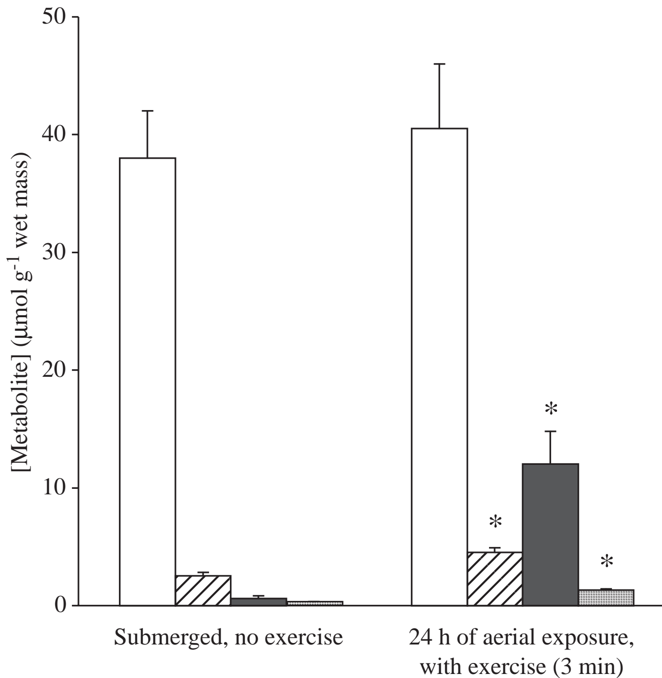

Fig. 3. Effects of 3 min of exercise on land after 24 h of aerial exposure in a 12 h:12 h dark:light regime on the contents of glycogen (open bars), lactate (hatched bars), alanine (shaded bars) and ammonia (black bars) in muscle of *Periophthalmodon schlosseri*. Values are means ± S.E.M. (N=4). *Significantly different from submerged condition, P<0.05.

in the body. This can be achieved by decreasing production (Lim et al., 2001), maintaining or enhancing excretion (Randall et al., 1999; Wilson et al., 1999) and/or by converting ammonia to less toxic compounds for storage or excretion (Ip et al., 1993).

Mudskippers (*P. schlosseri* and *B. boddaerti*) do not detoxify ammonia to urea during aerial exposure (Lim et al., 2001). Instead, they reduce proteolysis and amino acid catabolism to slow down the build-up of ammonia internally during aerial exposure in constant darkness. Under those experimental conditions, TFAA concentrations decrease in certain tissues, and there is no accumulation of alanine (Lim et al., 2001). In contrast, when *P. schlosseri* were exposed to terrestrial conditions for 24 h in a dark:light regime, alanine levels increased in the muscle, liver and plasma, and TFAA concentrations increased significantly in the muscle and plasma.

One of the primary sources of metabolic energy in carnivorous fishes is protein (Moon and Johnston, 1981). The main storage house of utilizable protein is white muscle. Amino acids released through proteolysis can either be oxidized for energy production or converted to other utilizable forms by anabolic pathways. Before an amino acid can be oxidized through the Krebs cycle, the amino group has to be removed by either transamination or deamination (Campbell, 1991). Ammonia is not produced during transamination, but deamination produces either NH_3 or NH_4^+ (Mommsen and Walsh, 1991).

Certain amino acids (e.g. arginine, glutamine, histidine and proline) can be converted to glutamate. Glutamate can undergo deamination catalyzed by glutamate dehydrogenase, producing NH_4^+ and α-ketoglutarate (Campbell, 1991). The latter is then fed into the Krebs cycle. Glutamate can also undergo transamination with pyruvate, catalyzed by alanine aminotransferase, producing α-ketoglutarate without the release of ammonia (Fig. 4). If there were a continuous supply of pyruvate, transamination would facilitate the oxidation of carbon chains of some amino acids without polluting the internal environment with ammonia.

Under normal circumstances, the carbon chain of an amino acid can be completely oxidized to carbon dioxide and water through the Krebs cycle and the electron transport chain, producing ATP and/or its equivalent. For *P. schlosseri* exposed to terrestrial conditions, the carbon chain may undergo only partial oxidation (Hochachka and Guppy, 1987). α-Ketoglutarate can be metabolized through portions of the Krebs cycle to malate, which can then be turned into pyruvate in the presence of malic enzyme (Fig. 4). This would cause a reduction in the efficiency of ATP production, because amino acids are not fully oxidized. Since *P. schlosseri* has difficulty in excreting ammonia when active on land, transamination of glutamate and pyruvate to form alanine would provide energy in the form of ATP without NH_4^+ release (Fig. 4). This would allow the utilization of certain amino acids as energy sources and, at the same time, minimize ammonia accumulation.

In fish, alanine alone constitutes 20–30 % of the total amino acid pool (Hochachka and Guppy, 1987). Most of the FAAs can be converted to alanine, and the overall quantitative energetics would appear to be quite favourable. The net

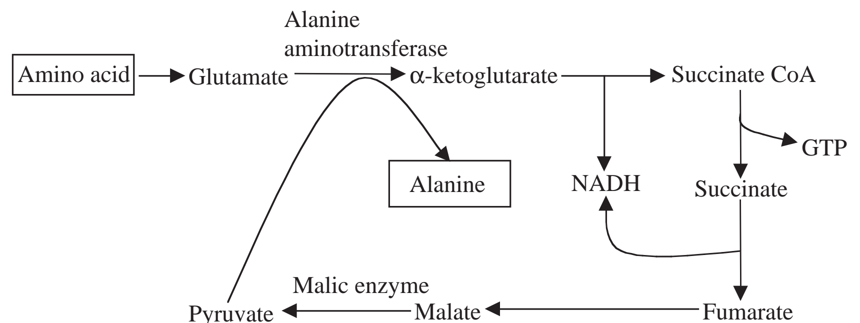

Fig. 4. Proposed pathway of partial catabolism of certain amino acids, producing alanine without releasing ammonia, in *Periophthalmodon schlosseri* when it is active on land.

conversion of glutamate to alanine would yield 10 moles of ATP per mole of alanine formed. This favourable ATP yield from amino acid catabolism is not accompanied by a net release of ammonia. Such a system would, therefore, be advantageous to *P. schlosseri* when it is active on land and confronted with the problem of ammonia excretion.

Increases in the concentrations of some essential amino acids (e.g. isoleucine, leucine, proline, serine, lysine and valine) were found in the tissues of *P. schlosseri* exposed to terrestrial conditions under a dark:light regime. Since specimens were not fed during the experiment, this might indicate the mobilization of amino acids through proteolysis under such experimental conditions. Such a phenomenon was not observed in *B. boddaerti* exposed to similar conditions.

To correlate alanine accumulation with locomotory activities in *P. schlosseri*, specimens were forced to exercise under specific experimental conditions. Exercise in water led to a decrease in glycogen content, and increases in the levels of lactate, ammonia and alanine in the muscle. It can be deduced that both glycogen and amino acids were mobilized in this situation. In contrast, when *P. schlosseri* was forced to exercise on land after 3 h exposure to aerial conditions, there was no change in the glycogen level despite an increase in the lactate content of the muscle. Muscle ammonia and alanine levels also increased, with ammonia accumulating to a level twice that of the fish exercised in water. It was noted that the excess amount of alanine that accumulated in the exercised fish under both submerged (1.22–0.42=0.80 μmol g^{-1}) and terrestrial (2.16–1.33=0.83 μmol g^{-1}) conditions were comparable. We therefore suspected that the efficiency of alanine production through partial amino acid catabolism was dependent on the period of aerial exposure. Indeed, for *P. schlosseri* forced to exercise after exposure to terrestrial conditions for 24 h, there was no change in glycogen level and no further accumulation of ammonia. However, muscle alanine content increased fourfold, and the amount accumulated (11.99–3.21=8.78 μmol g^{-1}) was much higher than the amount found in fish exercised in water. Taken together, these results lend further support to the proposition that *P. schlosseri* uses protein and amino acids to support locomotory activities on land. This would reduce its dependence on carbohydrate and spare the glycogen store. This shift in the metabolic pathway could, therefore, sustain the higher metabolic rate of *P. schlosseri* on land (as observed by Kok et al., 1998). Indeed, there was no significant change in the muscle ATP content and energy charge after the fish was forced to exercise on land or in water.

When *P. schlosseri* are exposed to terrestrial conditions, partial amino acid catabolism of certain amino acids is apparently coupled with a reduction in the rate of amino acid catabolism in general. Ip et al. (Ip et al., 1993) overlooked this phenomenon as they did not examine the reduction in ammonia and urea excretion rates in this mudskipper when exposed to terrestrial conditions. An examination of the balance between nitrogenous excretion and nitrogenous accumulation in a 70 g *P. schlosseri* (Table 7) reveals that degradation of amino acids in general was likely to be suppressed to slow down the build-up of endogenous ammonia. There was a deficit of −761 (−913+152) μmol N between nitrogenous excretion and nitrogenous accumulation. However, increases in TFAA concentrations in the muscle and plasma of these experimental fish indicate that proteolysis might not have been reduced as in the case of fish exposed to terrestrial condition in constant darkness (Lim et al., 2001). The absence of any significant accumulation of alanine, glutamine or glutamate in the muscle of *B. boddaerti* exposed to terrestrial conditions in a dark:light regime suggests that it did not rely on protein as an energy source during aerial exposure. The activities of alanine aminotransferase in the muscle and liver of this mudskipper are indeed significantly lower than the respective values for *P. schlosseri* (Y. K. Ip and S. F. Chew, unpublished results). However, *B. boddaerti* was also capable of reducing the rate of amino acid catabolism when exposed to terrestrial condition (Table 8). Consequently, the decrease in ammonia excretion during aerial exposure led to only a small increase in ammonia in its tissues. Glycogen levels in the muscle of *B. boddaerti* decreased significantly after 24 h of aerial exposure followed by exercise. Thus, unlike *P. schlosseri*, *B. boddaerti* used glycogen as a metabolic fuel to support activity on land. This strategy offers a limited amount of energy for a short period, and, as a consequence, the muscle ATP content decreased to 1/10 of the control value. Together with the high levels of ammonia that accumulated in the muscle of the exercised specimens, these results suggest that it is highly unfavourable for *B. boddaerti* to stay away from water for long periods.

When *P. schlosseri* and *B. boddaerti* are confronted with an ammonia-loading situation, TFAA concentrations in the brain, liver and muscle of the experimental fish increase, largely due to increases in glutamine levels (Peng et al., 1998). In contrast

Table 7. *A balance sheet of nitrogenous accumulation and excretion in a 70 g* Periophthalmodon schlosseri *exposed to submerged or terrestrial conditions (12 h:12 h dark:light regime)*

	Nitrogen excretion/accumulation (μmol)		
	Submerged	Aerial	Difference
Excreted from 70 g fish			
Ammonia	1127	321	−806
Urea	138	30.6	−107
Reduction in nitrogenous excretion			−913
Retained in muscle (42 g)			
Ammonia	31.9	64.9	+32.8
Urea	26.0	34.4	+8.40
Amino acids	44.9	135	+89.9
Retained in liver (2 g)			
Ammonia	2.68	4.98	+2.30
Urea	0.92	2.36	+1.44
Amino acids	20.5	38.0	+17.5
Increase in nitrogenous accumulation			+152

Table 8. *A balance sheet of nitrogenous accumulation and excretion in a 7 g* Boleophthalmus boddaerti *exposed to submerged or terrestrial conditions (12 h:12 h dark:light regime)*

	Nitrogen excretion/accumulation (μmol)		
	Submerged	Aerial	Difference
Excreted from 7 g fish			
Ammonia	76.6	8.06	−68.5
Urea	10.4	1.04	−9.07
Reduction in nitrogenous excretion			−78
Retained in muscle (4.2 g)			
Ammonia	3.78	9.28	+5.50
Urea	NS	NS	NS
Amino acids	NS	NS	NS
Retained in liver (0.2 g)			
Ammonia	NS	NS	NS
Urea	NS	NS	NS
Amino acids	0.03	0.044	+0.014
Increase in nitrogenous accumulation			+6

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

to the production of alanine, the formation of glutamine is energetically expensive. One mole of ATP is required for the production of every amide group of glutamine *via* glutamine synthetase. If the starting point is ammonia and α-ketoglutarate, every mole of ammonia detoxified would result in the hydrolysis of 2 moles of ATP-equivalents. Hence, *P. schlosseri* apparently varies its dependence on these two biochemical pathways (alanine formation or glutamine formation) to permit activity during aerial exposure and ammonia loading. To sustain activity on land, partial amino acid catabolism becomes the main metabolic pathway, that is forming alanine and reducing ammonia production. When exposed to ammonia, the penetrating NH_3 is preferentially detoxified to glutamine. How these two biochemical pathways are regulated is not clear.

For fishes that have difficulty in excreting internally produced ammonia, partial catabolism of certain amino acids, leading to the formation of alanine, coupled with a slowing of amino acid catabolism in general, would be the most cost-effective way to minimize endogenous ammonia build-up. It allows amino acids to be used as an energy source during adverse conditions without polluting the internal environment. The presence of similar phenomena in fishes, especially the obligatory air-breathers, should be sought to establish whether this is a widely adopted biochemical strategy to avoid ammonia intoxication during aerial exposure.

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