The Journal of Experimental Biology 211, 2969-2975
Published by The Company of Biologists 2008
doi:10.1242/jeb.019695

Urea loading enhances freezing survival and postfreeze recovery in a terrestrially hibernating frog

Jon P. Costanzo* and Richard E. Lee, Jr
Department of Zoology, Miami University, Oxford, OH 45056, USA
*Author for correspondence (e-mail: costanjp@muohio.edu)

Accepted 21 July 2008

SUMMARY

We tested the hypothesis that urea, an osmolyte accumulated early in hibernation, functions as a cryoprotectant in the freeze-tolerant wood frog, *Rana sylvatica*. Relative to saline-treated, normouremic (10 μmol ml^{-1}) frogs, individuals rendered hyperuremic (70 μmol ml^{-1}) by administration of an aqueous urea solution exhibited significantly higher survival (100% *versus* 64%) following freezing at –4°C, a potentially lethal temperature. Hyperuremic frogs also had lower plasma levels of intracellular proteins (lactate dehydrogenase, creatine kinase, hemoglobin), which presumably escaped from damaged cells, and more quickly recovered neurobehavioral functions following thawing. Experimental freezing–thawing did not alter tissue urea concentrations, but did elevate glucose levels in the blood and organs of all frogs. When measured 24 h after thawing commenced, glucose concentrations were markedly higher in urea-loaded frogs as compared to saline-treated ones, possibly because elevated urea retarded glucose clearance. Like other low-molecular-mass cryoprotectants, urea colligatively reduces both the amount of ice forming within the body and the osmotic dehydration of cells. In addition, by virtue of certain non-colligative properties, it may bestow additional protection from freeze–thaw damage not afforded by glucose.

Key words: amphibian, freeze tolerance, osmolyte, hibernation, *Rana sylvatica,* wood frog, cryoprotection.

INTRODUCTION

Urea accumulation is a generalized amphibian response to osmotic challenge that has been most extensively studied in animals undergoing salt acclimation, aestivation and transient exposure to arid environments (Shpun et al., 1992; Jørgensen, 1997). As a consequence of environmental water deficit, cold exposure and anuria, amphibians might also accumulate urea in terrestrial hibernation, although this possibility has received little study.

Our investigation of the wood frog (*Rana sylvatica* LeConte), a northern species that hibernates beneath forest duff, showed that plasma urea was ~50 μmol ml^{-1} in autumn and early winter, when soil moisture was scarce, but only ~2 μmol ml^{-1} in late winter and spring, after moisture availability increased (Costanzo and Lee, 2005). In laboratory experiments, we also determined that hibernating *R. sylvatica* can accumulate urea to at least 90 μmol ml^{-1} under relatively dry, warm conditions. Subsequent research showed that urea is the major solute contributing to increased plasma osmotic pressure during progressive dehydration (Muir et al., 2007). In nature, urea accumulation in *R. sylvatica* probably is facilitated by their preference for overwintering in relatively dry, upland habitats (Regosin et al., 2003). Urea is of obvious importance in the osmotic homeostasis of terrestrial amphibians, but this solute may play additional roles in overwintering survival, particularly in freeze-tolerant anurans such as *R. sylvatica*.

Freeze tolerance in anurans is supported by a host of molecular, biochemical and physiological responses that ameliorate the various stresses induced by the freezing and thawing of water in extracellular compartments of the body. Foremost among these is the synthesis of low-molecular-mass carbohydrates (glucose in *R. sylvatica*; glycerol and/or glucose in hylids) during the early hours of freezing. These permeable osmolytes, or 'cryoprotectants', colligatively lower the freezable fraction of body water and reduce cell dehydration and shrinkage, thereby limiting iono-osmotic and mechanical injuries. They may also act directly to stabilize macromolecules, membranes and other cellular structures, and possibly have other protective functions (Carpenter and Crowe, 1988; Storey and Storey, 2004).

Purportedly the sole cryoprotectant in *R. sylvatica*, glucose contributes to freezing survival at the cellular, organ and whole-animal levels of organization (Canty et al., 1986; Costanzo et al., 1993). However, recent studies have shown that physiological levels of urea (40–80 μmol ml^{-1}) enhance tolerance of *R. sylvatica* cells and organs to *in vitro* freezing–thawing (Costanzo and Lee, 2005; Costanzo et al., 2008), suggesting that urea also plays a role in amphibian freeze tolerance. In the present investigation, we tested this hypothesis by determining whether *R. sylvatica* experimentally rendered hyperuremic, *via* urea injection, demonstrate reduced cryoinjury and an enhanced tolerance to freezing–thawing.

MATERIALS AND METHODS

Experimental animals and acclimatization regimen

Male wood frogs were collected in mid February 2006 from a vernal pool in southern Ohio, USA. They were transported to our laboratory under refrigeration and placed inside covered, opaque boxes containing damp moss. Frogs were kept in darkness at 4°C and used in experiments within 6 weeks of capture. Prior research has shown that frogs collected and maintained in this manner readily tolerate freezing at –3°C, but become moribund or die at temperatures between –4 and –5°C (Layne and Lee, 1987; Costanzo et al., 1993; Costanzo et al., 1997a).

Urea loading

We prepared frogs for experimentation by draining any urine from the bladder through a polished glass cannula inserted into the cloaca.

Frogs were then weighed on a digital balance, kept in darkness at 4°C on dry paper inside covered plastic cups, and reweighed after 2–3 days. Because water vapor was permitted to escape through perforations in the cover, frogs lost ~0.5 g of their standard body mass (mean ± s.e.m.=14.8±0.3 g, N=48) whilst inside the cups. We randomly assigned frogs to either of two groups to which we administered 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; 230 mOsmol kg^{-1}, pH 7.4 at 23°C) or PBS containing 1.5 mol l^{-1} urea. Using a 27.5-gauge needle, the dorsal lymph pad of each frog was injected with a volume of the appropriate solution equal to 3.3% of standard body mass. Thus, the average frog received ~0.5 ml of injectate, which restored its initial hydration level. Frogs remained in darkened cages at 4°C for 3–5 h prior to being experimentally frozen or euthanized for tissue sampling (see below).

Freezing–thawing protocol

Following an earlier study (Costanzo et al., 1991b), our experimental freezing–thawing protocol simulated chilling episodes to which *R. sylvatica* are exposed during hibernation. Briefly, each frog was outfitted with a copper–constantan thermocouple placed against its abdomen and cooled inside a 50 ml polypropylene centrifuge tube. Groups of tubes (N=38) were submerged in a refrigerated ethanol bath programmed to cool from 0 to –4°C over a 40 h period. During chilling, body temperature was recorded on a multichannel data logger. Once the frogs had reached –0.5°C, freezing was initiated by placing small ice crystals against their skin. Cooling then resumed and, after reaching –4°C, they were kept *in situ* for an additional 2 h to ensure they attained thermoequilibrium. Fully-frozen frogs were transferred to an incubator at 4°C and, after 1–2 h, gently removed from their tubes and individually placed on damp paper inside plastic cups.

Recovery time course and survival assessment

For both urea-loaded (N=14) and saline-treated (N=14) frogs, we monitored the time course for restoration of neurobehavioral functions following freezing–thawing by examining each frog daily, at 08:30 h and 17:00 h, for 1 week. At each observation we recorded whether or not the subject exhibited each of the following behaviors: corneal reflex, pulmonary breathing, alert posture (i.e. limbs trunk, and head held in characteristic manner), retraction of hindlimb within 2 s of manual extension, and righting reflex (i.e. inversion within 2 s of being placed on dorsum). On day 7, we assessed survival on the basis of whether or not each frog met the righting-reflex criterion. Frogs failing to meet this criterion were double-pithed, dissected, and the blood was sampled and assayed as described below.

Sublethal cryoinjury

We investigated freeze–thaw injury in separate groups of urea-loaded (N=5) and saline-treated (N=5) frogs by measuring circulating levels of three intracellular proteins. Blood samples were collected (see below) 24 h after thawing was initiated. Hemoglobin (Hb) concentration in the cell-free plasma, an index of hemolytic damage, was assayed as cyanmethemoglobin (Sigma, no. 525; St Louis, MO, USA). Plasma samples were also assayed for the ubiquitous cytoplasmic enzyme, lactate dehydrogenase (LDH), and creatine kinase (CK), an intracellular enzyme found primarily in skeletal and cardiac muscle. LDH activity was quantified using a colorimetric assay kit (Sigma, no. TOX-7), whereas CK activity was assayed at 25°C using a kinetic procedure (Pointe Scientific, C7512; Canton, MI, USA). In order to establish baseline levels of these proteins, we also analyzed blood sampled from unfrozen frogs (urea-loaded, N=5; saline-treated, N=5) ~4.5 h after receiving the injections.

Tissue sampling and osmolyte assays

Frogs were double-pithed and quickly dissected. Blood was drawn from an incision in the aortic trunk into heparinized capillary tubes and centrifuged (2000 ***g***, 5 min). The resultant plasma was isolated and reserved on ice for subsequent analysis. We removed and sagittally bisected the heart, and equally divided a 50–100 mg portion of both the liver and hindlimb muscle (gastrocnemius). One set of samples was used in osmolyte assays; remaining samples were weighed to 0.1 mg, thoroughly dried in a 65°C oven, and reweighed in order to determine tissue water content. We estimated body water content by drying pre-weighed carcasses and determining the mass of water that had evaporated; values were expressed as a percentage of fresh mass.

We prepared tissue extracts by homogenizing pre-weighed organ samples in 7% perchloric acid. After removing the proteins by centrifugation (4000 ***g***, 5 min), the clear supernatant was neutralized with potassium hydroxide and the resulting precipitate was removed by a second centrifugation. Glucose and urea in these extracts and in blood plasma were assayed using glucose oxidase (Sigma, no. 510) and urease (Pointe Scientific, no. B7551-120) procedures, respectively. Plasma osmolality was determined on 10 µl samples by vapor-pressure osmometry (Wescor, model 5500, Logan, UT, USA).

Statistical inferences

Fisher's exact test was used to compare ratios of urea-loaded and saline-treated frogs fully recovering from freezing and meeting behavioral criteria at select time points. We used two-factor analysis of variance (ANOVA) to test for effects of urea loading and freezing–thawing on plasma and organ osmolyte levels and water content. Analyses involving percentage data were performed on values after arcsine-root transformation. Significance of statistical analyses was accepted at P<0.05. Mean values are reported as ± s.e.m.

RESULTS

Recovery from freezing–thawing

All urea-loaded frogs (N=14) survived freezing–thawing, whereas full recovery occurred in only 9 of 14 (64.3%) saline-treated frogs. These percentages differed significantly (P=0.020). Mortality statistics for the saline-treated group included two frogs that showed no vital signs after thawing, as well as one frog that exhibited the corneal reflex and pulmonary breathing but failed to meet other recovery criteria, and two others that ultimately showed normal posture and/or hindlimb retraction reflex but were unable to right themselves. The three moribund frogs were euthanized on day 7. We immediately sampled their blood (but not organs) for later analysis of urea, glucose, osmolality and intracellular marker proteins.

Dynamics of recovery from freezing–thawing differed between urea-loaded and saline-treated frogs (Fig. 1). Most urea-loaded frogs exhibited the limb-retraction reflex and normal posture by 24 h after thawing, all doing so within 48 h. By contrast (P=0.008), only eight of 14 (57.1%) saline-treated frogs demonstrated these behaviors within 48 h after thawing began. Righting reflex, our most rigorous test of neurobehavioral function, was exhibited by all urea-loaded frogs within 56 h after thawing, whereas only four saline-treated frogs (28.6%) could right themselves by this time (P=0.002). The last of the latter group to meet the righting-reflex criterion did so after 80 h of postfreeze recovery.

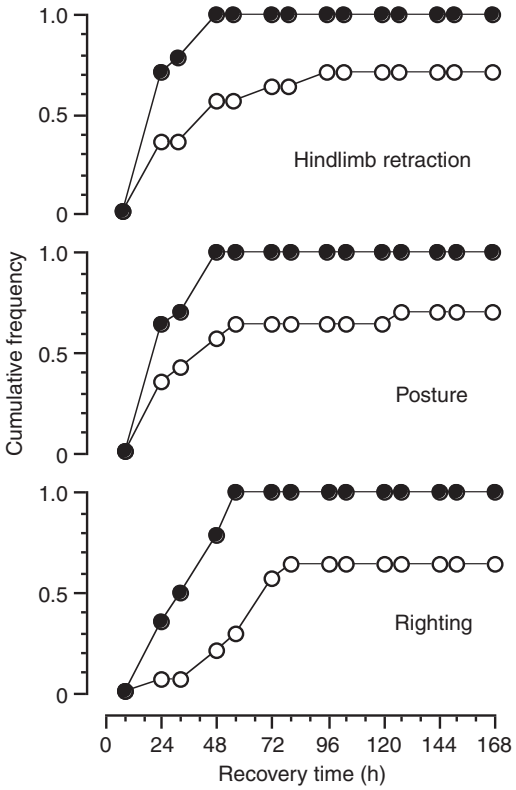

Fig. 1. Dynamics of recovery from freezing at –4°C in *Rana sylvatica* administered saline (open symbols) or saline containing urea (filled symbols). Data are presented as the cumulative frequency of the sample (N=14) exhibiting limb retraction reflex, normal posture and righting reflex at various time intervals following the onset of thawing.

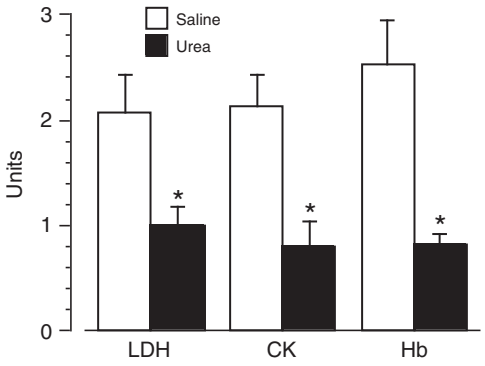

Fig. 2. Indices of cryoinjury in *Rana sylvatica* administered saline (open bars), or saline containing urea (filled bars), prior to freezing at –4°C. Response variables are plasma LDH (lactate dehydrogenase) activity (i.u. ml^{-1}), plasma CK (creatine kinase) activity (mi.u. ml^{-1}), and plasma Hb (hemoglobin) concentration (mg ml^{-1}). Values are means ±1 s.e.m., N=5 frogs per group. Asterisk indicates that means differed at P<0.05.

Sublethal cryoinjury

Expectedly, we found little of the intracellular marker proteins in plasma of unfrozen frogs. Free Hb was absent. We detected low levels of LDH activity, which did not differ (P=0.139) between urea-loaded (0.4±0.08 i.u. ml^{-1}) and saline-treated (0.6±0.1 i.u. ml^{-1}) individuals. Levels of CK activity in plasma of unfrozen frogs also were low, but inexplicably differed (P<0.0001) between urea-loaded and saline-treated frogs (0.02±0.001 *versus* 1.1±0.1 mi.u. ml^{-1}, respectively).

As evidenced by their rhythmically contracting hearts, all frozen–thawed frogs used for cryoinjury analysis were alive when examined 24 h after thawing commenced. These frogs physically resembled specimens used in the survival experiments at the same stage of recovery, although it is impossible to know whether they would have met our ultimate survival criterion. Experimental freezing–thawing was associated with higher plasma levels of intracellular proteins as compared to those in unfrozen frogs (P<0.01, all cases), indicating that cell damage occurred in both urea-loaded and saline-treated frogs. However, among frozen–thawed frogs, saline-treated animals had markedly higher (1.7- to 3.2-fold) levels of plasma LDH activity (P=0.027), CK activity (P=0.008) and Hb (P=0.005) relative to urea-loaded frogs (Fig. 2).

Effect of urea loading and freezing–thawing on osmolytes and water balance

Administration of urea solution caused urea levels in plasma and organs to increase 3- to 7-fold (P<0.001, all cases) (Table 1) over those in saline-treated frogs. The latter had modest levels of urea in plasma (9 μmol ml^{-1}) and organs (12–16 μmol g^{-1}), and these levels were unaltered (P>0.05, all cases) by experimental freezing–thawing. Urea-loaded frogs subjected to freezing–thawing were still strongly hyperuremic when sampled 24 h after thawing commenced, although they had slightly lower urea concentrations in plasma (P=0.021), heart (P=0.002) and liver (P=0.028) as compared to their unfrozen counterparts.

Glucose levels in plasma and organs of frozen–thawed frogs were substantially higher (P<0.0001, all cases) than those of unfrozen frogs (Table 1), indicating that experimental freezing–thawing induced the characteristic hyperglycemic response. Among unfrozen saline-treated frogs, glucose levels were uniformly low in plasma (2 μmol ml^{-1}) and organs (1–4 μmol g^{-1}), whereas 5- to 18-fold higher concentrations (P<0.001, all cases) were found in their frozen–thawed counterparts. Glucose levels also differed (P<0.0001, all cases) between unfrozen and frozen–thawed groups of urea-loaded frogs; however, the interaction terms of the two-factor ANOVAs (P<0.006, all cases) indicated that the magnitude of the glycemic response differed between urea-loaded and saline-treated individuals. Among frozen–thawed frogs, glucose levels in plasma and organs of urea-loaded frogs were up to 3.5-fold higher than those of saline-treated animals. Within the liver, the difference, 70 μmol g^{-1} *versus* 20 μmol g^{-1}, was especially striking.

Plasma osmolality was strongly influenced by urea loading (P<0.0001). Relative to the frogs receiving only isotonic saline, plasma osmotic pressure was ~55 mOsmol kg^{-1} higher in urea-loaded frogs in both the unfrozen (277±5 *versus* 220±4 mOsmol kg^{-1}) and frozen–thawed groups (308±8 *versus* 255±5 mOsmol kg^{-1}). This increment reflected the measured increase in plasma urea concentration (49–60 μmol ml^{-1}) achieved with the injections. Plasma osmolality markedly increased (P<0.0001) with freezing–thawing over that in the corresponding unfrozen frogs, with the increase being of similar magnitude (33–35 mOsmol kg^{-1}; P=0.840) in urea-loaded and saline-treated animals. In both groups, the increment in osmotic pressure was primarily attributed to net differences in glucose and urea concentrations between unfrozen and frozen–thawed frogs (Table 1).

Urea loading had no effect (P>0.05, all cases) on the hydration state of *R. sylvatica* organs and carcass, and, with the sole exception of liver, neither did freezing–thawing (P>0.05, all other cases)

Table 1. Concentrations of urea and glucose in plasma and organs of *Rana sylvatica* administered saline or saline containing urea, and sampled before or after freezing at –4°C

	Urea		Glucose	
	Saline treated	Urea loaded	Saline treated	Urea loaded
Unfrozen				
Plasma	9.8±0.8	69.6±2.5*	1.9±0.4	2.4±0.2
Heart	14.6±1.5	80.6±5.3*	1.7±0.4	1.7±0.3
Liver	15.7±2.8	72.1±5.3*	3.9±0.4	4.1±0.3
Muscle	12.1±1.7	47.8±5.9*	1.0±0.2	1.3±0.1
Frozen/thawed				
Plasma	11.7±1.5	60.4±2.0*	28.1±2.0	42.3±3.8*
Heart	18.7±3.5	56.2±1.1*	18.6±2.1	53.7±3.9*
Liver	21.1±3.6	57.2±1.6*	19.9±2.1	69.9±10.9*
Muscle	19.5±3.3	56.1±2.2*	18.1±2.6	40.9±2.9*

Concentrations of urea and glucose in plasma are μmol ml^{-1} and in organs are μmol g^{-1} fresh tissue. Values are means ± s.e.m.; N=5 frogs per group.

Asterisk denotes that the mean for the urea-loaded group differed significantly (P<0.05) from its saline-treated counterpart.

Table 2. Water content in organs and carcasses of *Rana sylvatica* administered saline or saline containing urea, and sampled before or after freezing at –4°C

	Saline treated	Urea loaded
Unfrozen		
Heart	82.8±0.5	82.2±0.6
Liver	71.2±0.7	71.7±1.0
Muscle	79.2±0.2	78.5±0.4
Carcass	78.2±0.3	78.9±0.5
Frozen/thawed		
Heart	83.9±0.3	83.4±0.7
Liver	78.8±0.4*	78.3±0.5*
Muscle	79.2±0.4	78.3±0.5
Carcass	79.4±0.2	78.2±0.5

Water content is given as % fresh tissue mass. Values are means ± s.e.m.; N=5 frogs per group.

Asterisk denotes that the mean for the frozen/thawed group differed significantly (P<0.05) from its unfrozen counterpart.

(Table 2). Water concentration in liver of frozen–thawed frogs was ~1.5 times greater (P<0.0001) than that measured in unfrozen frogs, this difference being unaffected (P=0.892) by urea loading.

Hematocrit values determined for frozen–thawed frogs (urea-loaded: 36±3%; saline-treated: 45±2%) were substantially higher (P<0.0001) than those determined for unfrozen frogs (urea-loaded: 26±1%; saline-treated: 29±1%). Among frozen–thawed frogs, the mean hematocrit value for saline-treated frogs was higher (P=0.014) than that for urea-loaded frogs. However, among unfrozen animals, this variable did not differ (P=0.121) between the groups.

DISCUSSION

In temperate North America, *R. sylvatica* and several tree frogs (Hylidae) overwinter beneath forest duff where they potentially encounter low environmental water potential and subzero temperatures. They can survive these conditions by virtue of their profound tolerances to dehydration and somatic freezing (Storey and Storey, 2004). Because urea accumulation is a universal amphibian response to osmotic challenge (Shpun et al., 1992; Jørgensen, 1997), and because low temperature promotes urea retention through diminished renal function (Nielsen and Jørgensen, 1990), hyperuremia may be common among terrestrially hibernating frogs. Indeed, elevated urea has been found in winter *R. sylvatica* (Layne and Rice, 2003; Costanzo and Lee, 2005) and *Hyla versicolor* (J. R. Layne, personal communication). MacArthur and Dandy reported that plasma osmolality increased by ~50 mOsmol kg^{-1} in winter-acclimatized *Pseudacris triseriata* (MacArthur and Dandy, 1982). Although they did not assay for specific solutes, we suspect that urea accounted for much of the osmotic increase.

Long known as an osmoprotectant, urea also serves numerous other functions in various ectotherms (Griffith, 1991; Withers, 1998). In terrestrially hibernating frogs, urea undoubtedly is important in osmotic homeostasis, but may also contribute to winter survival in other ways. For example, urea accumulated during autumn apparently functions as a metabolic inhibitor, reducing the energetic cost of overwintering (Muir et al., 2007; Muir et al., 2008). We hypothesize that urea also promotes survival of natural freezing episodes by acting as a cryoprotective agent. This contention draws support from experiments in which urea pretreatment of *R. sylvatica* cells and organs enhanced their tolerance to freezing–thawing *in vitro* (Costanzo and Lee, 2005; Costanzo et al., 2008). The present study extends these findings by demonstrating that physiological levels of urea enhance freezing survival and postfreeze recovery *in vivo*.

Hyperuremia enhances freezing survival and postfreeze recovery

Tissue urea levels in urea-loaded frogs remained high during freezing and thawing, although concentrations in the frozen–thawed specimens were lower than those in unfrozen frogs (Table 1), apparently because some of the solute was lost *via* renal filtration. We found no evidence that urea loading altered tissue hydration, plasma osmolality or glycemia, and previous studies (Shoemaker, 1965; Taylor et al., 1999) have shown it affects neither cutaneous water uptake nor behavior. Thus, we are reasonably confident in ascribing the observed variation in survival and postfreeze recovery rates of *R. sylvatica* to differences in uremia between treatment groups.

Experimentally augmenting tissue glucose levels improves freezing survival of *R. sylvatica*, providing strong evidence for the cryoprotective function of this osmolyte (Costanzo et al., 1991a; Costanzo et al., 1993). Similarly, urea loading enhanced survival of *R. sylvatica* subjected to freezing at a potentially lethal temperature, –4°C. This finding is biologically relevant because urea levels in these frogs (50–80 μmol g^{-1}) were within the range that can be achieved during hibernation (i.e. to at least 90 μmol ml^{-1} in blood) (Costanzo and Lee, 2005). By contrast, mortality occurred among saline-treated frogs, whose tissues contained more modest levels of urea (12–16 μmol g^{-1}).

Leakage of intracellular proteins is a useful index of sublethal cryoinjury in intact organisms (Costanzo et al., 1991a; Costanzo et al., 1993; Costanzo et al., 1997a; Irwin et al., 2003; Costanzo et al., 2006). In our experiment, hyperuremia not only enhanced freezing survival, but also reduced sublethal cryoinjury, as urea-loaded frogs had lower extracellular levels of the marker proteins LDH, CK and Hb. Minimizing insult to cells and tissues is an important function of cryoprotectants because even freeze-tolerant animals die if injury is excessive. This concept is underscored by the responses of three saline-treated frogs that were alive after thawing but ultimately failed to meet the survival criterion. We do not know why these particular individuals (and two other saline-treated frogs, which were dead upon thawing) succumbed to freezing. Although cryoprotectant

levels were not measured whilst these frogs were frozen, when assayed 7 days after thawing commenced, their plasma concentrations of urea (11.1±1.4 μmol ml^{-1}) and glucose (2.0±0.5 μmol ml^{-1}), as well as plasma osmolality (213±5 mOsmol kg^{-1}), were comparable to those of unfrozen, saline-treated animals. Thus, possibly they amassed only meager amounts of cryoprotective solute and thereby incurred greater injury. Indeed, plasma collected from these moribund frogs contained substantial amounts of LDH (2.9±0.8 i.u. ml^{-1}), CK (3.3±0.2 mi.u. ml^{-1}) and Hb (2.9±0.4 mg ml^{-1}) that were 14–58% higher than those measured in frozen–thawed, saline-treated frogs (Fig. 2). However, these data should be interpreted cautiously because the moribund frogs were sampled much longer (7 days *versus* 24 h) after thawing commenced; thus, their higher protein levels could partly reflect protracted leakage, rather than more extensive damage.

Additional evidence for urea's cryoprotective efficacy is the finding that recovery from freezing–thawing stress was expedited in urea-loaded frogs. In *R. sylvatica*, restoration of complex neurobehavioral functions following freezing–thawing requires from several hours to many days, depending on the severity of the freezing exposure with respect to its rapidity, duration and minimum temperature (Costanzo et al., 1991a; Layne, 1992; Costanzo et al., 1993; Layne et al., 1998). This relationship implies that recovery attends resolution of homeostatic perturbations and repair of injuries. Given that freezing impairs function in both nerve and muscle (Kling et al., 1994; Irwin et al., 2003), complex behaviors, including ones we examined, are highly sensitive to freezing–thawing stresses. Relative to saline-treated frogs, hyperuremic frogs recovered rapidly, suggesting that urea somehow limits freezing-induced perturbations to neurobehavioral function.

Physiological responses to freezing–thawing

Results of the present investigation, like those of earlier studies with *R. sylvatica* (Layne and Rice, 2003; Costanzo and Lee, 2005), show that freezing stimulates synthesis of glucose, but not urea. Mediated by adrenergic stimulation of hepatocytes, somatic freezing initiates glycogenolysis and production of glucose, which is then distributed throughout the body (Storey and Storey, 2004). Apparently there is no equivalent system for mobilizing urea; therefore, frogs must accumulate this osmolyte in anticipation of freezing.

Because glucose clearance begins soon after thawing (Layne et al., 1996; Costanzo et al., 1997a), glycemic levels in our frogs (sampled 24 h after thawing began) were markedly lower than those usually found in still-frozen frogs (Storey and Storey, 2004) [see also table 4 in our earlier report (Costanzo and Lee, 2005)]. Filtered glucose is not necessarily lost from the body, but can be reabsorbed by the bladder epithelium and ultimately reconverted to liver glycogen (Costanzo et al., 1997b). We found differences in glycemic state between urea-loaded and saline-treated frogs, tissues of the former containing considerably more glucose. Although we cannot rule out the possibility, it seems unlikely that urea-loaded frogs had synthesized more glucose, which could have contributed to their enhanced survival. Rather, we suspect that hyperuremic and saline-treated frogs differed in efficacy of glucose clearance subsequent to thawing. Conceivably, differential clearance rates could result if urea, a well-known perturbant of many proteins, inhibited glycogenesis through its interaction with one or more glycolytic enzymes, such as phosphofructokinase (Hand and Somero, 1982). Unlike the case with cartilaginous marine fishes, amphibians apparently do not co-accumulate methylamines to levels that would counteract perturbing effects of urea (Wray and Wilkie, 1995; Withers and Guppy, 1996). In any case, hyperglycemia persisting longer during postfreeze recovery could benefit *R. sylvatica* by fueling repair processes and limiting injury due to subsequent, rapid freezing (Costanzo et al., 1991b).

Aside from using protective solutes, freeze-tolerant anurans minimize freeze–thaw stress by re-compartmentalizing extracellular water in a manner that reduces the amount of ice forming within the tissues and microvasculature. During freezing, much of the water inside organs (up to 60%) is translocated to the coelom and lymph sacs, where it freezes innocuously (Lee et al., 1992). After thawing, circulation resumes and organs quickly rehydrate to prefreeze levels (Costanzo et al., 1997a). Accordingly, with the exception of the liver (which tends to hyperhydrate, probably owing to high solute content), the organs of our frozen–thawed frogs, sampled 24 h after thawing began, resembled those of unfrozen frogs. Tissue water contents of urea-loaded and saline-treated frogs were similar, suggesting that hyperuremia has no influence on the organ dehydration response; however, studies of fully-frozen animals are needed to draw a definitive conclusion.

Despite the cryolytic loss of some erythrocytes, the hematocrit of frozen–thawed frogs commonly is higher than that of unfrozen animals whilst excess water remains within extravascular spaces (Costanzo et al., 1991b; Costanzo et al., 1997a; Irwin et al., 2003). Accordingly, both urea-loaded and saline-treated frogs were hypovolemic following thawing. However, hematocrit in the urea-loaded frogs more closely matched that of unfrozen frogs, suggesting that urea aided restoration of blood volume. We speculate that this could occur if urea functions as a vasodilator (Vajragupta et al., 1996) and/or if urea enhances water flux by mediating increased expression of water channels (Storm et al., 2003; Umenishi et al., 2005).

Possible mechanisms of cryoprotection by urea

Freeze-tolerant organisms commonly use cryoprotectants representing a diverse array of organic compounds that share certain attributes, including low molecular mass, high solubility and permeability, stability, ready availability and compatibility with macromolecules (Storey and Storey, 2004). By virtue of the colligative properties of small solutes, these agents reduce both the osmotic loss of cell water and the amount of ice forming at any given temperature. Because urea undoubtedly functions in this manner, its cryoprotective efficacy is, at least to some extent, concentration dependent (Costanzo et al., 2008). However, pretreating *R. sylvatica* erythrocytes with 80 or 40 μmol urea ml^{-1} afforded virtually the same margin of protection from *in vitro* cryoinjury (Costanzo and Lee, 2005), suggesting that this solute also has special protective properties.

Various organic osmolytes function as cryoprotectants by mollifying freeze–thaw damage to macromolecules and cellular structures (Carpenter and Crowe, 1988; Göller and Galinski, 1999). Contrary to its reputation as a destabilizing agent, urea, especially when present in low concentrations, can actually enhance hydrophobic interactions, perhaps by increasing the solvent structure, and thereby stabilize certain proteins (Bhuyan, 2002; Kumar et al., 2004; Chakraborty et al., 2005; Gull et al., 2007). Urea may even counteract deleterious effects of elevated ionic solutes on biopolymers (Tian and Cohen, 2001). Contrary to its protein denaturing effect at high (i.e. molar) concentration, physiological levels of urea may be even less perturbing than some 'compatible' osmolytes (e.g. Yancey and Burg, 1990).

Our results suggest that urea also provides cryoprotection at the cellular (and perhaps higher) level of biological organization, although its precise mode(s) of action remains to be determined.

Pretreatment with urea protects certain cells from hyperionic stress leading to apoptotic death (Santos et al., 1998; Zhang et al., 2000). Urea, which is used in the clinical treatment of hyponatremia, also prevents brain damage and neurobehavioral aberrations caused by fluctuations in ion levels, such as may occur with freezing–thawing (Soupart et al., 2007). That the *R. sylvatica* heart is particularly amenable to cryoprotection by urea (Costanzo and Lee, 2005) is consistent with this solute's high capacity to scavenge certain reactive oxygen species, thereby protecting cardiocytes from post-ischemic reperfusion injury (Wang et al., 1999). Although urea may be less effective against hydroxyl radicals (Halliwell, 1978), its high permeability, ubiquity and ease of mobilization are advantages over other natural antioxidants.

A compound cryoprotectant system

Like many freeze-tolerant animals, *R. sylvatica* apparently uses a mixture of osmolytes in its cryoprotectant system. This may be advantageous because each component offers unique benefits, but also has certain limitations. For example, urea accrues in autumn, chiefly in response to environmental water deficit, such that all tissues become laden before freezing begins. On the other hand, frogs may fail to amass much urea if environmental moisture is abundant. Glucose accumulation, triggered directly by tissue freezing, is far more predictable and potentially more robust, but tissue levels rise meagerly if freezing proceeds rapidly or if hepatic glycogen reserves are low (Costanzo and Lee, 2005). Furthermore, levels of glucose, which must be synthesized in the liver, exported to distant organs, and transported into cells whilst freezing proceeds, vary markedly between core and peripheral organs (Storey and Storey, 2004). The net effect is that cells in some tissues could contain as much (or more) urea as glucose (Table 1) (Costanzo and Lee, 2005).

Another consideration is that the combination of glucose and urea may provide benefits not bestowed individually. Furthermore, certain osmolytes may excel at performing functions to which others are not particularly well suited. For example, in one experiment, urea was better than glucose at reducing freeze–thaw injury to *R. sylvatica* erythrocytes, perhaps because it was superior at raising the fraction of unfreezeable cell water and/or protecting macromolecules and cellular structures (Costanzo and Lee, 2005). Future studies aimed at identifying specific mechanisms of protection conferred by these osmolytes, both individually and in combination, would be instructive.

Our present results, together with findings of recent studies (Costanzo and Lee, 2005; Costanzo et al., 2008), marshal compelling evidence for a role of urea as a natural cryoprotective osmolyte in *R. sylvatica*. Collectively, this work establishes a previously undocumented role for this 'waste product' of nitrogen metabolism, and also identifies a new class of natural cryoprotectant. In addition, the notion that an osmolyte accumulated preparatory to freezing contributes to freezing survival challenges the longstanding view that freezing-induced mobilization of cryoprotective solute is the hallmark physiological adaptation of amphibian freeze tolerance.

We thank P. Baker, M. Elnitsky and T. Muir for collecting the research specimens, A. Rosendale for critically reading the manuscript, and J. R. Layne, Jr for sharing with us his unpublished data. We also thank two anonymous referees for providing suggestions that improved this paper. This work was supported by a National Science Foundation Grant IOB 0416750.

REFERENCES

Bhuyan, A. K. (2002). Protein stabilization by urea and guanidine hydrochloride. *Biochemistry Mosc.* **41**, 13386-13394.

Canty, A., Driedzic, W. R. and Storey, K. B. (1986). Freeze tolerance of isolated ventricle strips of the wood frog, *Rana sylvatica*. *Cryo Letters* **7**, 81-86.

Carpenter, J. F. and Crowe, J. H. (1988). The mechanism of cryoprotection of proteins by solutes. *Cryobiology* **25**, 244-255.

Chakraborty, A., Sarkar, M. and Basak, S. (2005). Stabilizing effect of low concentrations of urea on reverse micelles. *J. Colloid Interface Sci.* **287**, 312-317.

Costanzo, J. P. and Lee, R. E. (2005). Cryoprotection by urea in a terrestrially hibernating frog. *J. Exp. Biol.* **208**, 4079-4089.

Costanzo, J. P., Lee, R. E. and Wright, M. F. (1991a). Glucose loading prevents freezing injury in rapidly cooled wood frogs. *Am. J. Physiol.* **261**, R1549-R1553.

Costanzo, J. P., Lee, R. E. and Wright, M. F. (1991b). Effect of cooling rate on the survival of frozen wood frogs, *Rana sylvatica*. *J. Comp. Physiol. B* **161**, 225-229.

Costanzo, J. P., Lee, R. E. and Lortz, P. H. (1993). Glucose concentration regulates freeze tolerance in the wood frog *Rana sylvatica*. *J. Exp. Biol.* **181**, 245-255.

Costanzo, J. P., Irwin, J. T. and Lee, R. E. (1997a). Freezing impairment of male reproductive behaviors of the freeze-tolerant wood frog, *Rana sylvatica*. *Physiol. Zool.* **70**, 158-166.

Costanzo, J. P., Callahan, P. A., Lee, R. E. and Wright, M. F. (1997b). Frogs reabsorb glucose from urinary bladder. *Nature* **389**, 343-344.

Costanzo, J. P., Baker, P. J. and Lee, R. E. (2006). Physiological responses to freezing in hatchlings of freeze-tolerant and -intolerant turtles. *J. Comp. Physiol. B* **176**, 697-707.

Costanzo, J. P., Marjanovic, M., Fincel, E. A. and Lee, R. E. (2008). Urea loading enhances postfreeze performance of frog skeletal muscle. *J. Comp. Physiol. B* **178**, 413-420.

Göller, K. and Galinski, E. A. (1999). Protection of a model enzyme (lactate dehydrogenase) against heat, urea and freeze-thaw treatment by compatible solute additives. *J. Mol. Catal., B Enzym.* **7**, 37-45.

Griffith, R. W. (1991). Guppies, toadfish, lungfish, coelacanths and frogs: a scenario for the evolution of urea retention in fishes. *Environ. Biol. Fishes* **32**, 199-218.

Gull, N., Sen, P., Kabir-Ud-Din and Khan, R. H. (2007). Effect of physiological concentration of urea on the conformation of human serum albumin *J. Biochem.* **141**, 261-268.

Halliwell, B. (1978). Superoxide-dependent formation of hydroxyl radicals in the presence of iron chelates: is it a mechanism for hydroxyl radical production in biochemical systems? *FEBS Lett.* **92**, 321-326.

Hand, S. C. and Somero, G. N. (1982). Urea and methylamine effects on rabbit muscle phosphofructokinase. Catalytic stability and aggregation state as a function of pH and temperature. *J. Biol. Chem.* **257**, 734-741.

Irwin, J. T., Costanzo, J. P. and Lee, R. E. (2003). Postfreeze reduction of locomotor endurance in the freeze-tolerant wood frog, *Rana sylvatica*. *Physiol. Biochem. Zool.* **76**, 331-338.

Jørgensen, C. B. (1997). Urea and water economy. *Comp. Biochem. Physiol.* **117A**, 161-170.

Kling, K. B., Costanzo, J. P. and Lee, R. E. (1994). Post-freeze recovery of peripheral nerve function in the freeze-tolerant wood frog, *Rana sylvatica*. *J. Comp. Physiol. B* **164**, 316-320.

Kumar, S., Parveen, N. and Kabir-ud-Din. (2004). Effect of urea addition on micellization and the related phenomena. *J. Phys. Chem. B* **108**, 9588-9592.

Layne, J. R. (1992). Postfreeze survival and muscle function in the leopard frog (*Rana pipiens)* and the wood frog (*Rana sylvatica*). *J. Therm. Biol.* **17**, 121-124.

Layne, J. R. and Lee, R. E. (1987). Freeze tolerance and the dynamics of ice formation in wood frogs (*Rana sylvatica*) from southern Ohio. *Can. J. Zool.* **65**, 2062-2065.

Layne, J. R. and Rice, M. E. (2003). Postfreeze locomotion performance in wood frogs (*Rana sylvatica*) and spring peepers (*Pseudacris crucifer*). *Can. J. Zool.* **81**, 2061-2065.

Layne, J. R., Lee, R. E. and Cutwa, M. (1996). Post-hibernation excretion of glucose in urine of the freeze tolerant frog *Rana sylvatica*. *J. Herpetol.* **30**, 85-87.

Layne, J. R., Costanzo, J. P. and Lee, R. E. (1998). Freeze duration influences postfreeze survival in the frog *Rana sylvatica*. *J. Exp. Zool.* **280**, 197-201.

Lee, R. E., Costanzo, J. P., Davidson, E. C. and Layne, J. R. (1992). Dynamics of body water during freezing and thawing in a freeze-tolerant frog (*Rana sylvatica*). *J. Therm. Biol.* **17**, 263-266.

MacArthur, D. L. and Dandy, J. W. T. (1982). Physiological aspects of overwintering in the boreal chorus frog (*Pseudacris triseriata maculata*). *Comp. Biochem. Physiol.* **72A**, 137-141.

Muir, T. J., Costanzo, J. P. and 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.

Muir, T. J., Costanzo, J. P. and Lee, R. E. (2008). Metabolic depression induced by urea in organs of the wood frog, *Rana sylvatica*: effects of season and temperature. *J. Exp. Zool.* **309**, 111-116.

Nielsen, K. H. and Jørgensen, C. B. (1990). Salt and water balance during hibernation in anurans. In *Biology and Physiology of Amphibians* (ed. W. Hanke), pp. 333-349. New York: Gustav Fischer Verlag.

Regosin, J. V., Windmiller, B. S. and Reed, J. M. (2003). Terrestrial habitat use and winter densities of the wood frog (*Rana sylvatica*). *J. Herpetol.* **37**, 390-394.

Santos, B. C., Chevaile, A., Hébert, M.-J., Zagajeski, J. and Gullans, S. R. (1998). A combination of NaCl and urea enhances survival of IMCD cells to hyperosmolality. *Am. J. Physiol.* **274**, F1167-F1173.

Shoemaker, V. H. (1965). The stimulus for the water-balance response to dehydration in toads. *Comp. Biochem. Physiol.* **15**, 81-88.

Shpun, S., Hoffman, J. and Katz, U. (1992). Anuran amphibia which are not acclimable to high salt, tolerate high plasma urea. *Comp. Biochem. Physiol.* **103A**, 473-477.

Soupart, A., Schroëder, B. and Decaux, G. (2007). Treatment of hyponatraemia by urea decreases risks of brain complications in rats: brain osmolyte contents analysis. *Nephrol. Dial. Transplant.* **22**, 1856-1863.

Storey, K. B. and Storey, J. M. (2004). Physiology, biochemistry, and molecular biology of vertebrate freeze tolerance: the wood frog. In *Life in the Frozen State* (ed. B. J. Fuller, N. Lane and E. E. Benson), pp. 243-274. Washington, DC: CRC Press.
Storm, R., Klussmann, E., Geelhaar, A., Rosenthal, W. and Maric, K. (2003). Osmolality and solute composition are strong regulators of AQP2 expression in renal principal cells. *Am. J. Physiol.* **284**, F189-F198.
Taylor, K., Mayer, L. P. and Propper, C. R. (1999). Intra- and extracellular dehydration-induced thirst-related behavior in an amphibian. *Physiol. Behav.* **65**, 717-721.
Tian, W. and Cohen, D. M. (2001). Signaling and gene regulation by urea in cells of the mammalian kidney medulla. *Comp. Biochem. Physiol.* **130A**, 429-436.
Umenishi, F., Yoshihara, S., Narikiyo, T. and Schrier, R. W. (2005). Effect on stability, degradation, expression, and targeting of aquaporin-2 water channel by hyperosmolality in renal epithelial cells. *Biochem. Biophys. Res. Commun.* **338**, 1593-1599.
Vajragupta, O., Pathomsakul, A., Matayatsuk, C., Ruangreangyingyod, L., Wongkrajang, Y. and Foye, W. O. (1996). Synthesis and antihypertensive activity of N-(alkyl/alkenyl/aryl)-N-heterocyclic ureas and thioureas. *J. Pharm. Sci.* **85**, 258-261.
Wang, X., Wu, L., Aouffen, M., Mateescu, M.-A., Nadeau, R. and Wang, R. (1999). Novel cardiac protective effects of urea: from shark to rat. *Br. J. Pharmacol.* **128**, 1477-1484.
Withers, P. C. (1998). Urea: diverse functions of a “waste” product. *Clin. Exp. Pharmacol. Physiol.* **25**, 722-727.
Withers, P. C. and Guppy, M. (1996). Do Australian desert frogs co-accumulate counteracting solutes with urea during aestivation? *J. Exp. Biol.* **199**, 1809-1816.
Wray, S. and Wilkie, D. R. (1995). The relationship between plasma urea levels and some muscle trimethylamine levels in *Xenopus laevis*: a ^{31}P and ^{14}N nuclear magnetic resonance study. *J. Exp. Biol.* **198**, 373-378.
Yancey, P. H. and Burg, M. B. (1990). Counteracting effects of urea and betaine in mammalian cells in culture. *Am. J. Physiol.* **258**, R198-R204.
Zhang, Z., Tian, W. and Cohen, D. M. (2000). Urea protects from the proapoptotic effect of NaCl in renal medullary cells. *Am. J. Physiol.* **279**, F345-F352.