

Metabolic Depression Induced by Urea in Organs of the Wood Frog, *Rana sylvatica*: Effects of Season and Temperature

TIMOTHY J. MUIR*, JON P. COSTANZO, AND RICHARD E. LEE JR.
Department of Zoology, Miami University, Oxford, Ohio

ABSTRACT It has long been suspected that urea accumulation plays a key role in the induction or maintenance of metabolic suppression during extended dormancy in animals from diverse taxa. However, little evidence supporting that hypothesis in living systems exists. We measured aerobic metabolism of isolated organs from the wood frog (*Rana sylvatica*) in the presence or absence of elevated urea at various temperatures using frogs acclimatized to different seasons. The depressive effect of urea on metabolism was not consistent across organs, seasons, or temperatures. None of the organs from summer frogs, which were tested at 20°C, or from winter frogs tested at 4°C were affected by urea treatment. However, liver, stomach, and heart from spring frogs tested at 4°C had significantly lower metabolic rates when treated with urea as compared with control samples. Additionally, when organs from winter frogs were tested at 10°C, metabolism was significantly decreased in urea-treated liver and stomach by ~15% and in urea-treated skeletal muscle by ~50%. Our results suggest that the presence of urea depresses the metabolism of living organs, and thereby reduces energy expenditure, but its effect varies with temperature and seasonal acclimatization. The impact of our findings may be wide ranging owing to the number of diverse organisms that accumulate urea during dormancy. *J. Exp. Zool.* 309A:111–116, 2008. © 2008 Wiley-Liss, Inc.

How to cite this article: Muir TJ, Costanzo JP, Lee Jr RE. 2008. Metabolic depression induced by urea in organs of the wood frog, *Rana sylvatica*: effects of season and temperature. *J. Exp. Zool.* 309A:111–116.

Accumulation of particular organic solutes is a common strategy for enhancing water conservation in animals faced with unfavorable hydric conditions. Urea, an end product of nitrogen metabolism, is amassed by a diverse array of organisms as a means of conserving water. Whereas some animals, such as marine elasmobranchs, maintain constitutively high levels of urea to combat chronic water loss to the environment, others accumulate urea only intermittently. This response often coincides with a period of extended dormancy, which in turn is accompanied by a substantial metabolic depression (Yancey et al., '82).

Mechanisms underlying the hypometabolic state are not well understood and likely vary depending on the environmental conditions that trigger or persist during dormancy (Guppy and Withers, '99). However, the realization that urea retention and metabolic depression often occur in concert, along with the fact that urea is generally perturbing to protein structure, led to the hypothesis that accumulated urea contributes to metabolic regula-

tion in dormant animals by reversibly inhibiting key metabolic enzymes (Hand and Somero, '82; Yancey et al., '82). Support for this hypothesis comes from a handful of in vitro enzyme kinetic studies (Hand and Somero, '82; Fuery et al., '97; Kaji et al., '98; Cowan and Storey, 2002), but a paucity of tests at higher levels of biological organization has made it difficult to assess the actual metabolic impact of urea accumulation. However, recent studies (Costanzo and Lee, 2005; Muir et al., 2007) of hibernating wood frogs (*Rana sylvatica*) suggest a link between urea accumulation and metabolic depression in living systems.

Grant sponsor: National Science Foundation; Grant number: IOB 0416750.

*Correspondence to: Timothy J. Muir, Department of Zoology, Miami University, Oxford, OH 45056. E-mail: muirtj@muohio.edu

Received 15 November 2007; Revised 13 December 2007; Accepted 18 December 2007

Published online in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/jez.436

R. sylvatica, a terrestrially hibernating frog, is likely exposed to dehydrating conditions during late fall and winter when soil moisture is at an annual minimum and freezing of the soil further reduces its water potential (Spaans and Baker, '96). In response, these frogs can amass high levels of urea (to at least 90 mM) in their blood plasma and tissues during hibernation (Costanzo and Lee, 2005). Dehydration not only increases urea concentration, but also decreases resting metabolic rate (Muir et al., 2007). Although the observed correlation between hyperuremia and low metabolism in dormant *R. sylvatica* supports the urea-induced metabolic depression hypothesis, definitive evidence for a causal relationship is still lacking. Our objective in this study was to test the urea-hypometabolism hypothesis by measuring metabolic rates of isolated *R. sylvatica* organs in the presence or absence of urea and determine the efficacy of urea as a metabolic depressant in different seasons and temperatures.

METHODS

Animals and acclimation

We collected male *R. sylvatica* at a breeding pond in southern Ohio in February 2006. Frogs were brought to Miami University, transferred to boxes containing damp moss, and held at 4°C. Some frogs (spring frogs; $N = 11$) were kept at 4°C until used in April and May, whereas others were transferred in early March to a 48-m² 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 fall. One set of these animals (summer frogs; $N = 5$) was sampled from the outdoor enclosure in mid-August. In mid-November, the remaining animals (winter frogs; $N = 12$), on the verge of dormancy, were recaptured and held on damp moss in darkened boxes at 4°C until they were used in late December and January.

Organ preparation

Frogs were double pithed and immediately dissected. The heart, liver, stomach, right and left semitendinosus muscles, and a portion of dorsal skin from the trunk were excised and placed in Ringer's solution (RS; in g/L; 6.10 NaCl, 0.15 KCl, 0.88 Na₂HPO₄, 0.15 KH₂PO₄; 230 mOsm/kg, pH 7.4 at 21°C) kept at 4°C (winter and spring frogs) or 21°C (summer frogs). We then bisected each

sample of heart, liver, stomach, and skin and placed one-half of each in 5-mL fresh RS (control) and the other half in RS augmented with 80 mM urea (urea treated). Most organ samples were further cut into several ~2-mm pieces to increase surface area for diffusive exchange of dissolved gases, although muscles were kept intact, so as not to cut their fibers. One muscle from each frog was placed into fresh RS, whereas the other was placed into RS with 80 mM urea. We chose the semitendinosus because it is a long and slender bifurcated muscle with a relatively high surface-to-volume ratio. Prepared samples were incubated in the appropriate solution for 16 hr (spring and winter frogs at 4°C), 12 hr (winter frogs at 10°C), or 5 hr (summer frogs at 20°C) before testing. Initial respirometry trials showed that organ metabolic rates remained steady over those incubation periods.

In an attempt to better understand the mechanism by which urea regulates metabolism, we carried out an additional experiment. After respirometry trials at 10°C had been conducted on muscles from winter frogs ($N = 2$), urea was "washed out" of the urea-treated muscles by placing them in RS for 6 hr. The RS was replaced several times throughout the 6-hr period to facilitate urea removal. Control muscles, which had been tested in RS without urea, were treated identically. Respirometry trials were repeated on each muscle after the 6-hr period.

Heart, liver, stomach, muscle, and skin samples were collected from a separate group of winter frogs ($N = 3$) to determine post-incubation concentrations of urea and lactate. Organ samples were prepared as described above and incubated for 16 hr at 4°C. They were removed from the solution, blotted free of excess moisture, weighed to the nearest 0.1 mg, and stored at -80°C for future analysis of metabolites. Subsequently, deproteinized organ extracts were prepared by homogenizing thawed samples in 1 N HClO₄, centrifuging the homogenate at 2000g for 5 min, and neutralizing the supernatant with an equal volume of 1 N KOH. Urea and lactate concentrations of the extracts were measured using the Berthelot method (Pointe Scientific, Canton, MI) and a colorimetric lactate dehydrogenase procedure (Sigma, St Louis, MO) according to the manufacturer's instruction.

Respirometry

Rate of oxygen consumption ($\dot{V}O_2$) of each organ sample was measured via closed-system

respirometry using a fiber optic oxygen monitoring system in a 1-mL titanium chamber (No. 110T, Instech, Plymouth Meeting, PA). The chamber was filled with either RS or RS with 80 mM urea, as appropriate, which was continuously mixed by a magnetic stir bar. We held chamber temperature constant by circulating water from a temperature-controlled bath through the chamber jacket. Dissolved oxygen concentration decreased ~ 1 mg/L over the course of each respirometry trial that lasted 15–90 min, depending on the temperature. After each trial, the organ sample was removed, blotted free of excess moisture, and dried to a constant mass at 60°C. $\dot{V}O_2$ values were expressed as microliters of O_2 consumed per gram of organ dry mass per hour.

Statistical analyses

Within each season $\dot{V}O_2$ data were analyzed using a two-factor (treatment \times organ) repeated measures analysis of variance. When there was a significant treatment effect, paired Student's *t*-tests were used to compare mean $\dot{V}O_2$ values between control and urea-treated samples of a given organ. To test for a season effect on metabolic rate, we compared $\dot{V}O_2$ of control organs from spring frogs at 4°C with those of control organs from winter frogs at 4°C using a two-factor (season \times organ) analysis of variance; post hoc Student's *t*-tests were used as before. We used paired *t*-tests to compare urea and lactate concentrations in control vs. urea-treated organs. Mean values are reported \pm SE and significance was accepted at $P < 0.05$.

RESULTS AND DISCUSSION

Urea concentrations of organ samples after incubation confirmed that urea treatment markedly increased urea content within the organs (Table 1). Furthermore, low lactate concentrations suggested that the organ samples remained aerobic during incubation in either RS or RS with urea (Table 1). Therefore, any difference in $\dot{V}O_2$ between these groups likely reflected a change in metabolic rate, rather than a shift to anaerobic metabolism.

The effect of urea on the metabolic rate of organ samples varied among seasons and organs. There was no effect of urea treatment on $\dot{V}O_2$ measured in organs from winter frogs tested at 4°C ($F_{1,20} = 3.27$, $P = 0.086$; Fig. 1) or from summer frogs tested at 20°C ($F_{1,20} = 2.04$, $P = 0.169$; Fig. 1). However, organs from spring frogs tested at

TABLE 1. Urea and lactate concentrations of *Rana sylvatica* organs after incubation in Ringer's solution (control) or Ringer's solution with 80 mM urea (urea treated)

Organ	Urea (mM)		Lactate (mM)	
	Control	Urea treated	Control	Urea treated
Muscle	1.9 \pm 0.3	79.9 \pm 0.7	3.0 \pm 1.5	3.1 \pm 0.6
Liver	3.2 \pm 0.7	82.1 \pm 1.1	1.8 \pm 0.5	1.3 \pm 0.2
Heart	2.1 \pm 0.3	84.2 \pm 2.1	2.4 \pm 0.2	3.6 \pm 0.2
Skin	1.7 \pm 0.2	94.9 \pm 1.1	1.2 \pm 0.1	1.0 \pm 0.2
Stomach	2.1 \pm 0.1	85.0 \pm 1.4	0.1 \pm 0.1	0.4 \pm 0.1

Reported values are mean \pm SE ($N = 3$).

4°C had significantly lower $\dot{V}O_2$ in the presence of urea compared with control organs ($F_{1,36} = 40.95$, $P < 0.0001$; Fig. 1). The interaction between treatment and organ was also significant ($F_{4,36} = 7.74$, $P = 0.0001$), suggesting that the magnitude of urea's effect varied among organs. Specifically, mean $\dot{V}O_2$ was significantly lower by 10, 18, and 34% for urea-treated liver ($t_9 = 2.50$, $P = 0.034$), stomach ($t_{10} = 3.22$, $P = 0.009$), and heart ($t_4 = 4.20$, $P = 0.014$), respectively, compared with those of control samples. Urea had no effect on $\dot{V}O_2$ of muscle ($t_9 = 0.67$, $P = 0.521$) or skin ($t_4 = 1.49$, $P = 0.210$) from spring frogs.

That urea had a depressive effect on metabolism for some, but not all, organs, and only in those from spring frogs, suggests there were season- and organ-specific differences in urea sensitivity. The underlying reason for that result is unknown. However, if urea inhibits metabolism by perturbing the function of certain regulatory enzymes (Hand and Somero, '82; Yancey et al., '82), then differential expression of those enzymes and their isoforms among organs and seasons could result in metabolic pathways that are more or less susceptible to perturbation by urea. Significant differences in $\dot{V}O_2$ between control organs from winter frogs tested at 4°C to those of spring frogs tested at 4°C ($F_{4,56} = 22.11$, $P < 0.0001$) suggest that intrinsic metabolic regulation changed between seasons, possibly via expression of different metabolic isozymes (Fig. 1). In these comparisons, liver ($t_{13} = 2.96$, $P = 0.011$), muscle ($t_{13} = 2.36$, $P = 0.035$), and stomach ($t_{14} = 2.48$, $P = 0.026$) had a significantly higher mean $\dot{V}O_2$ in spring than in winter. Heart had a lower mean $\dot{V}O_2$ ($t_8 = -5.16$, $P = 0.001$) in spring, whereas skin $\dot{V}O_2$ did not differ ($t_8 = -0.04$, $P = 0.970$) between the two seasons. Several metabolic enzymes have isoforms that are differentially sensitive to urea (Cowan and Storey, 2002). Mashino and Fridovich

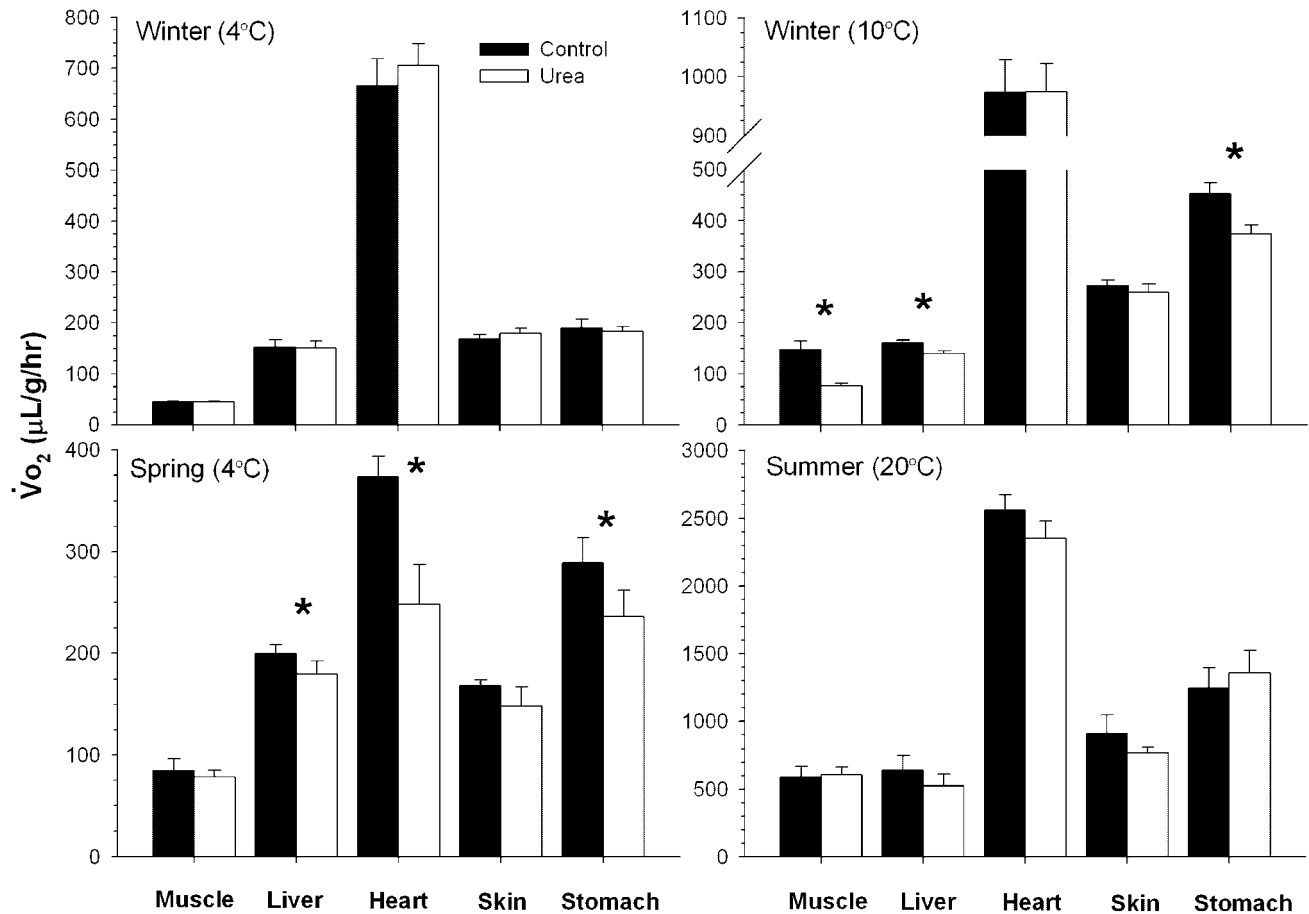


Fig. 1. Rates of oxygen consumption ($\dot{V}O_2$) of isolated organs from winter (4 and 10°C), spring, and summer *Rana sylvatica* incubated in Ringer's solution with or without 80 mM urea. Data are presented as mean \pm SE ($N = 5$, except for winter samples tested at 10°C, where $N = 6$ for heart and $N = 7$ for others; and spring samples, where $N = 10$ for muscle and liver, and $N = 11$ for stomach). An asterisk denotes a significant difference ($P < 0.05$) between control and urea-treated organs using a paired Student's *t*-test.

('87) posited that urea affects the function of some enzymes, but not others because native state enzymes exist in a continuum of structural flexibility, and although urea typically increases flexibility, it does not necessarily shift the conformation away from a more active state. Thus, urea can have different functional effects depending on the relationship between the native structural flexibility of an enzyme and that of its most active state (Baskakov et al., '98).

Given that high levels of urea decrease metabolism of winter *R. sylvatica* at 4°C only when the animals are also moderately dehydrated (Muir et al., 2007), it was not surprising that urea did not affect metabolism in our well-hydrated organs from winter frogs tested at that temperature (Fig. 1). Muir et al. (2007) speculated that cellular dehydration favors a less stable enzyme structure such that further perturbation by urea alters the

structure enough to reduce function. In this study, we hypothesized that a slightly higher test temperature would cause a similar urea susceptibility in organs from winter frogs because higher temperatures increase enzyme flexibility (Hochachka and Somero, 2002). Indeed, when organs from winter frogs were tested at 10°C, we found a pronounced effect of urea treatment on organ $\dot{V}O_2$ ($F_{1,29} = 33.67$, $P < 0.0001$; Fig. 1).

Results of our urea-washout experiments provided additional evidence of urea's depressive effect on organ metabolism. In the first experiment, flushing the urea-treated muscle sample with RS coincided with a rise in $\dot{V}O_2$ from 72 to 136 $\mu\text{L/g/hr}$. In the second experiment, $\dot{V}O_2$ of muscle again increased, from 92 to 355 $\mu\text{L/g/hr}$, after urea removal. We attribute this marked rebound in metabolism to the release of urea's inhibitory effect because, in sharp contrast,

corresponding control samples exhibited a slight decrease (by 20 and 13%, respectively) in $\dot{V}O_2$. Although these results strongly suggest that elevated urea directly modulates metabolic processes, additional experimentation is needed to bolster this conclusion.

Temperature coefficients (Q_{10}) were calculated for control and urea-treated organs from winter frogs using mean $\dot{V}O_2$ at 4 and 10°C. Q_{10} values were highly variable among organs (Fig. 2). Whereas heart, skin, and stomach generally exhibited the typical Q_{10} values of 2–3, liver and muscle had extraordinary temperature coefficients. $\dot{V}O_2$ of control liver showed very little difference between 4 and 10°C. This small thermal influence on the metabolic rate of liver was surprising and deserves further study. However, a low Q_{10} may be the result of liver being highly active at low temperatures (Lewis and Driedzic, 2007), perhaps because in *R. sylvatica* it must synthesize large amounts of cryoprotectant at the onset of natural freezing (Storey, '90). In contrast, muscle metabolism exhibited unusually high thermal sensitivity, although this response was lessened by the inhibitory effect of urea treatment at 10°C (Fig. 2).

Although skeletal muscle had the lowest $\dot{V}O_2$ of all the organs studied, its contribution to whole-animal metabolism is probably considerable, as it accounts for such a high proportion of total body mass. In fact, any substantial metabolic depression of the whole animal must include decreased energy use by muscle (Flanigan et al., '91). Flanigan et al. ('91) calculated that skeletal muscle made up 50–65% of the standard metabolic rate of the Australian desert frog, *Neobatrachus pelobatoides*. If the same is true for *R. sylvatica*, then the observed ~50% metabolic depression of urea-treated muscle at 10°C would by itself result in a 25–33% decrease in whole-animal metabolism. Because *R. sylvatica* overwinter in superficial hibernacula that are poorly insulated (especially when snow cover is lacking), body temperatures fluctuate with changes in ambient temperature throughout winter (e.g., Costanzo et al., '97). Urea's effect of reducing an otherwise large increase in energy use during warm periods would not only save energy, but may also prevent premature arousal from hibernation under these conditions.

In conclusion, we showed that elevated urea concentration can depress the in vitro metabolism of several organs from hibernating *R. sylvatica*, but the effect is not consistent among organs,

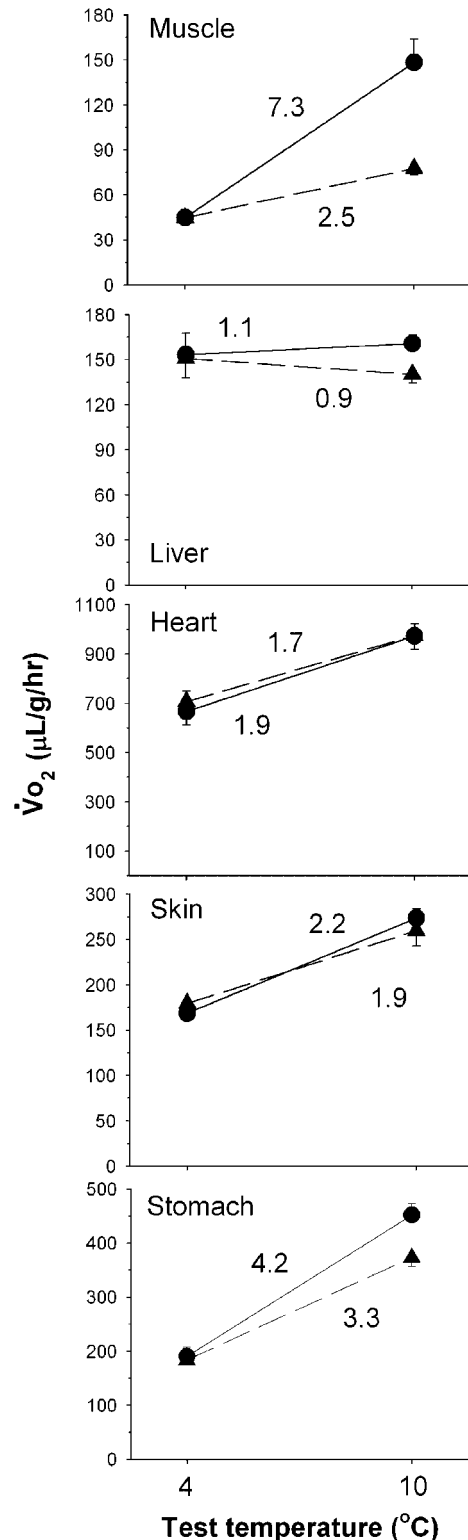


Fig. 2. Rates of oxygen consumption ($\dot{V}O_2$) of control (circles) and urea-treated (triangles) organs from winter *Rana sylvatica* tested at 4 or 10°C. Data are presented as mean \pm SE. Sample sizes are as reported in Figure 1. Temperature coefficients (Q_{10}) for each organ in each treatment are shown next to their respective lines.

seasons, or temperatures. Metabolic sensitivity to urea may be mediated by structural properties intrinsic to a given enzyme as well as those imparted by the intracellular environment. Future studies addressing the mechanism underlying the metabolic effect of urea are needed. Moreover, given that urea accumulation during extended dormancy occurs in several species of amphibians (Pinder et al., '92; Costanzo and Lee, 2005), gastropods (Rees and Hand, '93; Arad, 2001), reptiles (Peterson and Stone, 2000; Costanzo et al., 2006), and lungfishes (Chew et al., 2004), it is conceivable that other organisms also exhibit a metabolic response to hyperuremia.

ACKNOWLEDGMENT

We thank P. Baker and M. Elnitsky for aid in the frog collections and R. Schaefer for assisting with the statistical analyses. Experimental procedures were approved by the Institutional Animal Care and Use Committee of Miami University (protocol 629). This work was supported by the National Science Foundation Grant IOB 0416750 to J. Costanzo.

LITERATURE CITED

- Arad Z. 2001. Desiccation and rehydration in land snails—a test for distinct set points in *Theba pisana*. *Isr J Zool* 47:41–53.
- Baskakov I, Wang A, Bolen DW. 1998. Trimethylamine-*N*-oxide counteracts urea effects on rabbit muscle lactate dehydrogenase function: a test of the counteraction hypothesis. *Biophys J* 74:2666–2673.
- Chew SF, Chan NKY, Loong AM, Hiong KC, Tam WL, Ip YK. 2004. Nitrogen metabolism in the African lungfish (*Protopterus dolloi*) aestivating in a mucus cocoon on land. *J Exp Biol* 207:777–786.
- Costanzo JP, Lee RE. 2005. Cryoprotection by urea in a terrestrially hibernating frog. *J Exp Biol* 208:4079–4089.
- Costanzo JP, Irwin JT, Lee RE. 1997. Freezing impairment of male reproductive behaviors of the freeze-tolerant wood frog, *Rana sylvatica*. *Physiol Zool* 70:158–166.
- Costanzo JP, Baker PJ, Lee RE. 2006. Physiological responses to freezing in hatchlings of freeze-tolerant and -intolerant turtles. *J Comp Physiol B* 176:697–707.
- Cowan KJ, Storey KB. 2002. Urea and KCl have different effects on enzyme activities in liver and muscle of estivating versus nonestivating species. *Biochem Cell Biol* 80:745–755.
- Flanigan JE, Withers PC, Guppy M. 1991. *In vitro* metabolic depression of tissues from the aestivating frog *Neobatrachus pelobatoides*. *J Exp Biol* 161:273–283.
- Fuery CJ, Attwood PV, Withers PC, Yancey PH, Baldwin J, Guppy M. 1997. Effects of urea on M₄-lactate dehydrogenase from elasmobranchs and urea-accumulating Australian desert frogs. *Comp Biochem Physiol B* 117:143–150.
- Guppy M, Withers PC. 1999. Metabolic depression in animals: physiological perspectives and biochemical generalizations. *Biol Rev* 74:1–40.
- Hand SC, Somero GN. 1982. Urea and methylamine effects on rabbit muscle phosphofructokinase. *J Biol Chem* 257:734–741.
- Hochachka PW, Somero GN. 2002. Biochemical adaptation. New York: Oxford University Press. 466p.
- Kaji DM, Lim J, Shilkoff W, Zaidi W. 1998. Urea inhibits the Na-K pump in human erythrocytes. *J Membr Biol* 165:125–131.
- Lewis JM, Driedzic WR. 2007. Tissue-specific changes in protein synthesis associated with seasonal metabolic depression and recovery in the north temperate labrid, *Tautoglabrus adspersus*. *Am J Physiol* 293:R474–R481.
- Mashino T, Fridovich I. 1987. Effects of urea and trimethylamine-*N*-oxide on enzyme activity and stability. *Arch Biochem Biophys* 258:356–360.
- Muir TJ, Costanzo JP, Lee RE. 2007. Osmotic and metabolic responses to dehydration and urea-loading in a dormant, terrestrially-hibernating frog. *J Comp Physiol B* 177:917–926.
- Peterson CC, Stone PA. 2000. Physiological capacity for estivation of the Sonoran mud turtle, *Kinosternon sonoriense*. *Copeia* 2000:684–700.
- Pinder AW, Storey KB, Ultsch GR. 1992. Estivation and hibernation. In: Feder ME, Burggren WW, editors. Environmental physiology of the amphibians. Chicago, IL: The University of Chicago Press. p 250–274.
- Rees BB, Hand SC. 1993. Biochemical correlates of estivation tolerance in the mountainsnail *Oreohelix* (Pulmonata: Oreohelicidae). *Biol Bull* 184:230–242.
- Spaans EJA, Baker JM. 1996. The soil freezing characteristic: its measurement and similarity to the soil moisture characteristic. *Soil Sci Soc Am J* 60:13–19.
- Storey KB. 1990. Life in a frozen state: adaptive strategies for natural freeze tolerance in amphibians and reptiles. *Am J Physiol* 258:R559–R568.
- Yancey PH, Clark ME, Hand SC, Bowlus RD, Somero GN. 1982. Living with water stress: evolution of osmolyte systems. *Science* 217:1214–1222.