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Persistence of Freeze Tolerance in Terrestrially Hibernating Frogs after Spring Emergence

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Freeze tolerance is an adaptation for winter survival in various species of terrestrially-hibernating frogs. We assessed the persistence of freeze tolerance in four species collected at breeding ponds after the spring emergence from hibernation. *Rana sylvatica, Hyla versicolor, H. crucifer,* and *Pseudacris triseriata* were as tolerant of whole body freezing in early spring as they were in autumn or winter, based on survival at −2.5 C for periods ranging up to 8 d. In later spring, after animals had begun to feed, tolerance to freezing declined sharply. Whole animal supercooling points (−1.2–−2.5 C) were the same in spring as in autumn but the capacity for producing cryoprotectants in response to the initiation of freezing was generally reduced in spring animals. Levels of glucose or glycerol produced in spring animals during freezing were often at least 10 fold lower than amounts which typically accumulate in animals in autumn or winter under equivalent freezing exposures. This reduced capacity for cryoprotectant synthesis may have resulted from lower rates of cryoprotectant synthesis in spring animals or the commitment of liver glycogen reserves to other uses in spring animals.

FOR poikilothermic vertebrates hibernating on land, the subzero temperatures of winter seriously threaten survival. However, a number of terrestrially-hibernating amphibian species occur in the northern United States and southern Canada. Toads and salamanders supercool only slightly (to about −2 C) and are killed by internal freezing (Storey and Storey, 1986a). These animals avoid exposures to low subzero temperatures by behavioral means; for example, *Bufo americanus* digs about 1 m down into the soil (Froom, 1982).

A natural tolerance for the freezing of extracellular water has developed in four species of frogs: the wood frog, *Rana sylvatica,* the grey tree frog, *Hyla versicolor,* the spring peeper, *H. crucifer,* and the chorus frog, *Pseudacris triseriata* (Schmid, 1982; Storey, 1984a, 1985). These animals spend the winter at the soil surface hidden under leaves, logs, tree roots, or rocks (Froom, 1982) and covered with a blanket of snow; the microclimate of such a protected hibernation site is not often severe but temperatures of −5–−7 C occur (Schmid, 1982; MacArthur and Dandy, 1982). Since whole animal supercooling points are only −2–−3 C (Schmid, 1982; Storey, 1985; Storey and Storey, 1986a) animals often face one or more bouts of freezing in a winter. Freezing occurs in extracellular compartments producing large masses of ice in the abdominal cavity and abundant crystals under the skin all over the body. Ice contents of 35–48% of total body water are tolerated (Schmid, 1982; Storey, 1984a). When frozen, limbs are no longer extendible, and breathing, blood flow, and heart beat are temporarily suspended. Low molecular weight cryoprotectants are accumulated and used within cells to limit dehydration

and protect/stabilize cellular structure and function in the frozen state. Three species produce glucose as the cryoprotectant; *H. versicolor* accumulates high levels of glycerol instead (Storey and Storey, 1984, 1985a, 1986a). All species derive cryoprotectant from the catabolism of liver glycogen reserves. Over the last several years, we have extensively studied the wood frog, *R. sylvatica*, as a model for vertebrate freeze tolerance focusing on topics such as the regulation of cryoprotectant synthesis and degradation, tissue metabolism in the frozen state, and the physical effects of cryoprotectant in vitro in the freezing protection of isolated cells and tissues (Storey and Storey, 1985b, 1986b; Canty et al., 1986).

To date we have examined only frogs captured in the autumn prior to entry into hibernation. An advantage to hibernation on land is the ability to exit hibernation sites very early in the spring and begin breeding well before breeding ponds are completely free of ice. Indeed, *R. sylvatica*, *H. crucifer*, and *P. triseriata* are often found near the breeding ponds in late March and early April (Froom, 1982; Rosen, 1986). At this time, frogs could still be exposed to periods of subzero temperature. The present study examines the persistence of freeze tolerance and its associated metabolic adaptations during the spring.

Materials and Methods

Frogs were collected on damp evenings in spring at or near breeding ponds. *Rana sylvatica* (male $\bar{x}$ (±SD) = 7.0 ± 1.0 g, N = 33; female $\bar{x}$ = 9.6 ± 2.3 g, N = 10) were collected at Bishops Mills, Ontario on the first nights that animals were active, 7 April 1983, 6 April 1984, and 28 March 1985. Adult male *H. versicolor* ($\bar{x}$ = 8.6 ± 0.4 g, N = 8) were collected from breeding ponds on 20 May 1984. *Hyla crucifer* were collected from Ottawa suburbs on 9 May 1984 (male $\bar{x}$ = 1.2 ± 0.5 g, N = 20) and 7 April 1986 ($\bar{x}$ = 2.34 ± 0.38 g 2 male, 8 female). Male *P. triseriata* ($\bar{x}$ = 0.75 ± 0.13 g) were collected on 9 May 1984. *Hyla crucifer* and *P. triseriata* collected on 9 May 1984 had been present at breeding ponds for about 1 mo by this time (F. Cook, pers. comm.). In most instances, frogs were brought to the laboratory the following morning, having been left outdoors or in a refrigerator at 3 C overnight. In the laboratory, animals were transferred to plastic boxes containing damp sphagnum moss and placed in a cold room at 3 C. They were then used in one of three ways: a) immediate sampling; b) immediate transfer to an incubator at subzero temperature for freezing; or c) long-term holding at 3 C followed by various tests of freeze tolerance.

To determine whole animal supercooling point, individual frogs were placed on a pad of paper toweling with a thermocouple in contact with the abdomen of the animal. A broad band of masking tape was used to secure both animal and thermocouple to the toweling. Each animal was then placed in an incubator set at −2.5 C and the course of cooling and freezing was monitored with a YSI telethermometer attached to a linear recorder.

To test long-term survival of freezing, frogs were transferred, in their closed plastic boxes, to a constant temperature incubator set at −2.5 C. Frogs were frozen for varying times up to 8 d and were then thawed at 3 or 23 C and tested for survival.

For blood and tissue sampling, animals were killed by double pithing. The neck was then cut open, the aorta severed, and blood was sampled with a heparinized capillary tube. Blood was immediately mixed with perchloric acid as described previously (Storey and Storey, 1984). Tissues were rapidly excised, frozen in liquid nitrogen, and then transferred to −80 C for long-term storage.

Perchloric acid extracts of blood and tissues were prepared as described previously (Storey and Storey, 1984), and neutralized extracts were restored at −80 C until analysis. Metabolites were analyzed spectrophotometrically with coupled enzyme assays as described previously (Storey and Storey, 1984).

Results

Hyla versicolor.—Adult male frogs were transferred to −2.5 C within 16 h of capture and were given 12 or 36 h of freezing exposure. Animals readily survived both freezing exposures. Frogs frozen 12 h were very active after thawing at room temperature for 2 h; those frozen for 36 h were fully active after 4–5 h. Table 1 shows levels of metabolites in blood, liver, and leg muscle of *H. versicolor*. Both glycerol and glucose were produced as cryoprotectants, the ratio of glycerol : glucose accumulation in blood being 3:1. The two compounds also accumulated in liver but only glycerol was found in significant amounts in leg muscle.

TABLE 1. LEVELS OF METABOLITES IN BLOOD, LIVER, AND LEG MUSCLE OF SPRING *Hyla versicolor*: CONTROL VS FREEZING-EXPOSED. Data are means ± SEM in μmol/ml for blood and μmol/g wet weight for tissues with glycogen expressed in glucose units.

			Freezing-exposed	
		Control	12 h	36 h
Blood:	Glycerol	0.2 ± 0.03	9.0 ± 2.8	19.3 ± 0.3
	Glucose	1.5 ± 1.0	2.1 ± 0.4	8.3 ± 4.7
Liver:	Glycerol	0.2 ± 0.07	4.5 ± 1.6	12.3 ± 0.8
	Glucose	1.3 ± 0.5	4.1 ± 1.3	10.7 ± 3.6
	Glycogen	322 ± 14	325 ± 79	62 ± 9
Muscle:	Glycerol	0.5 ± 0.07	3.6 ± 0.3	14.7 ± 0.7
	Glucose	0.9 ± 0.1	0.9 ± 0.3	2.5 ± 2.1
	Glycogen	17 ± 6.5	2.4 ± 1.1	0
	N	3	2	2

Glycerol content appeared to be in equilibrium between the 3 tissues.

Hyla crucifer.—Responses of *H. crucifer* collected in early spring were virtually identical to those of autumn frogs (Storey and Storey, 1986a). Survival of freezing was complete. Seven frogs were held for 2.5 d at 3 C and were then transferred to −2.5 C. Four readily survived 20 h of freezing and were active within 30 min when thawed at room temperature. The remaining three were frozen for 4 d and were then thawed at room temperature for 3 h; all survived. All frogs had empty guts. Table 2 shows levels of glucose in tissues of these frogs. After 4 d of freezing, average levels were 197 μmol/ml in blood and ranged from 39–164 μmol/g in tissues. These amounts were equivalent to those found after freezing exposure of autumn animals (an average of 175 μmol/ml in blood and 86 to 228 μmol/g wet weight in tissues [Storey and Storey, 1986a]). Glucose production utilized the liver reserves of glycogen; levels of glycogen were 955 ± 59, 508 ± 69, and 235 ± 21 μmol/g wet weight (as glucose) ($\bar{x}$ ± SEM) in control, 20 h frozen, and 4 d frozen frogs, respectively. Spring *H. crucifer* did not produce glycerol (blood levels less than 0.9 μmol/ml in all cases).

Male *H. crucifer* which were collected later in the spring (calling at breeding ponds had been noted for about 1 mo) showed lower freeze tolerance. After 3 d at −2.5 C, only two of five survived when thawed at room temperature for 3 h. A second group of five frogs were thawed slowly overnight at 3 C followed by warming to room temperature for 2 h; only one survived. Freezing exposure was repeated with four more animals frozen for only 15 h; again only one survived. Thus, by late spring, freezing tolerance of this species becomes very poor. Of note, however, was the fact that all frogs had food in their guts and gall bladders were full, indicating that animals were feeding regularly. Supercooling points were determined for individual frogs collected in late spring; one frog had a supercooling point of −1.2 C, another showed −2.5 C but both showed an animal freezing point of −0.5 C. These two animals readily survived the brief freezing exposures (100 and 70 min post-exotherm, respectively) given. Cryoprotectant content differed substantially in late spring frogs, compared to the early spring. Blood glucose was measured in those animals which survived freezing exposures; only minor accumulations were found, an average of 4.1 ± 0.7 μmol/ml (N = 6) compared to 0.8 ± 0.3 μmol/ml (N = 4) in blood of controls. Glycerol and fructose were not accumulated.

Pseudacris triseriata.—Freeze tolerance of male *P. triseriata* was tested on animals which had been out of hibernation for approx. 1 mo before collection. Frogs were held at 3 C for 2 d and then frozen at −2.5 C for 3 d. Four frogs were thawed at room temperature for 3 h; only one survived. Four others were thawed overnight at 3 C; all were lively the next day. Cryoprotectants were measured. Blood glucose levels in the five animals which survived freezing averaged 1.0 ± 0.2 μmol/ml; not significantly different from the content of control animals, 0.8 ± 0.2 μmol/ml (N = 4). Fructose and glycerol were not present. Whole animal super-

TABLE 2. LEVELS OF GLUCOSE IN TISSUES OF SPRING *Hyla crucifer*: CONTROL VS FREEZING-EXPOSED. Values as in Table 1.

	Control	Freezing-exposed	
		20 h	4 d
Blood	4.2 ± 0.85	144.5 ± 18.7	196.6 ± 28.8
Liver	3.0 ± 0.53	91.4 ± 12.8	164.3 ± 14.2
Heart	2.8 ± 0.45	61.4 ± 12.2	148.0 ± 12.7
Kidney	3.0 ± 0.41	66.0 ± 5.4	66.8 ± 10.2
Leg muscle	2.3 ± 0.48	19.3 ± 2.4	38.9 ± 10.3
N	3	4	3

cooling points were measured for two animals; values of −2.0 and −2.2 C were found with freezing points of −0.5 C, these data are equivalent to those found for autumn frogs (Storey and Storey, 1986a).

Rana sylvatica.—To determine whether cryoprotectant persisted in nature after frogs left hibernation, blood samples were taken within 12–18 h of capture in frogs collected on the first night that *R. sylvatica* were heard calling at breeding ponds (many animals were sitting on the snow when collected). In all cases, animals had low blood glucose contents, equivalent to those of control animals sampled in the autumn (Storey and Storey, 1984, 1985b); averages were 4.3 ± 1.2 (N = 6), 4.6 ± 1.6 (N = 3), and 5.4 ± 2.5 (N = 2) μmol/ml in 1983, 1984, and 1985, respectively. No other cryoprotectants were found in blood; sorbitol and fructose were not detected and glycerol levels were minimal (0.09 ± 0.02 μmol/ml, N = 6, 1983 frogs).

Individual cooling/freezing curves were monitored for *R. sylvatica* from the 1984 sample within 14–18 h of capture. Animals supercooled to −2.0 ± 0.1 C (N = 3) and, when nucleation began, all froze at −0.5 C. Both supercooling and freezing points were the same as those recorded for autumn animals using the same methodology (Storey and Storey, 1985b). The three individuals used in these determinations were sampled for blood and tissues at 5, 90, and 150 min after the appearance of the freezing exotherm. At 5 min post-exotherm, there was no visible sign of freezing; at 90 min the skin was icy all over but limbs were still extendible; and at 150 min the body cavity felt semi-solid and limbs were stiff.

Other animals were subjected to longer periods of freezing at a constant −2.5 C. Survival of freezing was as good as that found for autumn/winter animals. Of the 1983 frogs placed in the incubator 1 d after capture, five out of five survived 2 d frozen and four out of four survived 1 wk frozen, assessed as recovery after 24 h thawing at 3 C. Frogs caught in 1984 were held for 3 wk at 3 C without feeding before freezing survival was tested; these readily survived 2 or 5 d of freezing (N = 3 for each). In 1985, three out of three frogs survived 48 h of freezing and eight out of 11 survived for 1 wk frozen, survival based on good limb movement and gulping responses after 2 h thawing at room temperature.

Levels of cryoprotectants and other metabolites in blood, liver, and leg muscle of *R. sylvatica* collected in spring 1984 are shown in Table 3. Some animals were given freezing exposure within 12–16 h of capture, others were held first for 3 wk at 3 C before freezing. In all cases, freezing stimulated cryoprotectant synthesis. Levels of glucose in liver were elevated within the first 5 min post-exotherm. However, amounts of glucose produced were substantially less than the cryoprotectant contents typical of autumn/winter animals (e.g., in winter blood levels are generally 200–500 μmol/ml [Storey, 1985, 1986; Storey and Storey, 1984, 1986b]). This was particularly true for animals held first for 3 wk at 3 C and then given long-term freezing exposure (2–5 d).

Similar results were found for *R. sylvatica* sampled in 1985; animals given freezing exposure within 24 h of collection showed an average glucose content of 69 ± 24 μmol/ml in blood and 57 ± 19 μmol/g in liver after 48 h freezing. Other frogs, collected in mid-April 1985 and frozen for 8 d had a mean liver glucose content of 12.9 ± 2.6 μmol/g (N = 8) compared to control values of 1.9 ± 0.3 μmol/g (N = 8).

Liver glycogen is the source of cryoprotectant glucose in *R. sylvatica* collected in autumn

TABLE 3. EFFECT OF FREEZING ON LEVELS OF METABOLITES IN BLOOD, LIVER, AND LEG MUSCLE OF *Rana sylvatica* COLLECTED IN SPRING 1984. Values as in Table 1.

		Immediate freeze exposure		Freeze exposure after 3 wk at 3 C	
	Control, 3C	5, 90, 150 min freeze	12 h freeze	2 d freeze	5 d freeze
Blood glucose	4.6 ± 1.6	6.6, 15.7, 28.5	34.2 ± 2.4	56.3 ± 12.7	57.0 ± 13.1
Liver					
Glucose	8.1 ± 2.5	19.6, 24.2, 51.2	45.2 ± 1.8	29.7 ± 7.4	27.8 ± 15.3
Glycogen	661 ± 63	724 ± 60	810 ± 11	74 ± 50	26.1 ± 1.1
Glycerol	0.4 ± 0.1	0.4 ± 0.1	0.1 ± 0.1	0.7 ± 0.5	0.8 ± 0.1
Lactate	1.7 ± 0.6	1.5 ± 0.3	1.1 ± 0.2	4.4 ± 1.3	3.3 ± 1.3
Alanine	1.4 ± 0.2	1.1 ± 0.1	0.8 ± 0.2	5.1 ± 1.6	3.5 ± 0.5
Leg muscle					
Glucose	2.3 ± 0.4	2.5, 4.4, 5.4	6.0 ± 2.0	15.6 ± 1.1	19.3 ± 1.5
Glycogen	36 ± 10	48 ± 8	58 ± 9	55 ± 5	30 ± 7
Glycerol	0.3 ± 0.1	0.3 ± 0.1	0.2 ± 0.1	0.4 ± 0.1	0.5 ± 0.1
N	3	3	2	3	3

(Storey and Storey, 1986b). Liver glycogen contents of spring frogs were measured. Table 3 shows that animals collected in early April and sampled just after capture had high liver glycogen (661 μmol/g as glucose). Levels were slightly lower than those in autumn frogs (600–1000 μmol/g) (Storey and Storey, 1984, 1986b), but this is likely due to a dependence on liver glycogen as an energy source for winter survival. *Rana sylvatica* given freezing exposure within 12–16 h of capture also had high liver glycogen levels, but those which were first held for 3 wk at 3 C before freezing exposure had very low liver glycogen contents, only 4–11% of the content in control frogs sampled 12–16 h after capture (Table 3). This approx. 600 μmol/g decrease in liver glycogen content was not balanced by large increases in tissue glucose contents (blood, liver, and leg muscle contents increased by only about 50, 20, and 20 μmol/g, respectively). This suggests that the loss of liver glycogen in spring frogs is only partially due to its mobilization for cryoprotectant synthesis.

These results were further confirmed in 1985. *Rana sylvatica* collected in early April had a high liver glycogen content, 610 ± 55 μmol/g in animals sampled within 18 h of capture. Frogs collected in mid-April, however, had much lower liver glycogen contents, 283 ± 83 μmol/g in animals held at 3 C for 1 wk and 112 ± 47 μmol/g in frogs frozen for 1 wk at −2.5 C (both control and frozen animals sampled 23 April) with liver glucose contents of 1.9 ± 0.3 and 12.9 ± 2.6 μmol/g, respectively. Of note is the large range of values for liver glycogen in these animals, from 13–643 μmol/g in controls and from 12–378 μmol/g in frozen frogs.

During long-term freezing, liver of spring *R. sylvatica* accumulated both lactate and alanine, products of fermentative glycolysis (average net increases of 2.15 and 2.95 μmol/g, respectively, Table 3). Both the total amounts produced and the near equimolar accumulation of the two products were the same as occurs during freezing exposure of winter animals (Storey and Storey, 1984, 1986b).

Levels of glycogen, glucose, and glycerol in leg muscle of *R. sylvatica* collected in spring 1984 are also shown in Table 3. Levels of glycogen were low, equivalent to those reported for autumn frogs (Storey and Storey, 1984), and did not change over freezing exposure. As suggested before (Storey and Storey, 1985b, 1986b), this indicates that cryoprotectant glucose is not produced from endogenous reserves in tissues other than liver. Glucose accumulated in leg muscle of freezing exposed frogs, again to levels similar to those reported in autumn/winter frogs (Storey and Storey, 1984, 1985b). Glycerol was not produced. Muscle response to freezing appeared, therefore, to be the same in spring animals as in autumn/winter animals.

DISCUSSION

Frogs of all four species are freeze tolerant in the early spring just as they are in autumn and winter. Thus, frogs brought out of hiber-

nation by the first warm days of spring can endure subsequent bouts of subzero temperatures. In 1985, for example, the first *R. sylvatica* were caught at breeding ponds on 28 and 29 March, both nights being warm and rainy with temperatures as high as 8 C. On the night of 30 March, however, temperatures plunged to −8 C with a snowstorm. Frogs were not active again for over a week. Thus, freeze tolerance is still a valuable adaptation in early spring.

The data for *H. crucifer* suggest, however, that freeze tolerance begins to decline later in the spring. Frogs sampled in early April were fully freeze tolerant while those sampled from breeding ponds in mid-May were much less tolerant, only four out of 14 survive freezing exposures. Loss of tolerance may be correlated with the beginning of feeding because animals in mid-May had food in their guts. In insects, a key adaptation for winter cold hardiness is the evacuation of the gut; this eliminates unpredictable and uncontrolled nucleation initiated by gut contents (Zachariassen, 1985). The same is probably true in frogs; nucleation by gut contents may result in lethal freezing as opposed to the nonlethal freezing caused by nucleation in the body extremities (Storey, 1985, 1986). If feeding was prevented, however, freeze tolerance was retained into late spring. Thus, our results for *R. sylvatica* showed a continued freeze tolerance in animals collected in early spring and held without feeding in the laboratory for several weeks before testing.

Although freeze tolerance was retained in starved animals in the spring, the capacity for cryoprotectant production was progressively lost. Except for *H. crucifer* captured in early spring (although not in late spring), cryoprotectant synthesis during freezing exposure was substantially reduced in spring frogs in comparison to the autumn/winter situation. For example, in winter, freezing of adult female *R. sylvatica* for 1 wk produced an average blood glucose content of 342 μmol/ml and a liver glucose content of 216 μmol/g (Storey, 1986). Comparable values for spring frogs were 57 μmol/ml in blood and 30 μmol/g in liver (Table 3) in 1984 animals frozen 5 d and 12.9 μmol/g in liver for 1985 animals frozen 8 d. The same was true of *H. versicolor* (blood glycerol in spring frogs was less than 20 μmol/ml compared to levels up to 423 μmol/ml in freezing-exposed autumn animals [Storey and Storey, 1985a]) and *P. triseriata* (blood glucose in freezing-exposed frogs was 1 μmol/ml in spring animals and 60 μmol/ml in autumn animals [Storey and Storey, 1986a]).

At least two factors may be responsible for this reduced cryoprotectant response in spring: 1) changes to the triggering and regulating mechanisms controlling glucose synthesis or to the regulatory mechanisms which permit high glucose contents to be sustained; and 2) a commitment of liver glycogen to other uses. To support the first proposition, the data for spring *R. sylvatica* indicate a much reduced rate of glucose synthesis during freezing exposure in these animals. Thus, levels of blood glucose in spring *R. sylvatica* rose to 6.6, 28.5, and 34 μmol/ml after 5 min, 175 min, and 12 h of freezing exposure, respectively (Table 3). Comparable values in autumn frogs were 16.3, 65.7, and 132 μmol/ml after 4 min, 175 min, and 18 h of freezing exposure (Storey and Storey, 1985b, 1986b). The same result is apparent when liver glucose concentrations are considered. In support of the second proposition, the data show that liver glycogen reserves change independently of cryoprotectant content during the spring. Liver glycogen reserves are high in animals collected early in the spring but decline rapidly over the first few weeks out of hibernation (for *R. sylvatica* levels were 610 μmol/g in control animals sampled 1 April but only 283 μmol/g in control animals sampled 23 April 1985). This suggests that liver glycogen is being utilized as a fuel source to support animal activity over the first few weeks of spring, a time when cold weather would make insect food scarce. Schlaghecke and Blum (1978) have similarly noted a progressive loss of liver glycogen over the spring months in *R. esculenta*. During winter, the store of liver glycogen can be interconverted with a pool of cryoprotectant glucose (Storey and Storey, 1986b). During spring, however, the apparent commitment of liver glycogen reserves to other uses appears to limit the amount of cryoprotectant that can be synthesized in response to freezing exposure, even when liver glycogen content is still high (as in early April).

These results might put into question the role of cryoprotectants in freezing survival. Proposed roles for cryoprotectants include: 1) limitation of intracellular dehydration via osmotic and water-binding effects; and 2) stabilization of protein and membrane structure and function (Storey, 1984b). The present studies used only mild freezing exposures of spring frogs (−2.5 C and short time periods). However, win-

ter frogs may face prolonged freezing at lower temperatures. Cryoprotectants then assume an increasingly important role in preventing intracellular dehydration beyond a critical limit. Future experiments will have to address the limits (low temperature, time, and percentage of body water as ice) of freezing survival in winter vs spring frogs.

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