

Cooling Rate Influences Cryoprotectant Distribution and Organ Dehydration in Freezing Wood Frogs

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ABSTRACT Ice formation in the freeze-tolerant wood frog (*Rana sylvatica*) induces the production and distribution of the cryoprotectant, glucose. Concomitantly, organs undergo a beneficial dehydration which likely inhibits mechanical injury during freezing. Together, these physiological responses promote freezing survival when frogs are frozen under slow cooling regimes. Rapid cooling, however, is lethal. We tested the hypothesis that the injurious effects of rapid cooling stem from an inadequate distribution of glucose to tissues and an insufficient removal of water from tissues during freezing. Accordingly, we compared glucose and water contents of five organs (liver, heart, skeletal muscle, eye, brain) from wood frogs cooled slowly or rapidly during freezing to -2.5°C . Glucose concentrations in organs from slowly cooled frogs were significantly elevated over unfrozen controls, but no significant increases occurred in rapidly cooled frogs. Organs from slowly cooled frogs contained significantly less water than did those from controls, whereas water contents from rapidly cooled frogs generally were unchanged. Rapid cooling therefore inhibited the production and distribution of cryoprotectant and organ dehydration during freezing. This inhibition may result from an accelerated, premature failure of the cardiovascular system. © 1992 Wiley-Liss, Inc.

Several vertebrate ectotherms are termed *freeze tolerant* because they survive extracellular ice formation under ecologically relevant temperatures and exposure periods. Since the geographic ranges of most freeze-tolerant vertebrates extend to high latitudes (Storey, '90), this capacity is likely a physiological adaptation promoting winter survival. Some of these species (e.g., box turtles, *Terrapene carolina*) inhabit more southerly latitudes where at least occasional freezing of body tissues is possible (Costanzo and Claussen, '90).

The mechanisms of vertebrate freeze tolerance are most extensively studied at subcellular levels (see reviews by Storey and Storey, '88; Storey, '90); hence, comparatively little is known about the specific adaptations to freezing and thawing of cells, tissues, and organs. However, investigations concerning the distribution of ice and the time-course of its formation (Layne and Lee, '87; Layne and Lee, '89), organ water balance (Lee et al., '90a), and dynamics of cardiac function (Layne et al., '89) during freezing have provided important clues concerning the physiology of freeze tolerance.

The wood frog, *Rana sylvatica*, is the most commonly studied among all freeze-tolerant vertebrates. This species tolerates the freezing of up to two-thirds of its body water and survives freezing for two weeks or more (Schmid, '82; Storey and Storey, '84; Layne and Lee, '87). The onset of freezing initiates a rapid production of glucose, via glyco-

genolysis, which is mobilized from the liver and distributed throughout the body. Glucose concentrations in some tissues rise noticeably within a few minutes and may ultimately reach 0.5 M (Storey and Storey, '84); hence, in *R. sylvatica* glucose may function as a cryoprotectant.

Another important physiological response to freezing in *R. sylvatica* is the sequestration of large amounts of water, as ice, within the coelomic cavity and between the integument and musculature (Storey and Storey, '84; Layne and Lee, '87). Presumably this water originates from adjacent organs since large quantities of water are not normally found in these compartments; the dynamics of organ water during freezing and thawing (Lee et al., '90a) support this notion. A dehydration of organs and tissues is beneficial insofar as it reduces the amount of ice forming within them (Lee et al., '90b). Mechanical injury, a deleterious consequence of organ freezing (Pegg, '88; Rubinsky and Pegg, '88), may thus be mitigated.

Recently we showed that rapid cooling of *R. sylvatica* during freezing is injurious, whereas slow cooling to the same temperature is readily tolerated (Costanzo et al., '91). We hypothesize that rapid-cooling injury is attributable to an inadequate distribution of glucose to tissues and an insufficient

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removal of water from tissues during the freezing episode. The present investigation evaluated this hypothesis by comparing glucose and water contents of organs from wood frogs cooled slowly (survivable treatment) and rapidly (lethal treatment) during freezing to -2.5°C .

MATERIALS AND METHODS

Male wood frogs were collected in Adams County, southern Ohio, during mid-February, 1990, as they initiated breeding activities. Frogs (mean mass \pm SEM = 13.9 ± 0.5 ; $N = 17$) were kept in laboratory cages containing damp moss and exposed to 4°C in total darkness, conditions simulating hibernation, for at least 2 wk prior to testing. Food was withheld as these frogs do not eat during hibernation.

Frogs were randomly assigned to one of three groups: 1) control, unfrozen; 2) slowly cooled, frozen; and 3) rapidly cooled, frozen. Those in the two latter groups were frozen inside 50 ml, plastic centrifuge tubes. Slow and rapid cooling rates were produced by submerging tubes, either insulated externally with plastic foam (slow cool) or uninsulated (rapid cool), in an alcohol bath (RTE 210, Neslab Instruments, Inc.) maintained at ca. -3°C . A thermocouple probe positioned on the abdomen of each frog provided a continuous temperature recording on a multichannel data logger (OM500, Omega Engineering, Inc.). Probes were insulated from the tube wall with a small piece of plastic foam. After frogs supercooled to between -1.0°C and -1.5°C , ice nucleation (verified for each frog by a recorded exotherm) was induced by lightly applying aerosol coolant to the tube's exterior. Frogs subsequently cooled to -2.5°C during freezing; they were kept at this temperature for 2 to 4 hr to allow the entire body to reach thermoequilibrium. Cooling rates during freezing, based upon temperature recordings in the interval -1°C to -2°C , and the time required to reach the equilibrium temperature, were calculated for each frog.

Frozen frogs were removed from their tubes, whereas unfrozen control frogs were taken from their cages (4°C) and killed by double-pithing, prior to their rapid dissection on ice. Visceral organs were exposed by a mid-sagittal incision in the ventral body wall; cranial organs were excised following decapitation. Tissue samples from the heart, liver, and skeletal (gracilis) muscle were bisected with one portion (ca. 100 mg) being used for glucose assays and the other for water content determinations. One eye, removed intact from its orbit, was used in each test. Only water content was measured on brain owing to the very small quantity of tissue available.

All tissue samples were gently blotted to remove surface ice and moisture prior to weighing. Samples assayed for glucose were rapidly homogenized in 10 volumes of ice-cold, buffered saline (230 mmol) and centrifuged at 2,000g. Extracts were immediately frozen to -80°C . Glucose assays followed an enzymatic, spectrophotometric method for deproteinized extracts (no. 510, Sigma Chemical Co., St. Louis, MO). The remaining samples were dried at 65°C to constant mass; water content, expressed as a percentage of tissue fresh mass, was calculated on the basis of mass loss. Mean values (reported \pm 1 SEM) for tissue glucose and water content from control (unfrozen), slowly cooled, and rapidly cooled frogs were statistically compared using ANOVAs. Mean separation tests (Fisher's PLSD) were applied where appropriate. Significance was judged at $P < 0.05$.

RESULTS

During freezing, frogs in the slowly cooled group ($N = 6$) cooled at $-0.2 \pm 0.01^{\circ}\text{C}/\text{hr}$ and reached the equilibrium temperature, -2.5°C , in 27.1 to 28.8 hr. Those in the rapidly cooled group ($N = 6$) cooled at $-1.6 \pm 0.2^{\circ}\text{C}/\text{hr}$ and reached the equilibrium temperature in 5.3 to 7.5 hr. When extracted from their tubes, all frogs exhibited external and internal characteristics indicative of substantial ice formation (e.g., Layne and Lee, '87).

The mean glucose concentrations in organs of control frogs ($N = 5$) ranged from $0.7 \pm 0.1 \mu\text{mol}/\text{g}$ in the eye to $16.3 \pm 3.5 \mu\text{mol}/\text{g}$ in the liver. The organs of frozen frogs also varied considerably in glucose content. Comparisons among control, slowly cooled, and rapidly cooled frogs showed that freezing elevated glucose concentrations, although concentrations in organs from rapidly cooled and control frogs were statistically indistinguishable (Fig. 1A). In contrast, organs from slowly cooled frogs contained much more glucose than did those from control and rapidly cooled frogs; these differences were statistically significant for both skeletal muscle and the eye (Fig. 1A). Slow cooling resulted in glucose concentrations ranging from 2.3-fold in the heart to 5.8-fold in skeletal muscle over control values (Table 1).

The mean water contents in organs from control frogs ranged from $75.0 \pm 1.0\%$ in liver to $84.1 \pm 0.9\%$ in eye, and were generally higher than those of the frozen frogs (Fig. 1B). Water contents in organs from rapidly cooled frogs were similar to those from control frogs (except for the heart). However, all organs from slowly cooled frogs contained significantly less water than did those from control and rapidly cooled

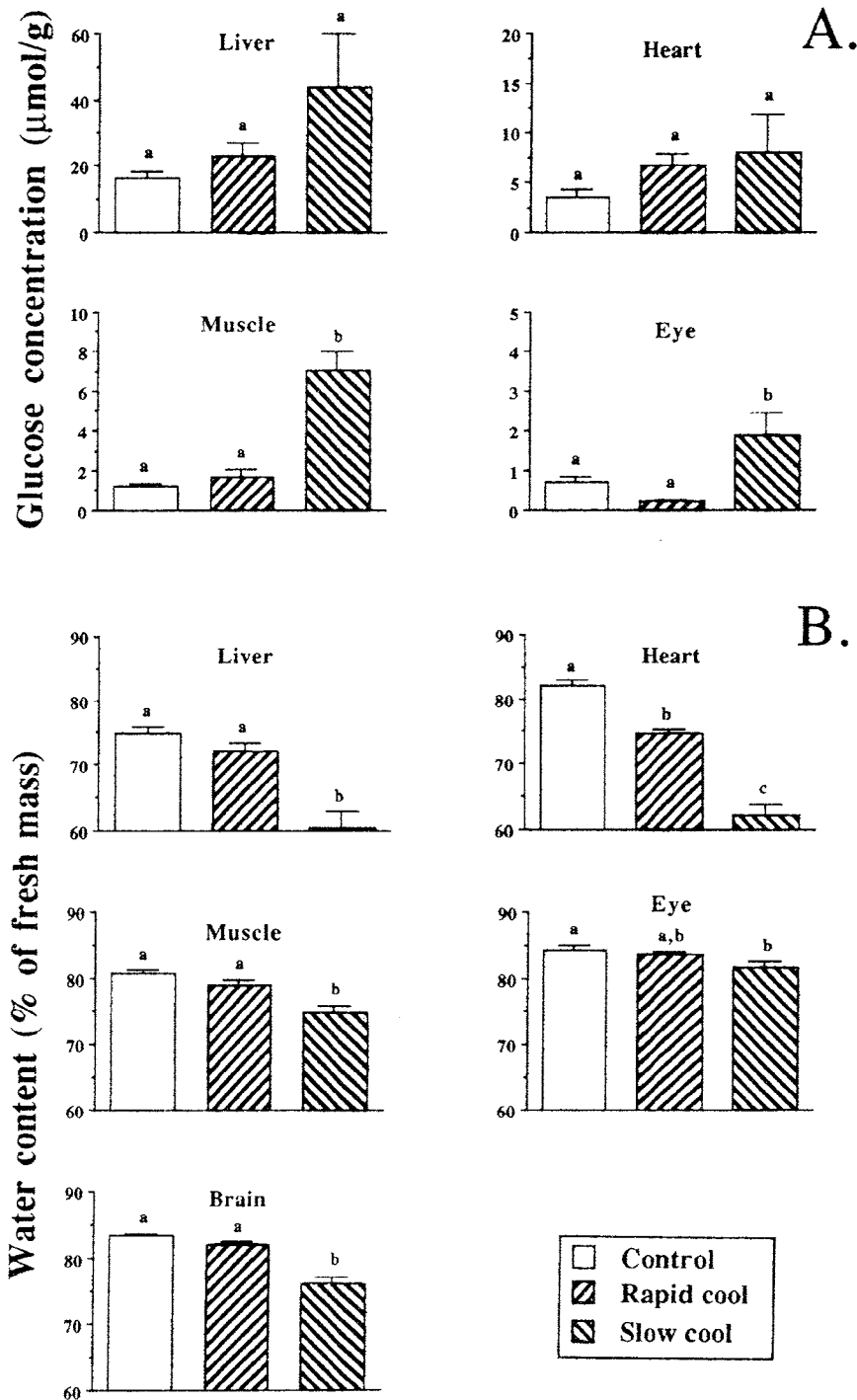


Fig. 1. A: Glucose concentrations in wood frog (*Rana sylvatica*) organs following slow (N = 6) and rapid (N = 6) cooling during freezing to -2.5°C , relative to unfrozen controls (N = 5). B: Water content in wood frog organs following slow (N

= 6) and rapid (N = 6) cooling during freezing to -2.5°C , relative to unfrozen controls (N = 5). Within tissues, means (shown \pm 1 SEM) identified with different letters were statistically distinguishable ($P < 0.05$).

TABLE 1. Increases in glucose concentrations and decreases in water contents in organs of wood frogs (*Rana sylvatica*) cooled rapidly or slowly during freezing to -2.5°C , relative to unfrozen controls

Organ	Glucose increase (%)		Water loss (%)	
	Rapidly cooled	Slowly cooled	Rapidly cooled	Slowly cooled
Liver	39.2	168.3	3.8	19.5*
Heart	89.8	127.5	8.8*	24.2*
Muscle	35.2	475.4*	2.1	12.7*
Eye	—	158.1*	0.6	2.8*
Brain	—	—	1.7	8.7*

*Values so indicated were calculated from sample means that differed statistically ($P < 0.05$).

frogs (Fig. 1B). Decreases in mean organ water contents associated with slow cooling ranged from 2.8% in the eye to 24.2% in the heart (Table 1).

DISCUSSION

Freezing survival in *R. sylvatica* is demonstrably dependent upon the rate of cooling during the freezing episode (Costanzo et al., '91). All frogs survive cooling at -0.2°C/hr and some survive cooling at rates up to -1.0°C/hr ; however, none recover from cooling at -1.2°C/hr . Frogs cooled at the latter rate are behaviorally incapacitated upon thawing and die within 2 d (Costanzo et al., '91). In the present study, we quantified physiological responses of frogs cooled slowly and rapidly during freezing. The rapid-cooling protocol (-1.6°C/hr) used in the present study, doubtless a lethal treatment, clearly inhibited the production and distribution of glucose and organ dehydration, responses likely critical to freeze tolerance in *R. sylvatica* (Lee et al., '90b). Rapid cooling is harmful because it promotes intracellular ice formation (Mazur, '63), transmembrane osmotic disequilibrium (Levin, '88), and, in the case of organs, extensive ice formation within the vasculature (Pegg, '88).

It is doubtful that the higher cooling rate used in the present study would be encountered by *R. sylvatica* in nature, since thermal fluctuations are dampened by the forest debris under which these frogs hibernate (Schmid, '82). However, wood frogs exposed to severely cold temperatures in autumn or during spring migrations to breeding ponds

(Layne and Lee, '87) may be injured or killed if cooled rapidly during freezing.

Earlier we reported (Costanzo et al., '91) that glucose concentrations in the blood and liver of rapidly cooled *R. sylvatica*, measured 2 hr after thawing, were markedly elevated above those of unfrozen controls and similar to those of slowly cooled frogs (Table 2). This result implied (erroneously) that rapid cooling did not diminish cryoprotectant production and distribution, yet we reasoned that the elevated glucose was established during thawing rather than freezing. This explanation is plausible because glucose production in the liver and its mobilization to tissues requires only that ice be present in the body, and that tissue perfusion occur (Storrey, '90); both conditions are met during thawing. Our present data for still-frozen frogs confirm that little glucose was produced and distributed during rapid cooling (Table 2). On evaluating data for thawed *R. sylvatica* we reported (Costanzo et al., '91) that organ dehydration did not occur with either slow or rapid cooling (Table 2). However, on the basis of our current data for still-frozen frogs we suggest the earlier result was an artifact of methodology, as the tissues must have rehydrated prior to sampling. These discrepancies serve to illustrate the importance of the timing of sample collection when measuring freezing-induced changes in tissue components.

Assuming that the dehydration of organs during freezing involves pure water, solutes become concentrated in tissues. Thus, the elevation of glucose

TABLE 2. Glucose concentrations and water contents in livers, sampled frozen and thawed, of wood frogs (*Rana sylvatica*) subjected to rapid or slow cooling during freezing to -2.5°C

Sample state	Liver glucose ($\mu\text{mol/g}$)		Liver water (% fresh mass)	
	Rapidly cooled	Slowly cooled	Rapidly cooled	Slowly cooled
Frozen ¹	22.7 \pm 4.5	43.7 \pm 16.1	72.1 \pm 1.3	60.4 \pm 2.5
Thawed ²	41.5 \pm 10.3	45.4 \pm 17.5	78.5 \pm 1.3	77.6 \pm 1.2

¹Tissue sampled from frozen frogs (present study).

²Tissue sampled following a 2-hr thaw at 4°C (data from Costanzo et al., '91).

accompanying freezing reflects both the de novo synthesis of cryoprotectant and the reduction of solvent volume owing to dehydration. Our data for slowly cooled frogs, recalculated using mean values for organ water contents of control frogs, illustrate the magnitude of this effect. The increases in mean glucose concentration in frozen organs over corresponding controls were from 4.5% (in eye) to 50.2% (in heart) greater owing to dehydration. This effect would be less important in rapidly cooled frogs because in these, organ dehydration was minimal. Glucose data expressed as $\mu\text{mol/g}$ fresh tissue (e.g., those in Table 1) do not account for changes in hydration states and should be interpreted accordingly.

Glucose is produced in large quantities during freezing in some freeze-tolerant vertebrates. Glycogen concentrations in liver are reduced dramatically, but those in other tissues are preserved; thus, liver appears to be the sole source of cryoprotectant (Storey and Storey, '84). The glucose is then distributed throughout the body via the vasculature. Not unexpectedly, glucose was elevated in the tissues of our slowly cooled *R. sylvatica*; however, the increases in liver and heart were not statistically significant owing to high sample variances. Indeed, individuals differ markedly in their capacity for cryoprotectant synthesis (e.g., Storey and Storey, '84, '88).

Limited evidence suggests that in *R. sylvatica* glucose functions as a cryoprotectant. First, glucose may enhance freeze tolerance by reducing injury to cells and tissues. For example, glucose added to a suspension medium mitigates damage otherwise occurring in hepatocytes (Storey and Storey, '88), erythrocytes (Costanzo and Lee, '91), and myocardium preparations (Canty et al., '86) frozen and thawed in vitro. Secondly, elevated concentrations of glucose in tissues apparently reduce the quantity of ice forming within them (Layne and Lee, '90). Finally, latitudinal differences in freeze-tolerance limits (see Storey and Storey, '84; Layne and Lee, '87) roughly correspond with the quantity of glucose produced during freezing. Assuming the elevation in glucose concentration during freezing is a critical adaptive response, rapid cooling of *R. sylvatica* may be injurious, at least in part, because insufficient glucose is produced and distributed to tissues. Frogs subjected to multiple cycles of freezing and thawing might better tolerate rapid cooling if previously mobilized cryoprotectant is already present within tissues.

An additional adaptation promoting freeze tolerance in *R. sylvatica* is the evacuation of water

from organs and its innocuous sequestration beneath the skin and in the coelomic cavity. Rubinsky and Pegg ('88) determined that excessive ice expansion within the vasculature is a major cause of cryoinjury to vertebrate organs. Lee et al. ('90a) first described organ dehydration during freezing in *R. sylvatica* and hypothesized that this response reduces mechanical injury to visceral tissues (Lee et al., '90b). Our present results confirm that slow cooling facilitates water loss from organs, including the eye and brain, but the specific source of the fluid (i.e., cellular versus extracellular; vascular versus extravascular) and the mechanism of its movement remain unknown. Rapid cooling clearly inhibited organ dehydration, very possibly contributing to the injurious effects of this treatment.

The production and distribution of glucose and the evacuation and translocation of organ water are events probably effected by the cardiovascular system. During a survivable, 24-hr freeze to -2.5°C , cardiac activity in *R. sylvatica* persists for 20 hr even though 50% of the body water has been frozen (Layne et al., '89). Nevertheless, these adaptive responses must occur in a timely manner since the cardiovascular system is ultimately susceptible to freezing. Cardioacceleration, induced by the liberated heat of fusion (Layne et al., '89), likely facilitates glucose distribution and the relocation of organ water during the early stages of freezing. Since the ice front passes from the integument toward the core, ice progressively occludes the microvasculature in peripheral tissues and perfusion is concomitantly diminished (Storey, '90). Accordingly, regardless of cooling rate, peripheral tissues must have a relatively shorter time to receive glucose and to evacuate water than do those located more internally. Our current data support this conjecture: the disparity in organ glucose concentrations between slowly and rapidly cooled frogs was greatest in (peripherally located) skeletal muscle, but minimal in visceral organs (i.e., liver and heart; Fig. 1A). Concordantly, differences in organ water contents between the groups were less pronounced in skeletal muscle than in visceral organs (Fig. 1B). These observations imply that adaptive responses (i.e., an increase in glucose, decrease in water) are dependent upon prevailing tissue perfusion during freezing, and that rapid cooling inhibits adaptive responses by accelerating cardiovascular failure, thus reducing the time available for them to occur. Additional research should provide a direct comparison between the time-courses of cardiovascular function during slow and rapid cooling.

Our data strongly imply that rapid cooling inhib-

its the production and distribution of glucose and organ dehydration, physiological responses promoting freezing survival. Through the experimental manipulation of cooling rates, the normally freeze-tolerant *R. sylvatica* may be rendered freeze intolerant. We submit this protocol as an instructive tool for investigating the mechanisms of natural freeze tolerance.

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