

Inoculative Freezing by Environmental Ice Nuclei in the Freeze-Tolerant Wood Frog, *Rana sylvatica*

JON P. COSTANZO,* JAIME M. BAYUK, AND RICHARD E. LEE, JR.
Department of Zoology, Miami University, Oxford, Ohio 45056

ABSTRACT Efficacy of inoculative freezing by ice nuclei in a simulated winter environment was studied in the wood frog (*Rana sylvatica*), a freeze-tolerant species that overwinters on the forest floor beneath organic detritus. Adult frogs were confined to plastic canisters and cooled to -2°C over 24 hr with their ventral skin in contact with substrate (humic soil hydrated to 40, 10, or 5%, or soil/peat mixture hydrated to 20 or 10%, w/w), or their dorsal skin in contact with damp leaf mould. Whereas only 20% of control frogs cooled in dry, plastic canisters froze, freezing occurred in nearly all (98%) frogs contacting soil or leaf mould. Inoculation was briefly delayed in frogs exposed to drier substrates. Frogs exposed to an unfreezable substrate (humic soil, 5% moisture) themselves froze, apparently due to the action of constituent nuclei which commonly occur in natural materials. Although the surface over which inoculation can occur is greater in larger frogs, inoculation susceptibility was not correlated with body mass in our frogs (mean \pm SE body mass = 14.0 ± 0.2 g; range, 9.8–17.8 g). We conclude that the high susceptibility to inoculative freezing in *R. sylvatica*, which is conferred by its moist, highly permeable integument, promotes freeze tolerance by ensuring that inoculation commences at relatively high temperatures. *J. Exp. Zool.* 284:7–14, 1999. © 1999 Wiley-Liss, Inc.

The wood frog (*Rana sylvatica*) inhabits mesic woodlands in the central and eastern United States and much of Canada (Martof and Humphries, '59) where it commonly overwinters in shallow depressions on the forest floor, covered by leaf litter or other cover objects, and possibly snow (Kirton, '74; Licht, '91). Because it uses a superficial, relatively unprotected hibernaculum, the body temperature of *R. sylvatica* may fall below the equilibrium freezing point (FP_{eq}) of its tissues, ca. -0.4°C , thus resulting in somatic freezing. Frogs survive the freezing of their extracellular body fluids by virtue of a host of physiological responses to the formation of ice within tissues (reviewed by Storey and Storey, '96).

In the absence of endogenous ice nuclei, many vertebrate ectotherms, including anurans, supercool (i.e., remain unfrozen) to several degrees Celsius below the tissue FP_{eq} (see Lee and Costanzo '98 and references therein). Although supercooling may be advantageous in some vertebrate ectotherms, extensive supercooling prior to ice nucleation is deleterious for most freeze-tolerant animals because nucleation of deeply supercooled tissues hastens ice formation which, in turn, interferes with cryoprotective responses that are invoked only after freezing begins. Thus, many freeze-tolerant animals employ protective mechanisms, such as biosynthesis and accumulation of

various ice nucleating agents, interaction with ice-nucleating microbes within the alimentary canal, and inoculation by contact of the animal with ice or other nuclei in the environment, that reliably initiate tissue freezing at relatively high temperatures (Duman et al., '95; Lee and Costanzo, '98).

The mechanism initiating somatic freezing of *R. sylvatica* (and other freeze-tolerant frogs) in nature has not been thoroughly investigated. Laboratory studies suggest that anurans are highly susceptible to inoculation by contact with environmental ice (Layne et al., '90; Layne, '91), but whether such inoculative freezing is effective under the usual array of environmental conditions in nature is unknown. The efficacy of inoculative freezing may be influenced by prevalent microenvironmental conditions (e.g., soil water content, water potential, texture, organic content, etc.) as well as the animal's morphology (e.g., surface area, structural composition, and osmotic permeability of the skin) and physiology (e.g., osmotic concentration of body fluids, hydration status). The pur-

Grant sponsors: Sigma Xi and Miami University; Grant sponsor: National Science Foundation; Grant number: IBN 9507437.

*Correspondence to: Dr. Jon P. Costanzo, Department of Zoology, Miami University, Oxford, OH 45056. E-mail: costanj@muohio.edu
 Received 27 March 1998; Accepted 14 September 1998.

pose of the present study was to investigate the influence of some of these factors on the susceptibility of *R. sylvatica* to ice inoculation under simulated overwintering conditions.

MATERIALS AND METHODS

Animals and natural materials

Male wood frogs were collected by hand and with pit-fall traps on February 20 and 21, 1997, near a breeding pond in Adams County, south-central Ohio. Frogs were chilled and transported to laboratory facilities where they were placed in darkened cages containing damp moss. Frogs were denied food and refrigerated (4°C) until use in experiments 2–3 weeks later. Immediately prior to use, frogs were briefly submerged in cold water and blotted dry with a paper towel to ensure that their skin was free of excess surface moisture, moss, and other debris.

The principal substrate used in inoculation trials was a humic, clay-loam soil indigenous to a deciduous woodlot at Miami University's Ecology Research Center, Butler Co., southwestern Ohio. We used the upper, 2-cm layer of soil to which frogs are exposed during hibernation. Although *R. sylvatica* are not presently abundant in Butler Co., we selected this collection site on the basis of its similarity to habitat used by this species in neighboring areas and its proximity to an enclosure used in our ongoing studies of anuran hibernation (Costanzo et al., unpubl. data).

Soil was dried in an oven (65°C), sieved to remove large aggregates, and then thoroughly mixed with water to produce the desired moisture level (40, 10, or 5% w/w). We also used an organic-enriched substrate that was prepared by mixing equal amounts (w/w) of humic soil and horticultural peat and hydrated to 10 or 20%. Leaf mould collected in the same area was thoroughly dried and then rehydrated by soaking in an equivalent mass of water. All natural materials were autoclaved prior to drying and hydrated with deionized, autoclaved water.

Experimental apparatus

Inoculation trials were conducted by individually cooling 5–15 frogs in contact with a prepared soil substrate or leaf mould inside a plastic, cylindrical canister (diameter, 5.5 cm; height, 5.5 cm; volume, 125 mL). Control runs consisted of cooling 20 frogs in the canisters without substrate or leaf mould present. All frogs were purged of bladder fluid using a cloacal catheter and weighed to

0.01 g before being placed inside the canister. In trials using substrates, prepared material (40 mL) was tamped into a 2-cm thick layer in the bottom of the canister and the chilled frogs were placed in a slight depression formed by pressing two juxtaposed thumbs into the substratum's surface. This concavity simulated the shallow form in which *R. sylvatica* hibernates (Kirton, '74; Licht, '91). Other frogs were placed directly on the canister's smooth floor and then covered with prepared leaf mould. Frogs were then placed in the characteristic, flattened posture in which they hibernate (Licht, '91; Costanzo et al., unpubl. data) and covered with a disk (diameter, 5.5 cm) of flexible plastic foam which both insulated the superjacent space and applied slight downward pressure assuring intimate contact between the frog and the material. Direct contact between the frog and the foam was prevented by an intervening, circular piece of nonwetable film (Parafilm, American National Can). Preliminary tests showed that contact with the film did not influence supercooling capacity. Each canister was then covered with a tight-fitting lid.

Although in nature bulk soil supercools little, if at all (Hillel, '71), it may do so under laboratory conditions. We thus took measures ensuring that substrates and leaf mould would be frozen during inoculation trials. Substrates were frozen by keeping canisters in a freezer (–22°C) for 15–30 min, and then partly thawed in an ice bath (final substrate temperature, ~0°C) before frogs were introduced. Leaf mould was prepared by loosely overlaying material and trimming stems and margins to form a circular pad (diameter, 5.5 cm; thickness, ca. 1 cm). The pads were wrapped in plastic film to preserve moisture, frozen (–22°C), and partially thawed in an ice bath before placement over frogs inside the canisters.

Inoculation trials

Up to ten canisters were inserted fully into cylindrical recesses bored into a thick block of polystyrene foam. This block, whose function was to promote thermal stability and uniformity among replicates during cooling, was then placed in the cabinet of a custom-designed incubator (model I-35X, Percival) set to cool frogs to an ultimate equilibrium temperature of –2°C. The block was removed from the incubator ca. 25 hr later. Canisters were opened and frogs were reweighed.

Temperature of each frog during chilling was monitored by a single-channel data logger (Stow-Away XTI, Onset Computer) and thermistor probe.

The sensing end of the probe was passed through a small hole in the canister's lid and positioned directly beneath the frog. Data loggers were calibrated against a melting-ice bath (0°C) and programmed to record temperature to the nearest 0.25°C at 5-sec intervals. From each temperature record we determined the supercooling duration (time elapsed after cooling below -0.4°C and up to the onset of freezing or 24 hr) and, where appropriate, the temperature at which the tissues crystallized (T_c ; the lowest temperature recorded before appearance of the freezing exotherm).

Physical characteristics and ice-nucleating activity of natural materials

Organic content of humic soil, soil/peat mixture, and leaf mould was determined from the mass of residue remaining after incinerating dried samples at 550°C (n = 3 replicates). Water contents of these materials at saturation were determined from the amount of absorbed deionized water samples (n = 3 replicates) held against gravity. Water potential was determined using thermocouple psychrometry, in the dew point mode of operation, for humic soil, soil/peat mixture, and leaf mould hydrated to the prescribed levels. Measurements were made on four replicate samples using a sample chamber (C-52, Wescor) and microvoltmeter (HR-33T, Wescor).

We determined the relative ice-nucleating activity of humic soil, soil/peat mixture, and leaf mould by comparing the mean T_c of small volumes of water containing the material against that of equivalent volumes of deionized water, which lacks heterogeneous nuclei (Vali, '91). A 100 mm³ volume of the material was placed in a 0.5 mL, polypropylene microfuge tube to which 12.5 µL water were added. The contents were thoroughly mixed by vortexing and then sedimented by low-speed centrifugation (180g, 2 min). The sensing junction of a 36-gauge copper/constantan thermocouple was taped to the exterior of the microfuge tube, which was then inserted into a dry, 20-mL test tube. Samples (n = 12 replicates) were chilled by submerging the test tubes in a refrigerated ethanol bath (RTE 140, Neslab) and, after equilibrating at ca. 0°C, further cooled at 1.5°C/min until the water within each had produced a freezing exotherm. The corresponding T_c was read from the output of a data logger (RD3752, Omega Electronics) to which the thermocouples were connected. Samples were then thawed, an additional volume of water was added, and the procedure was repeated. We collected data for sample water vol-

umes ranging from 12.5 to 100 µL; mean values for samples containing natural materials were plotted with values for samples containing only water. All natural materials, deionized water, microfuge tubes, and utensils were autoclaved before use.

RESULTS

Organic contents, moisture-holding capacities, and water potentials of the experimental materials to which frogs were exposed during inoculation trials are given in Table 1. Augmenting humic soil with organic matter (peat) substantially increased the water-holding capacity and decreased the water potential of the substrate. Water potentials of humic soil hydrated to 5% and soil/peat mixture hydrated to 10% were very low, below the limit of detection of our psychrometry system. Preliminary tests with prepared natural materials, in which we recorded freezing exotherms for bulk samples chilled to -20°C, suggested that humic soil hydrated to 5% was unfreezable.

Each of the 77 frogs used in the experiment survived chilling to -2°C, regardless of whether they remained supercooled or froze. Only 4 (20%) of the 20 control frogs cooled in the absence of external ice nuclei froze during the trial (Table 1). In contrast, all frogs cooled with their ventral skin in contact with humic soil or soil/peat mixture ultimately froze. Ice inoculation also occurred in all but 1 of the 12 frogs (92%) used in trials with leaf mould.

Although no differences in freezing incidence occurred among treatment groups, subtle variation in inoculation susceptibility can be discerned from patterns in the delay before freezing began. For example, whereas nucleation of four control frogs occurred at a temperature ($T_c = -1.8 \pm 0.2^\circ\text{C}$) near the ambient temperature of the incubator, -2°C, after these frogs had been supercooled for 189 ± 74 min (mean \pm SE), frogs cooled in contact with natural materials either nucleated immediately upon reaching the FP_{eq} (i.e., without supercooling) or supercooled briefly before freezing. Thus, marked differences occurred in mean supercooling duration (ANOVA; $F_{6,70} = 33.0$, $P < 0.0001$) and mean T_c (ANOVA; $F_{6,53} = 18.2$, $P < 0.0001$) among the control and treatment groups.

Frogs exposed to moister substrates froze promptly, without supercooling, whereas inoculation was delayed in some frogs exposed to drier substrates, which had relatively lower water potentials (Table 1). Although water potential was lower in substrates augmented with organic matter (i.e., peat), the incidence of su-

TABLE 1. Susceptibility to inoculative freezing of wood frogs (*Rana sylvatica*) relative to hydric properties of natural materials to which they were exposed during cooling

	Organic content (%)	Moisture content at saturation (%)	Water potential (kPa) ¹	Body mass (g)	No. frogs tested	No. frogs freezing (% of sample)	T_c (°C)	No. frogs supercooling (% of sample)	Supercooling duration (min)
Control	–	–	–	14.0 ± 0.4	20	4 (20)	–1.8 ± 0.2	20 (100)	1190 ± 116
Humic topsoil	12.2	80							
40% moisture	–	–	–90	13.4 ± 0.5	10	10 (100) [†]	–0.8 ± 0.03	0 (0) [†]	0 ± 0
10% moisture	–	–	–990	14.1 ± 0.5	15	15 (100) [†]	–0.9 ± 0.1	6 (40) [†]	19 ± 7
5% moisture	–	–	< –5000	14.2 ± 0.5	10	10 (100) [†]	–1.3 ± 0.1	9 (90)	54 ± 8
Soil/peat mixture	58.1	534							
20% moisture	–	–	–3750	13.8 ± 1.4	5	5 (100) [†]	–0.6 ± 0.1	0 (0) [†]	0 ± 0
10% moisture	–	–	< –5000	14.4 ± 0.7	5	5 (100) [†]	–0.5 ± 0.1	1 (20) [†]	7 ± 7
Leaf mould	65.9	280							
100% moisture	–	–	–730	13.8 ± 0.5	12	11 (92) [†]	–1.2 ± 0.1	10 (83)	170 ± 116

¹1 kPa = 0.01 atm.

[†]Frequency differed from that of control group within the same column (Fisher's exact test, $P < 0.002$).

percooling in frogs exposed to humic topsoil (40%) and frogs exposed to the organic-enriched, soil/peat mixture (20%) did not differ statistically (Fisher's Exact Test: $P = 0.61$). Neither did these groups differ in mean supercooling duration (Scheffe: $P = 0.99$) or mean T_c (Scheffe: $P = 0.23$), suggesting that substrate organic content per se did not influence inoculation susceptibility. Inoculation was also delayed in most frogs exposed to leaf mould (Table 1).

Frogs lost 1–3% of their body mass (presumably as water) during the inoculation trials, irrespective of treatment (ANOVA: $F_{6,51} = 1.3$, $P = 0.28$). Within the control group, no differences (student's t -test: $t = 0.4$, $P = 0.69$) in mass loss occurred between frogs that remained supercooled ($1.4 \pm 0.5\%$) and those that froze ($1.7 \pm 0.1\%$). Because larger frogs have a greater skin surface over which inoculation can occur, we performed a correlation between (pretrial) body mass and supercooling duration (i.e., delay in inoculation). This relationship was examined for frogs exposed to leaf mould, which, as a group, exhibited the most varied responses. Although the smallest frog in the group was the only one to remain supercooled throughout the trial, supercooling duration and body mass were not correlated (linear regression: $r^2 = 0.22$, $F_{1,11} = 2.9$, $P = 0.12$).

Ice nucleating activity in bulk samples of humic soil, soil/peat mixture, and leaf mould relative to deionized water is depicted in Fig. 1. The influence of fluid volume on T_c was clearly evident in samples containing only water (ANOVA: $F = 6.8$, $P = 0.0009$), but this relationship was supplanted by the activity of potent ice nuclei in samples containing humic soil ($F = 0.5$, $P > 0.70$) or leaf mould ($F = 2.2$, $P > 0.10$). A significant ($F = 5.0$, $P = 0.005$) volume-dependence was found for the soil/peat mixture, but these samples nevertheless exhibited potent ice nucleating activity over a relatively narrow range (range of sample means, -5.8 to -6.4°C).

DISCUSSION

One central tenet in our understanding of animal freeze tolerance is that freezing survival depends on having extracellular water nucleate at a relatively high temperature. This occurrence moderates the rate of tissue freezing, restricts the formation of ice to extracellular compartments, and facilitates cryoprotective responses (Costanzo and Lee, '96; Storey and Storey, '96; Lee and Costanzo, '98). The deleterious effect of marked supercooling prior to ice inoculation has been di-

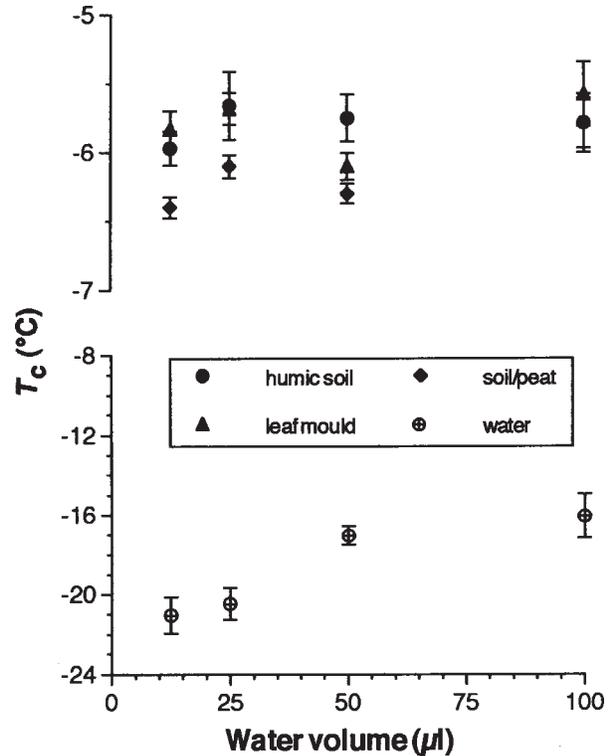


Fig. 1. Ice nucleating activity of natural materials used in tests of inoculative freezing of wood frogs (*Rana sylvatica*) relative to deionized water. Samples contained the prescribed volume of water only, or water and 100 mm³ substrate or leaf mould. Mean values, shown ± 1 SE, are based on $n = 12$ replicates per group.

rectly demonstrated in freeze-tolerant amphibians (Swanson et al., '96) and reptiles (Costanzo et al., '95a). In *R. sylvatica*, cryoinjury is avoided only if ice propagates slowly through the body because rapid freezing not only traps within organs water that would otherwise be translocated and sequestered innocuously within lymph spaces, but also hampers the mobilization and distribution of cryoprotectant (Costanzo et al., '92).

Given that the probability of spontaneous ice nucleation is lower in smaller aqueous volumes than in large ones (Vali, '91), it follows that supercooling capacity of animals lacking potent endogenous ice nuclei is strongly determined by body size (Lee and Costanzo, '98). Relative to most cold-hardy ectotherms, *R. sylvatica* is moderately large (up to 21 g for adult breeding males from southern Ohio) and thus has limited ability to supercool (in a dry environment, T_c is typically -2 to -3°C ; see Costanzo and Lee, '96). Although Layne found no correlation between T_c and body size in his sample of *R. sylvatica* (Layne, '95b), juveniles, and perhaps the much smaller adults in north-

ern populations (Martof and Humphries, '59), might be at risk of supercooling under certain environmental conditions.

Physiological factors influencing ice inoculation

Inoculative freezing was reported over 35 years ago (Salt, '63), yet the events whereby ice nuclei in the environment gain access to and seed the freezing of animal body fluids remain poorly understood (Lee and Costanzo, '98). In the case of anurans, inoculative freezing may be encouraged by the presence of isotonic secretions of the skin glands (e.g., Bjerregaard and Nielsen, '87; Ussing et al., '96) on the surface of the integument. Once an ice nucleus catalyzes the freezing of moisture on the skin, it is likely that the ice lattice penetrates pores or channels involved in osmotic water exchange, ultimately contacting and inoculating body fluids (see Layne et al., '90). If this model is valid, then the efficacy of ice inoculation may be influenced by osmotic permeability of the integument, which is markedly higher in anurans than in other vertebrate animals (Shoemaker et al., '92). Osmotic permeability of anuran skin, which is largely mediated by water channels homologous to the mammalian aquaporins (Harvey et al., '91; Verkman, '92; Ma et al., '96), is affected by temperature, season, and various physiological factors. In *R. pipiens*, for example, skin permeability markedly decreases at low temperature (Parsons and Lau, '76) and in winter relative to other seasons (Parsons et al., '78), due collectively to thermal effects on bulk and diffusive flows, and changes in endocrine status and responsiveness of the integument to antidiuretic hormone. Reduced skin permeability in winter presumably benefits *R. pipiens*, an aquatic hibernator, by curbing water influx. Skin permeability to water is also reduced in *R. sylvatica* during winter (Hadley, '69), although our results indicate that this species nevertheless remains highly susceptible to ice inoculation.

Ice inoculation was delayed in frogs cooled in contact with frozen leaf mould relative to frogs cooled in contact with frozen soil (Table 1). This result may simply reflect a less intimate contact between the skin and overlying sheets of leaf mould, as compared to a particulate substrate that may better conform to body contours. Alternatively, this difference may stem from variation in permeability of the skin surfaces involved. Under appropriate endocrine stimulation, permeability may be higher in skin of the ventral surface, par-

ticularly that of the so-called "pelvic patch," which facilitates cutaneous imbibition (Bentley and Main, '72). However, Layne et al. ('90) detected no differences in the rate of ice propagation across samples of dorsal and ventral skin from *R. sylvatica* mounted in a diffusion chamber.

Environmental influences on susceptibility to inoculative freezing

In the laboratory, contact of the skin of supercooled frogs with ice crystals on frozen filter paper, or in frozen soil, catalyzes freezing of the body fluids within 60 sec, suggesting that anurans are highly susceptible to inoculative freezing by environmental ice (Layne et al., '90; Layne, '91). The freeze-tolerant treefrogs *Hyla versicolor* and *Pseudacris crucifer*, for example, freeze virtually on contact with wet soil (60% moisture; Layne, '91). However, under field conditions, inoculation susceptibility of some burrowing animals may be strongly influenced by physical characteristics of the substrate, such as texture, porosity, water content, organic content, and water potential. (Salt, '63; Layne et al., '90; Lundheim and Zachariassen, '93; Costanzo et al., '95a,b; Costanzo et al., '97). Some ectotherms better avoid ice inoculation when cooled in substrates containing relatively high amounts of organic matter or clay, as these materials adsorb water and reduce soil water potential which, in turn, limits the amount of ice forming within soil pores (Forge and MacGuidwin, '92; Costanzo et al., '97). However, organic content of the substrate made no difference in inoculation susceptibility of our frogs. Whether water potential would more strongly influence inoculation susceptibility of anurans with less permeable skin remains to be determined.

The winter microenvironment to which *R. sylvatica* is exposed undoubtedly hosts, in addition to ice crystals, various potent ice nuclei. Ice nuclei commonly occurring in soil, vegetative detritus, and the atmosphere are likely complexes of inorganic and organic elements formed during decay of organic materials (Vali, '91). Thus, it is not surprising that nucleating agents were found in the natural materials we studied. Freezing of frogs exposed to humic soil hydrated to 5% may have been initiated by contact with these nuclei, given that this substrate was unfreezable and thus lacked ice crystals. Substrates and leaf mould used in our inoculation trials inoculated small volumes of supercooled water at ca. -6°C (Fig. 1). Considering the greater water surface represented by the moist integument, it is conceivable that they may

nucleate frogs at markedly higher temperatures. The roles of environmental nuclei other than ice crystals in the cold hardiness of ectothermic animals are poorly understood (Lee and Costanzo, '98).

Efficacy of ice inoculation as a cryoprotective mechanism

Given that anurans generally must hibernate in moist microenvironments to prevent excessive desiccation, and that soil and organic detritus associated with the hibernaculum may contain nuclei in addition to ice crystals, it seems likely that in nature somatic freezing usually occurs at body temperatures near the tissue FP_{eq} . However, ice inoculation was somewhat less effective in drier soils, which *R. sylvatica* apparently prefers for overwintering (Hadley, '69; see also Licht, '91). We did not test juvenile frogs, which, by virtue of their relatively small skin surface, might be less susceptible to inoculative freezing (albeit this relationship was not significant over the limited variation in body mass in our sample). Furthermore, we did not explore the possibility that inoculation may vary with physiological condition. Notably, supercooling capacity may be enhanced, and inoculation susceptibility reduced, by an increase in the osmotic concentration of body fluids (Lee and Costanzo, '98). In *R. sylvatica*, such changes may result from organismal dehydration or prior freezing, which mobilizes cryoprotective solutes. Perhaps under these circumstances, ice-nucleating bacteria harbored in the gut or skin of overwintering frogs may fortuitously promote freezing at relatively high temperatures (Layne, '95a; Lee et al., '95). Curiously, the blood of *R. sylvatica* (and other freeze-tolerant and freeze-intolerant vertebrates; Costanzo and Lee, '96) contains a nucleator that exhibits activity in vitro at -7 to -8°C (Wolanczyk et al., '90), but this agent apparently plays no role in ice inoculation. Although many freeze-tolerant animals employ physiological mechanisms that reliably initiate freezing at high subzero temperatures and thereby promote survival (Duman et al., '95; Lee and Costanzo, '98), our present results suggest that the moist, relatively permeable skin of amphibians effectively serves this purpose by promoting inoculative freezing.

ACKNOWLEDGMENTS

We thank C. Kostizen, J. Litzgus, and C. Wentzel for providing technical assistance, and J. Layne, H. Ramlø, and M. Wright for critiquing the manuscript. This work was supported by a

Sigma Xi Grant-in-Aid and Miami University Undergraduate Research Grant to J.M.B., and a National Science Foundation grant (IBN 9507437) to J.P.C.

LITERATURE CITED

- Bentley PJ, Main AR. 1972. Zonal differences in permeability of the skin of some anuran amphibia. *Am J Physiol* 223:361–363.
- Bjerregaard HF, Nielsen R. 1987. Prostaglandin E_2 -stimulated glandular ion and water secretion in isolated frog skin (*Rana esculenta*). *J Membrane Biol* 97:9–19.
- Costanzo JP, Grenot C, Lee RE. 1995a. Supercooling, ice inoculation, and freeze tolerance in the European common lizard, *Lacerta vivipara*. *J Comp Physiol* 165:238–244.
- Costanzo JP, Iverson JB, Wright MF, Lee RE. 1995b. Cold hardiness and overwintering strategies of hatchlings in an assemblage of northern turtles. *Ecology* 76:1772–1785.
- Costanzo JP, Lee RE. 1996. Mini review: ice nucleation in freeze-tolerant vertebrates. *Cryo-Lett* 17:111–118.
- Costanzo JP, Lee RE, Wright MF. 1992. Cooling rate influences cryoprotectant distribution and organ dehydration in freezing wood frogs. *J Exp Zool* 261:373–378.
- Costanzo JP, Moore JB, Lee RE, Kaufman PE, Wyman JA. 1997. Influence of soil hydric parameters on the winter cold hardiness of a burrowing beetle, *Leptinotarsa decemlineata* (Say). *J Comp Physiol* 167:169–176.
- Duman JG, Olsen TM, Yeung KL, Jerva F. 1995. The roles of ice nucleators in cold tolerant invertebrates. In: Lee RE, Warren GJ, Gusta LV, editors. Biological ice nucleation and its applications. St. Paul, MN: American Phytopathological Society Press. p 201–219.
- Forge TA, MacGuidwin AE. 1992. Effects of water potential and temperature on survival of the nematode *Meloidogyne hapla*. *Can J Zool* 70:1553–1560.
- Hadley RS. 1969. The effects of season and temperature on certain aspects of the physiology of the Alaskan wood frog, *Rana sylvatica*. M.S. Thesis, University of Alaska, College, AK. 42 p.
- Harvey B, Lacoste I, Ehrenfeld J. 1991. Common channels for water and protons at apical and basolateral cell membranes of frog skin and urinary bladder epithelia: effects of oxytocin, heavy metals, and inhibitors of H^+ -adenosine triphosphatase. *J Gen Physiol* 97:749–776.
- Hillel D. 1971. Soil and water: Physical principles and processes. In: Kozlowski TT, editor. Physiological ecology: a series of monographs, texts and treatises. New York: Academic Press. p 288.
- Kirton MP. 1974. Fall movements and hibernation of the wood frog, *Rana sylvatica*, in interior Alaska. M.S. Thesis, University of Alaska, Fairbanks, AK. 57 p.
- Layne JR Jr. 1991. External ice triggers freezing in freeze-tolerant frogs at temperatures above their supercooling point. *J Herpetol* 25:129–130.
- Layne JR Jr. 1995a. Crystallization temperatures of frogs and their individual organs. *J Herpetol* 29:296–298.
- Layne JR Jr. 1995b. Seasonal variation in the cryobiology of *Rana sylvatica* from Pennsylvania. *J Therm Biol* 20:349–353.
- Layne JR Jr, Lee RE, Huang JL. 1990. Inoculation triggers freezing at high subzero temperatures in a freeze-tolerant frog (*Rana sylvatica*) and insect (*Eurosta solidaginis*). *Can J Zool* 68:506–510.

- Lee MR, Lee RE, Strong-Gunderson JM, Minges SR. 1995. Isolation of ice-nucleating active bacteria from the freeze-tolerant frog, *Rana sylvatica*. *Cryobiology* 32:358–365.
- Lee RE, Costanzo JP. 1998. Biological ice nucleation and ice distribution in cold-hardy ectothermic animals. *Annu Rev Physiol* 60:55–72.
- Licht LE. 1991. Habitat selection of *Rana pipiens* and *Rana sylvatica* during exposure to warm and cold temperatures. *Am Midl Nat* 125:259–268.
- Lundheim R, Zachariassen KE. 1993. Water balance of overwintering beetles in relation to strategies for cold tolerance. *J Comp Physiol* 163:1–4.
- Ma TH, Yang BX, Verkman AS. 1996. cDNA cloning of a functional water channel from toad urinary bladder epithelium. *Amer J Physiol* 40:C1699–C1704.
- Martof BS, Humphries RL. 1959. Variation in *Rana sylvatica*. *Am Midl Nat* 61:350–389.
- Parsons RH, Brady R, Goddard B, Kernan D. 1978. Seasonal effects on osmotic permeability of *Rana pipiens* in vivo and in vitro. *J Exp Zool* 204:347–352.
- Parsons RH, Lau Y-T. 1976. Frog skin osmotic permeability: effect of acute temperature change in vivo and in vitro. *J Comp Physiol* 105:207–217.
- Salt RW. 1963. Delayed inoculative freezing of insects. *Can Entomologist* 95:1190–1202.
- Shoemaker VH, Hillman SS, Hillyard SD, Jackson DC, McClanahan LL, Withers PC, Wygoda ML. 1992. Exchange of water, ions, and respiratory gases in terrestrial amphibians. In: Feder ME, Burggren WW, editors. *Environmental physiology of the amphibians*. Chicago: University of Chicago Press. p 125–150.
- Storey KB, Storey JM. 1996. Natural freezing survival in animals. *Ann Rev Ecol Syst* 27:365–386.
- Swanson DL, Graves BM, Koster KL. 1996. Freezing tolerance/intolerance and cryoprotectant synthesis in terrestrially overwintering anurans in the Great Plains, USA. *J Comp Physiol* 166:110–119.
- Ussing HH, Lind F, Larsen EH. 1996. Ion secretion and isotonic transport in frog skin glands. *J Membrane Biol* 152:101–110.
- Vali G. 1991. Nucleation of ice. In: Gotz G, Meszaros E, Vali G, editors. *Atmospheric particles and nuclei*. Budapest: Akademiai Kiado. p 131–192.
- Verkman AS. 1992. Water channels in cell membranes. *Annu Rev Physiol* 54:97–108.
- Wolanczyk JP, Storey KB, Baust JG. 1990. Ice nucleating activity in the blood of the freeze-tolerant frog, *Rana sylvatica*. *Cryobiology* 27:328–335.