Seasonal Variation in Freeze Tolerance and Ice Content of the Tree Frog *Hyla versicolor*

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ABSTRACT Freeze tolerance and ice content of *Hyla versicolor* showed pronounced variation between summer (June) and winter (December). Summer frogs survived freezing at −3°C for up to 9 hr and ice accumulation up to 50% of their total body water. A time course of ice formation indicated that an equilibrium level was reached in approximately 15 hr. Thus, the lethal ice content was less than the equilibrium ice content for these conditions (63.1%). A second group was induced to enter an overwintering condition by holding them through the summer and then subjecting them to a progressive reduction in temperature and photoperiod for 2 months. These frogs survived freezing for 48 hr at −3°C. Their equilibrium ice content at this temperature was significantly lower (52.5%) than comparably treated summer animals. In the winter acclimatized group, frozen frogs had substantially higher blood glucose levels than unfrozen frogs (22.7 μmol/ml vs. 1.33 μmol/ml), but glycerol levels were not elevated after freezing. Freezing frogs conditioned for overwintering at −7°C resulted in a higher equilibrium ice content (62.6%), but none survived. It is evident that in preparation for overwintering, frogs reduce the amount of ice formed at a given subzero temperature, but there is little indication of a substantial change in the total amount of ice tolerated.

Freeze tolerance exists among a few species of terrestrially overwintering frogs (Lotshaw, '77; Schmid, '82; Storey, '85, '86; Storey and Storey, '86, '87). These frogs produce glucose and, in some, glycerol from their glycogen reserves to protect body tissues from freezing injury (Storey, '84a,b; Storey and Storey, '84, '85a,b). Seasonal decline of glycogen leads to reduced cryoprotectant levels in frogs during the late winter/early spring (Storey and Storey, '87); however, these frogs still retain tolerance to prolonged freezing at high subzero temperatures (Layne and Lee, '87; Storey and Storey, '87). By late spring frogs lose their ability to mobilize cryoprotectant, and they succumb to prolonged freezing (Storey and Storey, '87).

In freeze-tolerant animals, it is generally believed that ice forms extracellularly with the exclusion of solutes from the ice lattice. As a result of the concentration of solutes, cells become progressively dehydrated. A second consequence of freezing is prolonged ischemia since circulation of body fluids stops as freezing progresses (Storey and Storey, '88).

The amount of ice formed then is an important determinant of the survival of freeze-tolerant organisms. For a number of invertebrates the onset of mortality is associated with the freezing of two-thirds of their total body water (Williams, '70; Zachariassen et al., '79; Lee and Lewis, '85) but different limits exist in other species (Scholander et al., '53; Asahina, '69; Crisp et al., '77). The frog *Rana sylvatica* tolerates the freezing of two-thirds of its body water at high subzero temperatures (Layne and Lee, '87). However, information correlating ice content and survival in frogs is sparse especially when considered in light of the seasonal nature of freeze tolerance in these animals.

For the freeze-tolerant frog *Hyla versicolor*, adults are easily obtained during the summer when they breed; however, at this time they are "freeze intolerant." Few adults can be collected in the autumn when freeze tolerance is fully exhibited. Some investigators have taken newly metamorphosed frogs in the late summer and successfully induced freeze tolerance in the late autumn (Storey and Storey, '85b). However, their small size makes certain systemic and organismic level studies difficult. Therefore, it is essential to document whether adult *H. versicolor* can be taken in the summer and entrained in the laboratory to an overwintering, freeze-tolerant state.

This study documents freeze tolerance in *H. versicolor* from southeastern Indiana, which represents the most southern population of this species examined for freeze tolerance. Seasonal differences in ice content and freeze tolerance are reported. Successful
MATERIALS AND METHODS

Adult male *H. versicolor* were collected from a small breeding pond in Fayette Co., Indiana in June 1987. Some frogs were acclimated at 5°C for 1 week for subsequent testing of their freezing responses. A second group of frogs were maintained through the summer for testing in the winter. In early October, frogs were fasted for 1 week and then placed in a constant-temperature chamber set at 15°C with a 12:12 light-dark (LD) photoperiod. Overwintering was simulated by progressive reduction in temperature and photoperiod (Fig. 1). Freezing experiments commenced after 4 weeks at 1°C.

The protocol for freezing frogs was similar to procedures used by Layne and Lee (87). Frogs were placed in a metal canister that was immersed in a Neslab refrigerated bath. Each individual was held separately in a plastic centrifuge tube (50 ml capacity) with a wrapping of 1.5 cm thick foam rubber. Most freezing experiments were done at −3°C, but a few winter-conditioned frogs were frozen at −7°C. A thermocouple passed through the cap of each vial or tube and came to rest against the abdomen of a frog. Temperatures were compiled on a multichannel recorder.

Survival was assessed after thawing frogs for 2 days at 5°C. Recovery was judged complete if frogs showed regular breathing, normal posture, and jumped following light prodding.

Calorimetric determinations of body ice were made using a glass vacuum thermos containing 100 ml of water (Layne and Lee, 87). The change in water temperature following the introduction of a frozen frog was measured using a thermocouple connected to a digital thermometer. Properties of both wet and dry mass were considered in calculating body ice with the following equation (see also Murphy and Pierce, 75; Crowe et al., 81; Lee and Lewis, 86):

\[
Wi = \frac{F(Ww)(Sw)(T_t - T_0) + (T_s - T_0)(Wd)(Sd) + (Wd)(Sw)l}{(T_s - T_0)(Sw) + Q + Si(Mp - Ts) + Sw(T_t - Mp)}
\]

![Fig. 1. Acclimatization schedule used to achieve an overwintering state in *Hyla versicolor*. Temperature is indicated by open squares and photophase is indicated by solid circles.](image1)

![Fig. 2. A: The time course of ice accumulation and freeze survival of summer frogs subjected to freezing at −3°C. The hyperbolic plot (y = −4.271 + 8.785X − 0.351 X², R = 0.98) represents the best fit to the ice accumulation in the frogs over time. B: The survival data are represented by the frequency histograms set at specific time intervals during the freezing process. The fractions represent the number of surviving frogs/number of frogs tested at each interval.](image2)
mean values were made using Mann-Whitney U tests. Statistical comparisons of paired 
et al. ('83).

Glucose was determined using spectrophotometric analysis via Sigma glucose kit No. 510. Glycerol was measured using high-performance liquid chromatography (HPLC) using methods described by Lee et al. ('87) and Baust et al. ('83).

Mean values are reported with one standard error where appropriate. Statistical comparisons of paired mean values were made using Mann-Whitney U tests.

RESULTS

Seasonal conditioning markedly affected freeze tolerance of frogs. Summer frogs survived freezing at $-3^\circ C$ for up to 9 hr and accumulated ice in proportion to the length of time frozen (Fig. 2). Recovery took several hours, but frogs resumed normal behaviors and bodily functions (e.g., breathing, feeding, defecation) over the remaining weeks in captivity. No mortality occurred among winter frogs when frozen at $-3^\circ C$, but they often required 2 full days for recovery (Table 1). Frogs gradually became responsive to external stimuli, and eventually they engaged in normal spontaneous activities. In contrast, freezing at $-7^\circ C$ invariably proved lethal; even frogs initially frozen at $-3^\circ C$ before being exposed to $-7^\circ C$ failed to recover. Not even partial recovery such as breathing or responsiveness to stimuli was observed in any frog frozen for 48 hr.

Ice accumulation in body tissues similarly showed seasonal variations. The equilibrium ice content of summer frogs held at $-3^\circ C$ was $63.1 \pm 2.2\%$ (N = 5) of their total body water. This was significantly ($P = .009$) higher than the equilibrium ice content of similarly frozen winter frogs (Table 1). Freezing at a lower temperature also led to a significantly ($P < .01$) greater amount of ice formation in winter frogs (Table 1).

The time course of ice formation was measured in the summer frogs (Fig. 2A). Freezing progressed quickly and produced a close fit to a third-order hyperbolic curve ($r = 0.98$). Ice formed at a rate of slightly less than 6% of the total body water per hour frozen during the early stages of the time course, and the one-half equilibrium point was reached in approximately 6 hr. The equilibrium point for ice accumulation was approximately 15 hr, which differed from the duration of the exotherm produced by the release of the latent heat of fusion ($10.1 \pm 0.4$ hr, N = 5). The lethal amount of ice for summer frogs was between 50% (9-hr freeze) and 58% (11-hr freeze) of their total body water content (Fig. 2B). Survivorship and ice content data for winter frogs indicated that the lethal ice content ranged between 52% and 62% in this season (Table 1).

Winter frogs frozen at $-3^\circ C$ had significantly ($P = .007$) higher blood glucose than unfrozen controls ($22.7 \pm 3.4$ $\mu M$ml, N = 5 vs. $1.3 \pm 0.4$ $\mu M$ml, N = 4). However, blood glycerol was not detectable in the plasma of three freezing exposed frogs and two of three unfrozen controls. A measurable accumulation of glycerol was found in an unfrozen frog ($100 \mu M$ml). The minimum detectable limit for glycerol was $10 \mu M$ml. Blood glucose and glycerol were not assayed in the summer.

### TABLE 1. Survivorship and ice content of frogs prepared for overwintering following freezing at $-3^\circ C$ and $-7^\circ C$

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Survivorship</th>
<th>Ice content</th>
</tr>
</thead>
<tbody>
<tr>
<td>$-3^\circ C$</td>
<td>7/7</td>
<td>$52.5 \pm 3.1%$</td>
</tr>
<tr>
<td>$-3^\circ C$ to $-7^\circ C$</td>
<td>0/3</td>
<td>$-62.8 \pm 3.1%$</td>
</tr>
<tr>
<td>$-7^\circ C$</td>
<td>0/6</td>
<td></td>
</tr>
</tbody>
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*The single temperature freezes lasted at least 24 hr. The two-step temperature freeze lasted 24 hr at each temperature. The mean value and 1 standard error (n = 6) is reported for the ice content.

where $F =$ calorimeter constant (1.03); $M_p =$ melting point of body fluids ($^\circ C$); $Q =$ heat of fusion of water (79.7 cal/g$^\circ C$); $S_d =$ specific heat of the dry tissue (cal/g$^\circ C$); $S_i =$ specific heat of ice (0.5 cal/g$^\circ C$); $S_w =$ specific heat of water at 20$^\circ C$ (0.9988 cal/g$^\circ C$); $T_f =$ final temperature of the water in calorimeter ($^\circ C$); $T_i =$ initial temperature of the water in calorimeter ($^\circ C$); $T_s =$ temperature of the body tissues ($^\circ C$); $W_d =$ weight of dry mass in body tissues (g); $W_i =$ weight of ice in body tissues (g); $W_w =$ weight of water in body tissues (g); and $W_s =$ weight of water in the calorimeter (g).

Ice content was expressed as a percentage of the total water content of a frog. For *H. versicolor*, standard values for the melting point and specific heat content of the dry mass were $-0.4^\circ C$ and 0.29 cal/g$^\circ C$, respectively. Body water content was similar between summer (82.2 $\pm 2.5\%$) and winter (83.3 $\pm 3.0\%$) groups.

The maximal ice content was determined in frogs following freezing to completion of the exotherm. Animals were frozen for no less than 14 hr and in most cases for 24 hr or longer. The time course of ice formation also was assessed by measuring ice content of frozen frogs at set time intervals following the onset of freezing.

Cryoprotectant levels in the blood were assayed in frozen and unfrozen frogs. All blood samples were centrifuged to remove the cell fraction. The plasma was then used immediately for glucose determinations or frozen at $-80^\circ C$ for later assessment of glycerol. Blood was extracted from frozen frogs following rapid and complete thawing. Glucose was determined using spectrophotometric analysis via Sigma glucose kit No. 510. Glycerol was measured using high-performance liquid chromatography (HPLC) using methods described by Lee et al. ('87) and Baust et al. ('83).
DISCUSSION

*Hyla versicolor* ranges broadly over much of the eastern half of North America. Freeze tolerance is known for populations from Minnesota (Schmid, 1982) and Ontario, Canada (Storey and Storey, '85b). Indiana frogs were also tolerant of prolonged freezing at -3°C, but only after they were conditioned for overwintering. More extensive freezing at lower temperatures proved lethal; whereas *H. versicolor* from Minnesota survived freezing to -9°C (Schmid, '82). This likely reflects adaptive differences between populations that are separated by nearly 600 km in latitude, but this assumes that full overwinter conditioning was achieved by Indiana frogs using the present protocol.

Unlike studies by Schmid ('82) and Storey and Storey ('85b, '86), *H. versicolor* from Indiana did not substantially elevate glycerol content of the blood in response to freezing. The sensitivity limit (10 μmol/ml) for HPLC may have failed to detect a modest rise in glycerol. Indeed, Storey and Storey ('85b, '86) noted that some *H. versicolor* failed to attain blood glycerol levels in excess of 10 μmol/ml following freezing, but these were immature frogs. Glucose values for both frozen and unfrozen frogs correspond closely with values reported by Storey and Storey ('85b). Differences in glycerol production by frogs correspond with the greater susceptibility to freezing of Indiana frogs versus Minnesota frogs.

Invertebrates that seasonally acquire freeze tolerance do not necessarily die upon initial ice formation when exposed to freezing during the summer or following warm acclimation (Murphy, '79; Zachariaassen et al., '79), although the gastropod *Melampus bidentatus* succumbs to freezing of only 3% of its body water during the summer (Loomis, '85). Freeze-tolerant frogs lose their capacity to survive prolonged freezing and the ability to mobilize cryoprotectants by the late spring (Schmid, '82; Storey and Storey, '87). Our summer frogs also died after prolonged freezing; however, frogs were killed only after large amounts of ice accumulated in their body fluids (>50%).

Freezing leads to substantial cellular dehydration (Mazur, '84). The magnitude of ice accumulation and subsequent cellular dehydration is an important constraint on freeze-tolerant animals, although long-term survival may be determined by metabolic limitations (Storey and Storey, '88). It might be concluded that freeze tolerance may be facilitated by an ability to survive extensive dehydration. It is not surprising that even in the summer *H. versicolor* can survive substantial freezing without apparent benefit from cryoprotectants. Perhaps most intriguing is the close correspondence between the lethal ice content for summer frogs and the dehydration limit of 50% previously reported by Schmid ('85) for this species.

Freeze tolerance in anurans is not linked solely to their tolerance of dehydration. For example, *Bufo americanus* is highly terrestrial and dehydration tolerant, but it dies upon prolonged freezing since its main response to freezing involves burrowing below the freezing line in the soil (Storey and Storey, '86). Nevertheless, a comparative analysis of partial freezing tolerances of bufonids and less dehydration-tolerant ranids might prove interesting and enhance our understanding of vertebrate cryobiology.

The lethal ice content of some invertebrate species is not adjusted as a consequence of their conditioning for freeze tolerance; instead, this limit is reached at a lower temperature in winter (Williams, '70; Lee and Lewis, '85). *H. versicolor* show a similar response between summer and winter, although the present data cannot conclusively rule out a modest seasonal change in lethal ice content. Nevertheless, frogs survived freezing of at least 50% of their body water content in both summer and winter, but freezing of more than 60% proved lethal regardless of conditioning. Thus, winter frogs survive long-term freezing largely by reducing the amount of ice formed at a given subzero temperature and, thereby, avoid the lethal limit.

*H. versicolor* completed freezing to an equilibrium level nearly 50% faster than did *Rana sylvatica* (Layne and Lee, '87). The difference in ice accumulation rates for the two species undoubtedly is linked to their respective body masses: *H. versicolor* averaged only 7.3 g compared to 14.4 g for *R. sylvatica* (Layne and Lee, '87). As expected, large frogs freeze more slowly than small frogs.

Frogs survive prolonged freezing using only modest amounts of cryoprotectant. *Rana sylvatica* collected during the spring in Ohio elevate blood glucose only five- to tenfold in response to freezing; nevertheless, these animals survive lengthy freezing at high subzero temperatures (Layne and Lee, '87). Comparable results were observed here for *H. versicolor*.

It is doubtful that the colligative effects of cryoprotectants accounts for the seasonal difference in ice content. For example, the amount of bound water in the freeze-tolerant insect *Eurosota solidaginis* may change in response to winter conditioning (Storey et al., '81). Frogs may similarly manipulate the amount of unfreezable water to control the amount of ice forming, but crucial documentation is lacking. Indeed, this may require specialized cellular preparations in conjunction with the ability to mobilize
low molecular weight carbohydrates from glycogen reserves.

The acclimatization protocol used here undoubtedly enhanced freeze tolerance of frogs when compared to the capabilities of summer frogs. Laboratory conditioning has been successfully used to induce freeze tolerance in juvenile *H. versicolor* collected in the late summer and fall (Storey and Storey, '85b). In this study, adult frogs were obtained early in the summer and maintained for a much longer period than previously done. However, it is unclear whether full overwinter conditioning was achieved by these frogs. Certainly a comparison of entrainment programs is merited. Nevertheless, freeze tolerance can be induced in adult *H. versicolor* by laboratory acclimatization, which enhances the potential for using this species as a model system for studying vertebrate cryobiology.

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**LITERATURE CITED**


