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NATURAL FREEZING SURVIVAL IN ANIMALS

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ABSTRACT

Natural freeze-tolerance supports the winter survival of many animals including numerous terrestrial insects, many intertidal marine invertebrates, and selected species of terrestrially hibernating amphibians and reptiles. Freeze-tolerant animals typically endure the conversion of 50% or more of total body water into extracellular ice and employ a suite of adaptations that counter the negative consequences of freezing. Specific adaptations control the sites and rate of ice formation to prevent physical damage by ice. Other adaptations regulate cell-volume change: Colligative cryoprotectants minimize cell shrinkage during extracellular ice formation; other protectants stabilize membrane structure; and a high density of membrane transporter proteins ensure rapid cryoprotectant distribution. Cell survival during freezing is also potentiated by anoxia tolerance, mechanisms of metabolic rate depression, and antioxidant defenses. The net result of these protective mechanisms is the ability to reactivate vital functions after days or weeks of continuous freezing. Magnetic resonance imaging has allowed visual examinations of the mode of ice penetration through the body of freeze-tolerant frogs and turtles, and cryomicroscopy has illustrated the effects of freezing on the cellular and microvasculature structure of tissues. Various metabolic adaptations for freezing survival appear to have evolved out of pre-existing physiological capacities of animals, including desiccation-resistance and anoxia-tolerance.

INTRODUCTION

Seasonally cold temperatures are a reality over much of our planet, and both animals and plants have evolved many ways of coping with low temperatures and with the restriction of feeding, growth, and reproduction that typically

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accompany prolonged cold exposure (20, 49). Most organisms seek some refuge from the lowest extremes of air temperature by spending the winter underwater, underground, or under the snowpack, but others winter in sites that lack thermal buffering. Strategies of underwater or deep underground hibernation generally eliminate the probability of encountering freezing temperatures (53). The insulation of the snowpack typically holds temperatures in the subnivean (under the snow) environment close to 0°C, but lows of −6 to −8°C can occur (49, 60, 75), so animals wintering near the soil surface require some capacity to deal with subzero temperatures. Species wintering above the snowpack, however, may have to deal with prolonged periods of very low subzero temperatures.

To cope with exposure to temperatures below the freezing point (FP) of their body fluids (FP ranges from about −0.5°C for terrestrial species to −1.86°C for osmoconforming marine invertebrates in full strength seawater), ectothermic animals use one of two general strategies: freeze-avoidance or freeze-tolerance. Both include adaptations at behavioral, physiological, and biochemical levels, and indeed, some elements are shared between the two strategies (for review see 1–3, 22, 45, 53, 62, 70, 71, 74, 81). Their fundamental difference is that, whereas freeze-avoiding animals preserve the liquid state of body fluids even at very low temperatures (e.g. some Arctic insects can supercool to −50°C), freeze-tolerant animals defend only the liquid state of the cytoplasm, allowing ice to form in extracellular and extraorgan spaces of their bodies. The combination of adaptations used and the magnitude of their expression varies among species but deals with the specific winter habitat conditions of each.

In phylogenetic terms, both freeze-avoidance and freeze-tolerance have appeared many times in unrelated animal groups. The actual "choice" of strategy was probably a compromise that arose in each species due to the interplay of several factors including (*a*) the need to retain mobility and an active lifestyle at low temperatures, (*b*) the environmental realities of the species habitat or hibernation site, (*c*) pre-existing physiological capacities of the species, and (*d*) the ability to develop specific metabolic and physiological adaptations to perfect one form of cold hardiness. A few examples illustrate these points.

Some teleosts live year round in polar seas at a virtually constant water temperature of −1.86°C. If they were to freeze, they would never again thaw, for the melting point (MP) of their body fluids is above −1°C. However, contrary to expectations for most solutions, the FP of the blood of these fish is not equal to the MP but is pushed below −2°C by the action of specific antifreeze peptides or proteins (21). Various terrestrial invertebrates also benefit from a freeze-avoidance strategy to remain active under the snowpack in winter, and they also employ antifreeze proteins as their primary defense (22). The freeze-avoidance

strategy is very common among terrestrial insects and other arthropods (e.g. spiders, mites) (3, 62), and its development may have taken advantage of various pre-existing factors such as a waterproof cuticle, a protective silk cocoon, autumn elimination of gut contents that contain nonspecific ice nucleators (e.g. bacteria, food particles), and various metabolic changes associated with the cessation of feeding and entry into winter dormancy. Indeed, in summer many insects can supercool to at least -8°C in the absence of any apparent antifreeze or cryoprotectant, and winter hardening further extends this ability (22, 81).

Supercooling (that is, remaining liquid below the FP) is a metastable state in which the probability of spontaneous freezing increases with decreasing temperature until it reaches 100% at the crystallization temperature (T_c; also known as the supercooling point). Freeze-avoiding species have developed adaptations (including antifreeze proteins, high concentrations of colligative cryoprotectants, masking or eliminating nucleators, partial dehydration) that effectively lower whole animal T_c to a value well below the anticipated environmental minima (3, 22, 71, 81). For example, in midwinter the T_c of goldenrod gall moth caterpillars (*Epiblema scudderiana*) was -38°C in a locale where the lowest ambient air temperature was about -25°C (71). However, should freezing occur in such deeply supercooled animals, it is lethal because the instantaneous conversion of a very high percentage of total body water into ice allows no time for compensatory and protective responses by cells. Only a few exceptions to this are known, most notably an Arctic beetle that survived freezing after spontaneous nucleation at about -54°C (55). For other types of animals, the combination of winter environment with pre-existing physiology could make freezing virtually unavoidable; hence, to populate seasonally cold environments, they had to develop freeze-tolerance.

Many species are highly susceptible to inoculative freezing if they come in contact with environmental ice at or below the FP of their body fluids. This was probably the reason for the development of freeze-tolerance among intertidal marine invertebrates and terrestrially hibernating frogs. For example, aerial exposure at low tide can rapidly lower body temperatures of marine molluscs and barnacles to below 0°C (1). Although the animals close themselves off within strong shells, seawater is also trapped within the shell, and when this freezes, the animal comes into direct contact with ice that will seed freezing in tissues. However, an interesting advantage of freezing has been reported for intertidal species: Freezing actually minimizes the net subzero temperature stress on the animals, because the latent heat released during crystallization stabilizes body temperature at a high value (theoretically at the FP) until equilibrium ice content is approached. For animals that normally experience only a few hours of low-tide exposure per day, this can represent a significant percentage of the total

aerial exposure time. For example, the body temperature of mussels (*Mytilus edulis*) freezing in −10°C air remained at about −2°C for nearly 3 h before continuing a descent toward ambient (78). This same effect can apply on a larger scale to all animals in a tide pool that gain thermal buffering at low tide from the layer of surface ice growing on the pool (20).

Frogs that hibernate terrestrially also live in habitats where freezing may be unavoidable. Unlike salamanders and toads that retreat underground to hibernate, frogs remain at the soil surface in sites with a good cover of damp leaf litter to prevent desiccation (8). When ice penetrates these sites, frogs cannot avoid freezing because their highly water-permeable skin presents no barrier to the propagation of ice. Indeed, wood frogs supercooled to −1.5 to −2°C began freezing within about 30 sec when an ice crystal was dropped onto the damp paper on which they were resting (38); most frogs cooled in contact with damp paper or moss show no supercooling but begin to freeze at the FP of body fluids (K Storey, J Storey, unpublished observations).

FREEZE-TOLERANT ANIMALS

Although it is the most challenging method of winter survival, freeze-tolerance has, nonetheless, developed independently in many species (70). Freeze-tolerance is characteristic of hundreds of species of terrestrial insects (especially among Hymenoptera, Diptera, Coleoptera, and Lepidoptera) (22, 43, 55, 81), various intertidal marine invertebrates (including barnacles, bivalves, gastropods) (1, 45), and selected terrestrially hibernating amphibians and reptiles (74). Freeze-tolerance has also been reported for centipedes (76), one species of woodland slug (54), and nematodes (77). As first reported by Schmid (60), several species of terrestrially hibernating frogs are freeze-tolerant (*Rana sylvatica, Pseudacris crucifer, P. triseriata, Hyla versicolor, H. chrysoscelis*), but of urodeles that have been tested, only the Siberian newt, *Salamandrella* (formerly *Hynobius*) *keyserlingii*, with a range that extends onto the tundra, appears to tolerate freezing (74). Among reptiles, box turtles (*Terrepene carolina*), which hibernate in shallow burrows, and hatchling painted turtles (*Chrysemys picta*), which winter in shallow nests on exposed banks, show well-developed freeze-tolerance, surviving several days with more than 50% of body water frozen (5, 13, 16, 67, 75). Other reptiles, including garter snakes, hatchling *Pseudemys scripta*, and various lizards, endure some freezing (6, 7, 12, 14, 15, 41).

Overall, however, the capacity for freeze-tolerance is quite weakly developed in the Reptilia, and the widely variable limits of time, temperature, and ice content endured by reptiles have generated considerable debate about the relevance of freeze-tolerance to the winter survival of these species. Geographic variation in the freeze-tolerance of painted turtles seems to be considerable; both

C. p. marginata and *C. p. bellii* from Canadian populations (in Ontario and Manitoba, respectively) show good freeze-tolerance, whereas *C. p. bellii* from more southern populations (Nebraska) appear to rely on freeze-avoidance for winter survival (5, 52, 75). Furthermore, lizards, garter snakes, and red-eared sliders can endure only a few hours of freezing at relatively mild temperatures; often they cannot recover after longer times when ice content has reached its maximum.

Because temperature change is often slow in the protected hibernacula used by these animals, it becomes difficult to imagine situations in which freezing exposures in the hibernacula could be brief enough to permit recovery after thawing (remembering also that animals might supercool to -2 or -3°C before beginning to freeze but will not melt until temperature rises to the MP of about -0.5°C). It has been proposed that the ability to endure brief freezing stresses is adaptive in dealing with occasional low temperatures when these reptiles are active in the spring and fall, but for long-term hibernation, protected sites are chosen to avoid freezing temperatures (53, 74). At high latitudes, for example, garter snakes migrate large distances to hibernate by the hundreds in underground dens where temperature does not fall below 0°C (47).

Indeed, recent studies with vertebrates have indicated the need to differentiate between "ecologically relevant freeze-tolerance" as an integral component of the winter hardiness strategy of a species and the ability of an animal to endure brief freezing stress. The former probably grew out of the latter, and indeed, survival during and recovery after brief periods of freezing affecting the body extremities including skin and underlying musculature is possible in many species. Ecologically relevant or true freeze-tolerance, however, should be reserved to describe situations in which animals endure long periods (days, weeks) of continuous freezing at temperatures routinely encountered in the hibernaculum, with ice content rising to its maximum, and with ice penetration throughout the core of the body such that vital functions (movement, breathing, heart beat) are interrupted.

Given the physiological challenges of freeze-tolerance, as well as the fact that an animal is totally helpless while frozen, one wonders what advantages are to be gained from wintering in freezing sites. There are probably at least three. The first is early spring emergence. Animals in less protected hibernation sites can detect and respond to the warming temperatures of spring sooner than those in underground or underwater sites. Wood frogs and spring peepers, for example, are active at breeding ponds very early in the spring, weeks before aquatic-hibernating frogs. From this, they gain a long growing season for tadpoles and can make good use of temporary ponds. The second advantage is predator avoidance. Throughout their range, painted turtles that hatch late in the

season remain in their subterranean nests over the first winter, living off stored internal yolk. Delayed emergence offers protection from predation until such time as conditions are favorable for rapid juvenile growth (24), but in the north, this behavior requires mechanisms for enduring subzero temperature. The third advantage is range extension—the ability to penetrate into environments that are not compatible with a freeze-avoidance strategy. Thus, the diversity of invertebrate species in the intertidal zone falls dramatically approaching the polar seas, but freeze-tolerant macrofauna such as mussels, littorines, and barnacles abound. Freeze tolerance has also allowed insects to penetrate some very harsh environments such as the high Arctic or to winter in exposed sites above the snowpack. The woolly bear caterpillar of Elsemere Island, for example, is freeze-tolerant throughout the year, actively feeds for only about one month in the summer, requires as much as 14 years to reach adulthood, and can withstand temperatures as low as -70°C in winter (33).

FREEZING INJURY

To understand the complexity of natural freeze-tolerance and the adaptations that are needed for survival, a brief examination of the damage done by freezing to nontolerant organisms is required. Intracellular freezing is apparently lethal for all organisms in nature due to the physical damage done to subcellular architecture by growing ice crystals. Under laboratory conditions, using isolated cells and extremely high rates of freeze/thaw, some cases of survivable intracellular ice formation have been documented (50). Although some evidence of natural intracellular freezing has been reported in insect fat body cells and nematodes (41, 77), the evidence remains unsatisfying. Ice can also do physical damage in extracellular spaces because the expansion of water when it crystallizes can break delicate capillaries; indeed, the loss of vascular integrity after thawing is a critical problem in cryomedical attempts at freezing organs (57). Rapid rates of ice formation (as occur when there is extensive pre-freeze supercooling) are also highly injurious because of the extreme osmotic stress placed on cells and the very limited time available to make metabolic adjustments before organ functions are shut down by advancing ice. For example, although their overall ability to endure freezing is marginal, European wall lizards uniformly died if the instantaneous ice surge upon nucleation was greater than 5% of total body water (12).

As extracellular ice forms, the osmolality of remaining extracellular fluids rises, and this in turn causes an efflux of water from cells and a reduction of cell volume. Ice continues to accumulate and cells continue to dehydrate and shrink until the osmolality of the remaining fluids rises to a level at which its melting point is equivalent to the subzero temperature of the tissues. The

osmotic shock and cell-volume collapse that this causes are probably the most devastating effects of freezing on unprotected cells. Membranes are highly vulnerable, for these can withstand only so much compression before the lipid bilayer collapses irreversibly into a gel state. Cellular proteins and metabolic functions can also be adversely affected by the increase in intracellular ion concentrations that are the consequence of sequestering a high percentage of water as ice. Other injuries during freezing can result from ischemia, since freezing halts the circulation of blood or hemolymph. Furthermore, the rapid reintroduction of oxygen during thawing may initiate a burst of damage by oxygen free-radicals, similar to the well-documented injuries associated with recovery from ischemia in other systems (28). Finally, freezing halts vital functions including skeletal and smooth muscle movements, breathing, and heart beat.

ADAPTATIONS SUPPORTING FREEZE TOLERANCE

From this list of freezing injuries, we can identify the types of adaptations needed for freezing survival (22, 50, 70, 81). These include: 1. ice control—mechanisms to induce extracellular ice formation, modulate its rate of accumulation, and minimize the physical damage that it can cause (17, 22, 38, 56, 58); 2. cell-volume regulation—including colligative mechanisms that prevent shrinkage below the critical minimum cell-volume (CMCV), transporters for the movement of cryoprotectants and water across membranes, and stabilizers of membrane structure (30, 50, 59, 66); 3. mechanisms of anoxia/ischemia tolerance and of metabolic arrest to sustain cellular viability over long-term freezing (28, 29, 70); and 4. mechanisms for the spontaneous reactivation of vital signs after thawing (34, 35, 67, 74).

Successful freeze-tolerant animals can typically endure days or weeks of continuous freezing with at least 50%, and very often about 65%, of total body water frozen (70, 74); up to 80% ice has been reported in barnacles (1). However, the temperature at which this amount of ice accumulates can vary widely as can the lower lethal temperature (LLT) endured. For example, gall fly larvae reached 64% ice when frozen at -23°C and had an LLT of -27.5°C (42), whereas wood frogs accumulated 65% ice at only -2 to -3°C and did not survive freezing at -5.5°C (36). The LLT seems to be determined primarily by the temperature at which the maximal amount of tolerable extracellular ice is formed, or more correctly, by the CMCV that can be endured. The freezing temperature at which the CMCV is reached is inversely proportional to the osmolality of body fluids, and the CMCV may also be lower in freeze-tolerant than in nontolerant species due to adaptations that stabilize membrane bilayer structure. LLT decreased progressively when marine bivalves were

acclimated to progressively higher seawater salinities (51). Indeed, marine molluscs seem to need only the naturally high osmolality of their body fluids (which are isosmotic with seawater) to defend cell volume during freezing as no specific cryoprotectant is produced.

Freeze-tolerant frogs as well as most insects elevate cellular osmolality by the synthesis of low molecular weight cryoprotectants. Wood frogs and spring peepers produce glucose in rapid response to ice forming in body extremities (11, 63), whereas insects slowly accumulate polyhydric alcohols (glycerol is the most common) over several weeks of autumn cold hardening and sustain polyol pools throughout the winter (for review see 71, 74). Some amphibians (*H. versicolor, S. keyserlingii*) also accumulate glycerol (74), but the pattern of accumulation and clearance of the polyol has not been examined. Perhaps, like insects, these sustain glycerol pools throughout the winter, whereas other frogs, because they use glucose as the cryoprotectant, clear the sugar after each thaw (64). Glucose clearance may be necessary because of the many negative effects of sustained high glucose on metabolism (as occur in diabetes) (23). Apart from glycerol, some insects use other polyols including sorbitol, mannitol, myoinositol, ribitol, erythritol, threitol, and ethylene glycol; some also employ sugars like trehalose as cryoprotectants (70, 73).

No studies have yet determined whether a phylogenetic pattern to cryoprotectant choice can be discerned, but glycerol clearly has metabolic advantages over the others, the most important being that glycerol production maximizes the number of osmotically active particles produced (two C3 glycerol molecules per one C6 hexose-phosphate unit cleaved off glycogen) without any loss from the total carbon pool (syntheses of C2, C4, or C5 polyols all involve CO_2 release) (73). Various freeze-tolerant insects accumulate both glycerol and sorbitol, each synthesized and catabolized with different seasonal patterns. Glycerol, whose synthesis is ATP-dependent, is accumulated early in the fall, well before freezing could impede aerobic energy metabolism, whereas sorbitol can be produced under anaerobic conditions (73). However, the cryoprotective advantage of the dual polyol system, compared with glycerol alone, is unknown. The dual system may benefit the repartitioning of carbohydrate reserves in the spring, since sorbitol carbon is quantitatively reconverted into glycogen, but glycerol carbon has other fates (oxidation, lipid biosynthesis) (70, 73).

Given that freeze-tolerance has arisen in numerous species from diverse groups, it is reasonable to suggest that the capacity arose, at least in part, by potentiating one or more pre-existing physiological capacities. Three of these are immediately obvious. The first is the capacity to deal with wide variations in cell volume and in the osmolality and ionic strength of body fluids. It is not surprising, then, that two groups that are highly tolerant of these

variables (amphibians and intertidal invertebrates) (25, 61) also include within their ranks numerous freeze-tolerant species. From the point of view of the cell, extracellular freezing is simply a form of water stress; whether water is lost to the external environment (as during desiccation or exposure to hypersaline conditions) or temporarily sequestered in extracellular ice masses makes no difference to cells. The second capacity that aids freeze-tolerance is good ischemia-resistance. Again, the ability to survive for extended periods of time without oxygen is well developed in various lower vertebrates (particularly freshwater turtles) and many invertebrates (46). Gill-breathing intertidal invertebrates are particularly good facultative anaerobes for they must deal with oxygen deprivation during each low tide aerial exposure (65). The third factor is metabolic rate depression, the ability to lower basal metabolic rate manyfold and so gain a comparable extension of the time that a fixed reserve of endogenous body fuels can support metabolism. Metabolic depression is always a component of facultative anaerobiosis but is also a widespread response to stresses including heat, cold, and dryness, and indeed, winter dormancy is common for many animals (72). Diapausing insects, air-exposed mussels, and submerged turtles typically have metabolic rates that are only 10% or less of their nondiapausing or aerobic resting rates at the same temperature. A state of metabolic arrest, whether pre-existing or induced during freezing, would both increase the potential survival time while the organism is frozen and minimize the accumulation of deleterious metabolic end products.

In several previous studies we have shown that well-developed mechanisms of anoxia tolerance are important for sustaining cellular energy metabolism during freezing (69, 70). Thus, freeze-tolerant wood frogs and insects show slow declines in tissue ATP content and energy charge over time during freezing, along with accumulation of lactate and alanine as glycolytic end products, but these are readily reversed upon thawing (69, 70). Recently, we have also analyzed the ischemia/reperfusion event of freeze/thaw from a different perspective. Studies with mammalian ischemia/reperfusion models have identified injuries to cellular macromolecules due to a burst of reactive oxygen species (ROS) generation when oxygen is reintroduced at the end of an ischemic episode (26). All animals maintain antioxidant defenses in the form of enzymes such as superoxide dismutase, catalase, and glutathione peroxidase, and metabolites such as glutathione and vitamin E, but these can apparently be overwhelmed by sudden bursts of ROS generation. To determine how freeze-tolerant animals deal with this problem, we compared antioxidant enzyme activities, glutathione levels, and the accumulation of lipid peroxidation damage products in freeze-tolerant (*R. sylvatica*) and freeze-intolerant (*R. pipiens*) frogs (29). Using two different methods, we found no evidence for accumulated lipid peroxidation

damage products in four tissues of wood frogs after 24 h of freezing or up to 4 h of thawing. Furthermore, freeze/thaw had little effect on the glutathione status of wood-frog organs, and together, these results indicate that wood frogs experience little or no oxidative stress over this ischemia-reperfusion event.

The metabolic basis for the lack of oxidative damage during freeze/thaw can be traced to high constitutive activities of antioxidant enzymes in wood-frog tissues; activities of superoxide dismutase, catalase, and glutathione peroxidase in wood-frog liver and skeletal muscle were two- to threefold higher than in the same organs of leopard frogs (29) or weakly freeze-tolerant garter snakes (28). Freezing-induced modification of enzyme activities also occurred in some wood-frog organs (29). Thus, it appears that mechanisms to deal with potential damage due to ROS formation during thawing are another of the important metabolic adaptations supporting natural freeze-tolerance.

In the remainder of this review, we deal with some new advances in the understanding of how freeze-tolerant animals control ice formation and regulate cell volume, and the influence of cell-volume changes on the expression of metabolic adaptations for freeze-tolerance.

ICE CONTROL

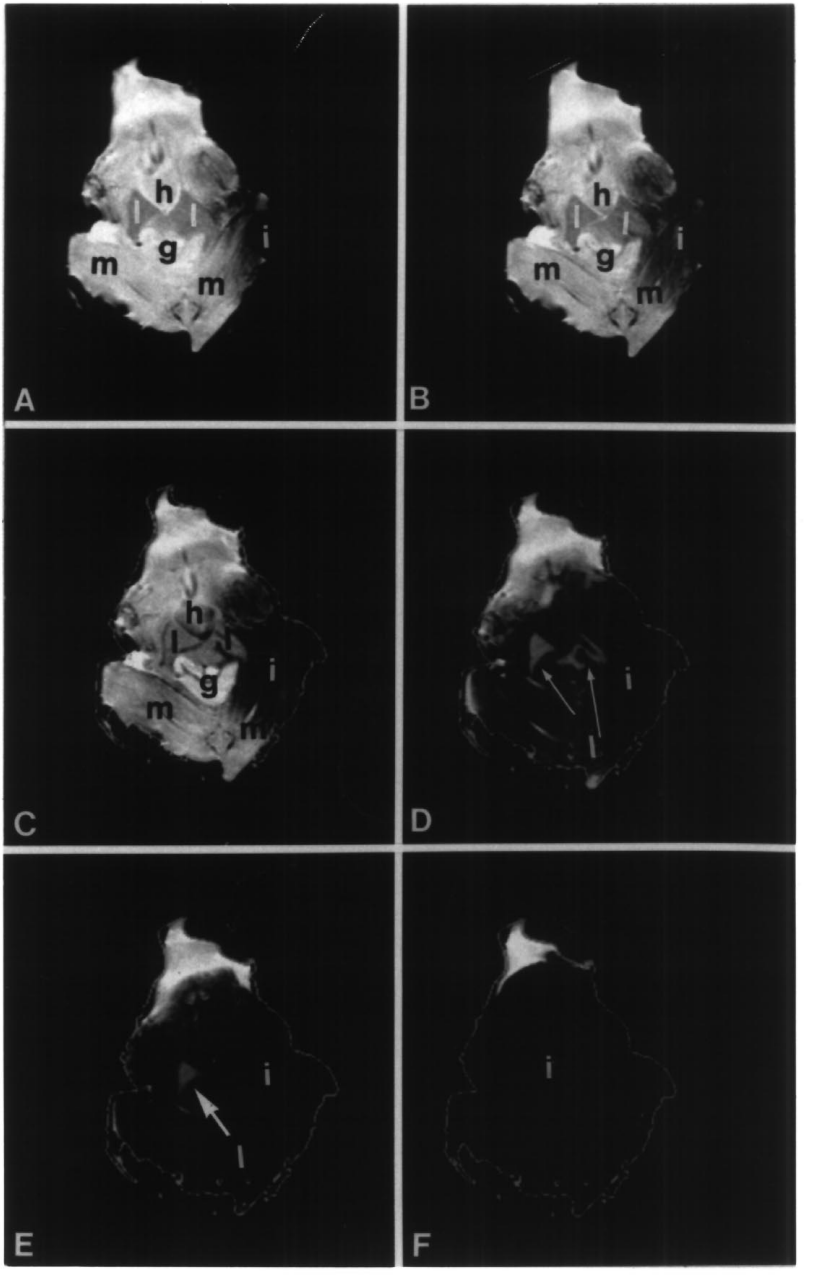
Ice growth can be initiated in two ways. Body fluids can be "seeded" across the epidermis when an animal comes in contact with environmental ice at or below the freezing point of body fluids. Freezing can also occur spontaneously in supercooled body fluids. For many species, freezing generally begins by seeding. The advantages of initiating freezing at a high subzero temperature are time (the slower the rate of ice formation, the longer the time available to make metabolic adjustments) and minimal osmotic shock, for the equilibrium content of ice will be low. Frogs frozen at -2.5°C, for example, take about 24 h to reach maximum ice content (36). The lower the temperature at which freezing begins (compared with the FP), the faster the rate of freezing, the greater the percentage of water that will freeze in the initial ice surge, and the less the time that will be available to implement cryoprotective measures. For example, when wood frogs were frozen at -2.5°C, there was plenty of time for a wide distribution of glucose from the liver to all core organs, but on a subsequent freeze, when frogs were held at -4°C, the rate of ice formation was too fast for extensive glucose distribution, and most cryoprotectant remained locked in the liver where it was made (64).

To minimize the freezing stress and maximize the ability to implement protective responses, freeze-tolerant animals, if not seeded by contact with environmental ice, stimulate crystallization themselves by employing ice nucleators. These initiate freezing usually only a few degrees Celsius below the FP of body

fluids. Sometimes nucleators are nonspecific, and in different species they have been linked to bacteria on the skin surface or in the gut, other gut contents, or frass (22, 39). In other species, specific plasma or hemolymph proteins are synthesized seasonally, and these reproducibly nucleate at a precise temperature. Hemolymph ice-nucleating proteins occur widely in insects and have been reported in some marine snails, wood frogs, and painted turtle hatchlings (22, 48, 68, 79, 80).

The process of freezing and the mode of ice propagation through the body of a freeze-tolerant animal have been examined in detail in wood frogs and painted turtles. It is apparent that ice formation is carefully controlled to ensure survival. Using proton magnetic resonance imaging (MRI), ice formation can be monitored in real time in intact, living animals. Figure 1 shows selected images taken over the course of freezing and thawing an individual frog (58). The freezing front moves directionally through the body of the frog. The striated appearance of skeletal muscle (Figure 1A, B) shows that extracellular freezing is constrained by the morphology of the tissue, with crystals growing along the length of the muscle fibers. Within the abdominal cavity, the dark outlines around organs (Figure 1B, C) show that freezing occurs first in the extraorgan fluid. Close examination of the liver shows that the lobes shrink in size as freezing progresses and that the liver is the last organ to freeze fully (Figure 1C–E). Freezing monitored at another cross section that highlighted the brain and spinal cord of the frog revealed similar phenomena, with freezing occurring first in the spinal fluid and within the ventricles of the brain before moving into the tissue itself (58). A similar pattern appeared when the freezing of painted turtle hatchlings was monitored by MRI (56).

In contrast to the directional mode of freezing, the pattern of thawing revealed by MRI was quite different. In both wood frogs and painted turtles, thawing began uniformly throughout the entire body with images from all organs lightening in concert (56, 58). Organs clearly melted while still surrounded by extraorgan ice, a phenomenon that was particularly striking for the core organs of frogs. The same phenomenon was seen in the brain and spinal cord; tissues thawed before ice melted in brain ventricles, spinal fluid, or the vitreous humor of eyes (in turtles) (56, 58). The reason for this pattern of thawing can be traced to the higher osmolality of fluids in contact with ice within the organ vasculature (due to the presence of cryoprotectants as well as normal plasma solutes) compared with the large mass of nearly pure ice in extraorgan spaces. While a frog freezes, glucose is rapidly produced by the liver and distributed by the blood to other organs (70). As the freezing front moves inward, circulation (and cryoprotectant distribution) is progressively cut off first to peripheral, and then to core, sites. Final cryoprotectant levels are highest, therefore, in liver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and heart, somewhat lower in brain and other abdominal organs, and lowest in skeletal muscle and skin (64). During thawing, the high glucose in core organs causes these to melt first. Melting from the inside out seems odd, but it has the physiological benefit of allowing the vital functions of the heart and lungs to reactivate as soon as possible. Not surprisingly, then, cardiac function is the last vital sign to cease during freezing (arrest occurs 11–21 h after freezing starts) (37) and is the earliest vital sign detected during thawing, occurring within 1 h at 3–5°C (32, 67). Following the resumption of heart beat, blood flow to the skin is detected soon thereafter, followed by spontaneous breathing, and finally skeletal muscle reflexes recover (35). Sciatic nerves regained excitability with 5 h of thawing, and recovery times for hind limb retraction and righting responses were 8 and 14 h, respectively (32). Differences in the physiology of nerve function between cold-sensitive, cold-resistant, and freeze-tolerant anurans appear to be important in the ability to recover after freezing exposure (19).

The MRI images suggested that liver and other organs shrink in size during freezing, and this can also be observed when dissecting frozen frogs. Huge masses of ice fill the abdominal cavity, and organs are visibly shrunken and encased in ice. Large flat crystals are also sandwiched between the skin and skeletal muscles of body and limbs. This extraorgan sequestration of ice has been quantified for frogs frozen slowly at −2.5°C (17, 40); organ water contents decreased by 2.8, 8.7, 12.7, 19.5, and 24.2% for eye, brain, skeletal muscle, liver, and heart, respectively, compared with organs from unfrozen animals. This appears to be an important method of avoiding physical damage by ice. By evacuating large amounts of water from organs and sequestering it innocuously as ice in extraorgan sites, the potential for damage due to excessive ice expansion within microvasculature of organs is greatly reduced. Such damage is a recognized problem in cryomedical organ preservation (57). Cryomicroscopy of tissue slices from both mammals and freeze-tolerant frogs shows that freezing

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Figure 1 Proton magnetic resonance imaging showing the progress of ice formation during simulated natural freezing in a wood frog *Rana sylvatica.* Shown is a dorsal cross section through the frog during freezing at −7°C. The frog was placed in a cylinder around which the radio-frequency coil was wound, and then the cylinder was cooled from the bottom through contact with circulating chilled fluid. Frozen areas of the frog are darker because the protons in ice are invisible to standard proton MRI. In this individual, at this cross section highlighting the abdominal core, the freezing front can be seen moving inward from the right side (*A–C*). The liver is the last organ to freeze (*D, E*). Timed from the initiation of freezing, images were taken at 1 h 16 min (*A*), 1 h 28 min (*B*), 1 h 48 min (*C*), 2 h 10 min (*D*), 2 h 23 min (*E*), and 4 h 24 min (*F*). Labels are: (*h*) heart, (*l*) liver, (*m*) skeletal muscle, (*g*) gut, (*i*) ice; photographs are full size. Edge detection was added to some of the images during data processing. Taken from Rubinsky et al (58).

results in shrunken cells with much ice accumulated in an expanded vasculature space (66). Good freeze-tolerant animals, then, appear to move water out of their tissues, minimize the amount of ice forming within organ vasculature, and pack their cells with cryoprotectant to maintain the CMCV.

CELL-VOLUME REGULATION

The formation of ice in extracellular fluid spaces places an osmotic stress on cells that results in the net outflow of water from cells, a net influx of low molecular weight solutes, and a net decrease in cell volume. To be survivable, the reduction in cell volume cannot exceed the CMCV, which is usually associated with about 65% of total body water frozen as extracellular ice. This amount of cellular dehydration still leaves considerable intracellular free water, as illustrated by cryomicroscopy of tissue slices from freeze-tolerant frogs and turtles (56, 66). Indeed, image analysis of cryomicrographs of turtle organs indicated that extracellular ice constituted 36% of the total tissue volume in liver at -4°C, and 61% in skeletal muscle and heart (56); at these levels of dehydration, intracellular structure was clearly maintained. However, when tissue slices were frozen at -20°C (not survivable in nature), total tissue ice values rose to 65% , 79% , and 72% in the three tissues, respectively, and the severe cellular dehydration that ensued disrupted subcellular organization (56).

At least three types of adaptations appear to be required for cell-volume regulation in freeze-tolerant animals: 1. mechanisms that stabilize membrane bilayer structure under the compression stress of cell-volume reduction, 2. mechanisms that limit cell-volume reduction and prevent shrinking below the CMCV within the range of naturally encountered freezing temperatures, and 3. adaptations of membrane transport systems to allow rapid redistribution of water and solutes between intra- and extracellular compartments. Membrane stabilization is achieved through the action of specific low molecular weight cryoprotectants, such as trehalose and proline, that interact directly with the polar head groups of membrane lipids to stabilize the bilayer structure. The actions of these compounds have been well studied in species that endure extreme low water stress (anhydrobiosis) (18) and confirmed for freezing preservation of isolated membranes (59). Notably, both trehalose and proline levels are elevated in freeze-tolerant insects during the winter, and proline is often one of the major intracellular free amino acids in euryhaline marine invertebrates, one whose concentration can change rapidly in response to osmotic stress (25, 70).

The second component of volume regulation is to minimize cell-volume decrease during freezing via the colligative actions of low molecular weight solutes, generally specifically synthesized cryoprotectants (sugars, polyols).

Regulation of cryoprotectant biosynthesis (glucose in frogs; glycerol, sorbitol, or other polyols in insects) and the actions of these compounds in regulating cell volume and stabilizing macromolecules have been extensively reviewed (18, 70, 71, 73, 80).

The third facet of volume regulation, which has only recently received attention, is the regulation of water and solute fluxes across the plasma membrane during freezing and thawing. The lipid bilayer of the plasma membrane allows few compounds to cross by simple diffusion. For most compounds, entry into or exit from cells is gated by transport proteins that span the membrane and provide facilitated transport for compounds moving in the direction of an osmotic gradient and active transport to move compounds against their concentration gradient. During extracellular freezing, the osmotic and ionic imbalance set up by the exclusion of solutes from rapidly growing ice crystals requires a redistribution of water, ions, and cryoprotectants across cell membranes; reverse movements accompany thawing. Glucose movement across cell membranes is carrier-mediated by transporter proteins, and glucose has proven to be a poor cryoprotectant in cryomedical trials with mammalian tissues because it can not enter cells quickly. Similarly, most mammalian cells are impermeable to sorbitol, yet this is one of the major polyols accumulated by freeze-tolerant insects (73). Hence, adaptations of membrane sugar and polyol transporters must have accompanied the use of these compounds as cryoprotectants in nature.

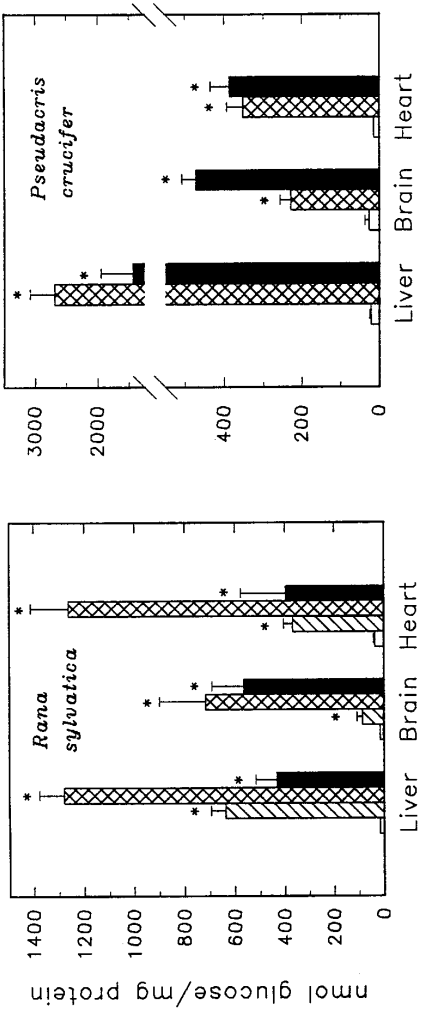
Recent studies have targeted glucose transporters in freeze-tolerant wood frogs. Within just a few hours after freezing begins, wood frogs catabolize a huge liver glycogen reserve (as much as 600–700 μmol/g wet weight (gww) in glucose equivalents) and export glucose to other organs to raise their sugar content as high as 200 μmol/gww before full freezing halts circulation (64, 70). Not surprisingly, the number of glucose transporters in *R. sylvatica* plasma membranes is specifically increased to deal with the demands of rapid cryoprotectant movement (30, 31). Membrane vesicles prepared from liver of autumn-collected wood frogs had an 8.2-fold greater rate of carrier-mediated glucose transport and a 4.7-fold higher number of glucose transporters (quantified by cytochalasin B binding) than did liver membrane vesicles from the freezing-intolerant, aquatic-hibernating leopard frog. Glucose transport rate by wood-frog skeletal muscle vesicles was also 8-fold higher than in leopard-frog vesicles, showing that transporter systems of both the cryoprotectant-producing organ and a receiving organ are modified in concert (30). Furthermore, the rate of carrier-mediated glucose transport by liver vesicles was 6-fold higher and the number of transporters 8.5-fold higher in liver vesicles from wood frogs collected in September, compared with June animals (31). The importance of elevated glucose transport capacity for freeze-tolerance in frogs raises the

question of whether other specific transporters are involved in cell-volume regulation during freezing. For example, water moves across cell membranes both by simple diffusion and by channel-mediated facilitated transport by aquaporins (4). An important next step in studying natural freeze-tolerance will be to determine whether adaptations of aquaporin numbers or activity aid water fluxes during freezing and thawing.

RELATIONSHIP BETWEEN FREEZE TOLERANCE AND DEHYDRATION TOLERANCE

The probable development of freeze-tolerance as an extension of pre-existing mechanisms of dealing with water stress in animals is further supported by some recent experiments on the metabolic responses to dehydration in frogs. Because ice forms extracellularly, the major stress perceived by cells during freezing is a sharp volume reduction. Hence, it seemed logical to predict that protective metabolic responses to freezing might be triggered or regulated by dehydration or changes in cell volume. Indeed, this seems to be the case for cryoprotectant synthesis in frogs. Two freeze-tolerant species, the wood frog *R. sylvatica* and the spring peeper *P. crucifer*, were subjected to controlled, whole-body dehydration stress at 5°C at a rate of 0.5−1% of total body water lost per hour (achieved by holding frogs in closed containers with desiccant in the bottom). Both species tolerated the loss of 50−60% of total body water, putting them among the best of the desiccation-tolerant anurans (61). Both species responded to dehydration with rapid glycogenolysis in liver and glucose export to other organs. In all six organs tested of autumn-collected wood frogs, glucose rose progressively as animals were dehydrated (Figure 2 shows liver, heart, and brain); the maximal increase ranged from 9-fold in gut to 313-fold in liver of frogs that had lost 50% of total body water. Final liver glucose was 127 μmol/gww, a value not much less than the 200–300 μmol/gww typically stimulated by freezing exposure (8). A similar response was seen with autumn-collected *P. crucifer*; glucose rose by 120-fold to 2690 $\pm$ 400 nmol/mg protein

Figure 2 Effect of dehydration and rehydration on glucose levels in liver, heart, and brain of the freeze-tolerant frogs *R. sylvatica* and *P. crucifer* and the freeze-intolerant *R. pipiens*. All frogs were autumn-collected, acclimated at 5°C, and then dehydrated at 5°C at a rate of 0.5−1% of total body water lost per hour in closed containers over a layer of silica gel desiccant. For rehydration, 50% dehydrated frogs were placed in containers with 1–2 cm of distilled water and sampled after 24 h at 5°C. Bars are: open, controls at 5°C; rising right, dehydrated to 25% of total body water lost; crosshatched, dehydrated to 50% of total body water lost; solid, 50% dehydrated then fully rehydrated. Data compiled from Churchill & Storey (8–10). Storey & Storey (30).


Pseudacris crucifer
Rana sylvatica
Rana pipiens
Liver Brain Heart
nmol glucose/mg protein
0
200
400
2000
3000
600
800
1000
1200
1400
40
80
240
320

or 220 μmol/gww in liver of 50% dehydrated frogs (Figure 2) (9). Glucose in other organs of *P. crucifer* rose by 3- to 60-fold. Glucose levels in both species fell when animals were rehydrated (Figure 2), which also occurs when frogs are thawed, and the hyperglycemic response to dehydration was much greater in autumn- versus spring-collected frogs, which again occurs with freezing-induced cryoprotectant synthesis (8, 9). Parallel experiments with the aquatic-hibernating leopard frogs showed that liver glycogenolysis and glucose output responses to dehydration were also shared by a freeze-intolerant species (10). Glucose rose progressively with dehydration in *R. pipiens* liver, rising by 24-fold overall to a final value of 20 μmol/gww in frogs that had lost 50% of total body water (Figure 2). Thus, although the magnitude of the dehydration-induced hyperglycemia is much lower in *R. pipiens* than in the freeze-tolerant species, the glycogenolytic response to dehydration is clearly in place in the freeze-intolerant species, and this suggests that the cryoprotectant response to freezing grew out of a more primitive hyperglycemic response to dehydration.

Also intriguing about this hyperglycemic response is that it is not a direct response to water loss by liver cells themselves. In all three species undergoing dehydration, water was lost first from extraorgan pools, and water content of core organs was defended until a high percentage of total body water was lost (8–10). Indeed, *R. sylvatica* excelled at this, and even when 50–60% of total body water was lost, liver water content remained unchanged (8). Rather, it appears that water loss (due to dehydration or freezing) is detected by peripheral target cells, perhaps in the skin, and the signal is transmitted by nervous or hormonal stimuli to the liver. The signal may be catecholamine-based since administration of the β-adrenergic antagonist, propranolol, suppresses the freezing-induced synthesis of glucose by wood frog liver (74). Recent research with mammalian systems has shown that cell-volume change can trigger numerous effects, including changes to intermediary metabolism and gene expression (27); insulin, for example, stimulates cell swelling in rat liver, whereas glucagon and catecholamines (glycogenolytic hormones) induce shrinkage (27). Thus, glycogenolysis has an ancient link to cell-volume reduction, and this may underlie the hyperglycemic response to freezing by frog liver. A key area for future research in freeze-tolerance will be to explore the range of cryoprotective responses stimulated and coordinated by changes in cell volume.

In summary, then, freeze-tolerance is one of the most fascinating and complex adaptations that has evolved among animals. Although much is known about some aspects of freeze-tolerance, notably cryoprotectant biosynthesis, many other areas are largely unexplored, and completely new elements of freeze-tolerance still remain to be identified. For example, in the newest studies by our lab, we have constructed and screened a cDNA library prepared from liver

of freezing-exposed wood frogs to identify genes that are upregulated during freezing. Expecting to identify genes related to one of the known areas of adaptation that have been the subject of this article, we found instead that the first two clones we identified are genes for the α and γ subunits of fibrinogen (Q Cai, KB Storey, submitted for publication). Fibrinogen is a plasma protein produced by liver that is key to the clotting process, and these results suggest that we have stumbled into a previously unrecognized area of adaptation in freeze-tolerance—the repair mechanisms that may be needed to deal with mechanical injuries to tissues by ice crystals. These new results suggest that the body's clotting defenses are potentiated while the animal is still frozen in order to deal swiftly and effectively with any damage to the vasculature that is detected upon thawing.

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