Temperature Sensitivity in Insects and Application in Integrated Pest Management

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Low temperatures pose a different set of challenges than those observed at high temperatures, albeit challenges every bit as formidable. While insects at high temperature are constantly threatened by high rates of water loss, at sub-zero temperatures insects are confronted with the obvious challenge of potential ice formation. To a small-bodied poikilotherm composed of roughly 70% water, management of body water becomes a critical issue at low temperatures. How can freezing be avoided or how can the body survive in a frozen state? And, numerous additional challenges to cell integrity and tissue function become evident as body temperature is lowered, even at temperatures well above 0°C.

Defense against low temperature injury is evident at several levels. For a few insects, such as the monarch butterfly, the low temperatures of winter in North America are simply avoided by migration to a more moderate clime in the mountains of subtropical Mexico or southern California. But, for most insects, such an escape is not an option. Yet, the first line of defense, even for insects remaining in cold regions during the winter, is a behavioral response, a response that directs the overwintering insect to a thermally-buffered microenvironment. In addition to the selection of a thermally-favorable environment, insects can invoke an impressive array of physiologic mechanisms to prevent injury at low temperatures.

Insects can also exploit low temperatures for their own benefit. Bumble bees carrying a heavy load of conopid parasitoids stay away from the colony on cool nights and expose themselves to low temperatures, thus retarding development of the troublesome parasitoids and reducing the chances of successful parasitoid development (Müller & Schmid-Hempel 1993). And, on a more regular basis, the cold temperatures prevailing in winter likely enable
many insects to escape pathogens that escalate in abundance during the favorable seasons of the year.

In this chapter we offer a brief overview of the injury inflicted by low temperature and the mechanisms used by insects to circumvent such injury. Protective mechanisms that might be disarmed to render the insect more vulnerable to low temperature injury are of particular interest in developing new strategies of insect pest management. Many aspects of low temperature responses were discussed previously in Lee & Denlinger (1991) and Leather et al. (1993). Diapause, a form of developmental arrest in common use by many overwintering insects, has been reviewed in considerable detail (Saunders 1982, Denlinger 1985, Tauber et al. 1986, Danks 1987, Zaslavski 1988).

Supercooling and Ice Nucleation

To understand the fundamental strategies of insects that overwinter at sub-zero temperatures it is necessary to consider the nature of supercooling and ice nucleation. These concepts have been treated extensively elsewhere (Angell 1982, Mazur 1984, Franks 1985, 1987, Karow 1991, Lee et al. 1991, 1993a, Vali 1995).

As an insect is cooled to sub-zero temperatures ice does not form at 0°C, indeed it cannot form until the temperature falls below the melting point of the insect’s body fluids (Fig. 3.1). For insects that have high concentrations of low-molecular-mass cryoprotectants the melting point of the blood may be colligatively depressed by many degrees. The beetle Pytho deplanatus, a species that dehydrates extensively during the winter, has a melting point of −20°C (Ring 1982).

Insects only begin to supercool when they are cooled to temperatures below their melting point (Fig. 3.1). Small volumes of water supercool more readily than larger ones (Angell 1982, Vali 1995). Consequently, the small size of insects has allowed them to exploit this physical characteristic of supercooling in their overwintering strategies, whereas larger ectotherms such as amphibians and reptiles cannot (Costanzo & Lee 1995, 1996). The limit of supercooling, termed the supercooling point or temperature of crystallization, is reached when ice begins to form within the body fluids. This limit is easily detected by the appearance of the exotherm caused by the release of the heat of crystallization as body water freezes.

The supercooling point is generally regulated by the presence of endogenous, non-water ice nucleating agents (Lee 1991, Lee et al. 1996).

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**FIGURE 3.1** Responses of insects cooled to low temperature. The bold line indicates insect body temperature in relation to the melting point of body fluids, the supercooling point and the temperature at which internal ice forms. The right side of the figure indicates the general temperature ranges for different categories of insect response to low temperature. From Lee (1989).
These agents function as catalysts to promote ice nucleation at higher temperatures than would occur in their absence. Some freezing tolerant species synthesize proteins or lipoproteins that induce ice nucleation in the range of -6 to -9°C (Zachariassen 1992, Duman et al. 1995). The induction of ice nucleation at high sub-zero temperatures promotes survival of freezing by slowing the rate of ice formation compared to that which would occur if the insect supercooled extensively before freezing began. Mognano et al. (1996) recently described a new class of insect nucleators in the form of endogenous crystals of calcium phosphate within the Malpighian tubules of the freeze tolerant fly larvae, Eurosta solidaginis. As discussed extensively in Chapter 4 recent reports of ice nucleating microorganisms isolated from insects identify yet another category of endogenous ice nucleators.

Classification of Insect Cold Hardiness

Investigations of cold tolerance typically place a given insect into one of two categories: the relatively few species that are freezing tolerant survive extensive internal ice formation, while freezing susceptible or intolerant insects succumb when their hemolymph freezes (Fig. 3.1). While these categories are useful in general discussions of insect cold-hardiness, they are simplistic when trying to describe the wide diversity of insect responses to low temperature (Baust & Rojas 1985, Lee 1991, Turnock & Bodnaryk 1991, Bale 1993). For example, some insects do not survive exposure to temperatures above 0°C. Others that supercool extensively and do not freeze until temperatures drop below -20°C may die at temperatures significantly above their supercooling points. Non-diapause pupae of the flesh fly Sarcophaga crassipalpis supercool to -23°C but do not survive exposure to -17°C for even 20 min (Lee & Denlinger 1985). In contrast, diapausia pupae readily survive chilling at all temperatures above the supercooling point. Chilling-induced injuries may also be expressed as a consequence of long-term exposure (weeks or months) to low temperature. Turnock et al. (1985) reported reduced survival to eclosion in Delia radicum, a species with a supercooling point of -23°C, when it was held continuously at -10.2°C for 80 days.

Determining the type and level of cold tolerance for a given insect empirically is difficult for a number of reasons (Zachariassen 1985, Baust & Rojas 1985, Bale 1987, Block 1991, Lee 1991). Biological factors that must be considered include the developmental stage, diapause status and age, as well as the acclimation status of the species. Survival depends on both the exposure temperature and the duration of exposure. Spontaneous ice nucleation in the supercooled body fluids of an insect has a stochastic component (Salt 1961). For example, if a freeze intolerant insect is held for 1 h at -20°C it might remain unfrozen and survive, however if the duration of exposure is extended to 24 h ice nucleation may occur and death results. To assess freezing tolerance the duration of exposure should be sufficient to allow an equilibrium level of internal ice formation to be attained; in the freeze tolerant gall fly E. solidaginis 2-3 days at -23°C are required to reach this level (Lee & Lewis 1985). Cooling and warming rates have also been shown to critically influence survival (Miller 1978). Lastly, the criteria for survival following low temperature exposure must be biologically meaningful; the ability of an insect to wiggle or walk does not mean that it can survive to the next developmental stage, and that does not mean that the individual can ultimately reproduce and leave viable offspring - the best criterion for assessing cold tolerance.

Developmental Alterations Caused by Low Temperature

Mortality and failure to reproduce are not the only consequences of low temperature exposure. Low temperature also influences several aspects of development, including adult size, the number of larval instars, tissue morphogenesis, and sex ratio (Schnal 1991).

As an insect larva grows, it normally progresses through a fixed number of instars. During its final larval instar, the larva attains a critical size that sets in motion the endocrine events that trigger metamorphosis (Nijhout & Williams 1974). The critical size may be reached very early in the final larval instar [e.g., Sarcophaga bullata (Zdarek & Slamka 1972)], or midway through the instar [e.g., Glossina morsitans (Denlinger & Zdarek 1991)]. Once the critical size has been reached, the larva is competent to initiate metamorphosis. For an insect such as S. bullata, which reaches its critical size early in the final larval instar, feeding can be halted long before the larva reaches its maximum size and it can still successfully proceed with metamorphosis. But, for larvae of G. morsitans, the critical size and maximum size are nearly the same: larvae removed from the food source prematurely fail to initiate metamorphosis. Since the final larval instar is frequently the longest instar and the period in which the most food is consumed, it is this instar that exerts a disproportionate influence on adult size. Adult size can thus be most readily influenced in species that attain their critical size early in the final instar.
Low temperatures that intercede after the critical size is attained may interfere with feeding processes and thus prevent the insect from reaching its maximum size, but the insect may still be able to initiate metamorphosis. Such a scenario results in the production of a small adult. For the carpet beetle, *Attagenus megatoma*, rearing at 20°C yields an adult that is only half the size of adults reared at 30-35°C (Baker 1983). Less time and energy are required to produce a small adult, and this may indeed be the basis for the small size characteristic of insects from alpine and polar environments (Danks 1981, Somme & Block 1991). But, lowering the rearing temperature does not always result in smaller individuals. Within the range of 16-25°C, females of *D. melanogaster* are larger when reared at the lower end of the range (David et al. 1983), and in this case, larger size implies higher fecundity (Robertson 1957).

Low temperature may also indirectly influence adult size by influencing the number of larval instars. Though this number is rigidly fixed in most species, the number of instars in some species can be altered in response to low temperature or other environmental stresses. The number of instars can either be decreased or increased by low temperature. While the moth *Ephesia kuehniella* reared at 25°C normally has 5 larval instars, it pupates at the end of the fourth larval instar at 18°C (Gierke 1932). In the wax moth, *Galleria mellonella*, a cold shock (0°C for 30 min) at the beginning of what is normally the final larval instar prompts the larva to molt into an additional larval instar rather than pupate (Cymborowski & Bogus 1976). A decrease in the number of instars results in smaller adults, while an increase in the number of instars usually produces larger adults.

The cold shock that induces supernumerary molts in *G. mellonella* somehow alters the response of the regulatory centers within the brain. As a consequence, allatropin is released at the wrong time (Cymborowski 1988), resulting in an elevated juvenile hormone titer (Sehnal & Rembold 1985), thus causing the subsequent molt to be a larval-larval molt rather than pupation.

Phenocopy defects, like those observed at high temperature (see Chapter 2), can also be elicited by low temperature, as demonstrated by the classic studies of Villee (1943, 1945) on temperature-sensitive homeotic mutants of *D. melanogaster*. In aristopedia (antennae are transformed into legs), low temperature rearing (15°C for several days after oviposition) shifts the direction of antennal development toward a tarsus, but formation of the normal antennal appendage (the arista) is favored at a higher temperature (29°C). In the homologous mutant, proboscipedia, exposure to the same low temperature regime causes the labial palps to be replaced with aristae, the antennal-like appendages. Interestingly, at higher temperatures (29°C) the labial palps are replaced with a tarsal-like appendage. The period during which the fly is susceptible to the effect of low temperature extends over several days and is most pronounced if the low temperature treatment is begun 5 days after oviposition. Thus, a much longer period of low temperature exposure is required to elicit phenocopy defects than is needed to elicit phenocopy defects at high temperature: while days of exposure are required at low temperature, only minutes are required at high temperature.

Different types of developmental defects can be elicited by low temperature at different developmental stages. Eggs of the chrysomelid beetle *Atractya menetriesi* exposed to low temperatures divide into multiple embryos (Miya & Kobayashi 1971), a condition that is lethal. Low temperature during postembryonic development may cause the production of individuals with a mixture of larval and adult features (Sehnal 1991). The yellow mealworm, *Tenebrio molitor*, is quite vulnerable to cold-induced developmental aberrations (Lengerken 1932, Stellwaag-Kittler 1954). Cold treatment of last instar larvae can cause a molt that will produce a larva-like individual, but one that possesses rudimentary pupal-like eyes and appendages. Similar effect can be achieved by administration of juvenile hormone to final instar larvae (Sehnal & Schneiderman 1973), thus suggesting that the cold treatments elicit this developmental response by boosting the juvenile hormone titer at a time when the hormone should be absent or present only at low levels.

As noted with high temperature (Chapter 2), low temperatures frequently distort sex ratios (Lauge 1985, Wrensch 1993). Males of the psychid moth *Talaeporia tubulosa* are produced by eggs containing two sex chromosomes (XX), and females develop from eggs having only a single sex chromosome (XO). In the optimal temperature range females produce a nearly equal proportion of oocytes with and without the X chromosome, but when the female is reared at 3-5°C, the X chromosome is displaced to the polar body, and consequently most of the resulting progeny are females (Seiler 1920). In many Hymenoptera, fertilization is controlled by the female, and eggs that are not fertilized develop into females. In the chalcid *Ooencyrtus* low temperature during development favors the production of nonfertilized (female) eggs (Wilson & Woolcock 1960). A shift toward production of a higher proportion of males is also well documented in response to low temperature. A distinct, but slight, increase in male production in response to low temperature was noted for the citrus red mite, *Panonychus citri* (Munger 1963) and the ichneumonid parasitoid *Campoplexis perdistinclus* (Hoelscher & Vinson 1971).
In the ant *Formica rufa* the spermatheca fails to release sperm at temperatures below 19.5 °C, thus only unfertilized eggs, in this case males, are produced (Gösswald & Bier 1955).

A shift in the autumn from parthenogenesis to sexual reproduction is common in thrips, aphids, cynipids, and many species of mites. Low temperature, in association with short daylength, frequently provides the cue triggering this shift to production of both males and females (Hardie & Lees 1985). In the autumn aphids also shift from apterous development to the formation of alates, a change that is again promoted by low temperature acting in concert with short daylength.

**Diapause and Cold Tolerance**

Most insects in the temperate zone are subjected to the lowest temperatures when they are in an overwintering diapause. The suppression of metabolism and purging of the gut (elimination of ice nucleators in the gut) are among characteristic features of diapause that can contribute to cold tolerance, yet diapause and cold hardiness are not consistently linked (Denlinger 1991). Diapause, by itself, does not necessarily imply cold hardiness, nor does cold hardiness imply that the insect is in diapause.

Diapause is not restricted to insects from temperate and polar regions, and it is not always limited to the winter season. Diapause is well documented in the tropics (Denlinger 1986) and can be expressed during the summer in temperate zones (Masaki 1980). In these situations diapause occurs in the apparent absence of cold hardening (Fig. 3.2). Metabolic suppression may result in altered carbohydrate metabolism in such cases (Pullin 1996), a feature often associated with increased cold hardening, but thus far no evidence is available demonstrating enhanced cold hardiness associated with either tropical or summer diapause.

At the opposite extreme, cold hardiness can readily be demonstrated in the absence of diapause. Examples include the development of cold hardiness in species that lack the capacity for diapause [e.g. *Tenebrio molitor* (Patterson & Duman 1978)], cold hardening in nondiapausing stages of insects that do enter diapause [e.g. cold hardening in adults of the flesh fly *Sarcophaga bullata*, a species that diapauses as a pupa (Chen et al. 1987b)], and rapid cold hardening, the hardening response that can occur at any developmental stage within a few minutes of exposure to an intermediately low temperature (Chen et al. 1987a, Lee et al. 1987).

**FIGURE 3.2** Relationship between diapause and cold hardness. The two events may be expressed independently or in association with each other. When associated, the relationship may be coincidental or linked. Adapted from Denlinger (1991).

But, quite frequently diapause and cold hardiness are associated. This association can have two forms: diapause and cold hardiness may be only coincidentally associated or cold hardiness may actually be a component of the diapause program. The relationship is considered to be coincidental if separate environmental cues regulate diapause and cold hardiness. This is the case, for example, in the European corn borer, *Ostrinia nubilalis* (Hanec & Beck 1960): the larva enters diapause in response to short daylength but it becomes cold hardy only after it is exposed to low temperature. Separate environmental cues thus dictate these two events, and a corn borer can be in diapause without being cold hardy. For corn borers in the field, however, the expression of diapause and cold hardiness normally closely coincide.

By contrast, a firm linkage between diapause and cold hardiness is exemplified in the flesh flies *S. bullata* and *S. crassipalpis* (Adedokun & Denlinger 1984, Lee & Denlinger 1985). In these flies cold hardiness is a component of the diapause program. Flies that enter pupal diapause are already much more cold hardy than nondiapausing pupae. Entry into diapause
is consistently linked to enhanced cold hardness. A separate set of environmental cues is not needed to initiate the cold hardening process. Of course, low temperature may further enhance the cold hardening, but even without exposure to low temperature, the pupae are cold hardened.

Whether the cold hardiness is associated with diapause coincidentally or is linked to the diapause program, it is during diapause that most insects exhibit the greatest cold hardness. For example, in *S. crassipalpis*, only diapausing pupae can survive at temperatures approaching its supercooling point (−23°C), and they can do so for many days (Lee & Denlinger 1985). Though the supercooling point is equally low in nondiapausing pupae, such pupae are readily killed following exposure to −10°C for less than an hour. In addition to the biochemical adaptations that contribute to cold hardiness during diapause, many diapausing species take refuge in thermally-buffered sites during diapause, and quite frequently they prepare special cocoons, hibernacula or other structures in which to overwinter (Danks 1991). This combination of developmental arrest, enhanced cold hardiness, selection and/or construction of a protected site results in an increased challenge when targeting diapausing individuals for thermal wounding.

**Variation in Tolerance**

A few classic examples illustrate the huge variation in cold tolerance that is evident among different species of insects and other arthropods. Certain species not only survive but remain active at temperatures near 0°C or lower. Snow fleas (Collembola) can be seen freely hopping over the surfaces of glaciers and snow fields at high altitudes. Likewise, gryllloblattids are most active at temperatures near 0°C and will succumb when temperatures exceed 12°C (Morrissey & Edwards 1979). An antarctic mite *Nanorchestes antarcticus* remains active down to −11°C (Sømme & Block 1991), and a midge living in glacial pools in the mountains of Himalaya remains active at temperatures as low as −16°C (Kohshima 1984). Winter active moths (several species of noctuids and geometrids) continue to fly even when air temperatures drop as low as 0 to 10°C. Though the thoracic temperature of the moths during flight (30-35°C) is similar to flight temperature of other moths, the extraordinary feature of the winter moths is their ability to initiate the shivering needed for preflight warm-up at temperatures as low as 0°C (Heinrich 1987).

Variation in cold tolerance within a single population is evident from the success of genetic selection experiments. Tucic (1979) succeeded in selecting for greater cold tolerance in *Drosophila melanogaster*. By selecting for increased cold tolerance in one particular stage, he was able to increase cold hardness in other stages as well, but the effect was diminished in stages more distant from the selected stage. In experiments by Chen & Walker (1994), separate lines of *D. melanogaster* were selected for greater tolerance against cold shock injury and long-term chilling injury. The cold-shocked line increased tolerance to cold shock, and the line selected for tolerance to long-term chill injury increased tolerance to long-term chilling injury. But interestingly, the increased tolerance to cold shock injury did not result in increased tolerance to long-term chilling injury, thus suggesting that these two forms of cold tolerance rely on distinct mechanisms.

Geographic variation is also evident. Though tropical species of flesh flies (Chen et al. 1990) and *Drosophila* (Hoffmann & Watson 1993) have some capacity for acclimating to low temperatures, the tropical species tend to be less cold tolerant than their temperate zone relatives, and even within the temperate region, populations at lower latitudes are less cold tolerant than those from higher latitudes (e.g., *Eurosta solidaginis* [Baust & Lee 1981, Lee et al. 1995]). Ample evidence suggests a genetic basis for such differences in cold tolerance. A cold hardy species, *D. lutescens*, crossed with a closely related species that is less cold hardy, *D. takahashii*, yields progeny with an intermediate level of cold hardness (Kimura 1982). Variation of cold hardiness that is inherent in a natural population can provide the grist for selecting strains of insects with increased cold tolerance, a feature that can be especially important for enhancing survival of predatory and parasitic species introduced into colder regions for biological control. Such naturally occurring variation, of course, also provides the capacity for pest species to expand their ranges into colder regions.

Crosses between selected lines of *D. melanogaster* suggest that the elements controlling cold hardiness in this species are dispersed over all chromosomes, but chromosome 2 makes the major contribution to cold hardiness in eggs and pupae, while chromosome 3 contributes most to cold hardness in larval and adult stages (Tucic 1979). Crosses between *D. takahashii* and *D. lutescens* suggest that the genes regulating cold hardiness are located on autosomes (Kimura 1982). One of the actetylcholinesterase mutants of *D. melanogaster*, Ace<sub>295</sub>, is a conditional mutation that is lethal if the fly is reared at temperatures below 20°C (Greenspan et al. 1980). A simple point mutation that replaces a single serine with a proline is responsible for this effect (Mutero et al. 1994). The mutation alters the secretion rate of
acetylcholinesterase, most likely by affecting its folding. This problem is exacerbated by low temperature and results in secretion of an insufficient amount of acetylcholinesterase.

Within the life of a single individual the capacity for cold tolerance also differs from one developmental stage to another. Among nondiapause individuals of *Sarcophaga crassipalpis*, the stage most tolerant of a cold shock at -10°C is the pupa, followed by pharate adult > adult > larva (Chen et al. 1991b). Interestingly, the stages most tolerant of high temperature stress are also the pupa and pharate adult. The most dramatic developmental differences, however, are associated with diapause. Diapausing pupae of *S. crassipalpis* survive for months at temperatures as low as -20°C (just above their supercooling point), while nondiapausing pupae and other developmental stages are killed by brief exposures to temperatures of -10°C or higher (Adedokun & Denlinger 1984, Lee & Denlinger 1985). Different melanic forms of the same developmental stage of the same species may also have different properties. Body color contributes to body temperature and the rate of warming. Radiant heat is more quickly absorbed by a dark body than by a light body, as illustrated in Fig. 3.3 by the more rapid rate of warming in a melanic form of the ladybird beetle *Adalia bipunctata* than in the non-melanic form of the same species (De Jong et al. 1996). Appreciating the profound differences in cold tolerance associated with different developmental stages and forms is, of course, critical for the design of pest management strategies that exploit low temperature.

**Causes of Low Temperature Injury**

Many insects do not survive chilling and die due to various forms of non-freezing injury. Although the actual mechanisms responsible for this form of injury remain largely unknown, information from cryobiological investigations using primarily microbial and mammalian cell models provides useful clues. Direct effects of chilling include decreases in the rate of enzymatic activity as well as changes in tertiary structure of proteins and disassembly of polypeptide subunits causing protein denaturation that may be irreversible upon warming (Morris & Clarke 1987). Low temperature induced depolymerization of cytoplasmic microtubules is frequently reported, however this phenomenon has received little attention with respect to insect cold-hardiness.

Nonfreezing injury due to low temperature exposure is frequently associated with damage to the plasma membrane (Steponkus 1984, Drobnis et al. 1993, Hazel 1995). At some point chilling induces fluid to gel phase transitions in cell membranes that result in major alterations in membrane permeability, reduction in the activity of membrane bound enzymes, and separation of membrane proteins and lipids into distinct domains that remain even after warming (Quinn 1985, Hazel 1995). Again, few investigations have explored the significance of these effects in insects despite the fact that these membrane related effects have received considerable attention in microorganisms, plants, and lower vertebrates.

To appreciate the nature of freezing injury it is first necessary to consider the dynamics of ice nucleation and freezing within the insect. It is commonly held that survival of freezing at temperatures naturally experienced requires that ice formation be restricted to the extracellular spaces (but see reports
describing survival of intracellular freezing in fat body cells by Salt 1959, 1962, Lee et al. 1993b). Initially ice nucleation occurs outside the cells, sometimes seeded by ice nucleating proteins or other nucleators. Because only water molecules can join the growing ice lattice, dissolved solute in the remaining unfrozen body fluids becomes concentrated. This freeze concentration of extracellular fluids causes the osmotic removal of cellular water. As more ice forms, more water leaves the cells.

Although mechanical injury due to internal ice formation can be a deleterious consequence of freezing, excessive concentration of body fluids and cellular dehydration are believed to be the primary stresses (Mazur 1984, Karow 1991). Freeze-concentration may elevate the levels of specific solutes, particularly electrolytes, to the point where they cause protein denaturation and extreme changes in pH. Excessive increases in the osmotic pressure of body fluids may also cause injury. The critical minimum cell volume hypothesis attributes freezing injury to excessive cellular shrinkage that damages the membrane to the point where it is unable to recover upon thawing (Meryman 1974).

Reports from the plant literature suggest that chilling injury may be attributed to oxidative stress (Jahnke et al. 1991, Walker & McKensie 1993, Prasad et al. 1994). Injury to the mitochondrial membrane and the proteins involved in electron transport could result in generation of free radicals and other prooxidants. Rojas and Leopold (1996) present intriguing evidence that a similar scenario may be operating in insects. In the house flies they examined, the most cold resistant stages, the pupa and pharate adult, have the highest activity of superoxide dismutase, the scavenging enzyme that represents the first line of defense against oxygen free radicals. Furthermore, they demonstrated elevation of superoxide dismutase activity in response to chilling. Superoxide dismutase converts oxygen free radicals into hydroxyl radicals and hydrogen peroxide, products that are then rendered less toxic to the cell by the action of glutathione. In house flies, the level of this important tripeptide, glutathione, declines during cold storage, further suggesting that oxidative stress may contribute to chilling injury.

Certain systems are more vulnerable to low temperature injury than others. The neuromuscular system appears to be particularly vulnerable. As temperatures decline, insects gradually lose their ability to fly and at slightly lower temperatures they lose their ability to walk. Chill coma, the point at which the insect loses its ability to walk, coincides with the temperature at which the muscles and nerves lose their electrical excitability (Goller & Esch 1990, Xu & Robertson 1994). This point is reached at 12.8°C in honey bee drones, at 10.6°C in honey bee workers, and at 7°C in adults of D. melanogaster (Hosler & Esch 1998). As temperatures drop toward the onset of chill coma several features of the muscle potential change. As shown in the example of honey bee queens (Fig. 3.4), the resting potential of the muscle membrane gradually decreases, amplitude of the muscle potential decreases and duration of the muscle potential increases (Hosler et al. 1998). A final burst of muscle potentials is observed just as the insect enters chill coma. The gradual loss of electrical activity is presumed to result from the loss in function of the ion channels needed to maintain the ionic balance essential for generating the potential difference across the membrane.

While the problems associated with brief periods of chill coma are readily reversible, more severe cold shock can produce nonreversible injury to the neuromuscular system. Flesh flies cold shocked as pharate adults continue to develop, but if the injury is sufficiently severe, adult flies fail to escape from the puparium (Yocum et al. 1994). Tensometric measurements of ecdision behavior demonstrate that the first signs of injury are reflected in an alteration of the contraction patterns (Fig. 3.5), rather than the intensity of the muscular contractions. This response is in contrast to the impairment observed at high temperature (Chapter 2, Fig. 2.4). With heat shock the patterns of the

![Figure 3.4](image-url) Temperature effects on the resting potentials and the amplitude and duration of the muscle potentials in thoracic muscles of queen honey bees, Apis mellifera. From Hosler et al. (1998).
contractions remained intact long after the intensity of the contractions was diminished. Though both heat shock and cold shock prevent eclosion, the nature of the injury differs. This suggests that the fly is more susceptible to CNS impairment at low temperatures and more susceptible to muscle injury at high temperatures. The circadian gate regulating the precise timing of eclosion within the daily light:dark cycle was altered by heat shock, delayed from dawn to mid-photophase, but no such alterations were observed by cold shock.

The proboscis extension bioassay was also used to evaluate neuromuscular injury in *S. crassipalpis* (Kelty et al. 1996). Adult flies that had been cold shocked as pharate adults fail to extend their proboscis in response to sucrose solutions and fail to groom properly. Cold shock decreases the resting membrane potential in leg muscle fibers and the conductance velocities of the motor neurons innervating the leg muscle. In addition, neuromuscular transmission is impaired as indicated by a lack of evoked end plate potentials (Fig. 3.6). Most likely all of these effects on the neuromuscular system can be traced to disruption in the integrity of the cell membrane, as discussed above.

The reproductive system may be even more vulnerable to low temperature injury. Flesh flies that have been cold shocked as pharate adults may successfully escape from the puparium, feed, mate, but still not reproduce normally. While cold shock injury is less dramatic than heat shock injury on the reproductive processes some impairment is still evident in both males and females: fewer eggs are produced and the fertility rate is lower (our unpublished results). In the house fly, *Musca domestica*, females cold shocked as pharate adults produced fewer eggs during their adult life than

**FIGURE 3.5** (Opposite page) Representative tensiometric records of ptitinum movements of eclosing adults of the flesh fly, *Sarcophaga crassipalpis*, that were held at (A) 25°C or were either cold shocked at -10°C for (B) 45 min, (C) 60 min, or (D) 75 min or (E) exposed to 0°C for 10 days as pharate adults. The time scale indicates 10 s intervals; vertical bars indicate a 0.1 mm displacement of the tensiometric sensor. POR, program for obstacle removal, a stereotypic behavior program used for removal of the cap of the puparium and for the removal of obstacles; PFM, program for forward movement, a stereotypic behavior program used to move forward when unobstructed by obstacles. In the least severe cold shock (B), the intensity of the muscular contractions remained strong, but the centrally generated patterns were altered, and the pattern alteration became more pronounced with cold shocks of longer durations (C and D). With long-term chilling (E), the patterns remained intact but the intensity of the muscular contractions decreased. From Yocum et al. (1994).
controls that were not cold shocked (Coulson & Bale 1992). Reduced lifetime fecundity was a result both of the female’s shorter life span and a reduction in the number of eggs she produced each day. In addition, viability of the eggs produced by the cold shocked females was lower. Similar reductions in fertility caused by cold injury have been reported for other insects including the aphids Sitobion avenae (Parish & Bale 1993) and Rhopalosiphum padi (Hutchinson & Bale 1994) and the lacewing Chrysoperla carnea (Chang et al. 1996).

Cold Hardening

The injury caused by low temperature can frequently be mitigated by prior exposure to less severe low temperatures. Like the acquisition of thermotolerance at high temperature (Chapter 2), cold hardening enables an insect to survive at low temperatures that would otherwise prove lethal. Cold hardening can be either a long term process attained after weeks or months at a low temperature or a very rapid process invoked within minutes or hours after exposure to low temperature.

The traditional view of cold hardening depicts a slow process that gradually increases the insect’s low temperature tolerance. This slow acquisition of low temperature tolerance appears to be common in field populations of insects. As temperatures gradually drop in the autumn, overwintering stages become progressively more cold hardy. For example, diapausing pupae of Sarcophaga bullata reared outside in central Ohio are not nearly as tolerant of an exposure to -17°C in September or October as they are from November to February (Chen et al. 1991a), and larvae of the goldenrod gall fly, Eurosta solidaginis, cannot tolerate -40°C in September or October, but do so in late autumn and winter (Baust & Nishino 1991). For diapausing insects this increase in cold hardiness may be in direct response to low temperature cues, as it is in the European corn borer, Ostrinia nubilalis (Hanec & Beck 1960), or it may simply increase with time at a constant temperature, as it does in S. crassipalpis (Lee et al. 1987b). Just as cold hardening in a diapausing insect increases gradually over time, it can also gradually decrease over an extended period of time. At the onset of development, a drop in cold hardness is quite striking. A rapid loss in cold hardness is usually noted within a few days, but a more subtle decline in cold hardness is frequently apparent toward the end of diapause, long before the termination of diapause is apparent. Diapausing pupae of S. crassipalpis gradually become less tolerant of -17°C during the 3-4 weeks before they initiate adult development (Lee et al. 1987b). This cold hardening process is thus characterized by both a slow acquisition and a slow decay of tolerance.

Rapid cold hardening, as the name implies, is in marked contrast to the slow, gradual attainment of increased cold tolerance. In this case, the hardening process occurs very fast and enables the insect to quickly respond to low temperature conditions. For example, in S. crassipalpis, pharate adults reared at 25°C cannot survive direct exposure to -10°C, but if they are first exposed to 0°C for 10 min or more, they readily survive a 2 h challenge at -10°C (Fig. 3.7). Rapid cold hardening prevents the neuromuscular damage inflicted by cold shock (Yocum et al. 1994, Kelty et al. 1997). This is the type of response that presumably enables an insect to track daily temperature changes and respond quickly to a drop in temperature. It is not a response restricted to any single developmental stage. Though rapid cold hardening was

![FIGURE 3.6 The effects of cold shock and rapid cold hardening on conduction velocities of the three motor axons (slow, medium, fast) innervating the tergotrochanteral muscle of Sarcophaga crassipalpis. For each motor neuron, cold shock was associated with a significant decrease in mean conduction velocity, a decrease which was prevented by rapid cold hardening. From Kelty et al. (1996).](image-url)
intermittent pulses was observed with *Musca domestica*, *Phaenicia sericata*, and *Lucilia cuprina* stored at 10°C and given periodic pulses of 25-28°C (R. A. Leopold & R. R. Rojas, unpublished observation, see Chapter 9). These results suggest the potential for using intermittent pulses of high temperature to sustain low temperature tolerance, a feature that may be especially valuable for maintaining stocks of insects in cold storage. The results also suggest that the natural pattern of temperature cycling may very well play an important role in maintaining low temperature tolerance. But, in contrast, interruption of low temperature exposure (−10 to −15°C) by a 14-day exposure to 2°C did not enhance survival in diapausing pupae of the cabbage root fly, *Delta radicum* (Turnock et al. 1985), nor did interruption of −10°C exposure with periods at 0 or −5°C prevent injury in the bertha armyworm, *Mamestra configurata* (Turnock et al. 1983). Post-stress temperatures, however, play a critical role in survival of diapausing pupae of *M. configurata*: pupae that were cold stressed for 3 days at −14.5°C survived much better at 0°C if they were briefly (1-24h) exposed to 20°C before being held at 0°C (Turnock & Bodnaryk 1993). Survival of cold-stressed pupae at 0°C was much lower for those not given a 20°C pulse. These examples suggest that intermittent pulses as well as post-stress pulses of a higher temperature may be important for both prevention of and recovery from low temperature injury. The precise conditions needed to generate or extend protection are likely to vary considerably with species and developmental stage.

**Mechanisms of Cold Hardening**

**Removal of Ice Nucleators**

A key cold hardening mechanism for freezing intolerant species is the removal of efficient internal ice nucleators that would otherwise limit the insect’s capacity to supercool. When insects empty their gut during the autumn their capacity to supercool frequently is markedly enhanced (Chapter 4, Cannon & Block 1988). This result suggests that gut contents harbor efficient ice nucleating agents, however in most cases the actual ice nucleating agent has not been identified. A number of freezing intolerant insects have ice nucleating active bacteria as normal flora in the gut (Lee et al. 1991, 1993a). Presumably these bacteria must be removed from the gut or their ice nucleating activity reduced during the winter. Alternatively insects could select protected hibernacula in which environmental temperatures do not decrease below that of their supercooling point.
Water Loss

Not surprisingly, the water relations of insects have a fundamental bearing on cold hardening. Absolute reduction in body water, as has been reported for a variety of overwintering insects (Ring 1982, Zachariassen 1991), decreases the chance of mechanical injury as ice forms in tissues and increases the relative concentration of cryoprotectants by reducing solvent volume. Seasonal changes in the level of “bound” or unfreezeable water have been reported (Storey et al. 1981). The nature of this binding remains controversial (Franks 1985) but appears to be associated with interactions between macromolecules and other cellular components (Clegg 1987). Such binding would function to resist cellular water loss to the extracellular space during freezing.

Winter low temperatures are closely tied to the reduced capacity of atmospheric air to carry water vapor and generally lower relative humidities. A recent review emphasized links between cold hardening and resistance to desiccation (Ring & Danks 1994). For example, the accumulation of cryoprotectants in the hemolymph decreases the vapor pressure of supercooled fluids, thereby reducing the gradient promoting water loss to external ice within the hibernaculum (Lundheim & Zachariassen 1993). Although the scientific literature has generally focused on the role of cryoprotectants and water balance for survival at low temperatures, cryoprotective adaptations also confer increased resistance to desiccation stress. A link is also evident between desiccation and cold stress in Tenebrio molitor (Kroeker & Walker 1991). In this species, a 28 kDa hemolymph protein increases dramatically in response to desiccation stress, and interestingly, also in response to cold stress. How such a protein may function in response to desiccation and cold hardiness remains unknown.

Polyols, Sugars, and Amino Acids

A particularly notable adaptation of overwintering insects is their synthesis and accumulation of exceptionally high concentrations of low-molecular-mass polyols and sugars. Hemolymph levels of these cryoprotectants commonly reach several tenths molar to multimolar levels. Glycerol levels in a larval wasp reached 5M and comprised 25% of its body weight (Salt 1961). Other species, like gall fly larvae of E. solidaginis, produce several cryoprotectants (glycerol, sorbitol, trehalose) as they cold harden in preparation for winter (Baust & Lee 1981, Storey & Storey 1981).

These low-molecular-mass polyols and sugars confer increased cold tolerance in several ways. In species that must avoid ice formation in their body fluids the accumulation of cryoprotectants increases their capacity to supercool (Duman et al. 1995). For freezing tolerant species these compounds cause a marked colligative depression (1.86 °C per osmole) of the hemolymph melting point. This effect is significant because it reduces the amount of ice that can form at a given sub-zero temperature and consequently decreases cellular dehydration (Karow 1991). Cryoprotectants that penetrate the cell membrane reduce the severity of osmotic gradients generated as ice forms outside the cells and help to retain cytoplasmic water, thereby avoiding excessive cellular dehydration. Cryoprotectants also function to protect cells by stabilizing proteins and cell membranes during freezing and thawing (Carpenter & Crowe 1988, Crowe et al. 1990). However, the accumulation of cryoprotectants does not completely explain the nature of cold hardening at the cellular level. A recent study by Bennett & Lee (1997) using logistic regression modeling revealed that freeze tolerance of E. solidaginis fat body cells frozen in vivo is consistently greater than for cells frozen in vitro, even when cryoprotectants are added to the culture medium.

Hemolymph concentrations of certain free amino acids, most notably alanine and proline, are also frequently elevated in response to low temperature (e.g., Mansingh 1967, Morgan and Chippendale 1983, Fields et al. 1998). Such increases directly correlate with increases in cold tolerance, and it is likely that these free amino acids contribute to cryoprotection. Yet, the precise manner in which this is achieved has not been carefully examined.

Thermal Hysteresis Proteins

Thermal hysteresis refers to a difference between the freezing and melting points of the body fluid. At equilibrium one would expect the freezing and melting points to be nearly identical, but this relationship can be altered by thermal hysteresis proteins (THPs), also known as antifreeze proteins (Duman et al. 1993). THPs depress the freezing point of water by a non-colligative mechanism while leaving the melting point unchanged. In the presence of THPs the freezing point may be lowered 5-6 °C below the melting point (Fig. 3.8), thus considerably expanding the organism’s low temperature tolerance. THPs were first discovered in cold water, marine fish (DeVries 1971) but were found soon thereafter in a tenebroid beetle (Duman 1977) and are now known in numerous species of beetles and representatives of many of the lower orders of insects (Duman et al. 1993). THPs appear to be rare in Lepidoptera (Hew et al. 1983) and have not yet been found in Diptera or Hymenoptera.
THPs from several species have been purified and partially characterized (Duman et al. 1993). Molecular masses are in the 14-20 kDa range, and multiple forms of very similar THPs may be present in a single species. Unlike the THPs found in fish, none of the THPs thus far examined in insects contains a carbohydrate component. Maximum activity of the THPs, at least in the beetle *Dendroides canadensis*, is attained when they are bound to a 70 kDa protein in the hemolymph (Wu & Duman 1991).

Synthesis of THPs is a seasonal event. They are produced by the fat body in response to short daylength and low temperature of autumn, persist during the winter and then disappear in response to long daylength in the spring (Fig. 3.8). In larvae of both *D. canadensis* (Horwath & Duman 1983) and *Tenebrio molitor* (Xu et al. 1992) synthesis of THPs is prompted by topical application of juvenile hormone. Changes in the hemolymph titer of juvenile hormone activity are also consistent with the idea that the autumn increase in THPs is mediated, at least partially, by the juvenile hormones.

The utility of THPs for avoiding freezing may have several dimensions. A drop in the freezing point obviously enhances the insect's supercooling capacity, an effect that appears to be achieved by masking ice nucleators present in the hemolymph. In addition, THPs may function to inhibit inoculative freezing by associating with the epidermal cells and thus constructing a barrier to external ice. The presence of THPs in some freeze-tolerant species is a bit more puzzling. It is normally assumed to be advantageous for a freeze-tolerant species to freeze at a relatively high temperature, thus the presence of THPs in such species is unexpected. Yet, THPs are evident in the freeze-tolerant centipede *Lithobius forficatus* where they appear to play a role in protecting the cells from injury during freezing of the extracellular fluids (Tursman & Duman 1995).

**Ice Nucleator Proteins**

Ice nucleator proteins function in just the opposite manner from thermal hysteresis proteins. Rather than inhibiting freezing, ice nucleator proteins promote freezing. Ice nucleator proteins facilitate the organization of water molecules into embryo crystals which, in turn, "seed" the supercooled solution, causing freezing at relatively high temperatures. As discussed above, elevation of the freezing temperature is advantageous for a freeze-tolerant species, and proteins with this property have now been identified in several insects (Duman et al. 1995).

The best characterized ice nucleator protein is a globular 800 kDa lipoprotein isolated from the hemolymph of the crane fly *Tipula trivittata* (Duman et al. 1985, Neven et al. 1989). This lipoprotein, consisting of 45% protein, 51% lipid, and 4% carbohydrate, contains two apolipoproteins. Unlike most insect lipophorins, this lipoprotein contains phosphatidylinositol, a component deemed essential for ice nucleating activity. An increase in concentration of the lipoprotein yields progressively higher nucleation temperatures, up to a maximum of 6°C at concentrations at or above 1.7 X 10^-7 M (Duman et al. 1992). This is possibly due to the fact that individual proteins appear to organize into chains (Yeung et al. 1991), a feature that may increase the availability of nucleation sites.

**Stress Proteins**

Synthesis of heat shock proteins is a well documented response to high temperature (Chapter 2). Some of the same proteins are synthesized in response to anoxia, heavy metals and other forms of metabolic stress, thus the term stress proteins more accurately captures the diversity of stresses that can stimulate their synthesis. More recently, cold shock was added to the list of

![Graph](image-url)
stressors capable of stimulating stress protein synthesis (Denlinger et al. 1991). Stress protein synthesis in response to cold shock has been documented in Drosophila melanogaster (Burton et al. 1988), Sarcophaga crassipalpis (Joplin et al. 1990), the gypsy moth, Lymantria dispar (Yocum et al. 1991, Denlinger et al. 1992), and several other insect species.

As with the heat shock response, the most prominent stress protein elicited by cold shock is a member of the heat shock 70 protein family. In *S. crassipalpis* the protein most highly expressed in response to both heat and cold shock is a 72 kDa protein, a protein recognized by an antibody to the 70 kDa heat shock cognate protein in *D. melanogaster* (Joplin et al. 1990). A 92 kDa protein is also synthesized by *S. crassipalpis* in response to both heat shock and cold shock. In addition, several potentially interesting proteins with molecular masses of 78, 45, and 23 kDa are synthesized in the integument, but not the brain, following cold shock. Such cold-shock specific proteins are likely to have special properties unique to the low temperature response. Differences in tissue responses also suggest the complexity inherent in the insect's adaptation to low temperature. The involvement of stress proteins in low temperature responses is not unique to insects. Spinach seedlings acclimated to 5°C also boosted synthesis of proteins in the 70 kDa heat shock family (Neven et al. 1992, Anderson et al. 1994), and like the flies, plants, and bacteria synthesize proteins that are unique to low temperature. One such protein found in *Escherichia coli* (Jones et al. 1987) and *Photobacterium* sp. (Clarke & Dowds 1994) is a polynucleotide phosphorylase.

Several aspects of stress protein synthesis in response to cold shock differ from the insect's response at high temperature. (1) Synthesis is observed during recovery rather than during the actual stress. A role in the events of recovery is likely. While this implies that stress proteins are unlikely contributors to rapid cold hardening, they may offer protection against subsequent low temperature injury. (2) Synthesis of the stress proteins is concurrent with normal protein synthesis. This is in contrast to the heat shock response. At high temperatures, synthesis of other proteins ceases while stress proteins are being produced. (3) Synthesis can persist for days rather than the brief (minutes or hours) interval of synthesis observed at high temperatures. The duration of the response is especially striking in diapausing pharate larvae of the gypsy moth (Yocum et al. 1991). In this case stress protein synthesis persists for at least 6 days after the cold shock.

The persistence of stress protein expression during gypsy moth diapause (Yocum et al. 1991) suggests a possible role in the cold hardening associated with diapause. In this species the diapausing pharate larvae become cold hardy only after they have been chilled, and it is this period of chilling that capacitates the gypsy moth to synthesize the stress proteins (Denlinger et al. 1992). Our unpublished results with flesh flies also indicate a persistent expression of certain stress proteins during pupal diapause. Both the gypsy moth and flesh flies are freeze intolerant species. The response differs in the goldenrood gall fly, *Eurosta solidaginis*, a freeze tolerant species. Though the gall fly readily synthesizes stress proteins in response to high temperature it does not do so when subjected to low temperature (Lee et al. 1995). Whether this represents a general trend distinguishing freeze-tolerant and freeze-intolerant species awaits validation from additional species.

The function of the stress proteins at low temperature remains unknown, but clearly several functions attributed to members of the 70 kDa heat shock protein family (Craig et al. 1993, Parsell & Lindquist 1993, Morimoto et al. 1994) could prove equally useful at low temperatures. Important enzymes are subject to denaturation at low temperature. Stress proteins could target denatured enzymes for elimination or serve to renature the enzymes. Roles in protein folding, assembly of oligomeric complexes, and chaperoning functions are all known functions for stress proteins and could very well contribute to maintaining cell function at low temperature.

**Vitrification**

Vitrification of body water is another possible mechanism of cold tolerance that may operate in insects. Vitrification refers to a physical state in which water becomes an amorphous solid or glass. Theoretically vitrification of body water avoids ice nucleation and growth of the ice lattice leading to mechanical injury. In woody plants high concentrations of sugars, particularly sucrose, raffinose, and stachyose, induce vitrification at temperatures as high as –20°C (Chen et al. 1995, Hirsh et al. 1985). Wasylyk et al. (1988) reported partial glass formation in a simulated hemolymph preparation and in intact larvae of *E. solidaginis*, and suggested that this vitrification may provide cryoprotection under natural conditions.

It is thus evident that cold hardening entails a complex suite of responses and can no longer be regarded as a process driven by a single biochemical event such as polyol synthesis. In addition to the mechanisms discussed above, such features as superoxide dismutase activity, glutathione concentrations, energy reserves, and other biochemical parameters are likely to be important contributors to cold hardening. Species differences are likely to dictate that one particular process may be more important in one species than
in another, but insects clearly have an array of responses at their disposal, and several mechanisms are likely to operate in any one species at the same time.

**Blockage of Cold Hardening**

A few studies have investigated ways to prevent or block protective mechanisms of cold hardening. One approach seeks to diminish the natural capacity of freezing intolerant insects to supercool by applying ice nucleating active bacteria and fungi (Fields 1993, Lee et al. 1993a, Chapter 4). These microorganisms are highly efficient ice nucleators that can markedly elevate the supercooling points of a variety of insects. Attempts are currently underway to develop methods using these ice nucleating microorganisms for the control of insect pests.

The capacity to rapidly cold harden is inhibited in *S. crassipalpis* by exposure to anoxic conditions (Yocum & Denlinger 1994). In this study pharate adults that were exposed to 0°C for 2 h prior to a 2-hour period at -10°C survived better than ones directly placed at -10°C. However, this rapid cold hardening at 0°C did not occur under anoxic conditions. This implies the rapid cold hardening that occurs at 0°C is an energy dependent process that can be blocked in the absence of oxygen. Suppressed oxidative metabolism can also prompt the synthesis of anaerobic by-products such as polyols (Wilhelm et al. 1961; Meyer 1980) and other compounds that may function as cryoprotectants. Exposure of the house fly to anoxia while it is within its normal temperature range will indeed stimulate cold hardening (Coulson & Bale 1991), but a similar treatment administered to the flesh fly was ineffective (Kukal et al. 1991). The impact of anoxia thus appears to vary, perhaps with species, developmental status or other experimental conditions. Further research is needed to understand the links between cold shock injury, rapid cold hardening and anoxia. Yet, two groups working with insect pests on cut flowers have effectively coupled exposure to low temperature and hypoxia for quarantine purposes (Seaton & Joyce 1993, Shelton et al. 1996). The advantage, of course, is that the coupling of a low temperature treatment with anoxia may permit the use of a less severe temperature, a feature that is likely to be both less costly as well as less damaging to the fruits or vegetables needing treatment.

Agents that could mask or otherwise incapacitate thermal hysteresis proteins, ice nucleator proteins, stress proteins, interfere with polyol production or other key biochemical processes, or disrupt behavioral responses associated with cold hardening have interesting potential application. The fact that bumblebees can be altered behaviorally by parasitoids to seek cold locations (Müller & Schmid-Hempel 1993) also suggests interesting possibilities for behavioral modification. For diapausing insects numerous tools can be exploited. Invariably the termination of overwintering diapause is associated with a pronounced loss of cold hardiness. By prematurely terminating diapause cold hardiness can also be prematurely lost, thus rendering the insect vulnerable to the low temperatures of winter. Although a diversity of hormonal mechanisms regulate insect diapause (Denlinger 1985), certain patterns are common: many cases of larval diapause can be terminated by a drop in the juvenile hormone titer and/or a pulse of ecystosteroids, pupal diapauses can usually be terminated with ecystosteroids, and most adult diapauses can be broken with juvenile hormone. In addition, diapause in a number of species can be broken with physical manipulations or chemical agents. For example, diapause in flesh flies can be broken by physically shaking the pupae or by exposing pupae to organic solvents such as hexane or ether (Denlinger et al. 1980). While the utility of such tools for breaking diapause have been well demonstrated in the laboratory, few attempts have been made thus far to control pest species with such manipulations.

**Future Directions**

Insects have a wealth of behavioral and physiological responses to counter the effects of low temperature, and if low temperature is to be used in an effective integrated pest management system, these mechanisms must either be overridden or disabled. The speed of the rapid cold hardening response can quickly subvert attempts to kill the insect if the transfer to low temperature is too gradual. The fact that most insects probably have at their disposal a complex suite of responses suggests a form of double assurance. The insect is not simply relying on a single mechanism for survival but instead involves a complex suite of responses. It is not at all clear how or whether such complex responses are linked. Are the responses somehow integrated through the expression of a master gene, or do distinct cues invoke different aspects of the response?

Overwintering mortality can be extremely high, presumably due both to the low temperatures experienced and to the length of time the insect must depend on energy reserves it has garnered prior to the onset of winter. Low temperatures that prevail during winter are frequently just a few degrees above
the insect's lower limit of tolerance. The low temperatures that already prevail during winter thus set the stage for manipulations that subject the insect to a lower temperature (e.g. destruction of its overwintering hibernaculum) or artificially elevate its lower limit of tolerance (e.g. elevation of the supercooling point).

Recent discoveries of ice nucleating bacteria and fungi, thermal hysteresis proteins, ice nucleator proteins, general stress proteins, and cold shock-specific proteins suggest that insects offer a rich source of material for pharmacological prospecting. The enormous diversity of insects suggests that many more such agents or compounds remain to be discovered. Molecular techniques make the small size of insects no longer an obstacle for isolation of interesting, new compounds. Recombinant DNA products that alter freezing or melting points, or offer protection against low temperature injury have potential commercial value as cryoprotective agents in the biomedical field and in agriculture as agents to increase cold tolerance in crops and for increasing the possibilities of cold storage. Transgenic cotton plants that overexpress the superoxide dismutase gene show increased cold tolerance (Allen 1995), and similar manipulations with superoxide dismutase genes or other genes associated with insect cold tolerance could have considerable utility for insects used in biological control or for long term storage of other species.

References


