

# Evidence for Urea-Induced Hypometabolism in Isolated Organs of Dormant Ectotherms

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## ABSTRACT

Many organisms endure extended periods of dormancy by depressing their metabolism, which effectively prolongs the use of their endogenous energy stores. Though the mechanisms of hypometabolism are varied and incompletely understood, recent work suggests that urea accumulation in autumn and early winter contributes to reduced metabolism of hibernating wood frogs (*Rana sylvatica*). Urea accumulation during dormancy is a widespread phenomenon, and it has long been presumed that numerous species from diverse taxa benefit from its hypometabolic effect. To investigate the phylogenetic prevalence of urea-induced hypometabolism, we studied four species of urea accumulators from the clades Amphibia (*Spea bombifrons* and *Ambystoma tigrinum*), Reptilia (*Malaclemys terrapin*), and Gastropoda (*Anguispira alternata*), and one amphibian species (*R. pipiens*) that does not accumulate urea during dormancy. We measured rates of oxygen consumption ( $\dot{V}O_2$ ) of excised organ samples from dormant animals in the presence or absence of physiological concentrations of urea. Three of the four urea-accumulating species had at least one organ whose  $\dot{V}O_2$  was significantly decreased by urea treatment. However,  $\dot{V}O_2$  of organs from *R. pipiens*, the one species tested that does not accumulate urea during dormancy, was not affected by urea treatment. Our results support the hypothesis that urea accumulation can reduce metabolic rate of dormant animals and provide a base for further investigation into the evolution of urea-induced hypometabolism. *J. Exp. Zool.* 313A:28–34, 2010. © 2009 Wiley-Liss, Inc.

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When faced with unfavorable environmental conditions such as low water availability or extreme temperature, terrestrial ectotherms often enter an extended period of dormancy. As dormant animals are aphagic and often have little opportunity for water uptake, they must reduce both energy use and water loss. As such, two physiological hallmarks of terrestrial dormancy are a profound metabolic depression and the accumulation of nonionic osmolytes (Storey, 2002). Although regulatory mechanisms underlying hypometabolism have received considerable study (Guppy, 2004), a comprehensive understanding of those processes is still lacking, likely due to their complexity at the organismal, cellular, and molecular levels (Storey and Storey, '90; Guppy et al., '94).

Accumulation of urea as a nonionic osmolyte often occurs in ureogenic ectotherms during dormancy (Yancey et al., '82; Withers, '98), thereby facilitating water retention. As urea generally perturbs protein structure, some researchers posited

that urea accumulation in the absence of so-called “counter-acting solutes” may beneficially depress metabolic rate by decreasing the activity of key metabolic enzymes (Hand and Somero, '82; Yancey et al., '82). Several studies of enzyme kinetics marshal support for this hypothesis by showing that physiological levels of urea can alter the functionality of key enzymes (e.g., Fuery et al., '97; Kaji et al., '98; Cowan and Storey, 2002). More recently, studies of hibernating wood frogs (*Rana sylvatica*) suggest that urea depresses the metabolic rate of

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isolated organs (Muir et al., 2008) and intact frogs (Muir et al., 2007).

Although there is growing support for the urea-induced hypometabolism hypothesis in the wood frog, the phenomenon may be more widespread given the number and diversity of animals that accumulate urea during dormancy. There are several mechanisms by which dormant animals achieve metabolic depression (Guppy and Withers, '99) and urea's effects may contribute to the beneficial hypometabolism. However, urea sensitivity of enzymes varies by species (Cowan and Storey, 2002) and by physiological state within a species (Fuery et al., '97); thus, the response may not be universal. We used four species, from the clades Amphibia, Reptilia, and Gastropoda, that accumulate urea during dormancy to investigate the prevalence of urea-induced hypometabolism among diverse taxa. Additionally, we investigated urea's effect on metabolism of one amphibian that does not typically accumulate urea during dormancy. Oxygen consumption rates of isolated organs were measured in the presence or absence of physiological levels of urea.

## METHODS

### Animals and Acclimation

Subjects were induced into dormancy (estivation or hibernation, as appropriate to the species' biology) before use in respirometry trials. Conditions varied by species because our aim was to induce a physiological state conducive to the expression of the urea-hypometabolic response.

Unsexed metamorphosing spadefoot toads (*Spea bombifrons*;  $N = 8$ ) were collected in summer from ponds in west-central Nebraska and transported to Miami University where they were held in water-filled plastic cages at 21°C (12:12, L:D). Upon completion of metamorphosis, toads were transferred to terraria containing moist soil and fed crickets thrice weekly. Beginning on October 1, temperature was decreased to 15°C, feeding was halted, and the substratum was permitted to dry, prompting the toads to burrow and become quiescent (cf., Jones, '80). Toads were used in experiments 2–4 weeks later.

Unsexed adult tiger salamanders (*Ambystoma tigrinum*;  $N = 6$ ) were collected in summer from west-central Nebraska and transported to Miami University where they were held at 21°C (12:12, L:D) in terraria containing moist soil. Salamanders were fed earthworms twice weekly. Beginning on October 1, temperature was decreased to 15°C, feeding was halted, and the substratum was permitted to dry, prompting the salamanders to burrow and become quiescent. Temperature was further decreased to 10°C on November 1, and salamanders were used in experiments 2–4 weeks later.

Eggs of diamondback terrapins (*Malaclemys terrapin*), collected from gravid females in coastal New Jersey, were transferred to Miami University and incubated at 28.5°C on a substratum of moist vermiculite until hatching. Unsexed

neonates ( $N = 10$ ) were transferred to fresh, moist vermiculite (0.5 g water:1 g dry vermiculite) and held at 20°C in an incubator (Percival I-35X, Boone, IA, USA). In order to simulate conditions within their terrestrial hibernacula, hatchlings were kept in darkness and exposed to stepwise reductions in temperature. Incubator temperature was sequentially decreased to 15, 10, and 4°C on October 1, November 1, and December 1, respectively (cf., Costanzo et al., 2006). Hatchlings were kept in darkness at 4°C for 4 months before use in experiments.

Snails (*Anguispira alternata*;  $N = 7$ ) were collected in summer from a woodlot in southwestern Ohio and transported to Miami University where they were held at 21°C (12:12, L:D) in terraria containing moist paper towel. Snails were fed dry dog food, lettuce, and carrots; chalkboard chalk, a source of calcium, was offered ad libitum. In early September feeding was halted and the paper towel was permitted to dry, prompting the snails to become quiescent and form an epiphragm. Snails were used in experiments 1–3 weeks later.

Male adult northern leopard frogs (*R. pipiens*;  $N = 8$ ) collected in autumn from populations in Northern Minnesota were obtained commercially (Trans-Mississippi Biological Supply) in early January. Leopard frogs are aquatic hibernators, which typically do not accumulate urea during the winter. Frogs were kept (unfed) at 4°C in aquaria filled to a height of 2 cm with dechlorinated tap water (10:14, L:D). A cloth was placed over each aquarium to reduce light transmission, simulating conditions in an ice-covered pond. Frogs were used in experiments 1–2 weeks later.

### Organ Sampling and Preparation

Animals were euthanized by double pithing (amphibians), cranial pithing (terrapins), or decapitation (snails) and immediately dissected. To determine endogenous levels of urea, we collected blood plasma (vertebrate specimens) from each individual and stored it at –80°C for future analysis (see below). From snails, we collected a sample of foot, rather than hemolymph, which might become contaminated with pallial water. Respirometry was conducted on liver and a matching pair of skeletal muscles (vertebrate specimens; Table 1), or mantle and foot (snails), which were excised and rinsed with Ringer's solution (RS). We selected specific skeletal muscles for their relatively high-surface area:volume and kept them intact, so as not to cut their fibers. One muscle from each animal was placed into fresh RS (reference), whereas the other was placed into RS augmented with urea (urea treated). Liver samples were bisected and one-half of each was placed in 5-mL fresh RS and the other half in RS augmented with urea. We selected urea concentrations (Table 1) at the high end of what the animals naturally accumulate in order to have the best chance of distinguishing a metabolic effect. These samples were further cut into several ~2-mm pieces to increase the surface area for diffusive uptake of urea and exchange of dissolved gases. Mantle and foot samples from snails were treated identically to

**Table 1.** Incubation conditions for organ samples, isolated from dormant ectotherms, before being used in respirometry trials.

Species	Organs tested	Incubation solution	Incubation temperature (°C)	Incubation time (h)
Spadefoot toad	Liver, gastrocnemius	RS <sup>1</sup> with or without 300 mM urea	15	8
Tiger salamander	Liver, ischioflexorius	RS <sup>1</sup> with or without 150 mM urea	10	10
Diamondback terrapin	Liver, iliofibularis	RS <sup>2</sup> with or without 80 mM urea	10	10
Snail	Mantle, foot	RS <sup>3</sup> with or without 150 mM urea	20	5
Leopard frog	Liver, semitendinosus	RS <sup>1</sup> with or without 80 mM urea	10	10

<sup>1</sup>Amphibian RS (mM): 104.4 NaCl, 2.0 KCl, 6.2 Na<sub>2</sub>HPO<sub>4</sub>, 1.1 KH<sub>2</sub>PO<sub>4</sub>; 230 mosmol kg<sup>-1</sup>. <sup>2</sup>Reptilian RS (mM): 137 NaCl, 2.7 KCl, 10 Na<sub>2</sub>HPO<sub>4</sub>, 2 KH<sub>2</sub>PO<sub>4</sub>; 280 mosmol kg<sup>-1</sup>. <sup>3</sup>Gastropod RS (mM): 138 NaCl, 32 NaHCO<sub>3</sub>, 6 KCl, 3 MgSO<sub>4</sub>, 6 NaH<sub>2</sub>PO<sub>4</sub>, 1 CaCl<sub>2</sub>; 240 mosmol kg<sup>-1</sup>.

liver samples. We then incubated organ samples in the appropriate RS for 5–10 h, depending on the incubation temperature, before using them in respirometry trials (Table 1).

We assessed the efficacy of our urea-loading protocol by incubating organ samples (collected from separate specimens;  $N=3$ , each species) as described above and determining postincubation concentrations of urea. Lactate concentrations were also measured to reveal any effect of urea loading on anaerobic metabolism. After incubation, samples were removed from the solutions, blotted free of excess moisture, weighed to the nearest 0.01 mg, and stored at  $-80^{\circ}\text{C}$  for future analysis of metabolites. Deproteinized organ extracts were prepared by homogenizing thawing samples in ice-cold 1 N HClO<sub>4</sub>, centrifuging the homogenate at 2,000g for 5 min, and neutralizing the supernatant with an equal volume of 1 N KOH. Urea and lactate concentrations were measured, according to the manufacturer's instruction, using the Berthelot method (Pointe Scientific, Canton, MI, USA) and a colorimetric lactate dehydrogenase procedure (Pointe Scientific), respectively. Concentrations are expressed as millimolar tissue water. Water contents of these samples were estimated from measured water contents of organs used in respirometry trials (see below).

### Respirometry

Rate of oxygen consumption ( $\dot{V}\text{O}_2$ ) was measured via closed-system respirometry using a fiber-optic oxygen-monitoring system (no. 110T, Insteck, Plymouth Meeting, PA, USA) in a 1-mL or 0.25-mL titanium sample chamber per Muir et al. (2008). The chamber was filled with the appropriate RS, which was continuously mixed by a magnetic stir-bar. We held constant the chamber temperature, which was identical to the incubation temperature (Table 1) for each sample, by circulating water from a temperature-controlled bath through the chamber's outer jacket. Dissolved oxygen concentration decreased  $\sim 1$  mg/L during the respirometry trial, which lasted 10–30 min, depending on the test temperature. After each trial, the organ sample was blotted free of excess moisture, weighed to the nearest 0.01 mg, thoroughly dried at  $60^{\circ}\text{C}$ , and reweighed. Water content was

determined by dividing the mass lost during drying by the initial mass of the sample.  $\dot{V}\text{O}_2$  values are expressed as microliters of O<sub>2</sub> consumed per gram of organ dry mass per hour.

### Statistical Analyses

Statistical analyses were performed using SAS 9.1 and significance was accepted at  $P \leq 0.05$ . Mean values (presented  $\pm$  SEM) were compared between reference and urea-treated groups within species using paired  $t$ -tests.

### RESULTS

Based on their behavior and physical appearance, we assumed that all specimens had entered into dormant states before use in experiments. All animals were quiescent. Spadefoot toads and salamanders had burrowed into the substrate within their cages and their skin appeared relatively dry and rough. Snails were retracted 5–10 mm into their shells and had formed at least a partial epiphragm. Endogenous urea levels in spadefoot toads ( $250 \pm 18$  mM), salamanders ( $53 \pm 4$  mM), terrapins ( $69 \pm 3$  mM), and snails ( $25 \pm 9$  mM) were relatively high compared with those found in nondormant individuals, which generally are  $< 10$  mM in salamanders (Delson and Whitford, '73), terrapins (Gilles-Baillien, '73), and snails (Riddle, '81), and  $\sim 30$  mM in spadefoot toads (McClanahan, '72). The fact that our animals had begun to accumulate urea further suggests they had entered into dormancy. In leopard frogs, urea levels were relatively low ( $7 \pm 1$  mM), as expected because they do not typically accumulate urea during hibernation.

Urea-treated organ samples had higher concentrations of urea than did reference samples (Table 2), suggesting our urea-loading protocol was successful. Generally, these urea levels matched those in the incubation media, suggesting urea flux had reached equilibrium, though some samples had higher concentrations. This disparity may stem from a slight inaccuracy of our estimates of organ water contents, which necessarily were based on a separate set of organs. Lactate levels were generally low and did not differ ( $P > 0.05$ ; all organs) between reference and urea-treated organ samples (Table 2).

**Table 2.** Urea and lactate concentrations of organ samples after incubation in Ringer's solution in the absence or presence of urea.

Species	Organ	[Urea] (mM)		[Lactate] (mM)	
		Reference	Urea treated	Reference	Urea treated
Leopard frog	Muscle	1 ± 0.2	97 ± 0.2	1 ± 1	2 ± 1
	Liver	5 ± 1	86 ± 2	2 ± 0.2	2 ± 0.4
Spadefoot toad	Muscle	2 ± 0.1	303 ± 2	2 ± 1	1 ± 0.03
	Liver	4 ± 1	318 ± 6	1 ± 0.2	1 ± 0.3
Tiger salamander	Muscle	1 ± 0.3	164 ± 8	1 ± 1	2 ± 0.4
	Liver	1 ± 0.1	164 ± 12	2 ± 1	2 ± 1
Diamondback terrapin	Muscle	1 ± 0.2	80 ± 2	1 ± 0.1	0.4 ± 0.2
	Liver	3 ± 1	76 ± 1	0.4 ± 0.1	0.3 ± 0.1
Snail	Foot	3 ± 1	152 ± 2	0.2 ± 0.1	0.2 ± 0.03
	Mantle	9 ± 2	172 ± 4	2 ± 0.3	2 ± 0.1

Values are mean ± SEM ( $N = 3$ ). Incubation conditions are given in Table 1. Metabolite concentrations (mM tissue water) were calculated based on water content of organs used in respirometry trials.

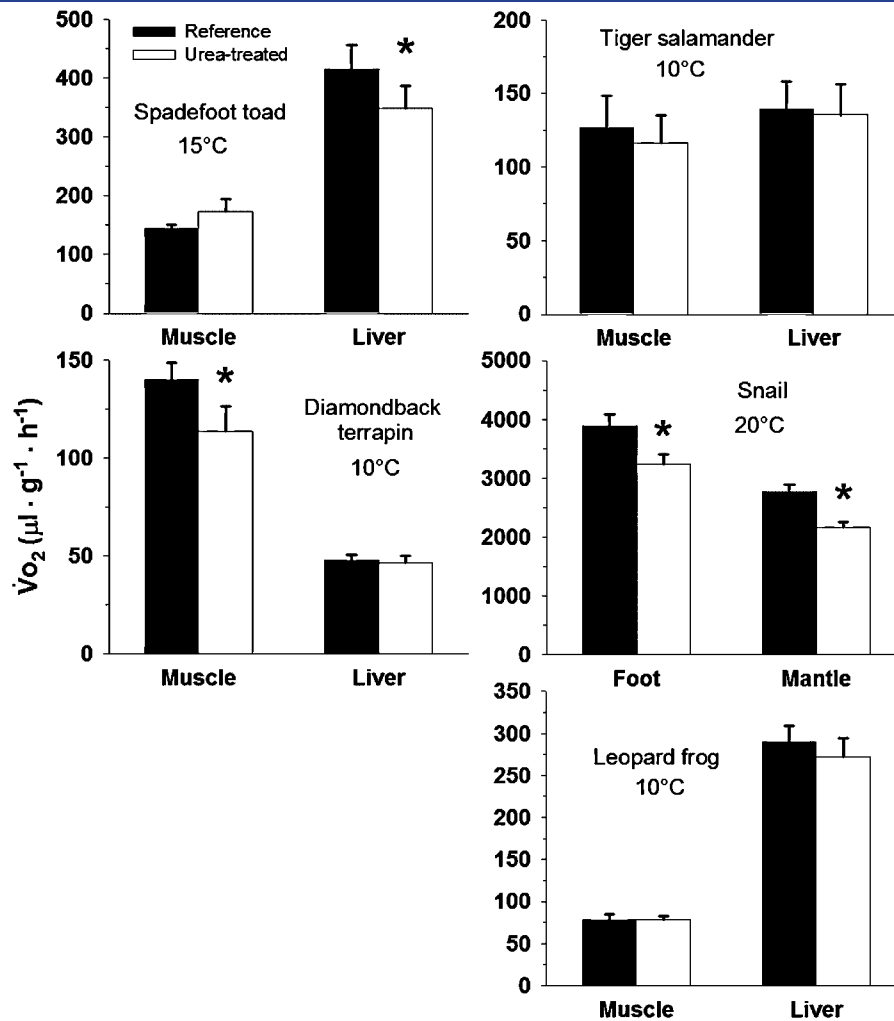
Organ  $\dot{V}O_2$  remained linear throughout each respirometry trial and resulted in a 10–15% reduction in dissolved oxygen within the chamber, suggesting the samples were not oxygen limited. The effect of urea treatment on organ  $\dot{V}O_2$  varied by species and organ (Fig. 1). Although there was no difference in mean  $\dot{V}O_2$  between urea-treated and reference liver ( $t_5 = 0.49$ ,  $P = 0.645$ ) or muscle ( $t_5 = 2.19$ ,  $P = 0.080$ ) from salamanders, the remaining three urea-accumulating species had organ samples that exhibited significantly lower mean  $\dot{V}O_2$  when treated with urea. Mean  $\dot{V}O_2$  of urea-treated liver ( $t_7 = 5.17$ ,  $P = 0.001$ ), but not muscle ( $t_7 = -1.35$ ,  $P = 0.218$ ), was significantly lower than that of respective reference samples from spadefoot toads, whereas urea-treated muscle ( $t_9 = 2.55$ ,  $P = 0.031$ ), but not liver ( $t_9 = 0.61$ ,  $P = 0.560$ ), had significantly lower mean  $\dot{V}O_2$  than their respective reference samples from terrapins. Mean  $\dot{V}O_2$  of urea-treated samples from snails was significantly lower than that of reference samples for both foot ( $t_6 = 3.06$ ,  $P = 0.022$ ) and mantle ( $t_6 = 8.40$ ,  $P = 0.0002$ ). Mean  $\dot{V}O_2$  was not significantly different between urea-treated and reference liver ( $t_7 = 1.82$ ,  $P = 0.111$ ) or muscle ( $t_7 = 0.07$ ,  $P = 0.945$ ) samples from leopard frogs, a species that does not typically accumulate urea during hibernation.

## DISCUSSION

Seasonal dormancy is accompanied by hyperuremia in many temperate-region organisms, including those we selected for study. Spadefoot toads inhabit arid grasslands and deserts of North America. In early fall, as water and food availability decline, they enter burrows and remain dormant for up to 10 months (Ruibal et al., '69), during which time urea accumulates in their body fluids, sometimes in excess of 300 mM (McClanahan, '72). Tiger salamanders have successfully colonized arid habitats throughout the Plains Region and southwestern United States, and during estivation can accumulate >200 mM urea (Delson

and Whitford, '73). Diamondback terrapins inhabit brackish estuaries and marshes along the Atlantic and Gulf coasts of the United States. Adults become hyperuremic during hibernation (Gilles-Baillien, '73), as do hatchlings (Costanzo et al., 2006), which often hibernate terrestrially (Baker et al., 2006). The pulmonate snail, *A. alternata*, inhabits deciduous woodlots in the eastern United States. In early fall, the snails become dormant and can accumulate 175 mM urea before arousal the following spring (Riddle, '81). The aim of our study was to investigate whether elevated levels of urea in these species might contribute to a hypometabolic state during dormancy. However, given that the physiological underpinnings of metabolic depression in ectothermic animals are diverse (Guppy and Withers, '99), we would not necessarily expect this response to be universal. Indeed, some anurans, including saline-adaptable species, can maintain high levels of activity whilst hyperuremic (e.g., Gordon and Tucker, '68; Katz, '73), whereas others, such as aquatic hibernators, accumulate little or no urea but are nevertheless capable of severely depressing metabolism (Donohoe et al., '98; St-Pierre et al., 2000; West et al., 2006).

The fact that urea is generally perturbing to protein structure led to the hypothesis that urea accumulating within the tissues of dormant animals may inhibit key metabolic enzymes and beneficially depress metabolic rate (Yancey et al., '82). In this study, physiological levels of urea decreased  $\dot{V}O_2$  of organs from three taxonomically diverse species that naturally accumulate urea during long-term dormancy (Fig. 1). The difference in mean  $\dot{V}O_2$  between urea-treated and reference muscle from a fourth urea-accumulating species, the tiger salamander, approached statistical significance. That postincubation concentrations of lactate in organs were low and did not differ with urea treatment suggests that the organ samples remained aerobic during incubation. Therefore, any difference in  $\dot{V}O_2$  between reference



**Figure 1.** Rates of oxygen consumption ( $\dot{V}O_2$ ), measured at the indicated temperatures, of isolated organs pre-incubated and tested in Ringer's solution with or without urea. Data are presented as mean  $\pm$  SEM ( $N=8$  for spadefoot toads and leopard frogs,  $N=6$  for tiger salamanders,  $N=10$  for diamondback terrapins,  $N=7$  for snails). An asterisk denotes a significant difference (paired  $t$ -test;  $P < 0.05$ ) between each pair of corresponding reference and urea-treated organs.

and urea-treated samples likely reflected a change in metabolic rate, rather than a compensatory shift from aerobic to anaerobic metabolism.

Although our findings for in vitro organs support the hypothesis that urea can contribute to hypometabolism in some dormant animals, it is difficult to determine the extent of any energetic savings realized at the organismal level. The  $\sim 20\%$  decrease we observed in organ  $\dot{V}O_2$  may not itself result in a large metabolic depression of the entire animal, but a reduced metabolic activity of some organs could secondarily affect that of others. The central role of liver in storage and distribution of metabolic substrates may make it a particularly influential organ in terms of whole-animal metabolic rate. Moreover, the organ samples we studied (harvested from dormant animals) were likely

hypometabolic before urea treatment, suggesting urea may act to complement other mechanisms of metabolic depression, thereby enhancing energy conservation. Further study at the organismal level is necessary to establish the energetic benefits of urea's effect in these and other species.

Urea concentration in the organs apparently came to equilibrium with that in the incubation medium before we made the respirometry measurements. Nevertheless, upon initial immersion of the samples, reference and urea-treated organs were exposed to hypotonic and hypertonic conditions, respectively. Given that cell swelling tends to inhibit catabolic processes and cell shrinkage tends to increase substrate catabolism (Häussinger et al., '94), the resultant transient changes in cell volume could potentially have had metabolic effects. Conse-

quently, persistence of any such effects during testing might have partially masked urea's hypometabolic effect. Obviating cell volume changes by inducing a more gradual influx of urea (e.g., step-transferring organs to solutions of incremented urea concentrations) may have resulted in more pronounced metabolic depression than was measured here.

Although urea treatment depressed  $\dot{V}O_2$  of several organs from urea-accumulating species, certain organs apparently were unaffected (Fig. 1). Muir et al. (2008) similarly found that urea's hypometabolic effect in the wood frog was prominent in some organs, but seemingly absent in others. The underlying reason why some organs are more responsive than others is unknown, but could stem from organ-specific isoforms of key metabolic enzymes having differential sensitivity to urea (see Cowan and Storey, 2002). Moreover, it appears that urea's effect is modified by factors such as temperature, physiological state, and seasonal acclimatization at the molecular (Fuery et al., '97), organ (Muir et al., 2008), and organismal (Muir et al., 2007) levels. Here, we endeavored to use experimental conditions (i.e., test temperature, RS composition) that were both ecologically relevant and conducive to expression of the urea-hypometabolic response. However, it is unclear whether the conditions we chose were optimal or, more importantly, a true representation of conditions the animals experience during natural dormancy. Urea's effect may be more (or less) pronounced under slightly different conditions.

The mechanisms underlying hypometabolism in dormant animals are varied and incompletely understood (Guppy and Withers, '99). However, our present data, together with previous findings for hibernating wood frogs (Muir et al., 2007, 2008), suggest that urea-induced hypometabolism may contribute to metabolic depression in several species that naturally accumulate urea during dormancy. Metabolism of organ samples from leopard frogs, a species that hibernates aquatically and, consequently, does not typically accumulate urea, was not affected by urea treatment. Aquatically hibernating frogs likely achieve hypometabolism via other means, such as hypoperfusing their skeletal muscles (West et al., 2006), lowering intracellular pH (Donohoe et al., '98), and reducing proton leak across the mitochondrial inner membrane (St-Pierre et al., 2000). As the evolution of osmolyte systems is believed to complement macromolecular adaptation (Somero, '86), it is tempting to speculate that metabolic pathways susceptible to control by urea might be selected for in species that naturally accumulate urea during dormancy as a means of conserving finite energy reserves. If that is indeed the case, it would be instructive to determine whether urea susceptibility has evolved multiple times in lineages of urea accumulators or if it is a primitive trait that has been subsequently lost in some groups.

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