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Urea synthesis in the African lungfish *Protopterus dolloi* – hepatic carbamoyl phosphate synthetase III and glutamine synthetase are upregulated by 6 days of aerial exposure

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Summary

Like the marine ray *Taeniura lymma*, the African lungfish *Protopterus dolloi* possesses carbamoyl phosphate III (CPS III) in the liver and not carbamoyl phosphate I (CPS I), as in the mouse *Mus musculus* or as in other African lungfish reported elsewhere. However, similar to other African lungfish and tetrapods, hepatic arginase of *P. dolloi* is present mainly in the cytosol. Glutamine synthetase activity is present in both the mitochondrial and cytosolic fractions of the liver of *P. dolloi*. Therefore, we conclude that *P. dolloi* is a more primitive extant lungfish, which is intermediate between aquatic fish and terrestrial tetrapods, and represents a link in the fish–tetrapod continuum. During 6 days of aerial exposure, the ammonia excretion rate in *P. dolloi* decreased significantly to 8–16% of the submerged control. However, there were no significant increases in ammonia contents in the muscle, liver or plasma of specimens exposed to air for 6 days. These results suggest that (1) endogenous ammonia production was drastically reduced and (2) endogenous ammonia was detoxified effectively into urea. Indeed, there were significant decreases in glutamate, glutamine and lysine levels in the livers of fish exposed to air, which led to a decrease in the total free amino acid content. This indirectly confirms that the specimen had reduced its rates of proteolysis and/or amino acid catabolism to suppress endogenous ammonia production. Simultaneously, there were significant increases in urea levels in the muscle (8-fold), liver (10.5-fold) and plasma (12.6-fold) of specimens exposed to air for 6 days. Furthermore, there was an increase in the hepatic ornithine–urea cycle (OUC) capacity, with significant increases in the activities of CPS III (3.8-fold), argininosuccinate synthetase + lyase (1.8-fold) and, more importantly, glutamine synthetase (2.2-fold). This is the first report on the upregulation of OUC capacity and urea synthesis rate in an African lungfish exposed to air. Upon re-immersion, the urea excretion rate increased 22-fold compared with that of the control specimen, which is the greatest increase among fish during emersion–immersion transitions and suggests that *P. dolloi* possesses transporters that facilitate the excretion of urea in water.

Key words: amino acid, ammonia, arginase, carbamoyl phosphate synthetase, dipnoan, lungfish, glutamine synthetase, ornithine–urea cycle, *Protopterus dolloi*, urea, urea transporter.

Introduction

The fish–tetrapod transition represents one of the greatest events in vertebrate evolution. Air breathing evolved in fish (e.g. lungfish), but prolonged terrestrial respiration is a tetrapod feature. Similarly, limbs with strong skeletal units appeared in sarcopterygian fish, but the loss of fin rays and the appearance of digits are features of tetrapods (Forey, 1986). Lungfish (or dipnoans, as they are 'dual breathers') are an archaic group of fish characterized by the possession of a lung opening off the ventral side of the oesophagus. They depend entirely on aerial respiration and can live for an extended period out of water. There are few similarities between lungfish and tetrapods, particularly amphibians, in aspects of gas exchange and excretory physiology, pulmonary circulation and heart structure (Forey et al., 1991; Schultze, 1994).

Unlike their Australian (*Neoceratodus forsteri*) and South American (*Lepidosiren paradoxa*) counterparts, the African lungfish *Protopterus aethiopicus* and *Protopterus annectens* can aestivate in subterranean mud cocoons for long periods of time (Smith, 1935; Janssens and Cohen, 1968a,b). On land, there would be a lack of water to flush the branchial and cutaneous surfaces, impeding the excretion of ammonia and consequently leading to the accumulation of ammonia in the

body. Ammonia is toxic (Ip et al., 2001a), and therefore African lungfish have to avoid ammonia intoxication when out of water. Previous work on *P. aethiopicus* and *P. annectens* revealed that they are ureogenic (Janssens and Cohen, 1966; Mommsen and Walsh, 1989). Similar to tetrapods, they possess mitochondrial carbamoyl phosphate synthetase I (CPS I), which utilizes NH_4^+ as a substrate, and an arginase that is present mainly in the cytosol of the liver (Mommsen and Walsh, 1989). By contrast, coelacanths, marine elasmobranchs and some teleosts are known to have carbamoyl phosphate synthetase III (CPS III; Mommsen and Walsh, 1989; Anderson, 1980; Randall et al., 1989), which utilizes glutamine as a substrate, and arginase in the hepatic mitochondria. It was suspected that the replacement of CPS III with CPS I, and that of mitochondrial arginase with cytosolic arginase, occurred before the evolution of the lungfish (Mommsen and Walsh, 1989).

Found in Central Africa in the lower and middle Congo River Basins, the slender lungfish *Protopterus dolloi* aestivates on land within a dry layer of mucus (Brien, 1959; Poll, 1961) instead of in a cocoon inside the mud like *P. aethiopicus* and *P. annectens.* It is likely that African lungfish evolved through a sequence of events, i.e. air breathing, migration to land and then burrowing into mud. Aestivation could occur on land or in mud, but the latter must have certain advantages, such as predator avoidance, over the former. This led us to suspect that burrowing into the mud could be a more advanced development during evolution and to hypothesize that *P. dolloi* might be a more-primitive extant lungfish that bore some of the characteristics and traits of its piscine ancestors. Therefore, the first objective of this study was to determine what type of CPS was present in the liver and to elucidate the compartmentation of hepatic arginase in *P. dolloi*. Furthermore, it has been reported that CPS III is present in extra-hepatic tissues of some teleosts (see review by Anderson, 2001), but no such information is available for lungfish. Hence, attempts were made to examine whether the muscle or gut of *P. dolloi* would also have CPS I or CPS III activity.

To date, no glutamine synthetase (GS) activity has been detected in the liver of African lungfish (Campbell and Anderson, 1991), probably because the African lungfish examined possess CPS I and not CPS III. If indeed *P. dolloi* possessed CPS III, then it would be essential for it to have GS in the hepatic mitochondria to supply the glutamine needed for urea synthesis *de novo*. Hence, the second objective of this study was to verify whether GS activity was present in the liver of *P. dolloi*.

During 78–129 days of aestivation out of water, *P. aethiopicus* accumulates urea in its body (Janssens and Cohen, 1968a). However, it was reported that urea accumulation did not involve an increased rate of urea synthesis (Janssens and Cohen, 1968a), even though the animals appear to be in continuous gluconeogenesis throughout aestivation (Janssens and Cohen, 1968b). This apparent controversy arose because of two counteracting factors: (1) increase in the rate of urea production and (2) decrease in the rate of ammonia production. During the initial phase of aerial exposure before the onset of a reduction in the rate of ammonia production, the rate of urea synthesis *de novo* theoretically has to be increased to detoxify ammonia that is produced at a normal (or slightly sub-normal) rate and cannot be excreted. After entering into aestivation for a relatively long period, ammonia production rate would have been suppressed (Smith, 1935; Janssens, 1964). This would subsequently result in a decrease in the rate of urea synthesis *de novo*, leading to those observations made in previous studies (Janssens and Cohen, 1968a,b). Therefore, the third objective of the present study was to elucidate whether there was actually a large increase in the rate of urea production in *P. dolloi* and whether increases in the hepatic CPS III and GS activities would occur during 6 days of aerial exposure without undergoing aestivation.

Detoxification of ammonia to urea does not appear to be a common strategy adopted by adult teleosts exposed to air, probably because it is energy intensive, having a stoichiometry of 4 moles (*via* CPS I) to 5 moles (*via* CPS III) of ATP per mole of urea (Ip et al., 2001a). Instead, several tropical teleosts accumulate alanine [e.g. mudskipper (*Periophthalmodon schlosseri*), Ip et al., 2001c; snakehead (*Channa asiatica*), Chew et al., 2003] and glutamine [e.g. marble goby (*Oxyeleotris marmoratus*), Jow et al., 1999; sleeper (*Bostrichthyes sinensis*), Ip et al., 2001b; mangrove killifish (*Rivulus marmoratus*), Frick and Wright, 2002; swamp eel (*Monopterus albus*), Tay et al., 2003]. The formation of glutamine from glutamate (only 1 mole of ATP per mole of ammonia detoxified) is also energy dependent and appears to be adopted mainly by teleosts that remain completely quiescent on land. By contrast, alanine formation through the partial catabolism of certain amino acids would lead to the production of ATP, which can support locomotory activities on land (Ip et al., 2001b) without releasing ammonia (Ip et al., 2001a). Unlike amphibious teleosts (e.g. *P. schlosseri*), *P. dolloi* is completely inactive on land. Therefore, we hypothesized that *P. dolloi* would not adopt the strategy of partial amino acid catabolism and would not accumulate alanine when exposed to air. Also, we hypothesized that it would not accumulate glutamine during aerial exposure, because glutamine is channelled into the OUC for urea synthesis *via* CPS III.

It has been suggested that suppression of proteolysis and/or amino acid catabolism may be a fundamental strategy adopted by some tropical teleost fish to decrease endogenous ammonia production during aerial exposure (Lim et al., 2001; Ip et al., 2001b,c; Chew et al., 2001). Since *P. dolloi* can tolerate aerial exposure better than any teleost, the fourth objective of this study was to verify that *P. dolloi* could indeed suppress ammonia production on land even before aestivation took place.

Materials and methods

Collection and maintenance of specimens

P. dolloi (Boulenger 1900), weighing 100–150 g body mass

and being collected from Nigeria, Africa, were imported through a local fish farm in Singapore. Identification of the specimens was performed according to Poll (1961). Specimens were maintained in plastic aquaria filled with dechlorinated water, containing 2.3 mmol l^{-1} Na^+, 0.54 mmol l^{-1} K^+, 0.95 mmol l^{-1} Ca^{2+}, 0.08 mmol l^{-1} Mg^{2+}, 3.4 mmol l^{-1} Cl^- and 0.6 mmol l^{-1} HCO_3^-, at pH 7.0 and at 25°C in the laboratory. Water was changed daily. No attempt was made to separate the sexes. The specimens were acclimated to laboratory conditions for at least one month. During the adaptation period, specimens were fed frozen blood worms. Food was withdrawn 48 h prior to experiments, which gave sufficient time for the gut to be emptied of all food and waste. All experiments performed in this study were under a 12 h:12 h light:dark regime unless stated otherwise.

Verification of the presence of CPS III and GS

The liver, muscle and gut of *P. dolloi* in the control condition were excised quickly and homogenized in 5 volumes (w/v) of ice-cold extraction buffer containing 50 mmol l^{-1} Hepes (pH 7.6), 50 mmol l^{-1} KCl and 0.5 mmol l^{-1} EDTA. The homogenate was sonicated (110 W, 20 kHz; Misonix Incorporated, Farmingdale, NY, USA) three times for 20 s each, with a 10 s break between each sonication. The sonicated sample was centrifuged at 10 000 ***g*** and 4°C for 15 min. After centrifugation, the supernatant was passed through a Bio-Rad P-6DG column (Bio-Rad Laboratories, Hercules, CA, USA) equilibrated with the extraction buffer without EDTA. The filtrate obtained was used directly for enzyme assay. Preliminary results indicated that OUC enzymes were present only in the liver of *P. dolloi*. For comparison, the livers excised from the marine blue-spotted fantailed ray *Taeniura lymma* (obtained from the local wet market) and the mouse *Mus musculus* (obtained through the Animal Holding Unit of the National University of Singapore) were processed at the same time with those from *P. dolloi* and the CPS activities assayed by the same batch of chemicals.

CPS (E.C. 2.7.2.5) activity was determined according to the method of Anderson and Walsh (1995), as applied to the mudskipper (Lim et al., 2001). Radioactivity was measured using a Wallac 1414 liquid scintillation counter (Wallac Oy, Turku, Finland). Enzyme activity was expressed as μmol [^{14}C]urea formed min^{-1} g^{-1} wet mass.

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

Argininosuccinate synthetase (E.C. 6.3.4.5) and lyase (E.C. 4.3.2.1) (ASS + L) 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. ASS + L activity was expressed as μmol [^{14}C]fumarate formed min^{-1} g^{-1} wet mass.

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

GS (E.C. 6.3.1.2) was assayed as transferase activity according to the method of Shankar and Anderson (1985). Its activity was expressed as μmol γ-glutamylhydroxymate formed min^{-1} g^{-1} wet mass.

Cellular fractionation of liver from *P. dolloi* was performed according to the methods of Anderson et al. (2002). Lactate dehydrogenase and cytochrome *c* oxidase were used as markers for cytosol and mitochondria, respectively.

Evaluation of the effects of 6 days aerial exposure on nitrogenous excretion and accumulation

Specimens were immersed individually in 2 litres of water at 25°C with aeration in separate plastic tanks (20.5 cm×14.5 cm×6 cm, length × width × height). 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, 3 ml of 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 water. After 24 h, the fish were sprayed thoroughly with water. The water collected was used for ammonia and urea analyses. The process was repeated for 6 days. The disturbance created by the daily collection and introduction of water prevented the experimental subject from initiating aestivation during this period. After 6 days of aerial exposure, specimens were re-immersed in water for 24 h to study the rates of ammonia and urea excretion upon recovery. A separate group of fish submerged in water for the same period of time served as the control. Ammonia and urea in water samples were determined according to the methods of Jow et al. (1999).

A preliminary study was performed to demonstrate that the rates of ammonia and urea excretion were not affected by bacterial actions. Small volumes (200 ml) of the external medium in which the control fish had been exposed for 24 h were set aside at 25°C. Water samples were collected 24 h later. The concentrations of ammonia and urea before and after this 24-h period of incubation were compared and were confirmed not to be significantly different from each other.

At the end of 6 days, specimens were killed with a strong blow to the head. The lateral muscle and liver were quickly excised. The excised tissues and organs were immediately freeze-clamped in liquid nitrogen with pre-cooled tongs. Frozen samples were kept at –80°C. A separate group of fish exposed to similar conditions was used for the collection of blood samples. The blood was collected in heparinized capillary tubes by caudal puncture. 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% trichloroacetic acid (TCA) and centrifuged at 10 000 ***g*** at 4°C for 15 min. The resulting supernatant was kept at –80°C until analyzed.

The frozen samples were weighed, ground to a powder in liquid nitrogen and homogenized three times in 5 volumes (w/v) of 6% TCA at 24 000 revs min^{-1} for 20 s each using an Ultra-Turrax homogenizer 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 obtained was kept at –80°C until analyzed.

For ammonia analysis, the pH of the deproteinized sample was adjusted to between 5.5 and 6.0 with 2 mol l^{-1} $KHCO_3$. The ammonia content was determined using the method of Bergmeyer and Beutler (1985). The reaction mixture, in a total volume of 1.55 ml, consisted of 115 mmol l^{-1} triethanolamine-HCl (pH 8.0), 11 mmol l^{-1} α-ketoglutarate, 0.56 mmol l^{-1} ADP, 0.19 mmol l^{-1} reduced NADH, 7.4 i.u. ml^{-1} glutamate dehydrogenase (Sigma Chemical Co., St Louis, MO, USA) and an aliquot part of sample. Glutamate dehydrogenase was added last to initiate the reaction. The change in absorbance at 25°C and 340 nm was monitored using a Shimadzu UV-160A spectrophotometer. Freshly prepared NH_4Cl solution was used as the standard for comparison.

The urea content in 0.2 ml of the neutralized sample was analyzed colorimetrically according to the method of Anderson and Little (1986), as modified by Jow et al. (1999). The difference in absorbance obtained from the sample in the presence and absence of urease was used for the estimation of urea content. Urea (Sigma Chemical Co.) was used as a standard for comparison. Results were expressed as μmol g^{-1} wet mass tissue or mmol l^{-1} plasma.

For FAA analysis, 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). FAAs were analyzed using a Shimadzu LC-6A amino acid analysis system with a Shim-pack ISC-07/S1504 Li-type column. Results for FAA analyses were expressed as μmol g^{-1} wet mass or mmol l^{-1} plasma.

Elucidation of whether the OUC capacity would be enhanced by aerial exposure

Specimens were exposed to the control (immersed) or terrestrial conditions individually in plastic aquaria as described above. OUC enzyme and GS activity in the liver was assayed according to the above-mentioned methods.

Statistical analyses

Results were presented as means ± S.E.M. Student's *t*-test and one-way analysis of variance (ANOVA) followed by Student–Newman–Keuls multiple range test were used to evaluate differences between means where applicable. Differences were regarded as statistically significant at $P<0.05$.

Results

OUC enzymes

A full complement of OUC enzymes was present in the liver of *P. dolloi* (Table 1). The *N*-acetylglutamate (AGA)-dependent CPS present in *P. dolloi* had characteristics similar to that in the blue-spotted fan-tail ray *T. lymma* but was dissimilar to that in the mouse *M. musculus* (Table 2). It could utilize glutamine as a substrate, was activated by AGA and was refractory to UTP inhibition. No CPS III activity was detectable (detection limit = 0.001 μmol min^{-1} g^{-1}) from the muscle or the gut of *P. dolloi*. GS activity was also detected in the liver (Table 1) but not the muscle.

Only 0.8% of lactate dehydrogenase activity was present in the mitochondrial fraction, indicating that mitochondria were quite free of cytosolic components. Eighty-five percent of the cytochrome *c* oxidase activity was present in the mitochondrial fraction, with 12% present in the nuclear fraction, indicating that the mitochondrial fraction represented a high percentage of the mitochondrial enzymes. CPS III was present exclusively in the liver mitochondria, but 90.8±4.6% (N=5) of the arginase

Table 1. *Effects of 6 days of exposure to terrestrial conditions on the activities of glutamine synthetase (GS), carbamoyl phosphate synthetase III (CPS III), ornithine transcarbamoylase (OTC), arginosuccinate synthetase + lyase (ASS + L) and arginase in the liver of* Protopterus dolloi

	Activity (μmol min^{-1} g^{-1} wet mass)	
Enzymes	Immersed	Terrestrial
GS	0.16±0.01	0.36±0.04*
CPS III (glutamine + AGA + UTP)	0.27±0.04	1.04±0.23*
OTC	9.86±1.33	16.2±2.85
ASS + L	0.47±0.07	0.87±0.15*
Arginase	143±36	250±51

Results represent means ± S.E.M. (N=5).
AGA, *N*-acetyl-L-glutamate; UTP, uridine triphosphate.
*Significantly different from the fish immersed in water ($P<0.05$).

Table 2. *Activities of carbamoyl phosphate synthetase (CPS) in the presence of various substrates and effectors from the livers of* Mus musculus *(mouse),* Protopterus dolloi *(lungfish) and* Taeniura lymma *(stingray)*

	CPS activity (μmol min^{-1} g^{-1} wet mass)		
Substrate and effectors	*M. musculus*	*P. dolloi*	*T. lymma*
NH_4Cl	n.d.	0.007±0.03	0.008±0.007
NH_4Cl+AGA	4.01±0.43	0.035±0.009*	0.09±0.02*
NH_4Cl+AGA+UTP	3.94±0.25	0.028±0.009*	0.06±0.02*
Glutamine	n.d.	n.d.	0.23±0.04
Glutamine + AGA	n.d.	0.28±0.04	0.63±0.10†
Glutamine + AGA + UTP	n.d.	0.27±0.04	0.60±0.11†

Results represent means+S.E.M. (N=5)
AGA, *N*-acetyl-L-glutamate; UTP, uridine triphosphate; n.d.; not-detectable (detection limit = 0.001 μmol min^{-1} g^{-1} wet mass).
*Significantly different from *M. musculus* ($P<0.05$); †significantly different from *P. dolloi* ($P<0.05$).

was present in the cytosol. GS activity was detected in both the mitochondrial (0.042±0.006 μmol min^{-1} mg^{-1} protein; *N*=4) and cytosolic (0.018±0.003 μmol min^{-1} mg^{-1} protein; *N*=4) fractions from the liver of *P. dolloi*.

Aerial exposure led to significant increases in the activities of CPS III (3.8-fold), ASS + L (1.8-fold) and GS (2.2-fold) in *P. dolloi* (Table 1).

Rates of ammonia and urea excretion

Aerial exposure significantly decreased the rate of ammonia excretion in *P. dolloi* (Fig. 1). During the 6 days of aerial exposure, the rate of ammonia excretion was approximately 8–16% of the control (immersed) value. Upon re-immersion, the ammonia excretion rate was still significantly lower than that of the immersed control (Fig. 1). The urea excretion rate was not affected during the first 3 days of aerial exposure (Fig. 2). However, there was a 3-fold and 2.8-fold increase in the rate of urea excretion on day 4 and day 5, respectively. Upon re-immersion after 6 days of aerial exposure, there was a 22-fold increase in the urea excretion rate (Fig. 2).

Ammonia and urea content in the tissues

There was no significant change in the ammonia content in the muscle, liver or plasma of *P. dolloi* exposed to terrestrial conditions for 6 days (Table 3). However, the urea content in the muscle, liver and plasma increased by 8-, 10.5- and 12.6-fold, respectively (Table 3).

FAAs in the tissues

There was no significant change in the contents of FAAs and total FFA (TFAA) in the muscle of fish exposed to terrestrial conditions for 6 days (Table 4). However, there were significant decreases in the glutamate, glutamine and lysine levels in the liver of these experimental specimens (Table 5). In addition, the TFAA in the liver decreased significantly. There were slight increases in the concentrations of leucine and tryptophan, but a slight decrease in the concentration of threonine, in the plasma (Table 6). Six days of aerial exposure had no significant effect on the concentration of TFAA in the plasma of *P. dolloi*.

Discussion

P. dolloi *possesses CPS III and GS – significance in biochemical evolution*

The relative systematic positions of the lungfish and the coelacanth (the only living

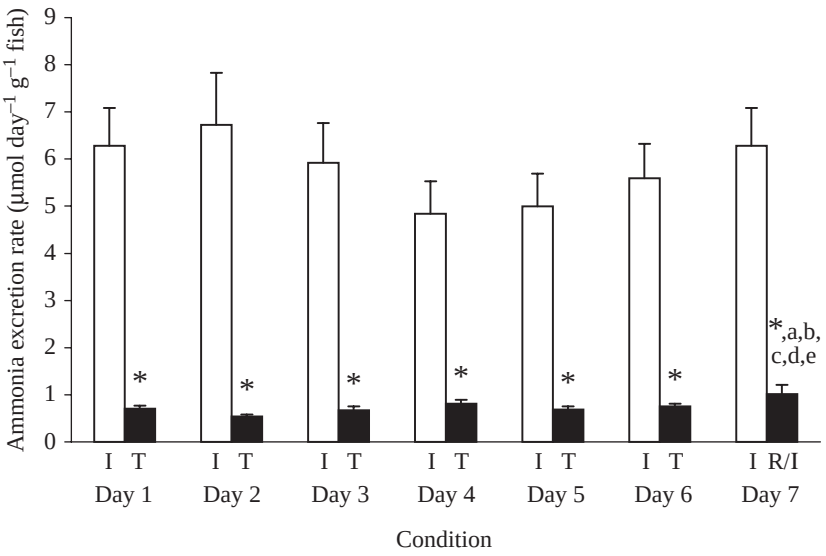

Fig. 1. Effects of exposure to terrestrial conditions on the rate of ammonia excretion of *Protopterus dolloi*. Values are means ± S.E.M. I, immersed (*N*=9); T, terrestrial (*N*=15); I/S, re-immersed (*N*=5). *Significantly different from the corresponding immersed condition ($P<0.05$); [a]significantly different from the corresponding Day 1 condition ($P<0.05$); [b]significantly different from the corresponding Day 2 condition ($P<0.05$); [c]significantly different from the corresponding Day 3 condition ($P<0.05$); [d]significantly different from the corresponding Day 4 condition ($P<0.05$); [e]significantly different from the corresponding Day 5 condition ($P<0.05$).

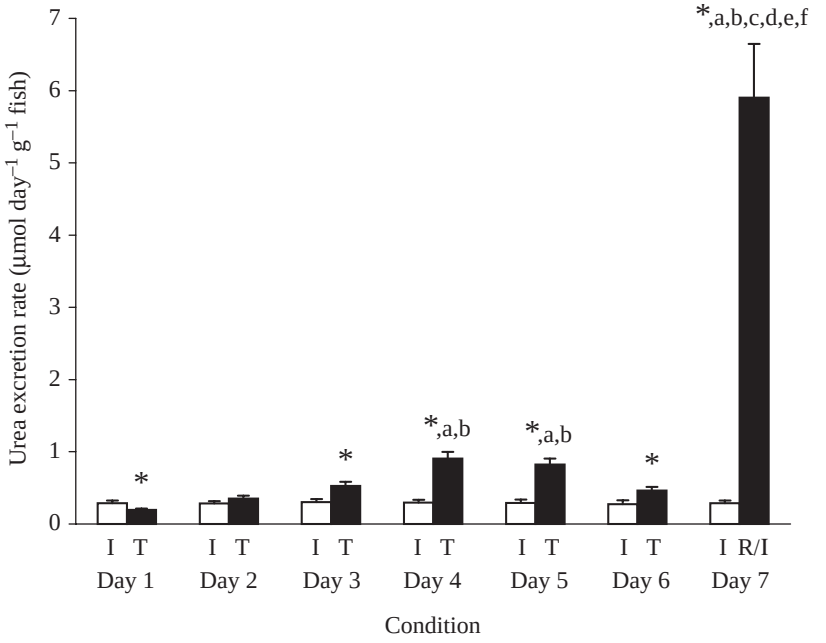

Fig. 2. Effects of exposure to terrestrial conditions on the rate of urea excretion of *Protopterus dolloi*. Values are means ± S.E.M. I, immersed (*N*=8); T, terrestrial (*N*=15); R/I, re-immersed (*N*=5). *Significantly different from the corresponding immersed condition ($P<0.05$); [a]significantly different from the corresponding Day 1 condition ($P<0.05$); [b]significantly different from the corresponding Day 2 condition ($P<0.05$); [c]significantly different from the corresponding Day 3 condition ($P<0.05$); [d]significantly different from the corresponding Day 4 condition ($P<0.05$); [e]significantly different from the corresponding Day 5 condition ($P<0.05$); [f]significantly different from the corresponding Day 6 condition ($P<0.05$).

Table 3. *Effects of 6 days of exposure to terrestrial conditions on the contents of ammonia and urea in the muscle, liver and plasma of* Protopterus dolloi

	Ammonia		Urea	
Tissue	Immersed	Terrestrial	Immersed	Terrestrial
Muscle (μmol g^{-1} wet mass)	0.671±0.107 (6)	0.988±0.079 (6)	2.25±0.89 (5)	18.6±1.4 (6)*
Liver (μmol g^{-1} wet mass)	2.59±0.349 (5)	2.70±0.16 (5)	1.83±0.63 (6)	19.2±2.6 (6)*
Blood plasma (mmol l^{-1})	0.163±0.015 (6)	0.173±0.008 (5)	2.58±0.40 (4)	32.7±8.4 (5)*

Results represent means ± S.E.M., with the number of determinations (*N*) in parentheses.
*Significantly different from the corresponding immersed condition (P<0.05).

representative of the crossopterygians) have been under debate for a long time (Forey et al., 1991; Schultze, 1994). They both belong to the subclass Sarcopterygii, or lobe-finned fish, are ureogenic and possess a functional OUC in the liver. Coelacanths, like other fish (elasmobranchs and some teleosts), possess a glutamine-dependent CPS III and an arginase in the hepatic mitochondria (Mommsen and Walsh, 1991). By contrast, the lungfish *P. aethiopicus* and *P. annectens*, like tetrapods, possess a mitochondrial ammonia-dependent CPS I and a cytosolic arginase in the liver (Mommsen and Walsh, 1989, 1991). Therefore, the water–land transition in vertebrates apparently involved the replacement of CPS III with CPS I in the mitochondria and the replacement of a mitochondrial arginase with a cytosolic enzyme in the liver. With respect to these two biochemical traits, lungfish appear to lead the way towards the evolution of other vertebrate groups and it has been accepted that these changes occurred before the evolution of the extant lungfish (Mommsen and Walsh, 1989).

Urea synthesis in *P. dolloi* involved mainly the OUC, as indicated by the presence of the full complement of OUC enzymes in the liver. Unlike *P. aethiopicus* and *P. annectens*, which possess CPS I (Janssens and Cohen, 1966; Mommsen and Walsh, 1989), the CPS activity from the liver of *P. dolloi*

Table 4. *Effects of 6 days of exposure to terrestrial conditions on the content of various free amino acids (FAAs) and total FAA (TFAA) in the muscle of* Protopterus dolloi

	Content (μmol g^{-1} wet mass)	
FAA	Immersed	Terrestrial
Alanine	0.153±0.035	0.214±0.026
Anserine	6.73±0.57	5.16±1.16
Arginine	0.070±0.007	0.054±0.008
Aspartate	0.097±0.012	0.113±0.026
Asparagine	0.053±0.004	0.044±0.004
β-alanine	0.055±0.009	0.043±0.003
Glutamate	0.394±0.087	0.209±0.031
Glutamine	0.465±0.101	0.352±0.034
Glycine	1.20±0.42	0.372±0.068
Histidine	0.102±0.008	0.08±0.01
Isoleucine	0.079±0.008	0.081±0.005
Leucine	0.148±0.013	0.152±0.012
Lysine	0.146±0.024	0.069±0.015
Proline	0.068±0.011	0.088±0.011
Phenylalanine	0.064±0.010	0.074±0.003
Serine	0.161±0.015	0.188±0.012
Taurine	0.365±0.052	0.569±0.100
Threonine	0.283±0.103	0.136±0.005
Tryptophan	0.121±0.028	0.188±0.016
Tyrosine	0.237±0.067	0.214±0.044
Valine	0.115±0.010	0.139±0.014
TFAA	11.1±1.1	8.53±1.40

Values are means ± S.E.M. (*N*=4).

Table 5. *Effects of 6 days of exposure to terrestrial conditions on the contentsof various free amino acids (FAAs) and total FAA (TFAA) in the liver of* Protopterus dolloi

	Content (μmol g^{-1} wet mass)	
FAA	Immersed	Terrestrial
Alanine	0.127±0.048	0.092±0.012
Anserine	0.009±0.003	0.003±0.001 (3)
Arginine	0.005±0.001	0.004±0.001
Aspartate	0.670±0.153	0.545±0.114
Asparagine	n.d.	n.d.
β-alanine	0.012±0.002	0.009±0.001
Glutamate	3.78±0.44	1.45±0.20*
Glutamine	0.587±0.049	0.104±0.013*
Glycine	0.195±0.024 (3)	0.342±0.048
Histidine	0.086±0.007	0.064±0.022
Isoleucine	0.042±0.006	0.034±0.003
Leucine	0.113±0.010	0.086±0.007
Lysine	0.085±0.012	0.025±0.008*
Proline	0.101±0.030	0.081±0.009
Phenylalanine	0.047±0.010	0.034±0.006
Serine	0.138±0.028	0.218±0.056
Taurine	1.51±0.32	0.961±0.187
Threonine	0.722±0.301	0.113±0.010
Tryptophan	0.079±0.020	0.073±0.013
Tyrosine	0.066±0.006	0.055±0.010
Valine	0.092±0.016	0.084±0.004
TFAA	8.69±1.21 (3)	3.22±1.35 (3)*

Values are means ± S.E.M. *N*=4, unless otherwise stated in parentheses.
*Significantly different from the corresponding immersed condition (P<0.05).
n.d.; not detectable (detection limit = 0.01 μmol g^{-1} wet mass).

had characteristics comparable with that of *T. lymma* but different from that of *M. musculus*. Thus, *P. dolloi* evidently possesses CPS III, which is known to be present in coelacanths, marine elasmobranchs and some teleosts (Mommsen and Walsh, 1989; Anderson, 1980; Randall et al., 1989) but not in lungfish (Janssens and Cohen, 1966; Mommsen and Walsh, 1989). Cellular fractionation studies revealed that CPS III was present exclusively in the mitochondria. However, similar to other African lungfish and amphibians (Mommsen and Walsh, 1989), the majority of arginase was present in the cytosol. To date, there is no report on the presence of GS activity in the livers of African lungfish. However, GS activity, which is essential to the supply of glutamine for the reaction catalyzed by CPS III, was detected in both the mitochondrial and cytosolic fractions from the liver of *P. dolloi*, with the specific activity in the former greater than that in the latter.

Taken together, these results indicate that the evolution of CPS from type III in fish to type I in tetrapods occurred within the Sarcopterygii, specifically within dipnoans. Aerial exposure could be an important factor leading to the substitution of NH_4^+ for glutamine as the substrate for CPS during evolution. These results also suggest *P. dolloi* as the more-primitive extant African lungfish, which is intermediate between aquatic fish (having mitochondrial CPS III and GS) and terrestrial tetrapods (having cytosolic arginase). Other extant African lungfish are likely to be more advanced (having CPS I and no detectable GS) and evolved later to aestivate in subterranean mud cocoons instead. An analysis of the relatedness of mitochondrial DNA in the coelacanth, lungfish and tetrapods (Zardoya and Meyer, 1996) supports the hypothesis that lungfish are the closest living relatives of terrestrial vertebrates. However, a re-analysis of the data led Rasmussen et al. (1998) to conclude that lungfish occupy a basal position among gnathostome fish as the sister-group to all other bony fish. Our results obtained from *P. dolloi* indeed support the proposition made by Rasmussen et al. (1998).

Table 6. *Effects of 6 days aerial exposure on the concentrations of various free amino acids (FAAs) and total FAA (TFAA) in the plasma of* Protopterus dolloi

	Concentration (mmol l^{-1})	
FAA	Immersed	Terrestrial
Alanine	0.349±0.036	0.403±0.048
Anserine	n.d.	n.d.
Arginine	0.042±0.014	0.045±0.003
Aspartate	0.139±0.025	0.112±0.012
Asparagine	0.153±0.034	0.110±0.008
β-alanine	0.760±0.363	0.986±0.152
Glutamate	0.221±0.036	0.223±0.058
Glutamine	0.812±0.155	0.770±0.031
Glycine	0.274±0.035	0.499±0.207
Histidine	1.05±0.44	1.30±0.18
Isoleucine	0.372±0.041	0.505±0.54
Leucine	0.837±0.098	1.12±0.13*
Lysine	0.265±0.017	0.262±0.012
Proline	0.198±0.030	0.113±0.007
Phenylalanine	0.423±0.022	0.389±0.012
Serine	0.581±0.76	0.408±0.032
Taurine	0.220±0.089	0.144±0.044
Threonine	0.931±0.195	0.530±0.025*
Tryptophan	0.879±0.034	1.36±0.09*
Tyrosine	0.231±0.011	0.318±0.028
Valine	0.670±0.057	0.807±0.065
TFAA	9.40±1.35	10.4±0.9

Values are means ± S.E.M. (N=4).

*Significantly different from the corresponding immersed condition (P<0.05).

n.d.; not detectable (detection limit = 0.01 mmol l^{-1}).

Ammonia excretion was impeded on land but did not lead to its accumulation in the body

In terrestrial conditions, no water current is available to take away the excreted ammonia from the gills; the partial pressure of NH_3 (P_{NH_3}) increases quickly in the boundary layer, leading to the reduction of the blood-to-boundary-water NH_3 gradient. Thus, branchial ammonia excretion by diffusion is repressed. Although the role of the branchial epithelium in NH_3 excretion in *P. dolloi* is unclear, the ammonia excretion rate in specimens exposed to terrestrial conditions decreased significantly. Theoretically, this would imply that ammonia was accumulated in these experimental specimens. However, there was no change in the ammonia content in the muscle, liver and plasma of these specimens after 6 days of aerial exposure. This is an extraordinary adaptation exhibited by *P. dolloi*, which apparently cannot be surpassed by any other teleosts (see review by Ip et al., 2001a; Chew et al., in press).

Ammonia was detoxified to urea during 6 days of exposure to terrestrial conditions

It has been established that *P. aethiopicus* and *P. annectens* have a full complement of OUC enzymes in the liver and are able to synthesize urea from ammonia and bicarbonate *in vitro* (Janssens and Cohen, 1968a; Mommsen and Walsh, 1989). In addition, it has been suggested that the capacity to synthesize urea during periods of restricted water availability, as demonstrated by African lungfish, would have pre-adapted the early vertebrates for their transition to land (Campbell, 1973; Graham, 1997). Indeed, *P. dolloi* detoxified ammonia to urea during aerial exposure, and the excess urea was mainly stored in the body. There was only a slight increase in urea excretion in specimens exposed to terrestrial conditions for ≥3 days. Although no information on the role of the kidneys of lungfish in urea excretion is available at present, it would be impractical for *P. dolloi* to excrete urea through its kidney due to the lack of water. Urea excretion might take place across the branchial/opercular epithelium or the skin; but without water to flush away the excreted urea, the excretion process would not be effective to compensate for the increased rate of urea production. Consequently, urea accumulates in the body and serves the secondary function of facilitating water retention through vapour pressure depression during desiccation.

Ammonia production was suppressed during aerial exposure

The deficit in ammonia excretion in a 100 g specimen during the 6 days of aerial exposure amounted to [(6.4–0.7)+(6.8–0.6)+(6.0–0.7)+(4.9–0.8)+(5–0.7)+(5.7–0.7)] μmol g^{-1}× 100 g, or 3060 μmoles. From Table 3, the excess amount of urea accumulated in the body of a 100 g specimen, which contains approximately 55 g muscle, 2 g liver and 1 ml plasma (Y.K.I. and S.F.C., unpublished data), can be calculated as [(18.6–2.25) μmol g^{-1}×55 g]+[(19.2–1.83) μmol g^{-1}×2 g]+[(32.7–2.58) mmol l^{-1}×1 ml], or 964 μmoles. Since there are two moles of N in one mole of urea, this is equivalent to 964×2=1928 μmoles of ammonia. The deficit (3060–1928=1132 μmoles) indicates indirectly the occurrence of a reduction in the rate of endogenous ammonia production in these experimental animals.

This proposition is further supported by the fact that the ammonia excretion rate of specimens re-immersed in water after 6 days of aerial exposure remained low. If we take the rate of ammonia production in an immersed specimen (control) to be the summation of the rate of ammonia and urea excretion (from Figs 1, 2), this amounts to [6.3+(0.27×2)], or 6.84 μmol N day^{-1} g^{-1}. During the 24-h of subsequent re-immersion, the rate of ammonia production is equal to the summation of the rate of ammonia excretion (from Fig. 1) and the normal rate of urea excretion (from Fig. 2), assuming that the increased rate of urea synthesis (see below) had returned to a normal level, which amounts to [1+(0.27×2)], or 1.54 μmol N day^{-1} g^{-1}. This estimated rate of ammonia production during the 24 h of re-immersion is only 22% of the control value.

The rate of urea synthesis increased >8-fold and the OUC capacity was enhanced during aerial exposure

In a submerged specimen, the steady-state level of urea in the body is maintained through a balance of urea production and urea loss (through excretion). Hence, it can be deduced that the rate of urea synthesis in a submerged *P. dolloi* was approximately 0.25 μmol day^{-1} g^{-1}. The amount of urea synthesized in a 100 g specimen during the 6-day period of aerial exposure is equal to the summation of urea excreted and stored, or [(0.25+0.4+0.5+0.9+0.8+0.45) μmol g^{-1}×100 g]+964 μmol=1294 μmoles. This is equivalent to a rate of 1294 μmoles/(100 g×6 days), or 2.16 μmol day^{-1} g^{-1}. In other words, in order to detoxify the endogenous ammonia, which could not be excreted as NH_3 during aerial exposure, the rate of urea synthesis was upregulated 8.6-fold. The normal rate of urea synthesis (0.25 μmol day^{-1} g^{-1}) was definitely inadequate to detoxify the amount of ammonia formed, even after a suppression of the rate of ammonia production to 0.7–1.0 μmol day^{-1} g^{-1} by day 6 of the experimental period.

Janssens and Cohen (1968a) induced *P. aethiopicus* to aestivation for 78–129 days in the laboratory. They reported that the rate of urea synthesis and the activity of OUC enzymes in these experimental specimens were comparable with those of the unfed control. Since then, it has been a general belief that the accumulation of urea in African lungfish during aestivation does not involve an increased rate of urea synthesis (Graham, 1997). In the present study, we confirmed that this was not the case for *P. dolloi* during 6 days of aerial exposure. Aerial exposure is a phase that *P. dolloi* (and presumably also other African lungfish) has to naturally go through before aestivation. Presumably, during aestivation, ammonia production decreases further (<0.7 μmol day^{-1} g^{-1}) so that the normal rate of urea synthesis (as in the submerged control) can adequately detoxify the ammonia produced, thereby preventing ammonia from reaching toxic levels. Indeed, 6 days of aerial exposure without aestivation led to significant increases in the activities of GS (2.2-fold), CPS III (3.8-fold) and ASS + L (1.8-fold) in *P. dolloi* (Table 1). Previous work on aestivating *P. aethiopicus* (Janssens and Cohen, 1968a,b) revealed a rate of urea synthesis comparable with that of the immersed control because the aestivating specimen had entered a profound metabolic rate reduction. Hence, this is the first report on the induction of OUC enzyme activities in the liver of an African lungfish.

The rate of urea excretion increased 22-fold during subsequent recovery in freshwater

Despite the lack of capability to excrete urea on land, the rate of urea excretion in *P. dolloi* increased 22-fold, which is probably the greatest increase known amongst fish, upon re-immersion. This suggests that, unlike marine elasmobranchs (Fines et al., 2001) and coelacanths (Yancey, 2001), which retain urea for osmoregulation, *P. dolloi* possessed transporters to facilitate urea excretion in freshwater, as observed in some teleosts [e.g. gulf toadfish (*Opsanus beta*), Wood et al., 1995; Walsh et al., 2000; Lake Magadi tilapia (*Alcolakia grahami*), Wood et al., 1994; Walsh et al., 2001]. It was known for a long time that the amphibian kidney can secrete urea actively (Balinsky, 1970). In addition, phloretin-sensitive and/or sodium-independent active urea transporters have been reported in the skin of several amphibians (see Sands et al., 1997 for a review). At present, the nature of these transporters and their location in the body tissues of *P. dolloi* is not clear, and therefore *P. dolloi* appears to be an ideal specimen for future studies on the regulation of urea transport.

Aerial exposure affected the contents of FAAs and TFAA in the liver

There were significant decreases in the TFAA content in the liver of specimens exposed to terrestrial conditions for 6 days. To slow down the build-up of ammonia internally (see above), it would be necessary to decrease the rate of amino acid catabolism. The steady-state concentrations of amino acids in the tissues depend on the rates of their degradation and production. In the case of the experimental subjects in this study, amino acids would be produced mainly through proteolysis because food was withdrawn 48 h before, and during, experiments. Under such conditions, it is logical to assume that the rate of protein degradation was higher than the rate of protein synthesis, which led to a net proteolysis. If the rate of proteolysis remained relatively constant and was

unaffected by aerial exposure, there would be accumulations of FAAs, leading to an increase in the internal TFAA content. Therefore, the decrease in TFAA content in the liver of *P. dolloi* exposed to air indicates that simultaneous decreases in the rates of proteolysis and amino acid catabolism would have occurred. Furthermore, the decrease in proteolytic rate must be greater than the decrease in the rate of amino acid catabolism, subsequently leading to decreases in the steady-state concentrations of some FAAs and, consequently, lowering the TFAA concentration.

Despite the 2.2-fold increase in GS activity in the liver, there was a significant decrease in the hepatic glutamine content in specimens exposed to air for 6 days. This suggests that the excess glutamine formed was completely channelled into urea synthesis *via* CPS III. Simultaneously, there was a significant decrease in the content of glutamate in the liver of these specimens, indicating that the utilization of glutamate for glutamine formation out-paced the formation of glutamate through glutamate dehydrogenase or its release *via* proteolysis. This would suggest that the decrease in the rate of ammonia production was achieved through the regulation of hepatic glutamate dehydrogenase activity in *P. dolloi* during aerial exposure.

Ip et al. (2001c) reported that the mudskipper *P. schlosseri* was capable of using certain amino acids as a metabolic fuel and avoided ammonia toxicity through partial amino acid catabolism during an excursion on land. However, a similar phenomenon was not observed in *P. dolloi*. In contrast to the mudskipper, the pectoral and pelvic fins of *P. dolloi* are filamentous and incapable of sustaining locomotion on land. On land, *P. dolloi* remains quiescent and is relatively motionless. It is probably because of this that partial amino acid catabolism was not adopted as a strategy by *P. dolloi* to survive aerial exposure.

Conclusion

P. dolloi possesses GS and CPS III in the liver, and not CPS I as has been shown previously in other African lungfish. Hence, in this regard, *P. dolloi* is a more-primitive extant African lungfish intermediate between aquatic fish and terrestrial tetrapods. Six days of exposure to terrestrial conditions without aestivation led to an increase in urea content in the body, accompanied by an increase in hepatic OUC capacity, in *P dolloi*. The accumulated urea was released to the external medium during the subsequent 24 h of re-immersion, during which the rate of urea excretion increased 22-fold.

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