J Comp Physiol B (1991) 161:225–229

Journal of Comparative Physiology B Biochemical, Systemic, and Environmental Physiology

Effect of cooling rate on the survival of frozen wood frogs, *Rana sylvatica*

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Accepted February 5, 1991

Summary. Wood frogs (*Rana sylvatica*) were frozen to −2.5 °C under five distinct cooling regimes to investigate the effect of cooling rate on survival. Frogs survived freezing when cooled at −0.16 °C · h^{-1} or −0.18 °C · h^{-1}, but mortality resulted at higher rates (−0.30 °C · h^{-1}, −1.03 °C · h^{-1}, and −1.17 °C · h^{-1}). Surviving frogs in the latter groups required longer periods to recover, and transient injury to the neuromuscular system was evident. Some of the frogs that died had patches of discolored, apparently necrotic skin; vascular damage, as indicated by hematoma, also occurred. It is concluded that slow cooling may be critical to the freeze tolerance of wood frogs. Additional studies examined the effect of cooling rate on physiological responses promoting freeze tolerance. Mean glucose concentrations measured in plasma (15–16 μmol · ml^{-1}) and liver (42–45 μmol · g^{-1}) following a 2-h thaw did not differ between slowly- and rapidly-cooled frogs but in both groups were elevated relative to unfrozen controls. Thus, freezing injury to rapidly-cooled frogs apparently was not mitigated by the presence of elevated glucose. Water contents of liver tissue, measured 2 h post-thawing, did not differ between slowly-cooled (mean = 77.6%) and rapidly-cooled (mean = 78.5%) frogs. However, the mean hematocrit of slowly-cooled frogs (48%) was significantly higher than that (37%) of frogs cooled rapidly, possibly owing to differences in the dynamics of tissue water during freezing.

Key words: Freeze tolerance – Cooling rate – Glucose – Cryoprotection – Dehydration – Frog

Introduction

Thermal rate phenomena in cryobiological systems have received considerable study over the past several decades. Rapid cooling of unfrozen tissues may produce cold shock, an injury to cells owing to respiratory damage, changes in the selective permeability of plasma membranes, or phase transitions in membrane lipids (Morris 1987). Rapid cooling also may be harmful to freezing tissues because it promotes intracellular ice formation (Mazur 1963), transmembrane osmotic disequilibrium (Levin 1988), or extensive ice formation within the vasculature (Pegg 1988).

Slow cooling is typically required for freezing survival of isolated cell preparations (Meryman 1966; Liebo and Mazur 1971; Diller 1975), as well as complex tissues and embryos (Pegg 1988). This principle extrapolates to intact organisms (Baust and Rojas 1985) the lethal effect of rapid cooling has been revealed in certain, otherwise freeze-tolerant, insect larvae (Bale et al. 1989) and adults (Miller 1978). Slow cooling is likely crucial to the survival of all freeze-tolerant animals, but in vertebrates this has yet to be conclusively demonstrated. However, some investigators (Layne and Lee 1987a; Costanzo et al. 1988) noted that, under laboratory conditions, well-insulated (i.e., slowly cooled) vertebrates survive freezing better. In a recent study, the failure of lizards to survive freezing was attributed, in part, to excessive rates of ice accumulation (Claussen et al. 1990).

Freezing survival requires that cells and tissues tolerate significant perturbations in physiochemical homeostasis. With freeze-tolerant vertebrates, two major adaptations promoting freezing survival have been hypothesized. First, glucose, which serves a cryoprotective function in most freeze-tolerant frogs (Storey and Storey 1988; Costanzo et al. 1992), is produced in the liver and mobilized to tissues in direct response to ice formation (Storey and Storey 1984). The cardiovascular system is the principal mechanism effecting glucose distribution and must accomplish this task in a timely manner, since it eventually succumbs to freezing-related stresses (Layne et al. 1989). Secondly, large quantities of water are withdrawn from organs during freezing and accumulate as ice in the coelomic cavity and beneath the skin

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(Lee et al. 1990a). This dehydration response may protect tissues and organs from mechanical damage owing to excessive ice formation (Lee et al. 1990b).

The freeze-tolerant wood frog, *Rana sylvatica*, was used to investigate the effect of cooling rate on survival. By measuring glucose and water contents of tissues following freezing, the influence of cooling rate on physiological responses promoting freeze tolerance was also assessed.

Materials and methods

Male wood frogs (*Rana sylvatica*) were collected during early February 1990, from breeding ponds in Adams County, southern Ohio. The frogs were transported to the laboratory, placed in cages containing damp moss, and exposed to conditions (4 °C, total darkness) simulating hibernation. Food was not provided. The frogs were tested 53–75 days following their capture.

All frogs were frozen inside plastic centrifuge tubes submerged in an alcohol bath (RTE 210, Neslab Instruments, Inc.). A thermocouple probe, placed against the frog's abdomen, was used in conjunction with a multichannel data logger (OM500, Omega Engineering, Inc.) to make continuous temperature recordings during cooling. Thermocouple probes were insulated from the tube wall with a small piece of plastic foam. Frogs were initially equilibrated to 0 °C in an ice bath, following which the tubes were transferred to the alcohol bath and the freezing episode was initiated. The onset of ice formation (verified by a recorded exotherm) was induced when body temperature reached −1.0 to −1.5 °C by lightly applying aerosol coolant to the tube's exterior. After subsequent cooling to −2.5 °C, frozen frogs were kept in the bath an additional 1–2 h to allow the entire body to reach thermoequilibrium.

Survival experiments. Frogs were chosen randomly from a pool of 24 animals (mean mass ± 1 SEM = 14.5 ± 0.5 g) for use in the survival studies. Groups of frogs were frozen under five distinct cooling regimes. Two of these were produced by submerging tubes, either insulated externally with plastic foam ("slow" cool) or uninsulated ("fast" cool), in the precooled alcohol bath. Three intermediate cooling regimes ("moderately slow", "moderate", and "moderately fast") were produced by submerging uninsulated tubes in a bath controlled by a temperature programmer (ETP-3, Neslab Instruments, Inc.). In these trials, the cooling program was initiated immediately following the onset of ice formation. Cooling rate (i.e., rate of heat loss) during the freezing episode, and the time required to reach thermoequilibrium at −2.5 °C, were determined for each frog.

Frozen frogs were transferred to a cold room (4 °C), removed from the tubes, placed individually in plastic containers on a wet paper substrate, and allowed to thaw; they were periodically monitored for recovery criteria over several days. We judged that frogs recovered fully from freezing and thawing if they ultimately showed normal neuromuscular reflexes (righting response and retraction of extended hindlimbs), maintained normal head and trunk postures, and were capable of spontaneous locomotion.

Physiological responses to freezing. Thirty additional frogs (mean mass ± 1 SEM = 14.5 ± 0.3 g) were randomly assigned to one of three groups. Two groups were frozen according to either the "slow" or "fast" cooling regimes described above and permitted to thaw at 4 °C for 2 h before tissues were sampled. The remaining group, which served as an unfrozen control, was taken directly from the cold room.

All frogs were quickly killed by double-pithing and dissected on ice. The heart and liver were exposed by a mid-sagittal incision in the ventral body wall. Blood was collected from the conus arteriosus and centrifuged; hematocrit was measured by standard methods and the plasma was stored (−80 °C). A portion of the liver (ca. 160 mg) was excised, lightly blotted, weighed, and homogenized in 1.0 ml ice-cold phosphate buffered saline (230 m*M*). The homogenate was centrifuged (2000 x*g*) and the supernatant immediately frozen to −80 °C. An additional liver sample (ca. 100 mg) was excised and dried at 65 °C to constant mass; water content of the tissue was calculated on the basis of mass loss. Glucose concentrations in deproteinized plasma and liver extracts were measured spectrophotometrically (procedure no. 510, Sigma Chemical Co.). Mean values for plasma and liver glucose content, hematocrit, and liver water content were compared statistically between slowly- and rapidly-cooled frogs using Student's *t*-tests. Significance was judged at $P < 0.05$.

Results

Effect of freezing rate on wood frog survival

Frogs in the "slow" and "moderately slow" treatment groups readily survived freezing when cooled at −0.16 °C · h^{-1} and −0.18 °C · h^{-1}, respectively, but cooling at −0.3 °C · h^{-1} or higher rates resulted in mortality (Table 1). All frogs in the "fast" group, which were subjected to the highest cooling rate (−1.17 °C · h^{-1}), died. Frogs in the "moderate" and "moderately fast" treatment groups had intermediate survival rates; transient, sublethal injury occurred in the survivors (Table 1).

Frogs in the "slow" treatment groups recovered rapidly. Within 12 h of thawing, rhythmic respiratory movements and cardiac activity were evident; all frogs showed typical head and trunk postures, normal hindlimb retraction and righting reflexes, and normal locomotor ability. Frogs in the "moderately slow" group responded similarly: one individual had poor motor control and difficulty in righting 24 h post-thawing, but all met recovery criteria by 48 h.

Table 1. Survival and recovery of male wood frogs (*Rana sylvatica*) subjected to different cooling regimes during freezing to −2.5 °C. Means are shown ± 1 SD

Cooling regime	Freeze[a] duration (h)	Cooling[b] rate (°C · h^{-1})	No. surviving/ no. tested	Recovery of survivors
Slow	24.9 ± 0.1	−0.16 ± 0.01	4/4	all within 12 h
Moderately slow	19.0 ± 0.1	−0.18 ± 0.02	5/5	most within 12 h
Moderate	13.0 ± 0.1	−0.30 ± 0.02	4/5	most within 24 h
Moderately fast	6.2 ± 0.2	−1.03 ± 0.06	3/5	most within 43 h
Fast	4.1 ± 0.3	−1.17 ± 0.24	0/5	(none)

[a] Time required for frogs to reach thermoequilibrium at −2.5 °C following the onset of ice formation

[b] Cooling rates of frogs were calculated in the interval −1 °C to −2 °C

Table 2. Physiological responses of male wood frogs (*Rana sylvatica*) cooled slowly and rapidly during freezing, relative to unfrozen controls. Means are shown ±1 SEM

Group[a]	*n*	Liver		Blood	
		glucose (μmol · g^{-1})	water content (% fresh mass)	glucose (μmol · ml^{-1})	hematocrit (%)
Unfrozen	10	13.3± 3.2	75.0±0.5	1.5±0.1	34.0±1.1
Slow cool	10	45.4±17.5	77.6±1.2	15.3±2.3	47.6±3.7
Fast cool	10	41.5±10.3	78.5±1.3	16.4±3.3	37.2±3.2
Student's *t*		0.191	0.547	0.282	2.121
P[b]		0.425	0.296	0.391	0.024

[a] Freeze durations and cooling rates of "slow cool" and "fast cool" treatments are similar to those given in Table 1

[b] Statistical comparisons, using Student's *t*-test ($df = 18$), were made between parameter means from slow- and fast-cool groups; $P < 0.05$

Frogs in the "moderate" group lacked limb retraction and righting reflexes, showed no respiratory movements, and lacked muscle tone 6.5 h post-thawing, but their condition later improved. By 24 h most had normal postures and only two lacked the righting response. One of these was later found dead (at 52.5 h) with a large hematoma beneath the thigh skin, but the other recovered fully.

Frogs in the "moderately fast" group were in poor condition 9.5 h post-thawing. All lacked muscle tone and failed to retract their limbs. The eyes of three frogs appeared opaque and one of these made spasmodic respiratory movements when its thoracic region was depressed. When examined 6 h later, one frog, which had a large patch of discolored skin on its dorsum, was dead. However, the remaining frogs, though clearly lacking the righting reflex, breathed rhythmically and all but one could retract their hindlimbs. The latter, which had large patches of darkened skin on its dorsal trunk and thighs (but apparently no hematoma), was found dead at 43 h post-thawing. Recovery of the remaining animals was nearly complete by this time, although one frog appeared to suffer from neuromuscular damage: it displayed poor body posture and carried its head in a tilted manner but ultimately survived.

Frogs in the "fast" treatment group were in poor condition following thawing. By 16.5 h they were limp (lacking muscle tone) and showed no righting or limb retraction reflexes; most were unresponsive to pinching. However, all had rhythmic cardiac activity and two frogs showed breathing movements in response to thoracic palpation. Within the next 5 h their condition improved slightly: some showed spontaneous breathing and one could move its eyelids and eyes. None regained the righting reflex. All frogs died by 41 h post-thawing, as no breathing or heart beat could be discerned and the frogs did not respond to pinching. Two frogs had very noticeable hematoma under the skin of the thigh; when incised, a small volume of fluid, which contained erythrocytes, was exuded.

Physiological responses to freezing in wood frogs

Frogs frozen under both "slow" and "fast" cooling regimes had higher plasma and liver glucose contents relative to unfrozen controls (Table 2). Freezing to −2.5 °C elevated plasma glucose about 10-fold and liver glucose about 3-fold. Regression analysis showed that plasma glucose concentration was directly related ($r^2 = 0.462$, $df = 18$, $P < 0.001$) to liver glucose concentration (Fig. 1); both parameters were highly variable among individuals. Concentration means were not statistically different between the "slow" and "fast" treatment groups for either plasma or liver glucose (Table 2).

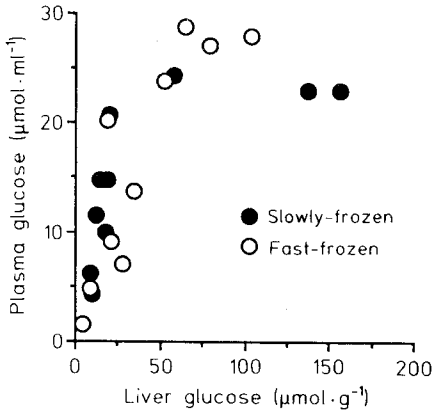

Fig. 1. Glucose concentration in plasma as a function of glucose concentration in liver, measured in male wood frogs (*Rana sylvatica*) cooled slowly (−0.16 °C · h^{-1}) or rapidly (−1.17 °C · h^{-1}) during freezing to −2.5 °C

Mean values for liver water content did not differ statistically between the "slow" and "fast" treatment groups and were similar to that of the unfrozen control, 75% of fresh mass (Table 2). In contrast, slowly-cooled frogs had hematocrits (mean = 47.6%) significantly higher than those (mean = 37.2%) of rapidly-cooled frogs (Table 2).

Discussion

Our experiments suggest that freeze tolerance in *R. sylvatica* depends upon slow cooling during the freezing episode. Freezing injury may result because ice accumulation is excessive (Storey and Storey 1988); however, the differential survival we observed is probably not due to differences in ice content. This is assumed because final body ice contents should have been comparable among frogs with similar equilibrium temperatures and tissue osmotic concentrations (e.g., Claussen and Costanzo

1990). Alternatively, injury associated with the higher cooling rates may be attributed to thermal effects and the location and rate of ice formation.

The physiological processes enabling animals to tolerate freezing doubtless have limits that are breached if cooling proceeds at an excessive rate. It is unlikely that the higher cooling rates used in the present study would be encountered by *R. sylvatica* in nature because thermal buffering is provided by the leaf litter under which these frogs hibernate (Schmid 1982). Nevertheless, experimentation using elevated rates is instructive in studies of physiological adaptations promoting freeze tolerance.

Rapid cooling during the freezing episode resulted in delayed recovery, transient sublethal injury, and, with the highest rates, death. Some frogs that died had discolored skin, probably reflecting localized necrosis and the destruction of melanophores. Lotshaw (1977) reported that epidermal necrosis was rare in frozen *R. sylvatica,* but common in *R. catesbeiana,* a freeze-intolerant species. Since hematoma was evident in several frogs that died, the vasculature also appears susceptible to rapid cooling. Minor hematoma in *R. sylvatica* was reported by Layne and Lee (1987b), although these frogs recovered fully. Our behavioral observations of frogs during recovery suggested that the neuromuscular system was particularly debilitated by rapid cooling. Interestingly, the heart initially recovered from rapid cooling, since rhythmic cardiac contractions were always evident during the early stages of recovery.

The mobilization of the cryoprotectant, glucose, is apparently an adaptation promoting freezing survival in *R. sylvatica* under natural conditions. Ice formation in the tissues of most freeze-tolerant frogs invokes a rapid production of glucose via glycogenolysis in the liver, which enters the blood and ultimately reaches other tissues (Storey and Storey 1984). Concordantly, we observed a high correlation between glucose concentrations in plasma and liver. Our mean value for plasma glucose, 15.3 $\mu mol \cdot ml^{-1}$, compares favorably with that (14.6 $\mu mol \cdot ml^{-1}$) reported for other slowly-cooled, Ohio *R. sylvatica* (Layne and Lee 1987b).

Rapid ice accumulation may conceivably hamper the distribution of glucose, perhaps by accelerating cardiovascular failure, with potentially damaging consequences. However, glucose concentrations in the plasma and liver of rapidly-cooled frogs, like those of slowly-cooled frogs, were elevated above controls, suggesting that a significant mobilization occurred. Thus, injury induced by rapid cooling apparently is not mitigated by the presence of elevated glucose.

Although our glucose data imply that cryoprotectant was adequately produced and mobilized during rapid cooling, this contention may be deceptive. Conceivably the elevated glucose measured in these frogs was synthesized in the liver and mobilized to the blood during *thawing,* rather than during freezing per se. This speculation is plausible because conditions requisite for this response [e.g., ice crystals within body fluids, effective tissue perfusion; Storey and Storey (1988)] were likely present during thawing. Accordingly, injury in the rapidly-cooled frogs may have resulted in part from insufficient cryoprotectant and consequent, excessive ice accumulation in tissues [without glucose's colligative depression of the freezing point, relatively more ice may have formed; see Claussen and Costanzo (1990)]. Further experimentation is needed to clarify the mechanisms of injury associated with rapid cooling.

During a slow freezing episode, large quantities of water are removed from tissues and redistributed throughout the coelomic cavity and beneath the skin (Lee et al. 1990a). This beneficial dehydration is believed to reduce mechanical freezing injury to the vasculature (Lee et al. 1990b). It is hypothesized that rapid cooling traps water in tissues that otherwise would be evacuated during slow cooling. Livers from slowly-cooled frogs therefore should contain less water than those from rapidly-cooled frogs. Unfortunately, potential rate differences were obscured because tissues apparently rehydrated during the 2-h thaw prior to sampling. This is evident because liver water contents of all thawed frogs approached those of the unfrozen controls (Table 2). Our hematocrit data nevertheless support the notion that rapid cooling inhibits the efflux of water from tissues. The marked hemoconcentration in slowly-cooled frogs probably resulted because much water remained outside the vasculature at the time of sampling. The comparatively lower hematocrits of rapidly-cooled frogs, which were thawed and sampled in a similar manner, conceivably reflect a lesser, overall redistribution of water (i.e., dehydration) during freezing.

Subsequent study of the mechanisms of rapid-cooling injury ought to include a direct evaluation of the response of the cardiovascular system to freezing. For example, the time-courses of cardiac output and tissue perfusion might be compared between slowly- and rapidly-cooled animals. These results, coupled with data on tissue glucose concentrations, hydration states, and ice contents, should elucidate factors critical to freezing survival.

Acknowledgements. We thank Rocky Knauff and Ted Easterling for granting access to their properties in our pursuit of frogs. This work is supported in part by the National Institutes of Health Grant 1 R15 HL-40535-01 and the Ohio Board of Regents Research Challenge Grant, Miami University (REL).

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