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Increased gene expression of a facilitated diffusion urea transporter in the skin of the African lungfish (*Protopterus annectens*) during massively elevated post-terrestrialization urea excretion

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SUMMARY

The full-length cDNA sequence of a putative urea transporter (*IfUT*) of the facilitated diffusion UT-A type has been cloned from the African lungfish *Protopterus annectens*. The *IfUT* cDNA is 1990 bp in length and its open reading frame encodes a 409 amino acid long protein, with a calculated molecular mass of 44,723 Da. The sequence is closest to those of amphibians (~65% amino acid homology), followed by mammals and elasmobranchs (~60%), and then teleosts (~50%). *IfUT* was clearly expressed in gill, kidney, liver, skeletal muscle and skin. Upon re-immersion in water after 33 days of air exposure ('terrestrialization'), lungfish exhibited a massive rise in urea-N excretion which peaked at 12–30 h with rates of 2000–5000 μmol-N kg^{-1} h^{-1} (*versus* normal aquatic rates of <130 μmol-N kg^{-1} h^{-1}) and persisted until 70 h. This appears to occur mainly through the skin. Total 'excess' urea-N excretion amounted to ~81,000–91,000 μmol-N kg^{-1} over 3 days. By real-time PCR, there was no difference in *IfUT* expression in the ventral abdominal skin between aquatic ammoniotelic controls and terrestrialized lungfish immediately after return to water (0 h), and no elevation of urea-N excretion at this time. However, skin biopsies revealed a significant 2.55-fold elevation of *IfUT* expression at 14 h, coincident with peak urea-N excretion. At 48 h, there was no longer any significant difference in *IfUT* mRNA levels from those at 0 and 14 h, or from aquatic fed controls. In accordance with earlier studies, which identified elevated urea-N excretion *via* the skin of *P. dolloi* with pharmacology typical of UT-A carriers, these results argue that transcriptional activation of a facilitated diffusion type urea transporter (*IfUT*) occurs in the skin during re-immersion. This serves to clear the body burden of urea-N accumulated during terrestrialization.

Key words: facilitated diffusion urea transporter, ammonia, terrestrialization, skin.

INTRODUCTION

Urea is a dipole which is not freely diffusible through lipid membrane bilayers (Wood, 1993; Walsh and Smith, 2001). Non-erythrocytic urea transporters of the facilitated diffusion type (UT-A) were first discovered in the mammalian kidney (You et al., 1993; Smith et al., 1995) and later in amphibian bladder (Couriaud et al., 1999; Konno et al., 2006), elasmobranch kidney (Smith and Wright, 1999; Morgan et al., 2003; Janech et al., 2003; Janech et al., 2006; Hyodo et al., 2004) and teleost gills (Walsh et al., 2000; Walsh et al., 2001a; Walsh et al., 2001b). A cDNA orthologue (UT-C) with relatively low sequence identity (35%) to the UT-As has been identified in eel kidney (Mistry et al., 2005). UT-A urea transporters have been shown to facilitate diffusive urea transport when expressed in *Xenopus laevis* oocytes (Smith and Wright, 1999; Fenton et al., 2000; Walsh et al., 2000; Walsh et al., 2001a; Shayakul et al., 2001; Janech et al., 2002; Stewart et al., 2006; MacIver et al., 2008).

The Dipnoi are facultatively ureotelic, but the possible occurrence of urea transporters in this important evolutionary group has not been explored to date. The African lungfishes (*Protopterus* spp.) are obligate air-breathers which possess a primitive lung as well as both internal and external gills; they are renowned for their ability to withstand prolonged periods of drought by going into a state of torpor or aestivation, covered by a cocoon to prevent dehydration (Smith, 1930; Janssens, 1964; DeLaney et al., 1974). While submerged in water, lungfishes are usually ammoniotelic, producing ammonia and excreting it across branchial and cutaneous epithelia (Graham, 1997; Wood et al., 2005). However, when water becomes limited and ammonia excretion becomes impeded, lungfishes convert toxic ammonia into a less toxic nitrogenous product, urea, and store it in the body (Smith, 1930; Janssens, 1964; DeLaney et al., 1974; Chew et al., 2003; Chew et al., 2004; Wood et al., 2005; Ip et al., 2005; Loong et al., 2005; Loong et al., 2008; Wilkie et al., 2007).

When *Protopterus dolloi* were returned to water after either aestivation in air (with complete cocoon formation) or 'terrestrialization' in air (with minimal water available on the ventral surface only, and less complete cocoon formation), the urea-N excretion rate was greatly elevated, reaching 2000–6000 μmol-N h^{-1} kg^{-1} at 10–24 h after return to water (Wood et al., 2005). A divided chamber experiment demonstrated that 72% of this urea-N efflux occurs through the posterior 85% of the body, with minimal involvement of the kidney, thereby implicating the skin as an important site of urea-N excretion (Wood et al., 2005). These urea-N flux rates in lungfish are two to three orders of magnitude higher than in most teleosts but comparable to two exceptions: the facultatively ureotelic gulf toadfish *Opsanus beta* (Wood et al., 1995; Wood et al., 1998; Walsh, 1997; Gilmour et al., 1998), and the obligately ureotelic Lake Magadi tilapia *Alcolapia grahami* (Randall et al., 1989; Wood et al., 1989; Wood et al., 1994; McDonald et al., 2003). These two teleosts both express UT-A type facilitated

urea transporters which are restricted to the gills (Walsh et al., 2000; Walsh et al., 2001a). Notably, during re-immersion after air exposure, urea excretion in *P. dolloi* exhibited the same pattern of pharmacological sensitivity to urea analogues (Wood et al., 2005) as for toadfish and Magadi tilapia, which is the same as reported earlier for UT-A transporters in higher vertebrates (McDonald et al., 2003; Walsh et al., 2001a).

In light of this background, we hypothesized that elevated urea-N excretion in lungfish during the post-terrestrialization period is mediated by a carrier, probably of the UT-A facilitated diffusion type, and that the transporter is expressed in the skin and perhaps other body tissues. Given the proposed evolutionary position of the Dipnoi as the closest living relatives to the tetrapods (Meyer and Dolven, 1992; Cao et al., 1998; Zardoya et al., 1998; Tohyama et al., 2000), we also postulated that such a carrier would show greatest similarity in sequence to the UT-As of amphibians. In higher vertebrates, transcriptional control of UT-A expression occurs only very slowly, in response to dietary N changes, for example (Bagnasco, 2005). In the gulf toadfish, the rapid (1–3 h) pulses in urea excretion are not accompanied by changes in UT-A gene expression (Walsh et al., 2000). However, given the relatively slow time course of the massive urea-N surge in the lungfish, peaking at 10–24 h post-terrestrialization (Wood et al., 2005), we also hypothesized that the expression of skin UT-A mRNA would increase in accordance with the pattern of urea-N excretion. We selected *P. annectens* for the present study, rather than the *P. dolloi* earlier studied by Wood and colleagues (Wood et al., 2005), because of the scarcity of the latter. It was therefore first necessary to establish the pattern of urea-N excretion after re-immersion in *P. annectens*.

MATERIALS AND METHODS

Experimental animals

West African lungfish, *Protopterus annectens* Owen 1839 (100–200 g), were imported from Africa through a fish farm in Singapore, and air-shipped to McMaster University. These fish were kept individually in plastic aquaria with about 21 of non-aerated dechlorinated water, supplemented with a small amount of commercial sea salts, which helped to prevent fungal infections. This resulted in a water composition of approximately Na^+ 2.0 mmol l^{-1}, Cl^- 1.8 mmol l^{-1}, Ca^{2+} 1.2 mmol l^{-1}, hardness 170 mg l^{-1} as $CaCO_3$ equivalents, and a pH of 7.8. Fish were held under a constant photoperiod (12 h:12 h L:D) at 27–29°C and were fed once every 2 days (3% body mass ration) with frozen bloodworms, and water was changed the day after feeding. The lungfish were kept for more than 1 year under these conditions before the present experiments. All experimental and holding procedures followed Canadian Council on Animal Care guidelines and were approved by the McMaster University Animal Care Committee. As far as practical, experimental protocols were designed so as to minimize the need to kill these valuable animals.

There has been confusion in the recent literature about the terminology used to describe the various air exposure and aestivation protocols used by different workers [see discussion by Loong and colleagues (Loong et al., 2008)]. In the experimental series of the present study, some of the lungfish were subjected to an experimental treatment similar to that employed by Wood and colleagues (Wood et al., 2005) and Wilkie and colleagues (Wilkie et al., 2007). This was originally described as 'terrestrialization' by Wood and colleagues (Wood et al., 2005) and the same term is used here; it should not be confused with true 'aestivation' (see Loong et al., 2008). Lungfish were induced to undergo terrestrialization in their aquaria by removing all the water except for 5–10 ml of fresh water. Throughout the terrestrialization period, the lungfish were kept under 24 h darkness with no feeding. Water (about 1 ml) was sprayed every 2 days to keep the aquaria moist. It took about 5–7 days before a brown cocoon was formed covering the body. Under these conditions, most fish exhibited very little movement, but in a few instances it was clear that fish had shifted position, with occasional slight damage to the cocoon. The total terrestrialization period was 33 days. To control for the effects of fasting, one control group was kept under standard aquatic conditions but without feeding for the same length of time, while another aquatic control group was fed every 2 days in the regular fashion.

Full-length cloning of urea transporter from *P. annectens*

One aquatic lungfish was killed for urea transporter cDNA cloning. The lungfish was anaesthetized with 0.5 g of MS-222 (Syndel Laboratories, Vancouver, BC, Canada) in 2 l of water (neutralized with NaOH), and then killed by a sharp blow to the head followed by decapitation, and immediately gill, kidney and skin were collected and frozen in liquid nitrogen. Total RNA was extracted from these three tissues using Trizol reagent (Invitrogen, Burlington, ON, Canada) according to the manufacturer's instructions and pooled together for cDNA synthesis. First strand cDNA was synthesized using Superscript reverse transcriptase II (Invitrogen, Carlsbad, CA, USA) with an adaptor oligo dT primer (T17AP2: gACTCgAgTCgACATCgAT_{17}).

Based on a partial cDNA sequence of a putative urea transporter, which we had earlier cloned from another lungfish, *P. dolloi* (EU852333) (see Wood et al., 2005), we designed a pair of cloning primers for the *P. annectens* urea transporter (Table 1). Polymerase chain reaction (PCR) was carried out in a PTC-200 MJ Research thermocycler with Platinum Taq DNA polymerase (Invitrogen) at 94°C (2 min), followed by 35 cycles of 94°C (30 s); 56°C (30 s); 72°C (45 s); and a final extension at 72°C (5 min). Electrophoresis on an ethidium bromide-stained 1% agarose gel revealed a single PCR product. This PCR product was excised and extracted using a Qiaquick gel extraction kit (Qiagen Inc., Mississauga, ON, Canada). Purified gel products were ligated into a pGEM-T easy vector (Promega, Fisher Scientific, Nepean, ON, Canada), transformed into competent *Escherichia coli* (XL-Blue, Stratagene, Mississauga, ON, Canada), and then grown on ampicillin LB agar plates at 37°C. Positive colonies containing the ligated product were

Table 1. List of primers used for cloning, screening and real-time PCR for urea transporter (*lfUT*) and actin of *Protopterus annectens*

Primers	5′– 3′
Cloning primers	
UT_F1	TgC AgT gTC AAC TCT TgT TgC
UT_R1	gTT gAA gTg TCC AgT TgC AgC
dgActin_F	CAg ggY gTS ATg gTK ggB AT
dgActin_R	KgT Tgg CYT Tgg gRT TSA g
RACE primers	
PA_UT_3′_GSP	ggC CTC CAT ggA TAC AAT ggg ATC CTT gTg g
PA_UT_3′_NGSP	CCC AgT ggT TgT CAT gTC TgC AgT CTg TCC
PA_UT_5′_GSP	gTg ATg TTA ggT ACT gAT gAC AAT CgC TgA
PA_UT_5′_NGSP	CAg TTg CAg CTA TAT gCA ggC ATA CTg CAg
Tissue screening primers	
PA_UT_SF	TgC CAC TgC TAg TCA gAg C
PA_UT_SR	AgC AgT gCT gAg Agg CAg A
Real-time PCR primers	
PA_UT_QF	CTg gTg gCT gAT TCT CCC AgT gg
PA_UT_QR	gTC CAg TTg CAg CTA TAT gCA ggC A
PA_Actin_QF	AgC CCA gAg CAA gAg Agg TAT CC
PA_Actin_QR	TTg ggg TTC AgT ggA gCC TCT g

inoculated into liquid LB media and grown overnight. Plasmids from the overnight culture were obtained and purified using GeneJet Plasmid Miniprep Kit (Fermentas Canada Inc., Burlington, ON, Canada) and sent for sequencing (ABI 3100 Gene Analyzer, MOBIX lab, McMaster University). A partial sequence (286 bp) of the putative *P. annectens* urea transporter was obtained. A partial *actin* sequence was also obtained using degenerate primers (Table 1) as described above.

Gene-specific primers (Table 1) were then designed to obtain the full-length sequence of the urea transporter by 3′ and 5′ RACE (Marathon cDNA Amplification Kit, Clontech, Mountainview, CA, USA). Messenger RNA needed for RACE cloning was purified from total RNA using an Oligotex Direct mRNA Mini Kit (Qiagen). The full-length sequence of *P. annectens* urea transporter *lfUT* (lungfish urea transporter) has been deposited in GenBank (accession number: EU716115).

Tissue distribution

Reverse-transcription (RT-) PCR was used to determine the mRNA expression pattern of *lfUT* in various tissues of fasted control and post-terrestrialized lungfish. Three lungfish were used per group.

On day 34, terrestrialized lungfish were moved to a lighted area and returned to aquatic conditions by adding 1 l of water. After re-immersion, it was about 45 min before the lungfish became active and struggled to break the cocoon to breathe. Therefore, before the lungfish were aroused, the aquaria were placed at an angle so that the nose of the lungfish was not covered by water, thereby preventing drowning. Residual cocoon pieces adhering to the skin of the lungfish were manually removed with a pair of blunt forceps. The lungfish were anaesthetized in neutralized MS-222 and killed within 10 min after they were aroused. Tissues were collected (gill, heart, kidney, liver, lung, muscle, dorsal and ventral skin) as described above. The fasted control group of lungfish were similarly killed and dissected on day 34 after 33 days of fasting.

Total RNA extraction and cDNA synthesis were done as described above. A DNase I (Invitrogen) digestion step was used (1 U μg^{-1} RNA, 15 min at room temperature) to ensure there was no genomic DNA contamination prior to cDNA synthesis. Sense and anti-sense screening primers (Table 1) were used to examine the tissue-specific expression of *lfUT*. *Actin* (the same primer pair as used for real-time PCR; Table 1) was used as a control gene to ensure that the cDNA of individual samples was successfully synthesized. PCR was carried out at 94°C (2 min), followed by 30 cycles of 94°C (30 s); 58°C (30 s); 72°C (45 s); and a final extension at 72°C (5 min).

Sequence analysis

Molecular mass calculations and analyses of amino acid sequence and sequence identity with other species were performed using BioEdit (Hall, 1999). Serine, threonine and tyrosine phosphorylation sites were predicted using NetPhos 2.0 Server (Blom et al., 1999). Hydropathy analysis to predict transmembrane domains was done through an online server (http://www.cbs.dtu.dk/services/TMHMM-2.0). Phylogenetic relationships were constructed by sequence alignment using ClustalW software (Thomson et al., 1994), followed by POWER software (Lin et al., 2005) using the neighbour-joining method for tree construction with an evaluation of 1000 bootstrap replicates.

Series 1: urea and ammonia excretion rates

To determine the amount of urea excreted by *P. annectens* under control and post-terrestrialization conditions, we terrestrialized six lungfish as described above, and subjected a second group of six lungfish to 33 days of fasting under standard aquatic conditions. A third group were fed regularly every 48 h; their excretion rates have been reported by Iftikar and colleagues (Iftikar et al., 2008). On day 34, the terrestrialized lungfish were moved to a lighted area and the aquaria were quickly flushed with 2×1 l of water to remove any accumulated waste. The lungfish were carefully handled as described above to prevent drowning. Both sets of lungfish were then put in water with a volume equal to 25× the animals' body mass, together with two air-stones in each aquarium to ensure thorough mixing. Starting at 2 h, and at 2 h intervals throughout the 72 h re-immersion, a 10 ml water sample was withdrawn from each aquarium and stored at –20°C until further analysis. Water was changed at 20, 40 and 60 h. Likewise, water samples were collected from 33 day fasted control fish at the same time intervals, using an identical protocol.

Water urea concentrations were measured by the diacetyl monoxime method (Rahmatullah and Boyde, 1980), and water ammonia concentrations were measured by the indophenol blue method (Verdouw et al., 1978), using freshly prepared urea (Sigma, St Louis, MO, USA) and NH_4Cl (Sigma) standards.

Series 2: urea excretion and skin *lfUT* mRNA quantification

After determining the pattern of urea flux from post-terrestrialization lungfish in series 1, we subjected another group of five lungfish to terrestrialization for 33 days and collected water for urea excretion rate measurement as described above, up to 60 h re-immersion. This time, we also collected ventral skin samples. Our skin sampling protocol avoided the need to kill these animals. Approximately 2 mm×1 mm biopsies of ventral skin were taken from all five individual lungfish at 0, 14 and 48 h re-immersion to investigate *lfUT* mRNA expression of *P. annectens*. Thus each fish was biopsied 3 times. Fed aquatic control animals (fed regularly at 48 h intervals) were similarly biopsied at 24 h after feeding. The lungfish were removed from the tank briefly for biopsy, which took about 1–2 min to complete and no bleeding was observed following each biopsy. The lungfish were not anaesthetized during the process and they were then kept in water supplemented with 20 $\mu g\,ml^{-1}$ of ampicillin to prevent any bacterial infection. Ventral skin samples collected were placed in Trizol reagent and frozen in liquid nitrogen immediately upon excision. A preliminary test was carried out to ensure that water urea concentration measurements were not affected by the addition of ampicillin.

Real-time PCR

Total RNA was extracted as described above, with the following modifications to increase the yield of RNA: after phenol–chloroform extraction, the upper aqueous phase containing RNA was transferred to a new, RNase-free 1.5 ml centrifuge tube and mixed well with 1 µl of glycogen (20 $\mu g\,\mu l^{-1}$, molecular biology grade, Fermentas), before isopropanol addition. The mixture was then stored at –80°C overnight, and centrifuged for 1 h at 4°C to collect the final RNA pellet. DNase digestion was performed before first-strand cDNA synthesis.

lfUT mRNA expression in aquatic, fed control lungfish as well as post-terrestrialization lungfish at 0, 14 and 48 h re-immersion was determined by quantitative real-time PCR (qPCR). qPCR primer (Table 1) specificity was tested in preliminary experiments to ensure only a single PCR product was obtained with the skin cDNA samples. The PCR product was purified and sequenced to confirm its identity. qPCR analyses of *lfUT* expression were performed on an Mx3000P QPCR System (Stratagene, Cedar Creek, TX, USA).

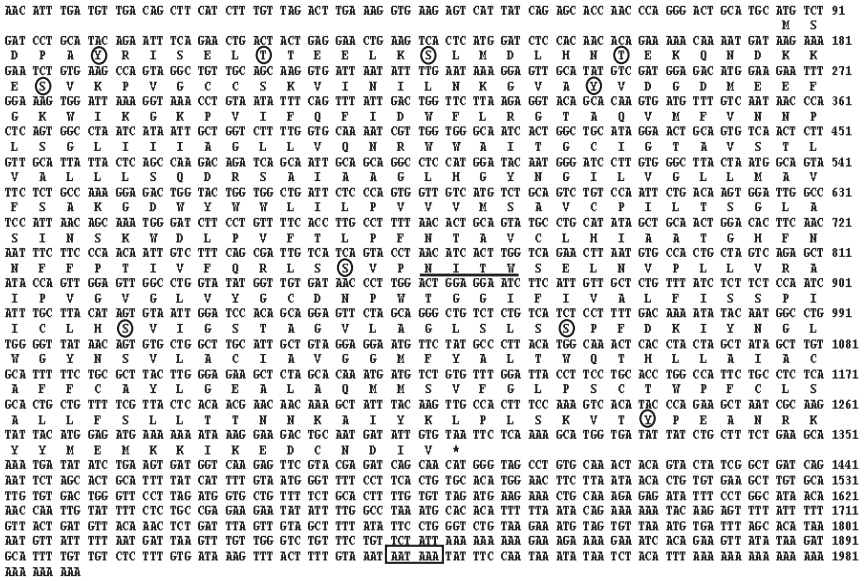

Fig. 1. Nucleotide sequence and translated amino acid sequence of the putative *Protopterus annectens* urea transporter (*lfUT*). The nucleotide sequence includes both 5′- and 3′-untranslated regions. The stop codon is indicated by an asterisk. A potential N-glycosylation site is underlined. Potential serine (S), threonine (T) and tyrosine (Y) phosphorylation sites are circled. The putative polyadenylation signal sequence (AATAAA) is boxed.

Each qPCR reaction (20 µl) containing 4 µl of DNaseI-treated (Invitrogen) cDNA (1:4 dilution), 4 pmol each of forward and reverse primer, 10 µl of Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen) and 0.8 µl of ROX (1:10 dilution) was performed at 50°C, 2 min; 95°C, 2 min; followed by 40 cycles of 95°C, 15 s; and 60°C, 30 s. Melt-curve analysis further verified that only one unique product was produced and gel electrophoresis confirmed the presence of a single band. Two negative controls were included: a non-reverse-transcribed RNA sample which controlled for possible genomic DNA contamination; and a water sample to ensure that primers and all reagents were free of contamination. In this study, *actin* was used as the normalization factor because it displayed consistent expression in aquatic, fed control samples and at all three time points post-terrestrialization. A standard curve was run for each of the genes in this study.

Statistical analyses

All data are expressed as means ± 1 s.e.m. (N=5–6). Friedman's repeated measures analysis of variance on ranks (for non-normally distributed data) was employed to detect significant variation in urea-N and ammonia-N excretion rates within control (starved for the same period but kept in water throughout) and post-terrestrialization lungfish, followed by a Student–Newman–Keuls *post-hoc* test to identify specific differences. Student's unpaired two-tailed *t*-test was used to compare overall excretion rates between 33 days starved aquatic control and fed aquatic control lungfish. The same test was also used to compare rates in control and post-terrestrialization lungfish at the corresponding time points. Student's two-tailed paired *t*-test with Bonferroni's correction was used to compare *lfUT* mRNA expression among the three re-immersion biopsy times in the same five terrestrialized lungfish, and a parallel unpaired test with Bonferroni's correction was used for comparison of each to the aquatic fed controls. A significance level of $P<0.05$ was used throughout.

RESULTS

Nucleotide sequence, protein sequence and predicted structure

We have obtained the full-length cDNA sequence of a putative urea transporter of the facilitated diffusion type, *lfUT*, from the African lungfish *P. annectens*. This is the first urea transporter cloned in full from the Subclass Dipnotetrapodomorpha. The *lfUT* cDNA is 1990 bp in length and its open reading frame encodes a 409 amino acid long protein, with a calculated molecular mass of 44,723 Da. Several putative phosphorylation sites (18-, 34-, 225-, 277-, 293-serine; 12-, 25-threonine; 6-, 54-, 386-tyrosine), and one N-glycosylation site, ^{28}NITW^{31}, are predicted (Fig. 1). Similar to other known urea transporters, a hydropathy analysis indicated that *lfUT* has nine membrane-spanning helices (Fig. 2), indicating that it is a membrane protein. Comparison of *lfUT* with other urea transporters showed that *lfUT* is of the UT-A type and shares highest amino acid sequence identity with amphibian urea transporters (63.9–67.8%), followed by mammalian and elasmobranch (58.8–62.5%), and then teleost urea transporters (48.1–50.7%; Table 2; Figs 2 and 3). Extensive cloning efforts using a variety of degenerate primers failed to detect other UT-A or UT-C sequences, although the present *lfUT* sequence was yielded several times by these approaches.

Tissue expression in control and post-terrestrialization lungfish

RT-PCR results revealed that *lfUT* was clearly expressed in a number of tissues, including gill, kidney, liver, muscle, skeletal muscle, dorsal skin and ventral skin but was only weakly expressed in or absent from heart and lung of control (aquatic) *P. annectens*, and after 33 days of terrestrialization (Fig. 4). There were no marked differences in tissue-specific expression by RT-PCR associated with terrestrialization; note that these animals were killed immediately after return to water.

Series 1: urea-N and ammonia-N excretion during re-immersion after terrestrialization

The rate of urea-N excretion in control *P. annectens*, which had been kept under aquatic conditions and fasted for 33 days, averaged 119.6±2.6 µmol-N kg^{-1} h^{-1} (Fig. 5). Urea-N excretion showed a distinct pattern that involved two daily maxima and minima, which, however, did diminish over time. The only significant difference was for 2 h *versus* 70 h. This was not significantly different from the rate of 129.9±9.6 µmol-N kg^{-1} h^{-1}, integrated over the 48 h feeding cycle, in the fed aquatic control animals.

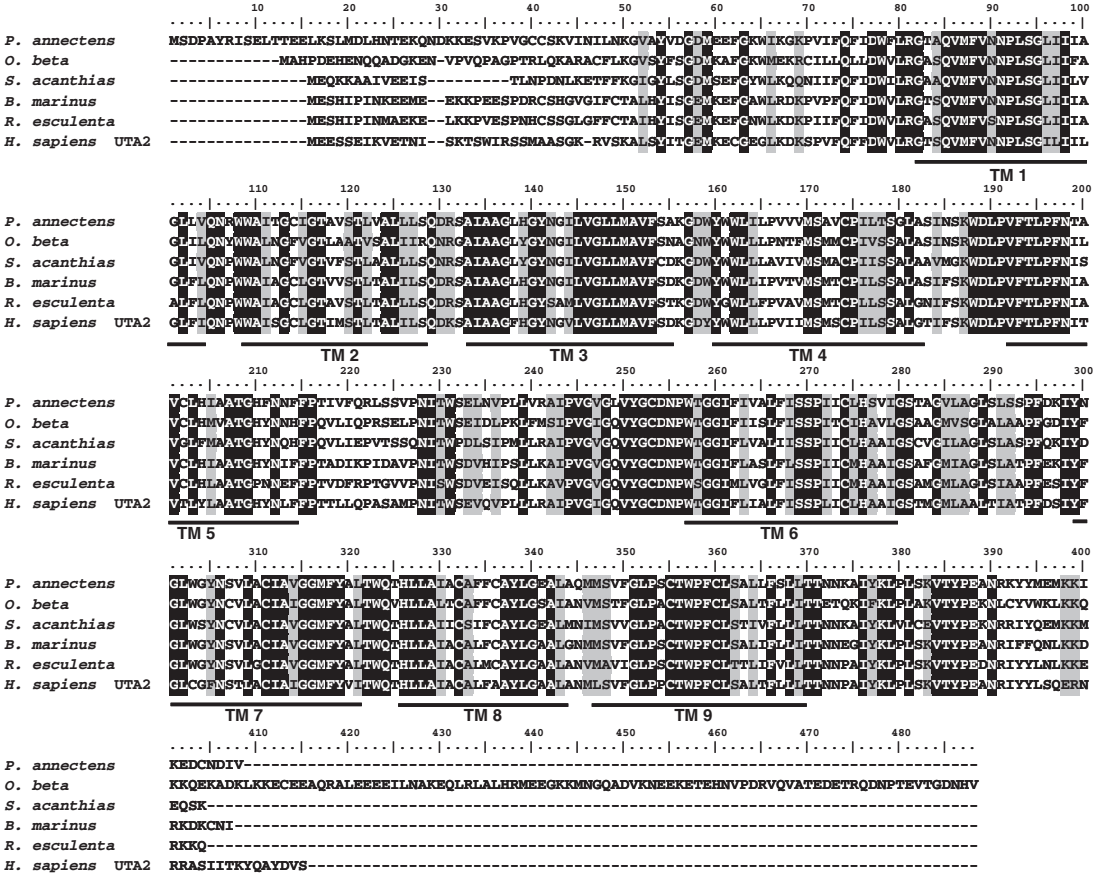

Fig. 2. Amino acid sequence alignment of the putative *P. annectens* urea transporter (*lfUT*) with several known urea transporters from other species. Urea transporter sequences are highly conserved across species from different taxa. Identical amino acids are highlighted in black and similar amino acids are shaded. Nine transmembrane (TM) regions were predicted for *P. annectens* and are underlined. Accession numbers: *P. annectens* (lungfish, EU716115); *Opsanus beta* (gulf toadfish, AAD53268); *Squalus acanthias* (spiny dogfish, AAF66072); *Bufo marinus* (giant toad, BAE16706); *Rana esculenta* (edible frog, CAA73322); *Homo sapiens* (human, CAA65657).

Following re-immersion after 33 days of terrestrialization, *P. annectens* exhibited a marked surge in urea-N excretion lasting approximately 3 days. The response was slightly delayed, inasmuch as urea-N excretion rates during the first 8 h of re-immersion were similar to those of control lungfish. The urea-N excretion rate was significantly higher at 14 h ($2156\pm834\,\mu mol\text{-}N\,kg^{-1}\,h^{-1}$) when compared with that at 2–8 h, and the rates remained significantly elevated up to 56 h (Fig. 5). The peak excretion rate occurred at 20 h ($5132\pm380\,\mu mol\text{-}N\,kg^{-1}\,h^{-1}$) and was significantly higher than the 2–18, 22, 26 and 30 h through to 72 h post-terrestrialization

Table 2. Percentage sequence identity (amino acid sequence) of *Protopterus annectens lfUT* in comparison with other UT-A sequences obtained from GenBank

Amphibian	Giant toad (BAE16706)	67.8%
	Edible frog (CAA73322)	63.9%
	Spiny dogfish (AAF66072)	62.5%
	Sei whale (BAF46916)	61.6%
	Sperm whale (BAF46918)	61.4%
Mammals and elasmobranch	Human (CAA65657)	61.2%
	Minke whale (BAF46914)	60.9%
	Banded houndshark (BAC75980)	60.7%
	House mouse (AAM21206)	60.4%
	Atlantic stingray (AAM46683)	58.8%
Teleost	Eel (BAC53976)	50.7%
	Lake Magadi tilapia (AAG49891)	50.0%
	Fugu rubipes (NP_001027896)	48.8%
	Gulf toadfish (AF165893)	48.1%
Prokaryotes	*Yersinia frederiksenii* (AAR15107)	20.7%
	Actinobacillus pleuropneumoniae (AAD49729)	14.4%

Sequences are arranged in descending order of similarity.

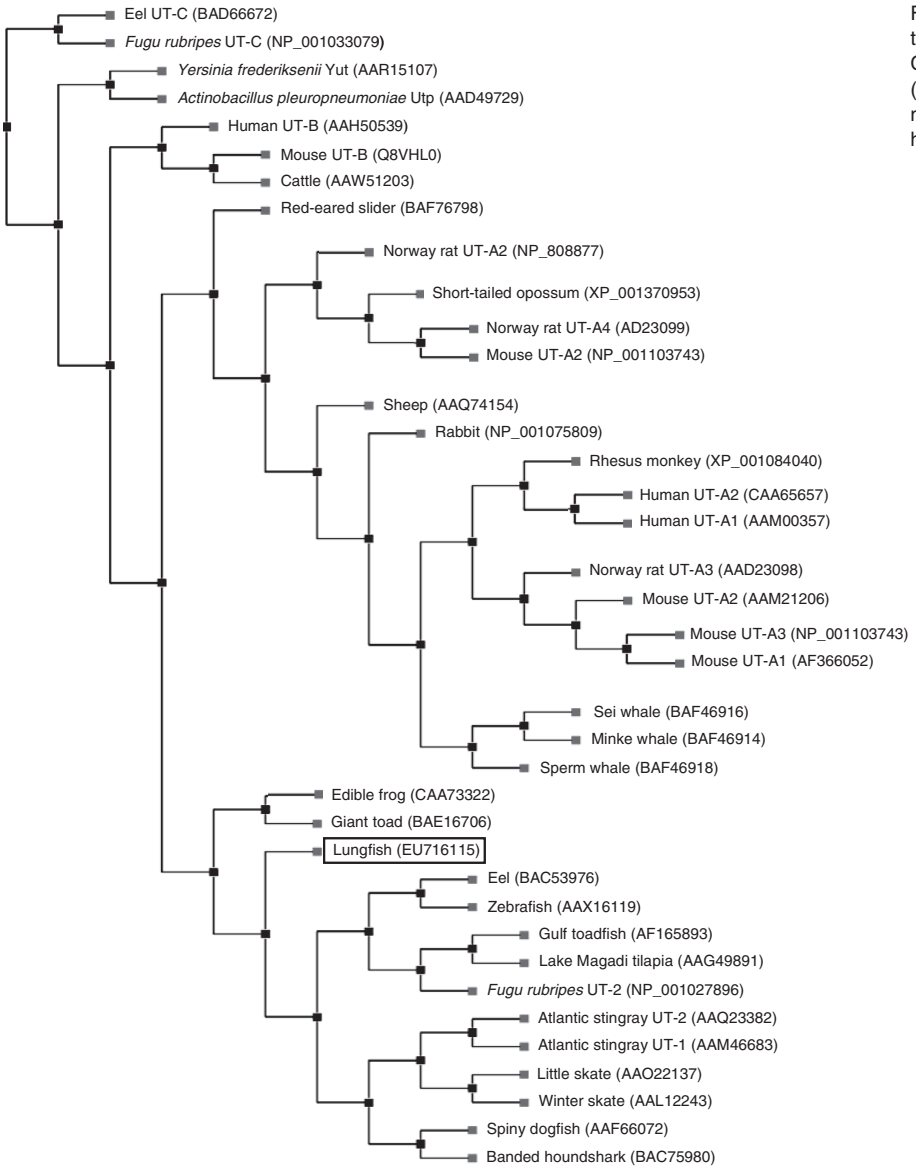

Fig. 3. Phylogenetic tree of urea transporters across taxa. The protein sequences were aligned using ClustalW software, followed by neighbour-joining (NJ) matrix for tree reconstruction and evaluated by means of a bootstrap of 1000 replicates at http://power.nhri.org.tw.

measurements. When compared with control *P. annectens*, urea-N excretion rates of re-immersed *P. annectens* were significantly higher from 10 h through to 70 h at every corresponding time point (Fig. 5). The total 'extra' urea-N excretion over the 3 day period was 81,070 μmol-N kg^{-1}.

Ammonia-N excretion rates, however, did not show any significant difference between fasted control and post-terrestrialization *P. annectens* or significant variation over time (data not shown). The mean ammonia excretion rate of fasted control lungfish was 48.9±4.4 μmol-N kg^{-1} h^{-1} and that of re-immersed *P. annectens* was 58.4±1.0 μmol-N kg^{-1} h^{-1}, respectively. By way of comparison, fed aquatic lungfish exhibited a significantly higher ammonia-N excretion rate of 155.2±11.8 μmol-N kg^{-1} h^{-1}, integrated over the 48 h feeding cycle.

Series 2: real-time PCR results

The only methodological difference in this experiment from series 1 was that the five terrestrialized lungfish were subjected to ventral skin biopsies at 0, 14 and 48 h re-immersion. The pattern of urea-N excretion following re-immersion was broadly similar to that in series 1 (Fig. 5), except for a variable elevation at 2 h (2100±1150 μmol-N kg^{-1} h^{-1}, in series 2 fish), although this rate was not significantly higher than that measured at this time in series 1 (372±77 μmol-N kg^{-1} h^{-1}). Urea-N excretion rates at 14–50 h post-terrestrialization were significantly higher than those measured at 6–10, 54 and 60 h. The total 'extra' urea-N excretion in this series was about 91,000 μmol-N kg^{-1} when compared with the total urea excreted by control aquatic lungfish in series 1 (up to 60 h).

lfUT mRNA expression in the ventral skin of aquatic fed control and re-immersed lungfish of series 2 was normalized to *actin* mRNA expression (which did not change) and the results are presented as a relative ratio of *lfUT* to *actin* mRNA levels (Fig. 6). There was no difference in *lfUT/actin* expression between fed control lungfish and 33 days terrestrialized lungfish. However, *lfUT/actin* expression was significantly higher (2.55-fold) in 14 h re-immersed lungfish skin when compared with that of aquatic, fed controls, and also significantly higher than in fish re-immersed at 0 h. At 48 h, relative *lfUT/actin* expression declined, so it was no longer significantly different from either the 14 or 0 h samples, or from the aquatic controls (Fig. 6).

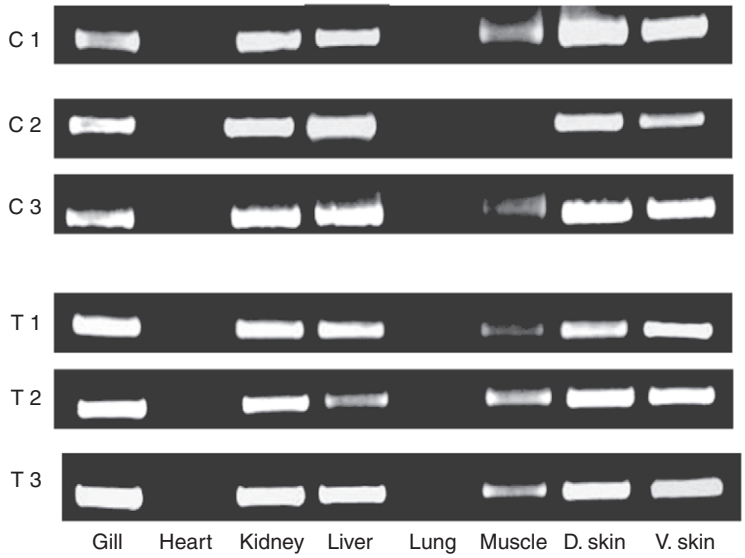

Fig. 4. Expression of the putative lungfish urea transporter gene (*lfUT*) in various tissues of aquatic control (C) and post-terrestrialization (T) *P. annectens*. Consistent expression of the urea transporter was detected in gill, kidney, liver, muscle, and dorsal and ventral skin, but it was only weakly expressed or absent in heart and lung of both control and post-terrestrialization *P. annectens*.

DISCUSSION

Sequence analysis

We have obtained the full-length cDNA sequence of a putative membrane-spanning urea transporter, designated *lfUT*, from the lungfish *P. annectens*, representing the first such sequence in the Subclass Dipnotetrapodomorpha. The translated amino acid sequence of *lfUT* exhibits greater than 45% sequence identity with other known urea transporters (Table 2), and contains two urea transporter signature domains: ^{188}WDLPVFTLPFN^{198} (known as the LP box) and ^{244}PVGXGXVXGCDNPW^{257} (where X indicates conservative substitutions; Fig. 1) (Rousselet et al., 1996; Bagnasco, 2003) but lacks the UT-B signature ALE domain. These findings suggest that this newly identified urea transporter from lungfish is a UT-A. A number of UT-A isoforms or transcripts have been identified in mammals, amphibians and elasmobranchs (see Introduction) (for reviews, see Bagnasco, 2003; Bagnasco, 2005; McDonald et al., 2006). It is possible that lungfish also possess more than one copy of UT-A in their very large genome. Repeated attempts to identify other UT-A as well as UT-C isoforms from the skin of *P. annectens* have proven unsuccessful to date.

Phylogenetic analysis based on amino acid sequences of the urea transporters available in GenBank indicated that *lfUT* is distinctively different from teleost, amphibian, elasmobranch, mammalian and prokaryotic urea transporters. Nevertheless, it is noteworthy that *lfUT* shares the highest homology with the amphibian orthologues, followed by mammals and elasmobranchs, and is only about 50% identical to the teleost UT-As (Table 2). The phylogenetic tree (Fig. 3) shows that the most primitive urea transporters separated into two groups: UT-C and all other urea transporters, similar to results reported earlier (Mistry et al., 2005). Prokaryotic (*Yersinia frederiksenii* and *Actinobacillus pleuropneumoniae*) transporters then divided from eukaryotic transporters. Eukaryotic urea transporters then branched into two main groups: 'erythrocytic' UT-Bs and 'renal' UT-As. Mammalian UT-As are furthered clustered as one group, separated from amphibian and fish UT-As. Interestingly, *lfUT* is branched out as a unique group, between amphibians and fish (teleosts and elasmobranchs).

The fact that the lungfish *lfUT* is more closely related to the amphibian UT-As is in accordance with reported phylogenetic relationships for other lungfish genes including somatostatin precursors I and II (Trabucchi et al., 1999), myelin DM20 (Tohyama et al., 2000) and urate oxidase (Anderson et al., 2006). In fact, recent evolutionary analyses have hypothesized that lungfish (Subclass Dipnotetrapodomorpha) are phylogenetically the closest relatives to tetrapods (Meyer and Dolven, 1992; Cao et al., 1998; Zardoya et al., 1998; Tohyama et al., 2000), though exact relationships between lungfish, coelocanths and tetrapods remain unclear (Takezaki et al., 2004; Friedman et al., 2007). Furthermore, the environmental circumstances of amphibians and lungfish (amphibious life style, frequent danger of dehydration, ability to aestivate) are similar. Therefore, it is possible that regulatory mechanisms (e.g. transcriptional, translational or post-translational modification, hormonal controls) of lungfish *lfUT* may also be more similar to those of amphibians than fish.

In teleost fish, gene expression of UT-A appears to be restricted to the gills (Walsh et al., 2000; Walsh et al., 2001a; Mistry et al., 2001), but in the amphibian *Bufo marinus*, UT-A is expressed in many tissues, including kidney, urinary bladder, intestine, brain, lung, spleen and testis (Konno et al., 2006). *P. annectens* also exhibits a broad distribution pattern, although the sites of expression are somewhat different from those in amphibians: *lfUT* is expressed not only in the skin but also in both gill and kidney, as well as in the liver and muscle (Fig. 4). This discrepancy in urea transporter expression probably indicates different major sites of urea excretion in amphibians *versus* lungfish. The absence of urea transporter expression in the skin of teleosts and amphibians but its presence in the lungfish signifies the importance of the skin as a major site for facilitated urea excretion in the lungfish.

Urea-N and ammonia-N fluxes during re-immersion after terrestrialization

When kept in water under normal fed conditions, *P. annectens* is ammoniotelic with >50% of nitrogenous waste excreted as ammonia-N (Loong et al., 2008; Iftikar et al., 2008). It is interesting therefore that the 33 day fasted control animals of the present study were actually ureotelic, excreting about 71% urea-N ($119.6\pm2.6\,\mu mol\ urea\text{-}N\,kg^{-1}\,h^{-1}$ *versus* $48.9\pm4.4\,\mu mol$ ammonia-$N\,kg^{-1}\,h^{-1}$). Notably, however, this apparent 'switch' to ureotelism was not due to an elevation in urea-N excretion, which remained unchanged relative to fed control rates ($129.9\pm9.6\,\mu mol\text{-}N\,kg^{-1}\,h^{-1}$), but rather was due to a reduction in ammonia-N excretion

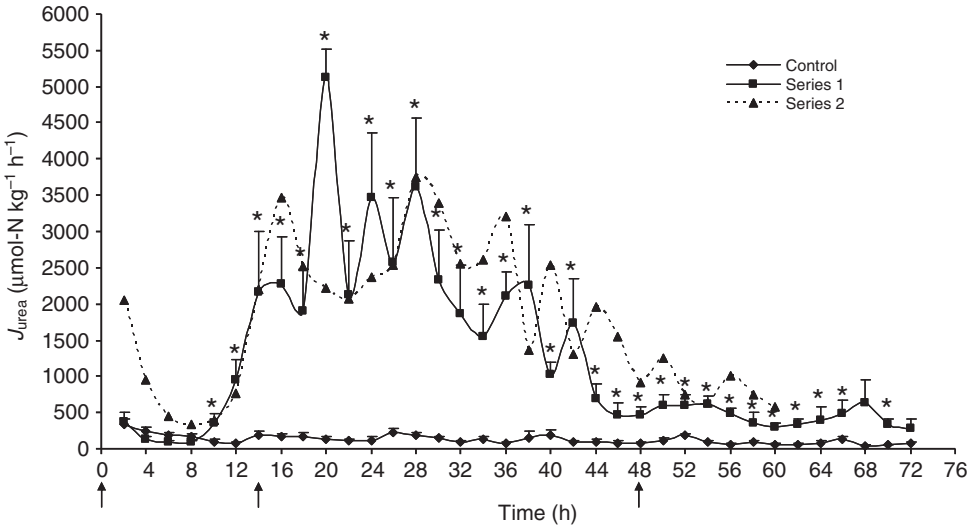

Fig. 5. Rates of urea-N excretion in *P. annectens* during re-immersion following 33 days of terrestrialization and in control (aquatic) lungfish following 33 days of fasting. Means and 1 s.e.m. (*N*=6) are shown for the control series and for the data of Series 1; asterisk indicates that the urea-N excretion rate in these post-terrestrialization animals was significantly different from the urea-N excretion rate in control (aquatic) animals at the respective time point ($P<0.05$). Means only (*N*=5) are shown for the data of Series 2, s.e.m. values have been omitted for clarity. Arrows indicate the times of biopsies in Series 2. Within treatment groups, multiple comparison testing revealed that in the control group only the 2 and 70 h points were significantly different ($P<0.05$). Within the post-terrestrialization group of Series 1, urea-N excretion was significantly higher at 14 h than at 2–8 h, and the rates remained significantly elevated up to 56 h. The peak excretion rate occurred at 20 h and was significantly higher than the 2–18, 22, 26 and 30 h through to 72 h post-terrestrialization measurements. Within the post-terrestrialization group of Series 2, urea-N excretion rates at 14–50 h post-terrestrialization were significantly higher than means at 6–10, 54 and 60 h ($P<0.05$).

significantly below fed control rates (155.2±11.8 μmol-N kg^{-1} h^{-1}). Loong and colleagues (Loong et al., 2008) noted a similar change, with ~57% urea-N excretion in *P. annectens* during a comparable fasting protocol. Thus the ratio between urea-N and ammonia-N excretion is very sensitive to feeding and fasting states in aquatic lungfish. Indeed the balance may also shift towards ureotelism during the digestion of a large meal (Lim et al., 2004; Iftikar et al., 2008). However, during prolonged aestivation or terrestrialization (when fasting also occurs), it is now clearly established that urea-N, and not the more toxic ammonia-N, progressively accumulates in the body tissues (see Introduction for references). Upon return to water, this accumulated urea-N burden is excreted.

The present N excretion data following re-immersion in terrestrialized *P. annectens*, showing a large but delayed and prolonged increase in urea-N efflux (Fig. 5) with only minor changes in ammonia-N efflux, are very similar to those reported earlier in the congeneric *P. dolloi* (Wood et al., 2005). In the present study, these measurements were continued for a much longer duration, showing that while the urea-N excretion surge reached its peak in the first 24 h, it did not fully attenuate until about 70 h post-terrestrialization (Fig. 5). Furthermore, the generally similar pattern between series 2 (where the fish were biopsied) and series 1 (where they were left undisturbed) suggests that the urea-N excretion mechanism was not greatly perturbed by handling (Fig. 5). It is possible that the initial transient elevation in urea-N excretion in series 2 could have resulted from disturbance, but the rates at this time were not significantly greater than those in series 1 (Fig. 5) and, furthermore, a similar transient initial rise was seen in undisturbed *P. dolloi* immediately after re-immersion (Wood et al., 2005). It seems likely that similar mechanisms are at play in the two species, and that the slow, prolonged surge represents increased activity of a UT-A facilitated diffusion urea transport system, mainly in the skin, as argued below.

The magnitude of the 'excess' urea-N excretion in both series was remarkable (~81,000–91,000 μmol urea-N kg^{-1}), in accordance with the early data of Smith (Smith, 1930) and Janssens (Janssens, 1964) on post-aestivation *P. aethiopicus*. This value is in line with plasma and tissue urea-N concentrations (50,000–200,000 μmol urea-N kg^{-1}) measured after comparable air exposure treatments in both *P. dolloi* (Chew et al., 2004; Wood et al., 2005; Wilkie et al., 2007) and *P. annectens* (Loong et al., 2008). The tests of Wood and colleagues (Wood et al., 2005) on *P. dolloi* indicated that simple diffusion or kidney excretion could explain only a small fraction of

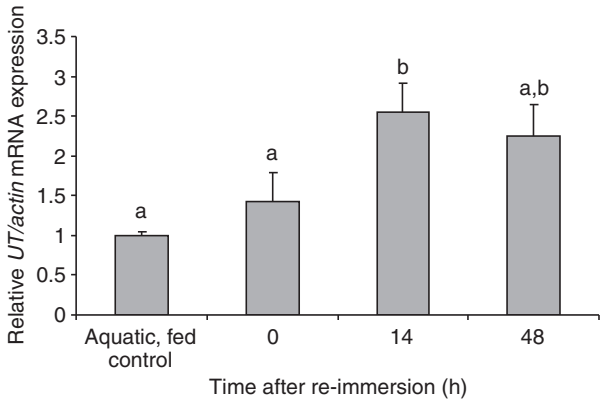

Fig. 6. Relative expression levels of the urea transporter gene (*lfUT*; relative to *actin*), in ventral skin of aquatic, fed control *P. annectens* and during re-immersion following 33 days of aestivation. Relative expression of *lfUT/actin* at 14 h following re-immersion was significantly higher than in aquatic fed control animals and in terrestrialized animals 0 h after re-immersion ($P<0.05$). At 48 h, expression was not significantly different from any other conditions. Values are means and s.e.m. (*N*=5).

this. Inasmuch as the majority (72%) of this high urea-N efflux occurred through the posterior 85% of the body, did not occur through the kidney, and occurred in concert with a greatly elevated excretion of two urea analogues but not the paracellular permeability marker PEG-4000, the evidence pointed to increased activity of a specific urea transporter in the skin (Wood et al., 2005). The measured analogue permeability order of urea>thiourea>acetamide was typical of UT-A type facilitated diffusion transporters in the gills of ureotelic teleosts (McDonald et al., 2000; Walsh et al., 2001a) and mammalian kidney (Chou and Knepper, 1989). The present study provides further evidence for this idea, inasmuch as *lfUT* was expressed in the skin, and exhibited a significant increase in mRNA expression level (Fig. 6) at the time of peak urea-N excretion (Fig. 5).

The role of *lfUT* in increased urea excretion

Although modest, the 2.55-fold increase in *lfUT* expression in lungfish skin at 14 h post-terrestrialization (Fig. 6) coincident with the surge of urea-N excretion (Fig. 5) was in accordance with our original hypothesis, and very different from the situation in teleost fish (McDonald et al., 2006). This difference may reflect the different time course of the response, which is slow enough to allow increased mRNA and protein synthesis in the lungfish. In mammals, transcriptional activation of UT-A is generally slow, and associated with the action of antidiuretic hormone (ADH), glucocorticoids and cAMP on promoter regions (Bagnasco, 2003; Bagnasco, 2005). Transcriptional activating mechanisms for UT-A expression are unknown in lungfish, but in amphibians the ADH analogue arginine vasotocin appears to be involved (Uchiyama, 1994; Konno et al., 2006). In toadfish, the expression of the branchial *tUT* mRNA transcript is constant, and not correlated with the rapid, short-lasting (1–3 h) pattern of pulsatile urea-N excretion across the gills (Walsh et al., 2000). McDonald and colleagues (McDonald et al., 2006) have reviewed the evidence that both a decline in circulating cortisol (as a permissive agent) and the release of serotonin (5-HT, as a neuroendocrine activating agent) may quickly turn on the toadfish gill *tUT* at the time of a urea-N pulse, probably by post-translational mechanisms. In the Magadi tilapia, the gill *mtUT* appears to be continually turned on, to deal with the very high rates of continuous urea-N excretion required by these 100% ureotelic teleosts (Walsh et al., 2001a). However, similar to the response in lungfish skin, modest transcriptional activation (~2-fold) of UT-As has been seen in the kidney and urinary bladder of the marine toad *Bufo marinus* (Konno et al., 2006), and in the kidney of the red-eared slider turtle *Trachemys scripta elegans* (Uchiyama et al., 2009) exposed to dehydrating conditions for 7 days. These slow increases in UT mRNA expression in the toad and the turtle were associated with the accumulation of urea in urinary bladders, while in lungfish urea was excreted across the skin (Wood et al., 2005).

In the present study there was no difference in *lfUT* expression (Fig. 6) between fed control lungfish and 33 days terrestrialized lungfish at 0 h (i.e. immediately after return to water) despite the fact that terrestrialized lungfish are known to have 4- to 11-fold greater plasma urea concentrations at this time (Wood et al., 2005; Wilkie et al., 2007; Loong et al., 2008). Furthermore, there was no elevation in urea-N excretion at this time or for the next 8 h (Fig. 5). This suggests that despite the large gradient, the availability of functional *lfUT* carrier proteins was initially limiting upon return to water for the flux of this poorly diffusible molecule, perhaps due to the general metabolic depression occurring during terrestrialization (Staples et al., 2008). Indeed it took at least 14 h for urea-N excretion to reach a peak (Fig. 5), and the modest increase in *lfUT* mRNA expression at this time (Fig. 6) was sufficient to facilitate an enormous amount of urea-N excretion. *lfUT* mRNA expression was no longer significantly elevated at 48 h (Fig. 6). Our interpretation is that, by this time, the functional UT protein level was probably sufficient for the remaining urea to be excreted without having to make more *lfUT* transcripts, and as accumulated urea was excreted and the gradient fell, the rate of excretion similarly fell through to 70 h (Fig. 5). However, it certainly remains possible that post-translational mechanisms acting at the *lfUT* protein level may also have been involved. In future experiments, it will be of interest to investigate the signalling mechanisms for these events.

Although RT-PCR is only a semi-quantitative technique, the absence of marked changes in *lfUT* expression in the various tissues immediately after terrestrialization (Fig. 3) is notable, and in accordance with the more sensitive real-time PCR results for the skin at 0 h (Fig. 6). In future studies, it will be of interest to use real-time PCR to evaluate whether *lfUT* expression increases later during re-immersion in other tissues as it does in the skin (e.g. at 14 h), coincident with greatly elevated urea-N excretion at this time.

In summary, in accordance with our original hypotheses (see Introduction) we have cloned a cDNA encoding UT-A in lungfish *P. annectens* (*lfUT*), shown that it bears greatest sequence similarity to the UT-As of amphibians, and demonstrated that the expression of *lfUT* in the skin is correlated with the greatly elevated rate of urea-N excretion during re-immersion after a prolonged period of terrestrialization in air.

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