Greatly Elevated Urea Excretion after Air Exposure Appears to Be Carrier Mediated in the Slender Lungfish (*Protopterus dolloi*)

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Accepted 4/22/2005; Electronically Published 9/21/2005

ABSTRACT

Under aquatic conditions, *Protopterus dolloi* is ammoniotelic, excreting only small amounts of urea-N. However, upon return to water after 30 d estivation in air, the lungfish excretes only small amounts of ammonia-N but massive amounts of urea-N. A similar pattern is seen after 21–30 d of terrestrialization, a treatment in which the lungfish is air exposed but kept moist throughout. After both treatments, the time course of urea-N excretion is biphasic with an immediate increase, then a fall, and finally a second larger increase that peaks at about 12 h and may be prolonged for several days thereafter. Urea-N excretion rates during the second peak reach 2,000–6,000 μmol N kg^{-1} h^{-1}, two to three orders of magnitude greater than rates in most fish and comparable only to rates in species known to employ UT-A type facilitated diffusion urea transporters. Divided chamber studies and measurements of the clearance rates of [^{3}H]-PEG-4000 (a glomerular filtration and paracellular diffusion marker) and two structural analogs of urea ([^{14}C]-acetamide and [^{14}C]-thiourea) were performed to characterize the two peaks of urea-N excretion. The smaller first peak was almost equally partitioned between the head (including internal and external gills) and the body compartment (including urinary opening), was accompanied by only a modest increase in [^{14}C]-acetamide clearance equal to that in [^{14}C]-thiourea clearance, and could be accounted for by a large but short-lasting increase in [^{3}H]-PEG-4000 clearance (to about fivefold the terrestrial rate). The delayed, much larger second peak in urea-N excretion represented an elevated efflux into both compartments but occurred mainly (72%) via the body rather than the head region. This second peak was accompanied by a substantial increase in [^{14}C]-acetamide clearance but only a modest further rise in [^{14}C]-thiourea clearance. The acetamide to thiourea permeability ratio was typical of UT-A type transporters in other fish. [^{3}H]-PEG-4000 clearance was stable at this time at about double the terrestrial rate, and excretion rates of urea and its analogs were many fold greater than could be accounted for by [^{3}H]-PEG-4000 clearance. We conclude that the first peak may be explained by elevated urinary excretion and paracellular diffusion across the gills upon resubmergence, while the second peak is attributable to a delayed and prolonged activation of a UT-A type facilitated diffusion mechanism, primarily in the skin and perhaps also in branchial epithelia.

Introduction

Traditionally, the N-waste molecule urea was thought to move by simple diffusion through biological membranes, but it is now clear that a variety of carrier-mediated urea transport mechanisms are present in vertebrate cell membranes and play important roles in the physiological regulation of urea movements (reviewed by Sands et al. 1997; Smith and Rousselet 2001; Bagnasco 2003; Sands 2003). The best characterized of these are members of the UT-A family (bidirectional facilitated diffusion type transporters), which were first discovered in the mammalian kidney (You et al. 1993; Smith et al. 1995) and later in amphibian bladder (Couriaud et al. 1999). The cDNA's for homologous members of this same family (reviewed by Walsh and Smith 2001) have now been cloned in part or in full from several ureotelic fish, including the elasmobranchs *Squalus acanthias* (Smith and Wright 1999), *Triakis scyllia* (Hyodo et al. 2004), and *Raja erinacea* (Morgan et al. 2003*a*), the facultatively ureotelic teleost *Opsanus beta* (Walsh et al. 2000), and the obligatory ureotelic teleost *Alcolapia grahami* (Walsh et al. 2001*a*). Furthermore, there is now direct or indirect evidence for similar transporters even in obligatory ammoniotelic teleosts such as *Anguillla japonica* (full-length cDNA; Mistry et

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Physiological and Biochemical Zoology 78(6):893–907. 2005. 1522-2152/2005/7806-4157$15.00

al. 2001), *Porichthys notatus* (Walsh et al. 2001*b*; McDonald et al. 2002), and *Oncorhynchus mykiss* (Pilley and Wright 2000; McDonald et al. 2003; McDonald and Wood 2004). In elasmobranchs, these appear to occur mainly in the kidney and, in teleosts, mainly in the gills.

Active urea transport mechanisms, at least some of which appear to be Na^+ coupled, also occur, though they have not yet been identified at the molecular level. Physiological evidence for active urea transport has been reported in mammalian kidney (reviewed by Sands 2003), amphibian kidney (Schmidt-Nielsen and Shrauger 1963) and skin (Garcia-Romeu et al. 1981; Rapoport et al. 1988; Lacoste et al. 1991), elasmobranch kidney (*S. acanthias*; Schmidt-Nielsen et al. 1972; Morgan et al. 2003*b*) and gill (Pärt et al. 1998; Fines et al. 2001), and teleost kidney (*O. mykiss*: McDonald and Wood 1998, 2003; *O. beta*: McDonald et al. 2000; *P. notatus*: McDonald et al. 2002).

In fish, our ability to differentiate these various transporters and our understanding of their physiology is fairly preliminary, but they are clearly involved in the regulation of urea excretion. The branchial UT-A transporter (tUT; Walsh et al. 2000) of the gulf toadfish (*O. beta*) has been best studied; tUT appears to be activated periodically to cause pulses of urea excretion through the gills (reviewed by Walsh and Smith 2001; Wood et al. 2003), a phenomenon first detected by fine timescale analysis of urea appearance in the external water (Wood et al. 1995). A particularly useful tool in demonstrating the carrier mediation of this phenomenon was the use of previously injected urea analogs ([^{14}C]-acetamide, [^{14}C]-thiourea), which were "pulsed" simultaneously to urea at different rates (Wood et al. 1998; McDonald et al. 2000). Indeed, the differential ability of various urea transporters to handle different analogs of urea has proven to be diagnostic of the presence and categorization of urea transporters in fish (Schmidt-Nielsen and Rabinowitz 1964; Wood et al. 1998; McDonald et al. 2000, 2002, 2003; Pilley and Wright 2000; Walsh et al. 2001*a*).

With this background in mind, we set out to investigate urea excretion in the slender lungfish, *Protopterus dolloi*, with a particular focus on possible carrier mediation of urea transport. Members of the class Dipnoi (the lungfishes) occupy a pivotal position on the pathway to tetrapod evolution and terrestriality, and the African genus *Protopterus* appears to be the most terrestrial of the class. These animals have both internal and external gills but are obligatory air breathers via a primitive lung, and they are capable of prolonged estivation when water availability becomes limiting (reviewed by Fishman et al. 1986). They are ammoniotelic when in water but rely on ureogenesis through the ornithine-urea cycle when air exposed (Smith 1930; Janssens 1964; Forster and Goldstein 1966; Janssens and Cohen 1966, 1968; Chew et al. 2003, 2004). Urea is accumulated internally, and after return to water, urea excretion is known to be greatly elevated for several days ("in spectacular quantities": Smith 1930; see also Janssens 1964; Chew et al. 2003). However, the temporal pattern (i.e., continuous or pulsatile), sites (i.e., gills, kidney, or skin), and mechanisms of urea excretion (simple diffusion vs. some type of carrier mediation) are unknown.

Protopterus dolloi was selected as a particularly useful model because it can be induced to estivate simply by removal of water from an otherwise empty aquarium. Under these conditions, it forms its own cocoon without the complicating presence of mud (Chew et al. 2004). In Zaire, it digs burrows in the raftlike substrate of floating swamps as a breeding nest and may use this same burrow for estivation during the dry season, without formation of a mud cocoon (Brien et al. 1959). We also found that it tolerates prolonged exposure to air in the continued presence of a thin film of water ("terrestrialization"; Chew et al. 2003), a less extreme condition that can be considered the precursor to true estivation. In this study, we exploited these treatments in combination with fine timescale recording of urea (and ammonia) excretion after return to aquatic conditions. Divided chamber and renal function studies ([^{3}H]-polyethylene glycol-4000 clearance) were performed to look for sites of urea excretion, and urea analog studies ([^{14}C]-acetamide, [^{14}C]-thiourea clearances) were carried out to look for evidence of possible carrier mediation.

Material and Methods

Experimental Animals

Protopterus dolloi with a mean weight of about 40 g (range 15–95 g) were collected in Nigeria and Zaire and air shipped to a commercial dealer in Singapore. After purchase, they were then either housed at the University of Singapore, where some of the experiments were performed, or else further air shipped to McMaster University in Canada, where other experiments were carried out. Holding and experimental conditions were essentially identical at the two locations. At both laboratories, they were held at 25°–27°C under a 12L : 12D photoperiod for several weeks before experimentation. During the holding period, lungfish were kept individually in small plastic aquaria containing approximately 2 L of dechlorinated tap water that was not aerated but supplemented with seawater to a salinity of 2 ppt, pH about 7.1–7.2. Approximate water composition was Na^+ 30, Cl^- 36, Mg^{2+} 3.1, and Ca^{2+} 2.6 mmol L^{-1}, with titration alkalinity (to pH = 4.0) 0.64 mmol L^{-1}. Since freshwater ponds evaporate during the dry season before estivation of the lungfish (Brien et al. 1959; Poll 1961), it is likely that similar elevations in ionic strength occur in nature. Water of this composition was used in all experiments. The lungfish were fed frozen bloodworms every second day, and the water was changed the day after feeding. These conditions were found to keep the animals very healthy, with the slightly saline water being important in preventing fungal infections. The animals were not fed for 48 h before the start of experiments.

Experimental Series

Experiments examining N-waste excretion after estivation (series 1), after terrestrialization (series 2), and in lungfish in divided chambers after various treatments (series 3) and ^{3}[H]-PEG-4000 clearance after terrestrialization (series 4) were performed at the University of Singapore. Experiments examining the clearances of ^{14}[C]-acetamide and ^{14}[C]-thiourea after terrestrialization (series 5), plasma urea-N and ammonia-N concentrations after terrestrialization (series 6), and some additional divided chamber studies (series 3) were performed at McMaster University.

Series 1: Return to Aquatic Conditions after Estivation

Lungfish were induced to estivate in their aquaria as described by Chew et al. (2004). All but 10 mL of water was removed, which was allowed to dry up over the following 3 or 4 d. By this point, the lungfish had curled up into the typical estivation posture, became motionless, and formed dry brown mucus cocoons. They were kept minimally hydrated thereafter by spraying with 1–2 mL water every 6 d until day 30. The aquaria were placed in 24-h darkness throughout the estivation period, and no food was given.

On day 30, the fish ($N = 7$) were returned to water. The aquaria were moved into lighted conditions and quickly flushed with 2 × 500 mL of water to remove any accumulated N-waste; then 1 L of water was added, together with an airstone for mixing, and 2 × 5-mL samples withdrawn for initial N-analyses (ammonia-N and urea-N). Within 5–10 min, the lungfish became active, breaking the cocoon and struggling to the surface to breathe. Any pieces of cocoon still adhering to the lungfish were manually removed to help this process. After 1 h, the water was sampled again for N-analyses and then replaced with a volume equal to × 25 the animal's mass. Sequential water samples were taken at 2-h intervals up to 15 h postestivation, then again at 21 h. The water was renewed, and sampling at 2-h intervals continued until 37 h postestivation, then again at 46 h, followed by water renewal. A final flux measurement covered the period from 49.5 to 68 h postestivation.

Series 2: Return to Aquatic Conditions after Terrestrialization

This treatment started identically to estivation, with all but 10 mL of water being removed at the start, when the aquaria were transferred to constant darkness and feeding was suspended. However, rather than being allowed to dry up, the water was replenished by daily spraying so as to maintain the volume at about 10 mL. Under these conditions, the lungfish's skin remained wet, and the animal lay in a thin film of water contacting its ventral surface. In general, these lungfish also secreted some cocoon material, though it tended to be incomplete or broken, probably disturbed by infrequent movements.

On day 21, the aquaria of these lungfish ($N = 6$) were rinsed with 2 × 500 mL of water, and then exactly 20 mL of water was added. Of this, 2 × 5 mL were immediately removed for initial N analyses. After 12 h, the remaining approximately 10 mL (into which N-flux had occurred) were removed for final N-analyses and the exact volume recorded. The procedure was repeated for another 12-h flux determination. The purpose was to monitor rates of ammonia-N and urea-N excretion into the water film under terrestrial conditions, before the return to aquatic conditions. On day 22, after two 12-h "terrestrial" flux periods, the animals were returned to aquatic conditions by replacing the water film with a volume equal to × 25 the animal's mass, together with an air stone for mixing. The aquaria were also moved into lighted conditions at this time. Water samples (2 × 5 mL) were taken at 1-h intervals until 12 h and then again at 24 h. The N-excretion rates in series 4 (see "Series 4: [^{3}H]-PEG-4000 Clearance Rates during and after Terrestrialization") were virtually identical to those of these series 2 fish, so the data were combined ($N = 13$).

To check for possible effects of starvation or disturbance, a control group ($N = 7$) was also transferred to constant darkness and starved for 21 d but kept under aquatic conditions. On day 21, the aquaria were flushed with 2 × 500 mL of water, the volume was then set to × 25 the animal's mass, and two 12-h flux measurements of N-excretion were made. On day 22, the water was then renewed, and the animals were transferred to lighted conditions, with water samples taken at 1-h intervals until 12 h and then again at 24 h.

Series 3: Divided Chamber Studies

In order to localize the sites of urea-N and ammonia-N excretion, the divided chamber approach pioneered by Smith (1929) was employed to separate excretion through the head region (containing the internal and external gills and comprising about 15% of the body mass) from excretion through the body (containing the bulk of the skin and the urinary opening and comprising about 85% of the body mass). Measurements were made in control lungfish, which had been kept throughout under aquatic conditions ($N = 10$), and in terrestrialized lungfish at approximately 0–3 ($N = 5$) and 12–15 h ($N = 9$) after return to aquatic conditions. [^{3}H]-PEG-4000 clearance rates through the head versus body regions were also measured in lungfish ($N = 7$) kept under aquatic conditions throughout.

Lungfish were placed for 1–2 h (precise time recorded) in an apparatus such that the head region was separated from the remainder of the body using a latex dental dam to make a water-tight seal. Water samples for N-analyses or [^{3}H]-PEG-4000 cpm were drawn at the start and end of the period. The rear chamber containing most of the body was a water-filled graduated cylinder, which was placed horizontally in a plastic tray tilted so as to have a reservoir of aerated water at one

corner into which the lungfish's head was submerged. The lungfish was able to easily raise its head to breathe air periodically. The volumes of the head and body reservoirs were typically about 200 mL each and were measured precisely at the end of the experiment.

Series 4: [^{3}H]-PEG-4000 Clearance Rates during and after Terrestrialization

The purpose of this experiment was to monitor possible changes in kidney function and paracellular diffusion associated with the time course of changes in urea-N excretion that had been observed in series 2. The animals were too small to be fitted with urinary catheters to measure urine flow rate, so instead the clearance of [^{3}H]-polyethylene glycol-4000 (PEG-4000) was used to monitor glomerular filtration rate (GFR). PEG-4000 is a well-established extracellular space, paracellular diffusion, and GFR marker in fish (Beyenbach and Kirschner 1976; Erickson and Gingerich 1986; Curtis and Wood 1991; Munger et al. 1991; Wood et al. 1998). Experimental lungfish ($N = 7$) were treated identically to those in series 2, with the exception that the animals received an injection of 50 μCi kg^{-1} of [^{3}H]-PEG-4000 (NEN-Dupont; specific activity = 2,000 μCi g^{-1}) in 5 mL kg^{-1} saline (140 mmol L^{-1} NaCl) into the caudal haemal arch approximately 4 h before the terrestrial flux measurements. Additional water samples (5 mL) were taken for scintillation counting to monitor [^{3}H]-PEG-4000 appearance into the water at each sample time, and at 24 h, a blood sample (200 μL) to monitor [^{3}H]-PEG-4000 cpm remaining in the extracellular fluid was drawn by caudal puncture. For injections and blood sampling, a #23 needle attached to a 1-mL syringe was used, and to prevent clotting, the needle and syringe were prerinsed with a 5,000 i.u. mL^{-1} lithium heparin solution (Sigma) made up in 140 mmol L^{-1} NaCl. [^{3}H]-PEG-4000 clearance rates were also measured in a control group ($N = 5$) kept under aquatic conditions throughout.

Series 5: [^{14}C]-Acetamide and [^{14}C]-Thiourea Clearance Rates during and after Terrestrialization

The goals of this experiment were to test whether changes in the clearance rates of two structural analogs of urea corresponded with the time course of changes in urea-N excretion that had been observed in series 2 and to look for differences in their clearance rates diagnostic of carrier-mediated transport. The protocol was the same as in series 4, except that the period of terrestrialization was 30 d, the initial injection was 10 μCi kg^{-1} of either [^{14}C]-acetamide ($N = 13$) or [^{14}C]-thiourea ($N = 14$; NEN-Dupont; specific activity 58.0 mCi mmol^{-1}), and water sampling (for N-analyses and [^{14}C]-analog counting) continued at 2-h intervals until 16 h after return to aquatic conditions, with final water and blood samples (as above) taken at 24 h.

Series 6: Plasma Urea-N and Ammonia-N Concentrations during and after Terrestrialization

Lungfish were put through the same protocol of terrestrialization and returned to aquatic conditions as in series 5, with blood samples (as above, only one per animal) being drawn at 0 ($N = 7$), 8 ($N = 7$), 12 ($N = 7$), or 24 h ($N = 8$) after return to aquatic conditions to establish the time course of changes in plasma urea-N and ammonia-N concentrations. Blood samples were also drawn from control lungfish ($N = 12$) kept under aquatic conditions. Additional blood plasma samples ($N = 27$) taken in series 5 at 24 h were also analyzed, yielding very similar data, for a total of $N = 35$ for the 24-h point. Plasma was immediately separated by centrifugation and stored in liquid nitrogen before analysis.

Analytical Methods and Calculations

The diacetyl monoxime method of Rahmatullah and Boyde (1980) and the indophenol blue method of Ivancic and Degobbis (1984) were used to measure the concentrations of urea-N and ammonia-N, respectively, in water, using freshly prepared urea and NH_4Cl standards made up in the test water. The diacetyl monoxime method was also used for plasma urea-N determinations, while plasma ammonia-N was measured by the enzymatic method of Kun and Kearney (1974). Water samples (5 mL) and plasma samples (25 uL diluted to 5 mL in the test water) were added either to 13 mL BCS fluor (Amersham) for [^{3}H]-PEG-4000 determination in a Wallac 1414 liquid scintillation counter or to 10 mL ACS fluor (Amersham) for [^{14}C]-acetamide and [^{14}C]-thiourea analysis in an LKB Rackbeta 1217 Counter, using onboard programs for quench correction.

Flux rates of urea-N and ammonia-N (μmol N kg^{-1} h^{-1}) were calculated from changes in concentration (μmol N L^{-1}), factored by the known lungfish mass (kg), volume (L), and time (h). To calculate clearance rates of [^{3}H]-PEG-4000, we assumed that this marker was distributed at plasma concentration in a space equivalent to 25% of the lungfish's mass, that is, a volume chosen to approximate the extracellular space (Holmes and Donaldson 1969). Similarly, we assumed that the urea analogs [^{14}C]-acetamide and [^{14}C]-thiourea were distributed at plasma concentration in a space equivalent to 75% of the lungfish's mass, that is, a volume chosen to approximate the body water space (Holmes and Donaldson 1969). Knowing the final measured plasma concentration in the blood sample taken at the end of the experiment, and by keeping track of the total amount excreted in each flux period, we calculated the amount present in the animal at the beginning and end of each flux period and therefore the average plasma concentration of the radiolabeled compound during the flux period in question. Clearance rate (mL kg^{-1} h^{-1}) was then calculated as the amount excreted during the flux period (cpm), factored by the lungfish mass, time (h), and estimated plasma concentration (cpm mL^{-1}).

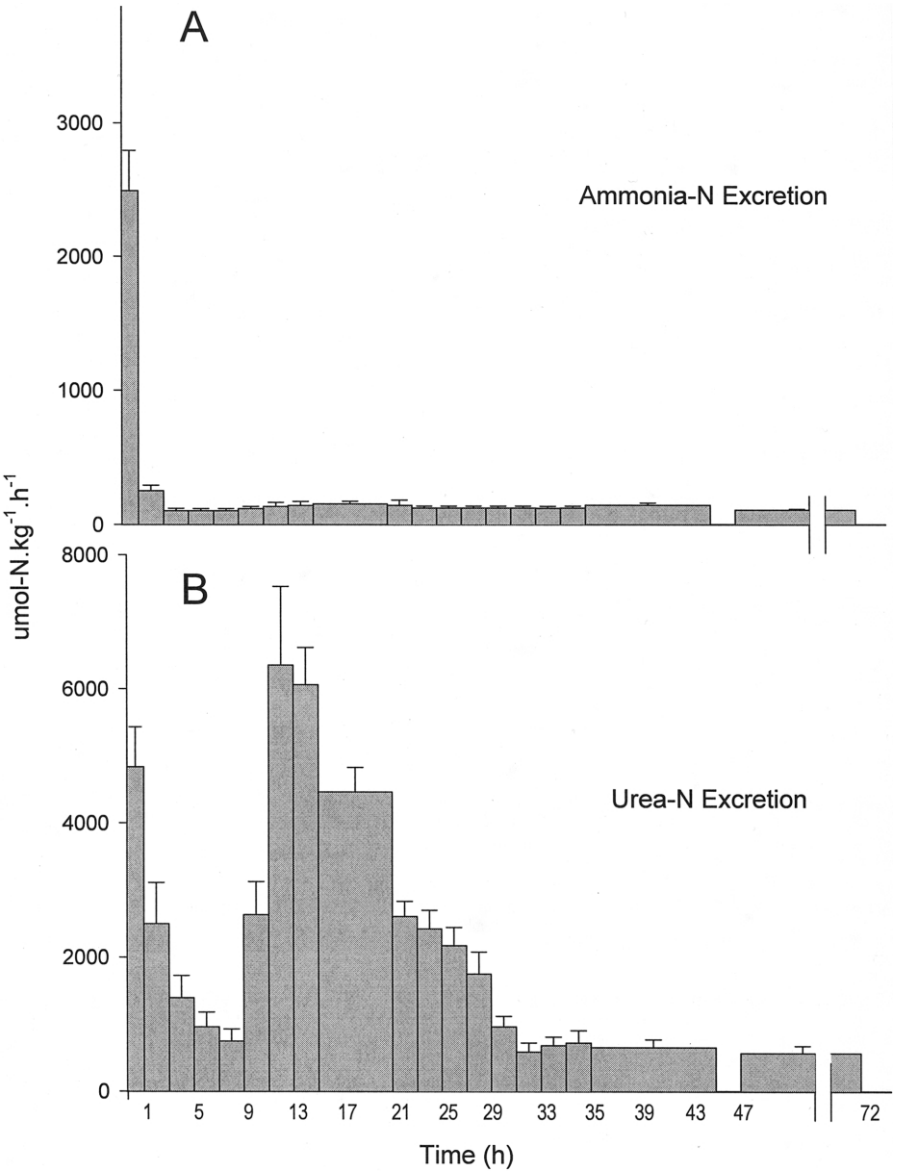

Figure 1. Rates of (*A*) ammonia-N excretion and (*B*) urea-N excretion in *Protopterus dolloi* of series 1 during return to aquatic conditions after 30 d of estivation. Note the biphasic pattern and sustained elevation of urea-N excretion relative to the monophasic pattern of ammonia-N excretion. In *A*, only the means at 0–1 and 1–3 h postestivation are significantly different ($P \leq 0.05$) from the reference aquatic rate of ammonia-N excretion. In *B*, means at all times are significantly different ($P \leq 0.05$) from the reference aquatic rate of urea-N excretion. In addition, two peaks are well defined by the following significant differences ($P \leq 0.05$) relative to the trough at 7–9 h postestivation: means at 0–1, 1–3, and 3–5 h (i.e., first peak) and all means at 9 through 29 h (i.e., second peak). Means ± 1 SEM ($N = 7$).

Statistical Analyses

Data have been expressed as means ± 1 SEM (N), where N represents the number of lungfish sampled. A Student's paired t-test (two-tailed) was employed for single comparisons within a treatment, and a Student's unpaired t-test (two-tailed) was employed for comparisons between treatments at the same time. The Bonferroni correction was used when more than two treatments were compared. A one-way ANOVA followed by Dunnett's test was used for multiple comparisons against a single reference value within a treatment. A significance level of $P \leq 0.05$ was used throughout.

Results

As a point of reference, lungfish kept under normal aquatic conditions and fasted for 48 h before measurement are strongly ammoniotelic, with ammonia-N excretion rates of 220.1 ±

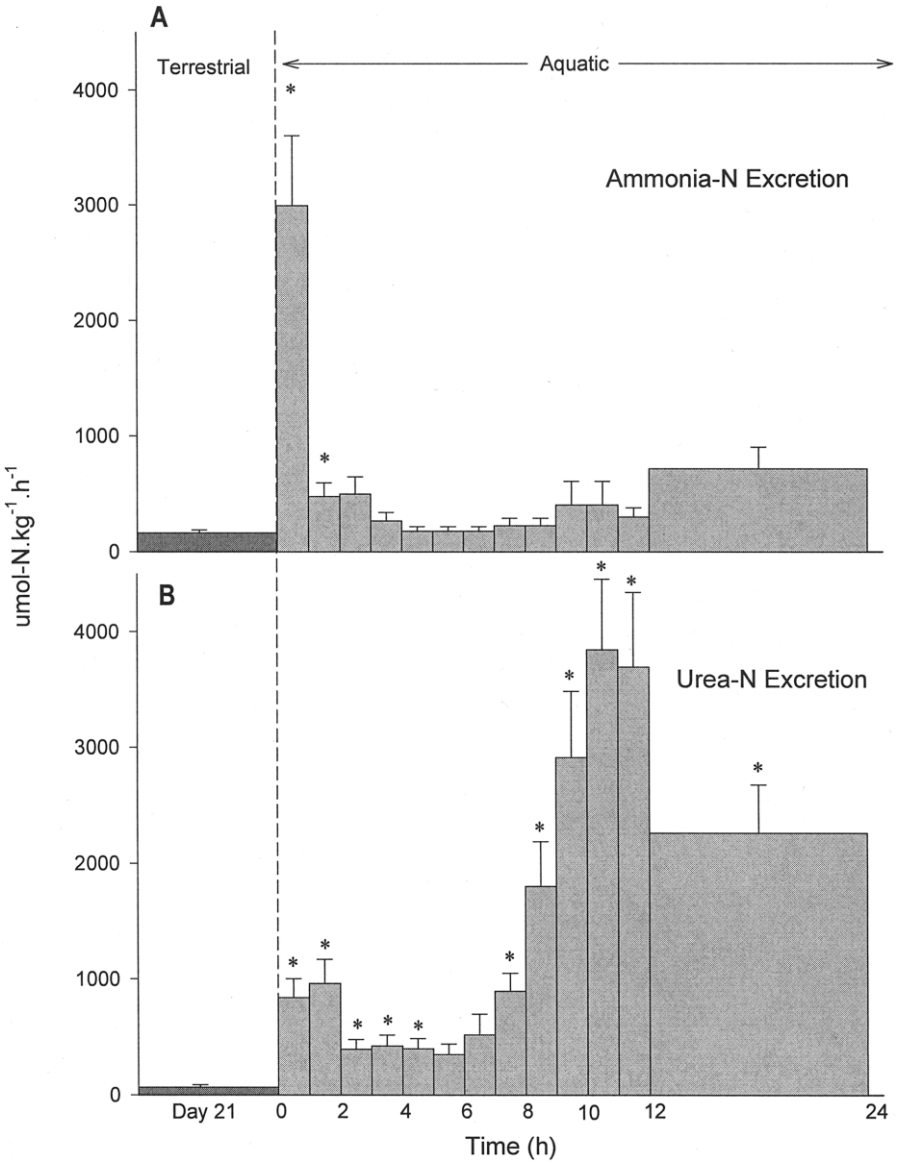

Figure 2. Rates of (*A*) ammonia-N excretion and (*B*) urea-N excretion in *Protopterus dolloi* of series 2 on day 21 of terrestrialization and during return to aquatic conditions thereafter. Note the biphasic pattern and sustained elevation of urea-N excretion relative to the monophasic pattern of ammonia-N excretion during return to aquatic conditions, qualitatively similar to the patterns seen after estivation (Fig. 1). Asterisks indicate means significantly different ($P \leq 0.05$) from the terrestrial rate on day 21. In *B*, two separate peaks of urea-N excretion are well defined by the following significant differences ($P \leq 0.05$) relative to the trough at 5–6 h postestivation: means at 0–1 and 1–2 h (i.e., first peak) and all means at 7 through 24 h (i.e., second peak). Furthermore, all means at 9 through 24 h are significantly higher than the initial peak. Means ± 1 SEM ($N = 13$).

31.2 μmol N kg^{-1} h^{-1} and urea-N excretion rates of 31.9 ± 10.8 μmol N kg^{-1} h^{-1} ($N = 7$; reported in Wood et al. 2005).

Series 1: Return to Aquatic Conditions after Estivation

In the first hour after lungfish were returned to water following 30 d of estivation, ammonia-N excretion was around 2,500 μmol N kg^{-1} h^{-1}, but by 3–5 h it had decreased to about 140 μmol N kg^{-1} h^{-1}, a value that was not significantly different from the reference aquatic rate (220.1 ± 31.2 μmol N kg^{-1} h^{-1}) and that remained unchanged until the end of the measurements at 68 h (Fig. 1*A*). In contrast, urea-N excretion rates were far higher and exhibited a clear biphasic pattern (Fig. 1*B*). The initial rate in the first hour was about 5,000 μmol N kg^{-1} h^{-1}, but this decreased progressively to about 750 μmol N kg^{-1} h^{-1} at 7–9 h before increasing to a second, even higher peak

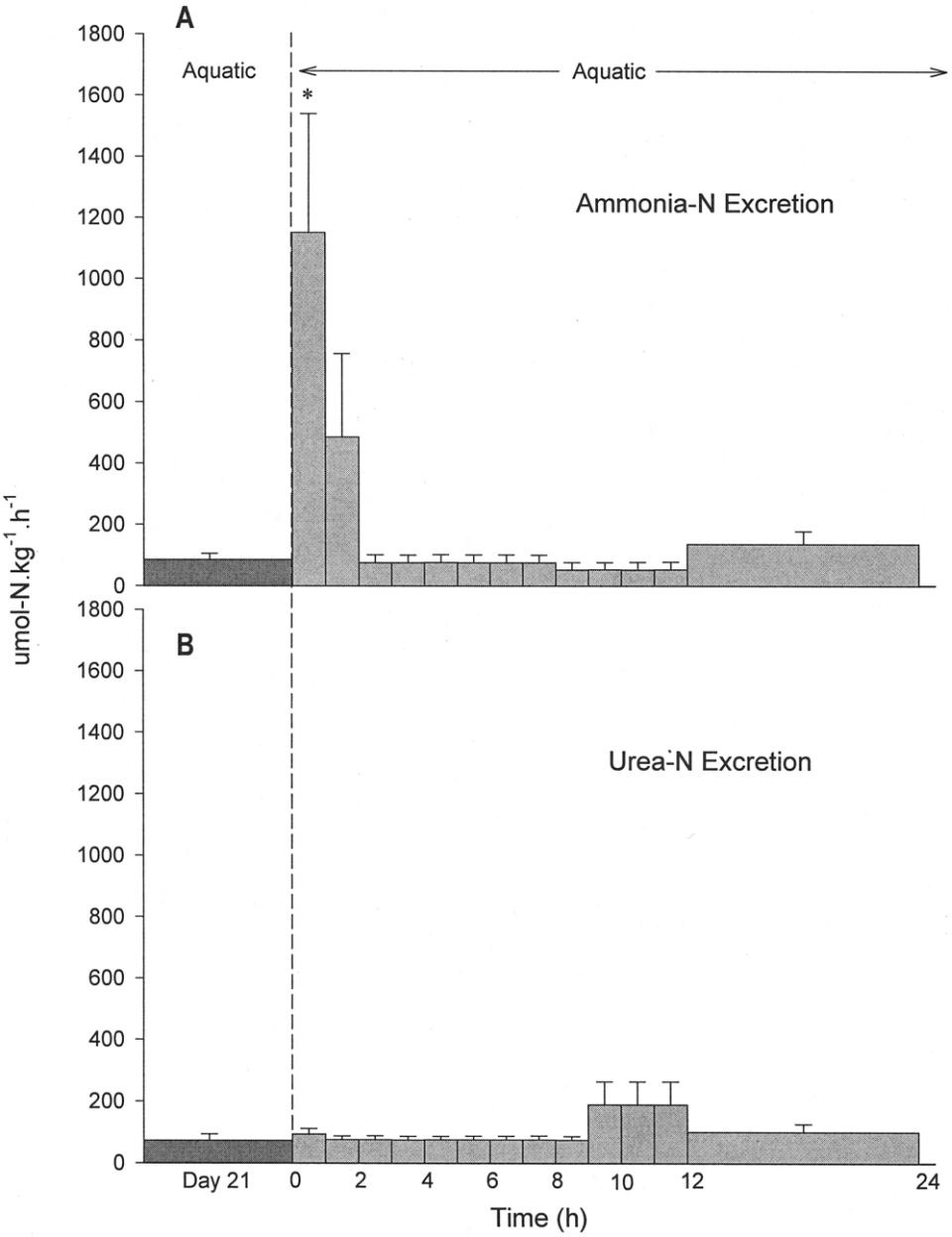

Figure 3. Rates of (*A*) ammonia-N excretion and (*B*) urea-N excretion in a control group of *Protopterus dolloi* of series 2 kept under aquatic conditions throughout and otherwise treated identically (same starvation and handling) as the experimental animals of the same series shown in Figure 2. Asterisks indicate means significantly different ($P \leq 0.05$) from the aquatic rate on day 21. Means ± 1 SEM ($N = 7$).

of around 6,300 μmol N kg^{-1} h^{-1} at 11–13 h. This second surge was prolonged, slowly falling to about 600 μmol N kg^{-1} h^{-1} at 31–33 h and remaining significantly elevated at this level relative to the reference aquatic rate right through 49–68 h.

Series 2: Return to Aquatic Conditions after Terrestrialization

For this treatment, a comparably handled and starved control group held under aquatic conditions throughout was available (Fig. 3). Qualitatively similar patterns were seen after terrestrialization (Fig. 2) as after estivation (Fig. 1). Interestingly, measured rates of ammonia-N (about 160 μmol N kg^{-1} h^{-1}) and urea-N excretion (65 μmol N kg^{-1} h^{-1}) into the 10-mL water film on day 21 of terrestrialization (Fig. 2) were comparable to reference aquatic rates (above) and also not significantly different from the rates on day 21 in the aquatic control group (Fig. 3).

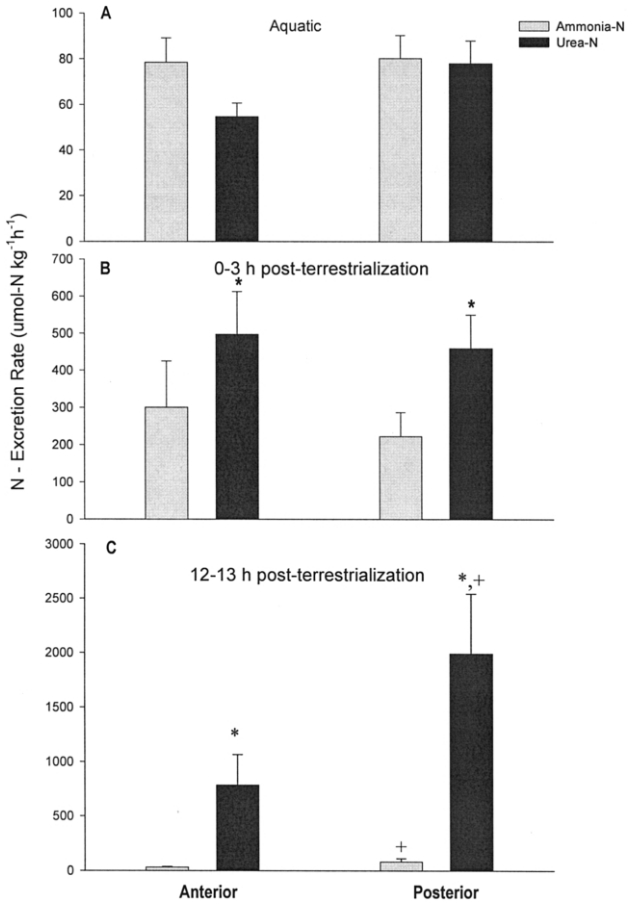

Figure 4. Partitioning of ammonia-N excretion and urea-N excretion between the anterior (i.e., head) and posterior (i.e., body) compartments in *Protopterus dolloi* of series 3 placed in divided chambers under (*A*) control aquatic conditions ($N = 10$), (*B*) the first 3 h after return to aquatic conditions following terrestrialization ($N = 5$), and (*C*) 12–13 h after return to aquatic conditions following terrestrialization ($N = 9$). Note the different scales in each panel, the similar increases in urea-N excretion in the two compartments in *B*, and the much larger increase in the posterior compartment in *C*. Asterisks indicate means significantly different ($P \leq 0.05$) from the aquatic rates in *A*. Crosses indicate means significantly different ($P \leq 0.05$) from the corresponding rates in the anterior compartment. Means ± 1 SEM.

In the first hour after return to aquatic conditions on day 22, ammonia-N excretion was elevated to around 3,000 μmol N kg^{-1} h^{-1}, but by 3–4 h it had dropped to about 200 μmol N kg^{-1} h^{-1}, which was sustained through 12–24 h when measurements were ended (Fig. 2*A*). This rate was not significantly different from the pretransfer terrestrial rate on day 21, the reference aquatic rate (above), or the simultaneous rate in the control group (Fig. 3*A*), despite a tendency for elevation at 12–24 h (Fig. 2*A*). At least part of the initial burst of ammonia-N excretion after transfer was attributable to disturbance, since the rate also increased significantly in the comparably handled control group (to about 1,150 μmol N kg^{-1} h^{-1} in the first hour; Fig. 3*A*).

Urea-N excretion during reimmersion after terrestrialization again exhibited a biphasic trend (Fig. 2*B*) very different from that in the control group, where there were no significant changes over time (Fig. 3*B*). Urea-N excretion reached about 1,000 μmol N kg^{-1} h^{-1} in the first 2 h after return to aquatic conditions, subsequently fell to around 400 μmol N kg^{-1} h^{-1} at 2–7 h, then rose again to about 3,800 μmol N kg^{-1} h^{-1} at 10–12 h, remaining strongly elevated through 12–24 h.

Terrestrialization (which caused no mortalities) was an easier and less stressful experimental treatment than estivation, in which several lungfish died apparently because the initial rate of dehydration was too high. Since terrestrialization revealed the same biphasic pattern in urea-N excretion (Figs. 1*B*, 2*B*) and monophasic pattern in ammonia-N excretion as estivation (Figs. 1*A*, 2*A*), this treatment was used in subsequent mechanistic studies.

Series 3: Divided Chamber Studies

Under control aquatic conditions ($N = 10$), both ammonia-N (49% ± 6% anterior) and urea-N excretion rates (41% ± 4% anterior) were partitioned almost equally between the anterior compartment, which contained the internal and external gills, and the posterior compartment, which contained the bulk of the skin and the urinary opening (Fig. 4*A*).

During the first 3 h after return to aquatic conditions following terrestrialization ($N = 5$), the partitioning did not change significantly (ammonia-N 57% ± 12% anterior, urea-N 52% ± 9% anterior; Fig. 4*B*). Notably, the absolute urea-N excretion rates into both compartments were significantly elevated at this time, in accord with the first peak in urea-N excretion seen in the unencumbered animals of series 2 (Fig. 2*B*). The increases in ammonia-N excretion rates in the fish confined in the divided chamber were rather variable at this time. They were significant overall, in accord with the pattern in Figure 2*A*, though not in either compartment alone.

At 12–13 h of resubmergence following terrestrialization ($N = 9$), the partitioning changed significantly for both N products (ammonia-N 28% ± 9% anterior, urea-N 28% ± 5% anterior; Fig. 4*C*). Notably, absolute ammonia-N excretion rates were no longer elevated, but urea-N excretion rates were massively elevated at this time in both compartments, in accord with the second peak in urea-N excretion alone seen in the unencumbered animals of series 2 (Fig. 2*B*). The absolute increase was much greater in the posterior chamber (Fig. 4*C*), so the bulk of urea-N excretion at this time clearly occurred via the body compartment rather than via the gills.

Series 4: [^{3}H]-PEG-4000 Clearance Rates during and after Terrestrialization

In view of this much greater contribution of the body compartment to the second peak of urea-N excretion, [^{3}H]-PEG-

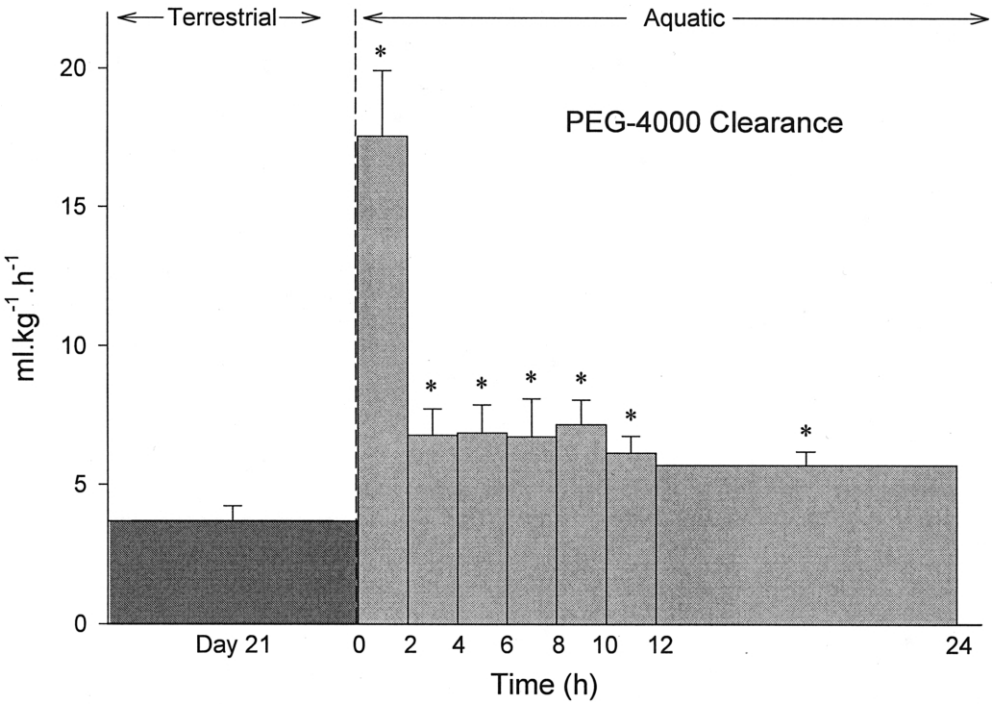

Figure 5. Rate of [^{3}H]-PEG-4000 clearance in *Protopterus dolloi* of series 4 on day 21 of terrestrialization and during return to aquatic conditions thereafter. Asterisks indicate means significantly different ($P \leq 0.05$) from the terrestrial rate on day 21. Means ± 1 SEM ($N = 7$).

4000 clearance studies were carried out to see whether either peak was associated with a change in renal function and urinary excretion. The rate of [^{3}H]-PEG-4000 clearance on day 21 of terrestrialization (Fig. 5) was 3.68 ± 0.55 mL kg^{-1} h^{-1} ($N = 5$), significantly lower than the rate in a control group (6.93 ± 0.52 mL kg^{-1} h^{-1}, $N = 5$) that had been kept under aquatic conditions throughout. In the first 2 h after return to aquatic conditions, the rate increased fivefold to about 17.5 mL kg^{-1} h^{-1}, but thereafter it immediately declined to around 7 mL kg^{-1} h^{-1} and remained stable at this value through 24 h (Fig. 5). This was significantly higher than the terrestrial rate throughout. However, this sustained [^{3}H]-PEG-4000 clearance rate of about 7 mL kg^{-1} h^{-1} was almost identical to the control aquatic rate (6.93 ± 0.52 mL kg^{-1} h^{-1}), so the latter was quickly reestablished. Notably, there was no increase in [^{3}H]-PEG-4000 clearance associated with the second, larger peak in urea-N excretion (cf. Fig. 2*B*).

The partitioning of [^{3}H]-PEG-4000 clearance between head and body was measured only in lungfish under control aquatic conditions ($N = 7$), where 2.60 ± 0.64 mL kg^{-1} h^{-1} (32%) occurred into the anterior compartment and 5.64 ± 1.34 mL kg^{-1} h^{-1} (68%) into the posterior compartment.

Series 5: [^{14}C]-Acetamide and [^{14}C]-Thiourea Clearance Rates during and after Terrestrialization

In these experiments performed at McMaster University (Fig. 6*A*), absolute magnitudes of postterrestrialization urea-N fluxes were lower than those performed at the University of Singapore (cf. Fig. 2*B*), but the temporal pattern of urea-N excretion was very similar, with a second, larger surge starting at 6–8 h, peaking at 12–16 h, and remaining strongly elevated through 16–24 h after return to aquatic conditions. The pattern of ammonia-N excretion (significantly elevated only at 0–2 h, data not shown) was also similar.

The rates of [^{14}C]-acetamide (Fig. 6*B*) and [^{14}C]-thiourea clearance (Fig. 6*C*) under terrestrial conditions were both about 4 mL kg^{-1} h^{-1} and not significantly different from each other or from the clearance rate of [^{3}H]-PEG-4000 measured under these conditions in series 4 (cf. Fig. 5). Upon return to aquatic conditions, the clearance rates of both analogs were comparably elevated to about 20 mL kg^{-1} h^{-1} at 0–2 h (Fig. 6*B*, 6*C*), again very similar to the increase in [^{3}H]-PEG-4000 clearance measured at this time. However, thereafter they both remained significantly elevated through 24 h, unlike [^{3}H]-PEG-4000 clearance. Notably, [^{14}C]-acetamide clearance exhibited a second, more pronounced peak reaching about 90 mL kg^{-1} h^{-1} by 16–24 h (Fig. 6*B*). This was approximately coincident with but slightly delayed behind the second peak in urea-N excretion (Fig. 6*A*). In contrast, the [^{14}C]-thiourea clearance rate increased gradually to only around 35 mL kg^{-1} h^{-1} at this time (Fig. 6*C*). [^{14}C]-acetamide clearance rate was significantly greater than [^{14}C]-thiourea clearance rate at 12–14 h and 16–24 h after return to aquatic conditions.

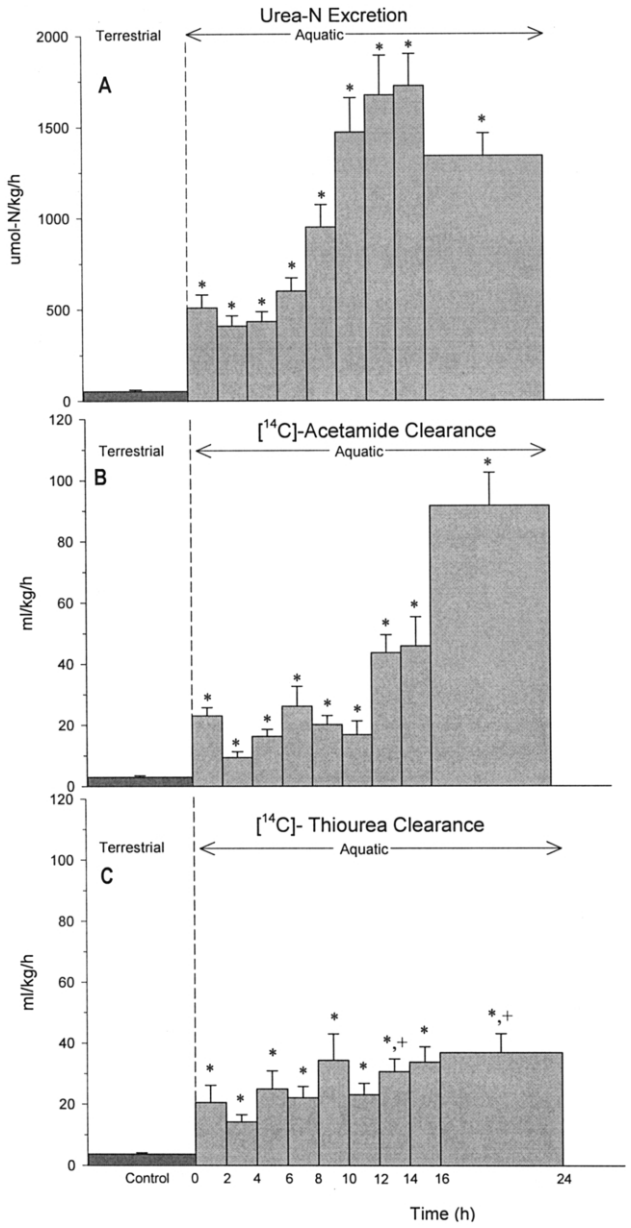

Figure 6. Rates of (*A*) urea-N excretion ($N = 27$), (*B*) [^{14}C]-acetamide clearance ($N = 13$), and (*C*) [^{14}C]-thiourea clearance ($N = 14$) in *Protopterus dolloi* of series 5 on day 30 of terrestrialization and during return to aquatic conditions thereafter. Asterisks indicate means significantly different ($P \leq 0.05$) from the corresponding rate under terrestrial conditions. Crosses indicate [^{14}C]-thiourea clearance rates significantly different ($P \leq 0.05$) from the clearance rates of [^{14}C]-acetamide at the same time. Means ± 1 SEM.

Series 6: Plasma Urea-N and Ammonia-N Concentrations during and after Terrestrialization

In these experiments at McMaster University, plasma urea-N concentration had increased about threefold after 30 d of terrestrialization relative to levels under control aquatic conditions (Table 1). Despite the marked activation of urea-N excretion during the first 24 h of return to aquatic conditions (e.g., Figs. 2*B*, 6*A*), plasma concentrations remained essentially unchanged at this significantly elevated level at 8, 12, and 24 h. In contrast, plasma ammonia-N concentrations, while variable, remained extremely low (about 0.2% of plasma urea-N levels) and showed no significant variation among sample times (Table 1).

Discussion

Our study confirms the "spectacular" increases in rates of urea-N excretion after estivation that were first reported by Smith (1930) in the congener *Protopterus aethiopicus* (see also Janssens 1964). Smith (1930) estivated his *P. aethiopicus* for a much longer period (>1 yr); his "example" data for daily urea-N excretion rates ranged from 3,000 to 16,000 μmol N kg^{-1} h^{-1} in individual fish on the first and second days after resubmergence, gradually declining over the following week. Janssens (1964) recorded about 1,700 μmol N kg^{-1} h^{-1} in the first 2 d of resubmergence following an unstated estivation period. In this study on *Protopterus dolloi* estivated for only 30 d, rates reached 5,000–6,000 μmol N kg^{-1} h^{-1} (Fig. 1*B*), while animals resubmerged after 21–30 d of terrestrialization exhibited rates of 2,000–4,000 μmol N kg^{-1} h^{-1} (Figs. 2*B*, 6*A*). Chew et al. (2003) reported about 500 μmol N kg^{-1} h^{-1} after only 6 d of terrestrialization in *P. dolloi*. Overall, these urea-N excretion rates are about two to three orders of magnitude higher than in most fish and are comparable only to rates in two teleosts that are known to express UT-A type facilitated diffusion type transporters in their gills (Walsh and Smith 2001). These are the obligatory ureotelic Lake Magadi tilapia *Alcolapia grahami*, which excretes urea-N through the gills at comparable rates all the time (Randall et al. 1989; Wood et al. 1994; Walsh et al. 2001*a*), and the facultatively ureotelic gulf toadfish *Opsanus beta*, which achieves these rates only when the branchial UT-A transporters are activated for a short period once or twice per day (Wood et al. 1995, 2003; Walsh et al. 2000). Contrary to popular belief, urea is a dipole and not freely diffusible though biological membranes (see Wood 1993; Walsh and Smith 2001), so the magnitude alone of these postestivation fluxes argues for the activation of some type of carrier-mediated mechanism to facilitate urea-N excretion at this time in the lungfish.

While it is generally agreed that ammonia-N production is turned down or off during prolonged air exposure in lungfish (note the absence of plasma ammonia-N buildup in Table 1), it has been controversial whether urea-N production is actually upregulated or whether urea-N simply accumulates because it cannot be excreted (e.g., Smith 1930; Janssens and Cohen 1968; Chew et al. 2003, 2004). The present results support the view that upregulated urea-N production occurs, in accord with measured increases of OUC enzyme activities (Chew et al.

Table 1: Concentrations of plasma urea-N (mmol L^{-1}) and plasma ammonia-N (μmol L^{-1}) in the blood plasma of *Protopterus dolloi*

Treatment	*N*	Plasma Urea-N (mmol L^{-1})	Plasma Ammonia-N (μmol L^{-1})
Control aquatic	12	17.7 ± 5.1	95.8 ± 16.8
Terrestrialization	7	51.0 ± 10.3*	88.3 ± 34.5
Resubmergence:			
8 h	7	57.4 ± 10.7*	53.1 ± 13.6
12 h	7	58.8 ± 5.3*	67.5 ± 25.6
24 h	35	58.7 ± 9.2*	122.0 ± 29.7

Note. Concentrations under control aquatic conditions, after 30 d of terrestrialization, and at various times after return to control aquatic conditions (resubmergence). Means ± 1 SEM (*N*).

* $P \leq 0.05$ relative to control aquatic value.

2003). Thus, protein metabolism may not be reduced during estivation as much as commonly believed. For example, following 30 d estivation, summated urea-N excretion into the water over the first 68 h of resubmergence (at which time excretion still remained elevated; Fig. 1*B*) amounted to about 125 d of urea-N production at the control aquatic rate or about 16 d of total-N production. Similar conclusions can be drawn from the terrestrialization experiments, although the situation was complicated there by some continued N-excretion into the water film (Figs. 2*B*, 6*A*). In these experiments, urea-N accumulated in the lungfish even though it continued to be excreted into the water film at close to control rates.

Factors such as nutrition, hydration levels, length of air exposure, and internal sequestration of urea-N may explain some of the variability between studies. Indeed, in this study, lungfish at McMaster University exhibited a smaller absolute urea-N excretion in the first 24 h of resubmergence than did animals at the University of Singapore (cf. Figs. 2*B*, 6*A*). The reason for this difference is not known, but it is noteworthy that the McMaster fish did not reduce their plasma urea-N levels during the resubmergence period (Table 1), despite the excretion of about 25,000 μmol kg^{-1} urea-N, suggesting that some of the urea-N was sequestered internally and/or that urea-N production rate was actually further increased during the first day of return to aquatic conditions. Measurements of metabolic rate, organ-specific urea-N distribution, and OUC activities during estivation, terrestrialization, and resubmergence should prove instructive in the future.

This study is the first to examine the detailed temporal pattern of urea-N excretion after resubmergence in lungfish; earlier studies measured only daily rates (Smith 1930; Janssens 1964; Chew et al. 2003). Clearly, the pattern is biphasic with an immediate increase, then a fall, and finally a second larger increase that peaks at about 12 h and may be prolonged for several days thereafter (Figs. 1*B*, 2*B*, 6*A*). This contrasts with the pattern of ammonia-N excretion, which shows only the immediate increase (Figs. 1*A*, 2*A*) at least partially attributable to disturbance, as demonstrated by the control experiment (Fig. 3*A*). Recently, we have found that some of this ammonia-N washes out from the cocoon, whereas virtually no urea-N is attributable to this source (R. Smith, M. Kajimura, A. Ip, and C. M. Wood, unpublished data). Thus, both peaks of urea-N appearance represent direct excretion by the animal, but their characteristics differ. The first peak is smaller (Figs. 1*B*, 2*B*, 6*A*), almost equally partitioned between the head (anterior compartment) and the body (posterior compartment) in divided chamber studies (Fig. 4*B*), and, most importantly, is accompanied by only a modest increase in [^{14}C]-acetamide clearance equal to that in [^{14}C]-thiourea clearance (Figs. 6*B*, 6*C*). The second peak is larger (Fig. 1*B*, 2*B*, 6*A*), occurs largely via the body rather than the head region (Fig. 4*B*), and, most importantly, is accompanied by a substantial increase in [^{14}C]-acetamide clearance but only a modest further increase in [^{14}C]-thiourea clearance (Fig. 6*B*, 6*C*).

In this regard, we have calculated the relative permeabilities of the lungfish to urea, acetamide, and thiourea at the times of the first and second peaks (setting urea permeability during the second peak arbitrarily to 100) and compared the results with data recently reported for UT-A type transporters in fish (Table 2). Clearly, during the first peak, the permeabilities to the three structural analogs are similar, whereas during the second peak, acetamide is transported much more effectively than thiourea. This pattern during the second peak fits well with the characteristics of other facilitated diffusion UT-A transporters in fish. Furthermore, it differs from the characteristics of apparent "active" urea transporters in the kidneys of amphibians (Schmidt-Nielsen and Shrauger 1963) and teleost fish (McDonald et al. 2000; McDonald et al. 2002), where thiourea is transported more effectively than acetamide.

The first immediate peak in urea-N excretion may not involve carrier mediation, given the similar apparent permeabilities of urea and its analogs at this time (Table 2). Coincident

Table 2: Relative permeabilities of *Protopterus dolloi* to urea, acetamide, and thiourea

Species	Urea	Acetamide	Thiourea	Reference
Lungfish (*P. dolloi*):				
Second peak	100	96	36	This study
First peak	28	23	21	This study
Magadi tilapia (*Alcolapia grahami*)	100	…	18	Walsh et al. 2001*a*
Gulf toadfish (*Opsanus beta*)	100	43	16	McDonald et al. 2000
Midshipman (*Porichthys notatus*)	100	74	55	McDonald et al. 2002
Rainbow trout (*Oncorhynchus mykiss*)	100	48	22	McDonald and Wood 2003

Note. Permeabilities during the first and second peaks of urea-N excretion when previously terrestrialized lungfish were returned to aquatic conditions (calculated from experiments of Fig. 6). The data are compared with those reported for UT-A type transport systems in other fish. In each case, the permeability to urea has been set to 100 (for the second peak in lungfish).

with this event, [^{3}H]-PEG-4000 clearance increased markedly (Fig. 5) and indeed was sufficient to account for the increases in rates of urea-N excretion and [^{14}C]-acetamide and [^{14}C]-thiourea clearances at this time (Fig. 6). This suggests that glomerular filtration and urine production, and therefore renal excretion rates, increased. However, the first peak was partitioned almost equally between anterior and posterior compartments, suggesting that branchial excretion also increased (Fig. 4*B*). In this regard, it must be noted that 32% of [^{3}H]-PEG-4000 clearance occurred into the anterior compartment, at least under control aquatic conditions. This is not surprising, because a comparable portion of [^{3}H]-PEG-4000 clearance also occurs across the gills of freshwater teleosts via paracellular diffusion (Curtis and Wood 1991; Scott et al. 2004). Blood flow to internal and external gills would likely have increased at the time of resubmergence (Burggren and Johansen 1986), so some of the increased flux of urea and its analogs may have occurred via passive paracellular diffusion through the branchial epithelia.

During the second peak, the major portion of urea-N was excreted from the body (Fig. 4*C*), but the involvement of the kidney or indeed of any [^{3}H]-PEG-4000 clearance route appeared to be minimal. The [^{3}H]-PEG-4000 clearance rate remained unchanged at this time (Fig. 5). If we assume that urine might have the same urea-N concentration as blood plasma (Table 1), then urine flow rate would have to have been at least sixfold greater than [^{3}H]-PEG-4000 clearance for the kidney to account for all the urea-N excretion via the body compartment at this time (Fig. 4*C*), which seems most unlikely since [^{3}H]-PEG-4000 clearance actually overestimates GFR (see above). Similarly, urine flow rate would have to have been about 12-fold and fourfold higher than [^{3}H]-PEG-4000 clearance to account for [^{14}C]-acetamide and [^{14}C]-thiourea clearances, respectively, which again seems unlikely. Smith (1930) and Janssens (1964) concurred that the role of the kidney is minimal in postestivation excretion of urea-N, although they reported only anecdotal evidence in this regard. However, it still remains possible that urea and acetamide could be secreted into the urine in copious amounts at this time rather than being washed out by an unreasonably high urine flow rate. Clearly, in future studies, it will be very useful to obtain actual measurements of urine urea concentrations to evaluate this possibility.

We therefore speculate that activation of a UT-A type facilitated diffusion transporter in the skin accounts for the second peak, the bulk of urea-N excretion, as well as the differentially elevated clearances of [^{14}C]-acetamide and [^{14}C]-thiourea at this time. Certainly, mitochondria-rich ionocytes are abundant in the skin of the congeneric *Protopterus annectens* (Sturla et al. 2001), so the skin is probably a transport epithelium. To our knowledge, urea transporters have not been reported in the skin of other fish, but they are certainly present in the skin of amphibians (Garcia-Romeu et al. 1981; Rapoport et al. 1988; Lacoste et al. 1991). We also speculate that the delay before the second peak occurs may have some adaptive value in the wild, since it may prevent the animal from responding to transitory rainfall events that are insufficient to restore aquatic conditions.

Such a transporter would presumably be expressed in the skin of the head also and perhaps also in the small external and internal gills, thereby accounting for the smaller but still significant elevation of urea-N excretion into the anterior compartment during the second peak (Fig. 4*C*). In this regard, we have recently cloned a 500-bp cDNA fragment from the internal gill that has high homology to other UT-As in fish and amphibians (F. Galvez, P. J. Walsh, A. Ip, and C. M. Wood, unpublished data). In addition to further physiological studies (e.g., tests for bidirectionality [Wood et al. 1998] and inhibitor profiles [Pilley and Wright 2000; Walsh et al. 2001*a*]), a critical next step is an expression study to examine UT-A tissue-specific distribution and to determine whether mRNA and/or protein levels change coincident with the second peak in which its involvement is suspected. In the gulf toadfish (*Opsanus beta*), pulsatile activation of the branchial UT-A transporter occurs without a change in mRNA levels (Walsh et al. 2000; Walsh and Smith 2001), so posttranslational modification of transporter function must also be considered.

Acknowledgments

This work was supported by grants to C.M.W. from the Natural Sciences and Engineering Research Council (Canada) Discovery Program, to P.J.W. from the National Science Foundation (IBN-0090355) and the National Institute of Environmental Health Sciences (ES 11005 and ES 05705), and to Y.K.I. from the National University of Singapore (Overseas Attachment Program) for the visit of P.J.W. and C.M.W. to his laboratory in December 2002. We thank Wong Wai Peng for excellent technical support at the University of Singapore; Tammie Morgan, Victoria Kjoss, Monika Patel, Mike Wilkie, and Chris Glover for help at McMaster University; all the students in the Ip lab for cheerful assistance; and two anonymous reviewers whose constructive comments improved the manuscript. C.M.W. is supported by the Canada Research Chair Program.

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