Seasonal Variation and Response to Osmotic Challenge in Urea Transporter Expression in the Dehydration- and Freeze-Tolerant Wood Frog, *Rana sylvatica*

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ABSTRACT   Urea accumulation is a universal response to osmotic challenge in anuran amphibians, and facilitative urea transporters (UTs) seem to play an important role in this process by acting in the osmoregulatory organs to mediate urea retention. Although UTs have been implicated in urea reabsorption in anurans, little is known about the physiological regulation of UT protein abundance. We examined seasonal variation in and effects of osmotic challenge on UT protein and mRNA levels in kidney and urinary bladder of the wood frog (*Rana sylvatica*), a terrestrial species that tolerates both dehydration and tissue freezing. Using immunoblotting techniques to measure relative UT abundance, we found that UT numbers varied seasonally, with a low abundance prevailing in the fall and winter, and higher levels occurring in the spring. Experimental dehydration of frogs increased UT protein abundance in the urinary bladder, whereas experimental urea loading decreased the abundance of UTs in kidney and bladder. Experimental freezing, whether or not followed by thawing, had no effect on UT numbers. UT mRNA levels, assessed using quantitative real-time polymerase chain reaction, did not change seasonally nor in response to any of our experimental treatments. These findings suggest that regulation of UTs depends on the nature and severity of the osmotic stress and apparently occurs posttranscriptionally in response to multiple physiological factors. Additionally, UTs seem to be regulated to meet the physiological need to accumulate urea, with UT numbers increasing to facilitate urea reabsorption and decreasing to prevent retention of excess urea. *J. Exp. Zool. 317A:401–409, 2012.* © 2012 Wiley Periodicals, Inc.

Due to their relatively permeable skin, amphibians are especially prone to dehydration and must utilize various strategies to conserve body water. Accumulation of urea, the major end product of nitrogen metabolism in amphibians, is one such strategy often employed to prevent excessive water loss. In hydrated amphibians, urea is usually maintained at low levels; however, when exposed to osmotic stress, they accumulate substantial amounts of urea (Jorgensen, '97). Increasing the tissue levels of urea improves survival by reducing the gradient for water loss to the environment (Jorgensen, '97) and replacing harmful ionic solutes (Grundy and Storey, '94). In addition, the high plasma urea levels found in some terrestrially hibernating anurans enhance freeze

tolerance and contribute to metabolic depression (Costanzo and Lee, 2005; Muir et al., 2007).

Among anurans, urea can be accumulated by two main mechanisms: increasing urea synthesis by altering urea-cycle enzymes (Balinsky, '81) and increasing urea retention. The latter is accomplished by reducing glomerular filtration rate (GFR), adjusting the secretion and reabsorption of urea in renal tubules, and reabsorbing urea from the urinary bladder fluid (Schmidt-Nielsen and Lee, '62; Shpun and Katz, '95). Evidence suggests that facilitative urea transporters (UTs) in the epithelium of osmoregulatory organs are involved in the reabsorption and retention of urea (Couriaud et al., '99; Konno et al., 2006).

UTs are present in various organisms including mammals (Sands, 2003), fish (McDonald et al., 2006), reptiles (Uchiyama et al., 2009), and anuran amphibians (Couriaud et al., '99; Konno et al., 2006). In mammals, two distinct UT groups are known. The UT-A group, with six isoforms (UT-A1–UT-A6), is found primarily in kidney; whereas, the UT-B group, with two isoforms (UT-B1, UT-B2), is known from erythrocytes and various tissues (Sands, 2003). Mammalian UTs are involved in diverse physiological processes ranging from urine concentration in kidney to nitrogen recycling in intestine (reviewed in Bagnasco, 2006). To coordinate the roles of the different UT isoforms, these proteins are subject to regulation by multiple processes. Short-term regulation, controlled by hormonal and osmotic cues, occurs by shuttling UTs between intracellular vesicles and the plasma membrane, and also by altering transport activity through changes in phosphorylation state (reviewed in Sands and Layton, 2009). Long-term regulation occurs through changes in protein abundance in response to hormones and certain physiological and pathological states (reviewed in Bagnasco, 2005).

Whereas mammalian UTs have been the focus of several studies, little is known about the roles and regulation of these transporters in amphibians. Amphibian UTs were first identified in urinary bladder of *Rana esculenta* (Couriaud et al., '99) and their presence has since been identified in various tissues of other anurans (Konno et al., 2006). Anuran UTs likely help modulate plasma urea levels and maintain osmotic balance, as these proteins are most abundant in the osmoregulatory organs, particularly urinary bladder. During osmotic challenge, these proteins likely play an especially important role in maintaining osmotic balance. Indeed, frogs exposed to dehydrating or hypersaline conditions show a marked increase in UT expression (Konno et al., 2006).

Although UT levels change in response to dehydration and hypersaline exposure, it is not known if other osmotic stresses, such as tissue freezing, also elicit changes in UT expression. To address this question, we examined a putative UT in the wood frog, *Rana sylvatica*, a terrestrial species that survives the loss of ∼50% of its body water during dehydration (Churchill and Storey, '93) and tolerates the freezing of up to 65% of its body water as a winter survival adaptation (Layne and Lee, '87). Because urea accumulation is thought to improve survival of both stresses, and because

this process is likely mediated by the action of UTs in osmoregulatory organs, we examined changes in UT protein and mRNA levels during the annual cycle and in response to experimental dehydration and freezing/thawing. Additionally, to clarify if changes in UT expression are triggered by increases in urea levels during osmotic stress or if some other physiological cue regulates UT expression, we measured changes in UT protein and mRNA levels in response to experimental urea loading in the absence of other osmotic challenge.

MATERIALS AND METHODS

Animals

Male *R. sylvatica* were collected from a vernal pool in southern Ohio (Adams County) during March, 2009. They were transported to our laboratory, placed inside boxes containing damp moss, and kept in darkness at 4°C. Within several weeks, presumably while they were still cold hardy (e.g., Layne and Lee, '87), some of these frogs were taken as the "spring" sample in a study of seasonal variation in protein expression, whereas others were used in urea-loading and freezing/thawing experiments (see below). The remaining frogs were transferred in early April to a 48 m^2 outdoor enclosure in a wooded area of the Miami University Ecology Research Center. They were provided with a pool of water and offered crickets three times each week until mid-October, when feeding waned due to the onset of cooler weather. A few of these frogs were sampled in autumn as part of the seasonal study, but most were transferred in late October to our laboratory and placed under conditions simulating hibernation. The latter were denied food and kept at 4°C on damp moss in darkened boxes. In early February, some of these frogs were taken to represent the "winter" sample in the seasonal study; others were used in a dehydration experiment (see below). Prior to receiving experimental treatment, standard body mass was determined by removing any fluid present in the bladder by inserting a polished glass cannula into the cloaca and applying gentle pressure to the lower abdomen, and weighing the frog on an electronic balance. All experiments were conducted in compliance with the Institutional Animal Care and Use Committee at Miami University.

Seasonal Variation in UT Expression

We sampled frogs on several occasions throughout the year to monitor variation in UT expression. "Spring" frogs were sampled on March 23, 2009 from groups kept in the laboratory, whereas frogs were taken from the outdoor enclosure in early fall (18th of October) and late fall (15th of November) to represent "fall" frogs. "Winter" frogs were sampled on February 10, 2010 from frogs in simulated hibernation in the laboratory. Both fall groups comprised frogs that had recently experienced frigorific weather and ceased feeding; however, frogs in the November sample were nearer to entering hibernation and had encountered drier

environmental conditions as compared to the frogs sampled in October.

Effect of Experimental Dehydration on UT Expression

Frogs were transferred from their holding boxes to individual plastic containers with perforated lids and dehydrated as described by Muir et al. (2007). Briefly, these frogs were placed on a substratum of dry paper towels, whereas control frogs were placed on wet paper towels and provided with enough moisture to remain fully hydrated throughout the experiment. All frogs were held in darkness at 4°C. Over the next 2 weeks, frogs were weighed daily to track any change in their body mass (through evaporative water loss) until the experimental frogs had lost ~50% of their body water.

Effect of Experimental Freezing and Thawing on UT Expression

Freezing experiments were performed using a protocol modified from Costanzo et al. ('93). Each frog was placed individually inside a 50-mL plastic tube and provided with a thermocouple probe that was placed against its abdomen. Throughout the experiment, body temperature was recorded on a multichannel data logger (OM500, Omega Engineering). Tubes containing these frogs were subsequently cooled to –0.7°C inside an ethanol bath. Aerosol coolant was applied to the tube's exterior; ice crystals forming inside the tube initiated the freezing of the frog through ice inoculation. Once freezing began, the bath was programmed to cool the frogs at $-0.0375°C\ hr^{-1}$ over 48 hr. Frogs were held at the equilibrium temperature, –2.5°C, for 6 hr, after which some were sampled in the fully frozen state and others, destined to be sampled after thawing, were held at 4°C for 24 hr. Control (unfrozen) frogs were held at 4°C in individual plastic tubes throughout the experiment.

Effect of Experimental Urea Loading on UT Expression

Blood urea concentrations of frogs were manipulated as described by Muir et al. (2007). Frogs were kept in dehydration chambers (as described in the preceding section) for 3 days, during which time they lost ~0.5 g of their body mass, and then administered isotonic phosphate-buffered saline (PBS; in g L^{-1}; 6.10 NaCl, 0.15 KCl, 0.88 Na_2HPO_4, 0.15 KH_2PO_4; 230 mosmol kg^{-1}) (control), or PBS containing 1.5 M urea (treatment). The volume (0.3–0.5 mL) of the solution, injected into the dorsal lymph sac using a 27.5-gauge needle, approximated 3% of standard body mass. Frogs were held in darkness at 4°C for 2 days prior to being sampled.

Tissue Sampling and Osmolytes Assay

Following experimental treatment and/or being collected from the field, standard body mass of the frogs was determined as described above. Frogs were then euthanized by double pithing and dissected. Aortic blood was collected into heparinized capillary tubes, which were centrifuged at $2,000 \times g$ for 5 min to isolate plasma. Excised urinary bladder and kidneys, and the plasma, were frozen in liquid N_2 and stored at –80°C until analyzed a few weeks later. Carcasses were dried in a 65°C oven and the mass of water evaporated was determined to estimate body water content (expressed as g water per g dry tissue). Plasma osmolality was measured by vapor-pressure osmometry (model 5520; Wescor, Logan, UT). Plasma urea concentrations were determined colorimetrically (urease, no. B7551–120, Pointe Scientific, Canton, MI).

Antibody

An oligopeptide was designed from the C-terminal amino acids 364–383 (LSKVTYPEDNRIYYLNLKKE) of the UT sequence from *Rana esculenta* (accession no. CAA73322). The peptide was synthesized with an amino-terminal cysteine residue, and a polyclonal UT antibody was raised commercially (Proteintech Group, Chicago, IL) in rabbit. Antiserum was purified with a SulfoLink Immobilization Kit (Pierce Protein Research Products, Rockford, IL) following the manufacturer's protocol.

Immunoblot Analysis

Tissues were thawed on ice and then homogenized in 1 mL Trizol[TM] reagent (Invitrogen, Carlsbad, CA) using a shearing-type homogenizer. Protein was extracted following a modified Trizol protocol. Briefly, following chloroform extraction of the RNA, DNA was precipitated by adding 0.3 mL of ethanol and centrifuging at $2,000 \times g$ for 5 min. The supernatant containing protein was transferred to a fresh tube, and protein was precipitated by adding 1.5 mL isopropanol and centrifuging at $12,000 \times g$ for 10 min at 4°C. The protein pellet was washed thrice by adding 2 mL of 0.3 M guanidine hydrochloride in 95% ethanol, incubating at room temperature (RT) for 20 min, and centrifuging at $7,500 \times g$ for 5 min at 4°C. After the final wash, 2 mL of ethanol was added and the pellet incubated at RT for 20 min, followed by a centrifugation at $7,500 \times g$ for 5 min at 4°C. The pellet was air-dried and the protein resuspended in 50–100 μL of 8 M urea.

Final protein concentration was determined using the Bio-Rad (Bradford) protein assay (Bio-Rad, Hercules, CA). To improve solubility, samples containing the protein (30 μg) were boiled for 15 min and then incubated at 37°C for 30 min prior to adding the buffer. Samples were mixed with Laemmli sample buffer (Bio-Rad) containing 5% β-mercaptoethanol, incubated at 50°C for 15 min, and analyzed by SDS-PAGE on a 4–15% gradient gel (Bio-Rad). Precision Plus protein standard (Bio-Rad) and MagicMark XP protein standard (Invitrogen) were used as size references. Following electrophoresis, proteins were transferred to a nitrocellulose membrane (Bio-Rad). To verify that the same amount of protein was loaded in all lanes and that transfer was equivalent across the gel, membranes were stained with 0.2% (w/v) Ponceau S (Sigma Chemical Company, St. Louis, MO) containing 5% acetic acid. Membranes were visually examined to confirm

uniform staining and then destained using 0.1 M NaOH. Nonspecific protein antigens were blocked at 4°C overnight in Western wash buffer (10 mM Tris, 100 mM NaCl, and 0.1% Tween 20 at pH 7.5) containing 10% nonfat milk. Membranes were incubated overnight at 4°C in a 5% nonfat milk solution containing 1 μg mL^{-1} of an anti-UT primary antibody. They were then washed with Western wash buffer 3 times for a total of 45 min, and then incubated for 2 hr at room temperature (~22°C) with goat anti-rabbit IgG-HRP conjugates secondary antibody (GE Healthcare, Piscataway, NJ) diluted 1:1000 in a 5% nonfat milk solution. Membranes were then washed in Western wash buffer, incubated 2 min in ECL (enhanced chemiluminescence) detection reagents (GE Healthcare), and exposed to autoradiography film. Radiography films were digitally scanned and bands were semiquantified using AlphaViewspot densitometry (Alpha Innotech, San Leandro, CA). All samples were run in duplicate; the average of the two densitometric values was then used in statistical analyses.

Quantitative Real-Time PCR

Total RNA was extracted from organ samples using Trizol reagent following the manufacturer's protocol. RNA quality was confirmed by checking the optical density ratio at 260 nm and 280 nm (Manchester, '96). RNA was reverse-transcribed using the GoScript Reverse Transcription System (Promega, Madison, WI). Each reverse transcription reaction contained GoScript reaction buffer, 1 μg RNA, 0.5 μg random primers, 4 mM MgCl$_2$, 0.5 mM dNTP, and GoScript Reverse Transcriptase. Quantitative polymerase chain reaction (qPCR) was performed using gene-specific primers for *R. sylvatica* UT (accession no. JF755889; sense 5′-CCTTTC-AATATCGCCGTTTGTCTGC-3′, antisense 5′-GTATACTTGCCCCA-CACCGACCG-3′) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (sense 5′-CTTG AAGGG AGGTGCCAAGCGT-3′, antisense 5′-ACCT TTGCA AGAGGAG CCAG-3′). qPCR reactions consisted of GoTaq qPCR Master Mix (Promega) with 5 μL of the cDNA sample (diluted 1:10) and UT (100 nM) or GAPDH (50 nM) primers. An iCycler thermal cycler (Bio-Rad) was used to run reactions that consisted of a hot-start activation at 95°C for 5 min, followed by 45 cycles of denaturation at 95°C for 10 sec, and annealing/extension at 60°C for 30 sec. Each sample was run in triplicate, and the average cycle at threshold (C_T) value was determined. A negative control (–RT) was run for each sample to check for genomic DNA contamination. The relative expression level for UT was normalized to the reference gene, GAPDH, using the $\Delta\Delta C_T$ method as previously described (Schmittgen and Livak, 2008).

Statistical Analysis

All values are reported as means ± SEM. Differences in mean protein, mRNA, and metabolite levels, considered significant at $P < 0.05$, were determined using Student's *t*-tests or analysis of variance (ANOVA), followed by Bonferroni multiple comparisons test. Some of the data were given square-root transformation to meet the assumptions of these parametric tests.

RESULTS

Seasonal Changes in Osmotic Physiology and UT Protein and mRNA Levels

Body water content varied ($F = 13.79$; $P = 0.0001$) among frogs ($N = 5$ frogs per group) sampled at several times throughout the year (Fig. 1). Water content was lowest (2.8 ± 0.1 g g^{-1} dry tissue) in November, but increased in winter, reaching a peak (3.7 ± 0.1 g g^{-1} dry tissue) in the spring. Plasma osmolality generally varied ($F = 169.26$; $P < 0.0001$) inversely with body water content, as levels were highest (244–266 mosmol kg^{-1}) in the late fall and winter. Mean plasma urea concentration ranged from 5 to 41 mM and was highest ($P < 0.001$) in the November sample.

UT protein amount varied seasonally, remaining low throughout the fall and winter, and then increasing in spring (Fig. 1D). In bladder, protein abundance increased ($F = 5.19$; $P = 0.011$) between 3- and 13-fold compared to February and November samples, respectively. In kidney, protein abundance increased ($F = 18.63$; $P < 0.0001$) between 5- and 75-fold compared to February and November samples, respectively. There was no significant ($F = 1.89$; $P = 0.18$) variation in UT mRNA levels in frogs sampled among the seasons (Fig. 1E).

Effect of Experimental Dehydration on UT Protein and mRNA Levels

Frogs ($N = 5$ frogs per group) exposed to dehydrating conditions for 14 days lost ~42% of their total body water; consequently, their body water content, 1.9 ± 0.1 g g^{-1} dry tissue, was lower ($P < 0.0001$) than that of the fully hydrated controls, 3.3 ± 0.1 g g^{-1} dry tissue. Dehydrated frogs had a 1.7-fold higher ($P < 0.0001$) plasma osmolality than control frogs (456 ± 13 vs. 266 ± 5 mosmol kg^{-1}), as well as a 3.7-fold higher ($P < 0.0001$) plasma urea concentration (48.1 ± 3.0 mM vs. 13.4 ± 0.3 mM). All frogs survived the dehydration and appeared healthy at the time they were sampled.

In response to experimental dehydration, UT protein amount increased in bladder ($P = 0.046$) to levels 4.4-fold higher than control values (Fig. 2A). In contrast, no change was detected in kidney ($P = 0.18$). UT mRNA levels did not differ between dehydrated and control frogs in either bladder ($P = 0.38$) or kidney ($P = 0.47$) (Fig. 2B).

Effect of Experimental Freezing and Thawing on UT Protein and mRNA Levels

Frogs ($N = 5$ frogs per group) reached an equilibrium temperature of –2.5°C during a 54-hr period of experimental freezing. Frogs sampled in the fully frozen state showed characteristics of substantial ice formation, including large ice crystals within the coelomic cavity, immobility, and lack of heart function. Because
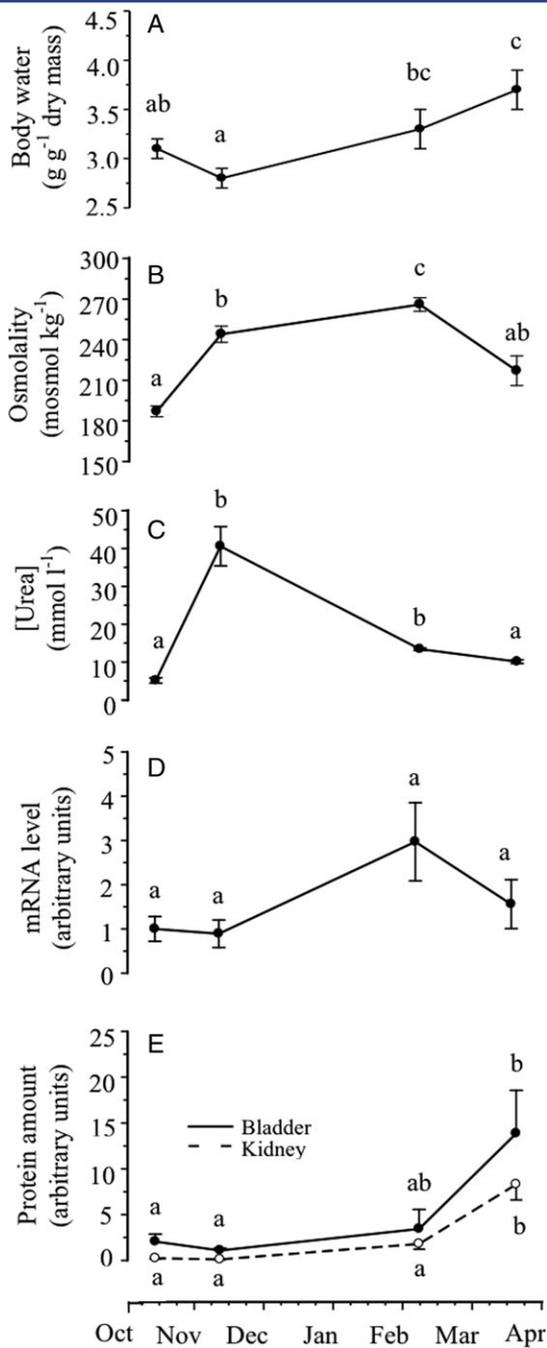
Figure 1. Seasonal variation in (A) body water content, (B) plasma osmolality, and (C) plasma urea concentration in relation to (D) urea transporter (UT) mRNA levels, normalized to glyceraldehyde 3–phosphate dehydrogenase (GAPDH), in bladder and (E) protein amount in bladder and kidney of *Rana sylvatica*. Data are means $\pm$ SEM (N = 5 frogs per group.) Within a dataset, mean values identified by different letters were statistically distinguishable (ANOVA/Bonferroni; $P < 0.05$).

we could not collect blood from these frogs, we report no data on their plasma urea concentration and osmolality. Frogs frozen under identical conditions and sampled 24 hr after thawing exhibited regular cardiac rhythm, tissue perfusion, normal posture, and locomotor ability.

Body water content did not differ ($F = 0.182$; $P = 0.836$) among unfrozen controls (3.6 ± 0.1 g g^{-1} dry tissue), fully frozen frogs (3.5 ± 0.4 g g^{-1} dry tissue), and frozen/thawed frogs (3.7 ± 0.1 g g^{-1} dry tissue). Although plasma urea concentrations were similar ($P = 0.99$) between unfrozen controls and frozen/thawed frogs (10.4 ± 0.2 vs. 10.4 ± 0.6 mM), these groups were distinct ($P = 0.0035$) in plasma osmolality (222 ± 6 vs. 255 ± 5 mosmol kg^{-1}). The higher value in the frozen/thawed frogs likely reflected the presence of additional solutes, such as glucose and lactate, which were mobilized during freezing.

UT protein levels, expressed as arbitrary densitometric units, in bladder of unfrozen controls (10.6 ± 2.2), fully frozen frogs (11.5 ± 3.1), and frozen/thawed frogs (12.5 ± 1.5) were statistically indistinguishable ($F = 0.16$; $P = 0.85$). Similarly, there was no significant variation ($F = 0.81$; $P = 0.47$) in protein amount in kidney among these groups (6.7 ± 0.5, 8.9 ± 1.8, and 7.3 ± 1.3, respectively). Levels of mRNA were not determined for kidney, but in bladder did not vary ($F = 0.24$; $P = 0.79$) among unfrozen controls (1.0 ± 0.3), fully frozen frogs (0.8 ± 0.3), and frozen/thawed frogs (0.9 ± 0.3).

Effect of Experimental Urea Loading on UT Protein and mRNA Levels

Plasma urea concentration was higher ($P < 0.0001$) in urea-loaded frogs (83.9 ± 5.3 mM; $N = 5$) than in saline-injected control frogs (10.3 ± 1.2; $N = 5$). This difference corresponded to a marked elevation ($P = 0.0021$) in plasma osmolality in urea-loaded frogs (356 ± 11 mosmol kg^{-1}) over that of control frogs (274 ± 14 mosmol kg^{-1}). There was no difference ($P = 0.534$) in body water content between urea-loaded and control frogs (3.3 ± 0.1 vs. 3.4 ± 0.1 g g^{-1} dry tissue). All frogs survived the experimental urea loading and appeared healthy at the time they were sampled.

In urea-loaded frogs, UT protein amounts in bladder and kidney were 76% and 90% lower ($P = 0.011$ and 0.0002, respectively) than corresponding levels in control frogs (Fig. 3A). Urea loading was not associated with a change in UT mRNA levels in either bladder ($P = 0.69$) or kidney ($P = 0.72$) (Fig. 3B).

DISCUSSION

Survival of amphibians under osmotic stress is enhanced by accumulating urea in part through its retention, a process that likely involves UTs in kidney and urinary bladder, two important osmoregulatory organs. In the present study, we found seasonal variation in UT protein abundance, as well as changes in UT abundance in response to organismal dehydration and urea loading, but not freezing. Neither seasonally nor
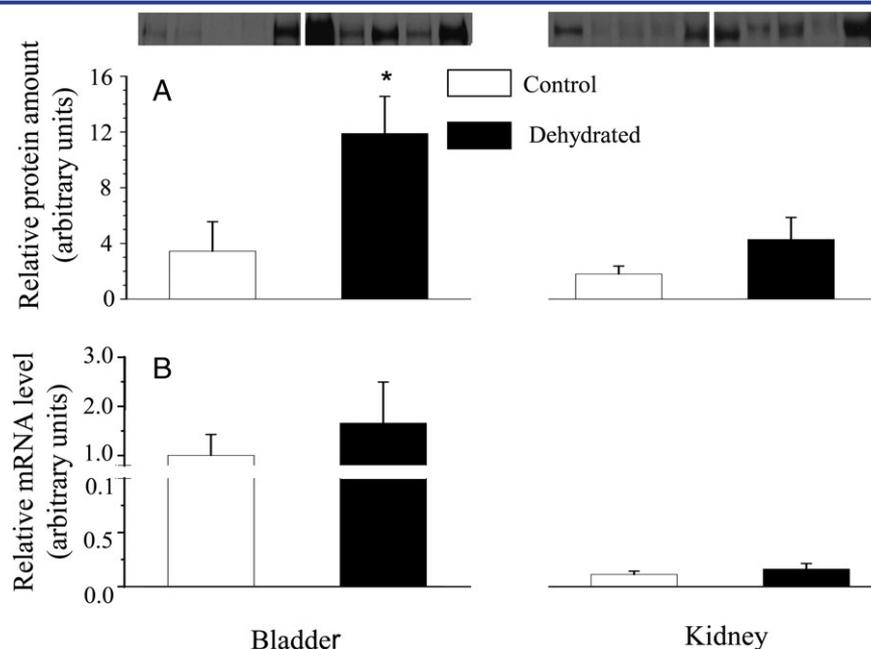
Figure 2. Effect of experimental dehydration of *Rana sylvatica* on (A) urea transporter (UT) protein amount, as indicated by immunoblots and densitometric values, and (B) UT mRNA level, normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH), in urinary bladder and kidney. Data are means ± SEM ($N = 5$ frogs per group.) *Asterisk* denotes a significant difference (*t*-test; $P < 0.05$) between groups.

in response to physiological stress did we observe changes in UT mRNA levels.

Seasonal Variation in UT Abundance

The urea-accumulation response to osmotic challenge enables some anurans to exploit terrestrial hibernacula and survive extended periods of dehydration and tissue freezing. In an earlier study of *R. sylvatica* overwintering in an outdoor enclosure, plasma urea levels were substantially higher in autumn and winter than in spring, and were inversely related to body water content (Costanzo and Lee, 2005). We observed similar dynamics in water and solute balance in the present study: body water content was lowest in autumn and highest in spring, and generally varied inversely with plasma osmolality. Plasma urea levels were highest in the frogs sampled in November, which also had the lowest body water contents. Dehydration and urea accumulation in these frogs likely owed to dry conditions and a paucity of rain during the 2 weeks preceding their sampling. To the contrary, frogs in the March sample had experienced several recent rain events; accordingly, they were well hydrated and had little urea in their blood.

Despite compelling evidence that urea serves as an osmo- and cryoprotectant, and that urea accumulation in fall and early winter contributes to the survival of hibernating *R. sylvatica* (Costanzo

and Lee, 2005; Muir et al., 2007), we found that UT protein was less abundant in frogs sampled during these seasons than in those sampled in spring, when urea was not retained. This result is perplexing, especially because fall frogs were in water deficit and, as our laboratory study showed, dehydration upregulated UT protein expression (Fig. 2). On the other hand, fall frogs were only moderately dehydrated (2.8 g g^{-1} dry tissue vs. 1.9 g g^{-1} dry tissue in the experimental frogs) and, furthermore, results from our urea-loading experiment indicated that, in the absence of extensive dehydration, elevated urea downregulated the UT protein (Fig. 3). Perhaps the abundance of these transporters in the osmoregulatory tissues of free-living frogs increases only if the degree (or rate) of dehydration achieves a certain threshold (e.g., McNally et al., 2002). Accordingly, in a preliminary study we found no change in UT abundance in experimentally dehydrated *R. sylvatica* that had lost only ~27% of their body water (data not shown). In the fall frogs, urea reabsorption apparently was adequately managed by the constitutive complement of UT proteins, perhaps because dehydration and urea accumulation proceeded gradually and/or because reduced GFR, which occurs at low temperatures, facilitates urea accumulation. Additionally, the high capacity for urea production exhibited by *R. sylvatica* during the fall (Schiller et al., 2008), when coupled with anuria, may be sufficient to enable urea to accumulate at the necessary rate.
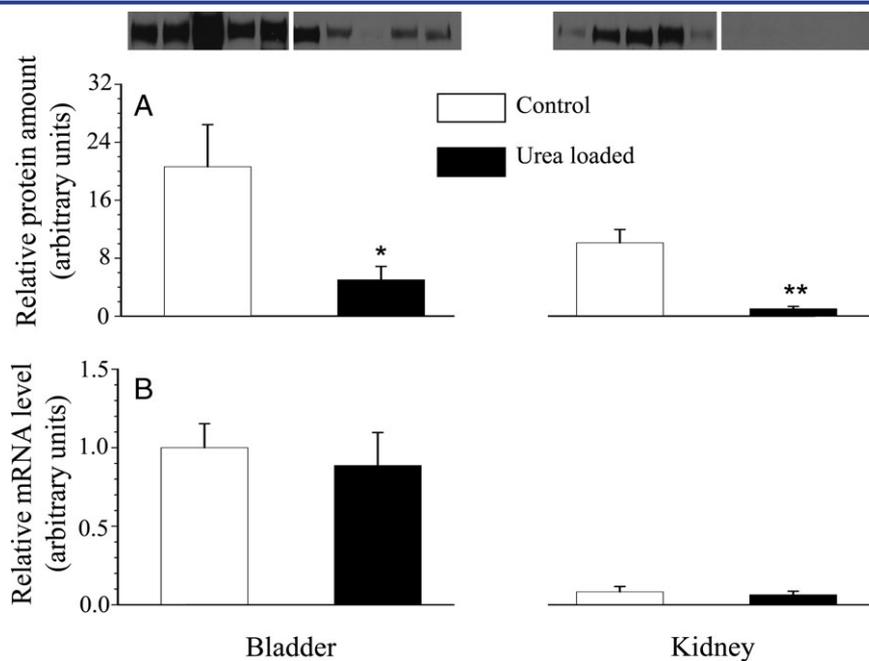
Figure 3. Effect of experimental urea loading of *Rana sylvatica* on (A) urea transporter (UT) protein amount, as indicated by immunoblots and densitometric values, and (B) UT mRNA level, normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH), in urinary bladder and kidney. Data are presented as means $\pm$ SEM ($N = 5$ frogs per group.) *Asterisk* denotes a significant difference (*t*-test; $^*P < 0.05$, $^{**}P < 0.001$) between groups.

Considering that in the spring, urea is no longer needed in a protective role, the biological significance of the relatively high UT expression in kidney and urinary bladder of spring frogs is not apparent. The exact cause(s) of the seasonal variation in UT abundance remains unclear, and a variety of factors could be involved. For example, perhaps feeding has an effect on UT expression, or it is possible that an unknown UT isoform may be present. Our findings underscore the notion that regulation of UT expression is complex and involves multiple stimuli, and further study is needed to explore possible explanations for changes in seasonal expression.

Effect of Experimental Dehydration on UT Abundance

Rana sylvatica is one of the most dehydration-tolerant of North American ranids, and can survive the loss of about half of its body water (Churchill and Storey, '93). Frogs in our dehydration experiment lost 42% of their initial body water, an amount approaching the limit of tolerance, and thus were likely to have mounted survival responses. Plasma osmolality in these frogs rose by 190 mosmol kg^{-1}, essentially all of this rise reflecting the solvent loss, although an additional quantity of urea (some 25 mM) was synthesized during the trial. The plasma urea concentration measured in these frogs, nearly 50 mM, is comparable to that found in *R. sylvatica* dehydrated in a similar manner (Muir et al., 2007) and in frogs overwintering in an outdoor enclosure (Costanzo and Lee, 2005).

In the dehydrated frogs, UT protein amounts increased 4.4-fold in bladder. Regulation of UT expression in response to changes in osmotic status occurs in various animals, including mammals, fish, and amphibians. In rats, water restriction results in an increase in UT-A2 mRNA in kidney, whereas water loading decreases expression of renal UT-A2 (reviewed in Sands, 2003). Similarly, hypoosmotic stress diminishes UT expression in kidney of marine elasmobranchs (Morgan et al., 2003; Yamaguchi et al., 2009). In anurans, reabsorption of urea in both kidney and urinary bladder increases under osmotic challenge (Shpun and Katz, '89; Shpun and Katz, '95) and our data suggest that this response is mediated by an upregulation of UTs.

In *R. sylvatica*, regulation of transporter expression in response to osmotic challenge is likely controlled by antidiuretic hormones. In mammalian kidney, antidiuretic hormones, such as arginine vasopressin (AVP), rapidly increase UT permeability by altering the protein's phosphorylation state (Blount et al., 2008), whereas chronic exposure to AVP increases mRNA expression and protein abundance (reviewed in Bagnasco, 2005). In amphibians, both antidiuretic hormones and hypertonicity increase urea

permeability of bladder (Parisi and Candia, '77; Verbavatz et al., '92). In isolated bladder cells of *Bufo marinus*, this change is due to an increase in the number of UT proteins (Konno et al., 2007). It is conceivable that increased arginine vasotocin (AVT) levels, known to increase during dehydration in anurans (Konno et al., 2005), may have caused the increase in UT proteins we observed in *R. sylvatica*.

Effect of Experimental Freezing and Thawing on UT Abundance

Rana sylvatica tolerates freezing of its body tissue due, in part, to accumulation of cryoprotective solutes, particularly urea and glucose (Costanzo and Lee, 2005). During freezing, various proteins are regulated in *R. sylvatica* (Storey, 2004) presumably to mitigate the stresses that provoke cryoinjury. However, we found that UT protein abundance did not differ among unfrozen controls, fully frozen frogs, and frozen/thawed frogs. The absence of an upregulatory response in the frozen frogs is consistent with the earlier finding (Costanzo and Lee, 2005) that, unlike the case with glucose, urea apparently is not synthesized during freezing. Perhaps no adjustment in UT numbers is necessary because urea, which is accumulated in late fall and early winter, reaches equilibrium throughout the body compartments before freezing is initiated.

Effect of Experimental Urea Loading on UT Abundance

Injecting *R. sylvatica* with a solution containing 1.5 M urea raised their plasma urea concentration to ~84 mM, about eightfold over that of saline-injected controls. This augmentation accounted for essentially all of the observed increase in plasma osmolality, indicating that no other solutes were mobilized, and, importantly, had no effect on hydration status. Urea levels in these frogs, although somewhat higher than those observed in frogs hibernating in the outdoor enclosure, were within the range reported for this species (Costanzo and Lee, 2005).

In response to urea loading, UT protein levels decreased to 24% and 10% of control values in bladder and kidney, respectively, suggesting that tissue urea concentration is a potent mediator of transporter expression. Similarly, in mammals made hyperuremic by a high protein or high urea diet, UT expression is regulated in an isoform- and tissue-dependent manner (reviewed in Bagnasco, 2005). Rendering fish hyperuremic through either direct urea loading or by altering environmental conditions causes UT mRNA levels in gill tissue to increase substantially (McDonald et al., 2009).). Toads acclimated to a concentrated urea solution show decreased urea permeability of bladder (Shpun and Katz, '95) and a nearly two-fold higher concentration of urea in bladder fluid than in plasma.

Downregulation of UT protein in response to elevated urea levels probably enables frogs to sequester excess urea in urinary bladder and thus facilitates its excretion when hydric conditions permit. In addition to limiting urea retention, urea loading of *R. sylvatica* also diminishes urea production capacity by reducing CPS I activity (Schiller et al., 2008). Thus, by curbing both the production and retention of urea, hydrated *R. sylvatica* can prevent accumulation of excess urea.

Relationship Between UT mRNA Expression and UT Protein Abundance

Changes in protein abundance commonly are preceded by corresponding changes in mRNA levels. This pattern is seen in *R. sylvatica* with respect to various proteins that respond to dehydration and/or freezing (McNally et al., 2002; De Croos et al., 2004). However, in the present study, changes in UT mRNA levels did not accompany the rather substantial adjustments in UT protein abundance. Although this phenomenon has been reported (Yamaguchi et al., 2009; Sands, 2000), our result for *R. sylvatica* was unexpected given that UT mRNA expression is sensitive to osmotic challenge in other anurans (Konno et al., 2006).

The observed incongruence between UT protein and mRNA levels suggest that UTs are regulated posttranscriptionally in *R. sylvatica*. Because we observed changes in UT protein abundance without complementary changes in mRNA levels, it is plausible that *R. sylvatica* controls UT protein abundance by regulating protein turnover. Other transporters, such as aquaporins, are regulated in anurans posttranscriptionally through phosphorylation and/or altering subcellular localization (reviewed in Suzuki et al., 2007). Further study is needed to fully elucidate the factors contributing to regulation of UT expression in amphibians.

Conclusions

Results of the present study suggest that UTs factor importantly in the ability of *R. sylvatica* to endure physiological stresses experienced in terrestrial habitats, likely by acting in the osmoregulatory organs to regulate tissue levels of urea. Due to its terrestrial habits, *R. sylvatica* can experience dehydration at various times throughout the year and tissue freezing during the winter. The contrasting effects of experimental dehydration and urea loading on UT protein abundance indicate that these transporters are regulated either to facilitate urea accumulation in tissues or to promote elimination of excess urea via urination. However, seasonal abundance of UT does not correspond to periods of urea accumulation, suggesting the regulatory system for UT in *R. sylvatica* is complex and is influenced by multiple physiological factors. Further study is needed to elucidate the mechanisms by which UT proteins are regulated during osmotic challenge and to more fully clarify their roles in hydroosmotic balance.

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