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Urea is not a universal cryoprotectant among hibernating anurans: Evidence from the freeze-tolerant boreal chorus frog (*Pseudacris maculata*)

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ABSTRACT

Freeze-tolerant organisms accumulate a diversity of low molecular weight compounds to combat negative effects of ice formation. Previous studies of anuran freeze tolerance have implicated urea as a cryoprotectant in the wood frog (*Lithobates sylvatica*). However, a cryoprotective role for urea has been identified only for wood frogs, though urea accumulation is an evolutionarily conserved mechanism for coping with osmotic stress in amphibians. To identify whether multiple solutes are involved in freezing tolerance in the boreal chorus frog (*Pseudacris maculata*), we examined seasonal and freezing-induced variation in several potential cryoprotectants. We further tested for a cryoprotective role for urea by comparing survival and recovery from freezing in control and urea-loaded chorus frogs. Tissue levels of glucose, urea, and glycerol did not vary significantly among seasons for heart, liver, or leg muscle. Furthermore, no changes in urea or glycerol levels were detected with exposure to freezing temperatures in these tissues. Urea-loading increased tissue urea concentrations, but failed to enhance freezing survival or facilitate recovery from freezing in chorus frogs in this study, suggesting little role for urea as a natural cryoprotectant in this species. These data suggest that urea may not universally serve as a primary cryoprotectant among freeze-tolerant, terrestrially hibernating anurans.

1. Introduction

The accumulation of osmolytes to prevent the harmful effects of both intracellular and extracellular ice formation is a common strategy among diverse groups of freeze-tolerant organisms. Further examination of the seemingly unrelated phenomenon of desiccation tolerance has uncovered a holistic view of evolutionarily conserved, physiological responses to abiotic stress within anurans (Churchill and Storey, 1993; Storey et al., 1997; Edwards et al., 2004). In particular, recent studies of anuran freeze-tolerance have implicated urea, a formerly overlooked osmolyte, as a cryoprotectant within the wood frog, *Lithobates sylvatica*, further supporting hypotheses of evolutionary conservation of physiological responses promoting desiccation and freezing tolerance in anurans (Costanzo and Lee, 2005, 2008). Indeed, urea conferred similar cryoprotection to red blood cells of the wood frog subjected to subzero temperatures when compared to glucose and glycerol (Costanzo and Lee, 2005), and blood levels of proteins indicative of cryoinjury were reduced in urea-loaded wood frogs versus saline-loaded controls (Schiller et al., 2008). However, a cryoprotective role for urea has been identified only for wood frogs (Costanzo and Lee, 2005, 2008), although urea accumulation appears to be a conserved mechanism for survival on land in diverse terrestrial organisms (Atkinson, 1992; Wright, 1995) and for coping with osmotic stress in several species of amphibians (Withers and Guppy, 1996; Cowan and Storey, 2002). Furthermore, glycerol, another known cryoprotectant, may be utilized by other species of freeze-tolerant frogs (Storey, 1997; Layne, 1999). However, further review of a widespread role for glycerol in anuran freezing tolerance is warranted due to low sample sizes in studies failing to detect glycerol as a cryoprotectant (Storey and Storey, 1986a), differences in acclimation protocols that may influence the degree of anticipatory glycerol production (Irwin et al., 2003), and a general recognition that several studied species of freeze-tolerant anurans utilize multiple cryoprotectants to survive freezing (Irwin et al., 2003; Costanzo and Lee, 2005).

To date, glycerol has been documented as a cryoprotectant only in the gray treefrogs, *Hyla versicolor* and *Hyla chrysoscelis* (Storey and Storey, 1985; Layne and Jones, 2001), and glycerol may increase seasonally in an anticipatory manner in these species prior to actual freezing (Layne and Jones, 2001; Irwin et al., 2003; Zimmerman et al., 2007) as well as upon freezing (Layne, 1999; Layne and Stapleton, 2009). Glycerol may accumulate during freezing in other freeze-tolerant frogs, although to lesser levels than in the gray treefrogs (Croes and Thomas, 2000), but the possible anticipatory accumulation of glycerol as a cryoprotectant is unstudied in other freeze-tolerant frogs.

The boreal chorus frog (*Pseudacris maculata*) is a small Hylid frog inhabiting the north-central, central, and east-central United States

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and south-central Canada (Kiesow, 2006; Lemmon et al., 2007) and is a species known to tolerate freezing of its body fluids using glucose as a cryoprotectant (MacArthur and Dandy, 1982; Storey and Storey, 1986b; Swanson et al., 1996; Jenkins and Swanson, 2005). Tissue glucose levels increase dramatically upon initiation of ice nucleation in this species and this hyperglycemic condition is facilitated by seasonal increases in hepatic glycogen phosphorylase activity (Swanson et al., 1996; Edwards et al., 2000; Jenkins and Swanson, 2005). Though boreal chorus frogs possess the physiological mechanisms to promote freezing survival, the capacity to survive freezing varies among years (Jenkins and Swanson, 2005). Precise mechanistic explanations for these differences are not understood and could involve cryoprotectants other than glucose. In addition, high plasma osmolality recorded in other species of frogs, including *P. maculata* (MacArthur and Dandy, 1982), may implicate urea and/or glycerol as cryoprotectants in addition to glucose (Costanzo and Lee, 2008).

We hypothesized that boreal chorus frogs may accumulate urea and glycerol in an anticipatory manner (i.e., seasonally) in addition to glucose accumulation upon freezing. In this study, we assayed boreal chorus frog liver, leg muscle, and heart tissue for seasonal differences in glucose, glycogen, urea, and glycerol concentrations. In addition, we experimentally tested whether urea-loading affected freezing survival or recovery, as well as tissue levels of glucose, glycogen, urea, and glycerol, following a 24-h freezing bout.

2. Materials and methods

2.1. Animals

We collected *P. maculata* for this study near Vermillion, Clay Co., South Dakota (42° 47′ N latitude), in March, July, September, and October of 2009 and 2010. For assays of seasonal osmolyte levels ($n=26$), we double-pithed frogs immediately in the field, and wrapped them in aluminum foil and stored carcasses on dry ice. However, fall-collected, winter-acclimated controls were brought back to the lab and maintained under simulated hibernation conditions until euthanasia in January. We then brought the carcasses back to the lab and stored them at −70 °C until tissue dissection and assays (less than 2 weeks). We recorded body mass (M_b) and snout-vent length (SVL) prior to dissection of various tissues. During the fall 2009 collecting effort, we temporarily kept frogs for the freezing survival ($n=22$) and cryoprotectant assays ($n=26$) communally in clear, plastic, paper towel-lined containers prior to our return to the lab for acclimation. Frogs captured in 2010 ($n=50$) were housed independently in both the field and the lab.

A housing protocol adapted from Swanson et al. (1996) was used following collection from the field. Briefly, frogs were housed in shallow terraria lined with moist paper towels and strewn with leaf litter and sphagnum moss. Frogs were kept at 25 °C with natural photoperiod (12L: 12D) until November and provided copious amounts of wingless fruit flies (*Drosophila melanogaster*) three times per week. Terraria were then transferred to a 10 °C environmental chamber with no light (0L: 24 D) for two weeks to allow frogs to digest food matter in the gut. Finally, the temperature was decreased to 2 °C and held constant until freezing experiments were conducted later in the winter. Routine terraria maintenance occurred as necessary throughout the acclimation process.

2.2. Urea-loading and freezing survival

Urea-loading followed a modified protocol of Costanzo and Lee (2008). Briefly, frogs were randomly selected, weighed to the nearest 0.01 g and SVL measured to the nearest 0.01 mm, injected with either cold, phosphate buffered saline (PBS, in grams per liter: 6.10 NaCl, 0.15 KCl, 0.88 Na_2HPO_4, 0.15 KH_2PO_4; pH 7.4 at 23 °C) or PBS containing 1.5 or 3.5 molal urea (hereafter, *m*). Using a 26 gauge needle, the dorsal lymph pad of each frog was injected with PBS or urea equal to 3.3% of standard body mass. Frogs were returned to their darkened terraria at 2 °C for 3–5 h before being euthanized for tissue sampling or commencing the experimental freezing bout. Initial injections of frogs with 1.5 *m* urea did not increase tissue urea to levels observed in *L. sylvatica* (Costanzo and Lee, 2008; Schiller et al., 2008), therefore, we conducted a second set of urea-loading experiments using 3.5 *m* urea to attempt to achieve similar urea levels to those observed in studies of *L. sylvatica*.

2.2.1. Freezing protocol

The experimental freezing protocol was adapted from Swanson et al. (1996) and is a freezing exposure routinely tolerated by chorus frogs in good condition. Freezing experiments took place from January through March of 2010 and 2011. Prior to freezing, we took M_b and SVL measurements to the nearest 0.01 g and 0.01 mm, respectively. Frogs were then placed in foam-lined, cylindrical plastic containers with a thermocouple (Yellow Springs Instruments 400 series temperature probe, Model 427, YSI telethermometer, Model 43 TB, Yellow Springs, OH) affixed to the foam substrate. A foam plug was then used to coerce frogs into position on top of the thermocouple, and the limited area ensured that the frogs' abdomens remained on top of the thermocouple probe. These smaller chambers were then inserted into foam-lined glass chambers and immersed in a bath of circulating water/ethylene glycol (Forma Scientific Inc., Model 2095, Marietta, OH) at −1 °C. Body temperature was recorded every 15 s for the duration of the freezing episode on Datacan 5.0 Data Acquisition Software (Sable Systems, Henderson, NV). Once temperature equilibrium was reached, the temperature was reduced to −1.5 °C and allowed to equilibrate again. Ice nucleation was initiated by placing an ice crystal against the hind limbs. Following the freezing exotherm, temperature within the bath was dropped at approximately 1 °C h^{-1}, until the bath reached −2.5 °C where it remained for the duration of the 24-h freezing episode. After freezing exposure, frogs were double-pithed and assayed for tissue osmolytes or returned to the environmental chamber at approximately 2 °C in a moist, paper towel-lined container to thaw. Frog survival was assessed once a day for 7 days after the 24-h experimental freezing bout. Only frogs that demonstrated a positive response for all four survival criteria (maintenance of normal posture, righting response, hind limb retraction, and capacity to locomote) were declared survivors. We recorded the time following the freezing bout that each of these criteria were achieved for those frogs surviving the freezing exposure.

2.3. Assay procedures and conditions

Tissues designated for osmolyte assays were quickly dissected on ice, weighed to the nearest 0.01 g, flash-frozen in liquid nitrogen, and wrapped in aluminum foil for storage at −70 °C until analysis. All tissues were prepared using the protocol of Storey (1984) adapted by Swanson et al. (1996). Briefly, we homogenized tissue (1:10 w/v) in ice-cold 0.6 N perchloric acid using a tissue homogenizer (Tekmar, Model SDT-1810 Tissuemizer, Cincinnati, OH) and divided the sample into two aliquots. One aliquot was used for determination of urea, glycerol, and glucose concentrations and was centrifuged at 20,900*g* for 6 min at 4 °C. Homogenates were neutralized by the addition of a few crystals (<1 mg) of solid $KHCO_3$ and centrifuged again at the previous settings for 6 min. The other aliquot was used for glycogen determination using the method of Keppler and Decker (1974) as adapted by Swanson et al. (1996). For glycogen assays, we added 100 μl of the aliquot to a mixture of 50 μl of 1 M $KHCO_3$ and 1 ml of amyloglucosidase solution (1 mg/ml enzyme in 0.2 M sodium-acetate buffer; Catalog No. 10115, Sigma). Glycogen digestion then proceeded for 2 h at 40 °C with shaking, followed by 15 min centrifugation at 20,900 *g* at 4 °C. The homogenate was neutralized with solid $KHCO_3$ crystals and centrifuged again at the previous settings for 6 min. We analyzed osmolyte concentrations of homogenates spectrophotometrically (Beckman DU-7400, Fullerton, CA) using procedures designated by each assay kit (glucose and glycogen: Autokit Glucose, Wako Diagnostics, Richmond, VA; urea: B7551, Pointe Scientific,

Inc., Canton, MI; glycerol: F6428 and G7793, Sigma). We performed assays in duplicate and means were used in the final calculation of osmolyte concentrations. The glycogen concentration (expressed as μmol glucosyl units g FW^{-1}) was calculated after subtraction of free glucose.

2.3.1. Statistics

All data are presented as means ± SE unless otherwise stated. Glucose, glycogen, urea, and glycerol data are presented on a wet mass basis to facilitate comparison with previous studies. Freezing survival within and among years for differing urea concentrations (1.5 or 3.5 *m*) and for saline-loaded controls were compared using a Fisher Exact Test. Glucose, glycogen, urea, and glycerol concentrations among seasons and between control and 24-h frozen groups were compared with a one-way analysis of variance (ANOVA) or a Kruskal–Wallis test, if parametric assumptions were not met. Equality of variance was assessed using Levene's test and normality was tested using a Shapiro–Wilks test. Non-normal, homoscedastic data were log transformed. When significant differences were detected, means were expressed as back transformations of the log-transformed means. Otherwise, upon detection of significant differences, either a Tukey's Studentized Range (HSD) test or a distribution-free multiple comparison for the Kruskal–Wallis rank sums by Dunn (1964) were employed to identify differences among treatments. Student's *t*-tests were employed to test significance of urea levels in individuals combined from non-frozen and frozen treatments versus frozen and non-frozen controls. We accepted statistical significance at $P<0.05$ for all tests.

3. Results

3.1. Seasonal metabolite assays

Summer frogs were smaller than those at other seasons, significantly so for fall and spring, both in terms of body mass ($F_{3,22}=4.99$, $P=0.009$) and SVL ($F_{3,22}=5.13$, $P=0.008$), but body size at other seasons did not differ significantly (Table 1).

Metabolite values for heart tissue were not significantly different among seasons ($P>0.2$; Table 2), and any apparent differences (e.g. glycogen concentrations) were nullified by large amounts of variation in the data (Table 2). This high variation was presumably due to the very small mass of chorus frog hearts (on the order of a few milligrams) and subsequent dilution effects for homogenization of this tissue compared to liver and leg muscle.

Differences in liver glucose concentrations among seasons approached significance ($F_{3,22}=2.59$, $P=0.079$) and were highest in spring (Table 2). Liver glycogen values were significantly elevated in fall, winter, and spring compared to summer glycogen levels ($F_{3,22}=11.1$, $P<0.01$ in all cases). Indeed, liver glycogen levels in fall-collected frogs were 7.1 times those of summer individuals (Table 2). Urea and glycerol concentrations in liver did not vary significantly among seasons.

Seasonal changes in metabolite concentrations in leg muscle mirrored changes in liver metabolite concentrations, with no significant differences in glucose, urea, or glycerol apparent among seasons (Table 2). Furthermore, glycogen concentrations in leg muscle were significantly higher in fall and winter than in spring and summer (Table 2, $F_{3,22}=7.35$, $P<0.05$), suggesting a pre-hibernation accumulation of glycogen in the leg muscles, in addition to the liver, for chorus frogs in this study.

3.2. Freezing effects on tissue metabolites

Boreal chorus frogs exposed to a 24-h experimental freeze at −2.5 °C had significantly elevated levels of glucose when compared to non-frozen controls in all tissues (Table 3). However, as for the seasonal assessment of cryoprotectant levels, heart tissue displayed significant differences in variation among groups, again probably due to the dilutions necessary for homogenization of their very small hearts. Although a *t*-test of pooled frozen and non-frozen heart glucose levels approached significance (Welch's 2 sample *t*-test, $t_{16,\ 0.05}=2.0251$, $P=0.06$), no definitive statements can be made about glucose levels in heart tissue between the frozen and non-frozen controls. Nevertheless, there was an 88-fold increase in glucose detected in heart tissue of frozen individuals loaded with 3.5 *m* urea compared to their non-frozen controls, and this increase was significant ($P=0.01$, Table 3). Heart glycogen concentration varied within and among treatment groups and did not show any consistent trends with freezing.

In liver and leg muscle, pooled glucose concentrations revealed 11.6-fold and 3.8-fold increases in glucose levels between non-frozen and frozen groups (21.1 and 12.2 μmol glucose g FW^{-1} for non-frozen liver and leg muscle, and 245 and 46.7 μmol glucose g FW^{-1} for frozen liver and leg muscle, respectively).

As observed for heart tissue, liver and leg muscle glycogen showed no consistent trends between frozen and non-frozen treatments within any groups. In all three tissues, no significant variation in urea or glycerol concentrations was detected between frozen and non-frozen treatments in any group.

Because tissue urea levels did not differ significantly between frozen and non-frozen frogs for either saline-injected controls or for 1.5 and 3.5 *m* urea-loaded groups, we pooled values for further statistical analysis. Urea-loading in liver and leg muscle significantly increased tissue urea levels compared to controls (Student's *t*-test, $t_{21,\ 0.05}=-4.19$, $P<0.001$, $t_{21,\ 0.05}=-3.81$, $P=0.001$, for 1.5 and 3.5 *m* urea-loaded groups, respectively) (Fig. 1). Urea levels were four-fold and three-fold higher for urea-loaded groups in liver and leg muscle tissue, respectively. Heart tissue experienced no significant increase in urea levels with urea-loading over controls (Fig. 1A), though values tended to be higher on average.

To determine if dehydrating conditions altered tissue urea concentrations in chorus frogs, we performed a pilot study exposing frogs ($n=3$) to dry substrate conditions (vented, plastic terraria lined with dry paper towels) for two weeks at 2 °C. No significant differences in

Table 1
Mean ± SD for body mass (M_b) (g), SVL (mm), and organ tissues (heart, liver, leg muscle) (g FW^{-1}) for fall, winter, spring, and summer-collected frogs and frozen versus non-frozen frogs.

	Body mass (M_b)	SVL	Heart	Liver	Leg muscle
Fall	$1.12\pm0.32^{\alpha}$ (7)	$23.4\pm1.75^{\beta}$	0.0084 ± 0.00050 (4)	0.12 ± 0.033	0.110 ± 0.021
Winter	0.93 ± 0.31 (6)	22.9 ± 2.89	0.0047 ± 0.0016 (5)	0.087 ± 0.033	0.0940 ± 0.042
Spring	$1.05\pm0.10^{\alpha}$ (7)	$23.8\pm1.1^{\beta}$	0.0058 ± 0.00073	0.073 ± 0.014	0.110 ± 0.021
Summer	$0.65\pm0.13^{\alpha}$ (6)	$20.0\pm1.5^{\beta}$	0.0062 ± 0.0022	0.050 ± 0.024	0.150 ± 0.039
Frozen	0.85 ± 0.26 (14)	23.0 ± 3.3	0.0055 ± 0.0030 (10)	0.065 ± 0.026	0.08 ± 0.030
Non-frozen	1.1 ± 0.40 (12)	24.8 ± 2.1	0.0056 ± 0.0014 (9)	0.093 ± 0.030	0.12 ± 0.020

[a] Sample sizes indicated in parentheses under M_b are for whole row unless other values in parentheses are given.
[b] α indicates M_b was significantly lower for summer-collected frogs compared to fall and spring-collected frogs.
[c] β indicates SVL was significantly lower for summer-collected frogs compared to fall and spring-collected frogs.

Table 2
Seasonal changes in mean ± SE concentrations of four metabolites in heart, liver, and leg muscle expressed in μmol g FW^{-1} or μmol glucosyl units g FW^{-1} for glycogen concentrations.

Tissue	Metabolite	Summer	Fall	Winter	Spring
Heart	Glucose	3.53 ± 1.6 (6)	8.23 ± 1.6 (4)	12.8 ± 6.8 (5)	3.73 ± 1.9 (7)
	Glycogen	265 ± 178 (6)	3.59 ± 3.6 (4)	180 ± 119 (5)	55.0 ± 34 (7)
	Urea	2.07 ± 1.3 (6)	2.56 ± 2.3 (4)	0.376 ± 0.24 (5)	1.30 ± 0.49 (7)
	Glycerol	1.46 ± 0.57 (6)	11.8 ± 7.8 (4)	0.846 ± 0.50 (5)	0.561 ± 0.16 (7)
Liver	Glucose	17.1 ± 3.2 (6)	15.7 ± 0.77 (7)	20.2 ± 3.5 (6)	25.6 ± 3.2 (7)
	Glycogen	129 ± 74.6α (6)	920 ± 54 (7)	815 ± 78 (6)	785 ± 163 (7)
	Urea	2.79 ± 0.51 (6)	3.24 ± 1.2 (7)	1.80 ± 0.75 (6)	1.03 ± 0.30 (7)
	Glycerol	1.08 ± 0.66 (6)	0.976 ± 0.18 (7)	0.698 ± 0.27 (6)	0.847 ± 0.52 (7)
Leg muscle	Glucose	2.68 ± 0.57 (6)	3.20 ± 0.63 (7)	3.30 ± 1.1 (6)	4.22 ± 0.88 (7)
	Glycogen	12.7 ± 8.3 (6)	76.8 ± 15β (7)	76.5 ± 22β (6)	8.43 ± 6.0 (7)
	Urea	3.40 ± 0.67 (6)	3.89 ± 1.4 (7)	4.67 ± 1.7 (6)	2.27 ± 0.34 (7)
	Glycerol	0.202 ± 0.09 (6)	0.632 ± 0.15 (7)	0.543 ± 0.31 (6)	0.606 ± 0.29 (7)

[a] Sample sizes are included in parentheses.
[b] α indicates summer average liver glycogen concentration significantly lower than fall, winter, and spring ($P<0.005$).
[c] β identifies fall and winter average muscle glycogen concentrations are significantly elevated compared to summer and spring ($P<0.01$).

urea levels were detected between saline-injected controls and dehydrated frogs for any tissue.

3.3. Urea-loading, freezing survival and recovery

Boreal chorus frogs injected with 1.5 or 3.5 *m* urea showed no significant differences in urea levels for any tissue between frozen or non-frozen control treatments (Table 3). Therefore, frogs injected with either 1.5 *m* urea or 3.5 *m* urea were combined and assessed for statistically significant differences in survival compared with PBS-injected controls. Only 3 out of 11 frogs in both control and urea-loaded groups (27 %) survived, and no significant difference was detected between treatments ($P=1$, two-sided Fisher's exact test). Thus, a total of 6 out of 22 (27 %) boreal chorus frogs survived a 24-h experimental freezing bout at −2.5 °C.

Thawing boreal chorus frogs showed signs of freezing related trauma, including capillary damage in hind limbs, lethargy, edema, swelling, and reduced capacity for locomotion and complex neuro-muscular behaviors. Therefore, the return of survival criteria behaviors with thawing followed an all-or-none pattern for both control and urea-loaded groups. After approximately 24 h of thawing at 2–3 °C, frogs that survived the 7-day assessment were already showing some signs of revival (i.e. hind limb retraction, normal posture, righting reflex, and locomotion). However, frogs not showing signs of recovery within the first 24 h of thawing sometimes displayed minimal improvement and subsequent performance of some behavioral criteria at later checks ($n=6$). Nevertheless, these frogs were not deemed survivors at the end of the 7-day recovery assessment because they did not show positive responses for all four survival criteria and survival in the wild would have likely been compromised.

Because body mass is positively correlated with freezing survival in chorus frogs (Jenkins and Swanson, 2005), we tested for body mass differences among treatment groups to examine its potential impact on freezing survival. PBS-injected controls and pooled urea-loaded frogs tested for freezing survival did not significantly differ in body mass (Welch's two-sample *t*-test, $t=1.26$, $df=19.1$, $P=0.22$), so mass effects cannot account for differences in survival between control and urea-loaded groups. Moreover, body mass of survivors (1.05 ± 0.18 g, $n=6$, reported as $M_b \pm SD$) and non-survivors (0.88 ± 0.19 g, $n=16$,

Table 3
Heart, liver, and leg muscle tissue metabolite levels of control and 24-h frozen frogs injected with saline, saline and 1.5 *m* urea, saline and 3.5 *m* urea, or acclimated to dry terrarium conditions.

Tissue	Osmolyte	PBS		1.5 m Urea		3.5 m Urea		Dry
		Non-Frozen	Frozen	Non-Frozen	Frozen	Non-Frozen	Frozen	Substrate
Heart	Glucose	16.8 ± 5.28† (3)	88.1 ± 28.2† (3)	51.6 ± 31.1† (3)	42.3 ± 35.8† (3)	3.41 ± 3.41†[b] (3)	202 ± 37.5†[b] (3)	0 (2)
	Glycogen	0 (3)	59.9 ± 33 (3)	126 ± 69 (3)	159 ± 87 (3)	179 ± 97 (3)	73.3 ± 58 (3)	0 (2)
	Urea	10.8 ± 2.0 (3)	2.90 ± 1.6 (3)	9.74 ± 6.3 (3)	25.5 ± 15 (3)	6.39 ± 3.7 (3)	9.51 ± 4.5 (3)	6.66 ± 6.66 (2)
	Glycerol	0.110 ± 0.11 (3)	0.360 ± 0.36 (3)	0.762 ± 0.52 (3)	1.91 ± 1.5 (3)	0.470 ± 0.26 (3)	0 (3)	0.258 ± 0.258 (2)
Liver	Glucose	10.0 ± 2.9†[c] (3)	239 ± 48†[c] (6)	41.3 ± 15†[d] (3)	164 ± 37†[d] (4)	12.0 ± 0.72†[e] (3)	332 ± 24†[e] (4)	27.1 ± 1.2 (3)
	Glycogen	725 ± 197 (3)	1207 ± 258 (6)	973 ± 155 (3)	850 ± 187 (4)	1514 ± 171 (3)	899 ± 200 (4)	1088 ± 22 (3)
	Urea	3.59 ± 0.82 (3)	3.76 ± 1.35 (6)	9.58 ± 0.84 (3)	22.0 ± 4.6 (4)	10.0 ± 3.4 (3)	15.9 ± 2.7 (4)	5.34 ± 1.4 (3)
	Glycerol	0.104 ± 0.063 (3)	0.475 ± 0.25 (6)	0.671 ± 0.21 (3)	0.413 ± 0.17 (4)	0.011 ± 0.011 (3)	0.634 ± 0.40 (4)	0.158 ± 0.046 (3)
Leg Muscle	Glucose	7.69 ± 2.5[f] (3)	44.2 ± 7.2[f] (6)	24.8 ± 8.4 (3)	37.7 ± 10 (4)	4.01 ± 1.5[g] (3)	58.1 ± 8.2[g] (4)	1.85 ± 0.44 (3)
	Glycogen	78.8 ± 41 (3)	58.7 ± 23 (6)	60.6 ± 36 (3)	98.9 ± 19 (4)	187 ± 8.8 (3)	38.6 ± 14 (4)	84.7 ± 29 (3)
	Urea	4.41 ± 1.3 (3)	4.72 ± 2.1 (6)	9.87 ± 3.1 (3)	15.2 ± 4.2 (4)	10.1 ± 2.7 (3)	16.7 ± 1.5 (4)	4.04 ± 0.30 (3)
	Glycerol	0.146 ± 0.13 (3)	0.319 ± 0.19 (6)	0.572 ± 0.33 (3)	0.990 ± 0.34 (4)	1.14 ± 1.1 (3)	0.242 ± 0.094 (4)	0.816 ± 0.58 (3)

[a] A † indicates means were log transformed prior to statistical analysis in one-way ANOVA. Means marked with the same letters indicate significant difference at $P<0.05$. Values in parentheses indicate sample sizes used in this study.
[b] Non-frozen individuals injected with 3.5 *m* urea had significantly lower levels of glucose in heart tissue ($F_{6,14}=5.13$, $P=0.006$, Tukey HSD $P=0.01$) than frozen individuals.
[c] Non-frozen, PBS-loaded individuals glucose levels were significantly lower ($F_{6,19}=36.2$, $P=2.06e-9$, Tukey HSD $P<0.001$) than frozen, PBS-loaded individuals.
[d] Frozen individuals, injected with 1.5 *m* urea had significantly elevated glucose ($F_{6,19}=36.2$, $P=2.06e-9$, Tukey HSD $P=0.007$) levels compared to non-frozen, urea-loaded controls.
[e] Frozen individuals, injected with 3.5 *m* urea had significantly higher levels of glucose ($F_{6,19}=36.2$, $P=2.06e-9$, Tukey HSD $P<0.001$).
[f] PBS-loaded, non-frozen individuals had significantly lower ($F_{6,19}=19.5$, $P=3.44e-7$, Tukey HSD $P=0.003$) glucose levels than PBS-loaded, frozen individuals.
[g] Frozen individuals, injected with 3.5 *m* urea in PBS had significantly higher ($F_{6,19}=19.5$, $P=3.44e-7$, Tukey HSD $P<0.001$) levels of glucose than non-frozen controls.

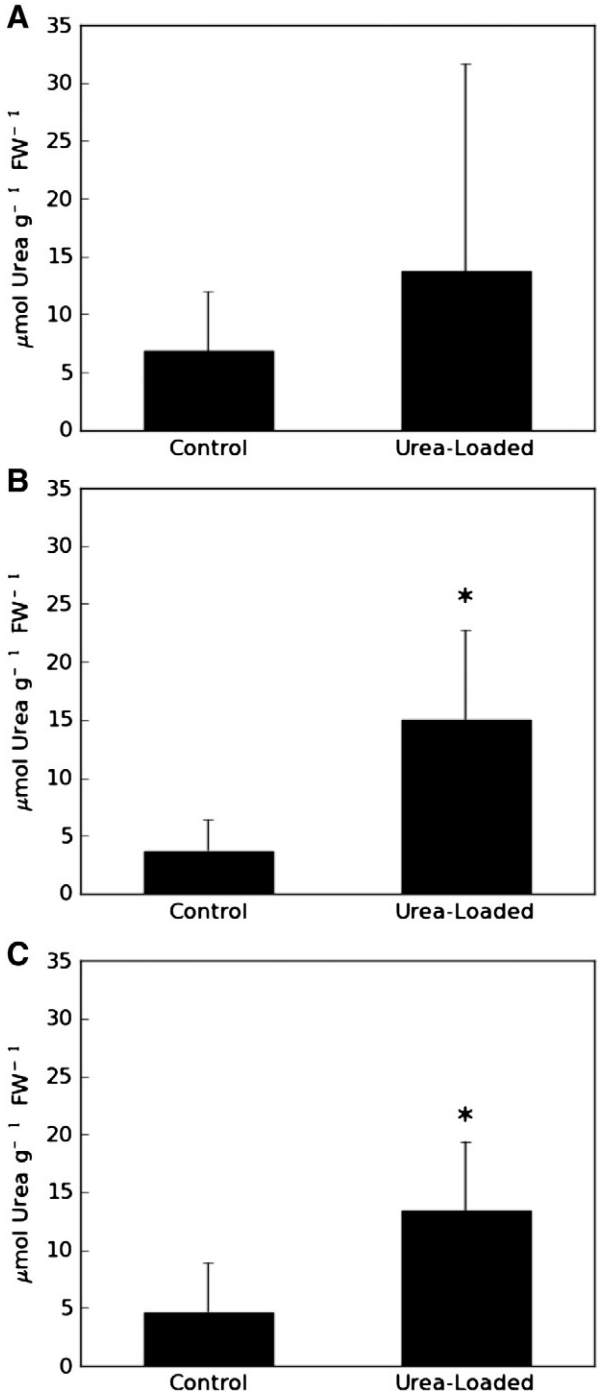

Fig. 1. Urea levels in saline-loaded and urea-loaded individuals in heart (A), liver (B), and leg muscle (C). Values are means ± SE. $n = 6$ saline-loaded heart, $n = 9$ saline-loaded for liver and muscle. $n = 13$ for urea-loaded heart, $n = 14$ for urea-loaded liver and muscle. Asterisk denotes means differed at $P \leq 0.05$.

reported as $M_b \pm SD$) of freezing bouts did not differ significantly, although survivors showed a non-significant tendency toward larger size (Welch's two-sample t-test, $t = -1.19$, df = 9.63, $P = 0.09$).

4. Discussion

4.1. Glucose and glycogen

Seasonal and freezing-induced patterns of tissue glucose and glycogen accumulation in chorus frogs in this study conformed to previous observations for this species (Swanson et al., 1996; Edwards et al., 2000; Jenkins and Swanson, 2005) and for other freeze-tolerant frogs (Storey and Storey, 1988; Storey, 1990). Glucose levels in liver and leg muscle increased 12-fold and 4-fold with freezing in liver and leg muscle, respectively, in this study, which is generally similar to freezing-induced elevations previously reported for this population, which range from 3.4-fold to 35-fold for liver and 5-fold to 22-fold for leg-muscle (Swanson et al., 1996; Edwards et al., 2000; Jenkins and Swanson, 2005). Both liver and leg muscle glycogen increased during fall and winter for frogs in this study (Table 2). Liver glycogen typically increases in fall and winter anurans, but the winter increase in leg muscle glycogen in this study is in contrast to a previous study of the boreal chorus frog, which reported no fall accumulation of glycogen in leg muscle (Dinsmore and Swanson, 2008). However, although average leg muscle glycogen concentrations in this study were greater than values reported by Dinsmore and Swanson (2008), they are in general accord with values of Edwards et al. (2000, 2004). Apparently, among-year variation in leg muscle glycogen storage occurs for chorus frogs, but whether this has implications for freezing tolerance or overwinter survival will require further study.

Seasonal and freezing-induced variation in glucose and glycogen levels in heart tissue have not been previously assessed for boreal chorus frogs and glucose levels tended to be higher following freezing, but both glucose and glycogen levels were highly variable, obscuring any significant seasonal or freezing induced trends (Tables 2 and 3). Some of this variation is probably due to methodological factors associated with very small heart masses and dilution errors associated with the homogenization protocol. Future research on heart tissue metabolites in this species should consider alternative homogenization protocols (e.g., pooling hearts from multiple individuals for homogenization).

4.2. Urea and glycerol

Urea and glycerol concentrations in all chorus frog tissues trended towards slightly higher values in the summer and fall, consistent with an autumn accumulation of urea in wood frogs (Costanzo and Lee, 2005, 2008) and glycerol in the gray treefrogs (Layne, 1999; Layne and Jones, 2001; Irwin et al., 2003), although they occurred to a much lesser degree in chorus frogs. Non-significant increases in urea and glycerol concentrations upon freezing suggest minimal freeze-induced alterations of these metabolites in the boreal chorus frog. Urea-loading treatments increased urea levels in boreal chorus frog tissues, and relative increases are consistent with the three to seven-fold higher urea levels in urea-loaded *L. sylvatica* compared to controls (Costanzo and Lee, 2005; Schiller et al., 2008).

The purpose of this study was to investigate whether the boreal chorus frog's repertoire of cryopreserving osmolytes consisted of molecules other than glucose, as observed in other freeze-tolerant frogs (Irwin and Lee, 2003; Costanzo and Lee, 2005). Costanzo and Lee (2005, 2008) hypothesized that generating an osmolyte (e.g., urea) to counteract perturbations from long-term, overwinter dehydration also benefits freezing tolerance for freeze-tolerant frogs. However, no long-term accumulation of urea was identified in any tissue for chorus frogs in this study (Table 2), and we observed no suggestive increases in urea levels in highly terrestrial fall frogs collected in late September and mid-October, just prior to hibernation, despite environmental conditions that may become drier at this time of year. Nevertheless, we cannot rule out urea accumulation under natural conditions in late winter, as we maintained winter-acclimated chorus frogs in this study under moist conditions in the lab. Although further study is required to confirm this result, our preliminary results for chorus frogs ($n = 3$) dehydrated on dry substrate for 14 days show no significant increases in tissue urea accumulation compared to PBS-injected, non-frozen controls (Table 2). The absence of microhabitat data providing soil moisture characteristics of natural overwinter hibernacula in this species, and for other species of freeze-tolerant anurans, limits our current understanding of the natural conditions facing freeze-tolerant frogs in winter

hibernacula, so documentation of hibernacula characteristics would be a profitable topic for future research.

We observed no substantive increases in tissue glycerol either seasonally or with freezing exposure (Tables 2 and 3). Glycerol does increase in an anticipatory manner before hibernation in the gray treefrogs *H. versicolor* and *H. chrysoscelis*, and likely represents a novel response to decreasing temperature or altered photoperiod and not to dry conditions (Layne and Jones, 2001; Zimmerman et al., 2007; Johnson et al., 2008). Our results confirm that glycerol is not a prominent contributor to freezing tolerance in the boreal chorus frog (Storey and Storey, 1986a). Boreal chorus frogs are Hylids, like the gray treefrogs, but unique evolutionary patterns of freezing tolerance have emerged within sympatric and related species occupying similar habitats (Voituron et al., 2009), and may help explain why uniform physiological responses to freezing do not occur in all species.

4.3. Urea-loading

Injecting chorus frogs with urea increased tissue urea levels but did not increase post-freeze survival or speed recovery from freezing (Fig. 1). In contrast, elevated tissue urea levels promoted post-freeze survival and freezing recovery in wood frogs (Costanzo and Lee, 2008). Relative increases in tissue urea levels in this study were comparable to those observed in urea-loaded and/or dehydrated wood frogs, but absolute urea levels were lower in chorus frogs, being similar to or only slightly higher than those for control, non-urea loaded, wood frogs (Costanzo and Lee, 2005; Muir et al., 2007; Costanzo and Lee, 2008; Schiller et al., 2008). This suggests that the lower urea levels for chorus frogs in this study were not sufficient to confer substantial cryoprotective benefits. Why the capacity for urea accumulation and its subsequent effects on freezing survival and recovery differs between wood frogs and chorus frogs is unknown, but could result from increased urinary excretion of urea in chorus frogs.

4.4. Factors impacting freeze survival

Chorus frogs in this study showed low levels of freezing survival relative to other studies of this population (Swanson et al., 1996; Jenkins and Swanson, 2005). Jenkins and Swanson (2005) found that body mass (M_b), hepatic glycogen levels and glucose mobilization during freezing exposure were positively correlated in chorus frogs. Moreover, among-year variation in freezing survival was related to variation in M_b, liver glycogen, and glucose mobilization, such that reduced survival occurred during winters in which frogs had low M_b, low liver glycogen, and low levels of glucose mobilization during freezing (Jenkins and Swanson, 2005). The low survival in this study was not associated with low liver glycogen levels or low glucose mobilization during freezing, as frogs in this study had relatively high levels of liver glycogen and glucose accumulation following freezing relative to other studies (Swanson et al., 1996; Edwards et al., 2000; Jenkins and Swanson, 2005; Dinsmore and Swanson, 2008). However, mean M_b for frogs measured for freezing survival in this study was less than 1 g for all treatment groups, which is lower than mean M_b for chorus frogs with higher freezing survival (1.1–1.7 g; Swanson et al., 1996; Edwards et al., 2000). Thus, the low M_b for frogs in this study may have contributed to the low freezing survival, although not through M_b-associated effects on liver glycogen stores or glucose accumulation.

The rate of freezing can negatively impact the amounts of glucose transported to tissues during ice formation and high cooling rates (e.g., >1 °C h^{-1}) during freezing may limit survival of freeze-tolerant frogs (Costanzo et al., 1992a). Small body size results in high surface area to volume ratios, which increases the rate of cooling during freezing for small frogs relative to large frogs. Average rates of cooling for 24-h frozen frogs in this study were 0.30 ± 0.03 °C h^{-1} ($n = 15$), double the rate of cooling in studies of the larger *L. sylvatica* (0.11–0.13 °C h^{-1}) (Layne, 1995) and *H. versicolor* (0.13 ± 0.01 °C h^{-1}) (Costanzo et al., 1992b), so the increased rate of cooling might help explain the low freezing survival (27%) experienced by boreal chorus frogs in this study. However, previous studies of this same population showed 50% (Jenkins and Swanson, 2005) and 100% (Swanson et al., 1996) survival with slightly higher rates (0.35 to 0.43 °C h^{-1}) of freezing. Therefore, freezing rate cannot completely explain the low freezing survival of boreal chorus frogs in this study.

An additional factor that might have impacted freezing survival in this study was high rates of infection by *Batrachochytrium dendrobatidis*, commonly referred to as chytrid, a fungal pathogen that has recently been implicated in the extirpation of several species of anurans worldwide (Berger et al., 1998; Collins and Storfer, 2003; Muths et al., 2003). As part of another study, we measured infection rates for frogs in this study and found that a high percentage of frogs were infected with chytrid (92%, $n = 50$; unpublished data).We do not know if chytrid infection played a role in the variable freezing survival observed among winters in this population of boreal chorus frogs, but viral infections may interact with other stressors to impact survival and physiological function in anurans (e.g., Kerby et al., 2011), so the potential exists for an effect of chytrid on freezing survival. Confirmation of such effects and elucidation of their mechanistic bases will require further investigation.

4.5. Is urea a 'natural' cryoprotectant?

A favored hypothesis concerning hibernacula conditions of *L. sylvatica* suggests that overwinter conditions for this species are relatively dry, which would favor urea accumulation in fall and winter (Costanzo and Lee, 2005, 2008; Costanzo et al., 2007; Muir et al., 2007). This hypothesis is partially supported by a mark-recapture study identifying the movement of *L. sylvatica* to upland habitat during winter (Regosin et al., 2003). In addition, Costanzo and Lee (2005) monitored seasonal urea levels in *L. sylvatica* kept in outdoor enclosures and found that drying conditions in fall and early winter resulted in elevated urea levels during this time, but urea levels later fell as environmental moisture increased. In contrast, Baldwin et al. (2006) suggest that upland habitats are moist havens during seasonally dry periods for *L. sylvatica* and other semi-terrestrial anurans. Because microclimate conditions within natural hibernacula are poorly known for terrestrially hibernating, freeze-tolerant frogs, it is unclear whether hibernaculum conditions are routinely dry enough to favor the production of urea in organs and tissues in response to dehydration.

The small body size and high surface area to volume ratios of chorus frogs should result in high rates of evaporative water loss (Duellman and Trueb, 1994; Seebacher and Alford, 2002), which could increase urea levels in response to dehydration relative to larger anuran species. However, elevated urea levels were not detected in fall-collected chorus frogs in this study. Behavioral selection of moist microhabitats by boreal chorus frogs could explain this lack of urea accumulation. Swanson et al. (1996) suggested that the use of moist hibernacula in boreal chorus frogs may promote inoculation by external ice, so that deep supercooling (which is lethal if freezing occurs) is avoided. Further research delineating microclimate characteristics of natural hibernacula for freeze-tolerant frogs, potentially incorporating recent advances in radio-telemetry, could identify precise overwintering microhabitats for some freeze-tolerant frogs (Baldwin et al., 2006; Johnson et al., 2008), and are needed to determine whether natural hibernacula characteristics generally promote urea accumulation in freeze-tolerant frogs.

Furthermore, it is uncertain whether urea contributes to freezing survival generally in freeze-tolerant frogs, as data in this study suggest little role for urea in cryoprotection in freeze-tolerant boreal chorus frogs. Further studies are necessary to examine the potential of a widespread cryoprotective role for urea in other freeze-tolerant frog species.

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References

Atkinson, D.E., 1992. Functional roles of urea synthesis in vertebrates. Physiol. Zool. 65, 243–267.
Baldwin, R.F., Calhoun, A.J.K., deMaynadier, P.G., 2006. Conservation planning for amphibian species with complex habitat requirements: a case study using movements and habitat selection of the wood frog *Rana sylvatica*. J. Herpetol. 40, 442–453.
Berger, L., Speare, R., Daszak, P., Green, D.E., Cunningham, A.A., Goggin, C.L., Slocombe, R., Ragan, M.A., Hyatt, A.D., McDonald, K.R., Hines, H.B., Lips, K.R., Marantelli, G., Parkes, H., 1998. Chytridiomycosis causes amphibian mortality associated with population declines in the rain forests of Australia and Central America. Proc. Natl. Acad. Sci. U. S. A. 95, 9031–9036.
Churchill, T.A., Storey, K.B., 1993. Dehydration tolerance in wood frogs: a new perspective on development of amphibian freeze tolerance. Am. J. Physiol. 265, R1324–R1332.
Collins, J.P., Storfer, A., 2003. Global amphibian declines: sorting the hypotheses. Divers. Distrib. 9, 89–98.
Costanzo, J.P., Lee, R.E., 2005. Cryoprotection by urea in a terrestrially hibernating frog. J. Exp. Biol. 208, 4079–4089.
Costanzo, J.P., Lee, R.E., 2008. Urea loading enhances freezing survival and postfreeze recovery in a terrestrially hibernating frog. J. Exp. Biol. 211, 2969–2975.
Costanzo, J.P., Lee, R.E., Wright, M.F., 1992a. Cooling rate influences cryoprotectant distribution and organ dehydration in freezing wood frogs. J. Exp. Zool. 261, 373–378.
Costanzo, J.P., Wright, M.F., Lee, R.E., 1992b. Freeze tolerance as an adaptation in Cope's Grey Treefrog (*Hyla chrysoscelis*). Copeia 1992, 565–569.
Costanzo, J.P., Marjanovic, M., Fincel, E.A., Lee, R.E., 2007. Urea loading enhances postfreeze performance of frog skeletal muscle. J. Comp. Physiol.B 178, 413–420.
Cowan, K.J., Storey, K.B., 2002. Urea and KCl have differential effects on enzyme activities in liver and muscles of estivating versus nonestivating species. Biochem. Cell Biol. 80, 745–755.
Croes, S.A., Thomas, R.E., 2000. Freeze tolerance and cryoprotectant synthesis of the Pacific Tree Frog *Hyla regilla*. Copeia 2000, 863–868.
Dinsmore, S.C., Swanson, D.L., 2008. Temporal patterns of tissue glycogen, glucose, and glycogen phosphorylase activity prior to hibernation in freeze-tolerant chorus frogs, *Pseudacris triseriata*. Can. J. Zool. 86, 1095–1100.
Duellman, W.E., Trueb, L., 1994. Biology of the Amphibians. John Hopkins University Press, Baltimore.
Dunn, O.J., 1964. Multiple comparisons using rank sums. Technometrics 6, 241–252.
Edwards, J.R., Koster, K.L., Swanson, D.L., 2000. Time course for cryoprotectant synthesis in the freeze-tolerant chorus frog, *Pseudacris triseriata*. Comp. Biochem. Physiol.A 125, 367–375.
Edwards, J.R., Jenkins, J.L., Swanson, D.L., 2004. Seasonal effects of dehydration on glucose mobilization in freeze-tolerant chorus frogs (*Pseudacris triseriata*) and freeze-intolerant toads (*Bufo woodhousii* and *B. cognatus*). J. Exp. Zool. 301A, 521–531.
Irwin, J.T., Lee, R.E., 2003. Geographic variation in energy storage and physiological responses to freezing in the gray treefrogs *Hyla versicolor* and *H. chrysoscelis*. J. Exp. Biol. 206, 2859–2867.
Irwin, J.T., Costanzo, J.P., Lee, R.E., 2003. Postfreeze reduction of locomotor endurance in the freeze-tolerant Wood Frog, *Rana sylvatica*. Physiol. Biochem. Zool. 76, 331–338.
Jenkins, J.L., Swanson, D.L., 2005. Liver glycogen, glucose mobilization and freezing survival in chorus frogs, *Pseudacris triseriata*. J. Therm. Biol. 30, 485–494.
Johnson, J.R., Mahan, R.D., Semlitsch, R.D., 2008. Seasonal terrestrial microhabitat use by gray treefrogs (*Hyla versicolor*) in Missouri oak-hickory forests. Herpetologica 64, 259–269.
Keppler, D., Decker, K., 1974. Glycogen: determination with amyloglucosidase. In: Bergmeyer, H.-U. (Ed.), Methods of Enzymatic Analysis. Verlag Chemie, Weinheim, pp. 1127–1131.
Kerby, J., Hart, A., Storfer, A., 2011. Combined effects of virus, pesticide, and predator cue on the larval tiger salamander (*Ambystoma tigrinum*). EcoHealth 8, 46–54.
Kiesow, A., 2006. Field Guide to Amphibians and Reptiles of South Dakota. Department of Game Fish, and Parks, Pierre, SD, USA.
Layne Jr., J.R., 1995. Crystallization temperatures of frogs and their individual organs. J. Herpetol. 29, 296–298.
Layne, J.R., 1999. Freeze tolerance and cryoprotectant mobilization in the gray treefrog (*Hyla versicolor*). J. Exp. Zool. 283, 221–225.
Layne, J.R., Jones, A.L., 2001. Freeze tolerance in the gray treefrog: cryoprotectant mobilization and organ dehydration. J. Exp. Zool. 290, 1–5.
Layne, J., Stapleton, M., 2009. Annual variation in glycerol mobilization and effect of freeze rigor on post-thaw locomotion in the freeze-tolerant frog *Hyla versicolor*. J. Comp. Physiol.B 179, 215–221.
Lemmon, E.M., Lemmon, A.R., Collins, J.T., Lee-Yaw, J.A., Cannatella, D.C., 2007. Phylogeny-based delimitation of species boundaries and contact zones in the trilling chorus frogs (*Pseudacris*). Mol. Phylogenet. Evol. 44, 1068–1082.
MacArthur, D.L., Dandy, J.W.T., 1982. Physiological aspects of overwintering in the boreal chorus frog (*Pseudacris triseriata maculata*). Comp. Biochem. Physiol.A 72, 137–141.
Muir, T.J., Costanzo, J.P., Lee, R.E., 2007. Osmotic and metabolic responses to dehydration and urea-loading in a dormant, terrestrially hibernating frog. J. Comp. Physiol.B 177, 917–926.
Muths, E., Stephen Corn, P., Pessier, A.P., Earl Green, D., 2003. Evidence for disease-related amphibian decline in Colorado. Biol. Conserv. 110, 357–365.
Regosin, J.V., Windmiller, B.S., Reed, J.M., 2003. Terrestrial habitat use and winter densities of the Wood Frog (*Rana sylvatica*). J. Herpetol. 37, 390–394.
Schiller, T.M., Costanzo, J.P., Lee, R.E., 2008. Urea production capacity in the wood frog (*Rana sylvatica*) varies with season and experimentally induced hyperuremia. J. Exp. Zool. A 309, 484–493.
Seebacher, F., Alford, R.A., 2002. Shelter microhabitats determine body temperature and dehydration rates of a terrestrial amphibian (*Bufo marinus*). J. Herpetol. 36, 69–75.
Storey, K.B., 1984. Freeze tolerance in the frog, *Rana sylvatica*. Experientia 40, 1261–1262.
Storey, K.B., 1990. Life in a frozen state: adaptive strategies for natural freeze tolerance in amphibians and reptiles. Am. J. Physiol. 258, R559–R568.
Storey, K.B., 1997. Organic Solutes in Freezing Tolerance. Comp. Biochem. Physiol. 117, 319–326.
Storey, J.M., Storey, K.B., 1985. Adaptations of metabolism for freeze tolerance in the gray tree frog, *Hyla versicolor*. Can. J. Zool. 63, 49–54.
Storey, K.B., Storey, J.M., 1986a. Freeze tolerance and intolerance as strategies of winter survival in terrestrially-hibernating amphibians. Comp. Biochem. Physiol. 83, 613–617.
Storey, K.B., Storey, J.M., 1986b. Freeze tolerant frogs: cryoprotectants and tissue metabolism during freeze–thaw cycles. Can. J. Zool. 64, 49–56.
Storey, K.B., Storey, J.M., 1988. Freeze tolerance in animals. Physiol. Rev. 68, 27–84.
Storey, K.B., Storey, J.M., Chruchill, T.A., 1997. De novo protein biosynthesis responses to water stresses in wood frogs: freeze–thaw and dehydration-rehydration. Cryobiology 34, 200–213.
Swanson, D.L., Graves, B.M., Koster, K.L., 1996. Freezing tolerance/intolerance and cryoprotectant synthesis in terrestrially overwintering anurans in the Great Plains, USA. J. Comp. Physiol.B 166, 110–119.
Voituron, Y., Barré, H., Ramløv, H., Douady, C.J., 2009. Freeze tolerance evolution among anurans: frequency and timing of appearance. Cryobiology 58, 241–247.
Withers, P.C., Guppy, M., 1996. Do Australian desert frogs co-accumulate counteracting solutes with urea during aestivation? J. Exp. Biol. 199, 1809–1816.
Wright, P.A., 1995. Nitrogen excretion: three end products, many physiological roles. J. Exp. Biol. 198, 273–281.
Zimmerman, S.L., Frisbie, J., Goldstein, D.L., West, J., Rivera, K., Krane, C.M., 2007. Excretion and conservation of glycerol, and expression of aquaporins and glyceroporins, during cold acclimation in Cope's Gray Tree Frog *Hyla chrysoscelis*. Am. J. Physiol. 292, R544–R555.