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BIOLOGICAL ICE NUCLEATION AND ICE DISTRIBUTION IN COLD-HARDY ECTOTHERMIC ANIMALS

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ABSTRACT

For many ectotherms, overwintering survival depends on the avoidance or regulation of ice nucleation and growth within their body fluids. Freeze avoidance via supercooling plays an important role in the cold hardiness of many small species, particularly terrestrial arthropods, that do not survive the freezing of their body fluids. In contrast, mechanisms that limit supercooling and initiate freezing at relatively high temperatures promote survival of the few invertebrates and vertebrates that tolerate freezing. These mechanisms include inoculative freezing, which results from contact with ice in the environment, and various ice nucleating proteins, microbes, and crystalloid compounds. In freeze-tolerant ectotherms, cold hardiness is influenced by complex, seasonally changing interactions among physiological factors, ice nucleators, and the physical microenvironment. Extraorgan sequestration of ice is a major adaptation of freeze tolerance. For most freeze-tolerant species, ice growth is primarily restricted to extracellular compartments; however, intracellular freezing also occurs in some species.

INTRODUCTION

A review article on the subject of biological ice nucleation and internal ice formation may seem to many physiologists, particularly ones that work with birds and mammals, as a rather bizarre topic with little relevance to the normal function and survival of animals. However, winter survival of many ectotherms critically depends on either avoidance or regulation of ice formation within their bodies.

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A number of recent reviews have dealt with various aspects of cold tolerance and winter survival; however, here we focus on endogenous and environmental factors that influence or regulate supercooling and ice nucleation within body fluids and compartments. It has become clear in recent years that studying an organism in isolation from its environment can result in major misconceptions about its means for winter survival. Consequently, we use several model systems to illustrate the critical and dynamic role that complex interactions between the organism and its particular microenvironment play in the survival of ectotherms at low temperature. We also examine sites of ice nucleation and growth, and their significance to organismal cold tolerance.

BIOLOGICAL ICE NUCLEATION AND SUPERCOOLING

A solution that remains unfrozen at temperatures below its equilibrium freezing point (FP_{eq}) is said to be supercooled. Water droplets of a few microliters in volume can supercool to −40°C before spontaneously freezing. The first step in ice nucleation is the aggregation of water molecules to form an embryo; once this cluster reaches a critical size, such that it grows rather than disperses, a nucleus is formed upon which an ice crystal can grow (1).

Ice nuclei may arise by two mechanisms. One, termed homogeneous nucleation, involves only the spontaneous aggregation of water molecules. The chance of an aggregation reaching critical size increases with decreasing temperature and increasing duration of chilling. The other mechanism, heterogeneous nucleation, occurs when some entity other than water forms the template upon which an ice crystal forms. These ice-nucleating agents facilitate the clustering of water molecules and increase the likelihood that embryos reach a critical size.

When exposed to subzero temperatures, many organisms can supercool, sometimes by many degrees, before ice nucleates within their body fluids. The lowest body temperature (T_b) reached before body fluids begin to freeze is called the supercooling point or temperature of crystallization (T_c). Within biological systems, ice nucleation is believed to begin by heterogeneous mechanisms in which nucleating agents catalyze ice nucleation at relatively high subzero temperatures. Organisms realize their innate capacity to supercool only in the absence of these agents. Ice nucleators vary in their potency to initiate freezing, with more efficient ones inducing freezing at higher T_bs. Although an organism may contain a variety of potential ice nucleators, only the most efficient one(s) actually catalyzes an ice nucleation event, because once freezing begins, the ice lattice grows throughout the body. Furthermore, the release of the latent heat of crystallization warms the body and thus decreases the chance that other

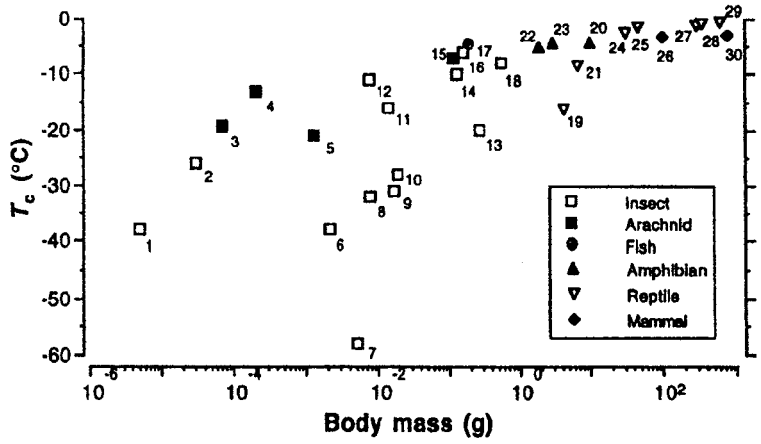

Figure 1 Relationship between body mass and temperature of crystallization (T_c) of animals representing Arthropoda and Vertebrata. Legend: 1. eggs of *Psylla mali* (81); 2. eggs of *Arcynopteryx compacta* (82); 3. *Hygroribates marinus* (6); 4. *Alaskozetes antarcticus* (6); 5. larvae of *Ixodes uriae* (83); 6. larvae of *Diplolepis bicolor* (8); 7. larvae of *Mayetiola rigidae* (8); 8. *Typhlocyba* sp. (8); 9. larvae of *Dendroctonus rufipenis* (8); 10. *Dendroctonus rufipenis* (8); 11. *Hippodamia convergens* (84); 12. *Pterostichus brevicornis* (8); 13. *Nymphalis antiopta* (8); 14. *Leptinotarsa decemlineata* (36); 15. *Ixodes uriae* (83); 16. *Upis ceramboides* (8); 17. juvenile *Carassius carassius* (85); 18. *Danaus plexippus* (86); 19. hatchlings of *Chrysemys picta* (JP Costanzo, JD Litzgus, JB Iverson & RE Lee, unpublished data); 20. *Bufo cognatus, B. woodhousei* (16); 21. *Sphenomorphus kosciuskoi* (26); 22. *Pseudacris triseriata* (16); 23. *Scaphiopus bombifrons* (87); 24. *Egernia saxatilis* (26); 25. *Thamnophis sirtalis* (88); 26. *Rattus norvegicus* (89); 27. *Chrysemys picta* (58); 28. *Terrapene carolina* (90); 29. *Alligator mississippiensis* (28) 30. *Spermophilus parryii* (91).

agents would catalyze freezing. In practice, the T_c measured for a given taxon, individual, or tissue may vary considerably depending on such factors as the quantity and potency of nucleators present, water content, cryoprotectant levels, and amount of freezable water present (2, 3).

The capacity to supercool decreases as body mass increases (Figure 1). This is due, in part, to the higher probability that an ice embryo will spontaneously form or that the larger volume of body fluid will contain a particularly active nucleator. Many insect eggs, springtails, and mites weighing <100 μg supercool extensively, while larger terrestrial arthropods freeze between −5 and −15°C. Larger still, most vertebrates supercool relatively little, if at all (4). The relationship between body mass and T_c has been observed intraspecifically (5, 6) and suggests that ontogenetic changes in supercooling capacity may influence

the winter survival strategy adopted during different life history stages (e.g. *Ixodes uriae*, *Chrysemys picta*; Figure 1).

It may seem contradictory that any animal could supercool to $\leq -60°C$ (Figure 1), given that the limit of homogeneous nucleation is $-40°C$. Some arthropods achieve exceptional supercooling by accumulating high concentrations of glycerol, sorbitol, or other cryoprotectants, which may reach multimolar levels and comprise up to 25% of their body mass. Several studies (7) indicate that accumulation of such osmolytes effectively reduces T_c by approximately two to three times as much as the FP_{eq} is colligatively depressed (1.86°C/osmol). Thus by producing 4.8 M glycerol, the Alaskan willow cone gall fly larva (*Rhabdophaga strobiloides*) achieves a corresponding FP_{eq} of $-19.3°C$ and the capacity to supercool to $-56.1°C$ (8). Vertebrates apparently do not use cryoprotectants for this purpose. Some species undergo a seasonal dehydration that serves to concentrate extant osmolytes and reduce water volume, thus enhancing supercooling capacity (4, 9).

Because most ectotherms do not survive extensive ice formation within body fluids, they must either avoid low temperatures, rely on mechanisms that promote supercooling, or attenuate ice embryos within the blood (e.g. antifreeze proteins in polar fishes; 10). In contrast, a relatively few species of terrestrial insects, intertidal invertebrates, amphibians, and reptiles that overwinter terrestrially survive freezing and do so daily or seasonally. For many of these species, it is critical that extensive supercooling be avoided and that ice nucleation occur at a T_b very near the FP_{eq}. This moderates the rate of ice growth and allows time to physiologically adjust to the osmotic and mechanical stresses associated with freezing. Low rates of ice growth are particularly important for freeze-tolerant vertebrates which, unlike invertebrates that prepare in advance of seasonal cold, initiate cryoprotective responses only after freezing begins. The wood frog (*Rana sylvatica*), for example, mobilizes the cryoprotectant glucose and undergoes protective organ dehydration during the first 12–24 h of freezing (see reviews 11–13); rapid freezing inhibits these cryoprotective responses and is lethal (14, 15). Similarly, the chorus frog *Pseudacris triseriata* readily survives freezing if ice nucleation occurs between -1 and $-2°C$, but mortality increases progressively at lower T_cs (16).

Classes of Ice Nucleators and Inoculative Freezing

Although the actual ice nucleating agent in a given organism is frequently unknown, distinct classes of ice nucleating agents have been identified. These include special proteins produced by the animal and microorganisms that become intimately associated with animals' tissues. In addition, crystalloid inorganic compounds, active in the range of -8 to $-12°C$, were recently discovered in a freeze-tolerant insect (17).

The best known class of biological ice nucleators is comprised of proteins and lipoproteins that occur in a variety of freeze-tolerant insects (see reviews 3, 7). Production of these hemolymph-borne agents, which typically are active in the range of -6 to $-9°C$, coincides with seasonal patterns of cold-hardening. Blood nucleators exhibiting activity at -7 to $-8°C$ occur in various freeze-tolerant vertebrates, although their adaptive significance is unclear because freezing at these temperatures is lethal (18).

The most potent ice nucleators known are an unusual group of bacteria and fungi (19), which were originally reported as epiphytes and often viewed as pathogens, that cause extensive frost damage to crops. Aggregations of ice-nucleating proteins within the bacterial cell wall serve as the nucleus upon which the ice crystal grows, with larger aggregations resulting in greater nucleating activity. The association between microbial ice nucleators and insect cold hardiness was suggested by the fact that gut evacuation frequently enhanced the supercooling capacity and, indeed, strains of the bacteria *Enterobacter taylorae*, *Enterobacter agglomerans*, and *Erwinia herbicola* expressing ice-nucleating activity as high as $-2°C$ have been isolated from the gut of insects (20, 21). A fungus (*Fusarium* sp.) with ice-nucleating activity matching that of the whole body T_c has been found in a freeze-tolerant moth larva (22); this finding is noteworthy because it suggests a mutualistic relationship in which these normal microbial flora insure protective freezing at high subzero T_bs (23, 24). Ice-nucleating microorganisms, which were recently isolated from a freeze-tolerant frog (25), may also play a role in the winter biology of vertebrate ectotherms. Microbial ice nucleators may be used to artificially elevate the T_c of freeze-intolerant insect pests and thus potentially offer a novel means of biological control (24).

Although some ice-nucleating agents have considerable activity, none matches the potency of ice itself. Ice in the animal's microenvironment may initiate inoculative freezing of body water. Ice apparently gains ingress through alimentary and respiratory orifices (26–28) or by directly permeating the integument (29–31). The moist skin of amphibians is a particularly poor barrier to the inward propagation of ice.

The vast majority of ectotherms do not survive freezing and must behaviorally avoid low temperature and contact with ice. For example, most aquatic freshwater invertebrates are freeze intolerant and have little capacity to supercool or resist inoculative freezing (32). In contrast, with freeze-tolerant animals, ice inoculation is beneficial because it allows freezing to begin when T_b falls to the FP_{eq} with little, if any, prior supercooling. Inoculative freezing is crucial to the survival of the centipede *Lithobius forficatus*, since despite having an ice nucleator active at $-3°C$, it dies if freezing begins after the centipede has supercooled to this temperature (33). When cooled in contact with ice, however,

inoculative freezing occurs at −1°C and the animal survives, even at T_bs as low as −6°C.

Recent work suggests that the susceptibility to inoculative freezing in some animals depends upon microenvironmental conditions. The likelihood of inoculative freezing increases with decreasing temperature (31) and increasing water potential of the microenvironment (34, 35). Characteristics of the substrate such as texture, water content, water potential, and hydraulic conductivity may be particularly important (36, 37).

Initiation of Freezing: Case Studies

There has been a growing appreciation that ice nucleation is influenced by the complex interplay of endogenous factors (e.g. ice nucleators, water balance, cryoprotectants), but also by interactions between the organism and its microenvironment. Furthermore, both physiological and physical factors influencing cold hardiness may vary seasonally. To illustrate this complexity, we discuss three model systems with which we are particularly familiar: an insect, an amphibian, and a reptile.

GALL-INHABITING FLY LARVA For approximately 11 months of the year, larvae of the goldenrod gall fly *Eurosta solidaginis* (Tephritidae) live within spherical galls on stems of goldenrod (*Solidago* sp.). This fly ranges from the Gulf of Mexico to central Canada. Because the galls provide little thermal buffering and frequently project above any surrounding snow, larvae often experience fluctuating and extreme temperatures, with the daily range commonly exceeding 25°C and occasionally reaching 35°C (38).

In the northern United States, first, second, and early third instar larva are intolerant of freezing and typically supercool to ≤−13°C (39). In autumn, larvae become freeze tolerant to ≤−40°C and accumulate cryoprotectants, principally glycerol and sorbitol, in response to low environmental temperatures and desiccation as the surrounding gall tissues senesce (40, 41); supercooling capacity at this time is limited to −8 to −10°C (39). During the subsequent larval-pupal metamorphosis, cold hardiness changes markedly as freeze tolerance is lost and the T_c of pupae decreases to −18°C (17).

The abrupt increase in T_c of larvae associated with the acquisition of freeze tolerance during the autumn suggests the presence of a relatively efficient endogenous ice nucleator. Some data suggest the presence of an ice nucleator in the hemolymph of this species, although a recent study has ascribed these results to contamination by external materials (42). The timing of this seasonal elevation in T_c is consistent with the production and action of ice-nucleating proteins that have been reported in other freeze-tolerant insects; however, proteinaceous nucleators have not been definitively identified in this species.

A recent report describes significant levels of ice-nucleating activity in fat body cells and a crystalloid compound isolated from *E. solidaginis* larvae (17). Fat body cell suspensions had a mean T_c of −10.9°C, with some samples freezing high enough (−6°C) to explain the T_c of intact larvae. In addition, within the Malpighian tubules of overwintering larvae are 25–45 crystalloid spherules that grow to a diameter of 300 μm. Scanning electron microscopy and X-ray diffraction studies reveal that these spherules are amorphous (i.e. lacking crystalline structure) conglomerates of round particles of tribasic calcium phosphate. As with the fat body cells, these spherules exhibit ice-nucleating activity at temperatures as high as −6°C (mean T_c, −10.9°C). Furthermore, during the larval-to-pupal transition, T_c drops to −18°C (17) coincident with the disappearance of these spherules.

Calcium carbonate, uric acid, potassium phosphate, and other crystalloid deposits are present in diapausing and overwintering insects. Commercial preparations of these compounds have ice-nucleating activity in the range of −8 to −11°C (17). These compounds represent a new class of endogenous, heterogeneous nucleators in freeze-tolerant insects that function to ensure that cryoprotective ice nucleation occurs at relatively high subzero T_bs.

Despite the identification of efficient internal ice nucleators in overwintering larvae, this story is not complete without considering interactions between the larva and the gall it inhabits. Layne and colleagues (30) investigated the possibility that ice in the gall tissues might inoculate the larva. In October, field-collected galls (with larvae removed) have a water content of 66%, and a mean T_c of −4.5°C. When galls containing larvae were held for 24 h at ≈−5.5°C, all the galls and the larvae within them froze. By November, the water content of the galls had fallen to 20%, although the T_c remained the same. When the galls with larvae were again held for 24 h at −5.5°C, all the galls froze but only 10% of the larvae did. The susceptibility of larvae to inoculative freezing was confirmed by cooling isolated larvae in contact with moist filter paper. The larvae froze when the external water did, whereas larvae on dry paper remained supercooled.

During early autumn in the northern United States, when galls are green, larvae apparently freeze at T_bs near 0°C by inoculation from ice in the surrounding plant tissues. Later in the season, when the plant has senesced and its tissues have dried, the action of internal ice nucleators predominates, causing freezing at lower T_bs of −8 to −10°C. Consequently, if only the T_c of isolated larvae and expected environmental temperatures were used to predict the first time that larvae freeze, the estimate would, in fact, be 1–2 months later than the actual first incidence of freezing.

Due to the daily thermoperiod and intermittent periods of warming, larva may undergo many cycles of freezing and thawing during the winter. Furthermore,

wetting of galls by rain may result in rehydration of the gall tissue and restoration of the potential to cause inoculative freezing of larvae in mid-winter (38). Consequently, whether a given larva freezes depends on dynamic interactions between a particular overwintering life stage, activity of internal ice-nucleating agents, and the hydration state of the surrounding plant tissues.

TERRESTRIAL HIBERNATION IN THE WOOD FROG The wood frog *R. sylvatica* is a common resident of mesic forests and ranges from the southern Appalachians north to the Maritime provinces and west to northern Alaska, even to the Arctic Circle (43). Its winter habits are known only from a few anecdotal accounts, which suggest that their hibernacula are shallow burrows in the forest floor, well within the frost zone, overlain by leaves and other organic detritus. In southern Ohio, *R. sylvatica* encounters infrequent freezing episodes that expose frogs to a minimum T_b of -2 to -4°C and may last several days (JP Costanzo, JT Irwin, RE Lee, unpublished data).

Freeze tolerance in *R. sylvatica* (and other vertebrate ectotherms) was reported only recently (44), yet several unique biochemical and physiological adaptations have been discovered (see current reviews, 11, 45–47). Generally, wood frogs can survive (*a*) the freezing of up to 65–70% of their body water, (*b*) a minimum T_b of -6°C, and (*c*) uninterrupted freezing for $\geq$4 weeks. Survival in the frozen state is promoted by an accumulation of cryoprotectant (glucose) and redistribution of water among body compartments; these responses mitigate the osmotic, mechanical, and metabolic perturbations of freezing and thawing.

Regulation of T_c is an important problem for freeze-tolerant animals that may suffer injury by spontaneous nucleation of deeply supercooled tissues. The intrinsic supercooling capacity of adult *R. sylvatica,* which weigh 5–20 g depending on geographic origin, is modest (Figure 1), but the smaller juveniles risk cryoinjury if they supercool extensively before nucleating (16). At least two efficient mechanisms ensure that freezing commences at relatively high T_b.

Owing to the highly permeable nature of amphibian skin, inoculative freezing of the body fluids of a supercooled frog commences virtually on contact with environmental ice (29, 30). To keep from desiccating extensively, *R. sylvatica* must hibernate in relatively moist microenvironments, which, during frosts, would provide an abundance of seed crystals. Thus freezing in nature likely occurs under most circumstances very near the FP_{eq}, -0.4°C.

Various strains of *Pseudomonas fluorescens, Pseudomonas putida,* and *Enterobacter agglomerans* expressing potent ice-nucleating activity have been cultured from intestines of winter-collected *R. sylvatica,* indicating that such bacteria are retained throughout hibernation (25). Feeding of *P. putida* to another freeze-tolerant frog, *Pseudacris crucifer,* markedly increased its T_c, demonstrating that these agents may promote ice formation in freeze-tolerant

frogs. The T_c of intact *R. sylvatica* corresponds closely with that of isolated intestine and skin, organs that likely harbor such bacteria, but not of other body organs, tissues, or fluids (18, 48). The adaptive significance of microbial ice nucleators in their winter biology remains uncertain, although in the absence of ice inoculation they may ensure that freezing begins at a relatively high T_b.

The blood of *R. sylvatica* contains a proteinaceous ice nucleator that retains full activity (-7 to -8°C) in 0.2% dilutions (49, 50). Such agents have been suggested to be a critical factor in the evolution of vertebrate freeze tolerance (51). However, there are problems assigning adaptive function to these blood nucleators: (*a*) They are not unique to freeze-tolerant species, but rather may occur in various animals, including mammals; (*b*) some freeze-tolerant vertebrates lack them; (*c*) blood-borne ice nucleators exhibit less activity in vitro than certain tissues (namely, skin and intestine, which may harbor microbial ice nucleators); (*d*) there is poor congruence between activity temperature of blood nucleators and the T_c of intact animals; and (*e*) the activity temperature of blood nucleators is substantially lower than the minimum T_b that can be survived in the frozen state (i.e. -4 to -6°C). Collectively these issues cast doubt that blood nucleators are important for initiating protective freezing of *R. sylvatica* or other vertebrates whose supercooling capacity is so limited (18, 52).

OVERWINTERING OF HATCHLING TURTLES Painted turtles (*Chrysemys picta*) are long-lived residents of quiet, shallow waters, which range from coast to coast in the northern United States and southern Canada. These turtles hatch during late summer but overwinter within the natal nest, only $\approx$10 cm beneath the ground surface, even in northern populations (43). Many emerge in spring after surviving exposure to minimum T_bs of -2 to -11°C (37, 53, 54). In the sandhills of Nebraska, winters are particularly severe and hatchling *C. picta* are intermittently exposed to subzero temperatures from late November through early March. Cooling episodes are usually mild (minimum $T_b > -4$°C) and brief (<24 h), but temperatures of -10 to -12°C occasionally occur (37, 53).

The remarkable cold hardiness of hatchling *C. picta* has been ascribed to supercooling (55, 56), as, indeed, they are among the few cold-hardy vertebrates whose bodies are small enough to permit extensive supercooling (Figure 1). However, these animals are also freeze tolerant (37, 54, 57, 58). Adaptations promoting freeze tolerance in *C. picta* are poorly understood (54, 57). A protein with ice-nucleating activity of -7 to -8°C occurs in its blood; however, as with *R. sylvatica*, it apparently plays no role in cold hardiness (18, 51).

Freeze tolerance and supercooling are generally regarded as dichotomous strategies for cold tolerance, yet both may be effective survival mechanisms in hatchling *C. picta* subject to certain constraints (43, 56). Turtles tolerate

freezing at T_bs ≥ -4 °C (37, 54, 57, 58), whereas survival at much lower T_bs (e.g. ≈ -12 °C) is possible only if freezing is avoided. Whether supercooling or freeze tolerance is employed during a particular cooling episode depends upon prevailing physiological and microenvironmental conditions (37). According to this model, supercooling predominates during periods of low environmental water potential, since the risk of ice inoculation is reduced and the turtles may partially desiccate. Alternatively, exposure to a damp substrate promotes ice nucleation via inoculation at a T_b near the FP_{eq}, a condition requisite for freezing survival. Although many cooling episodes may be endured by frozen turtles, survival of the extreme temperatures occurring in some nests can only be ascribed to supercooling (37, 53). The factors limiting supercooling capacity are thus of particular interest in the winter life history of this species.

Reptiles such as *C. picta* are much less susceptible to inoculative freezing than the moist-skinned amphibians (4, 18), yet inoculation may occur when external ice contacts mucous membranes of the cloaca, nostrils, or eyes (26, 28), or even the skin (31). The resistance of *C. picta* hatchlings to ice inoculation reportedly varies anatomically, as skin of the head and neck apparently is more impervious to ice than is skin of the inguinal and axial pouches (55). In nature, susceptibility to inoculative freezing depends on hydric characteristics of the substrate. Laboratory trials with Nebraska *C. picta* showed that hatchlings immersed in native sand containing as little as 2.3% moisture (w/w) could not avoid ice inoculation (37), but about half of those tested in "damp clayey soil" resisted inoculation at T_bs as low as -9°C (55). Data provided in a recent field study indicated that survival of hatchlings overwintering in loamy sand (94%) was substantially higher than that (65%) for animals overwintering in nests constructed in fine sand (53). Minimum nest temperatures did not differ between the groups, so one plausible explanation is that animals in the latter substrate were more susceptible to (lethal) inoculative freezing.

Given suitable environmental conditions it seems reasonable that hatchling *C. picta* may supercool as extensively in nature as they do under optimal laboratory conditions. By taking precautions to reduce contamination by free water and nucleating agents, turtles can be readily supercooled to ≈ -12°C (55) or even -20°C (JP Costanzo, JD Litzgus, JB Iverson & RE Lee, unpublished data). However, laboratory results for meticulously cleaned turtles may overrepresent supercooling capacity in nature because hatchling *C. picta* hibernate in intimate physical contact with soil, which conceivably harbors various ice nucleating agents. Recent work suggests that ice nucleators are indeed normal constituents of the nesting substrates and that they may constrain supercooling of hatchling turtles. For example, turtles hatched from eggs incubated in native sand supercooled much less than turtles hatched and reared on vermiculite (Figure 2). Material sampled in autumn from several nests of Nebraska

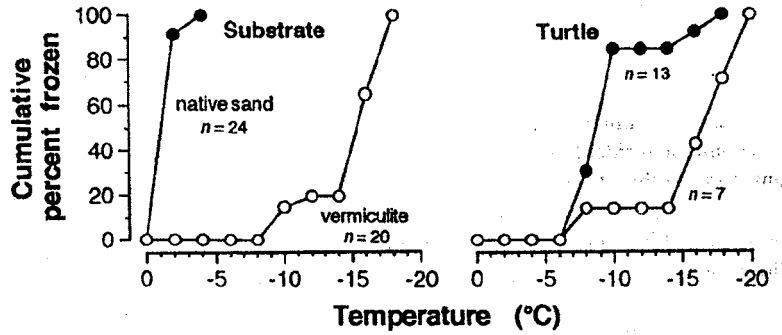

Figure 2 Cumulative freezing distributions of substrate washings (*left*) and hatchling painted turtles (*Chrysemys picta*) (*right*) that were hatched, reared, and cold acclimatized on native substrate (sand) or vermiculite. Substrate washings were cooled in 10-μl aliquots; turtles were individually cooled in dry vessels (10°C/h) after surface moisture was eliminated by evaporation. The data indicate that the substrate in which turtles overwinter contains a potent nucleator that markedly diminishes their supercooling capacity (JP Costanzo, JD Litzgus, JB Iverson & RE Lee, unpublished data).

C. picta contained ice nucleating agents that catalyzed the freezing of water at -3 to -5°C, exhibited increased potency with cold acclimation, and retained full activity in dilutions up to 10^{-3}. To date, ice nucleators have been found in nesting/overwintering substrates in the midwestern United States (Indiana, Nebraska) and Ontario, Canada. The identity of the nucleator is as yet unknown; however, preliminary results indicate that it is heat labile and, therefore, possibly of organic composition (JP Costanzo, JD Litzgus, JB Iverson & RE Lee, unpublished data). This discovery may reconcile some inconsistencies in the contemporary literature. Virtually all studies using animals hatching in natural nests (and thus potentially exposed to syntopic ice nucleators) have reported limited supercooling (e.g. $T_c \geq -4$°C; Reference 18), whereas extensive supercooling ($T_c < -8$°C) is known only in turtles hatched and reared in the laboratory (55).

Because hatchling *C. picta* must nucleate at high T_bs if they are to survive freezing, it is tempting to speculate a commensalistic role for the ice nucleator present in its winter microenvironment. Preliminary data do suggest that the nucleator may function in concert with available substrate water to promote (protective) inoculative freezing. However, under conditions favoring extensive supercooling (e.g. low environmental water potential), the nucleator catalyzes the freezing of turtles at -7 to -10°C, which do not survive. Interpopulational differences in winter survival may reflect not only the regional variation in

susceptibility to inoculative freezing associated with soil characteristics (37), but also patchy distributions of potent ice nucleators in the winter microenvironment. These interactions may ultimately influence both regional and local distributions of this species.

DISTRIBUTION OF ICE WITHIN THE BODY

Freeze-tolerant animals can survive the freezing of up to 65–70% of their body water, but only if ice gradually forms within the tissues (11, 13). In both invertebrates and vertebrates, an equilibrium ice content is attained many hours or days after freezing begins (59, 60). Such low rates of ice formation, which are promoted by insulation in the microenvironment (snow cover, organic detritus, etc), allow time for the activation of cryoprotective responses and permit cells to adapt to the ensuing physical and osmotic stresses. Magnetic resonance imaging has revealed that freezing of *C. picta* and *R. sylvatica* begins in peripheral tissues and gradually moves toward the core (61, 62). In contrast, thawing occurs simultaneously throughout the body with deep visceral organs (e.g. liver) melting relatively quickly due to their higher concentrations of cryoprotectant and consequently, lower FP_{eq} (61, 62).

It is believed that freeze tolerance requires ice growth to be restricted to the extracellular spaces (63, 64). One consequence of ice forming in these compartments is that cells, which remain supercooled, are subject to osmotic stress, a primary cause of freezing injury. Because only water molecules join the growing ice lattice, rejected solute accumulates in the as yet unfrozen water; in turn, this hypertonic solution osmotically draws water from within cells. As freezing progresses cells may become dramatically distorted and shrunken. Cryoprotectants mitigate osmotic stress by binding water within cells, increasing intracellular osmolality, and by stabilizing structural elements within cells (64).

Extraorgan Ice Sequestration

Ice formation within body fluids not only poses the threat of excessive cellular dehydration, but also the potential for mechanical injury by the growing ice lattice, particularly in compact and highly structured tissues and organs. Ice fronts may shear and separate tissues, disrupting intercellular communication systems. Within organs, ice forms preferentially in the vascular system (65). A gradual freezing of the blood causes the plasma to become progressively hypertonic, drawing in additional extravascular water. Ultimately, vessels may be damaged by excessive expansion of the ice within them even though surrounding tissues are unharmed. Prevention of this type of cryoinjury is a major challenge to the successful cryopreservation of mammalian organs (65).

Some freeze-tolerant plants are well adapted to avoid excessive ice formation within sensitive structures. Vegetative and flower buds, as well as seeds, survive by translocating water from frost-sensitive tissues to sites were ice crystals grow innocuously (66, 67). This temporary redistribution of water allows tissues to supercool extensively and avoid freezing injury. Because the extracellular fluids of plants are markedly hypotonic with respect to the cytoplasm, the withdrawal and translocation of cell water is affected along a vapor pressure gradient extending from supercooled tissues to the growing ice mass, rather than along an osmotic gradient.

Recent studies reveal that organs of some freeze-tolerant animals are also protected by the translocation of water to other compartments. Dissection of frozen wood frogs reveals a surprising and striking distribution of ice within the body. As frogs freeze, a process that may require >24 h for the crystallization of 65% of the body water, organ water is progressively translocated to subdermal lymph sacs and the coelomic cavity where it freezes (Figure 3). Appendicular skeletal muscles lose 20–30% of their initial water content, whereas the heart, liver, intestine, and peripheral nerves lose >50% (68, 69). Tissues are fully rehydrated within several hours after thawing begins.

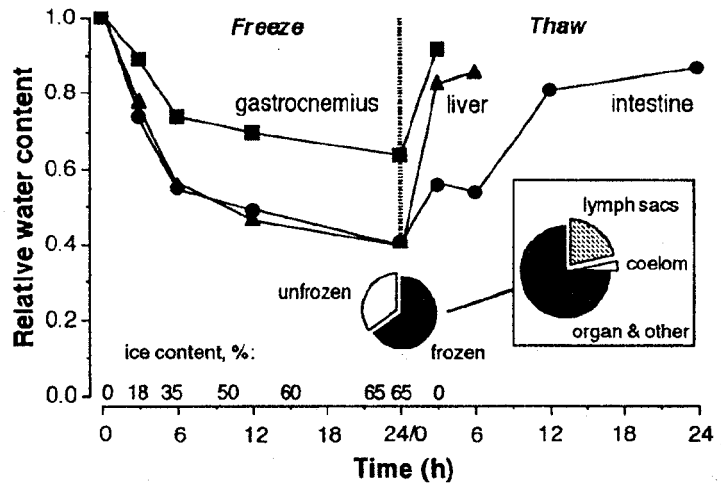

Figure 3 Dynamics of organ water and body ice during a routine 24-h freezing episode to −2.5°C, and subsequent thawing at 5°C, of the freeze-tolerant wood frog (*Rana sylvatica*). As up to 65% of total body water progressively freezes, over half of the water in some organs is relocated to spaces where it freezes innocuously; about 25% of all ice is sequestered within the coelom and lymph sacs. Most organs rapidly rehydrate upon thawing. Adapted from Lee & Costanzo (92).

Although some water inevitably freezes within organs, the partial dehydration apparently functions to reduce potential mechanical damage (69). An added benefit is that cryoprotectant becomes locally concentrated (15, 70). The significance of protective dehydration is evidenced by the fact that rapid freezing, which is lethal, inhibits organ dehydration in *R. sylvatica* (14, 15). In the freeze-tolerant box turtle (*Terrapene carolina*), some structures, particularly nervous tissues, dehydrate during freezing, although others (e.g. liver) do not (45). The freeze-intolerant leopard frog (*Rana pipiens*) also exhibits organ dehydration during freezing, but the amount of organ water lost (9–33%) is relatively low (71). Desiccation tolerance may thus be a chief preadaptation supporting the evolutionary development of freeze tolerance (69, 72).

The sequestration of ice in innocuous locations within the body may be facilitated by a lack of physical barriers and the presence of natural and/or potential voids. However, the specific mechanisms promoting translocation of bulk water and its redistribution are poorly known. The open circulatory system of arthropods and other invertebrates may facilitate the innocuous growth of ice external to compact organs. In *R. sylvatica*, an extended function of the heart, which continues to beat for many hours after freezing begins (73), likely affects the translocation of water from remote tissues. Osmotic forces may be involved, although hyperglycemia (as induced by administration of glucose) does not influence the degree of organ dehydration during freezing (70). Patterns of water loss among organs suggest that the basic mechanism is subordinate to regulation at the organ or tissue level. Extraorgan ice sequestration complements the action of cryoprotectants and plays a critical role in the freeze tolerance of some ectotherms.

Intracellular Ice Formation

Although cryobiologists generally believe that intracellular freezing is lethal to all organisms, some freeze-tolerant species naturally tolerate ice formation within certain cells. Working with *E. solidaginis*, Salt (74, 75) originally reported survival after freezing of cells in the fat body, an organ whose metabolic role is analogous to that of vertebrate liver. Recent investigation reveals that fat body cells are susceptible to inoculative freezing at a relatively high temperature (−4.6°C), a characteristic that markedly differs from that of mammalian cells, which resist inoculation at temperatures <−15°C (76). Upon thawing, a radical reorganization of cytoplasmic contents is evident. The formerly dispersed, uniformly distributed lipid droplets coalesce in each cell's center, whereas the remaining organelles, including the nucleus, are displaced to the periphery (77, 78). Salt observed lipid coalescence in fat body cells through the transparent cuticle of previously frozen larvae (that continued normal development to become pupae and adults), which suggests that these cells freeze internally during routine freezing of the organism.

In 1995, Wharton & Ferns (79) provided convincing evidence for survival of intracellular freezing in the nematode *Panagrolaimus davidi*. Using cryomicroscopy and freeze-fracture electron microscopy, they observed freezing within cells of intact individuals which later laid eggs that developed normally. In this species, intracellular freezing may be an adaptation for reducing transmembrane osmotic stress (79). The supernatant from homogenates of this nematode inhibits recrystallization, an action that may promote tolerance of intracellular freezing (80). Future studies using vital dyes, cryomicroscopy, and freeze-fracture electron microscopy are needed to determine whether intracellular freeze tolerance occurs more commonly in freeze-tolerant ectotherms and what special adaptations permit cells to tolerate these stresses.

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Literature Cited

1. Vali G. 1995. Principles of ice nucleation. See Ref. 19, pp. 1–28
2. Block W. 1990. Cold tolerance of insects and other arthropods. *Philos. Trans. R. Soc. London Ser. B* 326:613–33
3. Zachariassen KE. 1992. Ice nucleating agents in cold-hardy insects. In *Water and Life*, ed. GN Somero, CB Osmond, CL Bolis, pp. 261–81. Berlin: Springer-Verlag
4. Costanzo JP, Lee RE. 1995. Supercooling and ice nucleation in vertebrate ectotherms. See Ref. 19, pp. 221–37
5. Johnston SL, Lee RE. 1990. Regulation of supercooling and nucleation in a freeze intolerant beetle (*Tenebrio molitor*). *Cryobiology* 27:562–68
6. Pugh PJA. 1994. Supercooling points and water contents in Acari. *Acta Ecol.* 15:71–77
7. Duman JG, Olsen TM, Yeung KL, Jerva F. 1995. The roles of ice nucleators in cold tolerant invertebrates. See Ref. 19, pp. 201–19
8. Miller LK. 1982. Cold-hardiness strategies of some adult and immature insects overwintering in interior Alaska. *Comp. Biochem. Physiol.* 73A:595–604
9. Ring RA, Danks HV. 1994. Desiccation and cryoprotection: overlapping adaptations. *Cryo-Letters* 15:181–90
10. DeVries AL, Cheng C-HC. 1992. The role of antifreeze glycopeptides and peptides in the survival of cold-water fishes. In *Water and Life*, ed. GN Somero, CB Osmond, CL Bolis, pp. 301–15. Berlin: Springer-Verlag
11. Costanzo JP, Lee RE, DeVries AL, Wang T, Layne JR. 1995. Survival mechanisms of vertebrate ectotherms at subfreezing temperatures: applications in cryomedicine. *FASEB J.* 9:351–58
12. Storey KB, Storey JM. 1992. Natural freeze tolerance in ectothermic vertebrates. *Annu. Rev. Physiol.* 54:619–37
13. Storey KB, Storey JM. 1988. Freeze tolerance in animals. *Physiol. Rev.* 68:27–84
14. Costanzo JP, Lee, RE, Wright MF. 1991. Effect of cooling rate on the survival of frozen wood frogs, *Rana sylvatica*. *J. Comp. Physiol.* 161:225–29
15. Costanzo JP, Lee RE, Wright MF. 1992. Cooling rate influences cryoprotectant distribution and organ dehydration in freezing wood frogs. *J. Exp. Zool.* 261:373–78
16. 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.* 166:110–19
17. Mugnano JA, Lee RE, Taylor RT. 1996. Fat body cells and calcium phosphate spherules induce ice nucleation in the freeze-tolerant larvae of the gall fly *Eurosta solidaginis* (Diptera, Tephritidae). *J. Exp. Biol.* 199:465–71
18. Costanzo JP, Lee RE. 1996. Mini-review: ice nucleation in freeze-tolerant vertebrates. *Cryo-Letters* 17:111–18
19. Lee RE, Warren GJ, Gusta LV, eds. 1995. *Biological Ice Nucleation and Its Applications*. St. Paul, MN: Am. Phytopathol. Soc. 370 pp.
20. Lee RE, Strong-Gunderson JM, Lee MR, Grove KS, Riga TJ. 1991. Isolation of ice nucleating active bacteria from insects. *J. Exp. Zool.* 257:124–27
21. Kaneko J, Yoshida T, Owada T, Kita K, Tanno K. 1991. *Erwinia herbicola* ice nucleation active bacteria isolated from diamondback moth *Plutella xylostella* L. pupae (in Japanese). *Jpn. J. Appl. Entomol. Zool.* 35:247–51
22. Tsumuki H. 1992. An ice-nucleating active fungus isolated from the gut of the rice stem borer, *Chilo suppressalis* Walker (Lepidoptera: Pyralidae). *J. Insect Physiol.* 38:119–25
23. Lee RE. 1991. Principles of insect low temperature tolerance. In *Insects at Low Temperature*, ed. RE Lee, DL Denlinger, pp. 17–46. New York: Chapman & Hall
24. Lee RE, Lee MR, Strong-Gunderson JM. 1993. Insect cold-hardiness and ice nucleating active microorganisms including their potential use for biological control. *J. Insect Physiol.* 39:1–12
25. Lee MR, Lee RE, Strong-Gunderson JM, Minges SR. 1995. Isolation of ice nucleating active bacteria from the freeze-tolerant frog, *Rana sylvatica*. *Cryobiology* 32:358–65
26. Spellerberg IF. 1972. Temperature tolerances of southeast Australian reptiles examined in relation to reptile thermoregulatory behavior and distribution. *Oecologia* 9:23–46
27. Steigerwald KA, Lee MR, Lee RE, Marshall JC. 1995. Effect of biological ice nucleators on insect supercooling capacity varies with anatomic site of application. *J. Insect Physiol.* 41:603–8
28. Lowe CH, Lardner PJ, Halpern EA. 1971. Supercooling in reptiles and other vertebrates. *Comp. Biochem. Physiol.* 39A:125–35
29. Layne JR. 1991. External ice triggers freezing in freeze-tolerant frogs at temperatures above their supercooling point. *J. Herpetol.* 25:129–30
30. Layne 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.* 68:506–10
31. Packard GC, Packard MJ. 1993. Delayed inoculative freezing is fatal to hatchling painted turtles (*Chrysemys picta*). *Cryo-Letters* 14:273–84
32. Oswood MW, Miller LK, Irons JG. 1991. Overwintering of freshwater benthic macroinvertebrates. In *Insects at Low Temperature*, ed. RE Lee, DL Denlinger, pp. 360–75. New York: Chapman & Hall
33. Tursman D, Duman JG, Knight CA. 1994. Freeze tolerance adaptations in the centipede, *Lithobius forficatus*. *J. Exp. Zool.* 268:347–53
34. Holmstrup M, Zachariassen ZE. 1996. Physiology of cold hardiness in earthworms. *Comp. Biochem. Physiol.* 115A: 91–101
35. Forge TA, MacGuidwin AE. 1992. Effects of water potential and temperature on survival of the nematode *Meloidogyne hapla* in frozen soil. *Can. J. Zool.* 70:153–60
36. 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. B* 167:169–76
37. Costanzo JP, Iverson JB, Wright MF, Lee RE. 1995. Cold hardiness and overwintering strategies of hatchlings in an assemblage of northern turtles. *Ecology* 76:1772–85
38. Layne JR. 1993. Winter microclimate of goldenrod spherical galls and its effects on the gall inhabitant *Eurosta solidaginis* (Diptera: Tephritidae). *J. Therm. Biol.* 18:125–30
39. Morrissey RE, Baust JG. 1976. The ontogeny of cold tolerance in the gall fly, *Eurosta solidaginis*. *J. Insect Physiol.* 22:431–37
40. Rojas RR, Lee RE, Baust JG. 1986. Relationship of environmental water content to glycerol accumulation in the freezing tolerant larvae of *Eurosta solidaginis* (Fitch). *Cryo-Letters* 7:234–45
41. Baust JG, Lee RE. 1982. Environmental triggers to cryoprotectant modulation in separate populations of the gall fly, *Eurosta solidaginis* (Fitch). *J. Insect Physiol.* 28:431–36
42. Bale JS, Hansen TN, Baust JG. 1989. Nucleators and sites of nucleation in the freeze tolerant larvae of the gall fly *Eurosta solidaginis* (Fitch). *J. Insect Physiol.* 35:291–98
43. Ultsch GR. 1989. Ecology and physiology of hibernation and overwintering among freshwater fishes, turtles, and snakes. *Biol. Rev.* 64:435–516
44. Schmid WD. 1982. Survival of frogs in low temperature. *Science* 512:697–98
45. Costanzo JP, Wright MF, Lee RE. 1993. Physiological responses to freezing in the turtle *Terrapene carolina*. *J. Herpetol.* 27:117–20
46. Storey KB, Storey JM. 1996. Natural freezing survival in animals. *Annu. Rev. Ecol. Syst.* 27:365–86
47. Layne JR, Lee RE. 1995. Adaptations of frogs to survive freezing. *Climate Res.* 5:53–59
48. Layne JR. 1995. Crystallization temperatures of frogs and their individual organs. *J. Herpetol.* 29:296–98
49. Wolanczyk JP, Baust JG, Storey KB. 1990. Seasonal ice nucleating activity in the freeze tolerant frog *Rana sylvatica*. *Cryo-Letters* 11:143–50
50. Wolanczyk JP, Storey KB, Baust JG. 1990. Ice nucleating activity in the blood of the freeze-tolerant frog, *Rana sylvatica*. *Cryobiology* 27:328–35
51. Storey KB, McDonald DG, Duman JG, Storey JM. 1991. Blood chemistry and ice nucleating activity in hatchling painted turtles. *Cryo-Letters* 12:351–58
52. Storey KB. 1985. Freeze tolerance in terrestrial frogs. *Cryo-Letters* 6:115–34
53. Packard GC. 1997. Temperatures during winter in nests with hatchling painted turtles (*Chrysemys picta*). *Herpetologica* 53:89–95
54. Storey KB, Storey JM, Brooks SPJ, Churchill TA, Brooks RJ. 1988. Hatchling turtles survive freezing during winter hibernation. *Proc. Natl. Acad. Sci. USA* 85:8350–54
55. Packard GC, Packard MJ. 1995. The basis for cold tolerance in hatchling painted turtles (*Chrysemys picta*). *Physiol. Zool.* 68:129–48
56. Paukstis GL, Shuman RD, Janzen FJ. 1989. Supercooling and freeze tolerance in hatchling painted turtles (*Chrysemys picta*). *Can. J. Zool.* 67:1082–84
57. Churchill TA, Storey KB. 1992. Natural freezing survival by painted turtles *Chrysemys picta marginata* and *C. picta bellii*. *Am. J. Physiol.* 262:R530–37
58. Claussen DL, Zani PA. 1991. Allometry of cooling, supercooling, and freezing in the freeze-tolerant turtle *Chrysemys picta*. *Am. J. Physiol.* 261:R626–32
59. Lee RE, Lewis EA. 1985. Effect of temperature and duration of exposure on tissue ice formation in the gall fly, *Eurosta solidaginis* (Diptera, Tephritidae). *Cryo-Letters* 6:24–34
60. Layne JR, Lee RE. 1987. Freeze tolerance and the dynamics of ice formation in wood frogs (*Rana sylvatica*) from southern Ohio. *Can. J. Zool.* 65:2062–65
61. Rubinsky B, Hong J-S, Storey KB. 1994. Freeze tolerance in turtles: visual analysis by microscopy and magnetic resonance imaging. *Am. J. Physiol.* 267:R1078–88
62. Rubinsky B, Wong STS, Hong J-S, Gilbert J, Roos M, Storey KB. 1994. ^{1}H magnetic resonance imaging of freezing and thawing in freeze-tolerant frogs. *Am. J. Physiol.* 266:R1771–77
63. Storey KB, Bischof J, Rubinsky B. 1992. Cryomicroscopic analysis of freezing in liver of the free-tolerant wood frog. *Am. J. Physiol.* 263:R185–94
64. Mazur P. 1984. Freezing of living cells: mechanisms and implications. *Am. J. Physiol.* 247:C125–42
65. Pegg DE. 1988. The nature of cryobiological problems. In *Low Temperature Biotechnology: Emerging Applications and Engineering Contributions*, ed. JJ McGrath, KR Diller, pp. 3–21. New York: Am. Soc. Mech. Eng.
66. Quamme HA. 1995. Deep supercooling in buds of woody plants. See Ref. 19, pp. 183–99
67. Sakai A, Larcher W. 1987. *Frost Survival of Plants*. Berlin: Springer-Verlag. 321 pp.
68. Kling KB, Costanzo JP, Lee, RE. 1994. Post-freeze recovery of peripheral nerve function in the freeze-tolerant wood frog (*Rana sylvatica*). *J. Comp. Physiol.* 164:316–20
69. Lee RE, Costanzo JP, Davidson EC, Layne JR. 1992. Dynamics of body water during freezing and thawing in a freeze-tolerant frog (*Rana sylvatica*). *J. Therm. Biol.* 17:263–66
70. Costanzo JP, Lee RE, Lortz PH. 1993. Glucose concentration regulates freeze tolerance in the wood frog *Rana sylvatica*. *J. Exp. Biol.* 181:145–55
71. Costanzo JP, Lee RE, Lortz PH. 1993. Physiological responses of freeze-tolerant and -intolerant frogs: clues to evolution of anuran freeze tolerance. *Am. J. Physiol.* 265:R721–25
72. Costanzo JP, Wright MF, Lee RE. 1992. Freeze tolerance as an overwintering adaptation in Cope's gray treefrog (*Hyla chrysoscelis*). *Copeia* 1992:565–69
73. Layne JR, Lee RE, Heil TL. 1989. Freezing-induced changes in the heart rate

of wood frogs (*Rana sylvatica*). *Am. J. Physiol.* 257:R1046–49
74. Salt RW. 1959. Survival of frozen fat body cells in an insect. *Nature* 193:1426
75. Salt RW. 1962. Intracellular freezing in insects. *Nature* 193:1207–8
76. Lee RE, McGrath JJ, Morason RT, Taddeo RM. 1993. Survival of intracellular freezing, lipid coalescence and osmotic fragility in fat body cells of the freeze-tolerant gall fly *Eurosta solidaginis*. *J. Insect Physiol.* 39:445–50
77. Morason RT, Allenspach AL, Lee RE. 1994. Comparative ultrastructure of fat body cells of freeze-susceptible and freeze-tolerant *Eurosta solidaginis* larvae after chemical fixation and high pressure freezing. *J. Insect Physiol.* 40:155–64
78. Collins SD, Allenspach AL, Lee RE. 1996. Ultrastructural effects of lethal freezing on brain, muscle and Malpighian tubules from freeze-tolerant larvae of the gall fly, *Eurosta solidaginis*. *J. Insect Physiol.* 43:39–45
79. Wharton DA, Ferns DJ. 1995. Survival of intracellular freezing by the Antarctic nematode *Panagrolaimus davidi*. *J. Exp. Biol.* 198:1381–87
80. Ramlov H, Wharton DA, Wilson PW. 1996. Recrystallization in a freezing tolerant Antarctic nematode, *Panagrolaimus davidi*, and an alpine weta, *Hemideina maori* (Orthoptera; Stenopelmatidae). *Cryobiology* 33:607–13
81. Skanland HT, Somme L. 1981. Seasonal variation in cold-hardiness of eggs of the apple psyllid *Psylla mali* (Schmidb.) in Norway. *Cryo-Letters* 2:86–91
82. Gehrken U, Somme L. 1987. Increased cold hardiness in eggs of *Arcynopteryx compacta* (Plecoptera) by dehydration. *J. Insect Physiol.* 33:987–91
83. Lee RE, Baust JG. 1987. Cold-hardiness in the Antarctic tick, *Ixodes uriae*. *Physiol. Zool.* 60:499–506
84. Bennett VA, Lee RE. 1997. Modeling seasonal changes in intracellular freeze-tolerance of fat body cells of the gall fly, *Eurosta solidaginis* (Diptera: Tephritidae). *J. Exp. Biol.* 200:185–92
85. Kalabukhov NI. 1958. The problem of freezing, undercooling and vitrifying of animal organism. In *Institute of Biology, International Symposium on Freezing and Drying*. pp. 101–18. Oxford: Blackwell Sci. 2nd ed.
86. Larsen KJ, Lee RE. 1994. Cold tolerance including rapid cold-hardening and inoculative freezing in migrant monarch butterflies in Ohio. *J. Insect Physiol.* 40:859–64
87. Swanson DL, Graves BM. 1995. Supercooling and freeze intolerance in overwintering juvenile spadefoot toads (*Scaphiopus bombifrons*). *J. Herpetol.* 29:280–85
88. Costanzo JP, Claussen DL, Lee RE. 1988. Natural freeze tolerance in a reptile. *Cryo-Letters* 9:380–85
89. Andjus RK. 1955. Suspended animation in cooled, supercooled and frozen rats. *J. Physiol.* 128:547–56
90. Costanzo JP, Claussen DL. 1990. Natural freeze tolerance in the terrestrial turtle, *Terrapene carolina*. *J. Exp. Zool.* 254:228–32
91. Barnes BM. 1989. Freeze avoidance in a mammal: body temperatures below 0°C in an Arctic hibernator. *Science* 244:1593–95
92. Lee RE, Costanzo JP. 1993. Integrated physiological responses promoting anuran freeze tolerance. In *Life in the Cold: Ecological, Physiological, and Molecular Mechanisms*, ed. C Carey, G Florant, BA Wunder, B Horwitz, pp. 501–10. Boulder, CO: Westview