Key themes in the study of seasonal adaptations in insects I. Patterns of cold hardiness

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Abstract
Recent work on selected topics of particular interest for understanding insect cold hardiness is reviewed. Themes considered include the dynamic nature of cold hardiness, ice nucleation, connections between cold hardiness and desiccation, rapid cold hardening, seasonal changes in mitochondria, survival in nature, and selection for types of cold hardiness. Such seasonal adaptations have a wider range of components than has often been appreciated, including independently evolved elements. Some specific conclusions are drawn and suggestions are made for future work. Several adaptations, such as rapid cold-hardening and mitochondrial degradation, will probably prove to be much more widespread than has yet been realized. From a general perspective, understanding such diverse components and their differences requires an ecological approach that places the biochemical and timing adaptations in context with habitat conditions and demands related to the stresses of the adverse season, seasons favorable for development and reproduction, and the signals that are available in each habitat to predict future environments. Further understanding therefore depends especially on efforts to analyze the adaptations of individual species in the context of their natural environments.

Key words: Insects; seasonality; cold-hardiness; nucleators; rapid cold-hardening

INTRODUCTION
Insects respond to seasonal environments through a very wide range of adaptations such as cold-hardiness, dormancy, and life-cycle control. This review highlights some themes in the field of cold hardiness that I consider to be of current interest. In that way, it draws attention to key subject areas and concepts, a method that also serves to integrate knowledge in this broad field. Extensive background information, most of it not included here, is available in a number of earlier reviews (e.g. Danks, 1978, 1996, 2000b; Cannon and Block, 1988; Storey and Storey, 1988, 1992; Lee and Denlinger, 1991; Sømme, 1999; Bale, 2002; and others cited below).

COLD HARDINESS AS A DYNAMIC STATE
Insects do not simply enter a fixed cold-hardy state for the winter. For example, the levels of cryoprotectants such as glycerol and sorbitol can be adjusted markedly during the winter in larvae of the goldenrod gall fly *Eurosta solidaginis* (Fitch) (Baust, 1982; Baust and Nishino, 1991). The results of Meier and Zettel (1997) suggest that at least in some years the levels of antifreeze proteins (AFPs) in the collembolan *Entomobrya nivalis* (Linn.) also change according to winter conditions. These adjustments, as well as the manufacture of cryoprotectants in advance of the winter, respond to environmental changes in photoperiod, temperature and dehydration (e.g. Baust, 1982; Allmen and Zettel, 1984; Meier and Zettel, 1997; Layne and Kuharsky, 2000), and are distinct from the developmental changes that precede spring emergence.
Changes during winter in other aspects of cold hardiness are also known. For example, Bale et al. (2000, 2001) showed that individuals of the Antarctic beetle *Hydromedion sparsutum* Müller that have been frozen and thawed have lower supercooling points, reducing the likelihood that they will freeze again (although if these individuals are refrozen they are more likely to die than if they do not have such supercooling-point depression). A similar phenomenon is now known in the temperate fly *Syrphus ribesii* Linn. (Brown et al., 2004).

Diapause is another dynamic pattern of development responsive to environmental cues, and is linked to a varying degree—depending on species—to cold hardiness (reviews by Denlinger, 1991 and Hodkova and Hodek, 2004). In some species no relationship has been found. In others there is evidence for at least partial independence of cold hardiness and diapause (Saunders and Hayward, 1998), or independent evolution of traits (Tanaka, 1997, 1999). In many species diapause and cold hardiness have been reported as somehow linked, but without definitive experiments. Many such cases may simply reflect coincident timing or responses to similar inductive cues, but they include reports that cold hardiness or its response to cold temperatures depends on developmental stage including diapause (e.g. Li et al., 2002; Košt’ál et al., 2004a).

In some species, cold hardiness and diapause are more closely linked. Several species that overwinter in diapause cannot develop cold hardiness unless they are in the diapause stage (Šlachta et al., 2002b). In some other species that overwinter after a diapause that prevents premature emergence in fall but ends relatively early, cold hardiness increases only after the diapause ends (Goto et al., 2001a, b). Linkages between cold hardiness and diapause have also been made at the endocrine level: juvenile hormone appears to be involved in both, and both can be disrupted by hormone treatments (Watanabe and Tanaka, 2000; Zdarek et al., 2000).

The main lesson of recent work is that the processes are complex, because even in the same species some elements of cold hardiness and of diapause are linked but others are not. For example, alanine increases only in diapause larvae of the pyralid moth *Enosima leucotaeniella* (Ragonot); trehalose increases in both diapause and especially in non-diapause individuals (Goto et al., 1998). Diapause is needed for the decline of supercooling point in the bug *Pyrrhocoris apterus* (Linn.) (Hodkova and Hodek, 1997), but cold hardiness is not correlated with the intensity of diapause (Kalushkov et al., 2001). Moreover, in this species reduction of ice nucleators, specific polyol synthesis, increased palmitic acid levels in membrane phospholipids, and certain haemolymph ion adjustments depend on the diapause state, but features such as oxygen consumption, water loss, increased haemolymph osmolality and adjustments in other membrane phospholipids are instead controlled by acclimation at low temperature (Šlachta et al., 2002a).

Several kinds of evidence, from cryoprotectant profiles, supercooling points, and linkages with diapause, therefore confirm that insect cold hardiness reflects ongoing processes, environmental reactions, and varying degrees of linkage to other life-cycle stages. Consequently, investigators must be alert for programming by natural environments, for the inadvertent effects of experimental treatments even in winter, for the serial development of competence for some aspects of cold hardiness, and for shifts over winter in additional components of cold hardiness that have not yet been recognized.

**THE ACTION OF NUCLEATORS**

The roles of proteins that influence ice nucleation in insects have been well reviewed by Duman (e.g. 2001). Holt (2003a, b) recently reviewed nucleation from a more general perspective. In freezing-susceptible species, antifreeze proteins may inhibit inoculative freezing (e.g. Olsen et al., 1998; Zettel, 2000) as well as ice nucleation. Typically they occur in several body compartments, for example in the intracellular fluid and intestinal fluid as well as in the haemolymph (Kristiansen et al., 1999). In some freezing-tolerant insects, proteins made for the winter instead enhance nucleation (Lundheim, 2002), typically to avoid the rapid growth of ice that would take place if freezing began at low subfreezing temperatures. Other proteins in freezing-tolerant species may prevent damage by protecting membranes or inhibiting ice recrystallization. Other, smaller, specific recrystallization inhibitors are also known in a nematode (Ramlov et al., 1996).

These various protein molecules therefore have a
range of functions in different species, and the modes of action are complex; for example, the action of AFPs is enhanced by smaller molecules such as glycerol (e.g. Li et al., 1998; Duman, 2002). AFPs are relatively efficient in protecting against nucleation, so that low concentrations (too small to provide much thermal hysteresis) may nonetheless give significant protection against sub-freezing temperatures (e.g. Sinclair and Chown, 2002).

Conventional wisdom has been that nucleation in the environment and in insects is heterogeneous, induced by nuclei such as dust particles or surfaces around which water molecules can assemble in the general hexagonal configuration characteristic of the ice crystal. In contrast, homogeneous nucleation depends on appropriate organization of the water molecules themselves for long enough for freezing to be initiated, an event more likely at lower temperatures when the molecules are moving slowly, and thus supposed to take place in pure water—in the absence of heterogeneous nucleators—only at about −40°C, the spontaneous freezing temperature (Vali, 1995).

Work on antifreeze proteins, initially in Antarctic fish, showed that these substances inhibit ice nucleation at temperatures close to freezing and in habitats containing ice, preventing the growth of ice crystals at the ice-water interface (cf. Madura et al., 2000). Some students of insects supposed that AFPs in the haemolymph instantly inhibited the further growth of any embryonic ice crystals that formed as temperatures fell. However, Wilson and Leader (1995) asserted that AFPs act not on embryonic ice crystals but rather on any potential sites of heterogeneous nucleation. By binding to these sites (a function of their molecular structure, which appears to mimic the ice lattice: Liou et al., 2000; Graether and Sykes, 2004; Marshall et al., 2004; Strom et al., 2004), AFPs would preoccupy the most efficient triggers for nucleation and thus prevent freezing. Synthetic ice-blocking agents appear to act in this way (Wowk et al., 2000). The idea was supported by a review discounting the role of homogeneous nucleation in biological freezing (Wilson et al., 2003). However, Zachariassen et al. (2004b) took exception to this review. They concluded that homogeneous and not heterogeneous nucleation takes place in insects, based especially on the fact that the nucleation temperature in different systems depends on the volume of water itself but lacks any sort of correlation with potential heterogeneous nucleators. Moreover, depression of the melting point and depression of the nucleation temperature are about the same for homogeneous nucleation and for insects, but dissimilar (with the nucleation temperature depressed twice as much) for heterogeneous nucleation. Their analysis suggests that insects already have ways to offset heterogeneous nucleators (in addition to AFPs, of course), such as the shapes of structural proteins that have evolved, leaving homogeneous nucleation as the pathway for freezing in most freezing-susceptible species. Zachariassen et al. (2004b) also noted that water might be arranged in association with biological surfaces in ways that differ from the ice lattice but might contribute to depression of the nucleation temperature.

If nucleation is homogeneous, adjustment of available water volume would be the key method of reducing the nucleation temperature, using dehydration and comparable adaptations seen in most species over winter (see the next section). These recent analyses of homogeneous versus heterogeneous nucleation should serve as a springboard for further work on the freezing process in freezing-susceptible insects.

Of course, freezing-tolerant species behave differently. Most of them manufacture ice-nucleating proteins, heterogeneous nucleators that have similar properties to the AFPs (cf. Graether and Jia, 2001). A few species (perhaps those without their own ice-nucleators) rely on inoculative freezing, and this is the only route allowing survival in some freezing-tolerant species (review by Duman et al., 1991; Riihimaa, 1996).

COLD HARDINESS AND DESICCATION

There are many potential similarities and interactions between cold hardiness and desiccation. Ring and Danks (1994, 1998) pointed out that cold hardiness and desiccation resistance both rely on similar mechanisms, including elevated levels of polyols or sugars (notably trehalose), adjustments of water content, and choice of habitats. These themes have been reinforced by later work (e.g. Block, 1996, 2002; Danks, 2000a; Bayley et al., 2001; Block and Zettel, 2003), showing that there is indeed a wide range of adaptations to desicc-
tion, as well as interactions or coincident adaptations with cold hardness. As might be expected, the extent of the linkages varies among species and among habitats (e.g. Worland and Block, 2003 for 5 antarctic species; Kaersgaard et al., 2004 for 9 collembolan species), but three main themes have emerged: adaptation to dry winter environments by resisting water loss; freezing resistance in certain soil species with permeable cuticles by supporting water loss; and internal biochemical interactions.

Cold winter air is very dry. Most species overwinter in sheltered habitats, perhaps as much to escape the dryness as to escape the cold (Ring and Danks, 1994). Some structures, such as the shelters of bagworms and some pupal cocoons, limit water loss (Rivers et al., 2002; Danks, 2004b), but most species that overwinter above the snow have remarkable desiccation resistance, especially through cuticular waterproofing (e.g. Ramløv and Lee, 2000; Bauce and Han, 2001; Williams et al., 2002; Nelson and Lee, 2004). Tolerating extensive water losses or acquiring new supplies is not feasible in these habitats, and most adaptations (including biochemical mechanisms that make water unavailable for evaporation, see below) serve to ensure the retention of existing water (Danks, 2000a). Even in the soil, moisture interacts with temperature to create the conditions that must be tolerated over the winter (cf. Ellsbury and Lee, 2004), including variations from year to year or through the season. Species living in exposed soil habitats resist desiccation, as in some collembolans (e.g. some species studied by Worland and Block, 2003 and by Kaersgaard et al., 2004).

In soil microhabitats less subject to desiccation stress, so that small invertebrates can survive in summer without the need for waterproofing, dehydration itself can be used to prevent freezing during winter. This strategy, first described for earthworm egg capsules (reviewed by Holmstrup and Zachariassen, 1996), is now known from a variety of taxa, including enchytraeids (Sømme and Birke-moe, 1997), nematodes (Wharton et al., 2003) and collembolans (e.g. Holmstrup and Sømme, 1998; Worland et al., 1998), as reviewed by Holmstrup et al. (2002a). In these species, water is lost to surrounding ice (because of the vapour pressure differential between ice and unfrozen solutions, e.g. Lundheim and Zachariassen, 1993) and this process together with the manufacture of additional cryoprotectants is fast enough that the vapour pressure of the solute-rich body fluids soon reaches equilibrium with surrounding ice, minimizing the need for extensive supercooling and ensuring that freezing—even through inoculation from adjacent ice—cannot occur. Holmstrup et al. (2002a) point out that measurements of supercooling points for such species have little relevance to their cold hardness.

The third theme of interaction between cold hardness and desiccation, parallels at the molecular level, was first considered because dehydration and extracellular ice in the body create similar stresses by causing water to be removed from the cells. Not surprisingly, water available for such removal is reduced in many insects prior to the winter, reported as a reduction in free water or as an increase in “bound water” or “unfreezable water” (e.g. Block, 2002; Block and Zettel, 2003; and see Wolfe et al., 2002). Such dehydration is not always part of the cold-hardiness mechanism, but it does enhance cold hardness in many species. For example, water becomes unavailable to participate in the freezing process because of an increase in the number of molecules interacting with polyols, sugars or macromolecular antifreeze (Storey and Storey, 1988). Dehydration as well as cold resistance is also provided by the protection afforded to membranes and proteins by these sugars and polyols (review by Ramløv, 2000). Bayley et al. (2001) and Holmstrup et al. (2002b) have pointed out that cross-resistance against desiccation and cold is provided by the adjustment of membrane phospholipid fatty acids, which become desaturated in response not only to cold (cf. Koštál and Simek, 1998), but also to desiccation, therefore keeping the membranes fluid in the face of both stresses.

These recent findings confirm that further exploration of the links between cold hardness and desiccation is warranted from ecological, physiological and biochemical points of view.

RAPID COLD HARDENING

Insects showing rapid cold hardening acquire increased resistance to the adverse effects of low temperatures when they are first conditioned for a short period at a temperature lower than the temperature at which they are being maintained. A typical experiment would show that exposing individ-
uals reared at 20°C to temperatures between 0°C and 10°C for a short time (from a few minutes to a few hours) doubles their subsequent short-term survival at subfreezing temperatures. The hardiness so rapidly acquired (which protects against chilling, not freezing) is equally readily lost if individuals are returned to the higher rearing temperatures.

Early work established the existence of the phenomenon in several species of Diptera (Chen et al., 1987; Lee et al., 1987; Czajka and Lee, 1990; Coulson and Bale, 1990, 1992), and subsequently it was found to be widespread in many other taxa, including additional species of Diptera (e.g. Li et al., 1999; Koveos, 2001) as well as Orthoptera (Wang and Kang, 2003), Hemiptera (Powell and Bale, 2004), Coleoptera (Burks and Hagstrum, 1999), Thysanoptera (McDonald et al., 1997) and Lepidoptera (Larsen and Lee, 1994; Kim and Kim, 1997; Song et al., 1997). It occurs in both diapause and non-diapause individuals of the phytopсид mite Euisesius finlandicus (Oudemans) (Bróufas and Koveos, 2001a). These studies also showed that a period of gradual cooling as well as a period spent at cool temperatures effectively promotes the response (e.g. Koveos, 2001; Powell and Bale, 2004). Nevertheless, the response is not universal (e.g. Vandyk et al., 1996), and it has not been found in antarctic habitats where sudden cold spells are to be expected at any time (Sinclair and Chown, 2003). Another cold-hardiness feature potentially linked with rapid cold hardening, although it has not been addressed in this context, is the time required to recover from chill coma, which appears to be a complex trait (e.g. Macdonald et al., 2004).

Recent studies have focused on two aspects, the ecological relevance of the rapid increase in hardiness, and the possible biochemical pathways by which it can be acquired. Kelty and Lee (1999) and Powell and Bale (2004) showed that the response is induced at the sorts of cooling rates experienced in nature, confirming that the concomitant increase in survival is adaptive in coping with short-term temperature changes there. Furthermore, in Drosophila the rapid hardening responses reduce the chill coma (chill torpor) temperature, so that behavior such as courtship and mating can be maintained (Kelty and Lee, 2001; Shreve et al., 2004). The response differs among different life stages in the way that would be expected if it is adaptive to the natural temperatures likely to be experienced by those stages (cf. Wang and Kang, 2003; Powell and Bale, 2004). Unlike the longer-term changes for extreme cold hardiness, such as cryoprotectant production, it might confer particular advantages in spring and fall (Coulson and Bale, 1990).

The mechanism(s) for these responses have not yet been elucidated. As confirmed by patterns of mortality in earlier work, the process of rapid cold hardening is clearly different from the longer-term cold hardiness acquired for winter. For example, resistance to cold shock in Drosophila is harmed by the protein-synthesis-inhibitor cyclohexamide, but the rapid cold-hardening response produced by pre-conditioning is not (Misener et al., 2001). Moreover, unlike winter hardiness, short-term hardening (as opposed to severe cold exposure itself) seems to exact no metabolic cost in terms of development, fecundity or longevity (Powell and Bale, 2004). Although changes in supercooling points during the diel have been reported in some species (e.g. Worland and Convey, 2001; Sinclair et al., 2003), they are slower than the rapid responses originally reported and appear to have a different basis; and we do not yet know if all of these supercooling-point changes are adaptive or reflect other daily changes. Rapid cold hardening is assisted by cholesterol in the diet, so that a membrane-based process might be involved (Shreve et al., 2003). Koštál et al. (2004b) provided evidence that chilling injury is due to a breakdown of membrane-based ion gradients; see also Zachariassen et al. (2004a) for a wider review. Broader studies of rapid cold hardening are called for, especially because this form of adaptation appears to be very widespread and might even prove to occur in the majority of insect species.

**SEASONAL CHANGES IN MITOCHONDRIA**

In larvae of the high arctic lymantrid moth Gynaephora groenlandica (Wocke), mitochondria appear to break down over winter (Kukal et al., 1989; Bennett et al., 2000), although they are rapidly restored upon warming. Work on this species and on the gall fly Eurosta solidaginis, using molecular assays for mitochondrial nucleic acids, confirmed the winter losses and suggested that mitochondrial proteins can be regenerated rapidly in spring from stable RNAs that are stored during the winter (Levin et al., 2003).
Electron micrography by J. R. Byers (pers. comm.) in the 1970s failed to detect mitochondria in sections of overwintering larvae of arctic chironomids and cool-temperate moths, but it was considered likely that these results reflected an artifact of tissue preparation. Attempts have since been made to find similar losses of mitochondria in other species, especially using the DNA stain DAPI (O. Kukal and H. V. Danks, unpublished data). However, although the results were suggestive, technical difficulties made some results potentially unreliable. Therefore, although I suspect that these mitochondrial reductions are widespread in overwintering insects, it has not yet been possible to confirm that suspicion. In particular, although the changes might relate to cold hardiness (as assumed by Kukal et al., 1989), they may serve a wider purpose. For example, such a mechanism would temporarily disconnect the respiratory and metabolic machinery of the cells, protecting the mitochondrial proteins from possible damage caused by changes in osmolality and intracellular chemistry, as well as buffering the organism from potentially spurious uses of energy caused by short-term increases in temperature. The changes might even be linked to mechanisms effecting the depression of metabolism well known for insects in diapause.

**SURVIVAL IN NATURE**

The adaptive nature of many seasonal responses has been assumed without any explicit work on their effects on natural survival. For example, coverings such as cocoons are presumed to protect against ice or cold, but in the main only anecdotal observations of their effectiveness are available (reviews by Danks, 2002, 2004b).

On a longer time frame, winter survival is related to habitat choice (review by Danks, 1991). For example, Pfrimmer and Merkl (1981) showed that mortality of the boll weevil *Anthonomus grandis* Boheman in sites in Mississippi varied widely. Most strikingly, samples taken over a 15-year period recorded survival from 0% to 100%, depending on the geographic location, on the overwintering site, and on the year. Such findings suggest that winter mortality stems not only from overwintering-site selection and cold hardiness but especially from how winter conditions and their temporal variations interact with the nature of individual microhabitats. These interactions are complex (e.g. Danks, 1978). Evidently, relevant features of the organisms have been studied more diligently than relevant habitat features.

We do have some detailed data from winter habitats, including even prolonged temperature records from polar sites (e.g. Bennett et al., 2003; Worland and Block, 2003) despite the logistical difficulties of working there. There are also useful data on how survival is modified by the interaction of other winter conditions with temperature. For example, the high specific heat of water and its high heat of crystallization buffer wet soils against temperature changes and freezing, increasing survival of a moth that overwinters in the soil (Baskauf and McCauley, 2001). Cold winter environments assist survival of gall makers exposed above the snow because energy is conserved at cold compared with mild temperatures (Irwin and Lee, 2000, 2003; Williams et al., 2003; cf. Parry, 1986). Several studies hint at the need for realistic knowledge about the environmental conditions that apply to a particular species in order to interpret its cold-hardiness adaptations. For example, Convey and Worland (2000) showed that the supercooling point and mortality of an Antarctic springtail, unlike a mite species in the same habitat, did not change in contact with ice.

These sorts of findings and the key relevance of natural survival suggest that future work should focus to a greater degree on ecological aspects, because experiments designed in the virtual absence of natural environmental data may lack ecological relevance. For example, laboratory work on aspects of cold hardiness is most meaningful if field sites have been examined in winter to find overwintering populations and to measure the range of conditions they experience (e.g. Danks, 1991). Knowledge of natural conditions can help to determine many experimental requirements, including the cooling rates appropriate for supercooling-point determinations, the temperatures and durations used to test survival, and the conditions suitable for the preconditioning of individuals or for assessing the effects of size or energy level on long-term survival.

**SELECTION FOR TYPES OF COLD HARDINESS**

Attempts have been made to analyse why some
species are freezing tolerant and survive winter ice in their bodies while others supercool to similar low temperatures. Potential linkages with desiccation were considered in an earlier section. Vernon and Vannier (2002) proposed the partly phylogenetic explanation, from very limited evidence, that freezing tolerance evolved more recently than freezing resistance. Several papers point out that the moisture regime determines the likelihood of whether an insect can avoid freezing, because inoculative freezing is likely in wet habitats (cf. Klok and Chown, 1997). This is generally true also in fresh water, but because most of these habitats are buffered and insulated (e.g. Danks, 1971a) few species are actually freezing tolerant there (Frisbie and Lee, 1997), with the exception of species in phylogenetically cold-hardy groups such as chironomid midges (Danks, 1971b). However, in some species cuticular antifreezes (Olsen et al., 1998) or cocoons (Danks, 2004b) withstand inoculation by ice. The relative value of supercooling versus freezing has also been considered from an energetics viewpoint (e.g. Irwin and Lee, 2002). A theoretical treatment by Voituron et al. (2002) concluded that freezing tolerance, or a mixed strategy, is more energy-efficient than supercooling, depending on the extent and variability of the subzero temperatures experienced. Zachariassen and Kristiansen (2003) correlated the likelihood of freezing tolerance—as opposed to supercooling—with winter activity (because potent ice nucleators are likely to be present in the gut contents of active individuals), large size (because freezing by homogeneous nucleation is more likely in larger species, see Zachariassen et al., 2004b), low temperature (because supercooling is time-dependent, e.g. Sømme, 1996), and more permeable cuticles (because when the surroundings are frozen unfrozen body fluids lose water more rapidly than frozen ones because of the greater vapour pressure differential, see above).

In many species the supercooling point has little value for understanding cold-hardiness (e.g. Broufs and Koveos, 2001b; Koštál et al., 2001; Renault et al., 2002), especially when species suffer chill injury and hence mortality at temperatures well above the supercooling point (e.g. Knight et al., 1986; Bale, 1987). The supercooling point also varies according to the method of measurement, notably the rate of cooling, as recently confirmed by Gehrken and Southon (1997). The temperature at which individuals freeze is not meaningful for small permeable soil invertebrates that survive by winter dehydration (see Cold hardiness and desiccation, Holmstrup et al., 2002a). A few species employ mixed strategies: normally they supercool but can survive freezing if it is initiated by subsequent inoculation (e.g. Koštál and Havelka, 2000). Finally, changes in supercooling points during the diel have been reported (see Rapid cold-hardening).

Some ideas about the forces selecting for particular types of cold hardness are available. Bale et al. (2000, 2001) supposed that freezing was more costly than supercooling (other things being equal), because individuals of some species lower their freezing points if frozen once, apparently serving to make a second freezing much less likely. Variable temperatures improve cold survival of some species (Coulson and Bale, 1996; Renault et al., 2004), apparently because at warmer temperatures some repair of potential chilling injury caused by lower temperatures is possible. Therefore, the detailed patterns of temperature and other conditions are critical. However, questions that are especially helpful to understand adaptations to specific natural habitats—what are the conditions, how variable are they, and how easily can future conditions be predicted from available environmental signals—have not yet been addressed explicitly for cold hardness because biochemical mechanisms have attracted more attention than wider ecological relevance. Moreover, even at the molecular level cold hardness requires many integrated adaptations. For example, genetic transformation may allow the production of AFPs in species not normally capable of producing them, but this ability alone does not confer cold tolerance (Tyshenko and Walker, 2004).

**CONCLUSIONS**

This review of selected topics illustrates especially how diverse are the patterns of cold hardness in insects. For example, many components of cold-hardiness are known at the biochemical level, including solutes such as polyols and sugars, and proteins of several sizes and configurations, that serve to bind water, control osmolalities, protect membranes, stop transcutaneous inoculation by ice,
control or limit nucleation, and prevent recrystallization. Some of these substances play multiple roles (e.g. polyols can enhance the action of anti-freeze proteins). Unexpected patterns of cold hardiness are known in some species, as in some arctic insects (reviewed by Danks, 2000b, 2004a) that survive freezing without known cryoprotectants or that supercool extensively.

The diversity of time frames on which different adaptations operate is underappreciated. Rapid cold hardening takes effect in minutes, mid-term acclimatization to cold may take days, and overall control of the life cycle by diapause (often linked to some degree with cold hardiness) is structured over even longer time frames. These differences are important, because they suggest that adaptations on such different scales might involve completely different mechanisms. Rapid cold hardening, for example, evidently has a different biochemical basis from “ordinary” cold hardiness. Such differences are not unexpected, because the adaptations respond to very different environmental challenges (for example, short-term chilling injury or incapacitation versus freezing injury), but even resistance to chilling injury in the short term versus the longer term has not been much compared at the biochemical level in insects. The many differences suggest that different types of cold hardiness may well have evolved independently according to environmental demands, and so may use different routes and are not always linked to one another. The strategy of cold hardiness by dehydration shown by small permeable soil invertebrates has little in common with some other responses to potential freezing. Cold hardness is linked to diapause in some species but not in others. Such differences suggest that attempts to discover cold-hardiness features in a given species will require multiple experimental approaches to tease out the component elements.

A second general conclusion from this review is that some seasonal responses are probably more common than has been realized. For example, it seems likely that both rapid cold-hardening and mitochondrial degradation are widespread, but neither has been widely studied. We have learned only recently that preconditioning to desiccation stress is effective in increasing subsequent tolerance of desiccation (Sjursen et al., 2001; Holmstrup et al., 2002b for the springtail Folsomia candida (Willem)). Such findings suggest that other as yet unknown elements of seasonal adaptations (related to cold, to drought, to energy balance, and even to developmental timing), including especially anticipatory responses, remain to be discovered.

Although the different responses rely on different mechanisms, the adaptations are coordinated. Suitable timing, resistance and other requirements are all necessary for a given organism to survive in real environments, reinforcing the opinion that a broad ecological approach is needed for understanding seasonal adaptations. This context includes resistance to adversity, such as cold hardiness and desiccation resistance, but also development and reproduction under favorable conditions, and the control of both by environmental signals. However, recent discussions about the adaptive value of different kinds of cold hardiness have not yet been put into a suitable environmental framework. Nevertheless, the number of relatively novel cold-hardiness patterns now being explored suggest that many advances in the subjects and concepts introduced here, as well as new discoveries in many fields, can be predicted.

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