Survival of Frogs in Low Temperature

Abstract. *Anurans that hibernate at or near the ground surface can survive prolonged exposure to low winter temperatures of northern latitudes by tolerance to freezing. An accumulation of glycerol during winter was correlated with frost tolerance, indicating that this compound is associated with natural tolerance to freezing in a vertebrate.*

Many species of terrestrial anurans (*1*) apparently overwinter beneath leaf litter in moist uplands (*2, 3*) where the microclimate can be cold during winters with little snowfall (*3, 4*). I report that three species of terrestrial anurans, the gray tree frog (*Hyla versicolor*), the spring peeper (*H. crucifer*), and the wood frog (*Rana sylvatica*) can survive freezing. Such tolerance is not found in two anurans (*R. septentrionalis* and *R. pipiens*) that overwinter in aquatic sites. Accumulation of the cryoprotectant glycerol in the body fluids of *H. versicolor* is associated with frost tolerance.

Specimens were collected during late fall or late winter (*5*) and slowly cooled under controlled laboratory conditions; their body temperatures were recorded with a surface contact thermocouple (*6*). A cooling curve with an obvious exotherm (Fig. 1) shows that *H. versicolor* had little of the supercooling ability that characterizes some cold-hardy insects with antifreeze chemicals (*7*). Specimens were kept frozen for periods of 5 or 7 days at −6°C (range −4° to −9°C) in individual containers. Vital signs such as limb movement did not occur until 2 to 4 days after specimens were thawed in a refrigerator at 4° to 8°C. In contrast, two species of aquatic anurans, the mink frog (*R. septentrionalis*) and the northern leopard frog (*R. pipiens*) were killed by freezing under the same conditions. Cooling data for five species of frogs are shown in Table 1. The extent of internal ice formation was estimated by measuring latent heats associated with phase change of water between liquid and crystalline states.

Four *H. crucifer* and one *H. versicolor* that had survived freezing were refrozen in contact with a thermocouple inside small Analocups (Aloe Scientific, St. Louis) (*8*). The magnitude of each exotherm, integrated over time for complete freezing, was compared with that of a volume of water equivalent to the total water of the frog. The relative exotherms showed that an average of 33.9 ± 0.9 percent of the frog's body water had frozen. After 5 days at −6°C, frozen specimens were transferred to an insulated vessel with 10 ml of water at 20°C and allowed to thaw. The endothermic thawing of specimens and consequent cooling of the surrounding water was compared to those values from known amounts of ice or cold (−6°C) water to give an average of 35.6 ± 1.1 percent of body water as ice. Values of percent ice are based on the total water content of each specimen, which was derived from the difference between fresh and oven-dry (85°C) weights. The specimens had between 74 and 82 percent of their mass as water, an amount similar to that found in specimens collected in summer and treated the same way. These calorimetric data indicate that about 35 percent of the water of the frost-tolerant frogs was frozen at −6°C.

Three specimens of *H. versicolor* slowly cooled to −30°C did not recover, and although no second exotherm was apparent in the cooling curve, an estimate of the amount of ice that had formed was significantly higher, 58.3 ± 2.8 percent.

The lowest microclimate temperature recorded during the winter of 1980–81 was −7.2°C (*4*). The shallow freezing and recovery of the frogs therefore are important to survival in terrestrial sites of hibernation.

Variations in concentration of ions, carbohydrates, polyhydric alcohols, amino acids, and proteins have been correlated with development of frost tolerance in animals, especially insects (*9*). A modification of the paper chromatography technique of Somme was used [see (*10*)] on muscle extracts from the frost-susceptible *R. pipiens* and *R. septentrionalis* and the frost-tolerant *H. versicolor* in a search for cryoprotectant biochemicals. The crude extracts from *H. versicolor* indicated that approximately 0.3*M* glycerol was present in muscles

Table 1. Supercooling temperatures of winter frogs. The aquatic species did not survive freezing. Temperature values are mean ± 1 standard deviation. Abbreviation: S.C.P., supercooling point.

Species	Winter habitat	*N*	S.C.P. (°C)
Rana septentrionalis	Aquatic	3	−2.73 ± 0.25
Rana pipiens	Aquatic	2	−2.55 ± 0.35
Rana sylvatica	Terrestrial	3	−1.87 ± 0.25
Hyla versicolor	Terrestrial	6	−2.18 ± 0.32
Hyla crucifer	Terrestrial	4	−1.98 ± 0.22

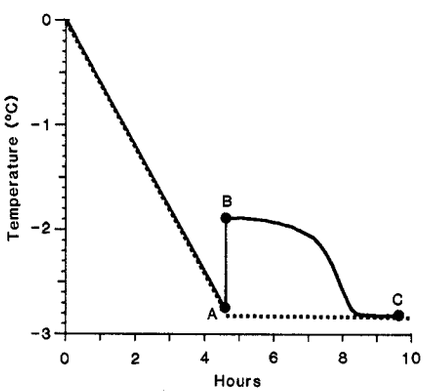
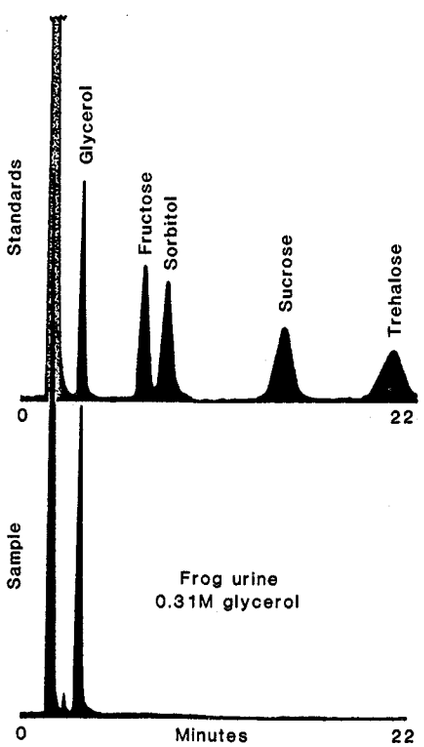


Fig. 1 (left). Cooling and freezing curve for a 1.8-g *H. versicolor* collected in October and tested in November 1980. Ice formation started at A and freezing continued from B to C, at which time specimen was placed in storage at −6°C for 7 days. Broken line is the temperature of the cooling bath and the solid line is the temperature of the frog. Point A is the supercooling point (−2.8°C) of this specimen. Fig. 2 (right). Chromatogram of urine from a winter frog. High-performance liquid chromatography showed the presence of glycerol in a sample of fluid taken from the urinary bladder of *H. versicolor* collected in February 1981. The small peak just before glycerol was not identified (*11*).



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from two specimens collected in the fall and two in late winter. The concentration of glycerol was calculated on the basis of total water content of the extracted muscles; that is, I assumed that glycerol was equally distributed in intra- and extracellular fluid spaces. Specimens of *H. versicolor* collected from terrestrial hibernation sites in late winter had full urinary bladders, and samples of their bladder fluid also contained approximately 0.3*M* glycerol (Fig. 2). Specimens collected in mid-May after migration from upland winter sites to breeding ponds had no glycerol and had also lost their tolerance to freezing. No glycerol was found in muscle extracts of *R. pipiens* or *R. septentrionalis*, so the correlation between glycerol content and frost tolerance was consistent both within and among species.

I had expected that winter survival of terrestrial frogs would involve frost avoidance through deep supercooling as is common among insects. I found instead an example of survival of a vertebrate after extensive freezing of body fluids. Complete freezing of extracellular water is consistent with my estimate of 35 percent of body fluid as ice (*12*). Frost tolerance is an important adaptation for survival of terrestrial frogs during winters of little snow when microclimate temperatures can easily fall below the supercooling point of these species. The occurrence of glycerol in association with frost tolerance suggests that this compound is a component of the cryoprotectant chemical system of these frogs.

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References and Notes

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2. R. P. Hodge, *Amphibians and Reptiles in Alaska, the Yukon and Northwest Territories* (Alaska Northwest, Anchorage, 1976), pp. 18 and 55.
3. W. O. Pruitt, *Arctic* **10**, 130 (1957); S. J. Coiner, thesis, University of Minnesota (1977).
4. T. Franke (personal communication) recorded soil-surface temperatures beneath leaf litter at a site of hibernating *H. versicolor* in Elm Creek Park Reserve, Hennepin County, Minnesota, through the winter of 1980–81 (model 1000 recording thermometer, Electric Auto-Lite Co., Toledo, Ohio). The minimum temperature was −7.2°C, and there were periods of 8 to 12 days when the temperature did not rise above −4°C. Frogs found at this site during February and March were at the same location as the sensing probe, that is, at the soil surface under leaf litter in the posture described by D. B. Ralin [*Comp. Biochem. Physiol.* **68**, 175 (1981)] for *H. versicolor* during desiccation.
5. Specimens of *R. sylvatica* were collected from upland forests in Itasca State Park, Clearwater County, Minnesota, in October 1979; *H. crucifer* from woods near Cedar Creek Natural History Area, Isanti County, Minnesota, in September 1980; *R. septentrionalis* from minnow traps in Itasca State Park in October 1980; *R. pipiens* from Rock Creek, Chisago County, Minnesota, in September 1980; and *H. versicolor* from upland woods in Elm Creek Park Reserve in October 1980 and in February and March 1981. All specimens collected during the fall were held in a refrigerator (4° to 8°C) for 20 to 30 days before testing, but the four specimens of *H. versicolor* collected during the winter were tested immediately; two of these frogs survived two cycles of freezing and thawing before being killed for extraction of muscles (*10*).
6. Individual specimens were placed in plastic containers with Styrofoam insulation below and above, with a copper-constantan (3-mil wires) thermocouple in contact with the mid-dorsal surface. Surface contact thermometry was used to measure supercooling points because invasive sensors usually bias results by providing artificial sites for nucleation of ice crystal growth inside the animals. The specimen holder was placed in a refrigerated bath (Forma Temp model 2025-2, Forma Scientific, Marietta, Ohio) with cooling rate controlled by manual adjustment of the thermostat. The thermocouple reference junction was held in distilled water and chipped ice at 0°C, and the thermoelectric voltage was used to measure and record specimen temperature (Omega 2809 digital thermometer, Omega Engineering, Stamford, Conn., and Gould 105 recorder, Gould, Inc., Cleveland) through the freezing cycle. A second thermocouple was used to monitor the bath temperature, which was lowered 0.6°C per hour until an exotherm occurred. Slow cooling was used to mimic rates of natural temperature change and to ensure thermal equilibrium between the specimen and bath before freezing. Temperatures of both the specimen and cooling bath were recorded to the nearest 0.1°C.
7. R. W. Salt, *Can. J. Zool.* **37**, 59 (1959); H. V. Danks, *Can. Entomol.* **110**, 1167 (1978).
8. The use of exotherms to estimate the amount of ice formed is similar to differential thermal analysis as described by M. J. Burke *et al.* [*Annu. Rev. Plant Physiol.* **27**, 507 (1976)]. The endothermic analysis of ice content is similar to that used by D. J. Murphy [*Physiol. Zool.* **52**, 219 (1979)].
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10. Frozen muscle was cut away from the hind legs, weighed, homogenized in cold 80 percent methanol (3 volumes per weight), and mixed with two volumes of chloroform from which the methanol-water phase was saved and centrifuged for 3 minutes at 11,600*g*. The supernatant was decanted and 5-μl samples were spotted on paper (Whatman No. 1 Chromatographic Paper) or cellulose thin-layer plates (Eastman 13255 Cellulose, Eastman Kodak Co., Rochester) together with mixed standards of glycerol-sorbitol, glycerol-glucose, and glycerol-fructose. After drying and development for 8 hours in either *n*-butanol:acetic acid:water = 12:3:5 or *n*-butanol:pyridine:water = 6:4:3, the chromatograms were dried and sprayed with periodate and iodide-starch as described by L. Somme [*Can. J. Zool.* **42**, 87 (1964)].
11. Technical details of high-performance liquid chromatography analysis (instrumentation, Waters Associates, Milford, Mass.) are described by D. L. Hendrix *et al.* [*J. Chromatogr.* **210**, 45 (1981)].
12. T. B. Thorson, *Physiol. Zool.* **37**, 395 (1964); H. T. Meryman, in *The Frozen Cell*, G. E. W. Wolstenholme and M. O'Connor, Eds. (Churchill, London, 1970), p. 51.
13. I thank J. C. Underhill, J. L. Howitz, and especially T. Franke for their aid in collecting specimens. P. J. Regal, D. J. Merrell, and R. E. Lee provided useful reviews of my writing, and R. E. Lee and J. G. Baust provided high-performance liquid chromatography analyses.

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Fossil Genes: Scarce as Hen's Teeth?

Kollar and Fisher (*1*) report that grafts of chick epithelium to mouse molar mesenchyme were induced to produce tooth enamel. Their experiments indicate that the loss of teeth in the class Aves should be attributed to factors other than the loss of genes for enamel production. We wish to draw attention to the profound implications of their results to modern evolutionary theory in ways not mentioned by them.

If we are to assume, as do Kollar and Fisher, that genes for enamel matrix proteins have no function in extant birds, and that such genes have remained unexpressed since toothed birds became extinct during the Cretaceous (*2*), we ask: what is the probability that any archaic gene could retain function after some 70 million years of unselected, random mutation?

Dormant DNA in living organisms is not frozen in time, remaining forever unchanged as a preserved fossil; instead it is transmitted from cell to cell and generation to generation via the same replicative machinery as any other DNA. Mutation is a slow but ineluctable part of this process that generates new alleles which must survive the pressure of negative selection to persist in time. Allelic variants of an unexpressed locus, however, are operationally neutral to the action of selection, and the accumulation of changes in wholly silent sites is expected to saturate after 100 million years (*3*).

According to conventional neutralist theory, proteins (the products of structural genes) evolve at a constant rate within the confines of selective constraints imposed by function (*4*). That is, realized amino acid substitutions are maximal when variants at a locus are independent of Darwinian fitness. Of the proteins surveyed thus far, fibrinopeptides appear to have evolved at the highest rate, with an estimated nine amino acid substitutions per amino acid site per 1 billion years (*4*). Kimura offers this particular example as one approaching selective neutrality; therefore, an equivalent expected rate for proteins of the resurrected bird-enamel locus is not unreasonably high. Using fibrinopeptides as a conservative baseline for near-neutral genes, we predict that the extant and putatively unexpressed bird-enamel locus would have experienced sufficient random mutations to effect substitutions at approximately 63 percent of the original amino acids in its protein product (*5*).

While it can be argued that numerous differences are known to exist within

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