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The wider integration of studies on insect cold-hardiness

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Abstract. Further progress in understanding insect cold-hardiness requires a broader perspective than hitherto. Such a broader approach is feasible, given the range of information now available on cold-hardiness and the variety and quality of equipment to study it. Comprehensive information on individual species is required, using the full range of available techniques, instead of piecemeal investigations. Comparisons of species must likewise be based on how their complete cold-hardiness strategies are structured, rather than on any particular contrasts between individual elements. Most importantly, a well based understanding of cold-hardiness requires wider linkages between cold-hardiness and other salient aspects of the life cycle, such as seasonal timing and control, water balance, and metabolism and energy budgets. Cold-hardiness is only one aspect of the adaptive trade-offs that structure the life cycle of any given species.

INTRODUCTION

Detailed information on insect cold-hardiness has accumulated rapidly in recent years, showing that cold-hardiness includes many complex adaptations and that no single or simple system is universal. As perspectives widened and the known diversity of elements increased, several authors challenged existing conventional wisdom. For example, cold-hardiness adaptations were recognized as more varied than previously thought, the meaning of the supercooling point was questioned especially given the influence of cooling rate and of subsequent temperatures on mortality, and the site of freezing was examined (Baust & Rojas, 1985). The occurrence of cold injury *above* the supercooling point was documented in freezing-susceptible species (Turnock et al., 1983; Lee & Denlinger 1985; Knight et al., 1986; Bale, 1987; Bale et al., 1988; Hutchinson & Bale, 1994).

Some authors realized that cold-hardiness should be studied in conjunction with related characteristics, such as winter habitats and ecology (Danks, 1978, 1991; Bale, 1987; Leather et al., 1993), growth and respiration (Block, 1994b), energy demands (Kukal, 1991; Danks et al., 1994), and other adaptations to cold environments (Danks, 1981; Sømme, 1989; Sømme & Block, 1991).

This paper points out that an even more holistic view is needed, considering cold-hardiness in the context of entire life cycles and of the trade-offs required by selection. In suggesting in this way that studies go one step further than hitherto, information on cold-hardiness is reviewed only relatively briefly because many detailed treatments are already available (e.g. Danks, 1978; Bale, 1989; Sømme, 1982; Zachariassen, 1985; Cannon & Block, 1988; Storey & Storey, 1988; Block, 1990; Duman et al., 1991a; chapters in Lee & Denlinger, 1991). Instead, this paper focusses on two main points. First, it emphasizes the many other elements contributing to the life cycles of cold-hardy species and how these

elements are integrated with demands for cold-hardiness. Second, it notes the new or refined techniques of great combined power now available that can be used together to obtain an integrated understanding of the cold-hardiness of individual species. Such a relatively broad view of cold-hardiness for a given species is a prerequisite both for proper comparisons among species and for still more comprehensive studies, recommended here, of cold-hardiness in the context of life-cycle adaptations as a whole.

THE ELEMENTS OF COLD-HARDINESS

The basic elements of insect cold-hardiness are surprisingly varied and are summarized in Table 1. Many insects prevent freezing through depression of the supercooling point of their body fluids. Nevertheless, injury can occur in unfrozen individuals both above and below 0°C. Other species survive winter in a frozen state (freezing tolerance). Vitrification, a glassy stable state resistant to freezing damage (Baust, 1990), has also been reported (e.g. Wasylyck et al., 1988; Chang & Baust, 1991). A few species are known to use supercooling with low supercooling points in some years or at some times of year, but are freezing tolerant at other times (Horwath & Duman, 1984; Duman, 1984; Kukal & Duman, 1989; Olsen & Duman, 1990, 1992; Gehrken et al., 1991).

When freezing occurs, it is normally initiated by nucleators, nuclei that favour ice formation by encouraging water molecules to adopt the hexagonal spatial configuration of the ice crystal. Small volumes of pure nucleator-free water otherwise supercool to –40°C. Because very rapid freezing after substantial supercooling causes mechanical injury from very fast-growing ice crystals, many freezing-tolerant species – but not all – manufacture proteinaceous ice nucleators to initiate freezing at relatively high subfreezing temperatures (Duman, 1982; Zachariassen, 1982; Baust & Zachariassen, 1983; Duman et al., 1991b). Such nuclei are internal, in haemolymph or the cell matrix. Food particles or other nuclei in the gut can also initiate freezing. External sources initiate freezing by inoculation through the cuticle, for example by ice contact in damp or frozen sites, soil particles (Sømme, 1982) or ice-nucleating bacteria (Strong-Gunderson et al., 1989, 1990; ice-nucleating bacteria are common on plant surfaces and presumably profit from growth on ice-damaged plant tissue). Some freezing-tolerant species, even in terrestrial habitats, may normally freeze by external inoculation (Shimada & Riihimaa, 1988). Contamination of haemolymph samples by surface (external) nucleators has also misled investigators into reporting internal nucleators when they are not present (references in Baust & Nishino, 1991, p. 212).

Several species rapidly acquire increased ability to survive the shock of encountering subfreezing temperatures, at least in the short term, when first conditioned by cold or other stresses (e.g. Coulson & Bale, 1990; Chen et al., 1987; Denlinger et al., 1991; Denlinger et al., 1992; Nunamaker, 1993; Chen & Walker, 1994). These responses appear to be linked to similar responses to heat shocks. The period of conditioning required to enhance subsequent survival can be very short, for example, less than one hour (Denlinger et al., 1991).

Many molecules manufactured for winter enhance cold-hardiness. Solutes of low molecular weight, acting colligatively, include polyhydroxy alcohols (polyols) such as glycerol, sorbitol, mannitol, threitol, ribitol and erythritol, carbohydrates such as glucose, sucrose, trehalose, and fructose, and amino acids such as alanine (Sømme, 1982; Storey & Storey, 1988, 1991, 1992; Lee, 1991).

TABLE 1. Basic elements of insect cold-hardiness. Not all elements are found in all species, of course.

Element	Notes
Physiological elements	
Supercooling	Remaining unfrozen below the incipient freezing point (through elimination of nucleators, and increase of cryoprotectants).
Avoidance of chilling injury	Avoiding injury by cold in the absence of freezing.
Freezing tolerance	Ability to withstand ice formation in the tissues.
Vitrification	Solidification of water (in concentrated solutions) into an amorphous glassy state with very low molecular motion.
Nucleators	Nuclei that encourage ice formation at high subfreezing temperatures. Nucleator proteins in many freezing-tolerant species prevent delayed rapid injurious freezing that would otherwise occur at very low subfreezing temperatures.
Avoidance of ice inoculation	
External	Inoculation through the cuticle by ice / ice-nucleating bacteria initiates freezing.
Internal	Elimination or masking of haemolymph nucleators in species that supercool lowers supercooling points.
Rapid cold-hardening	Exposure to moderate cold or other shocks rapidly confers increased ability to survive more severe cold; see also shock proteins.
Production of cryoprotectants	
Small molecular weight compounds	Relatively small solute molecules, chiefly sugars and polyhydric alcohols, that lower supercooling points, protect cell membranes, etc.
Thermal hysteresis proteins	Large antifreeze protein molecules that inhibit ice growth at the ice-water interface, and inhibit ice recrystallization during thaw.
Shock proteins	Shock or stress proteins, produced in response to rapid temperature changes, appear to play some role in cold tolerance.
Patterns of production	Multiple-cryoprotectant systems change the proportion of different molecules according to conditions.
Water relations: bound water	Water that is closely bound to cellular or other constituents is unavailable for freezing processes.
Resistance to mechanical damage by ice	Water expands by about 8% as it freezes, potentially causing injury.
Ecological Elements	
Climate and weather	Exposure to cold depends on seasonal patterns of cold temperatures, and the duration and intensity of cold in a given season.
Habitats and microhabitat selection	Temperatures are buffered in less exposed habitats; rates of freezing depend also on moisture in the habitat; habitats differ in exposure to inoculative freezing; some species move during winter to less severe sites.
Habitat modification	Production of cocoons, clustering in bees, etc.

Large antifreeze proteins (thermal hysteresis proteins) that inhibit ice-crystal growth also are widespread in both freezing-tolerant and freezing-intolerant species (Duman, 1982; Duman et al., 1991b). Several species rapidly produce "stress proteins" in response to thermal or other shocks, which appears to be linked to the rapid cold-hardening just noted. Finally, a number of species synthesize several different cryoprotectants, especially those of low molecular weight, simultaneously. Such multiple cryoprotectants perhaps provide protection through colligative properties (which are additive because they depend on the number not the nature of solute molecules) without exceeding the toxic concentration for individual compounds. Molecular interactions may also reduce the relative effectiveness of concentrated solutions of a single compound. In some species the proportions of different compounds also change during the cold season or in different populations (e.g. Baust et al., 1979; Baust & Lee, 1981, 1982, 1983; Rojas et al., 1983; Pio & Baust, 1988; Baust & Nishino, 1991).

The role of water in the cold-hardiness of insects has been considered for many years (e.g. Salt, 1955; Zachariassen, 1991). How much water is "bound", or unavailable to freeze, has most often been emphasized (Zachariassen et al., 1979; Storey et al., 1981; Storey, 1983). Water content also influences supercooling points (Pugh, 1994).

Ecological relationships provide a second set of basic elements of cold-hardiness (Table 1). Key data here are actual local temperatures and their variations through time, and especially the conditions in the different winter habitats and microhabitats occupied by insects. Unfortunately, actual microhabitat temperatures have seldom been measured for any species of "cold-hardy" insect during a whole winter, and ancillary information about overwintering site selection and winter biology is widely scattered. The synopsis below is chiefly based on this scattered information as cited by Danks (1978, 1991).

Most species overwinter in specific sheltered habitats protected by the very effective insulator snow. Nevertheless, other species overwinter exposed and a few species move during winter to less severe sites and back again. Habitat characteristics of various scales, including structure and moisture content, determine the temperatures experienced, the rate of freezing and the likelihood of inoculative freezing from external ice. For example, cooling rates are much slower in wet substrates, such as stumps and some galls (Baust, 1976; Baust & Nishino, 1991). Finally, a few species modify microhabitat conditions by constructing strong, impermeable or inoculation-proof cocoons, and even, in the case of honey-bee colonies, by generating substantial heat to maintain the temperature of the overwintering clusters.

THE DIVERSITY OF OTHER ELEMENTS

The realistic study of cold-hardiness includes many additional elements, beyond those listed in Table 1, that generally have not been formally addressed in previous studies. Any given species must survive a wide variety of environmental challenges in addition to winter cold; and in order to complete the life cycle it must simultaneously budget time, space and energy, involving a range of phylogenetic, physiological, biochemical, ecological and behavioural adaptations. Understanding how the fitness advantages of the various adaptations are integrated requires a knowledge of the whole life cycle, not just of means to survive the winter. As a simple example, a cold-temperate species that exploits resources exposed to winter cold, for example on the leading shoots of trees, typically requires

substantial cold-hardiness; but such exposure also brings increased predation, drying, and other pressures, so that the adaptations for cold-hardiness cannot be treated in isolation. Additional elements of this sort that must be considered for the integrated understanding of insect cold-hardiness are summarized in Table 2.

Information on phylogeny is useful to trace the evolution of adaptive features (e.g. Stearns, 1992; Brooks & McLennan, 1991). In particular, it may suggest whether observed adaptations are finely tuned to current ecological conditions, or whether they have merely resulted from shared features of a common ancestor that was subject to selection for cold-hardiness. For example, chironomid midges are supposed to have evolved in cold and even seasonally frozen habits (Brundin, 1966; Danks, 1971). Therefore, the wide distribution of cold-hardiness in the family (Danks, 1971) perhaps more likely results from shared basic features than from specific responses to local habitats. Recognizing this likelihood would avoid potentially erroneous interpretations that link cold-hardiness with local habitat conditions. Unfortunately, discussions about the evolution of cold-hardiness, such as the possible association of cryoprotectant production with desiccation tolerance or with diapause as a primitive character (rather than with low temperature) (Pullin, 1994) are still at an early stage.

Habitat determines the conditions experienced, and hence the nature of cold-hardiness required. But different habitats and microhabitats have different moisture regimens, they make their occupants differently available to predators, and potentially an individual with low mobility can occupy a harsh habitat with food when a milder habitat without food is unsuitable. Moreover, sheltered winter habitats thaw later in spring, forcing an unacceptable delay in the life cycle in cold regions where the summer is very short. Consequently, for example, several species of high-arctic insects overwinter on the cold exposed ridges that first become free of snow in spring, rather than in less severe but later-thawing sites (Danks, 1981). Species in cold sites, despite increased demands for cold-hardiness, also conserve metabolic energy (e.g. Duffy & Liston, 1985; Parry, 1986).

Protective structures such as cocoons can modify temperature to a small degree (for example through limiting wind exposure), as well as reducing the likelihood of inoculative freezing (review by Danks, 1991). At the same time, however, cocoons increase humidity around the occupant and may protect it from natural enemies.

Several properties of the surface of insects can alter temperature (Table 2). In the context of cold-hardiness, inoculation depends partly on surface features. These properties also are very important to water permeability, and hence desiccation resistance, as well as providing camouflage, protection or strength against predation.

Many aspects of life cycles pertain to the timing of growth, development and activity in relation to seasonal conditions. Studies of cold-hardiness have emphasized the necessity for preparation for winter, especially the build-up and later catabolism of cryoprotectants. Different overwintering instars of one species may suffer different winter mortality (e.g. Martyniuk & Wise, 1985). These events take place in the much wider framework of growth, dormancy, and related changes and controls (Table 2). Several authors have noted the relationship between cold-hardiness and diapause in some species (e.g. Denlinger, 1991; Pullin, 1994), or the frequent lack of a direct relationship (Danks, 1987, p. 41), and the similarity or dissimilarity of the cues and timing for these two sets of adaptations (Danks, 1987; Denlinger, 1991). Indeed, both cold-hardiness and diapause are complex

TABLE 2. Summary of some additional elements for the integrated understanding of insect cold-hardiness.

Element	Linkages and notes
Phylogeny	Cold-hardiness adaptations may be relatively recent, or simply reflect existing features of ancestors, or both. Consideration of phylogeny in comparisons suggests how cold-hardiness features of interest evolved.
Exposure	
Habitat	Habitat choice depends on habitat temperatures, including cold, but also on other conditions of humidity, temperature, etc., on further elements of the life cycle such as food, and on life-cycle timing.
Coverings	Cocoons or other structures can modify temperature and the likelihood of inoculative freezing, but also change humidity, susceptibility to parasitoids, etc.
Surface properties	
Colour	Colour determines solar heat gain, but also camouflage, etc.
Texture	Surface texture governs the likelihood of inoculation, but also rates of water loss, impact of pathogens and predators, etc.
Waterproofing	Cuticular thickness and surface lipids influence inoculation, but also govern permeability to water, structural strength, and other features.
Timing and seasonality	
Preparation	Timing of preparation for cold-hardiness, including winter habitat selection, build up of cryoprotectants and their control, may or may not coincide with cues and requirements for preparation for diapause, storage of reserves, etc.
Development	Visible interruptions in development (quiescence or diapause) may accord with cold, but also with heat, drought, lack of food, and other elements; development may be induced by various cues, not just temperature, during diapause. Development is a continuous process; elements including diapause may be integrated with cold-hardiness.
Growth	Growth or moulting stops at low temperatures. However, growth rates vary according to seasonal life-cycle timing, involving both direct effects (e.g. temperature) and cues (e.g. photoperiod), and some species show metabolic compensation for low temperatures.
Completion	The end of seasonal interruptions in development accord with the end of cold, but also with the start of the favourable season. They involve conversion of stored or sequestered substances (cryoprotectants, but also reproductive reserves, etc.) in haemolymph or various tissues, as well as habitat changes.
Water balance	
Water content	Levels of body water vary among species in relation to cold-hardiness and bound water, but also in relation to desiccation as well as to the basic body chemistry of a particular organism. One species may survive a range of levels ("desiccation tolerance").
Water availability	The availability of water to participate in freezing processes (bound water) varies, according to solute-influenced kinetics, association with structural components, and so on, that are not all associated with cold-hardiness, and that influence water availability for other metabolic processes.

TABLE 2 (continued).

Linkages and notes	Linkages and notes
Water conservation	Water, especially during dormancy including diapause, is conserved ("desiccation resistance") by low cuticular permeability, protective coverings and habitat selection, and by possible solute-based mechanisms parallel to some of those conferring cold-hardiness. Water can also be conserved or stored in lipids ("metabolic water").
Acquisition of water	Water is seldom acquired by dormant organisms at low temperatures. However, especially just before or just after dormancy, water can be acquired through drinking, feeding, and cuticular or spiracular absorption. Adaptations to flooding may also be required.
Respiration	
Suppression of metabolism	Reduced respiration may be associated with cold-hardiness (and see anhydrobiosis below), but also is a characteristic of diapause. Reduced mitochondria and mitochondrial enzymes may reflect either cold-hardiness or respiratory adaptations.
Anaerobiosis, anhydrobiosis	Oxygen supplies are limited for organisms encased in ice, but anaerobiosis is common in habitats with very low oxygen. Anhydrobiosis is linked to cold resistance (and may involve significant changes in ultrastructure), but also conserves energy and prevents biochemical imbalances by shutting down metabolism.
Acclimation	Respiratory adaptation compensates for lowered temperatures, but serves to maintain a variety of functions and activities rather than simply resisting cold.
Energy budget	
Energy storage	Sequestered materials may provide cryoprotection (e.g. "cryoprotectants" such as glycerol or trehalose), but also may act as energy reserves for non-feeding stages or for subsequent reproduction (e.g. glycogen, trehalose, lipids), as components of biochemical changes associated with metabolic interruptions such as diapause, and in desiccation resistance.
Respiratory balance	Temperatures selected by ecological or behavioural means influence the balance between respiration and assimilation in complex ways.
Thermoregulation	Microhabitat choice and basking behaviour modify body temperature, but this influences total energy balance.
Seasonality	Seasonal position of life-cycle stages, food specificity and seasonality of food, etc. influence the temperatures experienced, but also dictate seasonal energy budgets in terms of acquisition of food and other factors.
Hormonal states	
Hormonal control	Specific hormones control various processes, including elements of cold-hardiness. Also "cold" exposure (as well as starvation or other shocks) can disrupt hormonal balance, giving morphologically deranged individuals.
Production of key substances	Juvenile hormone (and also perhaps ecdysone) helps to control "cryoprotectant" production, and also nucleators. However, similar controls apply to many other processes, such as vitellogenesis.
Diapause	Absence or presence of juvenile hormone, ecdysone and other hormones is well documented to control diapause in different species.
Mortality	
Major elements	Cold, but also predation, desiccation, starvation, and many other factors all contribute to life-cycle mortality.
Key factors	Seasonal and life-stage patterns and variability of mortality factors determines their impact.

and continuous components of development. Diapause is a developmental process, not a simple cessation of development (Danks, 1987), involving the long-term integration of a variety of hormonal, biochemical and other responses (such as food storage and failure to metamorphose) triggered by external cues and internal programmes. Individuals in diapause continue to metabolize, albeit at a low rate, and change developmental potentiality largely in response to external cues. Information now accumulated suggests that cold-hardiness likewise should be viewed as a continuous programme through time, involving preparation, adjustments during winter in response to temperature (as in those multiple-cryoprotectant systems in which the levels of different substances respond to winter conditions, e.g. Baust & Lee, 1982), and readjustments in spring for normal development.

From the single viewpoint of temperature, growth rates have been adjusted through metabolic compensation to low temperatures in some species (e.g. Block & Young, 1978; Block, 1979). From the wider viewpoint of life cycles, growth rate, even under favourable conditions of food and temperature, can be modified through token stimuli such as photoperiod in many species, and so is part of the overall programme of life-cycle timing (Danks, 1987).

Because winter survival of so many species depends on whether freezing occurs, the status of water has been much studied in cold-hardy insects. Water content and the levels of bound water, which determine the availability of water molecules to participate in the freezing process and in cryoprotectant dynamics, have been emphasized. However, water participates in many other processes, including fluid and ionic balance, excretion, transportation, metabolism, and storage, which must be co-adjusted with aspects of cold-hardiness.

Water can be conserved for any of these uses by low cuticular permeability, by increased solute concentrations, and by choice of protected microhabitats. These three types of adaptations for protection against desiccation are the same three adaptations commonly emphasized in the context of cold-hardiness (Ring & Danks, 1994). In addition, greater water loss can be tolerated in some species from dry habitats, again raising the parallels that have often been drawn between the effects of freezing and those of dehydration in concentrating cell contents, stressing membranes and so on (e.g. Meryman, 1974), and emphasizing the roles of cryoprotectants in protecting membranes, enzymes, and other cell constituents against both freezing and dehydration (e.g. Crowe et al., 1983; Loomis, 1991).

Water can be acquired in diverse ways. Water acquisition may even be the purpose of feeding in some species (Wharton, 1985). Dormant individuals seldom actively drink or feed (although this is common immediately before or after diapause to sequester stored reserves and to develop eggs: Danks, 1987). However, diapause puparia of the flesh fly *Sarcophaga crassipalpis*, diapause larvae of the parasitoid *Nasonia vitripennis*, and diapause eggs of the stick insect *Extatosoma tiaratum* absorb water vapour directly from the atmosphere (Yoder & Denlinger, 1991, 1992; Yoder et al., 1994). These many wider aspects of seasonal water balance have seldom been addressed in the context of cold-hardiness.

Respiratory metabolism is depressed in many overwintering insects. Furthermore, insects encased in ice can receive no fresh supplies of oxygen, and must respire anaerobically (Conradi-Larsen & Sømme, 1973a,b; Sømme, 1974a,b; Meidell, 1983). Anhydrobiosis (Crowe & Clegg, 1973) confers tolerance to very low temperatures in

organisms as diverse as nematodes (Crowe & Crowe, 1982), tardigrades (Crowe & Higgins, 1967), midges (Hinton, 1960) and springtails (Poinsot-Balaguer & Barra, 1983). Such modifications of respiratory metabolism may be accompanied by mitochondrial degradation (Kukal, 1991; Kukal et al., 1989) and other ultrastructural changes (e.g. Poinsot-Balaguer & Barra, 1983) and decreased mitochondrial enzyme activity (Joanissse & Storey, 1994).

All of these changes may relate to cold-hardiness, but also or instead to respiratory adaptations, including conservation of energy and protection of metabolic systems. Such changes could also be linked with diapause, in which respiration typically falls to only a fraction of normal levels (Danks 1987, Table 5); or with habitat relationships – many insects in substrates with very high biological oxygen demand, especially in aquatic habitats, respire anaerobically, at least intermittently.

Respiratory metabolism is one aspect of the broader balance of energy in the organism. Cold-hardiness requires energy especially for habitat selection and cryoprotectant production. Food may therefore influence cold-hardiness directly or indirectly (cf. Verhoef et al., 1994). Tolerance of cold shock is related to energetic reserves in *Drosophila* (Chen & Walker, 1994). But all other elements of the life cycle have energy demands too. Table 2 outlines some of the physiological and behavioural aspects of the acquisition, assimilation and storage of energy in relation to some aspects of the life cycle beyond survival of winter cold. For example, acclimation to continue respiration at low temperatures is only selected for if assimilation can also take place at low temperatures (MacLean, 1975). As spring plant growth slows, the quality of many foodplants decreases (slowing assimilation) even as seasonal temperatures are increasing. Especially important, therefore, is the seasonal position of life-cycle stages other than the overwintering stage and how energy is acquired and stored for growth and reproduction. Such a conclusion confirms the importance, emphasized earlier, of overall life-cycle timing.

Specific hormones, their levels, and the temporal pattern of production govern development. According to so-far somewhat limited studies, several aspects of cold-hardiness are controlled by hormones, including the production of polyols (Tsumuki & Kanehisa, 1981; Horwath & Duman, 1983; Hamilton et al., 1986), antifreeze proteins (Xu & Duman, 1991; Xu et al., 1992) and nucleators (Xu et al., 1990), although direct short-term control of cryoprotectant dynamics though temperature-sensitive enzymes has also been demonstrated (e.g. Storey & Storey, 1991).

The same hormones govern a very wide variety of processes throughout the life cycle, including diapause and reproduction (mating, previtellogenesis, vitellogenesis, etc.). Juvenile hormone and ecdysone are the major hormones controlling insect development and reproduction. Their ability, through presence, absence or level, to suppress development has been captured in diapause programmes (Danks, 1987). Evidently, the same powerful insect-wide machinery contributes to the programming of cold-hardiness.

Finally, the selective value of all of these features is expressed through mortality or its corollary, survival. In particular, many individual factors such as the microhabitat or size of particular individuals (Danks, 1978; Hokkanen, 1993), biotic factors such as natural enemies (Leather, 1993), and abiotic factors other than temperature contribute to winter mortality. For example, mortality depends on humidity, not just supercooling ability, in some mites (Cenxuan & Shimada, 1991). The same suite of factors, and temperature in a

different role, contribute to mortality during the rest of the life cycle. Because the variability of a mortality factor, and not just its level, determines its importance, careful annual life-table assessments rather than spot measurements of winter mortality are required. In other words, the careful assessment of patterns of mortality in space and time provides the most useful measure of the likely impact of cold on local populations and on the maintenance of adaptations for cold-hardiness.

This summary suggests two major conclusions. First, it demonstrates strikingly how many elements are shared, or at least coincide, between cold-hardiness and other life-cycle adaptations. For example, sites and conditions (habitats, seasonal movements, cocoons) affect cold-hardiness but also many other life-cycle elements. Potential interactions between cold-hardiness and water relationships stem from features ranging from habitat choice to cuticular structure and protection of enzyme activity. The timing of cold-hardiness is coincident with dormancy, needs for protection, and metabolic energy demands including stored reserves. Other metabolic adjustments, notably the suppression of metabolism, pertain to general respiratory adaptations as well as to cold-hardiness. It is also worth repeating that adjustments for cold-hardiness appear to involve environmental monitoring, ongoing adjustment of cryoprotectants, and other responses, and are not simply a switch from an active summer state to an inert winter state. These ongoing processes provide a striking parallel to the continuous process of diapause induction, development and completion. Diapause likewise was once thought of as the mere suspension of development, whereas it is correctly viewed as a different developmental pathway (Danks, 1987).

A second major conclusion that follows from these shared elements is that *trade-offs* in life-cycle requirements are inevitable. For example, when hatch must occur early in a short season, late-thawing habitats that are best protected from winter cold cannot be used. The selective advantages of dormancy, food storage, and the seasonal timing of reproduction may differ between males and females in one species (e.g. Danks, 1987, pp. 174–175). Consequently, even the way energy is optimized in different sexes has different impacts on requirements for cold-hardiness.

THE DIVERSITY OF TECHNIQUES

Fortunately, a great many techniques are available to investigate the diverse elements of cold-hardiness in detail. Given the combined power of these techniques, as recently refined, and our knowledge that cold-hardiness is complex rather than composed of a few simple components, it is no longer especially useful simply to record supercooling points for example. Advanced equipment or techniques that can be used to obtain relevant information are listed in Table 3. In addition, there are general methods for detailed examination of tissues or ultrastructure, such as transmission electron microscopy, and molecular techniques for protein or DNA purification and sequencing.

Supercooling-point determinations record the latent heat of fusion as water freezes during programmed cooling in a cryostat or other device, as monitored by a thermocouple linked to a recording potentiometer. Modern equipment (e.g. Bale et al., 1984; Callan et al., 1986) has closely controlled accurate rates of cooling and accurate assessments. The ability to record from a number of specimens simultaneously, and convenient specimen holders in which the thermocouple has good contact with the specimen without injuring it

(e.g. Brunnhofer et al., 1991), also are important. Multiple specimens can be assessed from many individual traces (requiring multiple recorder channels), but also from one trace, allowing the separate effects of cold and of the freezing process to be compared among the specimens in a given cooling run (Nedvěd et al., 1995).

The cryomicroscope is a microscope in which high quality optics and a variable light source, such as polarized or phase-contrast light, are coupled with a stage equipped for programmable cooling in order to observe processes related to low temperatures. High accuracy, stable set points, and evenness of the changes in temperature require sophisticated, computer-governed, cooling and warming modules. Various processes related to freezing or freezing mortality can be observed directly, including ice nucleation (Shimada, 1989), intracellular ice formation (Myers et al., 1989), and membrane damage (McGrath, 1984).

Vital dyes are dyes that penetrate living cells and stain the cells or specific organelles within the cells. Different tissues or organelles can be stained with different dyes; some vital dyes also stain dead cells, though often differentially; and other dyes will stain only dead cells. Therefore use of vital and other dyes can indicate the survival of tissues after freezing, suggest where physiological processes have been disrupted, and show when cells are dead.

The differential scanning calorimeter (DSC) measures the heat of reaction or change of state by assessing the energy required to maintain the temperature of a sample at the same temperature as a reference as both are heated or cooled, again normally using sophisticated heating and cooling modules programmed by computer. The instrument is calibrated for temperature and energy by pure reference standards of known melting point, commonly decane (–29.66°C) and cyclohexane (+6.54°C). The DSC therefore can show supercooling points, but its sensitivity also allows detailed assessment of ice content, bound water, nucleator activity, and other phenomena. For detailed examples, see Block (1994a).

Several chromatographic methods are useful to identify or quantify compounds of interest, notably cryoprotectants (e.g. Storey & Storey, 1991, who give references to selected specific studies using these methods). Chromatography separates compounds through their differential movement when carried by a mobile phase (e.g. solvent) along a stationary phase (e.g. silica), and they can be identified and quantified by comparison with the movement and concentration of known standards. A wide variety of solids and liquids (usually held in a solid matrix) can be used as the stationary phase; various solvents or mixtures, or inert gases, can be used as the mobile phase. Both must be of the highest purity.

Most methods involve extensive pretreatment of samples (e.g. Ring, 1986). For example, multiple solvent extractions, centrifugation and filtration, all under refrigeration, are required to select particular classes of compounds from the raw specimen extracts. Otherwise, solution in the mobile phase is incomplete, the stationary phase is overloaded or contaminated, and there may be too many signals for separate detection. Samples for the gas chromatograph also have to be derivatized (subjected to specific molecular modification) before analysis.

Thin-layer chromatography (TLC), and paper chromatography, run the solvent along a flat substrate (e.g. silica gel), and therefore a second run at right angles may give further separation. Compounds separated on the matrix are visualized by developing them with appropriate chemicals. They can be identified and quantified by reference to the behaviour of known standards of known concentration run in the same system.

TABLE 3. Key techniques available to study physiological elements of cold-hardiness.

Equipment or technique	Can give information on	Advantages	Disadvantages
Supercooling point apparatus	Supercooling points; mortality	Information on whole insects	Relevance may depend on cooling rate, which may not have been chosen appropriately
Cryomicroscope	Supercooling point, melting point, ice formation, crystal structure, effects of ice nucleators, freezing damage, osmotic effects, etc.	Allows direct observations of events related to freezing	Observations depend on transparency or small size of specimen; hence easier with solutions or isolated tissues than with specimens
Vital dyes	Survival, sites of freezing damage	Assist in direct observations of freezing damage	As for the cryomicroscope, depend on transparency of specimen.
Differential scanning calorimeter	Water / ice content, bound water, supercooling points, rate of ice-crystal growth, ice nucleators, vitrification, antifreeze protein activity	Automated systems	Relevance may depend on the selected ranges and rates of cooling or heating
Thin-layer chromatography	Identification and quantification of cryoprotectants, etc.	Quantitative; relatively rapid, especially useful for general assessments and surveys	Complex sample preparation, limited sensitivity
High-pressure liquid chromatography	Identification and quantification of cryoprotectants, etc.	Multiple compounds can be assayed in one run; quantitative, with moderate sensitivity	Complex sample preparation
Gas or gas-liquid chromatography	Identification and quantification of cryoprotectants	Quantitative with good sensitivity; multiple compounds can be assayed in one run	Complex sample preparation; samples must be derivatized
Enzymatic analysis	Identification and quantification of cryoprotectants, etc.	Rapid and sensitive; perchloric acid extracts allow wide range of metabolites to be quantified	Limited to certain cryoprotectants only (glycerol, sorbitol, glucose, fructose)
Liposome fusion and leakage and enzyme denaturation assays	Occurrence of cryoprotectants in sub-cellular fractions	Can detect presence of novel cryoprotectants	Concentration of cryoprotectants must be high enough to protect against freezing, for example in liquid nitrogen
Mass spectrometry	Identification of cryoprotectants	Highly sensitive	Complex sample preparation; requires relatively concentrated samples
Nuclear magnetic resonance spectroscopy	Cryoprotectant metabolism; also bound water, etc.	Non-invasive	Typically qualitative, limited sensitivity

TABLE 3 (continued).

Equipment or technique	Can give information on	Advantages	Disadvantages
^{14}C radiotracers	Routes and rates of cryoprotectant production and metabolism	Typically rapid and sensitive	Dealing with radioactive compounds
Computer temperature loggers	Temperature exposures in the field	Precise information about microhabitat temperatures can be integrated	Field power source required
Programmable temperature chambers	Response to temperatures of ecological or physiological significance	Desired patterns of experimental conditions can be delivered precisely	Calibration requires verification with independent standards
Miniature temperature-sensing transmitters	Habitat temperatures	Actual microhabitat temperatures of living insects can be monitored	Effective attachment, small enough size and battery operation at very low temperatures are difficult to ensure

In high-pressure liquid chromatography (HPLC) the passage of different substances through a column packed with fine particles of the stationary phase takes different periods as a precisely controlled pump forces solvent through the system to carry the substances to a detector (e.g. spectrophotometer, refractometer), the output of which is modified by the presence of solutes in the solvent stream. Solvent quality (purity, freedom from dissolved gases, etc.) must be carefully controlled to avoid contamination and ensure accurate flow rates.

In gas or gas-liquid chromatography (G(L)C), the time for different compounds to enter the detector likewise varies, but here gas is the mobile phase, and a solid or a liquid (usually on a solid support) is the stationary phase. Numerous compounds can be run together for analysis. Modern HPLC's and G(L)C's have computer control of flow rates, sample injections, and the storage and integration of data.

Certain cryoprotectants and their metabolites can also be identified and quantified by enzymatic analysis (Bergmeyer et al., 1983–86), using commercial enzymes at fixed temperature in a spectrophotometer to assay glucose, fructose, glycerol and sorbitol. Additional metabolites can be identified in perchloric acid extracts (Lowry & Passoneau, 1972).

Another method (Loomis et al., 1988; Loomis, 1991) uses fluorescent markers or enzyme assays to detect how much damage to liposomes or enzymes occurs on freezing (relative to both unfrozen and completely damaged samples), damage that would be limited by the presence of cryoprotectants.

Substances can also be characterized by mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy. Mass spectrometers, after ionizing sample substances, separate those differing in their charge-to-mass ratio by passing them through electrical and magnetic fields to an electrical detector (e.g. Beynon, 1960). Extensive preparation, concentration, and purification of samples usually is required.

In nuclear magnetic resonance spectroscopy (Gadian, 1982; Abraham et al., 1988), the interaction of an applied magnetic field with the nuclear spin of a substance in the sample

gives resonance, electromagnetic radiation due to the transition of energy states in the nuclei, and resulting in a radiofrequency spectrum characteristic of the substance. Therefore no special sample extractions or preparations are required. Enrichment by and detection of markers, especially ^{13}C, provides structural and quantitative information, and allows subsequent metabolites to be tracked (e.g. Buchanan & Storey, 1983; Kukal et al., 1988). NMR can also be used for imaging tissues using computer-controlled scans (Morris, 1986). Freezing injury and other phenomena can be visualized in this way.

Metabolites can also be tracked through radiotracers, small amounts of radioactive elements, such as ^{14}C, incorporated into the metabolites (e.g. Tsumuki et al., 1987).

Computer-assisted recording and control of temperature is valuable for a variety of field and experimental studies. For example, computer data loggers allow temperatures from many probes to be sampled frequently and more-or-less simultaneously; the copious information is stored digitally, and subsequently can be analyzed by computer. In experiments, programmable temperature chambers allow daily or longer-term cycles of experimental temperatures to be programmed to any degree of complexity (and hence potential realism) desired.

Electronic devices of very small size are now available, so that it is feasible to attach or implant temperature-sensing ultrasonic transmitters into living insects. Transmissions can then be used to trace the temperatures experienced in the field (as has been done with uniquely coded transmitters in individual fish, for example: Snucins & Gunn, 1995), in order to assess actual winter microhabitat conditions.

A few common themes emerge from this variety of methods. Many techniques require extensive sample preparation and purification, and intelligently chosen standards. Most machines require careful and time-consuming set up, equilibration, and calibration. And computer control of the experimental system, as well as integration and analysis of data, is commonplace, allowing both automation in running samples and the rapid summary and printout of results. Despite this automation, many choices remain with the experimenter, depending on the questions being asked and the processes or substances being examined.

This brief review of equipment for the study of cold-hardiness confirms that many powerful techniques are available. The main disadvantage of most of these pieces of equipment is the expense to purchase and run them. Because analytical machines costing up to a million dollars have to be used on more than an occasional basis, and because equilibration and calibration have to be carried out before sets of results can be generated reliably, full-time technical expertise is required to run these modern machines most effectively.

Combining the techniques is especially valuable. For example, details of microhabitat conditions integrated by data loggers can be compared directly with cryoprotectant levels assayed by HPLC. Thin-layer chromatography can be used to separate unknown compounds of interest by cutting relevant areas from the gel after the run for further analysis by GC or MS. Because different compounds travel differently in different experimental systems, and therefore in a particular system a compound may be masked by another, or sometimes by compounds introduced during preparation, combining work with HPLC, GC and MS can be instructive.

The existence of these powerful machines leads to the temptation to make piecemeal studies capitalizing on the one particular technology locally available. It is more

appropriate, however, to focus on a given species chosen for its particular interest in the context of cold-hardiness, such as habitat or known adaptations to cold. Each species is adapted differently, but a broad framework of information on a given species is more helpful than isolated facts to understand that species, and also is more likely to suggest general lessons about cold-hardiness. Despite the cost of individual machines, a broad approach is feasible especially through joint work by cooperators from different institutions.

CONCLUSIONS

Many specific aspects of cold-hardiness have been identified, and substantial information has accumulated on each of these aspects. However, three potentially fruitful broader approaches now seem to be necessary. First, a focus on single species, using the full range of techniques available, is required to understand the coordinated adaptations of each species. For example, the simultaneous assessment of cryoprotectants, nucleators and their effects in supercooling and mortality, as well as information on the conditions experienced in nature and how they accord with simultaneous features of cold-hardiness, is required. Remarkably, no complete assessment for a single species, not even an important crop or forest pest, has ever been published, although substantial information is available for a few species, notably the goldenrod gall fly *Eurosta solidaginis* (Baust & Nishino, 1991 and references cited there; Layne, 1993). However, even in this species different populations differ, reinforcing the need for simultaneous assessments on a given population. Species suitable for intensive study would include very cold hardy, widely distributed species (such as *E. solidaginis*) that are not very small in size, are well known taxonomically and live in one or more habitats where the physical conditions have already been studied or can relatively easily be characterized.

A second key approach would compare different species, but comparisons must be made in terms of their individual complete cold-hardiness systems, and not simply by comparing isolated characteristics such as the types of cryoprotectants. In contrast to this approach, most species comparisons that are possible from published information allow differences to be looked for in only a small number of features. For example, some species tolerant of freezing atypically lack evident cryoprotectants (Ring, 1981). Some freezing-tolerant species atypically supercool to very low temperatures before freezing (Miller, 1978, 1982; Ring 1982, 1983; Grubor-Lajsic et al., 1991). Without other specific evidence about nucleators, proteins and habitat conditions, for example, let alone other aspects of the life cycle, the significance of these interspecific differences is uncertain.

A third key approach would examine wider linkages between cold-hardiness and other salient aspects of the life cycle. For example, cold-hardiness attributes, timing of feeding activity, and other information for the high arctic moth *Gynaephora groenlandica* show that at least summer temperatures, winter temperatures, cloud cover, foodplant quality and abundance, natural enemies and moulting, influence the energy budget in this species (Danks et al., 1994). The "cold-hardiness" of the species based partly on glycerol accumulation (e.g. Kukal, 1991) explains only a small part of the fully adapted 14-year life cycle.

The numbers of shared elements and of the potential trade-offs between cold-hardiness and other aspects of the life cycle reinforce the view that a broad study from the perspective of life cycles is now the most profitable way to further advance our understanding of insect cold-hardiness. Thinking laterally about the subject, without a narrow focus on

cryoprotectants for example, allows the interactions between cold-hardiness, water relationships, and life-cycle timing to be viewed as a co-evolved set of fitness attributes, not a series of isolated "adaptations". For example, it is instructive and worth repeating that even some of the "cryoprotectants" that have been the touchstone for physiological studies of cold-hardiness over many years appear to serve also in energy storage, desiccation resistance, or general protection against stress.

Broader perspectives of the sort just outlined also extend the relevance of work on cold-hardiness to a variety of social and scientific concerns. For example, how cold-hardiness is integrated with the life cycle in terms of the onset of activity in spring and the duration of the summer season determines the response of a species to potential global climatic change more fully than just the possible effects of warmer winters. In a broad scientific context, information on cold-hardiness and its relationships with energy budgets and timing provides further elements for the assessment of life-cycle trade-offs beyond the reproductive statistics (fecundity, reproductive rate, etc.) currently emphasized in the theoretical analysis of life-cycle strategies (e.g. Danks, 1994). Such a setting in the complete context of insect life-cycles as a whole in turn is likely to help in interpreting the origin and evolution of the cold-hardiness attributes themselves.

In summary, to advance the understanding of cold-hardiness further it is now necessary to undertake studies that assess many aspects of cold-hardiness simultaneously, but that moreover incorporate information about the rest of the life cycle, and how seasonal demands for energy, and for the timing of reproduction, feeding, activity, dispersal, and so on, interact with the demands of cold-hardiness.

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