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ORIGINAL PAPER

Urea-induced hypometabolism in the hibernating wood frog (*Rana sylvatica*) is not reflected in isolated mitochondria

Timothy J. Muir · Jon P. Costanzo · Richard E. Lee Jr

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Abstract It has long been speculated that urea accumulated during seasonal dormancy contributes to metabolic depression. Recent work suggests urea can indeed act as a metabolic depressant during dormancy in a number of taxonomically diverse species of ectotherms. The mechanisms by which urea exerts its hypometabolic effect are unknown, but potentially stem from inhibition of mitochondrial respiration. We isolated mitochondria from *Rana sylvatica* skeletal muscle, an organ that is metabolically responsive to urea, and measured respiration rates in the absence or presence of 80 mmol l^{-1} urea in the respiration medium. Because the effect of urea may be influenced by the intracellular milieu, in these experiments we varied substrate (pyruvate or palmitoylcarnitine), temperature (4, 10, or 15°C), and pH (6.8 or 7.4). Oxygen consumption of control and urea-treated mitochondria was sensitive to each of these variables, but neither state 3 nor state 4 respiration was reduced by urea treatment and, to the contrary, urea treatment slightly increased state 4 respiration at higher test temperatures. Although we did not test the efficacy of other incubation times or urea concentrations, the outcome of our experiment intimates that the urea-induced hypometabolism observed in hibernating *R. sylvatica* results from inhibition of energy-utilizing processes elsewhere in the cell, rather than a direct inhibition of mitochondrial respiration. Future investigation into urea's effects on non-mitochondrial metabolic pathways is necessary to uncover the mechanisms by which urea depresses metabolic rate.

Keywords Mitochondria · Metabolism · Urea · Hibernation · *Rana sylvatica*

Introduction

When faced with environmental conditions unfavorable for growth or reproduction, many animals enter and remain in a state of dormancy until conditions become more propitious. Because dormant animals are aphagic, they must rely on endogenous energy stores to fuel their metabolic processes; therefore, frugal use of those energy stores via metabolic depression is necessary for survival. In addition to energy conservation, animals occupying terrestrial habitats during dormancy must conserve water, which, for many ureogenic ectotherms, is facilitated by accumulating urea within their body fluids (Withers 1998; Yancey et al. 1982).

The mechanisms by which animals achieve hypometabolism are incompletely understood (Guppy 2004), but it has long been thought that in certain species urea accumulated during seasonal dormancy could contribute to metabolic depression. Because urea has a destabilizing effect on protein structure (Neurath et al. 1944), some authors hypothesized that urea, in the absence of so-called "counteracting solutes," might render key metabolic enzymes less active, thereby reducing organismal metabolism (Hand and Somero 1982; Yancey et al. 1982). Recent work showed that elevated levels of urea can decrease metabolism during dormancy in several taxonomically diverse species (Muir et al. 2010). The terrestrially

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T. J. Muir · J. P. Costanzo · R. E. Lee Jr
Department of Zoology, Miami University,
Oxford, OH 45056, USA

T. J. Muir (✉)
Biology Department, Augustana College, Rock Island,
IL 61201, USA
e-mail: timmuir@augustana.edu

hibernating frog *Rana sylvatica* accumulates urea in autumn and early winter, when environmental water potential is low (Costanzo and Lee 2005). Concomitant with moderate dehydration and urea accumulation (~50 mmol l^{-1}), the metabolic rate of hibernating *R. sylvatica* decreases by ~25% (Muir et al. 2007). Moreover, treatment of isolated skeletal muscles with 80 mmol l^{-1} urea results in a ~50% reduction of metabolic rate (Muir et al. 2008).

Although it was originally posited that urea may depress metabolism by inhibiting key enzymes (Hand and Somero 1982; Yancey et al. 1982), the mechanisms underlying urea's hypometabolic effect are as yet unknown. Presumably, urea must decrease energy use by inhibiting processes involved in ATP production and/or consumption. We sought to investigate urea's effect on mitochondrial respiration because of the mitochondrion's key role in ATP production and because metabolic depression at the organismal level is often reflected at the mitochondrial level (Guderley and St-Pierre 2002). Moreover, mitochondrial respiration appears to be particularly sensitive to intracellular solute composition (Ballantyne and Chamberlin 1988).

In this study we isolated mitochondria from skeletal muscles of hibernating *R. sylvatica* and measured state 3 and state 4 respiration in the absence or presence of urea. Because the effect of urea on mitochondrial respiration may be influenced by the intracellular environment, we performed experiments under different thermal and pH conditions. Furthermore, because urea may not affect all metabolic pathways equally, we measured mitochondrial respiration driven by two different oxidative substrates (pyruvate or palmitoylcarnitine).

Materials and methods

Animals and acclimation

We collected male *R. sylvatica* in late winter at a breeding pond in southern Ohio. Frogs were brought to Miami University, transferred to boxes containing damp moss, and held at 4°C. In early March, frogs were transferred to a 48-m^2 outdoor pen in a wooded area of the Miami University Ecological Research Center. Water was continually available to these frogs, and they were fed crickets three times weekly until early autumn. In mid-November, the frogs, on the verge of dormancy, were recaptured and held on damp moss in darkened boxes at 4°C. These conditions simulated natural hibernacula and facilitated the frogs' entrance into hibernation soon after transfer. They remained buried under a layer of moss and quiescent for 1.5–2.5 months until they were used in experiments in January and February.

Mitochondrial isolation and urea treatment

Frogs were killed by double-pithing and immediately dissected. Mitochondria from skeletal muscles were isolated following a protocol adapted from Hillman et al. (1991). For each mitochondrial preparation, we excised limb muscles from a single frog, minced them over ice, and placed them in 20 ml ice-cold isolation medium (mmol l^{-1}: 170 mannitol, 55 sucrose, 5 EGTA, 20 HEPES; 0.5% bovine serum albumin, 50 units ml^{-1} heparin) in a Potter-type homogenizer. The ice-cold suspension was ground (three complete strokes) with a Teflon pestle attached to a power drill (no. DC728, DeWalt, Baltimore, MD) set to high torque and low speed. The crude homogenate was centrifuged (750*g*, 10 min, 4°C) after which the supernatant was collected and centrifuged (17,000*g*, 10 min, 4°C) to sediment the mitochondria. The resulting pellet was rinsed and resuspended in isolation medium, and again centrifuged (8,200*g*, 10 min, 4°C). The final pellet, enriched with mitochondria, was rinsed and then resuspended in 75–100 μl of suspension medium (isolation medium lacking bovine serum albumin and heparin) yielding a concentration of 20–25 mg mitochondrial protein ml^{-1}. Protein concentrations were determined using the colorimetric Bradford assay (no. 500-0006, Bio-Rad, Hercules, CA, USA).

Each mitochondrial suspension was aliquoted into two portions, to one (urea-treated) of which we added ~3 μl of suspension medium containing 1.2 mol l^{-1} urea (final concentration, 80 mmol l^{-1} urea), and to the other (control) we added an identical volume of suspension medium lacking urea. Mitochondria were incubated for 2 h at 4°C before being used in respirometry trials.

Respirometry

We measured state 3 and state 4 respiration of isolated mitochondria in the presence or absence of urea via closed-system respirometry. To determine rates of oxygen consumption ($\dot{M}O_2$), we measured changes in dissolved oxygen (DO) concentration inside a 250-μl respirometry chamber using a fiber-optic oxygen-monitoring system (no. 110T, Instech, Plymouth Meeting, PA, USA). Chamber temperature was maintained at 4, 10, or 15°C, as desired (see below), by circulating coolant from a temperature-controlled bath through the chamber's outer jacket. The chamber was filled with respiration medium (mmol l^{-1}: 90 KCl, 55 mannitol, 24 sucrose, 10 HEPES, 10 K_2HPO_4, 1.5 malate; 0.5% bovine serum albumin), with or without 80 mmol l^{-1} urea, which was continuously mixed by a magnetic stir-bar. We then added ~10 μl of mitochondrial suspension, resulting in a final concentration of 1 mg mitochondrial protein ml^{-1}. Next, we added substrate

(5 mmol l^{-1} sodium pyruvate or 0.03 mmol l^{-1} palmitoylcarnitine; see below) to induce state 2 respiration. We closed the chamber and began monitoring DO concentration. After ~2 min, 0.5 mmol l^{-1} ADP was added through an injection port to stimulate state 3 respiration, and the decrease in DO concentration was recorded for 1–3 min.

State 4 respiration was effected by adding 0.02 μmol l^{-1} oligomycin, and the decrease in DO concentration was recorded for 4–8 min. We elected to induce state 4 respiration with oligomycin, rather than allow state 4 to be reached via depletion of ADP, to expedite data collection. However, in several trials we determined state 4 $\dot{M}O_2$ from data collected following ADP depletion and found that it was not further reduced by subsequent addition of oligomycin, suggesting oligomycin-induced state 4 respiration was an accurate representation of non-phosphorylating respiration.

Our primary goal for this study was to determine the effects of urea on mitochondrial respiration. However, because urea's effect may be modified by the intracellular milieu, we measured $\dot{M}O_2$, fueled by two different substrates (pyruvate or palmitoylcarnitine), in the absence or presence of urea under varied thermal and pH conditions. Specifically, in one experiment, we tested samples of mitochondria at pH 7.4, using pyruvate ($n = 3$, 7, and 3 at 4, 10, and 15°C, respectively) or palmitoylcarnitine ($n = 3$, 7, and 3 at 4, 10, and 15°C, respectively) as substrate. In another experiment we tested samples of mitochondria at 10°C in respiration media with pH 6.8, using pyruvate ($n = 11$) or palmitoylcarnitine ($n = 5$) as substrate. To avoid the confounding effects of pseudoreplication, the respiration rates were measured for each mitochondrial preparation at only one temperature and pH. $\dot{M}O_2$ values are expressed as nmol O min^{-1} mg mitochondrial $protein^{-1}$.

Statistical analyses

Statistical analyses were performed using SAS 9.1. Data for state 3 and state 4 respiration were separately analyzed. Effects of urea treatment, temperature, and substrate (or urea treatment, pH, and substrate) on mitochondrial respiration were analyzed using a three-factor, dependent-measures ANOVA after confirming that data sets met assumptions for parametric tests. Mean values (presented ± SEM) were compared using differences of least-squares means. Significance was accepted at $P < 0.05$.

Results

During respirometry trials, the decline in DO of the respiration medium remained linear, suggesting that mitochondria were not oxygen limited even at the highest test temperature, 15°C. The mean respiratory control ratio (state 3 $\dot{M}O_2$:state 4 $\dot{M}O_2$) for all 84 trials was 7.7 ± 0.3, which was high enough to suggest that the mitochondrial isolations were of good quality (Chappell and Hansford 1972). Furthermore, our values for state 3 and state 4 $\dot{M}O_2$ of control mitochondria at 4°C compare well with those measured at 3°C for muscle mitochondria isolated from hibernating *Rana temporaria* (St-Pierre et al. 2000).

Effects of urea treatment on state 3 $\dot{M}O_2$

Results from the statistical analysis of urea treatment, temperature, and substrate effects on state 3 $\dot{M}O_2$ are presented in Table 1. Urea treatment had no effect on $\dot{M}O_2$ regardless of which temperature or substrate was used (Fig. 1a, b). Expectedly, higher test temperatures resulted in higher ($P < 0.0001$) $\dot{M}O_2$. Mitochondria catabolizing pyruvate had higher ($P = 0.0002$) $\dot{M}O_2$ than did mitochondria catabolizing palmitoylcarnitine.

Results from the statistical analysis of urea treatment, pH, and substrate effects on state 3 $\dot{M}O_2$ are presented in Table 2. Similar to the trials at pH 7.4, there was no effect of urea treatment on $\dot{M}O_2$ when mitochondria were tested at pH 6.8, and altering the substrate did not affect this outcome (Fig. 2a, b). However, $\dot{M}O_2$ was higher ($P < 0.0001$) at pH 6.8 than at pH 7.4. Likewise, mitochondria catabolizing pyruvate had higher ($P < 0.0001$) $\dot{M}O_2$ than did mitochondria catabolizing palmitoylcarnitine, even at the lower pH.

Effects of urea treatment on state 4 $\dot{M}O_2$

Results from the statistical analysis of urea treatment, temperature, and substrate effects on state 4 $\dot{M}O_2$ are presented in Table 1. The effect of urea treatment on $\dot{M}O_2$ was strongly dependent ($P < 0.0001$) on temperature. For both substrates, $\dot{M}O_2$ of urea-treated mitochondria was significantly higher than that of control mitochondria when tested at 10 and 15°C, but not at 4°C (Fig. 1c, d). At 10°C, state 4 $\dot{M}O_2$ of urea-treated mitochondria was 25–30% higher than that of controls, whereas at 15°C it was 35–40% higher than that of controls. Higher test temperatures resulted in higher ($P < 0.0001$) $\dot{M}O_2$ of control and urea-treated mitochondria regardless of treatment, pH, or substrate. Mitochondria catabolizing pyruvate tended to have lower $\dot{M}O_2$ than did mitochondria catabolizing palmitoylcarnitine, but that effect was dependent ($P = 0.03$) on temperature, as it was present at 10 ($P = 0.02$) and 15°C ($P = 0.0003$), but not at 4°C ($P = 0.82$).

Results from the statistical analysis of urea treatment, pH, and substrate effects on state 4 $\dot{M}O_2$ are presented in Table 2. Similar to trials at pH 7.4, urea-treated

Table 1 Results from ANOVAs of mitochondrial rates of oxygen consumption measured in the absence or presence of 80 mmol l^{-1} urea (treatment) at 4, 10, and 15°C (pH 7.4) with pyruvate or palmitoylcarnitine as the substrate

	State 3			State 4		
	df	*F*	*P*	*df*	*F*	*P*
Treatment × temperature × substrate	2, 30	0.7	0.50	2, 30	0.1	0.88
Treatment × temperature	2, 30	0.2	0.79	2, 30	37.8	**<0.0001**
Treatment × substrate	1, 30	0.5	0.49	1, 30	0.5	0.50
Temperature × substrate	2, 30	2.6	0.09	2, 30	3.8	**0.03**
Treatment	1, 30	0.5	0.49	1, 30	96.8	**<0.0001**
Temperature	2, 30	74.8	**<0.0001**	2, 30	15.0	**<0.0001**
Substrate	1, 30	17.7	**0.0002**	1, 30	14.7	**0.0006**

$P < 0.05$ are given in bold

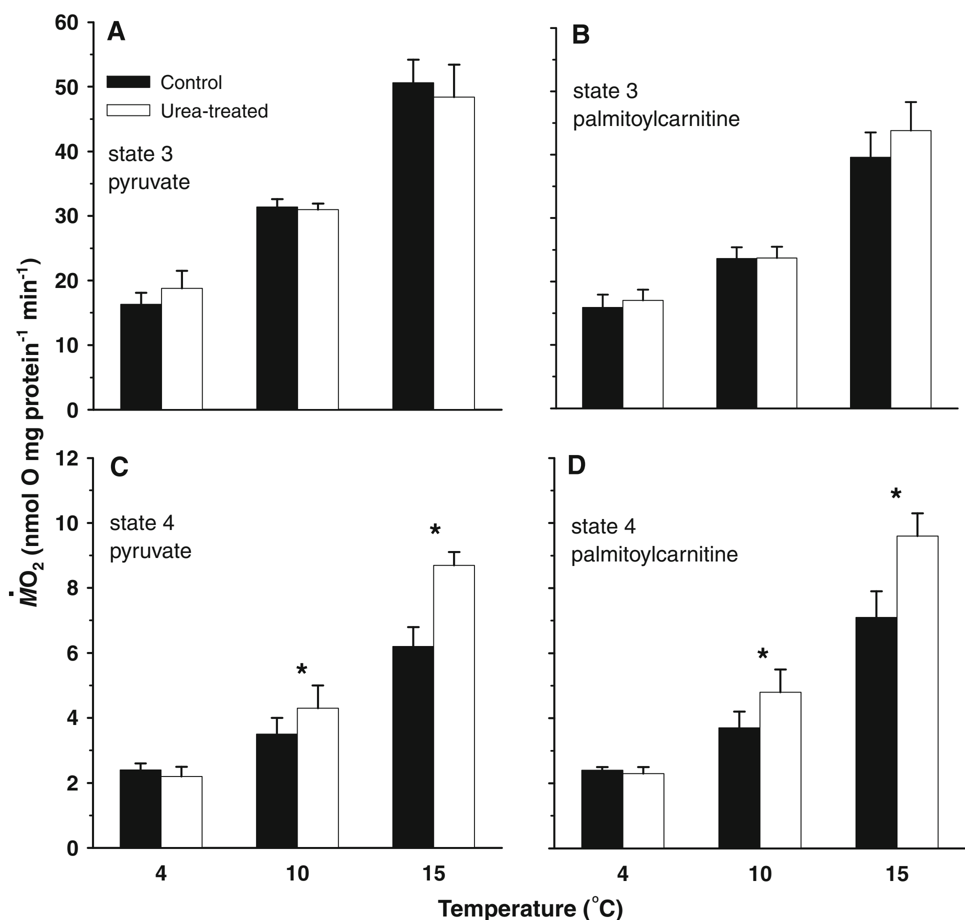

Fig. 1 Effect of urea treatment and temperature on state 3 and state 4 respiration of mitochondria catabolizing pyruvate or palmitoylcarnitine at pH 7.4. *Bars* represent group means (±SEM; $n = 7$ at 10°C; $n = 3$ at 4 and 15°C). *Asterisk* denotes a significant (least-squares means; $P < 0.05$) difference between respective control and urea-treated means

mitochondria had significantly higher $\dot{M}O_2$ than did control mitochondria when tested at pH 6.8, and altering the substrate did not affect this outcome (Fig. 2c, d). The ~15% increase in $\dot{M}O_2$ with urea treatment at pH 6.8 was lower than that found at pH 7.4. Similar to our results with state 3 $\dot{M}O_2$, state 4 $\dot{M}O_2$ was higher ($P = 0.02$) at pH 6.8 than at pH 7.4. Mitochondria catabolizing pyruvate had lower ($P = 0.02$) state 4 $\dot{M}O_2$ than did mitochondria catabolizing palmitoylcarnitine, even at the lower pH.

Discussion

Recent work suggests that elevated urea can induce reversible hypometabolism during dormancy in certain species of ureogenic ectotherms (Muir et al. 2007, 2008, 2010). The mechanisms underlying urea's hypometabolic effect in those species are unknown, but because metabolic rates at the cellular, organ, and organismal levels can be dictated by mitochondrial metabolism (Guderley and

Table 2 Results from ANOVAs of mitochondrial rates of oxygen consumption measured in the absence or presence of 80 mmol l^{-1} urea (treatment) at pH 6.8 or 7.4 (10°C) with pyruvate or palmitoylcarnitine as the substrate

	State 3			State 4		
	df	*F*	*P*	*df*	*F*	*P*
Treatment × pH × substrate	1, 32	0.0	0.90	1, 32	0.0	0.87
Treatment × pH	1, 32	0.0	0.86	1, 32	0.0	0.99
Treatment × substrate	1, 32	0.2	0.69	1, 32	0.7	0.40
pH × substrate	1, 32	1.7	0.21	1, 32	3.8	0.06
Treatment	1, 32	0.0	0.99	1, 32	21.5	**<0.0001**
pH	1, 32	27.3	**<0.0001**	1, 32	6.3	**0.02**
Substrate	1, 32	29.5	**<0.0001**	1, 32	6.4	**0.02**

$P < 0.05$ are given in bold

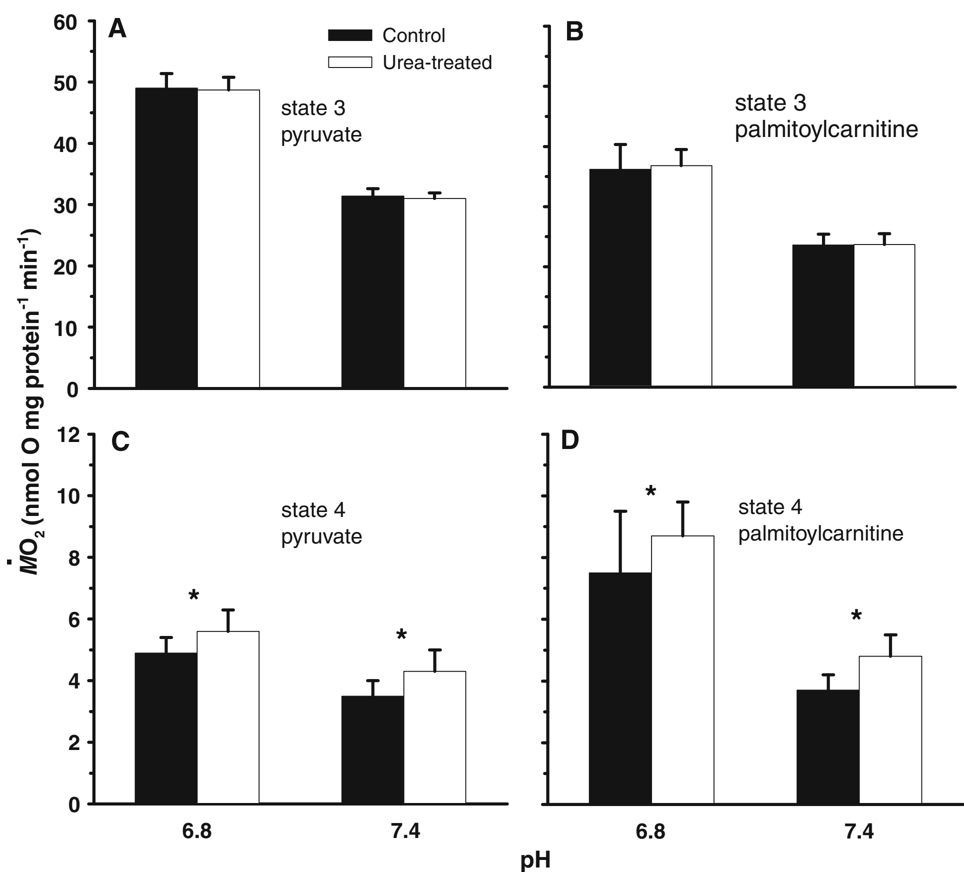

Fig. 2 Effect of urea treatment and pH on state 3 and state 4 respiration of mitochondria catabolizing pyruvate or palmitoylcarnitine at 10°C. *Bars* represent group means (±SEM; $n = 7$ at pH 7.4; $n = 11$ at pH 6.8 with pyruvate; $n = 5$ at pH 6.8 with palmitoylcarnitine). *Asterisk* denotes a significant (least-squares means; $P < 0.05$) difference between respective control and urea-treated means

St-Pierre 2002), we posited that urea may induce metabolic depression by decreasing rates of mitochondrial respiration. In this study we measured mitochondrial respiration in the absence or presence of urea in the respiration medium and because the intracellular environment may influence urea's effect, we varied the thermal, substrate, and pH conditions.

Respiration of control and urea-treated mitochondria from *R. sylvatica* skeletal muscle was affected by temperature, substrate, and pH. Not surprisingly, higher test temperatures resulted in higher $\dot{M}O_2$ for state 3 and state 4 respiration. The Q_{10} values (pyruvate: 3.2; palmitoylcarnitine: 3.7) for state 4 respiration of control mitochondria determined for the interval 10–15°C were greater than those (pyruvate: 1.9; palmitoylcarnitine: 2.1) determined for 4–10°C, suggesting that state 4 respiration is more thermally sensitive at higher temperatures. Control mitochondria fueled by palmitoylcarnitine also had

a higher Q_{10} (2.8) for state 3 respiration over the interval 10–15°C than that (1.9) determined for 4–10°C. However, control mitochondria fueled by pyruvate had a lower Q_{10} (2.6) for state 3 respiration over the interval 10–15°C than that (3.0) determined for 4–10°C. Similarly, Ballantyne et al. (1989) found that state 3 respiration of liver mitochondria from charr (*Salvelinus namaycush*) has a greater Q_{10} at higher temperatures when driven by acyl carnitines as compared to pyruvate. In the present study, state 3 $\dot{M}O_2$ was higher with pyruvate than with palmitoylcarnitine at all temperatures, as is usually the case for mitochondria isolated from skeletal muscle (Moyes et al. 1990). However, at 10 and 15°C, state 4 $\dot{M}O_2$ was lower with pyruvate than with palmitoylcarnitine, but there was no difference between substrates at 4°C. Guderley et al. (1997) similarly found little effect of varying substrate on state 4 respiration of mitochondria isolated from trout (*Oncorhynchus mykiss*) muscle at 4°C. Both state 3 and state 4 $\dot{M}O_2$ were higher at pH 6.8 than at pH 7.4. That the lower pH led to higher respiration rates was somewhat surprising as hibernating frogs, which must reduce energy use, are thought to be acidotic (Pinder et al. 1992). However, the actual intramuscular pH of hibernating *R. sylvatica* has, to our knowledge, never been reported and may be between 6.8 and 7.4.

Although urea treatment of isolated mitochondria had no effect on state 4 respiration at 4°C, it apparently increased $\dot{M}O_2$ at 10 and 15°C (Figs. 1c, d, 2c, d). This outcome seemingly opposes an earlier finding that skeletal muscle isolated from hibernating *R. sylvatica* exhibited a ~50% reduction in metabolic rate in the presence of elevated urea at 10°C (Muir et al. 2008). If the mitochondrial response we observed also occurs in vivo, then the overall energetic savings realized elsewhere in the cell must be great enough to overcome the energy "wasted" by an increased state 4 respiration. The mechanism by which urea treatment increased state 4 respiration is unknown, but presumably involves an increase in proton conductance of the inner mitochondrial membrane or production of a greater membrane potential driving proton leak across the membrane. Because an increase in membrane potential would necessarily elevate state 3 respiration under our experimental conditions, which we did not find, it is more likely that urea increased proton conductance of the membrane, possibly due to an increase in membrane fluidity (Barton et al. 1999).

We incubated the mitochondrial suspensions only briefly (2 h) as a precaution against their loss of functional integrity, but presumed that the urea-treated samples had accumulated significant amounts of urea before the respirometry trials began. Although we did not measure their internal levels of urea, the aforementioned rise in state 4 respiration following urea treatment suggests that they did indeed take up the solute. On the other hand, whereas the urea permeability of mitochondrial membranes is known to be high in at least some fishes (Ballantyne and Moon 1986; Rodela et al. 2008), it has not been investigated in amphibians; thus, our results for *R. sylvatica* should be interpreted with this limitation in mind.

Contrary to our hypothesis, exposing *R. sylvatica* mitochondria to elevated urea, such as occurs during hibernation, did not decrease state 3 or state 4 respiration. State 3 respiration was unaffected by urea treatment under any of the thermal, substrate, or pH conditions tested, suggesting that urea did not alter the activity of the so-called rate-limiting enzymes of pyruvate and palmitoylcarnitine catabolism, pyruvate dehydrogenase and carnitine palmitoyltransferase, respectively. That urea treatment also did not decrease state 4 respiration suggests that urea-induced hypometabolism observed at higher levels of organization does not result from direct inhibition of mitochondrial metabolism. Rather, urea may depress metabolism by inhibiting energy-utilizing processes (e.g., protein turnover, ion pumping) elsewhere in the cell. For example, lungfish (*Protopterus dolloi*) experimentally loaded with urea approximating levels reached during estivation apparently exhibited suppressed rates of proteolysis (Ip et al. 2005). Urea concentrations as low as 45 mmol l^{-1} can inhibit Na^{+}–K^{+}-ATPase activity (Kaji et al. 1998), which by itself can account for 28–75% of ATP use by the cell (Buck and Hochachka 1993). However, we must be cautious about interpreting responses of mitochondria operating outside their native environment. Although results from studies of isolated mitochondria are commonly extrapolated to in vivo conditions, using a more intact mitochondrial preparation, such as skinned muscle fiber (Saks et al. 1998), could potentially yield different results. Moreover, it is possible that urea regulates mitochondrial respiration in vivo indirectly, such as by limiting the availability of substrates upstream of the mitochondrion. Metabolic depression in mitochondria from estivating *Helix aspersa* is mostly accounted for by decreasing rates of processes that produce the membrane potential of the mitochondrial inner membrane, including those that supply the mitochondrion with substrates (Bishop et al. 2002). Investigation into urea's effects on energy-utilizing processes in hibernating *R. sylvatica* and into levels of key cellular metabolites in tissues that have been metabolically depressed by urea is necessary to better understand the mechanisms by which urea regulates metabolic rate in vivo.

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