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RAPID COMMUNICATIONS

Freeze Duration Influences Postfreeze Survival in the Frog *Rana sylvatica*

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ABSTRACT Survival, cryoprotection, and the time course for recovery of vital functions were studied in autumn-collected juvenile wood frogs (*Rana sylvatica*) following freezing at –1.5°C for various periods. Frogs readily tolerated freezing for 3 or 9 d, but only 50% survived a 28-d freezing trial. Generally, the postfreeze return of vital functions (vascular circulation, pulmonary breathing, righting reflex, jumping reflex) occurred later in frogs frozen for longer periods. Augmenting endogenous levels of the cryoprotectant glucose (via injections) prior to freezing substantially increased freeze endurance, as these frogs had excellent survival after remaining frozen for as long as 49 d. The improved freeze endurance of glucose-loaded frogs apparently was not associated with a reduction in ice content but rather may reflect the greater availability of energy substrate needed to support metabolism of frozen ischemic tissues. *J. Exp. Zool. 280:197–201, 1998.* © 1998 Wiley-Liss, Inc.

A few species of North American frogs, including the wood frog (*Rana sylvatica*), hibernate at or near the soil surface where they may encounter frost (Schmid, '82). These frogs tolerate an extensive freezing of their body fluids (for reviews, see Costanzo et al., '95; Layne and Lee, '95; Storey et al., '96). Freeze tolerance is promoted, in part, by the cryoprotectant glucose, which is synthesized and mobilized to tissues in response to ice crystallization within body fluids. Cryoprotectants preserve the integrity of cell membranes and macromolecules, limit osmotic shrinkage, and colligatively reduce the amount of ice that forms in the body (Mazur, '84).

One measure of the capacity for freeze tolerance is the minimum body temperature (= lower lethal temperature, LLT) that can be survived during freezing. This is a useful convention because temperature chiefly determines the quantity of ice that forms and, hence, the potential for cryoinjury. Although rigorous determinations of the LLT are lacking for many species, the value for *R. sylvatica* ranges from –5 to –6°C during the autumn and winter (Costanzo et al., '95; Layne and Lee, '95; Storey et al., '96). In spring, the LLT is higher (ca. –3°C), partly because less cryoprotectant is mobilized from the virtually depleted glycogen reserves (Storey and Storey, '87; Costanzo and Lee, '93; Layne, '95). Freeze tolerance of spring-collected frogs is substantially improved by augmenting natural cryoprotectant with exogenous glucose (Costanzo et al., '93).

Another useful, but relatively unstudied, index of freeze tolerance capacity is the length of time that sustained freezing can be tolerated (i.e., "freeze endurance"). This question has physiological and ecological relevance to *R. sylvatica* because this species lives in regions of North America (e.g., above the Arctic Circle) where freezing conditions may persist.

Our study investigates the effects of freeze duration on postfreeze survival and postfreeze recovery of physiological functions in *R. sylvatica*. The efficacy of glucose to protect frogs from lethal injury during prolonged freezing was also tested. Prolonged freezing (to 28 d) induced lethal injury and, to a lesser extent, slowed the rate of recovery in surviving frogs. Supplemental injections of glucose mitigated the lethal effects that were caused by prolonged freezes lasting 28 and 49 d.

MATERIALS AND METHODS

Rana sylvatica juveniles (mean ± SE body mass = 3.3 ± 0.08 g, n = 67) were collected from woodlands in Butler Co., PA, during the second and third weeks of October 1994 and 1995 and accli-

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mated under winterlike photothermal conditions in the laboratory. Frogs were sequentially exposed to 15°C (12:12 LD) for 1 week, 10°C (10:14 LD) for 1 week, and finally to 5°C (8:16 LD), under which conditions they were kept until used. Frogs were housed, unfed, on damp paper.

Our protocol for freezing frogs promotes survival and presumably mimics natural freezing and thawing episodes (i.e., slow freezing followed by gradual warming; Layne, '95). Frogs were frozen in 2-L jacketed beakers through which coolant was pumped by a refrigerated bath (Fisher 9001). Groups of 3–6 frogs were placed on water-saturated filter paper inside plastic petri fishes (diameter, 9 cm) that were stacked inside the jacketed beakers. Temperature was monitored by a thermocouple which had a sensing junction positioned inside each dish. After supercooling the dishes to ca. –1.5°C, a small ice crystal (< 1 g, –20°C) was dropped onto the water-saturated filter paper in each dish, leading to freezing of the paper. The frogs then quickly froze owing to the nominal resistance their integument offers to the growth of ice crystal into body fluids (Layne et al., '90). The frogs were kept frozen at the target body temperature (–1.5°C) for up to 49 d.

Frogs were thawed and permitted to recover in humidified plastic cages held in a cold room (5°C). To assess their survival, frogs were examined daily, for up to 7 d, for the maintenance of normal body posture and jumping in response to gentle prodding of the urostyle. Some frogs were also examined sequentially at intervals of 2, 4, 24, and 48 h after the onset of thawing to determine the time course for restoration of basic functions. These frogs were transferred from the cold room to the lab bench (20–25°C) 5 min before they were briefly examined for the presence of: (1) blood flow within superficial vessels in the skin of the ventral surface of the thigh and pelvic region (observed with the aid of a dissecting microscope); (2) pulmonary breathing (presence or absence of buccopharyngeal pumping motions); (3) righting reflex (return to upright posture within 60 s after being placed on its dorsum); and (4) jumping reflex (coordination of a forward jump in response to gentle prodding of the urostyle). Each frog was then returned to the cold room before use in subsequent examinations.

The importance of cryoprotectant in freeze endurance was investigated by administering glucose solution (1,500 mM, in Ringer's saline) to some frogs prior to freezing (Costanzo et al., '93). The chilled solution (100 μL/g) was injected into the intraperitoneal space with a 27-ga. needle 1 h before freezing commenced. Control experiments were performed to determine the freeze endurance of uninjected frogs as well as frogs receiving an equivalent volume of (glucose-free) Ringer's saline.

The ice content of frogs frozen at –1.5°C for 9 d was measured using calorimetric methods as detailed by (Layne and Lee, '87). The calorimetry apparatus consisted of a glass vacuum thermos containing 50 ml of distilled water at room temperature and a thermocouple/BAT-10 digital thermometer (Physiotemp Instruments) to record water temperature. Four replicate measurements were made on groups of 3–4 frogs. Placement of frozen frogs in the calorimeter caused its water to cool as a combined function of frogs' body temperature (–1.5°C) and their ice content. Water contents of the frogs used in calorimetric analyses were determined from the mass lost upon thoroughly drying the carcasses at 60–65°C.

Mean values (reported ± 1 SEM) were compared using ANOVA, with Tukey tests employed for multiple group comparisons. The proportions of samples surviving freezing episodes were compared among treatments using Fisher's Exact tests. Significance was judged at $P < 0.05$.

RESULTS

At the conclusion of the freezing trials, the frogs were inanimate, rigid, and extensively frosted. The postfreeze survival rate depended on freeze duration (Fisher's exact test: $P = 0.014$). All of the frogs kept frozen for 3 ($n = 6$) or 9 d ($n = 11$) readily recovered. However, survival was reduced to 50% in the group ($n = 12$) that was kept frozen for 28 d. Two-thirds of the frogs that ultimately died exhibited early signs of recovery (i.e., vascular circulation, pulmonary breathing).

Generally, physiological functions returned with 1–2 d of postfreeze recovery, although the timing of the return depended on the length of the freezing trial (Fig. 1). Basic vital functions (e.g., cutaneous perfusion) resumed before those requiring complex integration of the central nervous system. Return of pulmonary breathing and the righting reflex (but not the jumping reflex) was deferred by prolonged freezing (Fig. 1).

Freeze endurance was markedly improved by injecting frogs with glucose prior to freezing. Glucose loading permitted the survival of all of 11 frogs kept frozen for 28 d, and 9 of 10 frogs kept frozen for 49 d. In contrast, for animals used in

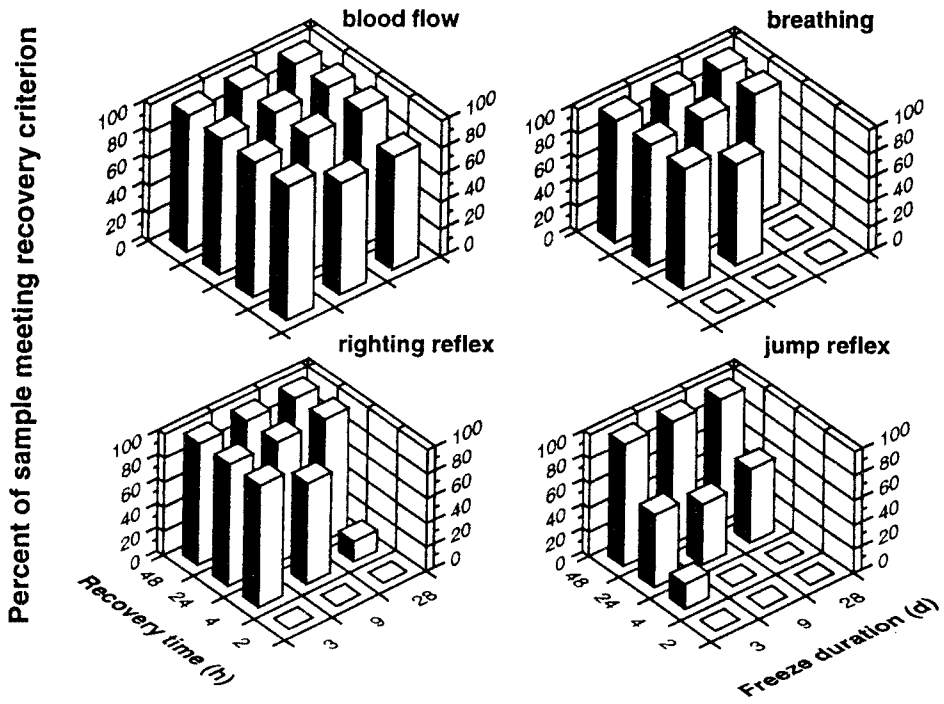

Fig. 1. Time course for the return of physiological functions in juvenile wood frogs (*Rana sylvatica*) after being frozen at −1.5°C for 3, 9, or 28 d. Indicated is the relative proportion of each sample exhibiting cutaneous perfusion, pulmonary breathing, righting reflex, and jumping reflex. Each treatment group consisted of $n = 6$ frogs.

28-d trials, the survival rate of frogs receiving only saline (1 of 5, 20%) was comparable to that of uninjected frogs (Fisher's exact test: $P = 0.34$). Glucose administration had no effect ($F = 0.90$, $P = 0.40$) on the body ice content of frogs frozen at −1.5°C for 9 d, as the mean value for glucose-loaded frogs (22.7 ± 1.2% of body water, $n = 4$) was comparable to that of the uninjected controls (30.4 ± 2.5% of body water, $n = 4$). The body water content of glucose-loaded frogs (82.7 ± 1.0% wet wt., $n = 4$) was similar ($F = 0.36$, $P = 0.73$) to that of the uninjected controls (83.1 ± 0.4% wet wt., $n = 4$).

DISCUSSION

Extensive freezing of the body fluids solidifies tissues, arrests vascular circulation, and deprives cells of oxygen. Upon thawing, basic physiological functions (e.g., heart beat and vascular circulation) return before functions that require complex neurological integration, such as righting and jumping reflexes (Layne and First, '91; Kling et al., '94). In the present study, the recovery sequence was similar although the time course was delayed in frogs exposed to protracted freezing.

Difficulty in evaluating the risk of freezing mortality in nature stems from uncertainty about the microenvironmental conditions to which frogs are exposed during winter. In the present study, autumn-collected frogs readily tolerated uninterrupted freezing episodes lasting several days, and many endured freezing for about 1 month. This time frame seems ample to promote winter survival in the midwestern United States, where freezing episodes are likely interrupted by occasional thaws. Possibly, the capacity for freeze tolerance varies geographically, with frogs from colder portions of the range (e.g., Canada and Alaska) tolerating lower temperatures and longer freezing episodes than frogs from more temperature locales.

For convenience, freezing protocols generally involve brief (e.g., 24–48 h) exposure to modest subzero temperature (e.g., −2.5°C), thus little is known about the tolerance of relatively long freezing episodes. Grey tree frogs (*Hyla versicolor*) recover after remaining frozen for 1 week at ca. −6°C (Schmid, '82), or 2 weeks at −2.5°C (Storey and Storey, '85). About 50% of *R. sylvatica* survive freezing at −4°C for 11 d (Storey and Storey, '84), although virtually all can survive ca. 2 weeks when frozen at −1.5°C (Layne, '95). Our present data indicate that, in autumn, *R. sylvatica* juve-

niles can survive in the frozen state for at least several weeks.

The capacity for freeze tolerance is strongly influenced by season (Storey and Storey, '87). Autumn-collected *R. sylvatica* in the present study endured substantially longer freezing episodes than did frogs collected in summer (Layne, '95). Our ancillary observations of *R. sylvatica* adults captured at breeding pools in Ohio and Pennsylvania, which tolerated freezing for periods of fewer than 5 d, also suggest that freeze endurance is diminished after emergence from hibernation (Table 1). Such seasonal variation in freeze tolerance capacity may partly reflect changes in the quantity of cryoprotectant that can be produced (Storey and Storey, '87; Costanzo and Lee, '93; Layne, '95). These frogs had meager hepatic glycogen concentrations (mean ± SEM = 137 ± 24 μmol/g wet wt.; $n = 8$) and accumulated only small amounts of glucose in the blood (8 ± 2 μmol/mL; $n = 4$). In contrast, the typically high concentration of liver glycogen (e.g., 800 μmol/g wet wt.) in autumn enables *R. sylvatica* to achieve blood glucose concentrations of 300–500 μmol/mL upon freezing (Storey et al., '96). Nevertheless, administering exogenous glucose to these spring-collected frogs did not appreciably increase freeze endurance (Table 1), perhaps owing to the diminished efficacy for cellular uptake of glucose at this time (King et al., '95) or changes in other, endogenous factors.

The cryoprotective effects of glucose during relatively brief freezing have been demonstrated in *R. sylvatica* at cellular, tissue, and whole-animal levels of organization (review in Costanzo et al., '95). Although a primary function of cryoprotectants is to reduce ice formation (Mazur, '84), in the present study, glucose enhanced freeze endurance apparently without influencing the body ice content. Rather, the role of cryoprotectant in conferring freeze endurance, as unequivocally demonstrated in the present study, may relate to the maintenance of the energy status of frozen tissues which rely exclusively on anaerobic production of ATP. Because glucose provides a readily fermentable substrate to support anaerobic metabolism, frogs having higher levels of glucose remain viable in the frozen state for longer periods. This hypothesis would not only provide a reasonable explanation for seasonal variation in freeze endurance, but would also suggest that the freeze endurance of a given individual is strongly influenced by the size of its hepatic glycogen reserve. The marked interindividual variability in carbohydrate stores in *R. sylvatica* (Storey and Storey, '87; Costanzo and Lee, '93) may thus have important consequences for winter survival and individual fitness.

*TABLE 1. Freeze endurance of control (uninjected) and glucose-loaded wood frogs (*Rana sylvatica*) collected in spring, cold acclimated for 2–6 weeks, and kept frozen for 1 to 5 d*

Exposure temp. (°C)	Group	Freeze duration (d) 1	2	3	5
–1.5[2]	Control	4/4	4/4	1/4	0/9
	Glucose-loaded	—	—	—	2/5
–2.5[3]	Control	20/20	4/5	3/5	0/5
	Glucose-loaded	—	—	3/5	—

The glucose solution was 1,500 mM glucose in Ringer's saline. Shown for each trial is the number of survivors/number of frogs tested.
[2]Collected in Butler Co., PA, during early April 1996.
[3]Collected in Adams Co., OH, during mid-February 1992.

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