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Urea loading enhances postfreeze performance of frog skeletal muscle

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Abstract The wood frog (*Rana sylvatica*) is a terrestrial hibernator that can accumulate urea as an osmoprotectant in autumn and winter. This study tested the hypothesis that elevated urea can also function as a cryoprotectant in this freeze-tolerant species. Performance characteristics (threshold stimulus voltage, maximal isometric twitch and tetanic contraction forces, and ½ fatigue time) of isolated gastrocnemius muscles were measured before and after experimental freezing at −1.5°C for 18 h, followed by thawing. Frozen/thawed muscles exhibited reduced function relative to baseline (prefreeze) levels; however, muscles preincubated in a saline solution containing urea (80 mmol l^{-1}) performed substantially better in some tests than muscles incubated without urea. Concentrations of urea in these treated muscles, ~65 mmol l^{-1}, were within the physiological range in winter *R. sylvatica*. Reducing tissue urea levels to ~33 mmol l^{-1} resulted in a similar pattern of response, although the differences between urea-incubated and saline-incubated muscles were not statistically significant. Tests of cryoprotective efficacy were also performed on gastrocnemius muscles from *R. pipiens*, a closely related, but freeze-intolerant species that hibernates aquatically and thus has little need to accumulate urea. Urea-treated muscles from this species performed no better than muscles incubated in saline, attesting that freeze tolerance cannot be conferred simply by augmenting cryoprotectant levels. Overall, these results bolster an earlier report that urea accumulated in response to low moisture availability can serve a cryoprotective role in freeze-tolerant ectotherms.

Keywords Cryoprotectant · Muscle · Freeze tolerance · Osmolyte · Wood frog · Leopard frog

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Introduction

Several species of frogs overwinter in temperate forests beneath a modest blanket of organic detritus. In northern regions, hibernating frogs become exposed to low environmental water potential and subzero cold, but they can survive these conditions by virtue of their tolerances to dehydration and somatic freezing. The wood frog (*Rana sylvatica*), which ranges farther north than any other anuran, tolerates extensive dehydration (Churchill and Storey 1993) and survives the freezing up to 65–70% of its body water at temperatures between −4 and −6°C (Storey and Storey 2004).

Natural freeze tolerance derives from various molecular, biochemical, and physiological responses that protect cells and tissues from osmotic and mechanical perturbations (Mazur 2004; Storey and Storey 2004). Certain organic osmolytes, or cryoprotectants, play a key role in freezing adaptation in diverse taxa. These agents colligatively lower the freezable fraction of body water and reduce cell dehydration and shrinkage, thereby limiting osmotic and mechanical injury to membranes and cellular infrastructure. In addition, cryoprotectants safeguard cellular functions by stabilizing membranes and intracellular macromolecules (Carpenter and Crowe 1988).

Cryoprotectants used by freeze-tolerant organisms constitute a diverse array of organic compounds (Yancey 2005), yet they share several important characteristics, including low molecular mass, high solubility and permeability, stability, ready availability, and compatibility with macromolecules (Storey and Storey 2004). Among amphibians, the better-known cryoprotectants are carbohydrates (glucose in *R. sylvatica*; glycerol and/or glucose in hylid tree frogs), which can accumulate to high concentrations during the early stages of freezing. Experimentally treating in vitro tissues and intact animals with exogenous cryoprotectants enhances freezing survival at cell, organ, and whole-animal levels of organization, providing strong evidence for their protective role in freeze tolerance (Costanzo et al. 1995).

Because terrestrially hibernating frogs potentially are exposed to dehydrating conditions (Costanzo and Lee 2005), and because urea accumulation is a universal amphibian response to hydro-osmotic challenge (e.g., Shpun et al. 1992; Jørgensen 1997), it is possible that this osmolyte, which possesses many of the physicochemical characteristics of cryoprotectants, contributes to freezing survival. In an initial test of this hypothesis, Costanzo and Lee (2005) found that experimentally treating *R. sylvatica* cells and tissues with urea limited in vitro freezing injury manifested as metabolic impairment and post-thaw leakage of intracellular proteins. However, the question of whether urea can preserve the more complex functions of organs and organ systems remains open. The current study extends these earlier findings by demonstrating urea's efficacy as a cryoprotectant in limiting freeze/thaw impairment of skeletal muscle performance.

Materials and methods

Animals and acclimatization

Male wood frogs (*Rana sylvatica*) were collected in February 2004 from a breeding congregation in southern Ohio, USA. They were kept outdoors from spring through early autumn in a 48-m^2 pen at the Ecology Research Center, Miami University. Frogs were provided with a pool of water and were fed crickets 2–3 times each week. In late October, near the onset of hibernation, feeding was suspended and the frogs were recaptured, brought to the laboratory, and kept in darkness at 4°C in opaque plastic boxes containing wet moss. In early March 2005, they were shipped under refrigeration to Eastern Illinois University where they were kept under similar conditions until used in experiments.

Northern leopard frogs (*Rana pipiens*) were used in companion experiments. Unlike the case with *R. sylvatica*, this species overwinters underwater, has little need to accumulate urea, and lacks freeze tolerance. Male *R. pipiens* were obtained in autumn from a commercial supplier (Charles D. Sullivan Co, Inc, Nashville, TN, USA), housed with water but without food, and acclimated at 5–6°C for 2–4 weeks before use in experiments.

Muscle isolation and performance testing

Frogs were euthanized by decapitation followed by spinal pithing. The hind limbs were skinned and the resting length of both gastrocnemius muscles was measured to the nearest millimeter. Each muscle was carefully separated from the tibia after inserting a hook through the Achilles tendon and cutting the tendon distal to the hook. The tibia was then severed below the knee and the femur was cut near the pelvis. Both muscles were rinsed and incubated for 1 h in ice-cold, aerated physiological saline (PS; 8.0 g NaCl, 0.14 g KCl, 2.0 g MOPS, 0.12 g $CaCl_2$, and 0.60 g glucose l^{-1}).

Muscle performance characteristics were measured at room temperature (~23°C) using a modification of the procedures described by Hillman (1982) and Layne (1993). Stimuli were delivered, and responses were recorded and analyzed, using a PowerLab data acquisition system (ADInstruments, Inc., Colorado Springs, CO, USA). Each muscle was suspended in air by securing the truncated femur in a clamp and attaching the distal tendon, via the hook and a thread ligature, to the blade of a force transducer (ADInstruments, Inc., model FT-100). The muscle was gently stretched to the measured resting length under optimal tension (~11 g). A pair of stimulating electrodes was inserted into the muscle belly at right angles to each other, such that virtually all fibers could be stimulated simultaneously. Threshold stimulus voltage was determined by gradually increasing the intensity of single-pulse stimuli (100-mV increments; 10-ms duration; 10-s intervals) until a contraction was elicited. Gradually increasing the single-pulse stimulus intensity further increased contractile force until the maximum twitch amplitude was reached. To determine the maximum amplitude of tetanus, the frequency of stimulation was increased (using the same maximal stimulus intensity) from 10 Hz, in 5-Hz increments, until the muscle achieved complete tetanus (~30 Hz). Finally, the time elapsed before reaching 50% decay in force generation (½ fatigue time) was determined whilst the muscle was in complete tetanus. Muscles were kept moist by occasionally rinsing them with PS throughout the testing period, which lasted no more than 5 min.

Following Hillman (1982), we normalized all tension values to muscle cross-sectional area. Computations assumed a tissue density of 1 g cm^{-3} and were made after adjusting each value of muscle mass (see below for details of mass measurements) to the equivalent mass of a fully hydrated muscle (80% fm) to correct for slight variations in water concentration. Therefore, the computed

cross-sectional area represented the average cross-sectional area of each muscle in the fully hydrated state.

Urea loading and experimental freezing/thawing

Immediately following these baseline performance tests, muscles were disconnected from the force transducer and one muscle from each pair was incubated in ice-cold PS (saline-incubated) or PS containing 80 or 40 mmol urea l^{-1} (urea-incubated). After 4 h, muscles were removed from the solutions, individually wrapped with a 3 × 3 cm piece of laboratory tissue paper, and wetted with PS, or PS containing urea, as appropriate. Muscles were inserted into separate glass tubes, which were placed upright inside an insulated beaker immersed in a refrigerated bath. During cooling, temperature was monitored with a type T thermocouple placed against each muscle.

Muscles were cooled from 4 to −1.5°C at a rate of 4–5°C h^{-1}. After remaining at the target temperature for 1 h, freezing of the tissue was induced by adding a small piece of ice to each tube. Muscles were kept at −1.5°C for 18 h and were thoroughly frozen; equilibrium ice content of muscles treated in this manner averages 72% (Layne 1993). Afterwards, they were soaked in ice-cold PS, or PS containing urea (as appropriate), for 1 h to permit thawing. Next, they were bathed in identical solutions at room temperature for 10 min and then immediately used in performance trials as described earlier. Following these trials, muscles were isolated from tendons and bone and blotted dry. They were measured to determine equilibrium length (nearest millimeter) and mass (nearest 0.01 g), and then frozen in liquid N_2 for subsequent analyses.

Tissue water and urea concentrations

The water content of each muscle was estimated using the gravimetric method. After the muscles were thawed on ice, a small portion (~20%) was weighed and then thoroughly dried in a 60°C-oven. The concentration of water in the fresh sample was calculated from the change in mass during drying and expressed as a percentage of total mass. Percentage data were transformed (arcsine-square root) before being used in statistical analyses.

Extracts of the muscle tissue were prepared according to Barany and Glonek (1982). Briefly, frozen muscles were finely pulverized under liquid N_2 and dissolved with stirring in cold 70% $HClO_4$. The supernatant resulting from centrifuging the mixture (43,000*g*, 4°C, 15 min) was neutralized with 5 M KOH and, following adjustment of the pH to 8–9, centrifuged to precipitate the $KCLO_4$. The clear, deproteinized extract was kept frozen at −60°C until analyzed. Urea concentration was measured in triplicate using a colorimetric procedure (BioAssay Systems, QuantiChrom™ Urea Assay Kit, DIUR-500). An average value was calculated for each sample and expressed as millimoles per liter of tissue water.

Control experiments

Control experiments were undertaken to determine the effect of in vitro cold storage and baseline performance testing on muscle integrity. In one experiment, baseline performance levels in both gastrocnemius muscles harvested from two *R. sylvatica* were determined. Subsequently, the muscles were removed from the apparatus and incubated at ~4°C for 12 h before being used in a final performance trial. One muscle of each pair was incubated in PS; the other was incubated in PS containing urea (80 mmol l^{-1}).

Another experiment was designed to reveal any effect of the baseline (prefreeze) testing on the postfreeze performance characteristics of isolated muscles. Both gastrocnemius muscles were isolated from *R. sylvatica* ($n = 3$) as described earlier, but only one muscle of each pair was subjected to performance testing. Afterwards, the muscle was removed from the apparatus and, together with the untested muscle (which for the same period also had been held in air) was incubated for 4 h in cold PS and frozen/thawed as described previously. Both muscles were then used in a final performance test so that the effect of prefreeze testing could be determined by comparing results for the muscles in each pair.

Statistical inferences

Data are presented as mean ± 1 SEM. Performance data collected from muscles before and after freezing/thawing were separately analyzed. In each set of analyses, matched values for each performance variable were compared using the dependent-measures *t* test. Extent of cryoinjury was determined by computing for each response variable the percentage change in performance associated with freezing/thawing; Wilcoxon matched-pairs signed-ranked tests were used to compare these values between saline-incubated and urea-incubated muscles. Additionally, performance values for baseline (prefreeze) and frozen/thawed muscles were compared between saline-incubated and urea-incubated groups using dependent-measures *t* tests. Statistical significance was accepted at $P < 0.05$.

Results

Postfreeze performance of muscles incubated in 80 mmol urea l^{-1}

For *R. sylvatica* muscles tested before freezing/thawing, there was no statistically significant difference between

paired gastrocnemius muscles for any of the measured performance parameters ($P > 0.70$, all cases). Therefore, an average of the two values was taken to represent the baseline muscle responses for each frog. Mean (±1 SEM; $n = 8$) values were as follows: threshold stimulus voltage, 0.4 ± 0.1 V; maximum twitch amplitude, 17.4 ± 2.4 mN mm^{-2}; maximum tetanus amplitude, 52.7 ± 3.7 mN mm^{-2}; and ½ fatigue time, 54.1 ± 7.4 s.

Incubation of *R. sylvatica* muscles in PS, or PS containing urea (80 mmol l^{-1}), followed by freezing/thawing strongly affected their isometric contractile properties (Fig. 1). Relative to baseline responses, frozen/thawed muscles were less excitable (indicated by a rise in threshold stimulus voltage) and generated less force in both twitch and tetanic contractions. After freezing/thawing, values for ½ fatigue time were two- to three-fold higher than that before, suggesting that resistance to fatigue increased; however, as will be discussed in the following, this result probably is anomalous. Although freezing/thawing clearly impaired the performance of muscles in both groups, muscles pretreated with urea generally retained a higher level of competence (Table 1).

With *R. pipiens* muscles tested before freezing/thawing, there was no difference between paired muscles in any performance parameter ($P > 0.20$, all cases); thus, the average of the two values was taken to represent the baseline muscle response for each frog. Mean (±1 SEM; $n = 8$) values were as follows: threshold stimulus voltage, 0.4 ± 0.02 V; maximum twitch amplitude, 23.4 ± 2.6 mN mm^{-2}; maximum tetanus amplitude, 55.4 ± 7.9 mN mm^{-2}; and ½ fatigue time, 32.4 ± 4.9 s.

Freezing/thawing strongly affected the function of *R. pipiens* muscles. In fact, to collect usable data from eight animals, it was necessary to replace some frogs, because after freezing/thawing, one ($n = 3$) or both ($n = 2$) of their muscles failed to respond to stimulation by up to 10 V. Relative to baseline metrics, frozen/thawed muscles were much less excitable, as stimulation intensity had to be increased approximately ten-fold to elicit a contraction. In addition, twitch and tetanus contractile strength was reduced to ≤15% of baseline values, although ½ fatigue time was essentially the same for muscles in prefreeze and postfreeze conditions. The magnitude of change with freezing/thawing did not differ between saline-incubated and

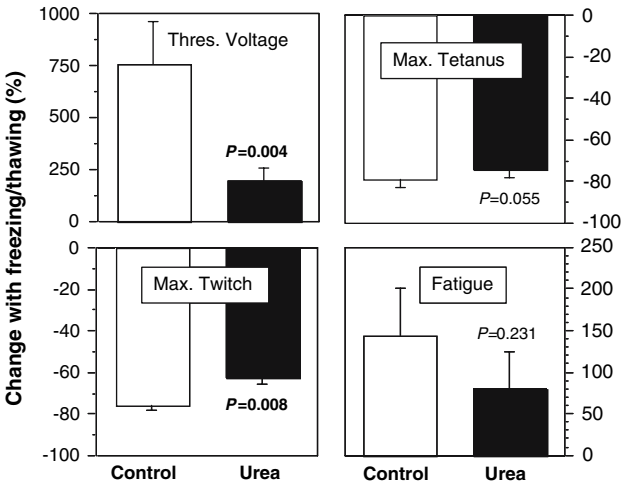

Fig. 1 Change in muscle performance associated with freezing/thawing after incubating isolated *R. sylvatica* muscles for 4 h in ice-cold physiological saline, or physiological saline containing 80 mmol urea l^{-1}. Response variables include threshold stimulus voltage, maximum twitch amplitude, maximum tetanus amplitude, and ½ fatigue time. Paired means (±1 SEM; $n = 8$) were compared using Wilcoxon matched-pairs signed ranks tests

Table 1 Performance characteristics of frog gastrocnemius muscles following incubation in physiological saline, or physiological saline containing urea, and in vitro freezing/thawing

Parameter	Saline incubated	Urea incubated	*t*	*P*
Rana sylvatica, 80 mmol urea l^{-1}				
Threshold stimulus voltage (V)	2.2 ± 0.4	1.0 ± 0.2	3.40	**0.006**
Maximum twitch amplitude (mN mm^{-2})	4.1 ± 0.5	6.1 ± 0.9	4.10	**0.002**
Maximum tetanus amplitude (mN mm^{-2})	10.9 ± 2.0	14.0 ± 2.6	1.50	0.088
½ Fatigue time (s)	150.1 ± 49.6	104.5 ± 28.0	1.42	0.099
Rana pipiens, 80 mmol urea l^{-1}				
Threshold stimulus voltage (V)	5.1 ± 0.8	3.4 ± 0.6	1.42	0.099
Maximum twitch amplitude (mN mm^{-2})	1.8 ± 0.6	3.5 ± 1.4	1.77	0.060
Maximum tetanus amplitude (mN mm^{-2})	4.5 ± 1.5	11.4 ± 5.7	1.58	0.079
½ Fatigue time (s)	24.3 ± 4.1	31.7 ± 10.9	0.93	0.192
Rana sylvatica, 40 mmol urea l^{-1}				
Threshold stimulus voltage (V)	1.6 ± 0.4	1.1 ± 0.2	1.43	0.098
Maximum twitch amplitude (mN mm^{-2})	5.5 ± 0.7	5.9 ± 0.8	0.52	0.310
Maximum tetanus amplitude (mN mm^{-2})	15.9 ± 2.3	17.4 ± 3.0	0.49	0.320
½ fatigue time (s)	110.0 ± 44.5	85.3 ± 39.6	0.67	0.261

Means (±1 SEM; $n = 8$) in each row were compared using the dependent-measures *t* test

urea-incubated muscles for threshold stimulation voltage ($W_+ = 24.0$, $P = 0.23$), maximum tetanus amplitude ($W_+ = 26.0$, $P = 0.16$), or ½ fatigue time ($W_+ = 17.0$, $P = 0.47$). The change in maximum twitch amplitude appeared less severe in urea-incubated muscles; however, the difference was not quite statistically significant ($W_+ = 29.5$, $P = 0.055$). Following freezing/thawing, urea-incubated muscles performed no better than their saline-incubated counterparts (Table 1).

Postfreeze performance of muscles incubated in 40 mmol urea l^{-1}

Cryoprotective efficacy of a lower urea concentration was investigated by repeating the muscle performance experiment in essentially the same manner, except that the urea-incubated muscle from each pair was incubated in PS containing 40 mmol urea l^{-1} before freezing. In baseline testing of the muscle pairs, there was no difference in performance for any of the parameters studied ($P > 0.20$, all cases); thus, the average of the two values was taken to represent the prefreeze muscle response for each frog. Mean (±1 SEM; $n = 8$) values were as follows: threshold stimulus voltage, 0.2 ± 0.03 V; maximum twitch amplitude, 20.5 ± 1.5 mN mm^{-2}; maximum tetanus amplitude, 53.9 ± 4.2 mN mm^{-2}; and ½ fatigue time, 34.2 ± 6.5 s.

Changes in muscle performance associated with freezing/thawing were comparable to those observed in our other experiments and were not impacted by urea treatment (Fig. 2). Comparisons between saline-incubated and urea-incubated muscles showed that, although the latter were somewhat more competent in performance tests, the differences were not statistically significant (Table 1).

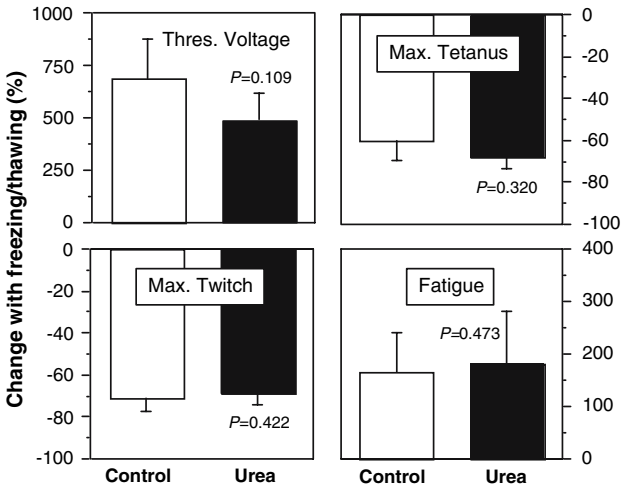

Fig. 2 Change in muscle performance associated with freezing/thawing after incubating isolated *R. sylvatica* muscles for 4 h in ice-cold physiological saline, or physiological saline containing 40 mmol urea l^{-1}. Response variables include threshold stimulus voltage, maximum twitch amplitude, maximum tetanus amplitude, and ½ fatigue time. Paired means (±1 SEM; $n = 8$) were compared using Wilcoxon matched-pairs signed ranks tests

Tissue concentrations of water and urea

The mean water content of urea-incubated muscles from *R. sylvatica* was slightly, albeit significantly, lower than that for corresponding saline-incubated muscles (Table 2). However, no such difference was found for *R. pipiens* muscles.

The typical urea concentration in saline-incubated muscles was 3–6 mmol l^{-1}. Incubation of isolated muscles in PS containing 80 mmol urea l^{-1} raised the concentration by ~60 mmol l^{-1} in both *R. sylvatica* and *R. pipiens* (Table 2).

Table 2 Attributes of frog gastrocnemius muscles used in performance tests following incubation in physiological saline, or physiological saline containing urea, and in vitro freezing/thawing

	Saline-incubated	Urea-incubated	*t*	*P*
Rana sylvatica, 80 mmol urea l^{-1}				
Dry mass (mg)	51 ± 3	55 ± 5	1.20	0.269
Fresh mass (mg)	207 ± 9	202 ± 1	1.05	0.331
Water content (% fm)	75.5 ± 0.6	73.1 ± 1.3	2.50	**0.041**
Urea (mmol l^{-1})	3.6 ± 0.2	67.0 ± 2.0	31.90	**<0.0001**
Rana pipiens, 80 mmol urea l^{-1}				
Dry mass (mg)	179 ± 69	189 ± 80	0.88	0.409
Fresh mass (mg)	501 ± 125	475 ± 119	3.44	**0.011**
Water content (% fm)	79.0 ± 0.7	77.7 ± 1.3	1.22	0.262
Urea (mmol l^{-1})	4.7 ± 0.5	49.9 ± 2.6	18.31	**<0.0001**
Rana sylvatica, 40 mmol urea l^{-1}				
Dry mass (mg)	44 ± 3	45 ± 4	0.37	0.723
Fresh mass (mg)	186 ± 10	187 ± 11	0.17	0.870
Water content (% fm)	76.0 ± 1.4	76.0 ± 1.2	0.06	0.955
Urea (mmol l^{-1})	4.9 ± 0.7	33.4 ± 1.2	21.86	**<0.0001**

Means (±1 SEM; $n = 8$) in each row were compared using the dependent-measures *t* test

Effect of cold storage, urea loading, and prefreeze testing

Incubating isolated *R. sylvatica* muscles in cold PS, or PS containing urea (80 mmol l^{-1}), for 12 h was associated with a decrease in all measures of muscle performance. However, the magnitude of these changes was little affected by urea loading (Fig. 3).

Subjecting an isolated muscle to baseline performance testing clearly influenced its contractile properties. Compared to muscles that were not tested, muscles used in baseline tests prior to freezing/thawing tended to be less sensitive to stimulation, generated less force in twitch and tetanic contractions, and fatigued more quickly (Fig. 4). Thus, baseline testing probably accounted for a portion of the measured decrease in performance of the frozen/thawed muscles in our cryoprotection experiments.

Discussion

The role of urea in amphibian osmoregulation has received considerable study over the past several decades (for a review, see Jørgensen 1997). As a general rule, amphibians tend to accumulate urea when under osmotic challenge, such as occurs with salt exposure, dehydration, and estivation (Shpun et al. 1992). Terrestrial hibernators, such as *R. sylvatica*, can accumulate urea during autumn and early winter (Layne and Rice 2003; Costanzo and Lee 2005), because environmental moisture is scarce and renal excretion is depressed by low temperature (Nielsen and Jørgensen 1990). This study addressed the question of whether this well-known osmoprotectant can also function as a cryoprotectant in species that rely on freeze tolerance for winter survival.

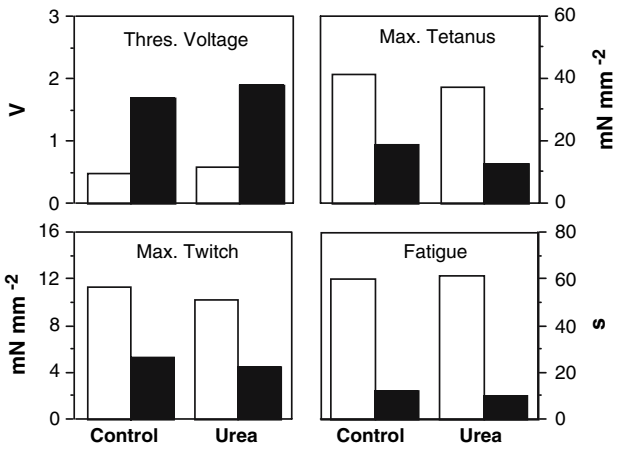

Fig. 3 Decrease in muscle performance associated with incubating isolated *R. sylvatica* muscles for 12 h in ice-cold physiological saline, or physiological saline containing 80 mmol urea l^{-1}. Response variables include threshold stimulus voltage, maximum twitch amplitude, maximum tetanus amplitude, and ½ fatigue time. *Bars* represent the average of two replicate experiments. Initial (time zero) responses are shown by *white bars*; final (12 h) responses are shown by *black bars*

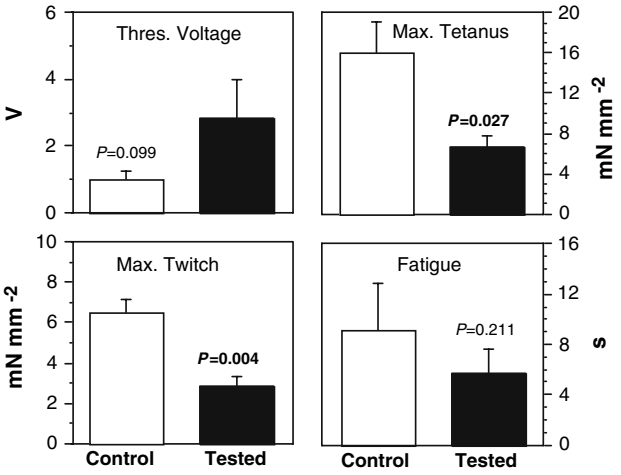

Fig. 4 Decrease in muscle performance associated with subjecting isolated *R. sylvatica* muscles to baseline testing prior to incubation in physiological saline and experimental freezing/thawing. Response variables include threshold stimulus voltage, maximum twitch amplitude, maximum tetanus amplitude, and ½ fatigue time. Paired means (±1 SEM; $n = 3$) were compared using dependent-measures t tests

In an earlier investigation, Costanzo and Lee (2005) reported that concentrations of urea (40–76 mmol l^{-1}) potentially found in winter *R. sylvatica* improved the viability of cells and tissues frozen/thawed in vitro at ecologically relevant temperatures. Measures of post-thaw integrity, based on rates of metabolic activity and/or LDH leakage, attested that urea limited freezing injury in heart, liver, and skeletal muscle (albeit not in kidney, which exhibited a high, innate resistance to cryoinjury). However, these experiments did not determine whether urea could also preserve the more complex functions of organs and organ systems.

The primary aim in this study was to compare measures of postfreeze muscle function between saline-incubated muscles, which exhibited relatively low concentrations of urea, typical of hydrated frogs (Costanzo and Lee 2005), and muscles augmented with exogenous urea. In one experiment, urea loading decreased tissue water concentration slightly, although this outcome probably had no effect on isometric tension development in the affected muscles (Howarth 1958; McClanahan 1964; Cleworth 1967). All muscles exhibited diminished performance after freezing/thawing; however, muscles pretreated with urea were more competent, suggesting that physiological levels of urea can limit cryoinjury.

It is noteworthy that the observed loss of muscle function was not solely due to freezing/thawing. Control experiments indicated that diminished muscle function resulted partly from *ex vivo* storage (Fig. 3; see also Cleworth 1967; Layne 1993) and partly from the initial testing (Fig. 4),

which potentially led to an unresolved accumulation of lactate and P_i, and depletion of phosphocreatine and glycogen. Postfreeze performance might have been improved had our muscles been tested whilst immersed in physiological saline. Furthermore, any impairment caused by hypoxia and/or lactacidosis might have been ameliorated had we tested muscles in the cold, rather than at room temperature. Despite these methodological limitations, it seems clear that the higher level of contractile performance exhibited by urea-incubated versus saline-incubated muscles reflected a measure of cryoprotection afforded by urea.

Freezing and thawing cause myriad changes in muscle biochemistry and ultrastructure that can lead to contractile dysfunction (Chambers and Hale 1932; Walter 1970). In this study, cryoinjury was manifested as a lower sensitivity to electrical stimulation and reduced twitch and tetanic contractile strength. Such impairment has been reported previously for frog muscles frozen/thawed in vitro (Moran 1929; Miller and Dehlinger 1969; Layne and First 1991) and in vivo (Layne 1992; but see Layne and First 1991); this effect may underlie the compromised locomotor function exhibited by intact frozen/thawed frogs (Costanzo et al. 1997; Irwin et al. 2003; Layne and Rice 2003). At the cellular level, diminished function potentially stems from an increase in P_i, and concomitant decrease in ATP, creatine phosphate, and intracellular pH, associated with tissue freezing and the accompanying anoxia (Storey and Storey 2004). Although a relatively mild freezing exposure little affects the energetic status of intact frogs (Layne and Kennedy 2002), muscles frozen/thawed in vitro would be more susceptible to this form of damage.

Irwin et al. (2003) found that in vivo freezing/thawing caused exercising *R. sylvatica* to fatigue sooner as compared to unfrozen animals. In contrast, our present results showed that experimental freezing/thawing resulted in longer ½ fatigue times in both saline-incubated and urea-incubated muscles, suggesting that they became more resistant to fatigue. We suspect this outcome is an anomaly caused by partial tetanus, a possible consequence of impaired calcium pumps and/or Ca^{2+} leaking from the sarcoplasmic reticulum. This damage can be attributed directly to freezing/thawing, as the expected reduction in endurance was observed in muscles subjected to cold, unfrozen storage (Fig. 3) and baseline performance testing (Fig. 4).

In contrast to our findings for *R. sylvatica*, our results offered little evidence that urea protects *R. pipiens* muscles from cryoinjury. Indeed, one or both muscles from 5 of 13 frogs (38.5%) were unresponsive to electrical stimulation after freezing/thawing; consequently, the performance metrics presented in Table 1 markedly overestimate populational characteristics of muscle function following experimental freezing/thawing. Given that urea concentrations in treated *R. pipiens* muscles were similar to those in *R. sylvatica* muscles, the lack of protection afforded to the former could reflect a reduced cryprotective efficacy and/or innate differences in freeze tolerance between these species (Layne 1992, 1993). As an aquatic hibernator (Emery et al. 1972; Cunjak 1986), *R. pipiens* has not evolved as an extensive capacity for freeze tolerance (Layne 1992; Costanzo et al. 1993) and this attribute is not conferred simply by raising internal levels of cryoprotectants (Costanzo et al. 1993).

Putative mechanism of action

Cryoprotective osmolytes help maintain cell volume and fortify membranes and intracellular macromolecules against ionic and osmotic perturbations (Storey and Storey 2004; Yancey 2005). Given that urea possesses the same attributes that render certain organic osmolytes well-suited as cryoprotectants, we hypothesized that urea accumulated as an osmoprotectant during autumn and early winter could also protect frogs from the deleterious effects of freezing/thawing that arise later in hibernation.

In this study, incubating muscles in the presence of 80 mmol urea l^{-1} raised the internal urea concentration to ~65 mmol l^{-1} tissue water, which is within the physiological range in winter *R. sylvatica* (Costanzo and Lee 2005). Perhaps muscles accumulating more urea would show an even greater postfreeze vitality. Although we did not test that possibility, we did investigate the cryoprotective efficacy of a lower urea concentration. Incubating muscles in PS containing 40 mmol urea l^{-1} raised the internal urea concentration to a level only one-half as high and, although the results of the postfreeze performance trials were in the expected direction, for no parameter was the nominally greater competence of urea-incubated muscles statistically significant (Table 1). This outcome suggests that the urea concentration in these muscles, ~33 mmol l^{-1}, was too low to elicit more than a subtle effect and indicates that this solute protects tissues through colligative means. On the other hand, Costanzo and Lee (2005) reported that treating *R. sylvatica* erythrocytes with 40 or 80 mmol urea l^{-1} afforded essentially the same degree of cryoprotection. This finding suggested that urea's mechanism of action is not purely colligative, but also involves protection of macromolecules and cellular structures (Carpenter and Crowe 1988). Contrary to its well-known denaturing properties in high (i.e., molar) concentrations, in more moderate concentrations, urea may be less perturbing than some "compatible" osmolytes and, indeed, may even stabilize proteins (Bhuyan 2002; Kumar et al. 2004).

Amphibian freeze tolerance, an adaptation for surviving winter within the frost zone, was first recognized some 25 years ago (Schmid 1982). Subsequent studies have focused on the underlying molecular, biochemical, and

physiological mechanisms of freezing survival, and much attention has been paid to the role of cryoprotectants, especially glucose, that are mobilized in response to tissue freezing (Canty et al., 1986; Costanzo et al. 1993). Findings of this study not only suggest that urea accumulated before freezing can contribute to freezing survival, but also that freeze-tolerant frogs use more than one class of cryoprotective agent. Some evidence suggests that urea is of equal or greater efficacy and, under certain circumstances, may accumulate to higher levels than glucose (Costanzo and Lee 2005); however, from a colligative standpoint, a combination of both solutes would provide the greatest cryoprotection. We suspect that the putative cryoprotective role of urea has been overlooked until only recently, probably because frogs destined for use in laboratory experiments are commonly kept in humid surroundings and thus retain little urea. In nature, however, environmental conditions in autumn and early winter can elicit an accumulation of urea to levels that offer a measure of protection from cryoinjury.

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References

Barany M, Glonek T (1982) Phosphorus-31 nuclear magnetic resonance of contractile systems. In: Frederiksen DW, Cunningham LW (eds) Methods enzymol. Academic Press, San Francisco, pp 624–676

Bhuyan AK (2002) Protein stabilization by urea and guanidine hydrochloride. Biochemistry (Mosc) 41:13386–13394

Canty A, Driedzic WR, Storey KB (1986) Freeze tolerance of isolated ventricle strips of the wood frog, *Rana sylvatica*. Cryo-Letters 7:81–86

Carpenter JF, Crowe JH (1988) The mechanism of cryoprotection of proteins by solutes. Cryobiology 25:244–255

Chambers R, Hale HP (1932) The formation of ice in protoplasm. Proc R Soc Lond B Biol Sci 110:336–352

Churchill TA, Storey KB (1993) Dehydration tolerance in wood frogs: a new perspective on development of amphibian freeze tolerance. Am J Physiol 265:R1324–R1332

Cleworth DR (1967) A comparative study on the effects of urea on contraction in skeletal muscle. Ph.D. Dissertation, University of California, Los Angeles, Los Angeles, p 71

Costanzo JP, Irwin JT, Lee RE (1997) Freezing impairment of male reproductive behaviors of the freeze-tolerant wood frog, *Rana sylvatica*. Physiol Zool 70:158–166

Costanzo JP, Lee RE (2005) Cryoprotection by urea in a terrestrially-hibernating frog. J Exp Biol 208:4079–4089

Costanzo JP, Lee RE, DeVries AL, Wang T, Layne JR (1995) Survival mechanisms of vertebrate ectotherms at subfreezing temperatures: applications in cryomedicine. FASEB J 9:351–357

Costanzo JP, Lee RE, Lortz PH (1993) Physiological responses of freeze-tolerant and -intolerant frogs: clues to evolution of anuran freeze tolerance. Am J Physiol 265:R721–R725

Cunjak RA (1986) Winter habitat of northern leopard frogs, *Rana pipiens*, in a southern Ontario stream. Can J Zool 64:255–257

Emery AR, Berst AH, Kodaira K (1972) Under-ice observations of wintering sites of leopard frogs. Copeia 1972:123–126

Hillman SS (1982) The effects of in vivo and in vitro hyperosmolality on skeletal muscle performance in the amphibians *Rana pipiens* and *Scaphiopus couchii*. Comp Biochem Physiol A 73:709–712

Howarth JV (1958) The behavior of frog muscle in hypertonic solutions. J Physiol (Lond) 144:167–175

Irwin JT, Costanzo JP, Lee RE (2003) Postfreeze reduction of locomotor endurance in the freeze-tolerant wood frog, *Rana sylvatica*. Physiol Biochem Zool 76:331–338

Jørgensen CB (1997) Urea and water economy. Comp Biochem Physiol A 117:161–170

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

Layne JR Jr (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 JR Jr (1993) Postfreeze recovery of gastrocnemius muscles of *Rana pipiens* and *R. sylvatica*. J Herpetol 27:478–480

Layne JR Jr, First MC (1991) Resumption of physiological functions in the wood frog (*Rana sylvatica*) after freezing. Am J Physiol 261:R134–R137

Layne JR Jr, Kennedy SD (2002) Cellular energetics of frozen wood frogs (*Rana sylvatica*) revealed via NMR spectroscopy. J Therm Biol 27:167–173

Layne JR Jr, Rice ME (2003) Postfreeze locomotion performance in wood frogs (*Rana sylvatica*) and spring peepers (*Pseudacris crucifer*). Can J Zool 81:2061–2065

Mazur P (2004) Principles of cryobiology. In: Fuller BJ, Lane N, Benson EE (eds) Life in the frozen state. CRC Press, Washington, DC, pp 3–65

McClanahan L (1964) Osmotic tolerance of the muscles of two desert-inhabiting toads, *Bufo cognatus* and *Scaphiopus couchi*. Comp Biochem Physiol 12:501–508

Miller LK, Dehlinger PJ (1969) Neuromuscular function at low temperatures in frogs from cold and warm climates. Comp Biochem Physiol 28:915–921

Moran T (1929) Critical temperature of freezing—living muscle. Proc R Soc Lond B Biol Sci 105:177–197

Nielsen KH, Jørgensen CB (1990) Salt and water balance during hibernation in anurans. In: Hanke W (ed) Biology and physiology of amphibians. Gutav Fischer Verlag, New York, pp 333–349

Schmid WD (1982) Survival of frogs in low temperature. Science 512:697–698

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

Storey KB, Storey JM (2004) Physiology, biochemistry, and molecular biology of vertebrate freeze tolerance: The wood frog. In: Fuller BJ, Lane N, Benson EE (eds) Life in the frozen state. CRC Press, Washington, DC, pp 243–274

Walter CA (1970) Ultrastructural and functional changes in smooth muscle associated with freezing and thawing. In: Wolstenholme GEW, O'Connor M (eds) The frozen cell. J. & A. Churchill, London, pp 271–293

Yancey PH (2005) Organic osmolytes as compatible, metabolic and counteracting cytoprotectants in high osmolarity and other stresses. J Exp Biol 208:2819–2830