

Post-hibernation Excretion of Glucose in Urine of the Freeze Tolerant Frog *Rana sylvatica*

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Wood frogs (*Rana sylvatica*) rely on a combination of adaptations to ensure survival of internal freezing (Lee and Costanzo, 1993; Layne and Lee, 1995). Extensive degradation of liver glycogen rapidly follows the onset of tissue freezing in these frogs, leading to massive hyperglycemia that protects their tissues from cryoinjury (Storey and Storey, 1985). It has been well documented that the magnitude of glucose mobilization directly correlates with the level of freeze tolerance developed by wood frogs at both tissue (Canty et al., 1986; Costanzo and Lee, 1991) and organismal levels (Costanzo et al., 1993; Layne, 1995). Upon thawing, glucose is slowly processed by the liver and stored via glycogenesis, but this process typically takes several days for completion (Storey and Storey, 1985). Because wood frogs reanimate major organ systems just hours after the onset of thawing (Layne and First, 1991), their bodily functions resume while body fluids still are substantially hyperglycemic.

Postfreeze urine composition has not been examined previously in these frogs. Such data are of particular interest since under normal physiological conditions glucose is readily reabsorbed from the renal filtrate, whereas excessive hyperglycemia causes the appearance of glucose in the urine (glucosuria) when-

ever the renal threshold is exceeded (Forester, 1942). Since unchecked glucosuria in thawing wood frogs would constitute a costly loss of glucose, it is reasonable to expect that they would be expected to avoid glucosuria altogether.

Adult wood frogs (N = 9) were collected from Ontario Co., New York during September and October, 1990. For the next 2-4 wk, frogs were maintained at room temperature (20 to 25 C). The frogs then were transferred to 15 C for 1 wk and step-acclimated (5 C/wk) to 5 C. Experiments on these 'winter frogs' were performed in December. In contrast, 'spring frogs' (N = 12) were collected from vernal breeding ponds in Athens Co., Ohio during February and March, 1990. These frogs were kept unfed at 5 C and used within two weeks of their capture. Freezing experiments were performed at -2.0 to -2.5 C in a Neslab RTE 210A refrigerated bath. All freezing episodes lasted 24 h.

The effect of freeze exposure/cryoprotectant mobilization on urine composition was studied by thawing frozen frogs on paper towels saturated with water at 20 C. After 4 and 24 h (spring frogs only) of thawing each frog was lightly blotted dry and a urine sample was collected by cannulating its bladder. A second group of frogs was quickly thawed by placing them in water (23 C) for 5 min. After the frogs had been killed by pithing, blood was drawn from an incision made in their hearts.

Recovering frogs produced urine in varying amounts. Quantitative measurements of urine volume were not taken; however, each frog yielded at least 50 microliters of urine and often much more than this amount. Plasma and urine glucose were assayed using a spectrophotometric assay (Sigma #510 glucose kit), and urine osmolality was determined with a Precision Instruments 5004 Osmometer. Urine Na⁺ and K⁺ concentrations were measured (winter frogs only) using a flame photometer (Instrumentation Laboratory Model 943).

Paired sample t-tests were used to compare groups where the same animals had been tested repeatedly (winter frogs), whereas, independent sample t-tests were used for comparisons of different groups (e.g., glucose concentration of blood). Finally, comparisons of glucose concentration and osmolality of urine from spring frogs were done using a repeated measures ANOVA and Tukey tests.

Frogs varied considerably in their plasma glucose values (Table 1). Seasonal conditioning of the frogs accounted for much of this variation; the winter frogs mobilized over three times as much glucose (independent sample t test: $t = 4.70$, $P < 0.001$, $df = 10$) as did spring frogs.

The composition of the urine changed substantially in response to freezing (Table 1). Its glucose concentration and osmolality in winter frogs increased 800 × (paired t test: $t = 7.081$, $P < 0.006$, $df = 3$) and 3 × (paired t test: $t = 8.17$, $P < 0.005$, $df = 3$), respectively, during the transition from the control condition to the 4 h thaw. However, the concentrations of sodium and potassium in the urine were essentially unchanged (paired t test, Na: $t = 2.96$, $P > 0.05$, $df = 3$; paired t test, K: $t = 0.92$, $P > 0.42$, $df = 3$) between these two sample periods. The composition of urine from spring frogs also showed differences among control and freeze treatments with respect to glucose concentration (repeated measures ANOVA: $F = 67.91$,

TABLE 1. The composition of urine and blood from unfrozen (control) and thawed wood frogs. Means \pm one standard deviation are reported for each parameter.

	Osmolality (mOsmolal)	Glucose (mM)	Sodium (mM)	Potassium (mM)
Urine				
Winter				
Control	39.0 \pm 8.7	0.1 \pm 0.1	4.6 \pm 1.2	0.2 \pm 0.2
4 H thaw	124.5 \pm 13.3	80.4 \pm 22.7	8.4 \pm 1.8	0.8 \pm 0.6
Spring				
Control	49.8 \pm 14.2	0.2 \pm 0.5	—	—
4 H thaw	112.7 \pm 14.6	43.9 \pm 10.3	—	—
24 H thaw	49.7 \pm 20.1	1.4 \pm 2.4	—	—
Plasma				
Control ¹	—	2.8 \pm 0.5	—	—
Frozen winter	—	88.2 \pm 29.7	—	—
Frozen spring	—	24.1 \pm 16.4	—	—

¹ This value is from Layne and Lee, 1987.

$P < 0.0009$, $df = 8$) and osmolality (repeated measures ANOVA: $F = 9.18$, $P < 0.04$, $df = 8$). The 4 h thaw sample had a much higher glucose content than either the control sample (Tukey test: $q = 14.45$, $P < 0.01$) or the 24 h thaw sample (Tukey test: $q = 14.09$, $P < 0.01$); the latter two samples did not differ from one another (Tukey test: $q = 0.36$, $P > 0.05$). The osmolality of the 4 h thaw sample was substantially higher than the value for the 24 h thaw sample (Tukey test: $q = 5.78$, $P < 0.05$) but other comparisons of the samples did not reveal any differences in osmolality (Tukey test, control vs. 4 h thaw: $q = 4.43$, $P > 0.05$; Tukey test, control vs. 24 h thaw: $q = 1.35$, $P > 0.05$).

Wood frogs restore the function of their kidneys within hours after thawing begins (see Layne and First, 1991). Recovering frogs, however, form urine that differs in composition from that of control frogs; it is laden with glucose. This does not seem to stem from a major alteration of renal tubule permeability or reabsorption capacities because highly regulated solutes like potassium and sodium did not show altered concentrations in the urine. The ultimate cause of postfreeze glucosuria, therefore, most logically results from the wood frog's need to induce massive hyperglycemia during freezing; this leads to an obligatory rise in glucose concentration in the renal filtrate and a subsequent overload of glucose transport by the renal tubules.

Since tubular reabsorption of glucose cannot compensate for freeze-induced hyperglycemia, only the urinary bladder could retard glucose excretion from the body. The frog bladder has substantial capacities to reabsorb water, electrolytes, and urea, but glucose recovery has not been studied (Shoemaker and Nagy, 1977; Micelli et al., 1983). Even if the glucose-laden urine is excreted it is possible that it may be recovered, at least in part, by the skin in contact with the excreted urine within the hibernaculum (Vanatta and Frazier, 1982; Lee and Costanzo, 1993). Lee and Costanzo (1993) estimated that *R. sylvatica* recovers glucose through the skin at a rate of 22.2 $\mu\text{moles/h/cm}^2$; however, the efficiency of this mechanism under natural conditions remains to be determined.

Two potential consequences of postfreeze glucosuria are: (1) excretion of valuable cryoprotectant and metabolic substrate from the body and (2) disruption

of water balance and the composition of body fluids since high glucose entry into the renal filtrate promotes diuresis. Wood frogs, however, begin glycolysis shortly after thawing; this would reduce the effects of postfreeze glucosuria since the levels of blood glucose return to normal within a few days. Nevertheless freeze-tolerant frogs show reduced glycogen reserves following repeated freeze/thaw episodes in the laboratory (Lee and Costanzo, 1993).

Other freeze-tolerant ectotherms, particularly insects, typically mobilize cryoprotectant in anticipation of tissue freezing at body temperatures well above 0°C (Lee and Denlinger, 1991). Wood frogs, however, only mobilize glucose after their body tissues begin to freeze. While delayed glucose mobilization seemingly risks cryoinjury, the biochemistry and physiology of wood frogs combine with the physical nature of their hibernacula to render this an ecologically viable response (Lee and Costanzo, 1993; Layne and Lee, 1995). Specifically, these frogs overwinter at the soil surface below forest floor debris and snow cover. This insulative covering slows the rate of freezing such that the crystallization of body fluids may require days to attain an equilibrium ice content. Finally, the susceptibility of wood frogs to glucosuria may render anticipatory cryoprotectant mobilization too costly, since the resultant glucosuria could diminish their pool of cryoprotectant.

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