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Physiological responses of freeze-tolerant and -intolerant frogs: clues to evolution of anuran freeze tolerance

JON P. COSTANZO, RICHARD E. LEE, JR., AND PETER H. LORTZ

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Costanzo, Jon P., Richard E. Lee, Jr., and Peter H. Lortz. Physiological responses of freeze-tolerant and -intolerant frogs: clues to evolution of anuran freeze tolerance. Am. J. Physiol. 265 (Regulatory Integrative Comp. Physiol. 34): R721-R725, 1993.—Freeze tolerance in the wood frog, Rana sylvatica, is promoted by multiple, integrated physiological responses to ice forming within body tissues. By analyzing the freezing responses of the sympatric, but freeze intolerant, leopard frog (R. pipiens), we sought clues to the evolution of anuran freeze tolerance. Physiological responses critical to R. sylvatica’s freeze tolerance, such as the synthesis and distribution of the cryoprotectant glucose, protective dehydration of organs, and deferred cardiac failure, were present, but comparatively less pronounced, in R. pipiens. Both species were innately tolerant of hyperglycemia. Glucose supplements did not enhance the freezing viability of R. pipiens, although in vitro tests of cryoprotectant efficacy revealed that glucose and glycerol provided comparable protection to erythrocytes of both species. We conclude that the evolution of freeze tolerance in R. sylvatica is not only promoted by its desiccation tolerance and the fortuitous biophysical consequences of freezing (e.g., exothermic induction of cardioacceleration and moderation of cooling rate) but also involves a progressive enhancement of fundamental physiological stress responses.

These anurans, derived from a common ancestor in the Eocene, are morphologically similar and occupy broadly comparable habitat and thermal niches (2, 17). However, the R. pipiens, which usually overwinters underwater rather than in terrestrial burrows, lacks freeze tolerance (10, 19, 20). For these reasons it was judged an appropriate freeze-intolerant model. Our intent was not to determine the specific basis for freeze susceptibility in R. pipiens, but rather to analyze its physiological responses to freezing under conditions that are readily tolerated by R. sylvatica.

**Materials and Methods**

Leopard frogs were commercially obtained from West Jersey Biological Supply during autumn 1990 and winter 1991. These frogs, field collected in eastern Canada during September and October, were maintained at supply house facilities in ~2-cm well water (5°C) until shipped to our laboratory. Wood frogs were field collected in Adams and Scioto counties, southern Ohio, in late winter 1991. Freeze tolerance in this R. sylvatica population is documented (6, 11). Specimens of both species weighed ~15 g.

All frogs were “winter-conditioned” by housing them in plastic boxes maintained at 4°C in total darkness for at least 12 days (usually 3-6 wk) before use. The boxes contained water in one end (R. pipiens) or a mat of damp moss (R. sylvatica). Food was not provided because hibernating frogs do not feed. Experiments involving R. sylvatica were conducted only when necessary because some data were available from published studies using identical methods.

**Freezing method.** Following the procedure of Costanzo et al. (5), frogs were placed inside plastic tubes and submerged in a precooled ethanol bath (RTE 210, Neslab Instruments). Temperature was monitored using a thermocouple probe, positioned against each frog’s abdomen, and a multichannel recorder (OMS300, Omega Engineering). After the frogs supercooled to approximately ~1.0°C, ice nucleation (verified by a recorded exotherm) was induced by lightly applying aerosol coolant to each tube. Freezing proceeded for ~24 h, during which time the specimen gradually cooled to the target temperature (usually ~2.5°C). Where appropriate, frogs were thawed at 4°C in an environmental chamber.

**Glucose synthesis and mobilization during freezing.** To investigate the glycogenolytic response to freezing, glucose concentrations were measured in organs from R. pipiens either frozen at −2.5°C or taken (unfrozen) directly from the environmental chamber at 4°C. After each frog was killed by double-pithing and dissected, its heart, brain, eyes, and portions (~50 mg) of the liver and skeletal (gracilis) muscle were excised, lightly blotted to remove surface moisture, and weighed to 0.1 mg. Extracts, isolated from tissue homogenates prepared in ice-cold perchloric acid (7%), were assayed in duplicate using an enzymatic, spectrophotometric procedure (5). We considered that the freeze susceptibility of R. pipiens might confound our interpretation of the glucose data owing to postmortem changes in tissue chemistry. However, some specimens remained alive for as long as 34 h of freezing (as evidenced by the persistence of cardiac activity; see Cardiac responses to freezing and thawing). Additionally, the low temperature likely retarded tissue deterioration.
Cryoprotection of erythrocytes. In accordance with the procedures of Costanzo and Lee (4), we tested the efficacy of the cryoprotectants, glucose and glycerol, in reducing freezing damage to erythrocytes from R. pipiens. Whole blood (250 μl) was collected from the aortae of double-pitched frogs and added to 1,500 μl heparinized, isotonic phosphate-huffered saline (PBS; 230 μM, pH 7.4). Erythrocytes from each sample were washed twice in PBS, resuspended in PBS containing 0, 15, 150, and 1,500 mM glucose or glycerol, frozen 30 min at -8°C, and passively thawed at 4°C. Cell damage was assessed based on the ratio of extracellular hemoglobin in the sample to that in a 100% hemolysis standard. The standard was a separate aliquot resuspended in PBS and frozen 2 h at -15°C.

Glucose loading. We investigated whether the freezing viability of R. pipiens could be improved by increasing the glucose levels via exogenous glucose supplements. According to the method of Costanzo et al. (6), three groups of frogs were administered PBS (volume, 6.7% of body mass, injected into the dorsal lymph pad) containing glucose in one of the following concentrations: 0 mM (control), 650 mM, or 1,500 mM. Subsequent to the injections, frogs (12.9 ± 0.3 g; n = 30) were kept 2 h in darkened cages (4°C) before further use.

Half the frogs in each group were taken from their cages, pithed, and dissected on ice. Concentrations of glucose in the plasma, heart, and portions (50 mg) of liver and skeletal (gracilis) muscle were measured as before. The remaining frogs were frozen at -1.5°C, passively thawed at 4°C, and periodically examined for recovery criteria (normal posture and neuromuscular reflexes) over several days. Hyperglycemia tolerance was studied in additional R. pipiens (14.6 ± 0.7 g; n = 5) administered 650 mM glucose, maintained at 4°C, and monitored 5 days for general health.

Dynamics of bulk water in organs during freezing. Water contents of organs from unfrozen (control) frogs and frogs frozen at either -2.5 or -5°C were compared to determine whether tissues of R. pipiens dehydrate during freezing. Frogs (13.3 ± 0.5; n = 25) were pithed and dissected; their hearts and two samples (50 mg) of liver, intestine, and skeletal (gracilis and gastrocnemius) muscle were excised, lightly blotted, and weighed to 0.1 g. The tissues were reweighed after drying 5–7 days at 70°C. Water content was calculated from the mass lost; values for the duplicate samples were averaged. The water lost during freezing was calculated from the difference in mean values for frozen and unfrozen frogs.

The change in hematocrit accompanying freezing provided an additional measure of the magnitude of water redistribution in both R. pipiens (21.9 ± 1.4 g; n = 20) and R. sylvatica (14.7 ± 0.4 g; n = 20). These frogs were frozen at -2.5°C and subsequently thawed for 2 h, or they were taken directly from the environmental chamber at 4°C, pithed, and dissected. Blood collected from the aorta was centrifuged in heparinized microcapillary tubes. An average hematocrit value, based on 3–5 aliquots, was obtained for each frog.

Measurement of body ice content. Calorimetric procedures detailed by Layne and Lee (12) were used to estimate body ice contents in R. pipiens (13.9 ± 0.3 g; n = 12) and R. sylvatica (14.6 ± 0.3 g; n = 12) frozen at -2.5°C. Frozen frogs were rapidly transferred to a calorimeter containing 150 ml of distilled water, and the mass of body ice (reflected by the decrease in water temperature) was quantified using the following constants: specific heat of water, 0.999 cal/g; specific heat of residual matter, determined calorimetrically, 0.219 cal/g; body water content, 83.8% of fresh mass for R. pipiens and 80.4% for R. sylvatica.

Cardiac responses to freezing and thawing. Thermal dynamics and indexes of the heart’s susceptibility to freezing were compared between R. pipiens (15.8 ± 1.3 g; n = 6) and R. sylvatica (15.5 ± 1.1 g; n = 4). Frogs, fitted with subdermal electrodes on the proximal forelimbs and left hindlimb and a thermocouple probe positioned on the abdomen, were wrapped in cotton gauze, placed in plastic tubes, and submerged in a cold bath. After the frogs supercooled to approximately -1°C, freezing was induced by applying aerosol coolant. Electrocardiograms of freezing R. pipiens and R. sylvatica were recorded at intervals with a polygraph (7D, Grass Instruments). Profiles of heart rate (each value calculated from a continuous series of 10 pulses) and body temperature were constructed only for R. pipiens, but for both species we recorded the time elapsed since ice nucleation and the body temperature associated with the onset of cardiac failure.

RESULTS

Glucose synthesis and mobilization during freezing. Concentrations of glucose were greater in the heart, liver, skeletal muscle, brain, and eye from frozen, relative to unfrozen, R. pipiens (Table 1). The statistically significant increases in glucose associated with freezing, which occurred in all organs except the eye, ranged from 2.6-fold in the brain to 11.9-fold in the liver.

Cryoprotection of erythrocytes. Freezing survival of erythrocytes from R. pipiens was significantly improved by adding glucose (P < 0.001) or glycerol (P < 0.001) to the suspension medium. Cryoprotectant concentrations ≥150 mM effectively reduced cell injury, but 15 mM had no effect (Fig. 1).

Glucose loading. The administration of 650 or 1,500 mM glucose significantly elevated tissue glucose concentrations (Table 2). Five R. pipiens, injected with 650 mM glucose and monitored 5 days, survived the 25-fold increase in blood sugar without apparent ill effects. Despite its producing high glucose concentrations in tissues, the loading procedure failed to improve organismal freeze tolerance, as none of the five frogs in either the 650 or 1,500 mM glucose injection groups survived freezing at -1.5°C. These frogs, like the five frogs in the saline-injected (control) group, failed to recover any neuromuscular reflexes, even after 6 days of thawing.

Dynamics of bulk water in organs during freezing. Freezing of R. pipiens to -2.5°C significantly reduced the water content of most organs, whereas all organs of frogs frozen at -5°C had water contents similar to those of unfrozen controls (Table 3). Hematocrits for unfrozen and frozen (-2.5°C) R. pipiens, 29.1 ± 1.8% (n = 10) and 30.9 ± 2.7% (n = 10), respectively, did not differ (P = 0.597). In contrast, hematocrits of R. sylvatica increased (P = 0.003), from 33.5 ± 1.6% (n = 10) to 47.6 ± 3.7% (n = 10), during freezing at -2.5°C. The mean values for

<table>
<thead>
<tr>
<th>Organ</th>
<th>Unfrozen</th>
<th>Frozen</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart</td>
<td>0.4±0.1</td>
<td>2.6±0.4</td>
<td>0.001</td>
</tr>
<tr>
<td>Liver</td>
<td>0.8±0.2</td>
<td>10.0±0.8</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Gracilis</td>
<td>2.3±0.5</td>
<td>7.5±1.2</td>
<td>0.004</td>
</tr>
<tr>
<td>Brain</td>
<td>0.6±0.2</td>
<td>1.0±0.3</td>
<td>0.040</td>
</tr>
<tr>
<td>Eye</td>
<td>0.1±0.05</td>
<td>0.2±0.04</td>
<td>0.234</td>
</tr>
</tbody>
</table>

Values are means ± SD in μmol/g; n = 5 frogs/group. Within rows, sample means were compared using analysis of variance.
FREEZING RESPONSES OF RANID FROGS

R723

0

Glycerol

Glucose

Cryoprotectant concentration (mM)

Fig. 1. Cryoinjury to erythrocytes, indicated by percentage of cells hemolyzing, from leopard frogs (Rana pipiens) and wood frogs (R. sylvatica) frozen at -8°C in cryoprotectant solutions. Column height indicates sample mean (n = 5 frogs/group, R. pipiens; n = 10 frogs/group, R. sylvatica); vertical lines represent SE. Sample means compared using 2-factor analyses of variance differed between species (glycerol, P < 0.001; glucose, P < 0.001) and concentrations (glycerol, P < 0.001; glucose, P < 0.001). Data for R. sylvatica were derived from Costanzo and Lee (4).

Table 2. Glucose concentrations in organs and plasma of leopard frogs, R. pipiens, measured 2 h after administering saline or glucose

<table>
<thead>
<tr>
<th>Organ</th>
<th>Saline</th>
<th>Glucose, 650 mM</th>
<th>Glucose, 1,500 mM</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart</td>
<td>1.4±0.3*</td>
<td>18.5±4.4†</td>
<td>46.3±4.6†</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Liver</td>
<td>4.8±0.9*</td>
<td>10.8±9.5†</td>
<td>34.6±1.9†</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Gracilis</td>
<td>6.0±0.2*</td>
<td>6.0±0.2†</td>
<td>26.0±1.3†</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Gastrocnemius</td>
<td>0.6±0.04*</td>
<td>15.0±0.2†</td>
<td>14.0±0.1†</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Values are means ± SE in μmol/g for organs and μmol/ml for plasma; n = 5 frogs/group. Within rows, sample means were compared using Fisher's least-significant difference tests; means identified by different symbols were statistically distinguishable.

unfrozen R. pipiens and R. sylvatica were indistinguishable (P = 0.089).

Body ice content. Freezing at -2.5°C produced significantly (P = 0.015) more ice in R. pipiens (69.8 ± 1.0% of total body water frozen; n = 12) than in R. sylvatica (65.4 ± 1.3%; n = 12). These values are directly comparable because mean values for body mass for the two groups were statistically indistinguishable (P = 0.093).

Cardiac responses to freezing and thawing. Generally, the heart rate of R. pipiens tracked changes in body temperature during prefreeze cooling. However, at the onset of freezing, the release of the latent heat of fusion abruptly increased heart rate from 4.7 ± 0.3 to 8.4 ± 0.6 beats/min (n = 6) and stabilized body temperature, thus deferring further cooling for ~8 h (Fig. 2). Subsequently, both heart rate and body temperature gradually decreased, and ultimately the heart ceased to function. The duration of cardiac activity during the freezing episode ranged from 12 to 34 h and was directly correlated (r² = 0.94; P = 0.002) with body mass. The mean duration of activity, 20.2 ± 3.0 h, was shorter (P = 0.016) than that measured for R. sylvatica (36.5 ± 4.8 h; n = 4) of statistically equivalent body mass (P = 0.093). The body temperature associated with cardiac cessation in R. pipiens, -1.5 ± 0.1°C (n = 6; range, -1.0 to -2.0°C), was higher (P < 0.001) than that for R. sylvatica, -2.6 ± 0.1°C (n = 4; range, -2.3 to -2.7°C). Cardiac activity temporarily resumed in one of the two R. pipiens thawed with electrodes in place (Fig. 2); however, this frog also did not recover.

DISCUSSION

The question of whether freeze-intolerant amphibians synthesize glucose and glycerol, the principal cryoprotectants used by freeze-tolerant anurans, in response to freezing has received only cursory attention with largely negative results (e.g., 19, 23). Freezing clearly induced glucose synthesis in our R. pipiens, although the highest

Table 3. Water contents of organs from leopard frogs (R. pipiens) sampled unfrozen or after freezing at -2.5 or -5°C

<table>
<thead>
<tr>
<th>Organ</th>
<th>Unfrozen</th>
<th>Frozen, -2.5°C</th>
<th>Frozen, -5°C</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart</td>
<td>86.7±0.4*</td>
<td>83.5±1.0†</td>
<td>84.0±1.5†</td>
<td>0.026</td>
</tr>
<tr>
<td>Liver</td>
<td>75.5±0.7*</td>
<td>70.3±1.2†</td>
<td>75.1±0.5†</td>
<td>0.001</td>
</tr>
<tr>
<td>Gut</td>
<td>83.7±0.6*</td>
<td>76.8±1.0†</td>
<td>80.9±0.8*</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Gracilis</td>
<td>82.1±0.4*</td>
<td>80.9±0.8*</td>
<td>80.7±0.6*</td>
<td>0.228</td>
</tr>
<tr>
<td>Gastrocnemius</td>
<td>81.0±0.4*</td>
<td>76.4±1.0†</td>
<td>79.0±1.1†</td>
<td>0.001</td>
</tr>
</tbody>
</table>

Values are means ± SE in % of fresh mass; n = 10 frogs/group, except those frozen at -5.0°C, where n = 5 frogs/group. Within rows, sample means were compared using Fisher's least-significant difference tests; means identified by different symbols were statistically distinguishable.

Fig. 2. Profile of heart rate and body temperature of leopard frog (R. pipiens) during freezing (initiated at 0 h) and thawing (onset indicated by arrow), illustrating transient recovery of cardiac activity.
concentration (10 μmol/g liver) was markedly lower than that typically achieved by R. sylvatica [e.g., 40-150 μmol/g (5, 22)]. Nevertheless, a common synthesis mechanism may be involved. Storey (21) suggested that the cryoprotectant response of freeze-tolerant anurans is an “exaggeration of the vertebrate ‘fight or flight’ response, the catecholamine-mediated activation of glucose output from the liver in response to stress.” Because R. pipiens showed a similar, albeit much reduced, production of glucose, our results are consistent with his hypothesis. Furthermore, in the reportedly (20, 23) freeze-intolerant toad, Bufo americanus, liver glucose was 6.7 ± 1.9 μmol/g in five unfrozen controls compared with 90.4 ± 8.7 μmol/g in five toads frozen at −2.5°C (Costanzo, unpublished data). Thus hyperglycemia may indeed represent a fundamental response of certain cells. In-fact, rapid cooling hampers organ dehydration in R. pipiens and R. sylvatica is not enigmatic. Rather it seems likely that the evolutionary development of anuran cryoprotectant systems exploited this fundamental response of certain cells. Interestingly, our data (Fig. 1) imply that relatively greater protection is afforded R. sylvatica red cells by equal amounts of cryoprotectant. However, additional study would be needed to determine whether this difference has biological significance.

The nature of its cryoprotectant system requires that R. sylvatica endures elevated, potentially harmful, levels of glucose. Because R. pipiens also survived extreme hyperglycemia, glucose tolerance may represent an important preadaptation to freeze tolerance. Possibly the freeze susceptibility of this species stems from its relatively inferior glucose production capabilities. However, supplementing putative cryoprotectant did not impair freeze tolerance to R. pipiens, even in specimens exposed to a very mild temperature (−1.5°C). Apparently the evolution of anuran freeze tolerance involves not only the development of effective glucose production mechanisms but also substantial improvements in the tolerance of cells and tissues to freezing-related stresses.

Ice accumulates under the skin and within the coelomic cavity of freezing R. sylvatica (11). This result, which involves a massive redistribution of bulk tissue water, is beneficial because it reduces mechanical injury to organs (16). Organs of R. pipiens also dehydrated during freezing, although they lost comparatively much less water. Decreases in the water content of the heart, liver, and gracilis muscle were 3.7, 6.9, and 1.5%, respectively, in R. pipiens, whereas corresponding values for similarly treated R. sylvatica were 24.2, 19.5, and 12.7% (7). The conspicuous absence of hemococoncentration in freezing R. pipiens, which in R. sylvatica presumably results from the loss of vascular water (5), further suggests that its dehydration response is less developed.

Although the magnitude of organ dehydration during freezing clearly differs between these species, the mechanism effecting the response may be the same. For example, rapid cooling hampers organ dehydration in R. sylvatica, probably by hastening cardiovascular failure (7); similarly, organs of rapidly cooled R. pipiens (i.e., those frozen at −5°C) retained more water than did organs from slowly cooled frogs (i.e., those frozen at −2.5°C). Perhaps organ dehydration is a generalized response to freezing that in R. sylvatica has been enhanced to promote freeze tolerance. Additionally, it is possible that this response is linked to desiccation tolerance, which among anurans generally increases with terrestriality (18). Desiccation tolerance may thus be requisite for the development of anuran freeze tolerance (8).

Both the quantity and location of ice within the body are critical determinants of freezing survival. Layne and Lee (11) determined that freezing of R. sylvatica to −2.5°C causes ~65% of its body water to freeze. Our study not only confirmed this value for R. sylvatica but also revealed that significantly more ice formed in R. pipiens frozen under similar conditions. This difference is likely due to the elevated glucose in R. sylvatica, which colligatively reduces ice formation (e.g., 9). Presumably R. sylvatica survives freezing only if the ice is restricted to extracellular spaces because intracellular freezing is reportedly lethal (21). The specific location of the ice forming in our R. pipiens was not determined; thus it is uncertain whether intracellular freezing contributed to its freeze intolerance.

Cardiovascular responses to freezing are of critical importance in promoting anuran freeze tolerance (13, 16). The cooling rate of R. sylvatica during freezing is moderated by the release of the latent heat of crystallization (i.e., exotherm). This effect is beneficial because rapid cooling is detrimental to freezing survival (5). Additionally, the vascular distribution of cryoprotectant in R. sylvatica is enhanced by the cardioacceleration associated with exothermy (13). Generally, the profile of body temperature and heart rate during freezing for our R. pipiens was similar to that reported for R. sylvatica (11, 13). Because cardioacceleration was also observed in R. pipiens, this response likely is a simple biophysical consequence of exothermy which, in certain species, fortuitously promotes freeze tolerance.

Layne and First (15) determined that cardiac activity ultimately ceases during freezing in R. sylvatica but resumes shortly after thawing. In contrast, sustained cardiac function did not return after thawing in our R. pipiens. Moreover, cardiac activity persisted longer and continued at substantially lower body temperatures in R. sylvatica than in R. pipiens of similar body size. These results strongly suggest that marked improvements in organ and tissue resistance to freezing stresses are fundamental to the evolution of anuran freeze tolerance.

**Perspectives**

Our comparisons of freezing responses between freeze-intolerant and freeze-tolerant species are simplistic but nevertheless suggestive of the origin of anuran freeze tolerance. Some factors promoting anuran freeze tolerance, such as the exothermy that induces cardioacceleration...
and moderates cooling rate, are merely fortuitous biophysical effects of freezing. Other attributes, such as hyperglycemia tolerance and cell sensitivity to cryoprotection by glucose, were shared by *R. pipiens* and thus may exemplify preadaptations to freeze tolerance. The prominent physiological dynamics associated with freezing of *R. sylvatica*, such as glucose mobilization and pronounced

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