The Journal of Experimental Biology 209, 4475-4489
Published by The Company of Biologists 2006
doi:10.1242/jeb.02557

Active ammonia transport and excretory nitrogen metabolism in the climbing perch, *Anabas testudineus*, during 4 days of emersion or 10 minutes of forced exercise on land

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Accepted 21 September 2006

Summary

The climbing perch, *Anabas testudineus*, inhabits large rivers, canals, stagnant water bodies, swamps and estuaries, where it can be confronted with aerial exposure during the dry season. This study aimed to examine nitrogen excretion and metabolism in this fish during 4 days of emersion. Contrary to previous reports, *A. testudineus* does not possess a functional hepatic ornithine-urea cycle because no carbamoyl phosphate synthetase I or III activity was detected in its liver. It was ammonotelic in water, and did not detoxify ammonia through increased urea synthesis during the 4 days of emersion. Unlike many air-breathing fishes reported elsewhere, *A. testudineus* could uniquely excrete ammonia during emersion at a rate similar to or higher than that of the immersed control. In spite of the fact that emersion had no significant effect on the daily ammonia excretion rate, tissue ammonia content increased significantly in the experimental fish. Thus, it can be concluded that 4 days of emersion caused an increase in ammonia production in *A. testudineus*, and probably because of this, a transient increase in the glutamine content in the brain occurred. Because there was a significant increase in the total essential free amino acid in the experimental fish after 2 days of emersion, it can be deduced that increased ammonia production during emersion was a result of increased amino acid catabolism and protein degradation. Our results provide evidence for the first time that *A. testudineus* was able to continually excrete ammonia in water containing 12 mmol l^{-1} NH_4Cl. During emersion, active ammonia excretion apparently occurred across the branchial and cutaneous surfaces, and ammonia concentrations in water samples collected from these surfaces increased to 20 mmol l^{-1}. It is probable that the capacities of air-breathing and active ammonia excretion facilitated the utilization of amino acids by *A. testudineus* as an energy source to support locomotor activity during emersion. As a result, it is capable of wandering long distance on land from one water body to another as reported in the literature.

Key words: ammonia, *Anabas testudineus*, emersion, nitrogen, metabolism, urea.

Introduction

The climbing perch *Anabas testudineus* (Bloch 1792) is a teleost belonging to the family Anabantidae and order Perciformes. *A. testudineus* can be found in tropical Asia (28°N to 10°S) from India to the Wallace line, including China. Found mostly in canals, lakes, ponds, swamps and estuaries, *A. testudineus* can tolerate extremely unfavourable water conditions (Pethiyagoda, 1991). It possesses a special accessory breathing organ (ABO), situated just above the gills in a large extension on the upper part of each gill chamber, which facilitates the utilization of atmospheric air (Graham, 1997). The ABO is composed of lamellae which are covered with an extremely vascular layer of skin and convoluted into numerous folds to maximize the surface area for respiration (Hughes and Munshi, 1973; Munshi et al., 1986). Because the ABO structure resembles a complicated maze, it has been dubbed the labyrinth organ. *A. testudineus* periodically gulps air from the water surface, and air is channelled to the labyrinth organs for gaseous exchange. During droughts, *A. testudineus* stays in pools associated with submerged woods and shrubs (Sokheng et al., 1999) or buries under the mud (Rahman, 1989). In addition, it is able to travel long distances on land (Das, 1927; Herre, 1935). It crawls about with its spiky gill covers propped by its pectoral fins, and the hind part of the fish

twitches violently to propel it forward (Liem, 1987; Davenport and Abdul Matin, 1990). When the air is sufficiently humid, it is able to cover several hundred meters per trip using this method of locomotion. To date, however, no information is available on the type of energy store utilized by the climbing perch to support locomotion on land.

Ammonia is produced in fish mainly through the catabolism of amino acids in the liver (Ip et al., 2001b), and is usually excreted as NH_3 through the gills (Wilkie, 2002; Evans et al., 2005). However, excretion of ammonia is impeded when a fish moves out of water, and ammonia subsequently accumulates in the body. Ammonia is toxic, and therefore amphibious air-breathing fishes have special adaptations to defend against ammonia toxicity (for reviews, see Ip et al., 2001b; Ip et al., 2004a; Chew et al., 2006). These adaptations include reduction in ammonia production through suppression of amino acid catabolism or *via* partial amino acid catabolism leading to the formation of alanine, conversion of ammonia to less toxic compounds such as glutamine for storage or urea for subsequent excretion, and volatilization of NH_3.

Saha and Ratha (Saha and Ratha, 1989) reported that *A. testudineus* possessed all the ornithine–urea cycle (OUC) enzymes, including carbamoyl synthetase I (CPS I) which uses ammonia as a substrate, in its liver. However, they (Saha and Ratha, 1989) should have determined the activity of CPS III, which uses glutamine as a substrate because fishes are known to possess CPS III instead of CPS I. These include both marine and freshwater elasmobranchs (Anderson, 2001; Tam et al., 2003), a few teleosts (Anderson and Walsh, 1995; Iwata et al., 2000; Randall et al., 1989), the coelacanth (Mommsen and Walsh, 1989) and African lungfishes (Chew et al., 2003b; Loong et al., 2005). Saha and Ratha (Saha and Ratha, 1987; Saha and Ratha, 1989) also reported the presence of hepatic CPS I in the Asian walking catfish *Clarias batrachus* and the Indian air-sac catfish *Heteropneustes fossilis*, but subsequently Saha et al. (Saha et al., 1997; Saha et al., 1999) suggested that both CPS I and CPS III were present in the liver. However, using a more sensitive radiometric assay, Ip et al. (Ip et al., 2004d) were unable to detect CPS I or CPS III activities from the liver of *C. batrachus* and *Clarias gariepinus* (for a review, see Chew et al., 2006). Therefore, this study was undertaken to determine whether a full complement of OUC enzymes, including CPS I and/or III, was indeed present in the liver of *A. testudineus*.

In addition, Saha and Ratha (Saha and Ratha, 1989) proposed that *A. testudineus* was able to detoxify ammonia to urea during emersion, but did not present any result to support their proposition. Although there was an earlier study (Ramaswamy and Reddy, 1983) on the same species (as *Anabas scandens*), these researchers did not examine nitrogenous excretion during emersion, nor did they determine the amount of ammonia and urea in the muscle, which constituted the bulk of the fish. Thus, another objective of this study was to examine whether urea accumulation occurred in *A. testudineus* during 4 days of emersion, and whether an increase in the rate of urea excretion would occur in fish re-immersed in water on day 5.

This study also aimed to elucidate whether *A. testudineus* would adopt, (1) a reduction in ammonia production, (2) partial amino acid metabolism leading to alanine formation, and/or (3) detoxification of ammonia to glutamine, to ameliorate ammonia toxicity during emersion. During the course of the study, it was discovered unexpectedly that *A. testudineus* was able to excrete ammonia continuously during emersion without difficulty, resulting in high concentrations of ammonia in a small volume of external medium. Therefore, an attempt was made to determine ammonia concentrations in water samples collected from the branchial or cutaneous surfaces of fish exposed to terrestrial conditions for 24 h. Efforts were also made to determine whether *A. testudineus* was capable of excreting ammonia against a concentration gradient in water containing 12 mmol l^{-1} NH_4Cl. Furthermore, because there were significant increases in tissue ammonia content and muscle total essential free amino acids in fish exposed to terrestrial conditions, experimental fish were exercised on land to explore the possibility that amino acids were utilized as substrates to fuel locomotor activities on land.

Materials and methods

Fish

Specimens of *Anabas testudineus* (Bloch 1792) (45–67 g body mass) were purchased from a local fish farm (in Singapore). Fish were maintained in plastic aquaria (22 cm × 11.5 cm × 13 cm, L × W × H) in 25 volumes (w/v) of dechlorinated tapwater at 25°C (control conditions; 0.68 mmol l^{-1} Na^+, 0.23 mmol l^{-1} K^+, 0.058 mmol l^{-1} Mg^{2+}, 0.51 mmol l^{-1} Ca^{2+}, and 1.6 mmol l^{-1} Cl^-; pH 6.8–7.0), and water was changed daily. No aeration was provided because *A. testudineus* is an air-breather. A layer of bubble wrap was attached to the inside surface of the lid, which acted as a cushion to prevent the fish from injuring themselves during jumping. Fish were acclimated to laboratory conditions for at least 1 month. During the adaptation period, *A. testudineus* was fed an artificial pellet diet. Food was withdrawn 2 days prior to experiments, which gave sufficient time for the gut to be emptied of all food and waste. The wet mass of the fish was obtained to the nearest 1 g using a Shimadzu animal balance (Shimadzu Co., Kyoto, Japan). All experiments in this study were performed under a 12 h:12 h dark:light regime.

Determination of activities of ornithine-urea cycle enzymes and glutamine synthetase from the liver

Fish in control conditions (N=4) were killed with a strong blow to the head. The liver was 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, 0.5 mmol l^{-1} EDTA, 1 mmol l^{-1} dithiothreitol and 0.5 mmol l^{-1} PMSF. The homogenate was then 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 and PMSF. The filtrate obtained was used directly for enzymes assay.

Carbamoyl phosphate synthetase I and III (E.C. 2.7.2.5; CPS) activities were determined according to published methods (Anderson and Walsh, 1995; Chew et al., 2003b; Loong et al., 2005). The CPS III activity was determined in the presence of glutamine, *N*-acetylglutamate and UTP in order to eliminate the activity of CPS II. 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 (E.C. 2.1.3.3) activity was determined by combining the methods of Anderson and Walsh (Anderson and Walsh, 1995) and Xiong and Anderson (Xiong and Anderson, 1989). Absorbance was measured at 466 nm using a Shimadzu 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 lyases (E.C. 4.3.2.1) activities were determined together assuming that both were present, by measuring the formation of [^{14}C]fumarate from [^{14}C]aspartate using the method of Cao et al. (Cao et al., 1991), and 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 (Felskie et al., 1998) and activity was expressed as μmol urea formed min^{-1} g^{-1} wet mass. Glutamine synthetase (E.C. 6.3.1.2) was assayed as transferase activity according to the method of Shankar and Anderson (Shankar and Anderson, 1985) and activity was expressed as μmol γ-glutamylhydroxymate formed min^{-1} g^{-1} wet mass.

Determination of effects of emersion on ammonia and urea excretion rates

After 2 days of fasting to clear the gut (0 h), control fish (*N*=12) were exposed individually to 25 volumes (w/v; approximately 1.4 l) of freshwater in plastic containers (22 cm×11.5 cm×13 cm, L×W×H). Water samples (3.6 ml) were collected every 24 h, acidified with 0.04 ml of 2 mol l^{-1} HCl, and stored at 4°C. Water was changed daily after sampling and the experiment was continued for 4 days. Ammonia and urea concentrations in water samples were determined within 1 week according to the method of Anderson and Little (Anderson and Little, 1986) and the method of Jow et al. (Jow et al., 1999), respectively.

Experimental fish (*N*=12) were transferred individually after 2 days of fasting to similar plastic containers (22 cm×11.5 cm×13 cm, L×W×H) but with only 80 ml of freshwater, which formed a thin film at the bottom. The experimental conditions were designed to examine effects of emersion, but not desiccation. Under such experimental conditions, the fish could not maintain its normal posture and usually lay on its side at the bottom of the tank. In spite of this, the gills and the ABOs were not in direct contact with the thin film of water due to the presence of spikes on the surface of the opercula, which were puffed open to facilitate air-breathing. Very often, the fish produced jerking movements through flipping of its tail, and occasionally it would be able to right itself by leaning against the side wall of the tank. This happened especially during the first day of emersion. After 24 h, 50 ml of water was poured over the fish for brief rinsing (<1 min), and the total amount of water was determined using a measuring cylinder. An aliquot of the water sample was acidified and stored at 4°C for subsequent ammonia and urea assays. Fish were then transferred to new plastic tanks containing 80 ml of freshwater. The experiment continued for 4 days.

To evaluate the effects of recovery in water on fish after 4 days of emersion, fish (*N*=6) were exposed to the control conditions, as described above, for 5 days. Experimental fish were exposed to terrestrial conditions, as described above, for 4 days, and on day 5, they were re-immersed in 25 volumes of freshwater and water samples were collected 24 h later for ammonia and urea assays.

To verify that ammonia and urea excretion rates obtained were not affected by bacterial activities, several water samples (20 ml without acidification) were left at 25°C in glass beakers for 24 h. Preliminary results confirmed that the ammonia and urea concentrations in the water sample before and after the 24 h of incubation at 25°C were not statistically different. Ammonia and urea excretion rates were expressed as μmol day^{-1} g^{-1} fish.

Because results obtained indicated unexpectedly that the climbing perch could excrete a large amount of ammonia into the thin film of water during emersion, a separate set of experiments was performed following the protocol described above, except that there was no change of water in the tank and the experiment lasted only 2 days. In this set of experiments, we aimed to determine how high the concentration of ammonia could reach in the external medium after 48 h. A 2-day period was chosen because preliminary experiments revealed that bacterial activities could become an important factor if the experimental period were extended beyond 2 days.

Determination of ammonia concentrations in water collected from branchial or cutaneous surfaces in fish after 15 min or 24 h of emersion

Control fish (hour 0; *N*=5) were taken out of water and anaesthetized immediately on land in a saturated atmosphere of diethylether, which took approximately 15 min. Experimental fish (*N*=5) were exposed to terrestrial conditions in plastic containers (14.7 cm×8.2 cm×9.1 cm, L×W×H) with 40 ml of freshwater for 24 h before being anaesthetized with diethylether. Because one side of the fish was in direct contact with the thin film of water, it was important to take note of which side of the fish was exposed to air. The opercular opening of the air-facing side of the anaesthetized fish was open and a small piece of pre-weighed glass microfibre paper (2 mm×10 mm; Whatman GF/C) was applied gently to the surface of each gill arch to absorb the water. The volume of water absorbed was determined gravimetrically. This

procedure was repeated a second time with a new piece of fibreglass paper. The two pieces of glass microfibre paper were transferred immediately into 0.5 ml of water acidified with 2 μl of 1 mol l^{-1} HCl for subsequent ammonia assay. Similarly, a water sample was collected from the surface of the air-facing skin using pre-weighed glass microfibre paper (4 mm × 10 mm) and transferred to 0.5 ml of acidified water. Efforts were made to absorb water from below the scales by pressing the fibreglass paper lightly along the body surface. Then, the fish was flipped over carefully, and the procedure of water collection was repeated. The whole process took approximately 10 min. Ammonia was assayed as described above, and results were expressed as mmol l^{-1}.

Determination of the capacity to excrete ammonia against a concentration gradient

The normal ammonia and urea excretion rates of fish fasted for 48 h (N=7) were determined as described above. They were then transferred individually into Ziplock plastic bags (double layered) containing 5 volumes (w/v) of 12 mmol l^{-1} NH_4Cl at pH 7.0. Under such conditions, the fish was completely immersed in the medium and maintained in the upright position inside the bag. The opening of the bag was partially clipped to prevent the fish from escaping. Water samples were collected as described above every 24 h for a total period of 2 days for the estimation of ammonia and urea excretion rates.

Determination of effects of emersion on content of tissue ammonia, urea and free amino acids

A total of 24 fish were kept in the control condition as described above. Fish [(N=6) for each time point] were killed with a strong blow to the head at the end of days 1, 2, 4 and 5, for tissue sampling. Blood was collected from the severed caudal artery into sodium heparin-coated capillary tubes. The collected blood was centrifuged at 4000 ***g*** at 4°C for 10 min to obtain the plasma. The plasma was deproteinized in an equal volume (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 for analysis of ammonia, urea and free amino acids (FAAs). The muscle, liver and brain tissues were excised and immediately freeze-clamped with tongs pre-cooled in liquid nitrogen. Frozen samples were stored at –80°C until analysis.

Experimental fish (N=24) were killed at the end of days 1, 2 and 4 of emersion (N=6 each) for tissue sampling as described above. After 4 days of emersion, the remaining six fish were immersed in 25 volumes (w/v) of freshwater, and were killed for tissue sampling after 24 h of immersion.

To determine ammonia, urea and FAA content, the frozen liver, muscle and brain samples were weighed and ground to a powder in liquid nitrogen. Five volumes of ice-cold 6% TCA were added and the mixture was homogenized three times for 20 s each (with intervals of 10 s) with an Ultra-Turrax (Staufen, Germany) homogenizer at 24 000 revs min^{-1}. The samples were then centrifuged for 15 min at 10 000 ***g*** at 4°C and the supernatant were stored at –80°C for subsequent analysis. Ammonia and urea assays as well as FAA analysis were performed within 3 weeks. For analysis of ammonia, the pH of the deproteinized sample was adjusted to 6.0–7.0 with 2 mol l^{-1} $KHCO_3$. Ammonia and urea content in the muscle, liver and plasma samples were determined as described by Bergmeyer and Beutler (Bergmeyer and Beutler, 1985) and Jow et al. (Jow et al., 1999), respectively. Results were expressed as μmol g^{-1} wet mass tissue or μmol ml^{-1} plasma. For FAA analysis (N=4), the supernatant of the deproteinized muscle and liver samples were diluted with an equal volume of 0.2 mol l^{-1} lithium citrate buffer (pH 2.2) and adjusted to pH 2.2 with 4 mol l^{-1} LiOH. The filtered samples were then analyzed for FAAs using a Shimadzu LC-6A Amino Acid Analysis System with a Shim-pack ISC-07/S1504 Li-type column (Kyoto, Japan). FAAs, total FAA (TFAA) and total essential FAA (TEFAA) were expressed as μmol g^{-1} wet mass tissue.

Determination of effects of 10 min of exercise on A. testudineus during emersion

Fish (N=10) were transferred individually after 2 days of fasting to plastic aquaria containing 10 ml of freshwater, which formed a thin film at the bottom. Control fish (N=5) for this set of experiment were left inside the container without any disturbance. The control fish naturally exhibited some jerky movements, albeit much less frequently than the experimental fish. After 10 min, 50 ml of water was added to briefly rinse the fish. Water samples were collected for ammonia and urea assays as described above. Fish were killed with a strong blow to the head for the collection of muscle samples. Experimental fish (N=5) were stimulated mechanically and continuously, which resulted in frequent jerky movements, within the plastic aquaria. After 10 min, water samples were collected and the fish was killed for muscle collection.

Ammonia, urea and FAA were determined in the muscle samples as described above. Despite analysing all the FAAs, only content of alanine, glutamate, lysine, TEFAA and TFAA are presented. Glucose and glycogen content were determined according to the method of Lim and Ip (Lim and Ip, 1987). Lactate and succinate content were determined by the method of Gutmann and Wahlefeld (Gutmann and Wahlefeld, 1974) and Beutler (Beutler, 1985), respectively. ATP, ADP and AMP were determined spectrophotometrically following the method of Scheibel et al. (Scheibel et al., 1968).

Determination of O_2 consumption rate

In this set of experiments, the volume of the fish was determined by water displacement. Control fish (N=4) were transferred to air-tight plastic boxes (13.5 cm × 7 cm × 8.5 cm, L × W × H) containing 400 ml of water with continuous aeration 24 h before the experiment. For the determination of O_2 consumption rate, the box, containing 400 ml of water, 120 ml of air and the fish was sealed. The air space was reduced to 120 ml by the inclusion of a piece of polystyrene (Styrofoam) which had a thickness of 1.85 cm and a volume of 100 ml. The polystyrene was positioned in the box so that the fish was restricted to respire through an air space closest to the O_2

sensor. Water in the box was stirred slowly with a magnetic bar. Changes in aerial and aquatic P_{O_2} were monitored using an Ocean Optics FOXY Fiber Optics O_2 sensing system S2000 with two FOXY-R O_2 electrodes (Ocean Optics Inc., Dunedin, FL, USA) inserted separately into the air and water compartments. For the control fish, two measurements were made for 30 min each, with a 60 min interval during which the water was aerated. As for the experimental fish (N=4), they were transferred directly from water into air tight plastic boxes (13.5 cm×7 cm×4.2 cm, L×W×H), which contained 210 ml of air and 3 ml of water. Two pieces of polystyrene which each had a thickness of 1.85 cm and a volume of 100 ml were put inside the box to reduce the air volume. The O_2 consumption rate in air was monitored with a FOXY-R O_2 electrode. Another set of experimental fish (N=4) was exposed to terrestrial conditions for 24 h before the determination of the O_2 consumption rate.

To calibrate the FOXY-R O_2 electrode for measurements of water P_{O_2}, it was immersed in sodium sulphite solution (2 g l^{-1}; 0%) or air-saturated water (100%) until stable readings were recorded. For air P_{O_2} measurements, the electrode was calibrated in a partially closed tube supplied continuously with humidified N_2 gas (0%) or air (100%).

Statistical analyses

Results are presented as means ± standard errors (s.e.m.). Results shown in Fig. 1 were analyzed using repeated-measures analysis of variance (ANOVA) followed by least-square means (LSMEANS) to evaluate differences between means. Results in Tables 1 and 2 were assessed using two-way ANOVA followed by Bonferroni's multiple range test to evaluate differences between means, and those in Fig. 2 and Tables 3–6 were analyzed by Student's t-test. Differences with P<0.05 were regarded as statistically significant.

Results

Activities of the ornithine-urea cycle enzymes and glutamine synthetase from the liver

No carbamoyl phosphate synthetase I or III activity was detected (detection limit=0.001 μmol min^{-1} g^{-1} tissue; N=4) in the liver tissue from *A. testudineus* immersed in water. As for ornithine transcarbamoylase, argininosuccinate synthetase + lyase, argainase and glutamine synthetase, only very low activities (0.10±0.02, 0.006±0.001, 3.3±0.9 and 0.054±0.012 μmol min^{-1} g^{-1} tissue, respectively; N=4) were detected.

Effects of emersion on rates of ammonia and urea excretion

With daily change of water, the ammonia concentration in the thin film of water (80 ml) reached approximately 4–5 mmol l^{-1} after 24 h throughout the 4-day period of emersion. This indicates that the rate of ammonia excretion in *A. testudineus* during emersion was high. Indeed, the daily ammonia excretion rates (N=12) in *A. testudineus* on days 1, 3 and 4 of emersion were not significantly different from those of the control immersed in water (Fig. 1A). Surprisingly, on day 2, the daily ammonia excretion rate of the fish exposed to terrestrial conditions was significantly greater than that of the control. When summed together over a 2-day period, however, the excretion rate of 11.5±2.1 μmol 2 $days^{-1}$ g^{-1} was not significantly different from the control value of 13.9± 0.7 μmol 2 $days^{-1}$ g^{-1}. Similarly, the total rate of ammonia excretion in the experimental (24.1±4.6 μmol 4 $days^{-1}$ g^{-1}) and control (32.2±1.9 μmol 4 $days^{-1}$ g^{-1}) fish over a 4-day period were comparable. Upon re-immersion on day 5, the daily ammonia excretion rate (N=6) of the experimental fish was not significantly different from that of the immersed control (Fig. 1A).

By contrast, in spite of significant increases, the urea concentration in the thin film of water remained relatively low throughout the 4-day emersion period. Emersion led to a significant decrease in the daily urea excretion rate on day 1, but had no significant effect on urea excretion thereafter (Fig. 1B). There was no significant change in urea excretion in fish during re-immersion on day 5 as compared with day 1 or day 5 controls (Fig. 1B).

In a separate set of experiments in which daily change of water was omitted for a 2-day period, the ammonia concentration (N=5) in the thin film of water reached 6.68±1.11

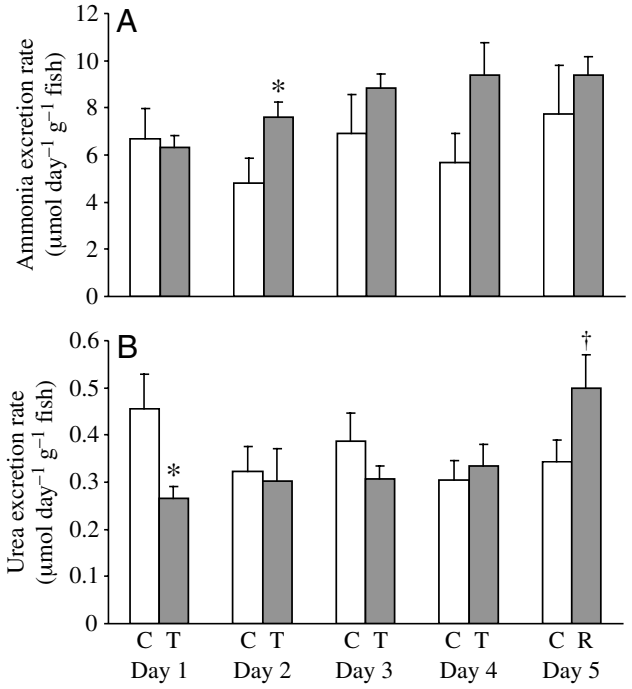

Fig. 1. Rates (μmol day^{-1} g^{-1} fish) of (A) ammonia and (B) urea excretion in *Anabas testudineus* immersed in water (control; C) for 5 days (N=12 for days 1 to 4 and N=6 for day 5) or exposed to terrestrial conditions (T) for 4 days (N=12) followed by 1 day (N=6) of re-immersion in water. Values are means ± s.e.m. *Significantly different from the corresponding freshwater control condition. †Significantly different from the corresponding day 1, terrestrial condition.

and 13.2±2.1 mmol l^{-1} at the end of day 1 and day 2, respectively. The respective daily ammonia excretion rates were 9.98±1.92 and 8.64±1.85 μmol day^{-1} g^{-1} fish, which were not significantly different from the corresponding values (7.45±1.32 and 7.54±1.21 μmol day^{-1} g^{-1} fish, respectively) of the control fish (*N*=5) immersed in water.

Ammonia concentrations in water samples collected from branchial or cutaneous surfaces of fish after 15 min or 24 h of emersion

The ammonia concentration in water samples collected from the branchial surface of the air-facing side of the fish after 24 h of emersion (*N*=5) was not significantly different from those of the control exposed to terrestrial conditions for 15 min during anaesthesia (*N*=5; Fig. 2). However, for the water-facing side of the experimental fish, the ammonia concentration in the branchial water increased to 21.5±2.4 mmol l^{-1}, which was significantly greater than that of the control fish (Fig. 2). In water samples collected from the air-facing cutaneous surface of the experimental fish, the ammonia concentration varied greatly and was not significantly different from the control value (Fig. 2). By contrast, the ammonia concentration (20.8±3.5 mmol l^{-1}) in water samples collected from the water-facing cutaneous surface was significantly greater than that of the control. The concentration of ammonia in the thin film of water at the bottom (80 ml) was 5.32±0.87 mmol l^{-1}.

Effects of environmental ammonia (12 mmol l^{-1}) on rates of ammonia and urea excretion

The ammonia excretion rate of *A. testudineus* (*N*=7) in normal freshwater without NH_4Cl was 7.12±1.04 μmol day^{-1} g^{-1} fish. At the beginning of the experiment, the ambient ammonia concentration without any fish was 12.3 mmol l^{-1}.

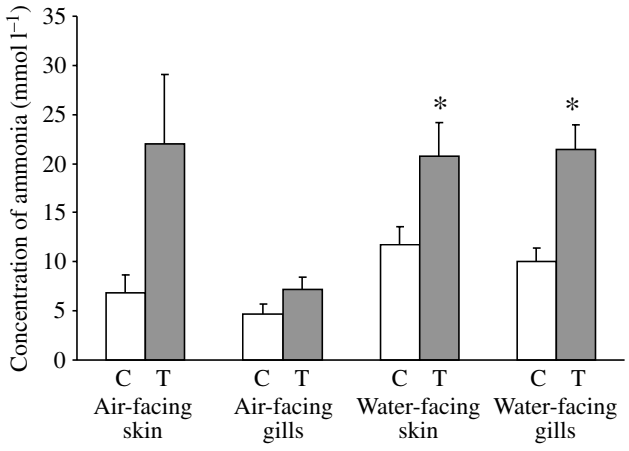

Fig. 2. Concentrations (mmol l^{-1}) of ammonia in water samples collected from the branchial or cutaneous surfaces of the air-facing side or the water-facing side of *Anabas testudineus* immersed in water and anaesthetized for 15 min on land (control; C) or exposed to terrestrial conditions for 24 h followed with 15 min of anesthetization on land (T). *Significantly different from the corresponding control condition.

With an initial pH of 7.0, the NH_4^+ and NH_3 concentrations were calculated to be 12.19 and 0.11 mmol l^{-1}, respectively. In comparison, the concentrations of NH_4^+ and NH_3 in the plasma were 0.188 and 0.012 mmol l^{-1}, respectively, taking the plasma ammonia concentration to be 0.2 mmol l^{-1} (from Table 1) and the blood pH to be 7.6 (Y.K.I., unpublished results). So, both the NH_4^+ and NH_3 gradients were directed inwards; but, surprisingly, the experimental fish could excrete ammonia against such a large ammonia gradient. As a result, the concentration of ammonia in the external medium increased to 13.1±0.2 mmol l^{-1} at the end of day 1. A simple calculation reveals that the ammonia excretion rate decreased significantly to 4.31±0.81 μmol day^{-1} g^{-1} fish (*N*=7) during this 24 h period. On day 2, the ambient ammonia concentration increased further to 14.7±0.3 mmol l^{-1}, and the ammonia excretion rate (8.32±1.44 μmol day^{-1} g^{-1} fish; *N*=7) returned back to a level comparable with the initial control value.

Effects of emersion on the contents of ammonia, urea and FAAs

Ammonia in the muscle and liver of *A. testudineus* (*N*=6) increased significantly during emersion and peaked at 4.06 μmol g^{-1} on day 2 (4.5-fold of the control value) and 10.7 μmol g^{-1} on day 1 (5.5-fold of the control value), respectively (Table 1). The ammonia concentration in the plasma of *A. testudineus* increased significantly during the first 2 days of emersion, but returned to the control level thereafter (Table 1). Although urea also increased significantly in the muscle and liver of *A. testudineus* during emersion, the peak levels (0.79 μmol g^{-1} and 0.81 μmol g^{-1} on day 2, respectively) attained were much lower than those of ammonia (Table 2).

Emersion led to significant increases in isoleucine, leucine, phenylalanine, tyrosine and valine in the muscle of *A. testudineus* (*N*=4) (Table 3). By contrast, the aspartate and glutamine content of muscle in fish emersed for 2 or 4 days were significantly lower than the corresponding control value. Although emersion had no significant effect on the muscle TFAA, there was a significant increase in the muscle TEFAA in fish exposed to 2 days of emersion. Two days of emersion led to a significant increase (8.8-fold) in the lysine content of the liver of *A. testudineus* (Table 4). However, 4 days of emersion resulted in significant increases in arginine and phenylalanine in the liver. In addition, there was a significant decrease in the glutamate in the liver of fish after 2 or 4 days of emersion. After 2 days of emersion, the brain glutamine content of *A. testudineus* increased significantly by 2.5-fold (Table 5). There were also significant decreases in alanine, aspartate and glutamate in the brain of fish during the first 2 days of emersion. However, the TFAA and TEFAA content in the liver and brain of *A. testudineus* were unaffected by emersion.

Effects of 10 min of forced exercise on land

Forced exercise for 10 min on land led to significant increases in excretion of ammonia (0.103±0.009 μmol 10 min^{-1} g^{-1} fish) and urea (0.015±0.002 μmol 10 min^{-1} g^{-1} fish) as compared with the control on land but without exercise

Table 1. *Ammonia content of muscle, liver, brain and plasma of Anabas testudineus immersed in freshwater for 5 days (control) or exposed to terrestrial conditions for 4 days followed by 1 day of re-immersion in water*

		Ammonia content			
Tissue	Condition	Day 1	Day 2	Day 4	Day 5 (recovery)
Muscle	Control	0.81±0.16	0.86±0.26	1.05±0.18	0.97±0.15
	Terrestrial	3.89±0.54*	4.06±0.71*	2.43±0.77	1.79±0.43
Liver	Control	1.95±0.42	3.81±0.80	3.28±0.50	3.05±0.30
	Terrestrial	10.7±1.9*	8.86±1.63*	5.88±0.67*,†	1.64±0.62†,‡,§
Brain	Control	1.33±0.11	1.56±0.13	2.23±0.39	1.29±0.09
	Terrestrial	3.11±0.13	4.10±0.76*,†	2.72±0.24‡	1.19±0.19†,‡,§
Plasma	Control	0.19±0.03	0.18±0.02	0.25±0.04	0.19±0.05
	Terrestrial	0.58±0.08*	0.67±0.13*	0.37±0.05†,‡	0.25±0.10†,‡

Values are in μmol g^{-1} tissue or μmol ml^{-1} plasma (means ± s.e.m., N=6).
*Significantly different from the corresponding control condition (P<0.05).
†Significantly different from day 1 terrestrial values (P<0.05).
‡Significantly different from day 2 terrestrial values (P<0.05).
§Significantly different from day 4 terrestrial values (P<0.05).

(0.047±0.014 μmol g^{-1} fish, and 0.003±0.001 μmol g^{-1} fish, respectively). As a result, the concentration of ammonia in the thin film of water in the container with the exercised fish reached 1.79±0.05 mmol l^{-1} which was significantly higher than that of the control (0.86±0.20 mmol l^{-1}). In addition, there were significant increases in ammonia, alanine, lysine and TEFAA in the muscle of fish after forced exercise on land as compared with the control fish exposed to terrestrial conditions for 10 min without disturbance (Table 6). However, forced exercise on land did not have any significant effect on ATP, ADP, AMP, glucose, glycogen, lactate and succinate in the muscle of *A. testudineus* (Table 6).

Rate of O_2 consumption

For the control fish in freshwater (N=4), the O_2 consumption rates in water and air were 1.38±0.13 and 3.10±0.54 μmol h^{-1} g^{-1} fish, respectively. Taken together, the total O_2 consumption rate was 4.47±0.48 μmol h^{-1} g^{-1} fish. For fish exposed to terrestrial conditions (N=4 for each group) for 1, 24 or 48 h, the O_2 consumption rates in air were 5.25±0.61, 4.67±0.22, and 6.43±0.53 μmol h^{-1} g^{-1} fish, respectively, and the value obtained from those exposed to terrestrial conditions for 48 h of emersion was significantly greater than that of the immersed control.

Construction of a balance sheet of ammonia and urea-N excretion and ammonia and urea-N retention in a 50 g fish

A 50 g fish contained approximately 30 g of muscle and 1 g of liver. Based on our results, a balance sheet was constructed for changes in nitrogen excretion and changes in ammonia-N and urea-N content in a 50 g fish after 2 or 4 days of immersion or emersion (Table 7). Although there was no significant

Table 2. *Urea content of muscle, liver brain and plasma of Anabas testudineus immersed in freshwater for 5 days (control), or exposed to terrestrial conditions for 4 days followed by 1 day of re-immersion in water*

		Urea content			
Tissue	Condition	Day 1	Day 2	Day 4	Day 5 (recovery)
Muscle	Control	0.25±0.06	0.11±0.02	0.19±0.03	0.29±0.06
	Terrestrial	0.59±0.10*	0.79±0.08*	0.77±0.12*	0.45±0.06
Liver	Control	0.20±0.04	0.15±0.03	0.29±0.03	0.39±0.08
	Terrestrial	0.64±0.09*	0.81±0.12*	0.68±0.09*	0.43±0.06‡,§
Brain	Control	0.13±0.03	0.15±0.01	0.53±0.04	0.36±0.06
	Terrestrial	0.58±0.11*	0.83±0.10	0.61±0.10	0.06±0.11‡,§
Plasma	Control	0.31±0.04	0.19±0.03	0.30±0.07	0.48±0.11
	Terrestrial	1.00±0.16	1.29±0.23*	1.26±0.18	0.55±0.07†,‡,§

Values are in μmol g^{-1} tissue or μmol ml^{-1} plasma (means ± s.e.m., N=6).
*Significantly different from the corresponding control condition (P<0.05).
†Significantly different from day 1 terrestrial values (P<0.05).
‡Significantly different from day 2 terrestrial values (P<0.05).
§Significantly different from day 4 terrestrial values (P<0.05).

Table 3. *Free amino acid, total free amino acid and total essential free amino acid content of muscle of Anabas testudineus immersed in freshwater (control) or exposed to terrestrial conditions for 2 or 4 days*

	Content (μmol g^{-1} tissue)			
	Day 2		Day 4	
FAAs	Control	Terrestrial	Control	Terrestrial
Alanine	1.31±0.24	2.01±0.53	3.20±0.24	2.12±0.42
Arginine	0.64±0.16	0.45±0.12	0.52±0.13	0.55±0.08
Asparagine	0.32±0.06	0.35±0.07	0.30±0.03	0.26±0.02
Aspartate	0.20±0.04	0.059±0.017*	0.12±0.06	0.028±0.010*
Glutamine	2.0±0.3	0.78±0.05*	1.7±0.1	1.36±0.18*
Glutamate	0.65±0.17	0.49±0.06	0.12±0.01	0.14±0.01
Glycine	11±3	10±2	11±2	7.5±1.0
Histidine	0.66±0.8	0.64±0.09	0.66±0.07	0.42±0.04
Isoleucine	0.097±0.031	0.49±0.08*	0.080±0.018	0.40±0.09*
Leucine	0.19±0.05	0.84±0.14*	0.16±0.02	0.71±0.15*
Lysine	1.01±0.24	0.46±0.13	1.57±0.36	1.40±0.26
Phenylalanine	0.073±0.021	0.33±0.07*	0.062±0.005	0.29±0.06*
Proline	n.d.	0.45±0.24	0.44±0.04	0.38±0.07
Serine	0.98±0.22	1.08±0.33	0.67±0.05	0.55±0.04
Taurine	14±2	10±3	10±3	11±2
Threonine	1.34±0.17	1.73±0.57	1.09±0.19	0.75±0.11
Tyrosine	0.052±0.013	0.25±0.05*	0.038±0.004	0.18±0.03*
Valine	0.16±0.04	0.64±0.09*	0.18±0.04	0.58±0.12*
TFAA	35±2	33±2	30±3	29±1
TEFAA	4.29±0.57	6.57±0.62*	3.89±0.70	5.48±0.85

FAAs, free amino acids; TFAA, total FAA; TEFAA, total essential FAA.
Values means ± s.e.m., *N*=4.
n.d., not detectable.
*Significantly different from the value of the corresponding control.

change in the overall ammonia excretion rate during 4 days of emersion, we took into consideration the small changes involved and presented them in Table 7. After considering the amount of ammonia-N and urea-N stored in the muscle and liver, it becomes apparent that there was an increase in nitrogen production in *A. testudineus* after 2 or 4 days of emersion.

Discussion

A. testudineus is ammonotelic and does not possess a functional ornithine-urea cycle in the liver

A. testudineus does not possess a functional OUC in its liver, as evidenced by the absence of detectable activities of CPS I or III based on the radiometric assay (Anderson and Walsh, 1995) adopted in this study. Thus, our results contradict those of Saha and Ratha (Saha and Ratha, 1989), and the discrepancy could be a result of differences in methodology. Saha and Ratha assayed for CPS I activity in the liver extract of *A. testudineus* using a colorimetric method that involved a coupled enzyme system, the accuracy of which depends heavily on the purity of the coupled enzymes obtained commercially. The radiometric assay adopted in this study is less prone to interference and more sensitive than the colorimetric method. Because of its high sensitivity, the radiometric assay is more specific, being able to differentiate CPS II and CPS III in the presence of UTP, and is therefore more reliable. Incidentally, using the same radiometric assay, Ip et al. (Ip et al., 2004d) were unable to detect CPS I or CPS III in the livers of *C. gariepinus* and *C. batrachus*, although their presence has been reported previously in the latter species based on a colorimetric method (Saha and Ratha, 1989; Saha et al., 1999). Similarly, activities of argininosuccinate synthetase and argininosuccinate lyase reported by Saha and Ratha (Saha and Ratha, 1989) for *A. testudineus* (0.32 μmol h^{-1} g^{-1} tissue and 0.21 μmol h^{-1} g^{-1} tissue, respectively) using two separate colorimetric methods were much higher than the combined activity of argininosuccinate synthetase + argininosuccinate lyase (0.006 μmol h^{-1} g^{-1} tissue) reported herein, based on a more sensitive and specific radiometric assay.

Unlike other ureogenic fishes which have CPS III in the liver, the hepatic glutamine synthetase activity of *A. testudineus* was very low. Thus, together with the absence of detectable CPS I or III activities (detection limit=0.001 μmol min^{-1} g^{-1} tissue), it can be concluded that *A. testudineus* is non-ureogenic and hence highly unlikely to be ureotelic. Indeed, *A. testudineus* is ammonotelic in water, excreting 94% of its nitrogenous wastes (ammonia-N + urea-N) as ammonia-N.

Contrary to the belief that there is a tendency towards predominance of ureotelism in amphibious air-breathing

Table 4. *Free amino acid, total free amino acid and total essential free amino acid content of liver of Anabas testudineus immersed in freshwater (control) or exposed to terrestrial conditions for 2 or 4 days*

	Content (μmol g^{-1} tissue)			
	Day 2		Day 4	
FAAs	Control	Terrestrial	Control	Terrestrial
Alanine	0.43±0.07	1.38±0.74	1.13±0.68	1.65±0.54
Arginine	0.069±0.020	0.19±0.06	0.13±0.04	0.24±0.01*
Asparagine	0.088±0.014	0.101±0.018	0.073±0.011	0.086±0.023
Aspartate	0.48±0.11	1.06±0.20	0.71±0.34	0.85±0.13
Glutamine	0.78±0.25	1.01±0.35	0.82±0.31	1.20±0.21
Glutamate	4.5±0.8	1.8±0.7*	5.2±0.6	2.6±0.5*
Glycine	0.73±0.17	0.74±0.07	0.61±0.10	0.88±0.07
Histidine	0.28±0.01	0.33±0.04	0.51±0.14	0.52±0.06
Isoleucine	0.075±0.009	0.080±0.024	0.17±0.04	0.18±0.05
Leucine	0.15±0.01	0.17±0.03	0.34±0.06	0.39±0.11
Lysine	0.077±0.017	0.68±0.17*	0.25±0.06	0.36±0.01*
Phenylalanine	0.061±0.006	0.10±0.01	0.060±0.009	0.13±0.03*
Proline	0.37±0.09	0.36±0.10	0.30±0.16	0.43±0.09
Serine	0.28±0.07	0.50±0.16	0.30±0.10	0.50±0.10
Taurine	19±1	14±2	17±4	18±1
Threonine	0.33±0.02	0.76±0.25	0.74±0.33	0.58±0.02
Tyrosine	0.037±0.005	0.043±0.009	0.059±0.022	0.082±0.009
Valine	0.13±0.01	0.18±0.05	0.29±0.05	0.34±0.10
TFAA	27.9±1.4	26.2±3.1	26.4±1.9	28.8±1.5
TEFAA	1.55±0.17	2.08±0.17	1.86±0.33	1.92±0.19

FAAs, free amino acids; TFAA, total FAA; TEFAA, total essential FAA.
Values are means ± s.e.m., *N*=4.
*Significantly different from the value of the corresponding control.

teleosts (Walsh, 1997; Saha and Ratha, 1998; Wright and Land, 1998; Sayer, 2005), most adult tropical amphibious teleosts studied so far are predominantly ammonotelic in water (Graham, 1997; Ip et al., 2004a). More importantly, they do not detoxify ammonia through enhanced ureogensis when exposed to terrestrial conditions (for reviews, see Ip et al., 2001b; Ip et al., 2004a; Ip et al., 2004b; Chew et al., 2006). In a recent review on tropical fishes (Sayer, 2005), results on increased urea excretion in a few species of mudskippers upon return to water after a period of emersion were cited as support for a physiological role of urea in ammonia detoxification in these fishes during emersion. However, the fact remains that these mudskippers are non-ureogenic and they do not possess a full complement of OUC enzymes in their livers (Gregory, 1977; Lim et al., 2001). Although a complete OUC with very low CPS activity is present in the liver of the giant mudskipper, *Periophthalmodon schlosseri* (Lim et al., 2001), increased urea synthesis has a very minor role in ammonia detoxification in fish exposed to terrestrial conditions (Ip et al., 1993; Ip et al., 2001c), alkaline pH (Chew et al., 2003a) or high concentrations of environmental ammonia (Peng et al., 1998; Randall et al., 1999). In another recent review (Eddy, 2005), it was reiterated that urea was produced by the African catfish *C. gariepinus* (as *C. mossambicus*) and the Indian air-sac catfish *H. fossilis* during air exposure. However, *C. gariepinus* is non-ureogenic and does not possess a functional OUC (Ip et al., 2004d); it does not accumulate urea during emersion either (Ip et al., 2005). As for *H. fossilis*, it is debatable if increased urea synthesis plays a major role in ammonia detoxification during emersion based on results reported in the literature (for a review, see Chew et al., 2006). More importantly, we were unable to detect CPS I or III activity from the liver of *H. fossilis* using the same radiometric assay as in this study (Y.K.I. and S.F.C., unpublished results).

Likewise, contrary to a previous proposition (Saha and Ratha, 1989), our results confirm that increased urea synthesis and excretion was not adopted by *A. testudineus* to detoxify ammonia during 4 days of emersion. In fact, the urea-N excreted by *A. testudineus* during emersion accounted for <4% of the total waste-N (ammonia-N + urea-N), and there was no significant increase in urea excretion when the fish was re-immersed in water on day 5. In addition, there were only minor increases in urea content in tissues of *A. testudineus* after 4 days of emersion despite large increases in tissue ammonia. Because a functional OUC is absent from the liver of *A. testudineus*, it is logical to deduce that the urea accumulated in the tissues of *A. testudineus* during emersion originated from argininolysis and/or purine catabolism followed with uricolysis.

Table 5. *Free amino acid, total free amino acid and total essential free amino acid content of the brain of Anabas testudineus immersed in freshwater (control) or exposed to terrestrial conditions for 2 or 4 days*

	Content (μmol g^{-1} tissue)			
	Day 2		Day 4	
FAAs	Control	Terrestrial	Control	Terrestrial
Alanine	0.43±0.03	0.20±0.02*	0.50±0.08	0.29±0.03*
Arginine	0.095±0.008	0.11±0.02	0.14±0.02	0.11±0.03
Asparagine	0.049±0.005	0.062±0.011	0.049±0.011	0.081±0.006
Aspartate	0.44±0.04	0.13±0.02*	0.48±0.06	0.19±0.03*
Glutamine	2.42±0.140	6.03±0.400*	2.77±0.393	4.64±0.189
Glutamate	5.77±0.37	3.33±0.16*	6.35±0.89	4.46±0.37*
Glycine	0.67±0.04	0.69±0.11	0.70±0.07	0.61±0.01
Histidine	0.62±0.15	0.72±0.14	0.68±0.02	0.67±0.12
Isoleucine	0.035±0.008	0.036±0.015	0.018±0.002	0.032±0.006
Leucine	0.086±0.009	0.099±0.024	0.060±0.006	0.089±0.011
Lysine	0.12±0.02	0.13±0.03	0.20±0.03	0.15±0.03
Phenylalanine	0.059±0.006	0.063±0.008	0.083±0.007	0.086±0.005
Proline	0.052±0.005	0.087±0.021	0.057±0.008	0.062±0.006
Serine	0.30±0.02	0.35±0.07	0.17±0.03	0.23±0.03
Taurine	13±1	13±2	12±1	12±1
Threonine	0.44±0.05	0.57±0.12	0.80±0.17	0.67±0.13
Tyrosine	0.042±0.005	0.043±0.009	0.049±0.002	0.043±0.013
Valine	0.063±0.007	0.082±0.028	0.044±0.004	0.059±0.008
TFAA	24.4±1.1	24.6±1.3	26.5±3.7	24.0±0.7
TEFAA	1.55±0.17	2.08±0.17	1.86±0.33	1.92±0.19

Values are means ± s.e.m., N=4.
FAAs, free amino acids; TFAA, total FAA; TEFAA, total essential FAA.
*Significantly different from the value of the corresponding control.

Emersion does not impede ammonia excretion in A. testudineus – a novel observation

In water, fishes excrete ammonia as NH_3 though their gills (Wilkie, 2002; Evans et al., 2005). Because no water current is available to flush the excreted ammonia away from the gills, the partial pressure of NH_3 (P_{NH_3}) increases quickly in the boundary layer, leading to a reduction in the blood to boundary water ΔP_{NH_3} during emersion. In addition, most fish gills collapse in air, and so branchial ammonia excretion *via* diffusion of NH_3 is repressed. Indeed, for the tropical air-breathing teleosts *Oxyeleotris marmoratus* (Jow et al., 1999), *Boleophthalmus boddarti* (Lim et al., 2001), *P. schlosseri* (Lim et al., 2001; Ip et al., 1993; Ip et al., 2001c), *Misgurnus anguillicaudatus* (Chew et al., 2001); *Bostrichyths sinensis* (Ip et al., 2001a), *Channa asiatica* (Chew et al., 2003a), *Monopterus albus* (Tay et al., 2003) and *C. gariepinus* (Ip et al., 2005) exposed to terrestrial conditions, ammonia excretion rates decreased to 10–25% of that of the corresponding immersed control fish. Therefore, the unique observation made in this study that *A. testudineus* was able to increase ammonia excretion on day 2 and sustain the normal (immersed) daily rate of ammonia excretion on days 1, 3 and 4 of emersion is unexpected. It would imply that *A. testudineus* was able to effectively excrete ammonia despite a lack of water to irrigate its branchial epithelial surfaces and a 50% reduction in the cutaneous surface being in direct contact with the external medium.

Results obtained in a separate experiment in which daily change of water was omitted during a 2-day period indicate that *A. testudineus* was able to continuously excrete ammonia into the small volume of external medium and increase the ammonia concentration therein to 13.2 mmol l^{-1}. Therefore, it can be deduced that *A. testudineus* was able to excrete ammonia against a concentration gradient during emersion.

High ammonia concentrations in water samples collected from brachial or cutaneous surfaces of fish exposed to terrestrial conditions

Although the contribution of the kidney to ammonia excretion is less than that of the gills in water-breathing fishes, it might have an important role in ammonia excretion for amphibious fishes. However, during emersion, water conservation is an important issue in *A. testudineus* and consequently urine flow rate could not be high. Therefore, it is unlikely that increased ammonia excretion occurred through the kidney in fish exposed to terrestrial conditions.

Because the ammonia concentration in the water collected from the branchial surface of the water-facing side of the fish after 24 h of emersion increased to 21.5±2.4 mmol l^{-1}, it can be concluded that *A. testudineus* was able to actively excrete

ammonia against a concentration gradient across its gills. However, the ammonia concentration in the branchial water collected from the air-facing side remained relatively unchanged compared with the control. It is probable that water in the buccal cavity was drained naturally from the air-facing side towards the water-facing side as the fish lay flat in the bottom of the container, facilitating the continual excretion of ammonia through the gills over that side of the body. Thus, it can be deduced that the presence of water in the buccal cavity is a prerequisite to active branchial ammonia transport. By contrast, the giant mudskipper, *P. schlosseri*, has a torpedo-shaped body and maintains an upright posture on land. Because of this, water would be drained away from the gills towards the ventral side of the buccal cavity; therefore, it had to develop gills with intrafilamentous interlamellar fusions (Low et al., 1988) to trap water in order to facilitate the continual and active excretion of ammonia during emersion. Unlike *P. schlosseri*, the skin of *A. testudineus* might also be involved in active ammonia excretion because the ammonia concentration (20.8 mmol l^{-1}) in the water samples collected from the water-facing cutaneous surface was greater than that (5.32 mmol l^{-1}) in the small volume of ambient water.

Table 6. *Content of adenosine triphosphate, adenosine diphosphate, adenosine monophosphate, total adenylate, glycogen, glucose, lactate, ammonia, alanine, glutamate, lysine, total essential free amino acid and total free amino acid in the muscle of Anabas testudineus exposed to terrestrial conditions for 10 min (control) or forced to exercise for 10 min on land*

	Control	Exercised on land
ATP	11.1±0.2	10.3±0.7
ADP	1.58±0.25	1.75±0.04
AMP	0.11±0.05	0.31±0.07
Total adenylate	12.59±0.25	12.28±0.13
Glycogen	134±20	98±10
Glucose	1.70±1.25	2.10±1.41
Lactate	11.9±2.8	14.7±1.8
Succinate	0.92±0.21	0.81±0.08
Ammonia	0.71±0.18	1.53±0.27*
Alanine	1.01±0.03	1.42±0.20*
Glutamate	0.97±0.04	0.51±0.14*
Lysine	0.43±0.12	0.71±0.16*
TEFAA	1.91±0.22	2.68±0.30*
TFAA	23.2±0.4	26.3±1.2

Values are in μmol g^{-1} tissue, except for glycogen which is in μmol glucosyl unit g^{-1} tissue; *N*=5.

Total adenylate = ATP + ADP + AMP.

TFAA, total free amino acid (FAA); TEFAA, total essential FAA.

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

A. testudineus can excrete ammonia against 12 mmol $^{-1}$ NH_4Cl

Indeed, *A. testudineus* was able to excrete ammonia continuously when immersed in water containing 12 mmol l^{-1} NH_4Cl, although there was an initial decrease in the rate of ammonia excretion on day 1. Hence, *A. testudineus* is one of a few fish species that are capable of excreting ammonia against a steep ammonia gradient; the others are the giant mudskipper *P. schlosseri* (Randall et al., 1999; Ip et al., 2004c) and the African catfish *C. gariepinus* (Ip et al., 2004d). *P. schlosseri*, which inhabits a brackish environment, is able to actively excrete NH_4^+ *via* a basolateral Na^+,K^+-ATPase and an apical Na^+/H^+ exchanger of the branchial epithelium (Randall et al., 1999; Wilson et al., 2000). However, Na^+/NH_4^+ exchange may not occur across the gills of *A. testudineus* because apical electroneutral Na^+/H^+ exchange is likely to be absent from gills of freshwater fishes (Wilkie, 2002). Therefore, some novel mechanisms may be involved in active ammonia excretion in

Table 7. *A balance sheet of changes in ammonia and urea excretion and changes in ammonia and urea content of the muscle and liver of a 50 g Anabas testudineus after 2 or 4 days of immersion (control) or emersion*

	Day 2			Day 4		
	Immersion	Emersion	Difference	Immersion	Emersion	Difference
In a 50 g fish						
Ammonia-N excreted	575	697	+122	1205	1608	+403
Urea-N excreted	77	53	–24	142	116	–26
Reduction in nitrogenous excretion (A)			+98			+377
In 30 g muscle						
Ammonia-N retained	26	122	+96	32	73	+41
Urea-N retained	7	47	+40	3	6	+3
In 1 g liver						
Ammonia-N retained	4	9	+5	3	6	+3
Urea-N retained	0.3	1.6	+1.3	0.6	1.4	+0.8
Increase in nitrogenous accumulation (B)			+142			+48
(A) + (B)			+240			+ 425

Values are μmol N.

A. testudineus during emersion or ammonia exposure. Taken together, our results confirm that the ability to excrete ammonia against an ammonia gradient facilitates the survival of *A. testudineus* during emersion because ammonia can continue to be excreted into an extremely small volume of external medium without being impeded.

Emersion also leads to accumulation of ammonia in tissues of A. testudineus

The steady state content of ammonia in the tissue is maintained by a balance between ammonia production and ammonia excretion/detoxification. Theoretically, the ammonia content in the experimental fish should remain relatively unchanged after 4 days of emersion, because they were able to excrete ammonia on land at a rate comparable to that of fish immersed in water. Surprisingly, results obtained proved otherwise, and emersion led to significant increases in ammonia content in the muscle, liver and brain. Thus, the only logical explanation is that an increase in ammonia production had occurred. Indeed, a balance sheet (Table 7) on changes in ammonia and urea-N excretion, and in the content of ammonia, urea and FAAs in various tissues revealed that an increase in ammonia production could have occurred in *A. testudineus* during 4 days of emersion. This is the first report of such a phenomenon because previous studies in various air-breathing fishes (*B. boddarti*, *B. sinensis*, *C. asiatica*, *C.* gariepinus, *M. albus*, *M. anguillicaudatusm*, *O. marmoratus*, *P. schlosseri* and the slender African lungfish *Protopterus dolloi*) revealed that the summation of debits in nitrogenous excretion and credits in nitrogenous accumulation in fish exposed to terrestrial conditions would lead to negative values, indicating the occurrence of a decrease in ammonia production to ameliorate ammonia toxicity (Jow et al., 1999; Lim et al., 2001; Tay et al., 2003; Chew et al., 2001; Chew et al., 2003a; Chew et al., 2003b; Ip et al., 2001a; Ip et al., 2005).

Since ammonia is produced mainly through amino acid catabolism, it can be deduced that amino acid catabolism increased in *A. testudineus* during emersion and the resulting carboxylic acid was fuelled into the tricarboxylic acid cycle for energy production. Glutamate holds an important position in amino acid metabolism because many amino acids can be channelled into glutamate for deamination through the reaction catalyzed by glutamate dehydrogenase. Glutamate could be catabolized to ammonia (or alanine through transamination) and α-ketoglutarate, a tricarboxylic acid cycle intermediate for energy production. Support for increased amino acid catabolism can be inferred from the decrease in glutamate content, which indicates that glutamate degradation exceeded its synthesis, in both the muscle and liver of fish during 4 days of emersion.

The brain is often the organ undergoing the largest increase in glutamine concentration in fish exposed to ammonia because it is the most vulnerable to ammonia toxicity (Mommsen and Walsh, 1992). The significant increase in the glutamine content in the brain of fish after 2 days of emersion is in agreement with the conclusion that an increase in ammonia production had occurred in extracranial tissues, and the brain was transiently confronted with ammonia toxicity. Glutamate is not only a substrate for glutamine synthesis catalyzed by glutamine synthetase, but also acts as a substrate for the formation of alanine or aspartate through transamination reactions. The decreases in alanine and aspartate content in the brain of *A. testudineus* exposed to terrestrial conditions for 2 days suggested that glutamate was selectively channelled into glutamine synthesis, detoxifying ammonia derived from the blood to protect the brain cells.

Accumulation of certain FAAs (essential and non-essential) in the muscles during emersion and its implication

In this study, food was withdrawn before and during the experiment. Therefore, the rate of protein degradation should be higher than the rate of protein synthesis, leading to a net proteolysis. Because there was an apparent increase in amino acid catabolism, it can be predicted that decreases in tissue FAA, and consequently TFAA, would theoretically occur if the rate of proteolysis remained unaffected during the emersion period. However, there were significant increases in several essential amino acids (isoleucine, leucine, lysine, valine and phenylalanine) and TEFAA in the muscle of *A. testudineus* after 2 days of emersion as compared with the immersed control. Thus, it can be confirmed that an increase in proteolysis had indeed occurred. Furthermore, the decreases in aspartate, glutamine and glutamate in the muscle suggested that certain amino acids were preferentially catabolized during emersion. The significant increase in muscle alanine content indicate that there might also be an increase in partial amino acid catabolism, which released carboxylic acids for energy production without producing ammonia (Ip et al., 2001c; Chew et al., 2003a). Hence, it can be concluded that emersion led to simultaneous increases in rates of proteolysis and amino acid catabolism in *A. testudineus*.

Most tropical air-breathing teleosts studied so far, with the exception of the small snakehead *C. asiatica* (Chew et al., 2003a), reduce the rate of amino acid catabolism/proteolysis to slow down the accumulation of endogenous ammonia during emersion (for reviews, see Ip et al., 2001b; Ip et al., 2004a; Ip et al., 2004b; Chew et al., 2006). However, such an adaptation naturally prevents the use of amino acids as energy sources when the fish is out of water. *A. testudineus* is unique with respect to nitrogen metabolism and excretion on land, because there was an apparent increase in ammonia production during 4 days of emersion (Table 7). This could be related to the fact that the fish frequently struggled with jerky movements under such conditions.

Effects of air-exposure on the rate of oxygen consumption

Carbon chains released after the deamination of amino acids are usually shuttled into the tricarboxylic acid cycle, which produces NADH in the mitochondria. The balance of mitochondrial redox involves the electron transport chain

which utilizes oxygen as the terminal electron acceptor. Therefore, it is highly unlikely that *A. testudineus* would reduce O_2 consumption or undergo anaerobic energy metabolism during emersion. Many amphibious air-breathing fishes can sustain aerial O_2 consumption rate at or near the same level or higher than in water (Graham, 1997). However, it had been reported previously that *A. testudineus* had a reduced O_2 consumption rate in air (by 10–24%) (Hughes and Singh, 1970; Natarajan, 1978), in spite of it being a triphasic breather with a demonstrated amphibious behaviour (Graham, 1997). By contrast, the non-amphibious anabantoid, *Trichogaster trichopterus*, is known to have the same O_2 consumption rate in air as in water (Burggren and Haswell, 1979).

Contrary to previous reports, our results, while comparable with those in the literature (Hughes and Singh, 1970; Natarajan, 1978), reveal for the first time that the O_2 consumption rate of *A. testudineus* exposed to terrestrial conditions for 2 days was significantly higher than that of the control. What leads to this discrepancy is uncertain at present, but based on our result, it can be concluded that the ABOs of *A. testudineus* functioned effectively to absorb O_2 from air and this could have facilitated *A. testudineus* to utilize amino acids as sources of energy for locomotor activities during emersion.

Effects of forced exercise on land

Lipid is the dominant fuel (35–68%) in resting non-fed fish; carbohydrate is the second most important fuel, and its contribution increases to a much greater extent during starvation (Lauff and Wood, 1996a; Lauff and Wood, 1996b). In comparison, the contribution of protein oxidation (14–30%) to the overall metabolic rate is low (for a review, see Wood 2001). When fish are made to swim at sustainable and submaximal velocities, the contribution of protein oxidation to overall fuel use stays the same or decreases with increasing velocity. Many tropical air-breathing fishes can actively move on land; and, out of these, the Boddart goggled-eye mudskipper, *B. boddarti* (Ip et al., 2001c), and the small snakehead, *C. asiatica* (Chew et al., 2003c), utilize glycogen to support bursts of locomotor activities during emersion. However, the giant mudskipper, *P. schlosseri*, which can actively excrete NH_4^+, utilizes amino acids to fuel locomotor activities on land (Ip et al., 2001c).

Similar to *P. schlosseri*, 10 min of forced exercise on land did not result in decreases in glycogen and glucose in the muscle of *A. testudineus*. In addition, there were no significant changes in lactate or succinate content in muscle. Taken together, these results indicate that carbohydrate was not the major fuel for locomotor activity in *A. testudineus* exposed to terrestrial conditions. Because 10 min of exercise resulted in no changes in ATP, ADP and AMP in the muscle, other energy stores must have been utilized. Forced exercise for 10 min on land led to both increased ammonia excretion and increased ammonia accumulation in *A. testudineus*. The excess ammonia produced during exercise was unlikely to be released from AMP deamination because there was no change in the muscle total adenylate content. Therefore, it can be concluded that the excess ammonia was released through increased amino acid catabolism in the exercised fish. In addition, there could be an increase in partial amino acid catabolism because there was a significant increase in the alanine content of the muscle. The simultaneous increases in lysine and TEFAA in the muscle confirms that there was an increase in proteolytic rate during the 10 min of exercise on land.

Proteins and amino acids are major fuels in anorexic salmon during the spawning migration (Mommsen et al., 1980), although lipid is the first substrate to be used (Wood, 2001). At present, whether lipid acts as a source of energy during exercise in amphibious fishes is uncertain, but using amino acids to fuel activity on land is not a common phenomenon among amphibious fishes (Ip et al., 2001c; Chew et al., 2003c). This is because provisions must be made to ameliorate ammonia toxicity during exercise. Results obtained from *A. testudineus* and *P. schlosseri* (Ip et al., 2001c) suggest that active ammonia excretion is an important prerequisite to the use of amino acid to support locomotor activities on land.

Why is A. testudineus so unique?

Its body shape, air-breathing capacity and ability to migrate on land during drought

Because of its laterally compressed body, *A. testudineus* usually lies on one side during emersion. Very often, it flips the body and the tail fin to produce jerky movements which constitutes, in effect, vaulting actions supported by the spiny edges of the gill plates (Davenport and Abdul Martin, 1990). Through such vaulting actions, fuelled by amino acid catabolism, it can ‘migrate’ long distances on land. Using amino acids as substrates for energy metabolism on land implies that it must respire in air effectively to remain aerobic. Indeed, like many other air-breathing fishes, *A. testudineus* has labyrinth organs (ABOs) which facilitate air-breathing during immersion or emersion. In general, amphibious fishes (e.g. catfishes, mudskippers, sleepers, snakeheads and swamp eels) develop torpedo-shaped bodies to maintain an upright posture on land, but *A. testudineus* possesses a laterally compressed body. Why would *A. testudineus* retain such a body shape during evolution? Our results suggest at least one advantage in doing so. Increased amino acid catabolism naturally leads to increased ammonia production, and excess ammonia must be excreted even in the absence of water to flush the gills. Therefore, it is imperative for *A. testudineus* to possess active ammonia transport mechanisms, which are apparently present in the branchial and cutaneous surfaces. Lying on one side of the body during emersion renders half of the cutaneous surface to be in direct contact with the external medium and water in the buccal cavity to drain towards the gills over that side. This would facilitate the continual and active excretion of ammonia through 50% of the total branchial and cutaneous surfaces. Although both *A. testudineus* and *P. schlosseri* can excrete ammonia actively on land, they apparently took different evolutionary paths with respect to branchial development. By developing intrafilamentous interlamellar fusions to facilitate active ammonia excretion during emersion, the gills of *P.*

schlosseri can no longer function effectively as a respiratory and/or osmoregulatory organ in water. By contrast, by maintaining a laterally compressed body, *A. testudineus* is able to achieve the same feat as *P. schlosseri*, but with unspecialized gills that may function effectively for osmoregulation and/or acid–base balance during immersion. Since *A. testudineus* is known to inhabit brackish water, efforts should be made in the future to determine the effects of increased salinity on its branchial functions and air-breathing capacity.

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