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Nitrogen metabolism in the African lungfish (*Protopterus dolloi*) aestivating in a mucus cocoon on land

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Summary

This study aimed to elucidate the strategies adopted by the African slender lungfish, *Protopterus dolloi*, to ameliorate the toxicity of ammonia during short (6 days) or long (40 days) periods of aestivation in a layer of dried mucus in open air in the laboratory. Despite decreases in rates of ammonia and urea excretion, the ammonia content in the muscle, liver, brain and gut of *P. dolloi* remained unchanged after 6 days of aestivation compared with the control fasted for 6 days. For specimens aestivated for 40 days, the ammonia contents in the muscle, liver and gut were significantly lower than those of the control fasted for 40 days, which suggests a decrease in the rate of ammonia production. In addition, there were significant increases in contents of alanine, aspartate and glutamate in the muscle, which suggests decreases in their catabolism. During the first 6 days and the last 34 days of aestivation, the rate of ammonia production was reduced to 26% and 28%, respectively, of the control rate (6.83 μmol day^{-1} g^{-1} on day 0). During the first 6 days and the next 34 days of aestivation, the averaged urea synthesis rate was 2.39-fold and 3.8-fold, respectively, greater than the value of 0.25 μmol day^{-1} g^{-1} for the day 0 control kept in water. No induction of activities of the ornithine-urea cycle (OUC) enzymes was observed in specimens aestivated for 6 days, because the suppression of ammonia production led to a light demand on the OUC capacity. For specimens aestivated for 40 days, the activities of carbamoyl phosphate synthetase, ornithine transcarbamylase and argininosuccinate synthetase + lyase were significantly greater than those of the control fasted for 40 days. This is in agreement with the observation that the rate of urea synthesis in the last 34 days was greater than that in the first 6 days of aestivation. *P. dolloi* aestivated in a thin layer of dried mucus in open air with high O_2 tension throughout the 40 days of aestivation, which could be the reason why it was able to sustain a high rate of urea synthesis despite this being an energy-intensive process. Our results indicate that a reduction in ammonia production and decreases in hepatic arginine and cranial tryptophan contents are important facets of aestivation in *P. dolloi*.

Key words: aestivation, *Protopterus dolloi*, lungfish, nitrogen metabolism, ammonia excretion, urea excretion, mucus, cocoon.

Introduction

Lungfishes (or dipnoans, as they are ‘dual breathers’) are an archaic group of fishes, characterized by the possession of a lung opening off the ventral side of the oesophagus. There are six species of extant lungfish, four of which (*Protopterus aethiopicus*, *Protopterus annectens*, *Protopterus amphibious* and *Protopterus dolloi*) can be found in Africa (Greenwood, 1987). African lungfishes depend entirely on aerial respiration and can live for an extended period out of water (Graham, 1997). Unlike their Australian (*Neoceratodus forsteri*) and South American (*Lepidosiren paradoxa*) counterparts, the African *P. aethiopicus* and *P. annectens* can aestivate in subterranean mud cocoons for long periods of time (Smith, 1935; Janssens, 1964; Janssens and Cohen, 1968a,b). On land, there is often 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 (Cooper and Plum, 1987; Hermenegildo et al., 1996; Ip et al., 2001; Brusilow, 2002; Felipo and Butterworth, 2002; Rose, 2002) and therefore African lungfishes have to avoid ammonia intoxication when out of water.

P. aethiopicus and *P. annectens* are ureogenic (Janssens and Cohen, 1966, 1968a; Mommsen and Walsh, 1989). Similar to tetrapods, they possess in their livers mitochondrial carbamoyl phosphate synthetase I (CPS I), which utilizes NH_4^+ as a substrate, and an arginase that is present mainly in the cytosol (Mommsen and Walsh, 1989). During 78–129 days of aestivation in a nylon bag designed to replace mud, *P. aethiopicus* accumulated 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 appeared to be in continuous gluconeogenesis throughout aestivation (Janssens and Cohen, 1968b). It was proposed that *P. aethiopicus* could undergo a profound suppression of ammonia production under such conditions (Janssens and Cohen, 1968a). However, it is difficult to envisage that the suppression of ammonia production would occur instantly when the fish was out of water. During the initial phase of aerial exposure, before the onset of a reduction in the rate of ammonia production, the rate of urea synthesis ought to be increased to detoxify the ammonia that is produced at a normal (or slightly sub-normal) rate and retained within the body. To date, no such information is available on African lungfishes.

It is important to point out that Janssens and Cohen (1968a; their table 3) also measured the rates of incorporation of [^{14}C]bicarbonate into urea during a 60-h period at the very end of a long period (78–129 days) of fasting or aestivation in *P. aethiopicus* and obtained comparable results between these two groups of specimens. These results were regarded as important evidence in support of the conclusion on the lack of an increase in the rate of urea synthesis in aestivating *P. aethiopicus*. However, later findings have identified two problems with such a conclusion. Firstly, decreases in metabolic rate in African lungfishes can actually be achieved through progressive starvation and emaciation (Fishman et al., 1987). Secondly, the lack of increased urea synthesis in specimens undergoing prolonged aestivation does not necessarily imply that an increased rate of urea synthesis would not occur during the initial period of (or short-term) aestivation.

Found in Central Africa in the lower and middle Congo River basins is the slender lungfish, *P. dolloi*, which can aestivate on land within a layer of dried mucus (Brien, 1959; Poll, 1961) instead of inside a cocoon in the mud like *P. aethiopicus* and *P. annectens.* Like elasmobranchs and some teleosts, *P. dolloi* possesses carbamoyl phosphate synthetase III (CPS III), which uses glutamine as a substrate, in the liver (Chew et al., 2003). Glutamine synthetase (GS) activity is present in both the mitochondrial and cytosolic fractions of the liver of *P. dolloi*. In the laboratory, *P. dolloi* can be induced to aestivate in a layer of dried mucus in a plastic or glass aquarium containing only 10–20 ml of water in open air. The water would dry up in approximately 3–4 days, and the specimen would enter a state of torpor in a layer of dried mucus on day 4 or day 5 (Fig. 1). By renewing the small amount of water in the container daily to prevent the specimen from entering into aestivation, Chew et al. (2003) indeed verified that the rate of urea synthesis increased 10-fold in *P. dolloi* exposed to air for 6 days. Aerial exposure also led to an increase in the hepatic ornithine-urea cycle (OUC) capacity (Chew et al., 2003), with significant increases in activities of CPS III (3.8-fold), argininosuccinate synthetase + lyase (1.8-fold) and, more importantly, GS (2.2-fold), which produces glutamine, the substrate required for CPS III activity.

However, there remain several fundamental questions. Would *P. dolloi* increase the rate of urea synthesis if it could enter into aestivation (i.e. after day 4) during the 6-day period? More importantly, would it sustain an increased rate of urea synthesis during a longer period (e.g. 40 days) of aestivation? Would there be a reduction in ammonia production, resulting from a reduction in catabolism of certain amino acids, in this lungfish during aestivation, and would the degree of reduction be constant throughout the 40-day period? The present study was therefore undertaken to examine nitrogen metabolism in *P. dolloi* that underwent a period of 6 or 40 days of aestivation in open air. The contents of ammonia, urea and free amino acids (FAAs) in various tissues and organs of the experimental specimens were determined. In addition, efforts were made to determine the activities of enzymes associated with the OUC in aestivating *P. dolloi*.

Fig. 1. A specimen of *P. dolloi* aestivating in a dried mucus cocoon on land in the laboratory.

Materials and methods

Specimens

P. dolloi (Boulenger 1900) (100–150 g body mass) were imported from Central Africa through a local fish farm in Singapore. 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, and water was changed daily. No attempt was made to separate the sexes. The specimens were acclimated to laboratory conditions for at least 1 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.

Determination of contents of ammonia, urea and FAAs in various tissues

Specimens were allowed to enter into a state of aestivation

individually in plastic tanks (29 cm×19 cm×17.5 cm, length × width × height) containing a thin film of 10 ml dechlorinated tap water. For those that were allowed to aestivate for 40 days, 1–2 ml of water was sprayed on the surface of the brown cocoon covering the body surface every 6 days. A preliminary experiment indicated that this was essential to reduce the rate of dehydration of the specimens in laboratory conditions (humidity, ~80%), which are likely to be drier than the natural habitat of *P. dolloi*. Another group of fish was placed in dechlorinated tap water at 25°C for the same period of time (i.e. 6 or 40 days) to serve as controls to evaluate the effects of fasting alone. At the end of 6 or 40 days, specimens were killed with a strong blow to the head.

The lateral muscle, liver, gut and brain 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.

It was essential to show that any change in the contents of ammonia, urea or FAAs in the tissues was not a result of a loss of water due to dehydration. Since the muscle comprises the bulk of the body, an attempt was made to determine the water content of muscle samples by estimating the difference in wet mass and dry mass. The wet mass of the muscle was determined to the nearest milligram using a Sartorius analytical balance. The dry mass was determined after the muscle had been dried in an oven at 90°C for ≥24 h until it reached constant mass.

The frozen samples were weighed, ground to a powder in liquid nitrogen and homogenized three times in five volumes (w/v) of 6% trichloroacetic acid (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 analysed.

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 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.

Urea contents in 0.2 ml of the neutralised sample were 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 (#U7127; Sigma Chemical Co., St Louis, MO, USA) was used for the estimation of urea content in the sample. Urea obtained from Sigma Chemical Co. was used as a standard for comparison. Results were expressed as μmol g^{-1} wet mass tissue.

For FAA analysis in muscle, liver and brain samples, 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 (Kyoto, Japan) with a Shim-pack ISC-07/S1504 Li-type column. Results for FAA analyses were expressed as μmol g^{-1} wet mass or μmol ml^{-1} plasma.

Determination of activities of OUC enzymes from the liver of experimental specimens

The liver was homogenized in five 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 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 CPS III activity was present only in the liver of *P. dolloi*.

CPS (E.C. 2.7.2.5) activity was determined according to the method of Anderson and Walsh (1995). Radioactivity was measured using a Wallac 1414 liquid scintillation counter (Wallac Oy, Turku, Finland). CPS 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 UV 160 UV VIS recording spectrophotometer. OTC activity was expressed as μmol citrulline formed min^{-1} g^{-1} wet mass.

Argininosuccinate synthetase (ASS; E.C. 6.3.4.5) and lyase (L; E.C. 4.3.2.1) activities were determined together (ASS + L), 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 . The 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.

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

Determination of ammonia and urea excretion rates of specimens

After 48 h of fasting, specimens (N=5) were kept individually in a plastic tank (20.5 cm×14.5 cm×6 cm, length × width × height) containing 2 litres of water at 25°C for 24 h (regarded as day 0 control). 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. Thus, 3 ml of water was sampled for ammonia and urea analysis after 24 h of exposure. The same individuals were then kept in plastic tanks containing 10 ml of water, i.e. experimental conditions that would induce aestivation (see above). At the end of day 3, only a very small amount of water was left. In order to find out the amounts of ammonia and urea excreted during this period before the onset of aestivation, a small volume of water was sprayed on the fish and the side of the tank. The water was then collected, made up to a known volume and used for ammonia and urea analyses. 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; N=4) 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.

Statistical analyses

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

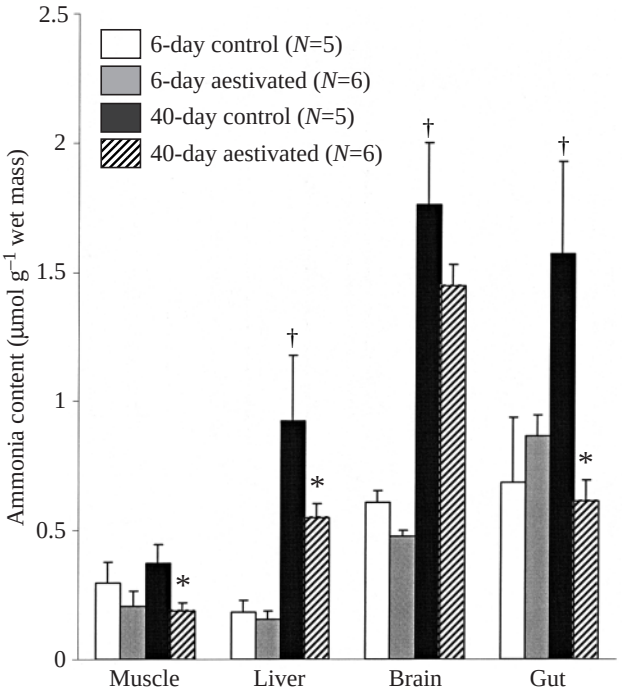

Fig. 2. Ammonia contents (μmol g^{-1}) in the muscle, liver, brain and gut of *P. dolloi* after aestivating for 6 days or 40 days. Values are means + S.E.M. *Significantly different from the corresponding control (P<0.05). †Significantly different from the 6-day control (P<0.05).

Results

Effects of aestivation on the water content of the muscle

The water content in one gram of muscle of *P. dolloi* after aestivating for 6 days was 0.813±0.012 g (N=6), which was not significantly different from controls kept in water for the same period of time (0.818±0.010 g). Similarly, the water content in the muscle of the 40-day aestivating lungfish (0.788±0.015 g) was not different from that of the controls fasted for 40 days (0.811±0.010 g).

Effects of aestivation on ammonia levels

There were no significant increases in ammonia content in the muscle, liver, gut or brain of *P. dolloi* after 6 days of aestivation compared with the control fasted for 6 days (Fig. 2). Surprisingly, ammonia levels in the muscle, liver and gut of specimens aestivated for 40 days were significantly lower than those of the 40-days fasted controls (Fig. 2). The specimens kept in water but fasted for 40 days had significantly higher ammonia levels in their liver, gut and brain compared with those fasted for 6 days only.

Effects of aestivation on urea levels

Urea contents in the muscle, liver, gut and brain of specimens aestivated for 6 days were 4.1-, 4.3-, 5.5- and 6.4-fold greater than the corresponding value of specimens fasted for the same period in water (Fig. 3). After aestivating for 40 days, the urea contents in the muscle, liver, gut and brain increased by 9.5-, 11.3-, 10.9- and 9.9-fold when compared with the corresponding values of specimens kept in water and fasted for 40 days. Again, the specimens fasted for 40 days had significantly higher levels of urea in their tissues than those fasted for 6 days only (Fig. 3).

Effects of aestivation on FAA levels

The content of total FAA (TFAA) remained unchanged in the muscle of *P. dolloi* aestivated for 6 or 40 days (Table 1). There was only a significant increase in isoleucine content in the muscle of specimens aestivated for 6 days. On the other hand, fish aestivated for 40 days showed significant increases in alanine, aspartate and glutamate levels in the muscle. By contrast, there was a significant decrease in the TFAA content in the livers of specimens that underwent 40 days of aestivation (Table 2), which was attributed mainly to decreases in contents of proline and glutamate. The arginine content in the liver of *P. dolloi* aestivated for 6 days was significantly higher than that of the control fasted for 6 days (Table 2). However, the hepatic arginine content became undetectable on day 40 of fasting or aestivation (Table 2). In addition, there was a significant decrease in the arginine content in the muscle of these specimens (Table 1). In the brain, there was a significantly higher TFAA content in specimens aestivated for 40 days, which was attributed mainly to an increase in glutamine (Table 3). Forty days of aestivation also led to a 57% decrease in the content of

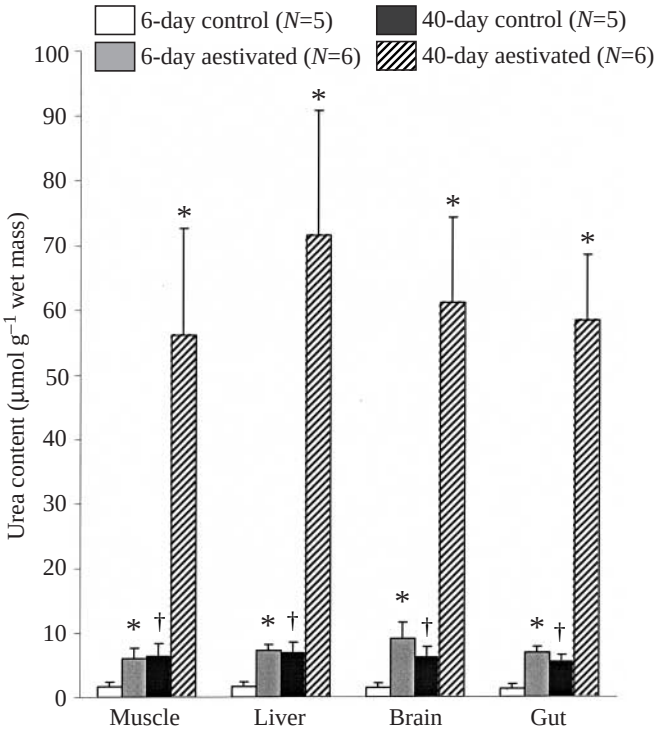

Fig. 3. Urea contents (μmol g^{-1}) in the muscle, liver, brain and gut of *P. dolloi* after aestivating for 6 days or 40 days. Values are means + S.E.M. *Significantly different from the corresponding control (P<0.05). †Significantly different from the 6-day control (P<0.05).

tryptophan in the brain compared with the 40-day fasted control (Table 3).

Effects of aestivation on OUC cycle enzymes

The activities of OUC enzymes, inclusive of CPS III, in the liver of *P. dolloi* were unaffected by 6 days of aestivation or 40 days of fasting in water (Table 4). There was a significant increase in hepatic GS activity in specimens aestivated for 6 days. Forty days of aestivation led to significant increases in the activities of GS, CPS III, OTC and ASS + L (Table 4).

Rates of ammonia and urea excretion

The rates of ammonia and urea excretion in specimens (N=5) kept in water at day 0 were 6.35±0.87 μmol day^{-1} g^{-1} fish and 0.25±0.03 μmol day^{-1} g^{-1} fish, respectively. The averaged rates of ammonia and urea excretion in the first 3 days of the aestivation period were 0.70±0.11 μmol day^{-1} g^{-1} fish and 0.21±0.02 μmol day^{-1} g^{-1} fish, respectively.

Discussion

Ammonia levels in tissues remained unchanged after aestivation for 6 or 40 days

Apparently, *P. dolloi* was able to excrete some ammonia and urea, albeit at much lower rates, during the first few days (up to day 4) of the experimental period before the water in the container totally dried up. Aestivation usually occurred on day 4 or day 5 with the formation of a dark brown cocoon that enclosed the whole specimen. By day 5, the container was absolutely dry and it was unlikely that ammonia or urea excretion would have occurred. *P. dolloi* was incapable of volatilising NH_3 during aestivation (S. F. Chew and Y. K. Ip, unpublished results). Yet, there were no significant increases in ammonia content in the muscle, liver, brain and gut of *P. dolloi* after 6 days of aestivation. More intriguing is the fact that the ammonia contents in the muscle, liver and gut of specimens aestivated for 40 days were significantly lower than those of the 40-days fasted control kept in water. It is important to note that these observable changes were unrelated to dehydration because 40 days of aestivation or fasting had no significant effect on the water content in the muscle of *P. dolloi*.

Fasting for 40 days led to significantly higher levels of ammonia in various tissues and organs of *P. dolloi*. This is probably due to the mobilization of protein and amino acids to sustain energy production during fasting, which led to an increase in the rate of ammonia production. However, the interesting observation here is that ammonia accumulation occurred in these fasted animals despite their being kept in water. When NH_4Cl was infused peritoneally into *P. dolloi*, >80% of the infused ammonia could be excreted to the external medium within the first 4 h, which indicates that *P. dolloi* has high capacity in ammonia excretion (Y. K. Ip and S. F. Chew, unpublished results), despite its gills being degenerate (Graham, 1997). Therefore, it would appear that *P. dolloi* was regulating the rate of ammonia (and urea) excretion during fasting with the aim of retaining it. Since fasting had been proposed as one of the initiating factors of aestivation (Fishman et al., 1987), it is possible that an initial accumulation of ammonia, leading to subsequent urea synthesis and its accumulation (see below), is essential for initiating those changes.

Compared with the controls fasted for 40 days, there were significant decreases in concentrations of ammonia in the muscle, liver and gut of specimens aestivated for 40 days. These results suggest that a decrease in the rate of ammonia production had occurred in specimens undergoing aestivation, especially considering the fact that ammonia excretion would have been completely impeded for 36 days.

P. dolloi *detoxified endogenous ammonia to urea with increased rates of urea synthesis during aestivation*

It has been suggested that the capacity to synthesize urea during periods of restricted water availability, as demonstrated by African lungfishes, would have pre-adapted the early vertebrates for their transition to the land (Campbell, 1973; Graham, 1997). Indeed, *P. dolloi* detoxified ammonia to urea during 40 days of aestivation on land, and the excess urea formed was mainly stored in the body. Urea excretion would not have occurred during aestivation due to a lack of water. Consequently, the accumulated urea could fulfil a secondary function of facilitating water retention through a reduction in vapour pressure, since aestivation prescribes desiccation.

Table 1. *Effects of 6 or 40 days of aestivation on the contents of various free amino acids (FAAs) and total free amino acids (TFAA) in the muscle of* P. dolloi

	Content (μmol g^{-1} wet mass)			
FAA	6 days in water (control)	6 days aestivation	40 days in water (control)	40 days aestivation
Alanine	0.167±0.029	0.237±0.047	0.232±0.040	0.437±0.090*
Anserine	4.87±1.90	4.71±0.98	2.51±1.11	4.06±1.37
Arginine	2.40±0.52	4.75±0.50	3.91±1.05	0.766±0.156*
Aspartate	0.075±0.018	0.154±0.021	0.094±0.010	0.377±0.087*
β-alanine	0.055±0.003	0.049±0.0045	0.065±0.013	0.042±0.006
Carnosine	0.118±0.018	0.117±0.012	0.167±0.028	0.177±0.044
Glutamate	0.144±0.030	0.240±0.033	0.166±0.035	0.887±0.137*
Glutamine	0.268±0.049	0.383±0.063	0.256±0.050	0.345±0.038
Glycine	0.662±0.459	0.411±0.100	0.234±0.028	0.233±0.066
Isoleucine	0.058±0.020	0.109±0.007*	N.D.	0.073±0.015
Leucine	0.103±0.027	0.201±0.019	0.117±0.011	0.186±0.035
Lysine	0.705±0.187	1.06±0.13	1.10±0.36	0.208±0.089
Proline	0.907±0.137	1.18±0.16	1.64±0.19	2.32±0.45
Serine	0.139±0.012	0.155±0.013	0.240±0.027	0.206±0.054
Taurine	0.419±0.051	0.248±0.033	0.361±0.048	0.287±0.045
Threonine	0.174±0.019	0.150±0.027	0.154±0.016	0.201±0.046
Tryptophan	0.092±0.004	0.104±0.008	0.136±0.045	0.039±0.012
Valine	0.042±0.034	0.050±0.041	0.176±0.015	0.135±0.050
TFAA	11.4±3.0	14.3±0.7	11.6±0.6	11.1±0.6

Results represent means ± S.E.M. (N=4).
*Significantly different from the corresponding control condition (P<0.05).
N.D., not detectable (detection limit=0.001 μmol g^{-1} wet mass).

Fasting also led to significant increases in the urea content in the body of *P. dolloi*. As mentioned above, this could be due to an increase in the degradation of protein, leading to increased ammonia production. However, why was urea retained in the body instead of being excreted despite the fish being immersed in water? For a long time, it was accepted that urea permeates biomembranes by diffusion. To date, five urea transporters have been identified, which aid in urea transport across biomembranes (Sands et al., 1997). It is uncertain if urea transporters exist in *P. dolloi*, but our results suggest that increased urea production was not accompanied immediately by an upregulation of urea transport when the fish was fasted in water. Alternatively, these results may suggest a physiological role of urea in initiating and/or perpetuating aestivation in *P. dolloi*, because fasting is known to affect the metabolic, circulatory and respiratory changes in ways similar to aestivation in African lungfishes (Fishman et al., 1987).

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). Therefore, it can be deduced that the rate of urea synthesis in a submerged *P. dolloi* (day 0 control) was 0.25 μmol day^{-1} g^{-1}. The amount of urea synthesized in a 100 g specimen during the 6-day period of aestivation is equal to the summation of urea excreted in the first 3 days and the urea stored in the body. The amount excreted was equal to 0.2 μmol×3 days×100 g=60 μmol. The excess amount of urea accumulated in the body of a 100 g specimen, which consists of 55 g muscle, 2 g liver, 0.3 g brain and 3 g gut, can be calculated (from Fig. 3) as [(6.43–1.58) μmol×55 g+(7.33–1.71) μmol×2 g+(8.99–1.40) μmol×0.3 g+(7.39–1.34) μmol×3 g], or 298.91 μmol. This is equivalent to an averaged urea synthesis rate of (60+298.91) μmol/(100 g×6 days) or 0.598 μmol day^{-1} g^{-1} during this 6-day period, which is 2.39-fold greater than the value of 0.25 μmol day^{-1} g^{-1} of the day 0 control in water. Chew et al. (2003) estimated the averaged urea synthesis rate for *P. dolloi* exposed to air for 6 days without undergoing aestivation as 2.21 μmol day^{-1} g^{-1}. They concluded that the rate of urea synthesis was upregulated 8.8-fold in order to detoxify the endogenous ammonia that could not be excreted as NH_3 during aerial exposure. The differences in results obtained in these two studies suggest that endogenous ammonia production was suppressed to a much greater extent when *P. dolloi* progressively entered the state of aestivation (especially on days 4–6) but there was still a substantial increase in the rate of urea synthesis. The latter was apparently necessary to maintain the ammonia contents in the body at low levels.

Janssens and Cohen (1968b) induced *P. aethiopicus* to aestivate for 78–129 days (but the exact duration was not given) 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 fasted control. Since then, it has been generally accepted that

Table 2. *Effects of 6 or 40 days of aestivation on the contents of various free amino acids (FAAs) and total free amino acids (TFAA) in the liver of* P. dolloi

	Content (μmol g^{-1} wet mass)			
FAA	6 days in water (control)	6 days aestivation	40 days in water (control)	40 days aestivation
Alanine	0.215±0.056	0.232±0.079	0.148±0.012	0.280±0.093
Anserine	0.040±0.022	0.015±0.005	0.011±0.005	0.010±0.003
Arginine	0.058±0.021	0.166±0.040*	N.D.	N.D.
Asparagine	0.055±0.008	0.086±0.031	0.038±0.005	0.047±0.009
Aspartate	0.728±0.119	1.15±0.44	0.636±0.208	0.627±0.115
β-alanine	0.034±0.005	0.032±0.012	0.033±0.006	0.012±0.001
Carnosine	0.175±0.011	0.207±0.020	0.135±0.007†	0.201±0.010*
Glutamate	2.75±0.37	2.01±0.32	1.80±0.30	1.07±0.12*
Glutamine	0.461±0.184	0.194±0.071	0.089±0.008	0.068±0.022
Glycine	0.348±0.038	0.373±0.052	0.278±0.040	0.405±0.040
Isoleucine	0.043±0.003	0.045±0.003	0.041±0.006	0.029±0.004
Leucine	0.116±0.012	0.170±0.044	0.141±0.035	0.121±0.021
Lysine	0.158±0.039	0.117±0.045	0.043±0.013	0.059±0.009
Proline	5.22±1.03	4.17±0.42	6.67±0.90	3.38±0.38*
Serine	0.174±0.019	0.282±0.061	0.152±0.025	0.253±0.038
Taurine	0.614±0.144	0.606±0.076	0.710±0.209	1.73±0.38*
Threonine	0.323±0.066	0.128±0.017*	0.094±0.015†	0.139±0.024
Tryptophan	0.105±0.025	0.108±0.049	0.135±0.038	0.035±0.008
Valine	0.035±0.018	0.078±0.007	0.085±0.007	0.064±0.022
TFAA	11.7±1.6	10.2±0.5	11.2±0.8	8.53±0.42*

Results represent means ± S.E.M. (N=4).
*Significantly different from the corresponding control condition (P<0.05).
†Significantly different from the corresponding 6 days in water condition (P<0.05).
N.D., not detectable (detection limit=0.001 μmol g^{-1} wet mass).

the accumulation of urea in African lungfishes during aestivation does not involve an increased rate of urea synthesis (Graham, 1997). In the present study, we have verified that this is not the case for *P. dolloi* during the first 6 days of aestivation on land nor is it the case for specimens aestivated on land for a 40-day period.

For specimens of *P. dolloi* that underwent aestivation for 40 days, the urea contents in the muscle, liver, brain and gut increased 9.48-, 11.3-, 9.96- and 10.89-fold, respectively, compared with fasted controls. The excess urea accumulated between day 6 and day 40 (a total of 34 days) of aestivation in a 100 g fish amounted to [(60.9–6.43) μmol×55 g]+[(77–7.33) μmol×2 g]+[(61.1–8.99) μmol×0.3 g]+[(58.3–7.39) μmol×3 g], or 3303.25 μmol. This would give an averaged rate of 3303.25/(100 g×34 days) or 0.97 μmol day^{-1} g^{-1} for urea synthesis during the latter 34 days of aestivation, which is 3.8-fold greater than the value of 0.25 μmol day^{-1} g^{-1} for the day 0 control in water, and 1.62-fold greater than the rate (0.598 μmol day^{-1} g^{-1}) obtained for specimens aestivated for 6 days only.

It might be argued that results would be different if the experiment for *P. dolloi* was prolonged to 78 days, as Janssens and Cohen (1968a) did for *P. aethiopicus*. However, that does not seem to be the case, because even if we made the assumption that absolutely no urea synthesis took place in the subsequent 38-day period (78–40 days), the averaged rate of urea synthesis for a total period of 38 days + 34 days (or 72 days) based simply on the urea accumulated by day 40 can be calculated as 3303.25 μmol/(100 g×72 days), or 0.46 μmol day^{-1} g^{-1}. This is still 1.8-fold greater than the day 0 control value (0.25 μmol day^{-1} g^{-1}).

The OUC capacity for urea synthesis remained unchanged during the first 6 days of aestivation but increased by day 40

A full complement of OUC enzymes was detected *in vitro* from the liver of *P. dolloi*, suggesting the occurrence of urea synthesis *de novo* in this lungfish. Chew et al. (2003) reported increases in activities of some OUC enzymes, including CPS III, in the liver of *P. dolloi* after 6 days of aerial exposure, which represents the initial phase that the lungfish has to go through before aestivation occurs. The maximal level of CPS III activity determined *in vitro* from the liver of the day 0 control fish was unable to sustain the rate of urea synthesis (2.21 μmol day^{-1} g^{-1}) during these 6 days. By contrast, no induction of CPS activity was observed in *P. dolloi* aestivated for 6 days in this study. The likely reason is that aestivation led to a greater reduction in the rate of ammonia production, which eventually exerted a smaller demand on the OUC and could be adequately handled by the control level of CPS III activity.

Table 3. *Effects of 6 or 40 days of aestivation on the contents of various free amino acids (FAAs) and total free amino acids (TFAA) in the brain of* P. dolloi

	Content (μmol g^{-1} wet mass)			
FAA	6 days in water (control)	6 days aestivation	40 days in water (control)	40 days aestivation
Alanine	0.222±0.025	0.301±0.060	0.180±0.016	0.154±0.009
Anserine	0.755±0.101	1.57±0.78	0.598±0.090	1.00±0.19
Arginine	0.385±0.020	0.740±0.243	0.332±0.024	0.424±0.053
Asparagine	N.D.	N.D.	N.D.	N.D.
Aspartate	0.670±0.085	0.891±0.238	0.555±0.218	0.696±0.181
β-alanine	0.038±0.006	0.033±0.003	0.035±0.006	0.029±0.007
Carnosine	0.074±0.005	0.124±0.042	0.084±0.006	0.112±0.014
Glutamate	3.33±0.37	3.74±0.36	3.20±0.62	3.68±0.33
Glutamine	2.38±0.18	3.48±0.28*	2.00±0.33	3.50±0.25*
Glycine	0.685±0.118	0.680±0.147	0.402±0.049	0.743±0.057
Isoleucine	0.017±0.008	0.043±0.011	0.016±0.007	0.021±0.003
Leucine	0.066±0.025	0.126±0.023	0.081±0.015	0.102±0.013
Lysine	0.050±0.030	0.153±0.049	0.056±0.019	0.174±0.027
Proline	0.883±0.209	1.82±0.22*	0.899±0.081	1.25±0.05
Serine	0.317±0.024	0.344±0.076	0.322±0.065	0.388±0.037
Taurine	0.161±0.023	0.233±0.031	0.187±0.039	0.606±0.025*
Threonine	0.217±0.030	0.231±0.048	0.155±0.038	0.270±0.021
Tryptophan	0.060±0.011	0.099±0.010	0.080±0.012	0.034±0.005*
Valine	0.024±0.010	0.065±0.016	0.030±0.014	0.025±0.014
TFAA	10.3±0.7	14.7±0.6*	9.22±0.42	13.2±0.5*

Results represent means ± S.E.M. (N=4).
*Significantly different from the corresponding control condition (P<0.05).
N.D., not detectable (detection limit=0.001 μmol g^{-1} wet mass).

For specimens aestivated for 40 days, the activities of GS, CPS III, OTC and ASS + L were significantly greater than the 6 days and 40 days fasted control in water. This is in agreement with the above analysis that the rate of urea synthesis in the latter 34 days was greater than in the first 6 days of aestivation.

P. dolloi *suppressed ammonia production during aestivation*

Since the ammonia and urea excretion rates of *P. dolloi* in water on day 0 were 6.35 μmol day^{-1} g^{-1} and 0.25 μmol day^{-1} g^{-1}, respectively, the total amount of nitrogen excreted theoretically by a 100 g specimen during a 6-day period in water was equal to [6.35+(0.25×2)] μmol×6 days×100 g, or 4110 μmol N. However, since only 2.1 μmol g^{-1} and 0.6 μmol g^{-1} of ammonia and urea, respectively, were excreted in the first 3 days before the external medium completely dried up, the deficit in nitrogen (N) excretion in a 100 g specimen during this period amounts to 4110 μmol – [2.1+(0.6×2)] μmol×100 g=3780 μmol. The excess amount of urea accumulated in the body of a 100 g specimen was equal to 298.9 μmol, which is equivalent to 298.9×2, or 597.8 μmol N. The deficit of 3780–597.8, or 3182.2 μmol N, indicates that a reduction in the rate of production of endogenous ammonia must have occurred, and this reduction is indeed much greater than that obtained for specimens exposed to air without undergoing aestivation for 6 days (1060 μmol; Chew et al., 2003). The deficit of 3182.2 μmol N corresponds to a reduction of 5.30 μmol day^{-1} g^{-1} in the ammonia production rate, which is equivalent to 77% (5.30×100/6.85) of the rate of ammonia + urea production (6.85 μmol N day^{-1} g^{-1}) in the day 0 control kept in water.

For the period between 6 and 40 days of aestivation, the reduction in ammonia excretion would theoretically amount to 6.85 μmol×34 days×100 g, or 23 290 μmol, for a 100 g fish, assuming that the rate of ammonia + urea production remained constant at 6.85 μmol N day^{-1} g^{-1} as in the day 0 control. However, the excess amount of urea accumulated during these 34 days was only 3303.25 μmol, or 6606.5 μmol N. The deficit of 23 290–6606.5 μmol N, or 16 683.5 μmol N, in 34 days implies a suppression of 4.91 μmol ammonia day^{-1} g^{-1} or 72% (4.91×100/6.85). This is in close approximation to the value of 77% obtained for the first 6 days of aestivation, indicating that ammonia production remained reduced during the 40-day period. These results confirm the validity of including the first 3–4 days of aerial exposure in the aestivation period.

Reduction in the rate of amino acid catabolism and changes in arginine and tryptophan contents during aestivation

In specimens aestivated for 40 days, there were significant increases in alanine, aspartate and glutamate content in the muscle. To slow down the build-up of ammonia internally (see above), it was necessary to decrease the rate of amino acid catabolism. The steady-state concentration of amino acids in

Table 4. *Effects of 6 or 40 days aestivation on the activity of GS, CPS, OTC, ASS + L and arginase in the liver of* P. dolloi

		Enzyme activity (μmol min^{-1} g^{-1} wet mass)			
Enzymes	Substrate and effector	6 days in water (control)	6 days aestivation	40 days in water (control)	40 days aestivation
GS	–	0.16±0.01	3.02±1.19*	0.19±0.02	0.42±0.04*
OTC	–	9.86±1.33	14.3±1.2	9.77±0.87	16.1±1.6*
ASS + L	–	0.47±0.07	0.50±0.04	0.37±0.05	0.82±0.07*
Arginase	–	143±36	124±10	73.9±11.1	96.8±6.1
CPS	Glutamine	N.D.	0.006±0.003	N.D.	N.D.
	Glutamine + AGA	0.28±0.04	0.27±0.07	0.43±0.07	0.78±0.16*
	Glutamine + AGA + UTP	0.27±0.04	0.26±0.07	0.41±0.06	0.75±0.16*

AGA, *N*-acetyl-L-glutamate; UTP, uridine triphosphate; GS, glutamine synthetase; CPS, carbamoyl phosphate synthetase; OTC, ornithine transcarbamoylase; ASS + L, arginosuccinate synthetase + lyase.

Results represent means ± S.E.M. (N=4).

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

the tissues depends on the rates of their degradation and production. In the case of the experimental subjects in this study, FAAs would be produced mainly through proteolysis because the specimen was undergoing aestivation (and fasting simultaneously). Hence, these results support the proposition that amino acid catabolism, specifically for alanine, aspartate and glutamate, in the muscle had been suppressed.

In the liver of specimens aestivated for 40 days, there was a significant decrease in the glutamate level. This suggests that glutamate was channelled into glutamine, which acted as a substrate for urea synthesis *via* CPS III. For specimens aestivated for 6 or 40 days, or those fasted for 40 days, increases in urea synthesis and urea accumulation were accompanied by a significant decrease in the glutamine content in the liver. Again, this is in support of the proposition that hepatic CPS III, which utilizes glutamine as a substrate, was involved in urea synthesis in *P. dolloi*.

Arginine is a powerful activator of *N*-acetylglutamate synthetase (K_a=5–10 μmol l^{-1}), and it increases the V_{max} of the enzyme with no effect on the K_m value for the substrates (Shigesada and Tatibana, 1978). *N*-acetylglutamate is the product of the reaction catalysed by *N*-acetylglutamate synthetase, and CPS III has an absolute requirement for it (Campbell and Anderson, 1991). The arginine content in the liver of *P. dolloi* aestivated for 6 days was significantly higher than that of the control fasted for 6 days. This is in support of the proposition that urea synthesis rate increased during this period. By contrast, the hepatic arginine content became undetectable on day 40 of fasting or aestivation. Therefore, despite an increase in the OUC capacity in the fish on day 40 of aestivation, hepatic CPS III activity *in vivo* might be activated to a lesser extent after long-term aestivation (i.e. ≥40 days). These results are also in agreement with the observations that fasting can initiate physiological and biochemical changes similar to aestivation (Fishman et al., 1987).

Aestivation for 6 or 40 days led to a slight but significant increase in glutamine content in the brain of *P. dolloi*, indicating that a small amount of ammonia was detoxified through glutamine formation. Forty days of aestivation also led to a 57% decrease in the content of tryptophan, which is the amino acid precursor of serotonin (5-hydroxytryptamine), a neurotransmitter in the brain. The rate of serotonin synthesis is normally restricted by tryptophan availability in mammals (Boadle-Biber, 1982). In rainbow trout, increased dietary tryptophan increases brain serotonin levels (Johnston et al., 1990), indicating that the rate of serotonin synthesis can also be dependent upon tryptophan availability in fish. Interestingly, stress has been reported to increase brain tryptophan concentrations in mammals (Neckers and Sze, 1975; Dunn, 1988; Dunn and Welch, 1991). In mice, stress leads to an increase in serotonin release that depletes the existing serotonin stores (Dunn, 1988), and Dunn and Welch (1991) argued that the increase in brain tryptophan concentration during stress could counteract this depletion. From this information, it can be deduced that the decrease in the concentration of tryptophan in the brain of aestivating *P. dolloi* may indicate a decrease in brain serotonin, which can be an important aspect of the aestivation process in African lungfishes.

A comparative perspective

It was reported that there was no change in the rate of urea synthesis in African lungfishes (e.g. *P. aethiopicus*) that aestivate naturally in a subterranean mud cocoon or artificially in nylon bags (Janssens and Cohen, 1968a). Urea synthesis is energy intensive and it is possible that previous observations made on those African lungfishes were related to a limited energy supply in hypoxia during aestivation in mud or in an artificial aestivation apparatus. By contrast, *P. dolloi* aestivates in a thin layer of dried mucus on land, where the O_2 tension is high. It is probably because of this that *P. dolloi* is able to sustain a high rate of urea production throughout the 40 days of aestivation. Our results indicate that *P. dolloi* reduces ammonia production during 40 days of aestivation, but this is not necessarily equivalent to a reduction in metabolic (catabolic and anabolic) rate. Whether *P. dolloi* suppresses its metabolic rate during aestivation awaits future investigation,

but it is obvious from this study that its rate of urea synthesis (anabolic) is increased during this period, although it is energy intensive to do so.

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