Insect Cold-hardiness: To Freeze or Not to Freeze

How insects survive low temperatures

by Richard E. Lee, Jr.

In temperate regions, few insects are able to avoid exposure to low environmental temperatures during the winter. Notable exceptions include the monarch butterfly, which may migrate more than 1000 miles to warmer climates, and the honeybee, which maintains constant hive temperatures near 35° C by behavioral and physiological activities of the colony (Heinrich 1981, Southwick and Heldmaier 1987). Other species avoid low-temperature extremes by vertical migration into the soil or the selection of protected hibernacula. However, for the many insects unable to avoid exposure to low temperatures, the capacity to cold-harden is required for overwintering survival.

Although many overwintering insects seek protected overwintering sites in leaf litter or beneath the bark of trees, the most cold-tolerant species are those that overwinter in exposed sites, such as the Arctic caterpillar (*Gynaephora*), which overwinters on rocky surfaces, or fly larvae in plant galls that extend above the snowline, thus exposing their inhabitants to the extremes of fluctuation in ambient temperatures (Ring and Tesar 1981).

Cold-hardiness or cold tolerance refers to the capacity of an organism to survive long-term or short-term exposure to low temperature. This capacity varies as a function of the specific developmental stage, season of the year, nutritional status, and the duration of exposure. Physiological and biochemical processes that enhance low-temperature tolerance result in cold-hardening.

The capacity to cold-harden is required for overwintering

The study of insect cold-hardiness is a comparatively new one. Based on his publications in the 1950s and 1960s, R. W. Salt is generally credited as the first to examine rigorously on biochemical and physiological levels the nature of low-temperature tolerance in insects (Salt 1961, 1969). Interest in this area has grown steadily, as evidenced by the number of publications listed in two recent bibliographies on this subject (Baust et al. 1982, Lee et al. 1986). This article provides a brief introduction to insect cold-hardiness with emphasis on some recent findings, including the description of an extremely rapid cold-hardening response protective against short exposures to cold.

Overwintering cold-hardiness

To prepare for the cold of winter, insects undergo a variety of physiological and biochemical changes. The process of winter cold-hardening requires at least several weeks to complete.

Two strategies. Overwintering insects appear to use two major strategies, depending on whether they can survive the freezing of their body water. Freeze-susceptible insects must avoid freezing; freeze-tolerant insects can survive extracellular ice formation within their tissues.

FREEZE-SUSCEPTIBLE INSECTS. Freeze-susceptible (or freeze-intolerant) species take advantage of supercooling. Small volumes of water, whether in organisms or not, can often be cooled many degrees below the melting point before spontaneous nucleation. For example, 5-microliter samples of tap water commonly can be cooled to −18° C or lower. The capacity to supercool decreases as volume increases and as the duration of exposure to subzero temperatures increases. Therefore, it should not be surprising that insects, being essentially small bags of water, have the capacity to supercool extensively. Insects can sometimes avoid freezing until their temperature has fallen many degrees below the melting point of the body fluids (Figure 1).

The supercooling point is the temperature at which spontaneous nucleation occurs and the ice lattice begins to grow. Even for small insects or their eggs, this value is easily measured by using thermocouples to detect the release of the heat of crystallization. The heat usually dissipates within a few minutes, and their body temperatures decrease to the ambient

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temperatures.

In preparation for overwintering, freeze-susceptible species, such as ladybugs and springtails (Collembola), commonly decrease their supercooling point, thereby increasing the chance of winter survival (Figure 1). For this group, supercooling points often range in winter between −15° and −35° C (Sømme 1982), although some species from the interior of Alaska supercool to approximately −60° C (Miller 1982). Evacuation of the gut, resulting in the removal of potential ice-nucleating agents, is believed to be one mechanism of enhancing supercooling capacity (Cannon and Block 1988). Also, endogenous ice-nucleating agents located in the tissues may be inactivated or masked during cold-hardening (Baust and Zachariassen 1983). In other species, water balance and desiccation appear to influence the supercooling point (Cannon et al. 1985). The insects also accumulate certain chemicals, called cryoprotectants.

FREEZE-TOLERANT INSECTS. Other insects are able to survive extensive extracellular ice formation within body tissues and organs when temperatures may fall as low as −70° C (Miller 1982, Storey and Storey 1988). These freeze-tolerant insects, such as the goldenrod gall fly (*Eurosta solidaginis*), commonly synthesize ice-nucleating agents during winter cold-hardening. These unique proteins or lipoproteins serve as catalysts for the extracellular nucleation of ice in body fluids at temperatures of −5° to −10° C (Duman et al. 1985). By limiting the amount of supercooling, these proteins apparently serve to avoid lethal intracellular freezing.

Freeze tolerance refers to a specific temperature range in which tissue freezing is tolerated; cooling below that range may result in mortality from excessive cellular dehydration (Figure 1). Because only water molecules join the ice lattice as it grows in the extracellular space, the remaining extracellular solute becomes more concentrated. This process results in an osmotic gradient that removes water from the cells. Injury due to freezing results from excessive concentration of solutes and cell shrinkage, and more recently it has been suggested that damage may be a function of the

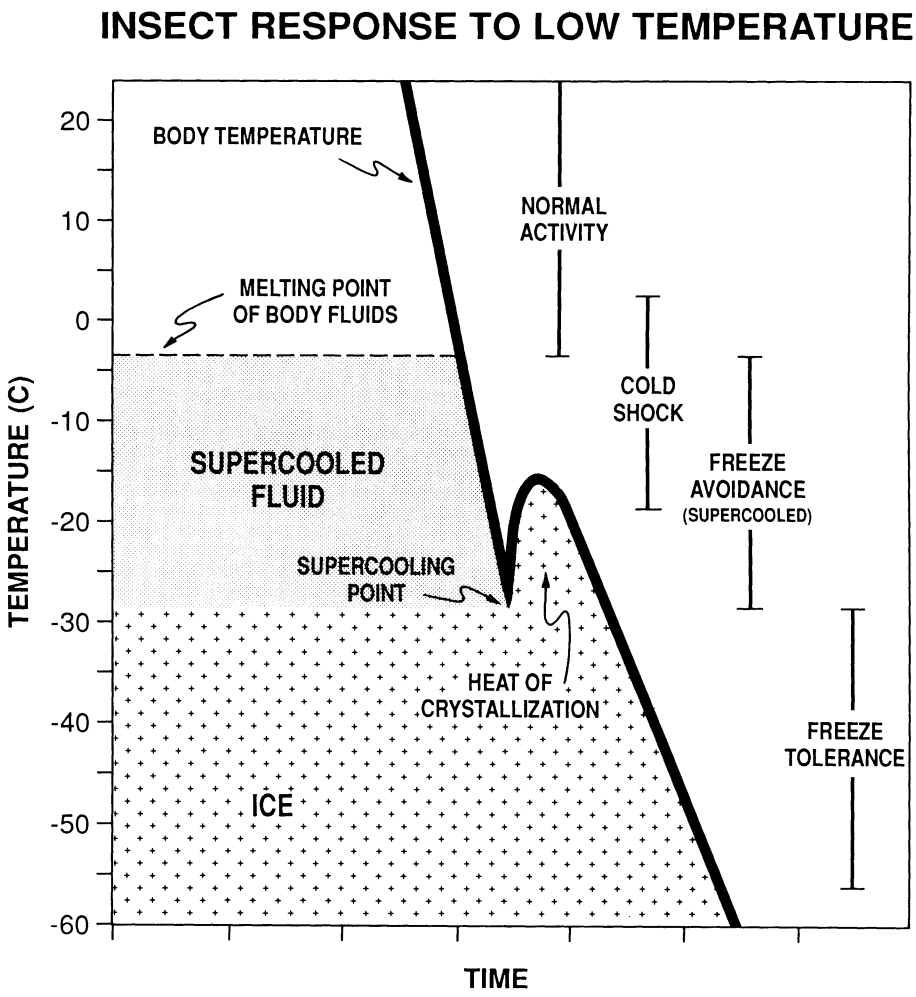

Figure 1. Responses of insects cooled to subzero temperatures. Insect body temperatures (heavy line) in relation to the melting point, the supercooling point, and the nucleation of ice in body fluids. Although the bars on the right convey general ranges of insect response to low temperatures, the top of the bar for the range of freeze tolerance and the bottom of the bar for freeze avoidance correspond to the supercooling point value illustrated in the center of the figure. However, a number of freeze-tolerant insects have supercooling points in the range of −8° to −10° C, whereas some freeze-susceptible species supercool extensively, to −60° C or below.

decrease in the size of unfrozen channels in the extracellular space (Mazur 1984).

Cryoprotectants. One of the most remarkable characteristics of overwintering insects is their capacity to produce both simple compounds and proteins that act as antifreeze. Insects can accumulate large amounts of glycerol, sorbitol, trehalose, or other low-molecular-weight carbohydrates or polyols. These compounds, made by both freeze-susceptible and freeze-tolerant species, are referred to as low-molecular-weight antifreezes or cryoprotectants. One larval wasp, *Bracon cephi,* produces 5-M glycerol concentrations corresponding to 25% of its body weight (Salt 1961). In some species, combinations of two or more of these compounds are accumulated during cold-hardening. In freeze-intolerant species, the traditional view held that these antifreezes functioned primarily in a colligative fashion (i.e., based on the number of solute particles in solution), causing a depression in both the hemolymph freezing point and the supercooling point (Salt 1961, Zachariassen 1985).

One of the most significant advances in the last few years is the realization that low-molecular-weight polyols and sugars can protect biological molecules and systems via non-

colligative mechanisms (Crowe et al. 1983, Crowe et al. 1987). The brine shrimp *Artemia,* the slime mold *Dictyostelium,* certain nematodes, and some other organisms can survive for years in a dry (anhydrobiotic) state, and this survival correlates closely with the presence of large amounts of sugars (Crowe et al. 1987). Furthermore, studies have demonstrated the direct action of sugars to preserve and stabilize enzymes, phospholipid bilayers, and liposomes during drying (Crowe et al. 1987). Potential cryoprotectants can be evaluated by their ability to protect freeze-labile enzymes and liposomes (Loomis et al. 1988).

Anhydrobiotic and freeze-tolerant survival are directly analogous; both require the tolerance of extensive cellular dehydration. Even relatively low concentrations of trehalose, glycerol, and other cryoprotectants may function to stabilize protein structure and membranes during cooling and/or freezing.

To date, most studies examining the function of endogenous nucleators and cryoprotectants required the extraction of these compounds from the insect. Baust and Rojas (1985) caution that this approach is limited for understanding in vivo mechanisms of cold tolerance. In contrast, nuclear magnetic resonance (NMR) spectroscopy provides a specific, but noninvasive, tool for studying cold-hardiness in living animals. With carbon-13 NMR, Buchanan and Storey (1983) detected cryoprotectants and lipids in freeze-tolerant larvae of the goldenrod gall fly. More recently, Kukal et al. (1988) obtained in vivo time-lapse carbon-13 spectra of glycerol metabolism in freeze-tolerant larvae of an arctic caterpillar.

In addition to accumulating low-molecular-weight cryoprotectants, a number of overwintering insects produce antifreeze proteins (Duman 1977, Duman and Horwath, 1983). Sometimes these proteins are referred to as thermal hysteresis proteins, because they cause a difference between the melting and freezing points of body fluids similar to that of antifreeze peptides and glycopeptides found in the blood of polar fish (DeVries 1971). In freeze-intolerant insects, these proteins appear to function as an antifreeze, lowering both the freezing and the supercooling points (Duman and Horwath 1983). Zachariassen and Husby (1982) offer an alternative explanation: the antifreeze proteins may function to stabilize the supercooled state by adsorbing to the surface of embryonic ice crystals, thereby preventing additional growth of the ice lattice. Later Knight and Duman (1986) demonstrated that these unique proteins inhibit recrystallization, growth of the ice lattice in tissue that is already frozen. This observation raises the possibility that antifreeze proteins may also protect freeze-tolerant insects from injury due to recrystallization.

Environmental regulation of winter cold-hardening. During the last decade, several investigators have begun to elucidate the mechanisms that synchronize cold-hardening with seasonal changes in the environment. The acclimatization process is anticipatory in that it occurs in advance of exposure to sub-zero temperatures in the field. The exposure of insects to low temperature, particularly in the range of 0° to 5° C, has been repeatedly demonstrated to trigger the accumulation of cryoprotectants and to enhance cold-hardiness (Baust and Lee 1981). Low temperature acts directly to increase the activity of specific enzymes (glycogen, phosphorylase, hexokinase, and phosphofructokinase) and thereby channel the flow of carbon into polyol synthesis (Storey and Storey 1981). Acclimation to high temperatures frequently results in the rapid loss of cryoprotectants and cold tolerance (Ring 1982).

Biological clocks also play a role in the cold-hardening process; exposure to short photoperiods induces the synthesis of thermal hysteresis proteins (Duman and Horwath 1983). Beck (1987) recently demonstrated that daily cycles of temperature (thermoperiods) interact with the photoperiodic cues in the regulation of dormancy. The possible role of thermoperiodism in cold-hardening should be examined.

Nonfreezing injury and cold shock

It has been assumed frequently that the supercooling point represents the lethal temperature for freeze-susceptible insects. Although the supercooling point represents the theoretical lower limit of temperature tolerance for this group, recent studies have shown that chilling, in the absence of tissue freezing, may result in lethal injury (Knight et al. 1986, Lee and Denlinger 1985). In some species, nonfreezing injury is evident only after extended periods of low-temperature exposure, as in the bertha armyworm, *Mamestra configurata* (Turnock et al. 1983). In his recent review, Bale (1987) emphasizes the importance that prefreezing mortality may have on survival in the field and urges future studies to determine carefully whether the supercooling point may be used reliably as a measure of the lower lethal temperature.

In other species, short-term expo-

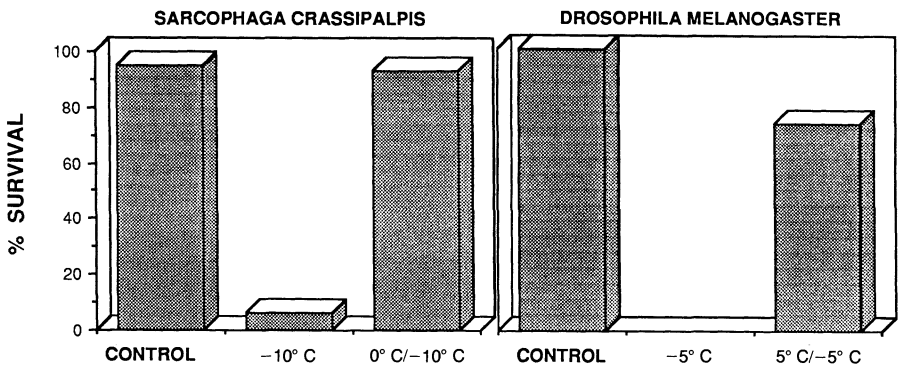

Figure 2. Cold shock and rapid cold-hardening in the flesh fly, *Sarcophaga crassipalpis* (Chen et al. 1987), and in the fruit fly, *Drosophila melanogaster* (Czajka and Lee 1988). Control flies were held continuously at 25° C, a second group was directly transferred to subzero temperatures for 2 hours, and a third group was chilled for 2 hours at 0° or 5° C, respectively, before subzero exposure. At least 50 flies were tested in each group.

sure to cold temperatures above the supercooling point is lethal. For example, even though active pupae of the freezing-intolerant flesh fly, *Sarcophaga crassipalpis*, have a supercooling point of −23° C, this stage does not survive as little as 20 minutes of exposure to −17° C (Lee and Denlinger 1985). Aphids are particularly susceptible to chilling injury (Knight et al. 1986). This type of injury is termed cold shock and occurs after rapid cooling, but in the absence of ice formation (Morris 1987).

This form of cellular injury is known for a wide variety of biological systems including bacteria, fungi, algae, and higher plants, as well as mammalian spermatozoa and embryos. The mechanism of cold-shock injury is thought to be the induction of phase transitions in the lipid membrane leading to the subsequent loss of membrane integrity (Quinn 1985). Alternatively, or perhaps concomitantly, membrane failure may be due to excessive thermoelastic stress (McGrath 1987).

Cold shock and chilling injury are of considerable economic interest, not only with respect to plants and agricultural products, but in the development of methods for the cryopreservation of semen, mammalian embryos, and other cells and organs (Morris 1987). Nearly all of these applications pertain to cells, tissues, or organs from tropical plants or from mammals, including humans, which do not naturally experience internal low temperature as a normal part of their life cycle. The study of insect model systems that are naturally able to protect themselves against injury due to cold shock may ultimately lead to methods of preventing chilling injury in the storage and transport of plant products and to more successful methods for the cryopreservation of human tissues and organs.

Rapid cold-hardening response

Historically, studies of insect cold-hardening have examined seasonal processes related to overwintering. In contrast, we have identified an extremely rapid cold-hardening response that protects insects from injury due to cold shock (Chen et al. 1987, Lee et al. 1987). This response is found even in nonoverwintering stages.

Table 1. Summary of characteristics associated with cold-hardening for winter and the rapid cold-hardening response that protects against injury due to cold shock. Adapted from Lee et al. 1988.

Characteristics	Winter cold-hardening	Rapid cold-hardening
Differences		
Cold tolerance	Long-term freeze tolerance and/or supercooling	Short-term prevention of non-freezing injury
Stage	Only in overwintering stage	Present in more than one developmental stage (larva, pupa, adult)
Activity	Inactive (diapause, quiescence)	Active feeding and reproduction (sometimes diapause)
Timing	Seasonal	All year
Rate of cold-hardening	Slow (weeks–months)	Rapid (minutes–hours)
Similarities		
Cryoprotectant	Present	Present
Induction trigger	Low temperature	Low temperature

If adults of the flesh fly, *S. crassipalpis*, are exposed directly to −10° C for two hours, few individuals survive (Figure 2). Because this stage has a supercooling point of −23° C, this result suggests that injury is due to cold shock. Remarkably, however, chilling at 0° C for as little as 10 minutes enables 50% of the flies to survive a two-hour exposure at −10° C. Two hours of chilling at 0° C allows nearly all flies to survive this treatment. Short-term chilling of larvae and pupae before −10° C exposure greatly enhances survival. Freeze-intolerant adults of *Drosophila melanogaster* have a supercooling point of −20° C, but they do not survive even short intervals of exposure to −5° C (Czajka and Lee 1988). Similarly, short-term chilling protects these fruit flies against injury due to cold shock (Figure 1).

Clearly, the phenomenon of overwintering cold-hardiness differs distinctly from the rapid cold-hardening response protecting against cold-shock injury (Table 1). Although both processes protect against forms of injury caused by exposure to low temperature, winter cold-hardening frequently includes the acquisition of freeze-tolerance or the capacity to survive exposure to temperatures near the supercooling point for a specific overwintering stage. In contrast, the rapid cold-hardening response protects against low-temperature injury that occurs as much as 15° C above the supercooling point. Furthermore, the rapid cold-hardening response occurs in various developmental stages, even when the insects are actively feeding and reproducing, and it may operate at any time of the year. The most unexpected and remarkable difference is the rapidity of the response: typically weeks are required for the completion of the seasonal hardening process in preparation for overwintering, whereas as little as 20 minutes of chilling is sufficient to elicit the rapid cold-hardening response.

Survival value and economical importance. Under natural conditions, the capacity to cold-harden rapidly may be important for survival throughout the year (Lee et al. 1987). In temperate regions, insects may be exposed to daily temperature cycles of 20° to 30° C. The capacity to cold-harden rapidly may allow even nonoverwintering life stages to almost instantaneously enhance cold tolerance as they track diurnal changes in temperature. This capacity may be of particular importance in the spring and autumn, when ambient temperatures may vary rapidly and without warning.

Mechanism: glycerol and heat-shock proteins. What is the underlying mechanism that results in the rapid cold-hardening response? One line of evidence suggests that a cryoprotective agent, glycerol, may be involved. Within two hours of transfer to 0° C, glycerol titers of larvae and pharate adults of the flesh fly are elevated two- to three-fold (Chen et al. 1987). This interpretation is consistent with glycogen phosphorylase being di-

rectly activated by exposure to cold in phylogenetically diverse insects (Lee et al. 1987). This enzyme catalyzes the breakdown of glycogen in the insect fat body, resulting in the production of glycerol. Quinn (1985) has suggested that cryoprotective agents such as glycerol may stabilize relationships between bilayer- and nonbilayer-forming lipids during rapid cooling. Thus, low-molecular-weight polyols may also play a cryoprotective role in preventing injury due to cold shock.

Another hypothesis suggests that rapid cold-hardening is related to an apparently universal biological response, known as heat shock. The heat-shock response is a cellular response to environmental stress, characterized by the synthesis of a specific set of proteins, the heat-shock proteins (Burdon 1986, Petersen and Mitchell 1985). Although the specific mechanism is unknown, heat-shock proteins are correlated with enhanced thermotolerance. This response is induced not only by exposure to sublethal heat treatments, but also by anoxia, transition-series metals, ethanol, teratogens, and some other chemicals. Due to the wide range of environmental inducers, heat-shock proteins are sometimes referred to as stress proteins.

Striking similarities exist between the heat-shock response and the rapid cold-hardening response against cold-shock injury. Both occur in less than an hour. Each provides protection against a form of temperature stress. Both responses are induced by exposure to specific temperature triggers, slightly below the upper lethal temperature for heat shock or slightly above the lower lethal temperature for rapid cold-hardening. The strongest tie is that protection against heat stress confers protection against chilling injury and vice versa. In the flesh fly, *S. crassipalpis,* transfer from 25° to 36° C for a short period increased survival at −10° C (Chen et al. 1987). Conversely, the production of heat-shock proteins has been linked to recovery from chilling in *Drosophila* (Burton et al. 1988).

Conclusions

The nature of insect low-temperature tolerance is clearly complex, and this complexity is further compounded by the great diversity of insects and the varying environments they inhabit. Furture studies must not only consider strategies of freeze-tolerance versus intolerance, but also cold shock and other forms of injury that occur at low temperature in the absence of tissue freezing. In the last decade, in-depth research efforts have begun to focus on a few select insect species and to search for mechanistic, as opposed to correlative, explanations of cold-hardiness at biochemical and physiological levels. Future advances in understanding are expected from tissue culture and molecular technology, as well as from NMR, differential scanning calorimetry, and cryomicroscopy.

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