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MINI-REVIEW: ICE NUCLEATION IN FREEZE-TOLERANT VERTEBRATES

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SUMMARY

The survival of freeze-tolerant vertebrates requires that ice nucleation in tissues be initiated at relatively high temperatures. Three current hypotheses for the initiation of freezing in these animals are: *a*) inoculation by contact with ice crystals in the external environment; *b*) nucleation by ice-nucleating bacteria associated with the animal; and *c*) nucleation by ice-nucleating proteins in the blood. The available evidence indicates that ice inoculation is the most important mechanism under natural conditions, although ice-nucleating bacteria harbored in the gut or skin during winter may function as an auxiliary system to promote crystallization, albeit at slightly lower temperatures. Despite the intriguing similarities between the blood ice-nucleating agents of some vertebrates and those of certain freeze-tolerant invertebrates, these agents seem unlikely to play an analogous, adaptive role in freeze tolerance.

KEY WORDS: Ice nucleation, inoculation, ice-nucleating bacteria, ice-nucleating proteins, vertebrate freeze tolerance

It is generally believed that the survival of freeze-tolerant animals is promoted by mechanisms that initiate tissue freezing at relatively high body temperatures. Theoretically, the amount of ice forming during nucleation is proportional to the degree of supercooling prior to nucleation (3). Thus, to minimize cryoinjury, ice nucleation should occur as near as possible to the equilibrium freezing point (i.e., FP_{eq}) of the body fluids, the highest body temperature at which ice crystallization and propagation is possible. Among the freeze-tolerant vertebrates this tenet derives empirical support from studies of lizards (4) and snakes (1) in which the ice crystallization temperature (T_c) is inversely related to the severity of cryoinjury. The rapid propagation of ice within tissues and organs allows little time for cells to adjust to the attendant osmotic stress and compromises survival by inhibiting normal cryoprotective responses (9, 10).

Owing to their relatively large size, most freeze-tolerant vertebrates do not supercool extensively (7, 8). However, young individuals (e.g., hatchling turtles), and the smaller species (e.g., hylid frogs and lizards), may be at considerable risk for this type of injury. Whereas the strategies used by various invertebrates for regulating T_c have received extensive study (13), relatively little is known about ice nucleation in freeze-tolerant vertebrates. We here review three current hypotheses concerning the mechanisms that initiate crystallization in these animals.

ICE INOCULATION BY CONTACT WITH AMBIENT ICE

Most freeze-tolerant vertebrates overwinter in shallow terrestrial hibernacula where physical contact with ice crystals may initiate the freezing of their tissues. Laboratory experiments using both isolated skin preparations and intact animals suggest that amphibian

skin is a poor barrier to inoculative freezing (Table 1). Indeed, supercooled frogs begin to freeze within 1 min after contacting a frozen paper or soil substrate (15, 19). Given that amphibians are restricted to moist hibernacula by virtue of their susceptibility to desiccation, it seems likely that they would supercool little, if at all, under field conditions. However, no study has demonstrated inoculative freezing of vertebrates in nature.

Reptiles are relatively less susceptible to inoculative freezing, probably because their dry, cornified skin better deters the inward propagation of ambient ice (albeit not as effectively as the skin of some fish; Table 1). Nevertheless, inoculation occurs in reptiles when ice permeates the skin, or accesses tissues via the major orifices (e.g., nostrils, eyes, cloaca) or integumentary wounds (22, 33). Inoculative freezing can occur rapidly (7) or can be deferred many hours or days (25). According to one hypothesis delayed inoculation results when saturated vapor, which is under high pressure in supercooled body fluids relative to the external environment, escapes through skin pores and condenses on adjacent ice crystals. Ice forming thusly propagates toward the vapor's source and ultimately seeds the freezing of the supercooled tissues (25, 31).

A few studies have addressed the efficacy of inoculative freezing and its implications for reptilian cold hardiness (6, 7, 24-27, 29). The thermal microenvironment exerts an especially strong influence on inoculation susceptibility, since the likelihood and timeliness of inoculation markedly increase with decreasing temperature (25, 27). Even among small cold-hardy reptiles, which have the greatest innate capacity for supercooling (7, 8), ice inoculation may limit the utility of supercooling as a freeze-avoidance strategy. For example, the incidence of inoculative freezing in hatchling *C. picta* cooled in frozen clayey soil increases from 10-12% at -2.6°C (27) to 25-30% at temperatures between -5 and -6°C (25). At -9°C, one half or more of the turtles begin to freeze (24-26). Thus, it appears doubtful that many turtles can avoid freezing at the minimum temperatures (e.g., -12°C) they may encounter during winter (7, 11, 28, 45).

Hydric and textural properties of the substrate also influence ice inoculation. For example, whereas hatchling painted turtles (*Chrysemys picta*) enveloped in frozen soil can remain supercooled for several hours or days if the substratum consists primarily of moist clay (25), specimens cooled in damp sandy soil are immediately inoculated (7). Both results were obtained using hatchlings from Nebraska populations that overwinter in sandy soils. Thus, tests of inoculation susceptibility should employ experimental protocols having ecological relevance to the species under investigation.

Table 1. Summary of reports regarding the susceptibility to inoculative freezing of vertebrate ectotherms.

	Susceptibility to inoculative freezing	Preparation studied	Reference
Fish			
Pseudopleuronectes americanus (winter flounder)	very low	isolated skin	(40)
Amphibians			
Rana sylvatica (wood frog)[a]	very high	isolated skin, intact animal	(19)
Pseudacris crucifer (spring peeper)[a]	very high	intact animal	(15)
Hyla versicolor(grey treefrog)[a]	very high	intact animal	(15)
Reptiles			
Lacerta vivipara (common lizard)[a]	moderate	intact animal	(6)
Chrysemys picta (painted turtle)[a]	moderate	intact animal	(25-27)
	high	intact animal	(7)
Chelydra serpentina (snapping turtle)	high	intact animal	(29)

[a]freeze tolerant

Susceptibility to inoculative freezing is also influenced by physiological and genetic factors. Some evidence suggests that the ability of hatchling *C. picta* to resist ice inoculation varies anatomically. The skin of the head and neck apparently is more resistant than the skin of the inguinal and axial pouches (27). Overall differences in inoculation susceptibility occur among (29) and within (25) species. It is plausible that susceptibility to inoculation is influenced by thermal acclimation and acclimatization, particularly as these processes may promote ultrastructural changes in the integument, such as lipid content and composition (23). The specific roles of these factors are unknown.

These various endogenous and exogenous factors have important implications for the cold hardiness strategy employed by some species, such as hatchling *C. picta* and the European common lizard, *Lacerta vivipara*. Under favorable conditions, these small reptiles may supercool to very low temperatures (< -12°C) or remain supercooled for long periods (e.g., > 3 wk). However, under conditions that promote inoculative freezing, survival is conferred by their tolerance for the freezing of tissues. In this scenario, supercooling predominates during periods of low ambient water potential, when inoculative freezing is unlikely. Conversely, exposure to damp substrate (e.g., after rain or snow melt) would promote inoculative freezing and a reliance on freeze tolerance. This system effectively promotes winter survival under dynamic physiological and microenvironmental conditions (6, 7).

ICE-NUCLEATING BACTERIA

Ice-nucleating bacteria are potent ice nucleators that are ubiquitous in various temperate habitats. Known primarily as plant epiphytes, ice-nucleating bacteria were recently described as components of the gut flora of the freeze-tolerant wood frog, *R. sylvatica* (20, 21). Nucleating activity was identified phenotypically in 13 strains of *Pseudomonas fluorescens*, 4 strains of *Pseudomonas putida*, and 2 strains of *Enterobacter agglomerans* cultured from the intestine of winter-collected *R. sylvatica*.

Feeding of *P. putida* suspensions to the freeze-tolerant frog, *P. crucifer*, caused each of 10 specimens to freeze during a 24-h exposure to -2 °C, and, indeed, ice nucleation in 5 frogs occurred within 10 min of administering the bacteria. By contrast, freezing occurred in only 20% of the control frogs ingesting only the vehicle. Nucleating activity of aqueous suspensions of the 4 *P. putida* strains (range, -1.6 to -3.0°C) was in close accord with the observed T_c of intact *R. sylvatica*.

Additional, indirect evidence also suggests that ice-nucleating bacteria may function to initiate tissue freezing at relatively high body temperatures. The T_c of intact *R. sylvatica* corresponds closely with that of isolated intestine and skin, organs which likely harbor such bacteria, but not of other body organs, tissues, and fluids (Table 2). Furthermore, our corroborating data for *R. sylvatica* also indicate greater nucleating activity in the intestine as compared with skeletal muscle (Figure 1). Centrifugation of these crude organ homogenates resulted in a marked loss of nucleating activity from intestine, but not from muscle, and these differences cannot be explained by changes in the concentrations of nonspecific proteins. Rather, the enhanced supercooling of the former suggests that the centrifugation removed a

Table 2. T_c (°C) of intact specimens of the freeze-tolerant wood frog (*R. sylvatica*) and the freeze-intolerant leopard frog (*R. pipiens*), and their isolated organs. Means are shown ± 1 SEM. Adapted from Ref. (17)

			Organ[a]			
	n	Intact specimen	gut	skin	liver	muscle
Rana sylvatica	8	-2.5 ± 0.1	-3.1 ± 0.2	-3.6 ± 0.3	-5.5 ± 0.5 [b]	-6.4 ± 1.0 [b]
Rana pipiens	8	-2.2 ± 0.1	-3.2 ± 0.3	-3.6 ± 0.4	-4.1 ± 0.5 [b]	-4.5 ± 0.5 [b]

[a]100 mg samples [b]mean was significantly lower than the corresponding mean for the intact specimens ($P < 0.05$)

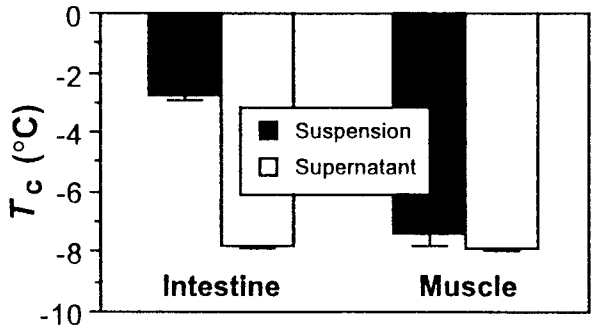

Figure 1. Ice-nucleating activity, as indicated by T_c, of homogenates of intestine and skeletal (sartorius) muscle of the freeze-tolerant wood frog (*Rana sylvatica*), before or after centrifugation. Mean (± 1 SEM) T_c, values are based on n = 5 frogs collected in late winter; the value for each individual was the average of 4 replicate determinations on 10-µl aliquots cooled in sealed, glass microcapillary tubes. Protein concentrations of the suspensions, prepared by homogenizing organs (20 mg/ml) in phosphate-buffered saline (235 mosM, pH 7.4), were statistically indistinguishable (Student's t = 0.23, df = 8, P > 0.82) between intestine (803 ± 118 µg/ml) and muscle (760 ± 155 µg/ml). Paired Student's t-tests indicated that centrifugation of the suspensions at 14,000 g for 5 min decreased the T_c of preparations from intestine (t = 29.9 df = 4, P << 0.01), but not muscle (t = 1.2, df = 4, P > 0.30). J. P. Costanzo, L. Smith, and R. E. Lee, Jr. (unpubl. data).

potent ice-nucleating agent. Although it appears that ice-nucleating bacteria have the potential to promote ice formation in freeze-tolerant frogs, this contention has not been tested under field conditions. Additional study is required to determine the prevalence of ice-nucleating bacteria in frogs (and other freeze-tolerant vertebrates), as well as the frequency with which these bacteria express the ice-nucleating phenotype. The exceptionally high supercooling capacity of hatchling *C. picta* reported by Packard and colleagues (25-27) suggests an absence of ice-nucleating bacteria, which may be an artifact of incubating and rearing specimens in the laboratory; generally, field-collected specimens supercool minimally (2, 5, 7, 11, 38).

ICE-NUCLEATING PROTEINS IN BLOOD

Among the freeze-tolerant vertebrates, blood-borne ice nucleators were initially reported for *R. sylvatica* (43, 44) and hatchling *C. picta* (36). Present in whole blood, serum, and plasma, these agents are proteinaceous (heat labile, acid sensitive) and retain nearly full activity at -7 to -8°C at dilutions of up to 1 in 500. The similarities in nucleating properties among such ice-nucleating proteins (INPs) and those found in the body fluids of certain tardigrades (42), molluscs (14), and insects (12, 13), which demonstrably function to initiate ice formation at high subzero temperatures, may indicate that the vertebrate agents have an analogous, adaptive role. Proponents of this hypothesis assert that the selective expression of specific blood INPs is one of the critical factors in the evolution of vertebrate freeze tolerance, and promulgate the presence of these agents as a criterion for freeze tolerance (36).

However, because these workers found blood INPs lacking in the box turtle (*Terrapene carolina*) and the garter snake (*Thamnophis sirtalis*), 2 of the 4 freeze-tolerant species they studied (36, 43, 44), such agents clearly would not be requisite for freeze tolerance. A corollary problem is that, contrary to certain claims (36), a substantial body of evidence indicates that blood INPs are not unique to freeze-tolerant vertebrates (Table 3). Spellerberg (33) reported nucleating activity at temperatures as high as -8°C in the blood and plasma of freeze-intolerant reptiles, and the data of Lowe *et al.* (22) suggest that the blood of various ectothermic and endothermic vertebrates may have even greater nucleating activity. Additionally, we found heat-labile blood nucleators in both freeze-tolerant and freeze-intolerant vertebrates, including endotherms (8), as did Layne (16). Collectively these studies cast doubt on the notion that blood INPs are unique to freeze-tolerant vertebrates.

One criticism of this analysis is the lack of uniformity among the experimental protocols used to investigate ice-nucleating activity in the blood and other tissues (Table 3). Indeed,

Table 3. Summarized results of laboratory studies measuring crystallization temperatures (T_c) of blood samples of various vertebrate animals.

Sample and cooling protocol	Species	Freeze tolerant?	T_c range (°C)	Reference
whole blood cooled in Warburg flask[a]	fish	no	-10.6	(22)
	amphibians (4 *spp.*)	no	-7.7 to -12.0	
	reptile	no	-12.0 to -12.9	
	endotherm	no	-10.5 to -13.5	
whole blood, plasma cooled in unspecified manner[a]	reptiles (11 *spp.*)	no	-8.0 to -18.0	(33)
50 or 100 µl whole blood cooled in microcentrifuge tube	amphibian	yes	-5.1 to -6.6	(16)
	amphibian	no	-6.9	
10 µl serum[b] used in differential scanning calorimetry	amphibian	yes	-7.8	(43)
cell-free blood, serum, plasma used in differential scanning calorimetry[a]	amphibian	yes	-6.3 to -7.4	(44)
7 µl plasma cooled in sealed capillary tube	fish	no	-13.4	(8)
	amphibians (2 *spp.*)	yes	-7.3 to -7.7	
	amphibians (3 *spp.*)	no	-6.8 to -8.4	
	reptiles (2 *spp.*)	yes	-8.1 to -9.1	
	endotherms (2 *spp.*)	no	-8.1 to -12.3	
1-µl aliquots of serum used in freezing droplet assay	fish	no	-14.6	(36)
	amphibians (5 *spp.*)	no[c]	-12.4 to -20.8	
	reptiles (3 *spp.*)	yes	-8.0 to -16.8	
	endotherms (13 *spp.*)	no	-15.3 to -21.5	

[a]sample volume not reported [b]erroneously reported as "plasma" [c]status of some species was presumed by us on the basis of life history traits

experimental techniques in some reports are inadequately described or may be undesirable because they encourage sample contamination. The available studies are also inconsistent with regard to the sample volume used, which in practice may be determined simply by the cooling technique employed (e.g., differential scanning calorimetry and droplet-freezing assays require very small samples). The use of very small volumes (e.g., 1-5 µl) facilitates the detection of potent nucleators, since such samples would otherwise supercool extensively. However, although they may be appropriate in studies of most insects and other small invertebrates, these samples probably do not adequately represent the volumes comprising the bulk fluid compartments of many vertebrates. Nucleation theory predicts that the influence of relatively large fluid volumes on the T_c may supersede the effects of relatively weak nucleators (41). In practice, however, considerable variation in sample volume may influence T_c minimally. Our reanalysis of Zachariassen and Hammel's (46) T_c data for fluid samples indicate no statistically significant differences among volumes spanning 2 orders of magnitude (i.e., from 10 to 1000 µl).

Despite the lack of uniformity in experimental protocol there is some striking agreement among the data on blood nucleators reported by various workers (Table 3). Using markedly different investigative techniques, 3 studies concluded that fish blood unequivocally lacks ice nucleating activity. Also, mean (± 1 SE) T_c values for *R. sylvatica* blood, -7.3 ± 0.6°C (8), -7.8 ± 0.1°C (43), and -6.6 ± 1.0°C (16), were in reasonably good accord among studies using sample volumes of 7 µl, 10 µl, and 50 µl, respectively. Two of these studies also reported comparable T_c means (-6.8°C and -6.9°C) for the freeze-intolerant leopard frog (Table 3).

The contention that the T_c of freeze-tolerant vertebrates is regulated by blood INPs stipulates that ice-nucleating activity in the blood must be at least as potent as that exhibited by other tissues. However, *R. sylvatica* blood actually exhibits less activity than do certain

other tissues (16). Given that the T_c of intestine and skin are higher than that of plasma by 3.0 and 3.5 degrees Centigrade, respectively, nucleation would occur preferentially in these tissues, rather than in the blood. Generally, the body compartment hosting the most potent nucleators determines the supercooling capacity of the intact organism (39). The marked disparity between the activity temperature of blood INPs and the T_c of intact specimens thus represents another fundamental problem with the blood INP hypothesis. For *R. sylvatica,* cooled in the absence of ambient ice, spontaneous nucleation usually begins at temperatures about 6 degrees Centigrade above those indicative of blood INP activity (Table 4). Indeed, the minimal supercooling capacity of *R. sylvatica* is probably the strongest argument against a need for INPs (35). In contrast, the T_c of hatchling *C. picta*, a species whose supercooling capacity depends on prevailing physiological and environmental conditions (7), ranges broadly, from -1 to < -13 °C (Table 4). Thus, freezing in this species can be initiated either well above or below the activity temperature of its blood INPs. The literature provides no evidence that the activity of blood INPs is expressed *in vivo*.

Finally, there is a crucial paradox in that the *in vitro* activity of blood INPs occurs at temperatures 2-4 degrees Centigrade lower than the minimum body temperature that can be survived in the frozen state by either species: -6 °C for *R. sylvatica* (18, 32, 34, 37), -4 °C for *C. picta* (2, 7, 37). Proponents of the hypothesis suggest that blood INPs might be more potent *in vivo* if: 1) INPs form cooperative interactions with cell membranes of the vascular epithelium; 2) the -8°C value is an artifact of small sample volume; or 3) the -8°C value is an artifact of freezing damage to the INPs sustained during storage at -80°C (43, 44). To date these explanations remain unsubstantiated.

Although the available evidence does not suggest an adaptive role for blood INPs in vertebrate freeze tolerance, the structure and behavior of these nucleators are of academic interest. Particularly intriguing is the recent finding of potent nucleating activity not only in body fluids (plasma and urine), but also in isolated organs (liver, skeletal muscle, skin, and

Table 4. Crystallization temperatures (T_c) of intact specimens and blood samples of wood frogs (*Rana sylvatica*) and hatchling painted turtles (*Chrysemys picta*). Means are shown ± 1 SEM.

		Population	*n*	T_c (°C)	Reference
Rana sylvatica					
	intact specimen	Minnesota	3	-1.9 ± 0.1	(32)
		Ohio	43	-2.2 ± 0.1	(18)
		Ontario	3	-2.0 ± 0.1	(37)
			9	-3.0 ± 0.04	(34)
		Pennsylvania	12	-3.0 ± 0.1	(17)
		West Virginia	8	-2.5 ± 0.1	(16)
	blood	n.r.	n.r.	-7.4 ± 0.2	(44)
		n.r.	n.r.	-7.8 ± 0.1	(43)
Chrysemys picta					
	intact specimen	Ohio	6	-1.1	(5)
		Ontario	4	-3.3 ± 0.2	(38)
			8	-2.5 ± 0.5 [a]	(2)
		Manitoba	6	-3.7 ± 0.3 [b]	(2)
		New Jersey	9	-4.8 ± 0.7	(11)
		Nebraska	6	-3.0 ± 0.1	(7)
			6	-2.8 ± 0.1	(7)
			16	< -10.9 [c]	(26)
			9	< -8.0 [d]	(30)
			4	-8.7 ± 0.08 [e]	(30)
	blood	Ontario	3	-8.0 ± 0.4 [f]	(36)

n.r., not reported
[a] *C. p. marginata* [b] *C. p. bellii* [c] data for turtles on platforms, Table 2 [d] run #1 [e] run #2 [f] data for serum

intestine) of both freeze-tolerant and freeze-intolerant frogs (16). The fundamental nature and structure of these agents remain essentially unknown.

In summary, inoculative freezing of amphibians and, under some conditions, reptiles is an efficient mechanism initiating the freezing of tissues at body temperatures very near FP_{eq}. In the event that inoculation fails, ice-nucleating bacteria harbored by the skin or gut of overwintering animals may function in an auxiliary capacity. The available evidence indicates that blood INPs do not play an adaptive role in promoting freeze tolerance among vertebrate ectotherms. Useful comparisons of the results from different studies would be facilitated by standardizing methodologies for measuring T_c, the thermal history and season of testing, procedures for sample procurement and preparation, and the volume of samples used in analyses. One challenge to workers in this field is the development of experimental protocols that are not only pragmatic, but that also are physiologically and ecologically relevant to the system under investigation.

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