THE ROYAL SOCIETY

Published online 17 June 2002

Insects and low temperatures: from molecular biology to distributions and abundance

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Insects are the most diverse fauna on earth, with different species occupying a range of terrestrial and aquatic habitats from the tropics to the poles. Species inhabiting extreme low-temperature environments must either tolerate or avoid freezing to survive. While much is now known about the synthesis, biochemistry and function of the main groups of cryoprotectants involved in the seasonal processes of acclimatization and winter cold hardiness (ice-nucleating agents, polyols and antifreeze proteins), studies on the structural biology of these compounds have been more limited.

The recent discovery of rapid cold-hardening, ice-interface desiccation and the daily resetting of critical thermal thresholds affecting mortality and mobility have emphasized the role of temperature as the most important abiotic factor, acting through physiological processes to determine ecological outcomes. These relationships are seen in key areas such as species responses to climate warming, forecasting systems for pest outbreaks and the establishment potential of alien species in new environments.

Keywords: insect cold hardiness; physiology; biochemistry; strategies; discoveries; applications

1. INTRODUCTION

'Insects survive at low temperature by adopting one of two main strategies: freeze tolerance and freeze avoidance by supercooling'. This statement, or one very similar to it, appears in the introduction of many papers on insect cold hardiness. As a framework within which to describe the function of the main biochemical compounds involved in insect overwintering, it is convenient to regard species as either freeze-tolerant or intolerant. Over the past 20 years, much more has become known about the synthesis and role of INAs, AFPs and polyols, including the fact that the same compounds can occur in both freeze-tolerant and freeze-avoiding species, but performing a different function. Progress in elucidating the molecular structure of these compounds has been more limited, but has provided valuable insight and confirmation of the structure–function relationships of INAs and AFPs (Duman 2001).

It is evident, but sometimes overlooked, that this 'two-strategy' concept relates primarily to the mechanisms by which insects tolerate or avoid freezing, whereas the majority of species live in climates where sub-zero temperatures are rare or never occur. Even where sub-zero temperatures are common in winter, the greatest threat to survival is 'cold' (rather than freezing), which varies in intensity depending on the temperature and the frequency and duration of exposure (Bale 1987, 1991).

This distinction between freezing and cold has led to recent 'reclassifications' of the effects of low temperature on insects (Bale 1993, 1996), the common aim of which is to introduce greater ecological reality to the narrow perspective of freeze tolerance and avoidance. This development has been stimulated by recognition of the problems that can arise when the cold tolerance of an insect is expressed in terms of the freezing temperature 'SCP' alone.

However, while on a global scale there are few insects that can tolerate freezing and similarly few that die only when they freeze, for those species inhabiting the temperate and colder climates, the ability to supercool is undoubtedly the most important component of the overwintering strategy. In most species, however, the SCP is the theoretical rather than the actual lower lethal limit, because death usually occurs from the cumulative effects of cold above the freezing temperature. Additionally, the long standing view of winter cold hardiness as a process of seasonal acclimatization linked to the synthesis of INAs and AFPs has recently been expanded with the discovery of rapidly inducible changes (within hours) in lower lethal limits (Lee *et al.* 1987) and the ability of some species to become more cold hardy by desiccation at sub-zero temperatures (Holmstrup & Sømme 1998; Worland *et al.* 1998).

A greater understanding of the ways in which temperature acts through physiological processes to determine ecological outcomes has formed the basis for a range of applications of insect thermal biology, including: species responses to climate warming (Bale *et al.* 2002), forecasting systems for pest and disease outbreaks (Werker *et al.* 1998), analysis of the establishment potential of alien species in new environments (Bale & Walters 2001), use of ice-nucleating behaviour as a novel method of pest management (Lee *et al.* 1998) and almost certainly in the near future, the transfer of insect genetic material to other taxonomic groups, including plants (Duman 2001).

This paper summarizes the principles of freeze tolerance and avoidance in insects, reviews recent progress in determining the molecular structure of the main

One contribution of 15 to a Discussion Meeting Issue 'Coping with the cold: the molecular and structural biology of cold stress survivors'.

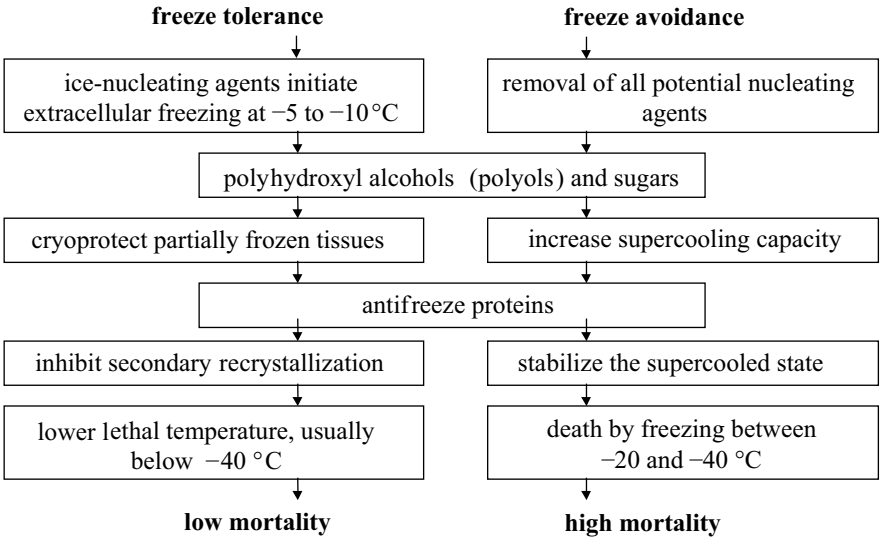

Figure 1. Schematic representation of the main biochemical components involved in the freeze-tolerance and freeze-avoidance strategies of insect overwintering.

biochemical compounds involved in these two strategies and outlines the ecological arguments underlying the introduction of new classification systems to describe the interactions between insects and low temperature. New insights on cold hardiness and overwintering are also described, together with examples of the applications of thermal biology in the wider fields of ecology and applied entomology. The paper is not intended to be a comprehensive review of the literature, but rather to reflect the current scope of insect cryobiology, especially with regard to natural systems, by reference to relevant examples and guidance to more specialized reviews.

2. STRATEGIES OF INSECT COLD HARDINESS

Cold hardiness is an attribute required by all insects that have to survive at certain times of the year or stages of the life cycle at temperatures below 0 °C (Bale 1989). For the two main strategies of cold hardiness, freeze tolerance and freeze avoidance by supercooling, the main biochemical components and their functions are now well understood, as summarized in figure 1.

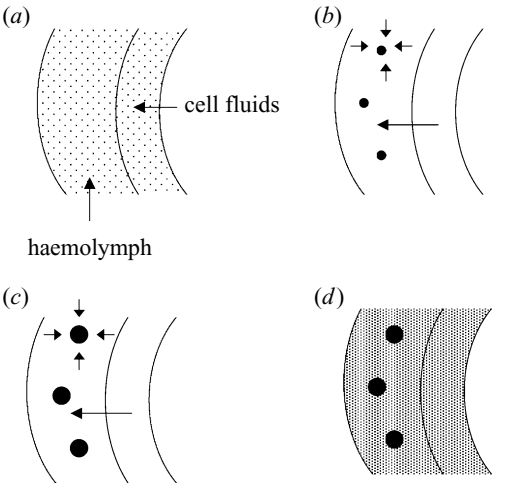

Figure 2. Schematic representation of the function of extracellular INAs in a freeze-tolerant insect. Black dots indicate sites of nucleation in extracellular areas and increasing ice masses at progressively lower sub-zero temperatures.

(a) *Freeze tolerance*

Freeze-tolerant insects contain three different types of compounds, INAs, polyols and sugars, and AFPs. Most freeze-tolerant species synthesize INAs (proteins) in autumn and early winter that initiate freezing in extracellular areas at temperatures above −10 °C. When ice freezes out of solution in extracellular spaces, water is moved progressively out of cells and across the cell membrane to re-establish the osmotic equilibrium, thus avoiding intracellular freezing, which is damaging and potentially lethal (figure 2), although fat body cells from the freeze-tolerant larva of *Eurosta solidaginis* can apparently survive intracellular freezing (Salt 1962; Lee *et al.* 1993*a*). Induction of freezing at high sub-zero temperatures also limits the level of supercooling such that the rate of ice growth and total amount of ice formed are both advantageously low. Nucleator activity and hence the freeze-tolerant condition is usually lost in spring, when insects then rely on supercooling for survival, though the risk of freezing under summer conditions is much reduced or absent.

Most freeze-tolerant insects accumulate polyols and sugars in winter, of which glycerol is the most common and abundant (Sømme 1964; Baust 1973; Zachariassen 1985). Other polyols found in freeze-tolerant species include sorbitol, threitol and erythritol and the sugars fructose, sucrose and trehalose. Polyols are able to counteract the suspected causes of freezing damage (mechanical damage at the site of freezing, electrolyte imbalance, critical cell volumes, recrystallization) by a range of mechanisms: increasing water binding capacity, thus reducing the rate of ice growth and total ice content; stabilizing protein structure and buffer electrolytes; reducing transmembrane water fluxes and maintaining cell volumes above critical minima (Baust 1973, 1982).

Polyols are an essential component in the freeze-avoidance strategy, but Zachariassen concluded from an

analysis of haemolymph osmolality and lower lethal temperatures that, above an osmolality of 1000 mosM kg^{-1}, freeze-tolerant species were increasingly more cold hardy, which is attributable to their more effective use of carbohydrate resources (Zachariassen 1985) and hence, freeze tolerance was likely to be the more successful strategy in the most extreme winter environments. The ability of some freeze-tolerant insects to survive in Alaska at temperatures below −80 °C (Miller 1982) supports this view.

AFPs lower the freezing point of water relative to its melting point (which is largely unaffected), thus producing a temperature difference between the freezing and melting points (sometimes described as 'thermal hysteresis'; Duman 2001). The discovery of AFPs in both freeze-tolerant and freeze-avoiding insects initially led to speculation about their role in the former group. AFPs may act to protect freeze-tolerant insects from the effects of sub-zero temperatures in autumn before INAs have been synthesized and the concentrations of colligatively active compounds, such as glycerol, are low. A similar effect would also be valuable in spring when higher temperatures lead to the loss of INAs (and hence the freeze-tolerant state) and the rapid metabolism of glycerol (Duman 1982; Duman *et al.* 1982).

An additional and arguably more important function of AFPs in freeze-tolerant insects has been identified following the discovery that similar compounds are highly effective inhibitors of ice recrystallization in polar fish (Knight *et al.* 1984). In freeze-tolerant insects, AFPs are thought to prevent the damaging effects of 'secondary recrystallization' that would otherwise occur during the warming phase at the end of winter.

As might be expected, given the diversity of insects, there are many exceptions to this general pattern of freeze tolerance. The most variable feature in freeze-tolerant insects is the difference between the freezing temperature and the lethal temperature. Thus, *Hydromedion sparsutum* in the Antarctic (Bale *et al.* 2000), *Syrphus ribesii* in the UK (Hart & Bale 1997) and *E. solidaginis* in North America (Bale *et al.* 1989*a*), all freeze above −10 °C, but the lethal temperatures of the three species are around 6, 20 and 40 °C, respectively, below the freezing temperature.

Other exceptions to this general pattern of freeze tolerance include species that retain ice-nucleating activity on a year-round basis, such as *Phyllodecta laticollis* (Van der Laak 1982) (although the lethal temperature is higher in summer than winter), species that are freeze-tolerant without any marked increase in polyols or sugars (*Xylophagus* spp (Ring 1982) and *Osmoderma eremicola* (Storey *et al.* 1993)) and species that freeze below −50 °C and survive (*Pytho deplanatus*; Ring & Tesar 1981; Ring 1982).

(b) *Freeze avoidance*

In freeze-avoiding insects, the winter hardy state is achieved as a two-stage process. First, there are a number of behavioural and physiological changes, including location of an overwintering site, reduced body water content, increased fat content and, particularly important, the cessation of feeding and evacuation of the digestive system to remove food material that might act as ice nucleators. At this time, other endogenous nucleators, such as those in the haemolymph, are also removed (Neven *et al.* 1986). In some species, these changes alone are sufficient to depress the SCP to −20 °C (Leather *et al.* 1993).

In the second stage, polyols and AFPs are synthesized that, in combination, depress the SCP relative to the melting point, increasing the supercooling capacity of the nucleator-free liquid compartments of the insect. Many freeze-avoiding insects possess a multicomponent cryprotectant system such as glycerol, mannitol and trehalose in *Cryptopygus antarcticus* (Sømme & Block 1982). These multifactor systems are thought to be advantageous, reducing the possible toxic effects associated with the concentration required for single components to produce the same level of cryoprotection (Baust 1973), or because different compounds may have different functions (e.g. increase supercooling capacity or reduce enzyme activity to promote energy conservation in winter (Zachariassen 1985)).

The concentration of polyols and sugars usually increases from autumn onwards, induced by progressively lower seasonal temperatures, with a corresponding decrease in the SCP through the colligative properties of the compounds, a pattern that is well illustrated in *Retinia resinella* (Hansen 1973); similar 'inverse' relationships between polyol concentration and SCP have been recorded in many other freeze-avoiding species (Sømme 1965).

In some insects, entry into diapause and the associated shifts in biochemical pathways lead to an initial accumulation of compounds that are known cryoprotectants (e.g. sorbitol), which then increase in concentration under a low-temperature trigger, as seen with diapausing pupae of *Pieris brassica*. The SCP of diapausing pupae reared at 2 °C, containing a higher concentration of sorbitol, was only 3 °C lower (−24 °C) than non-diapause pupae maintained at 20 °C. However, whilst both diapause and non-diapause pupae survived a two-week exposure at −5 °C, none of the latter group was able to develop when transferred to 20 °C (Pullin & Bale 1989).

It is now known that many species complete diapause in mid-winter, but development is suppressed by continuing low temperature. In such species, and those without a winter diapause, polyol concentrations and low SCP are usually maintained until spring, indicating that rising temperature triggers the reversible synthesis or degradation of the carbohydrates and consequent loss of cold hardiness (Leather *et al.* 1993). The relationships between diapause, cryoprotectant concentration, supercooling and survival are complex, but these data highlight the fact that the SCP should not be assessed in isolation as an indicator of cold hardiness in freeze-avoiding insects.

AFPs were originally described by Ramsay (1964) in *Tenebrio molitor* and thought to be involved in water balance. In freeze-avoiding insects, AFPs perform the dual roles of preventing inoculative freezing from external ice and inhibiting internal ice nucleators, both actions functioning to lower the SCP. The level of thermal hysteresis has also been shown to be inversely dependent on the size of the ice crystal in the observed sample.

The occurrence of AFPs in an overwintering insect was first reported by Duman (1977*a*,*b*) in the freeze-avoiding larvae of *Meracantha contracta*. The larvae do not accumulate polyols but show a thermal hysteresis in the haemolymph of 3.7 °C in mid-winter with a seasonal depression

of the 'whole body' SCP from −4 °C in summer to −11 °C in winter. The depression of the haemolymph freezing temperature to −5 °C in winter decreases the possibility of inoculative freezing across the cuticle from external ice in the overwintering site. Similar observations with other freeze-avoiding insects that overwintered in close proximity to ice, and knowledge from studies on polar fish (Raymond & DeVries 1977; DeVries 1980) led to the idea that AFPs function by adsorbing onto the surface of ice crystals, altering their structure, so preventing the crystals from acting as seeds for nucleation across the cuticle (inoculative freezing) until the temperature of the system reached its freezing point, thus producing the hysteretic effect. It is has also been shown that AFPs are able to inhibit ice nucleators in some freeze-avoiding species (e.g. *Dendroides canadensis*; Olsen & Duman 1997), again functioning to lower the SCP.

An analysis by Zachariassen (1985) showed that, in the majority of freeze-avoiding insects with AFPs, the haemolymph freezes between −5 and −10 °C in winter (hysteresis freezing point), whereas the whole-body SCP of many of the species was below −20 °C. Duman (1982) suggested that the ability of AFPs to 'poison' potential seed crystals may extend to embryo crystals, preventing them from reaching the critical size required to nucleate a supercooled liquid, thus lowering the SCP. Evidence to support this idea was obtained by Zachariassen & Husby (1982), who found a linear relationship between the hysteresis freezing point and ice-crystal size with haemolymph from the freeze-avoiding beetle *Rhagium inquisitor*, with smaller crystals producing a lower hysteresis freezing temperature. These studies identified the ability of AFPs to stabilize the supercooled state of overwintering insects.

3. STRUCTURE–FUNCTION RELATIONSHIPS

The structure–function relationships of insect cryoprotectants have focused on INAs and AFPs. To date, only a small number of species have been studied in any detail, so it is difficult to identify any general relationships. A recent review (Duman 2001) provides a comprehensive account in this area.

The first INA to be purified was from the freeze-tolerant hornet *Vespula maculata* (Duman & Patterson 1978), a 74 kDa hydrophilic protein found in the haemolymph, in which *ca*. 20% of the amino acids are either glutamate or glutamine. The most detailed studies have involved the freeze-tolerant cranefly *Tipula trivittata*, in which a haemolymph lipoprotein and two other proteins with ice-nucleator activity have been identified (Neven *et al.* 1989). The 800 kDa lipoprotein is 45% protein, 4% carbohydrate and 51% lipid, of which *ca*. 40% is neutral lipids and 12% phospholipids, including phosphotidylinositol. Both of the apoproteins and phosphotidylinositol are necessary for the lipoprotein to be ice-nucleator active. Cross reactivity has been detected via polyclonal antibodies raised to both the insect lipoprotein and bacterial ice nucleators, indicating some structural commonality (Duman *et al.* 1991). Also, both bacterial ice nucleators and the lipoprotein are only active above minimal concentrations, indicating that the nucleator activity is rare, or that aggregation and cooperation between molecules is required (Duman 2001). From the limited information currently available it appears that in *T. trivittata* both of the apoproteins (Apo-I and Apo-II) are necessary for the long chain structure of the lipoprotein to be formed with the correct positioning of the phosphatidylinositol, and that the inositol hydroxyls organize the embryo ice crystal (Duman *et al.* 1992; Duman 2001).

AFPs have so far been identified in over 40 species of insect, mainly beetles (Coleoptera), with only one report in butterflies and moths (Lepidoptera) and flies (Diptera). The consensus view is that AFPs adsorb onto the surface of potential ice crystals at specific sites, altering crystal growth into a highly curved (high surface free energy) front, thus requiring a lower temperature for crystal growth to proceed (Duman 2001). As with INA proteins, current information is limited and sequence data are available for only three species (*D. canadensis, T. molitor* and *Choristoneura fumiferana*). An analysis of the 22 known AFPs from *D. canadensis* and *T. molitor* showed similar structures, with varying numbers of 12- or 13-mer repeats with molecular masses of *ca*. 8.3–12.5; all of the cysteine residues were disulphide bridged and the repeats in the centre of the protein were highly conserved, indicating that they are important for AFP actvity. Over half of the residues are identical in all 22 AFPs with all C residues conserved. The C residues have the important role of stabilizing the protein and aligning the residues that bind (by hydrogen bonding) to ice or ice-nucleating sites (Duman 2001).

In studies on *D. canadensis,* thermal hysteresis actvity was observed to be greater in haemolymph than in a purified AFP from the insect. Following the observation that anti-AFP polyclonal antibodies increased the thermal hysteresis activity of the purified AFP (Wu *et al.* 1991), an enhancer protein was subsequently identified in *D. canadensis* that produced the same effect (Wu & Duman 1991). The enhancer protein was also INA active and bound to the AFP; INAs from other insects also produced the enhancing effect in the purified *D. canadensis* AFP (Lin *et al.* 1998). Duman (2001) suggests that the AFPs and INAs form an AFP–INA complex by binding at the ice-nucleating site, which blocks a larger surface of the ice crystal, thereby increasing the level of thermal hysteresis. In a further interesting discovery, glycerol, the most common polyol in overwintering insects (and present in *D. canadensis*), was also found to enhance the thermal hysteresis activity of purified AFP solutions, although the mechanism is unknown (Lin *et al.* 1998; Duman 2001).

4. NEW CLASSIFICATIONS OF INSECT OVERWINTERING STRATEGIES

The long-standing view that overwintering insects can be conveniently described as freeze-tolerant or freeze-intolerant (freeze-avoiding) (Salt 1961) has gained credence as the roles of INAs, polyols and AFPs in these 'alternative' strategies have become more clearly understood. There is no doubt that some insects are freeze-tolerant, other species die only when they freeze and many species would be unable to survive in temperate and colder climates without the ability to supercool.

The problem with the freeze-tolerant–intolerant system lies mainly in the reliability of the SCP as an indicator of cold tolerance, especially when measured in isolation. It

is now widely recognized that, even in polar species, the SCP of acclimatized insects is below the lowest winter temperatures (especially where snow cover provides a thermal buffer), hence the greatest threat to survival is the cumulative effect of prolonged exposure in the supercooled state rather than freezing *per se*.

In an attempt to combine the differential effects of 'freezing' and 'cold', and to include species across all climatic zones from the tropics to the poles, Bale (1996) proposed that insects could be conveniently 'classified' into five groups representing a continuum from the most to the least cold-hardy. The first two, freeze tolerance and freeze avoidance by supercooling, are identical to the two 'original' strategies, except that 'freeze avoidance' includes only those species in which there is little or no low-temperature mortality in the absence of freezing; the autumnal moth *Epirrita autumnata* that overwinters in the egg stage on birch trees in northern Scandinavia is one of few species so far described that exemplifies this group (Tenow & Nilssen 1990). The significance of this new system lies in the fact that on a worldwide basis, the vast majority of species are neither freeze-tolerant nor freeze-avoiding, within the strict definition of the latter group given above, as characterized by species such as *E. autumnata*.

The three 'new' groups proposed by Bale (1996), 'chill tolerant', 'chill susceptible' and 'opportunistic survival', all exhibit some 'pre-freeze' mortality. The chill tolerant category contains species with low SCP (typically −20 to −30 °C) and a high level of cold tolerance, but is distinguished from the freeze-avoidance group by the occurrence of some mortality above the SCP, which in most cases only becomes apparent with decreasing temperatures and increasing periods of exposure. Bale (1996) recognized that this was a very large group including polar species such as the mite *Alaskozetes antarcticus*, with a winter SCP of −30 °C and 73% survival after 100 days at −20 °C (Cannon & Block 1988) and the temperate weevil *Rhynchaenus fagi*, with an SCP of −25 °C, but only 26% survival after 56 days at −15 °C (Bale 1991). These two species differ in their relative cold hardiness but both show some mortality in prolonged exposures at sub-zero temperatures above the SCP and are therefore placed in the same category.

Chill-susceptible species may also supercool to very low temperatures, but die after very brief exposures (minutes or hours) at temperatures often substantially above the SCP. Overwintering anholocyclic clones of aphids such as *Myzus persicae* illustrate the characteristics of this group with lethal temperatures 15 to 18 °C above the SCP (−25 °C) in exposures of only 1 min (Clough *et al.* 1990; Howling *et al.* 1994).

The last group, opportunistic survival, describes species that are unable to survive below the threshold temperature for development and must therefore 'opportunistically' locate sheltered overwintering sites. Pupae of the housefly *Musca domestica* freeze at −15 °C, but there is 100% mortality after 5 days at 0 °C. Overwintering houseflies therefore seek out thermally buffered habitats such as animal housing (Coulson & Bale 1991).

The central argument underlying this 'reclassification' (Bale 1996) is that, for the majority of insects inhabiting climatic zones with a winter season, the risk of chilling injury and death is greater than the risk of freezing injury and death. The advantages of the system are that it classifies species according to the observed limits of their cold hardiness, distinguishes between freezing and chilling as a stress to be tolerated and a cause of death and has worldwide applicability. These 'new classes' have become widely used in recent literature and stimulated further discussions on the diversity of responses of insects to low temperature (Sinclair 1999; Nedvěd 2000).

5. RECENT DISCOVERIES

(a) *Loss of freeze tolerance after freezing*

Freeze-tolerant species often occupy overwintering sites where the temperature fluctuates at intervals above and below the SCP, indicating that these insects undergo irregular freeze–thaw cycles in winter, increasing the risk of cryo-injuries associated with these processes. The effect of repeated freeze–thaw events were recently investigated in two freeze-tolerant insects and produced an unexpected result. Larvae of the sub-Antarctic beetle *H. sparsutum* are weakly freeze-tolerant, with a mean SCP of -4.2 ± 0.2 °C and range from −1.0 to −6.1 °C; about 95% of larvae are killed by a 15 min exposure at −10 °C (Bale *et al.* 2000). When larvae were cooled to −6.5 °C (below the lowest SCP of the sample population) on 10 consecutive occasions at 1- and 4-day intervals, it was assumed that every larva would freeze every time. However, when the SCP of all surviving larvae was reassessed after the 10 exposures to −6.5 °C, some larvae remained unfrozen at −12 °C, a depression of 6 °C or more below their individual SCP values on day 1. Also, the lower the 'new' SCP, the more likely the larvae were to be killed by freezing (Bale *et al.* 2001). Evidently, when larvae freeze for the first time, a proportion of the population lower their SCP, such that they are then less likely to freeze on subsequent exposure to sub-zero temperatures, but are more likely to die if freezing occurs. To test this hypothesis, in a further experiment, larvae were cooled at daily intervals to their individual SCP to ensure that every larva froze every day. With this treatment, the SCP was again lowered in some larvae after one or more freezing events, but only by 2 to 4 °C, and no larva showed SCP around −12 °C or lower; in other larvae (which had the highest initial SCP around −2 °C), the SCP remained consistent in each daily freezing event, and these were the only larvae to survive the entire experiment (figure 3; Bale *et al.* 2001). It appears, therefore, that after undergoing one freezing event, further exposure to sub-zero temperatures lowers the SCP of *H. sparsutum* larvae, but the greatest depression occurs when the larvae do not actually freeze again. A further conclusion from these data is that after one or more sub-zero exposures or freezing events, the larval population becomes segregated into different groups; one group freezes on subsequent occasions at much the same temperature and retains the freeze-tolerant state, whilst another group freezes at a lower temperature and, given the greater likelihood of death should freezing occur, have in effect become 'freeze-avoiding' and are then reliant on the newly acquired extended supercooling for survival. Interestingly, when larvae supercool to below −12 °C, they may be more cold hardy than their freeze-tolerant

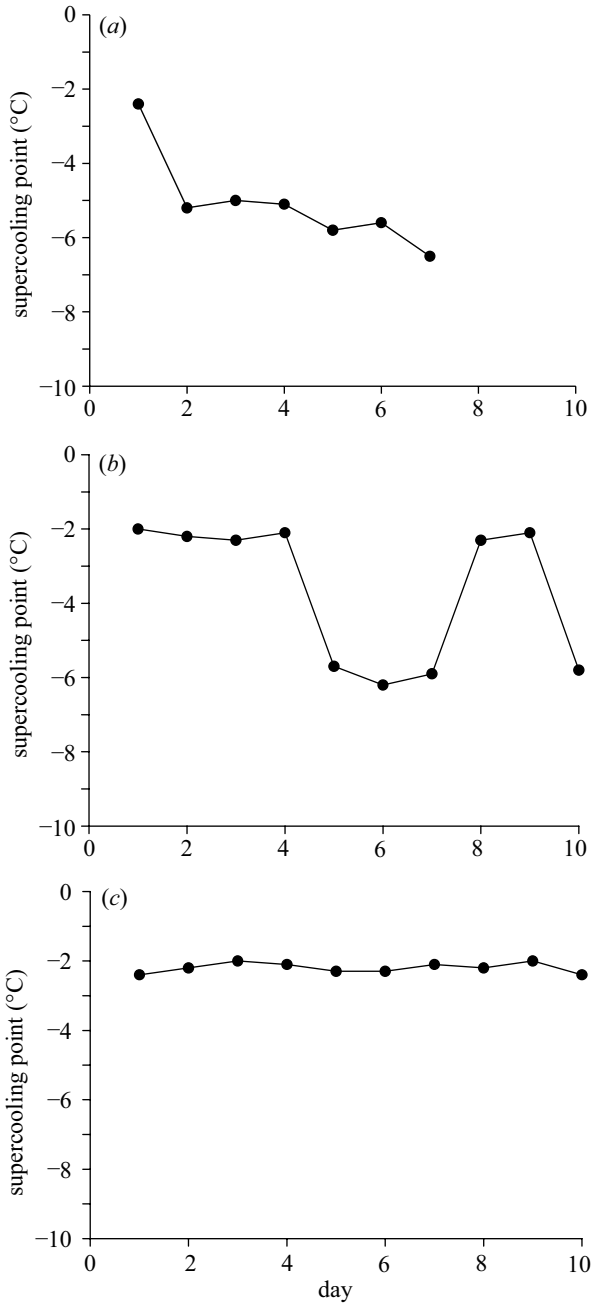

Figure 3. Patterns of change in the SCP of larvae of *Hydromedion sparsutum* frozen at daily intervals. Larvae in (*a*,*b*) both died after sequential freezing; the larva in (*c*) survived after freezing 10 times.

counterparts, where −10 °C is the lower lethal limit (Bale *et al.* 2000).

A similar pattern of results has now been found with the more strongly freeze-tolerant larvae of the hoverfly *S. ribesii.* The mean SCP of an acclimatized population was −7.6 ± 0.4 °C, with a range from −5.0 to −9.5 °C. When these larvae were cooled to their individual SCP at daily intervals on five occasions, the SCP of the majority of the sample was significantly depressed after the first freezing event. The mean SCP of larvae that were alive after being frozen five times was −8.3 ± 0.6 °C with a range from −5.4 to −21.5 °C, but only those larvae that froze at above −10 °C each day (17% of the original sample) survived to the end of the experiment (C. L. Harris and J. S. Bale, unpublished data).

It is known that some species switch strategy from freeze tolerance to avoidance in different years (e.g. *D. canadensis*; Horwath & Duman 1984), but with *H. sparsutum* and *S. ribesii* this change is inducible in a proportion of the population within one day, apparently in response to a single freezing exposure. It is unknown whether this is a common phenomenon in species that have been categorized as 'freeze tolerant' on the basis of one laboratory freezing event. The response appears to be an example of 'bet hedging' in which a dual overwintering strategy maximizes winter survival at the species level. Freeze tolerance may be the most efficient energy conservation strategy, but only if the insect remains frozen for the whole winter. In areas where temperatures fluctuate above and below the 'initial' SCP of the overwintering insects, there are likely to be costs associated with repeated freezing and thawing such that a switch to freeze avoidance in a proportion of the population may be ecologically advantageous.

(b) *Cold tolerance through desiccation over ice*

In a study on earthworms, Holmstrup (1992) found that overwintering cocoons lost water because of the difference in water vapour pressure between the supercooled body fluids and surrounding ice, leading to an increase in cold tolerance. This response was described as a 'protective dehydration strategy'. The ability to tolerate lower temperatures in a desiccated state has been recorded in a number of invertebrates, including nematodes, tardigrades and brine shrimps, and has recently been described in an insect, the arctic collembolan *Onychiurus arcticus* (Worland *et al.* 1998).

Summer populations of *O. arcticus* have a mean SCP of −6.1 °C and are killed by freezing (Block *et al.* 1994). Soil temperatures in the winter microhabitat can fall to around −25 °C, requiring a substantial seasonal increase in cold hardiness. When samples of *O. arcticus* were cooled from 0 to −5.5 °C over 14 days on moist paper, the body water content decreased from 70 to 40% of fresh weight, mainly due to the loss of osmotically active water, and the SCP dropped from −7 to −17 °C. Over the same time-period, glycogen levels deceased from 160 to 7.7 nmol glucose equivalents mg^{-1} protein and the trehalose concentration increased from 0.9 to 94.7 $\mu g\, mg^{-1}$ fresh weight. It was estimated that if exposure over ice had been continued to −6.7 °C, the SCP would have been further lowered to around −27 °C, below the minimum temperatures usually encountered in the Arctic winter habitat of *O. arcticus* (Worland *et al.* 1998).

At present, the extent to which overwintering insects utilize this desiccation-based strategy is unknown, but it may not be common. It is clearly most relevant to species that overwinter in close association with ice and in habitats with stable sub-zero winter temperatures. Interestingly, other polar Collembola (e.g. *C. antarcticus*) do not show the same response (M. R. Worland, personal communication).

(c) *Rapid cold hardening*

The process of rapid cold hardening was first described by Lee *et al.* (1987) in the flesh fly *Sarcophaga crassipalpis*,

and has since been reported in a number of other dipteran species (e.g. *M. domestica* (Coulson & Bale 1990) and *Drosophila melanogaster* (Czajka & Lee 1990)). Experiments designed to detect rapid cold hardening follow a similar format in which insects are transferred directly from their culture temperature (e.g. 25 °C) to a sub-zero temperature (e.g. −10 °C), at which 100% mortality occurs in an exposure of fixed duration e.g. 2 h. Similar samples are then transferred to progressively higher temperatures until a temperature is identified at which *ca.* 15% survival occurs after 2 h following direct transfer from the culture temperature (e.g. −8 °C). This is the 'discriminating' temperature at which to test for a rapid cold-hardening response. Further samples are then transferred from the culture temperature to 0 °C (the most commonly used 'rapid cold-hardening' temperature), for different periods of time, usually from 15 min up to 4–6 h, and then to −8 °C for 2 h. When the time spent at 0 °C is optimized in terms of the rapid cold-hardening response, survival on subsequent transfer to −8 °C is usually similar to unexposed controls at around 90%. It has also been shown that this rapid increase in cold tolerance can be induced by slow cooling from the culture temperature, such that the time spent between 5 and 0 °C, 2 h for instance, is the same as that required in a constant exposure at 0 °C to produce a significant increase in survival. The rapidly acquired increase in cold hardiness is, however, equally rapidly lost when insects 'acclimatized' at 0 °C are placed back at the culture temperature for a brief period of time before a direct transfer to −8 °C (Coulson & Bale 1990). In further experiments with the housefly *M. domestica*, Coulson & Bale (1991) found that exposure under conditions of anoxia at room temperature also produced a rapid cold-hardening response.

The physiological and biochemical processes involved in rapid cold hardening are as yet unresolved, although some changes in glycerol concentration have been detected in surviving insects. The induction of rapid cold hardening following anoxic exposure indicates that this may be a more generalized stress response, in which shifts in biochemical pathways result in the rapid synthesis of a compound that protects or desensitizes the insect to the otherwise lethal effects of the cold shock that arises from the sudden temperature change of more than 20 °C. Here, it is worth noting that the rapid increase in cold tolerance produced by 2–4 h at 0 °C is modest (*ca.* 3 °C), and although survival at −8 °C may be increased from *ca.* 10 to 90%, most insects are killed if transferred from 0 to −10 °C. Likewise, survival at −8 °C can only be extended from 2–10 h before mortality rapidly increases (Coulson & Bale 1990).

A different type of rapid cold hardening has recently been reported in certain Antarctic microarthropods (Worland & Convey 2001). One of these species, *C. antarcticus*, shows a 'classic' seasonal change in SCP between a summer 'high group' (mean −7 °C) and a winter 'low group' (mean −25 °C). This distinct bimodal pattern in SCP has been attributed in part to the role of food particles in the gut of summer feeding insects acting as ice nucleators and the absence of such material in the winter starved condition. Supporting evidence for this theory has been obtained by the starvation of summer-collected insects at low temperature with an increasing proportion of the sample moving from the high to the low group of SCP (Sømme & Block 1982). When the SCP of a summer field population was measured at hourly intervals, the mean SCP increased from around −21 to −10 °C over a 4 h period as the microhabitat temperature rose from 0 to 10 °C. On a different day with a similar rapid change in temperature from 0 to 11 °C, there was a shift in the mean SCP from a predominantly low to a predominantly high group over a 4 h period. These rapid changes in the SCP could also be produced by a simulated thermoperiod, but were not related to feeding status nor dehydration of the insects (Worland & Convey 2001). The response in these Antarctic species is also independent of the time of day, as there are no regular diurnal temperature fluctuations as seen in temperate climates. Apart from the observation that such large-scale shifts in the SCP in the absence of gut evacuation calls into question the 'gut clearance' hypothesis as a prerequisite for enhanced winter supercooling, this is a further illustration of the ability of insects to respond to rapid changes in their thermal environment by as yet, unidentified mechanisms.

(d) *Resetting of thermal thresholds*

The discovery of rapid cold hardening demonstrated the ability of insects to depress their lethal temperature in time-scales of a few hours under specific laboratory regimes. Whilst the response was potentially advantageous, two main reservations have been raised about the relevance of rapid cold hardening to natural environments (Coulson & Bale 1990). First, in nature, insects do not experience the rate of change in temperature that occurs during the direct transfer from 25 or 20 °C to 0 or −8 °C. Second, most species in which rapid cold hardening has been detected (*S. crassipalpis, M. domestica*) are killed by the 'cold shock' resulting from transfer to −10 °C, although such sub-zero temperatures would not normally be encountered in the overwintering habitats of these insects; and in any case, higher temperatures are known to become lethal if exposures are extended from a few hours to a few days (e.g. more than 90% of *M. domectica* pupae die after 5 days at 0 °C; Coulson & Bale 1990). The subsequent discovery that insects can 'reset' other critical thermal thresholds, such as flight and chill coma (coordinated movement), on similarly rapid time-scales is therefore arguably of wider ecological importance.

When samples of *D. melanogaster* were cooled at very slow 'ecologically relevant' rates (0.05 and 0.1 °C min^{-1}), survival after 1 h at −7 °C was higher than in flies cooled at 0.5 and 1 °C min^{-1} to the same exposure temperature. In addition, the chill coma (cold torpor) temperature was over 2.5 °C higher (6.5 °C) in flies cooled at 1 °C compared with 0.1 °C min^{-1} (3.9 °C) (Kelty & Lee 1999).

In further experiments, when *D. melanogaster* were maintained in a 24 h cycling thermoperiod between 23 and 9 °C (mimicking the diurnal high and low temperature variation in the local Ohio, USA habitat) and removed at the high-temperature point of the cycle (23 °C) and at the end of the 'cooling phase' (9 °C), survival after 1 h at −7 °C increased from 5 to 63%. In addition, this rapid cold hardening increased with each exposure to the thermoperiod, reaching 89% survival at −7 °C (1 h) after six daily cycles. Also, with the same diurnal thermoperiod, the chill coma temperature decreased

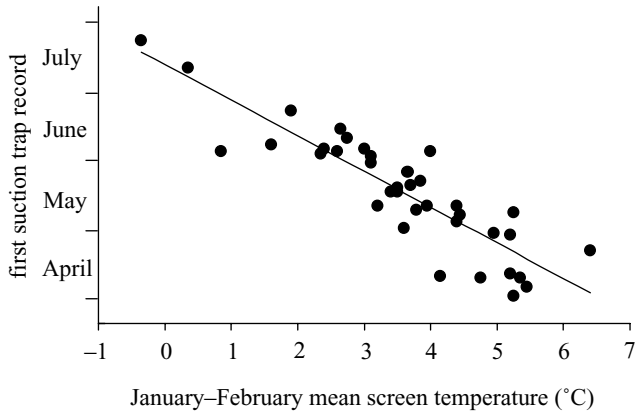

Figure 4. Relationship between the January–February mean temperature and the time of first flight in the aphid *Myzus persicae* from 1965–2001 ($r^2 = 0.786$, $p < 0.001$).

from 7.9 °C at the high point of the cycle to 6.0 °C at the end of the cooling phase, but unlike the lethal temperature, did not change with further daily cycles. The changes in the lethal and other thermal thresholds could not be related to levels of heat-shock proteins (Hsp70) or polyols and sugars (Kelty & Lee 2001).

Thermal thresholds for movement and flight are clearly important in population dispersal, migration and interactions with natural enemies, with relevance to the selection of species and strains for use in biological control and, in the longer term, the possible genetic modification of advantageous traits.

6. APPLICATIONS OF INSECT LOW-TEMPERATURE BIOLOGY

(a) *Forecasting pest outbreaks*

The annual abundance of some insects is strongly influenced by the severity of the preceding winter. This is particularly the case for the anholocyclic (asexual) clones of pest aphids such as *M. persicae* and *Sitobion avenae* (Bale 1989). The eggs of holocyclic (sexual) clones have a low SCP and are very cold hardy (Strathdee *et al.* 1995), to the extent that their survival is largely independent of interannual variablity in winter climate. However, the overwintering aphids of asexual clones, although having a low SCP (typically around −25 °C), die at much higher temperatures, with 50% mortality occurring around −7 to −10 °C, depending on the species, age group and acclimatization state (Knight & Bale 1986; Clough *et al.* 1990; Howling *et al.* 1994). The discovery of such extensive pre-freeze mortality in *M. persicae* and related species has provided an ecophysiological linkage between post-winter abundance, population increase and the crowding threshold leading to migration, explaining the differential abundance of these aphids after mild and severe winters (Harrington *et al.* 1991; Zhou *et al.* 1995, 1996). This interrelationship between winter climate and survival forms the basis for forecasting the timing of the annual 'spring' migration of *M. persicae* in the UK, and hence the likely damage and necessity of pesticide application (figure 4). The forecast is relevant to both potatoes and sugar beet (*M. persicae* is a virus vector to both crops), but is particularly important in the sophisticated prediction system now used for the 'yellows' viruses of beet (Werker *et al.* 1998).

(b) *Impacts of climate warming*

The impacts of climate warming on insects are complex for a number of reasons: (i) 'climate change' involves a combination of abiotic factors (temperature, carbon dioxide, precipitation), which do not necessarily operate in tandem, or produce a consistent response in insects from different feeding guilds; (ii) for herbivorous species, plant-mediated indirect effects such as changes in the C : N ratio may be as important as the direct effects of higher temperatures; and (iii) experimental systems sometimes exclude higher trophic levels, such as natural enemy species, from the investigation.

A recent review (Bale *et al.* 2002) focuses on the direct effects of rising temperatures on herbivorous insects with examples of the ways in which the predicted climate warming may affect species abundance, distribution, synchrony with host plants and interactions with photoperiod (the dominant entraining cue of life cycles). An interesting contrast was made between the probable main effect of climate warming on temperate insects (increased winter survival) and polar species (extension of the summer favourable season for growth and reproduction).

Studies on the arctic aphid *Acyrthosiphon svalbardicum* illustrate the role of temperature as a factor limiting both the annual abundance and local distribution of an insect. *Acyrthosiphon svalbardicum* has a highly specialized life cycle (Strathdee *et al.* 1993*a*) in which the fundatrix morph (which hatches from the overwintering egg) is able to give birth directly to the two sexual morphs (male and oviparae), a generation sequence that is prevented in temperate aphids by the interval timer mechanism. The sexual morphs then mate and lay the overwintering eggs. The fundatrix also produces a small number of viviparae as its first-born offspring, which on maturation produce exclusively a further generation of sexual morphs. The aphid therefore produces a minimum of two and a maximum of three generations in the arctic summer. There is a 'trade-off' between the fundatrix investing resources in some viviparae and the risk that the thermal budget (day degree requirement) for three generations will not be met, in which case the sexual morph progeny of the viviparae will die before reaching maturity and fail to produce the 'extra' overwintering eggs.

Monitoring the phenology of field populations of *A. svalbardicum* indicated that around 470 day degrees above 0 °C were required post-egg hatch for the dominant fundatrix → sexual morph → overwintering eggs life cycle route, but a minimum of 710 day degrees for the fundatrix → viviparae → sexual morphs → overwintering eggs sequence. Analysis of climate records for over 20 years indicated that only in 6 years was the thermal budget sufficient for the third generation to become reproductively active and there was only 1 year in which they would make a significant contribution to the number of overwintering eggs.

When populations of *A. svalbaridicum* were enclosed within perspex cloches, raising the average daily temperature by 2.8 °C, the total available summer thermal budget (day degrees) above 0 °C increased from around 600 to 800. As a consequence, all three generations of the aphid

matured inside the cloches, producing an 11-fold increase in the number of overwintering eggs compared with the control (Strathdee *et al.* 1993*b*).

The distribution of *A. svalbardicum* was found to be affected by temperature on different spatial scales. Over a distance of 10–15 km along Kjongsfjord (Svalbard), aphid abundance changes from abundant → rare → absent, although the host plant (*Dryas octopetala*) was common at all sites. The probability of site occupancy decreased from the inner to the outer fjord and from the shore to inland, both representing thermal gradients from higher to lower temperatures and, most importantly, earlier snow clearance, effectively increasing the length of the summer season for both plant and insect development. A striking demonstration of the same effect, but on a much smaller scale, was seen on a south-facing slope only 3.7 m high. In mid-summer, aphid population development was significantly more advanced at the top than at the bottom of the slope, representing the progressive pattern of snow melt (Strathdee & Bale 1995).

An example of the effects of winter climate on temperate aphids is discussed in § 6*a* and a more detailed comparative analysis of the impacts of climate warming on arctic and temperate species is presented in Bale (1999).

(c) *Establishment of invasive species*

The differential cold tolerance of tropical and polar insects is an indication of the stress that may be encountered when species are transferred between different climatic zones. Whilst low temperature is an obvious threat to tropical insects, there is also evidence that some polar species would not be able to survive the higher temperatures of a temperate climate; larvae of the sub-Antarctic beetle *H. sparsutum* died out after six weeks at 15 °C (Bale *et al.* 2000).

The concept of 'thermal screening' has been used to assess the establishment potential of non-native insects in the UK. Introductions of alien insects can occur accidentally, as with the transport of crop pest species on plant material. The western flower thrips *Frankliniella occidentalis* was first reported in the UK in 1986 and is now widely distributed throughout Britain as a naturalized pest on a range of glasshouse crops (McDonald *et al.* 1997, 1999).

A comparative analysis of the thermal requirements (for development) and tolerances (for winter survival) of closely related resident and non-resident pest insects with similar overwintering biologies identified a lack of winter cold hardiness as the probable explanation for the failure of the dark sword grass moth (*Agrotis ipsilon*) to establish permanent populations in the UK. Larvae of *A. ipsilon* are less cold hardy than the resident *Noctua pronuba* across a range of indices (freezing temperature or SCP, $LTemp_{50}$ = temperature at which 50% mortality occurs in a fixed-time exposure, $LTime_{50}$ = time after which 50% mortality occurs in a fixed-temperature exposure) and died out in the field in winter after only six weeks. A similar pattern of data was obtained with the resident whitefly, *Aleyrodes proletella* and the non-resident glasshouse pest, *Bemisia tabaci.*

A strong correlative relationship was found between survival in the laboratory at −5 °C and in the field in winter across UK resident (*N. pronuba, A. proletella*), non-resident (*A. ipsilon, B. tabaci*) and recently naturalized (*F. occidentalis*) species (figure 5; Bale & Walters 2001), which in future, may allow a rapid laboratory assessment of the establishment potential of alien pest species.

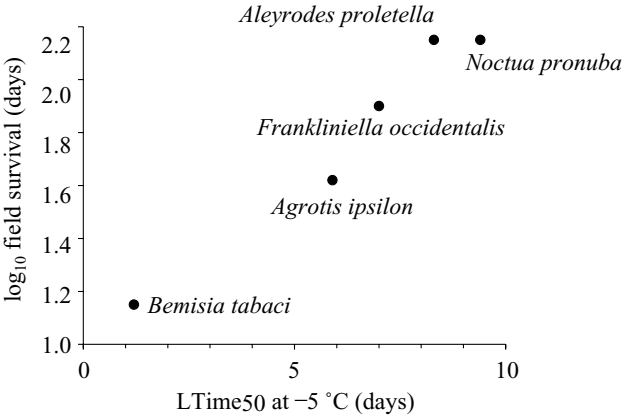

Figure 5. Relationship between duration of survival in the laboratory at −5 °C and in the field in winter for a range of resident and non-resident UK pest species.

In some instances, the introduction of alien species is intentional, such as the use of non-native predators and parasitoids to control glasshouse pests. The majority of these natural enemy species originate from tropical, semi-tropical or Mediterranean climates, leading to the assumption that any escaping individuals would rapidly die out in UK winters. In fact, this is not true, and in the case of the recently introduced predatory mite *Amblyseius californicus* (for the control of glasshouse red spider mite *Tetranychus urticae*), a diapausing ability present in a small proportion of the source population has become rapidly selected for in escaped individuals, leading to the local establishment of outdoor 'wild' populations in Britain (Jolly 2000).

A similar thermal-screening protocol is currently being applied to candidate biological control agents (the predatory coccinellid *Dephastus catalinae* and mirid *Macrolophus caliginosus* and the parasitoid *Eretmocerus eremicus*), involving an assessment of the developmental threshold, cold tolerance and diapausing ability so that 'high risk' species can be identified prior to release.

(d) *Ice-nucleating micro-organisms as biological control agents*

Ice-nucleating micro-organisms are known to cause frost damage in plants (Lindow 1983, 1995) and have also been found in decaying leaves. It might be expected therefore that such micro-organisms would form part of the natural flora of the digestive system of herbivorous and detritivorous insects and their predators, and this has now been confirmed for both bacteria and fungi in a range of insect species (Lee *et al.* 1993*b*). It has also been suggested that these intestinal micro-organisms may act as INA in freeze-tolerant insects in which they have been found, such as *Chilo suppressalis* (Tsumuki *et al.* 1992) and *H. sparsutum* (Worland & Block 1999).

Ice-nucleating active bacteria synthesize large outer-membrane proteins that are thought to aggregate and act as a template for the formation of ice nuclei (Wolber & Warren 1989; Mueller *et al.* 1990). This ice-nucleating

ability is retained after treatment with antibiotics or ultraviolet radiation (Maki *et al.* 1974; Kozloff *et al.* 1983). In a series of studies (summarized in Lee *et al.* 1993*b*, 1996, 1998), it was recognized that INA bacteria and fungi that raised the winter SCP of freeze-avoiding pest insects could be exploited in the biological control of such species. The model system used to develop and test the potential of this approach has been the Colorado beetle *Leptinotarsa decemlineata*, a major defoliating pest of potato in the USA and Europe, which is resistant to a wide range of insecticides. Following the senescence of the above-ground potato foliage (usually induced with a chemical desiccant), adult beetles burrow into the soil to overwinter.

Overwintering beetles supercool to between −7 and −9 °C. Although this is a high SCP for an insect killed by freezing, its winter soil habitat is apparently an adequate buffer against sub-zero air temperatures. When beetles were treated with a topical application of a killed preparation of INA *Pseudomonas syringae*, the mean SCP was raised from −7.6 to −3.7 °C (Lee *et al.* 1994). Subsequently, it was shown that application of live suspensions of *P. syringae*, *P. fluoresens* and *P. putida* were all effective in raising the SCP of *L. decemlineata* (Costanzo *et al.* 1998). It has now been demonstrated that ingested INA bacteria of *P. fluorescens* and *P. putida* fed to prehibernation beetles, retained similarly high nucleator activity 10 weeks later, even though the insects had evacuated their gut contents prior to burrowing into the soil (Costanzo *et al.* 1998). Also, field experiments have shown that INAs ingested by *P. fluorescens* in late summer are still active seven months later (Castrillo *et al.* 2001). The significance of these results is that the elevation of the SCP from −7 to −3 or −4 °C will cause the freezing death of many beetles when sub-zero temperatures are experienced in the soil microhabitat several months later.

The planned pest-management scheme envisages that after the normal application of chemical desiccants to potato foliage, beetles will move to unsprayed areas around the field margin and feed on plants treated with INA bacteria prior to overwintering. Current studies are aimed at increasing the retention time of INA bacteria in treated insects, developing molecular probes to identify ice-nucleating genes in insect digestive systems, selecting and enhancing of the most effective INA strains and minimizing any deleterious effects on non-target species (R. E. Lee, personal communication). The potential of this approach for the Colorado beetle has yet to be fully tested, but INA bacteria have also been shown to raise the SCP of other overwintering pest insects including the pear psyllid *Cacopsylla pyricola* (Lee *et al.* 1999) and the Russian wheat aphid *Diuraphis noxia* (Armstrong *et al.* 1998).

7. CONCLUSIONS

The rate of increase of an insect population is determined by a wide range of factors, the most important of which act directly on the rate of development, fecundity and length of reproductive life. The faster the development to adult, the more offspring are produced and the longer that individuals reproduce, the greater will be rate of population change. A deleterious effect on any one of these processes will reduce the potential rate of population increase.

This paper has primarily considered temperature as a mortality factor, which kills insects in their overwintering juvenile or adult stages, in both cases prior to reproduction, which normally occurs in the following spring and summer. The link between winter survival and subsequent population size can therefore be 'direct', as described for *E. autumnata* in Scandinavia and anholocyclic clones of aphids in the UK. However, the detrimental effects of low temperatures are much more extensive than such an immediate 'life or death' response. The mortality effect of cold exposure can be delayed, affecting later stages of development (Turnock *et al.* 1983), insects may survive but suffer sub-lethal damage affecting development, reproduction and longevity (Hutchinson & Bale 1994), or adults may emerge with morphological abnormalities, preventing flight and thus mate and food location and wider colonization (Bale *et al.* 1989*b*). Temperature is therefore arguably the most important abiotic variable, determining the limits to the distribution of species and exerting a strong influence on their success within the occupied range.

'Cold' has been described as the 'fiercest enemy of many forms of life' (Franks 1985), whilst Meglitsch (1972) regarded insects as the most successful animal group on Earth. It would therefore be surprising if insects had not developed effective means to 'cope with the cold'. Thus, without the physiological and biochemical mechanisms that allow insects to tolerate the partial freezing of their body tissues and fluids, or the ability to avoid such events, insects would be not be able to survive in extreme polar climates. The principles of insect cold hardiness via freeze tolerance or avoidance, first advanced by Salt (1961), have therefore remained a central theme in studies of insects and low temperature. The sequential discovery of the mechanisms of cryoprotection and the functions of polyols, INAs and AFPs has progressed from hypotheses regarding their roles to extraction and purification, and now, increasing knowledge of their molecular structure (Duman 2001).

At the same time, the ecological dimension of insect cold hardiness has been similarly advanced. It is now widely accepted that the interaction between an insect and its winter climate cannot be accurately represented by measuring its freezing temperature (SCP) at an unrealistic rate of cooling. Furthermore, as more experiments have been conducted with extended exposures at the less severe temperatures typically encountered in overwintering sites, the importance of cold-induced 'pre-freeze' mortality has been identified in many species (Bale 1996).

The early emphasis of research on the seasonal induction of the cold-hardy state has also been expanded by the discovery that insects can respond to changes in their thermal environment and depress their lethal temperature within time-scales of only a few hours and by different mechanisms (Lee *et al.* 1987; Worland & Convey 2001). The fact that such changes occur at ecologically relevant rates of cooling and affect critical thresholds other than death will provide a new perspective on the migratory behaviour of insects and predator–prey relationships.

The link between knowledge and application has also been increasingly evident in insect low-temperature

biology. The forecasting system for pest aphids and virus epidemics (Werker *et al.* 1998), predictions on the impacts of climate warming (Bale *et al.* 2002), assessment of the establishment potential of alien pests (Bale & Walters 2001) and the use of ice-nucleating bacteria in pest management (Lee *et al.* 1998) are individually complex and distinct areas of research, but each one depends for its success on a fundamental understanding of the thermal biology of the subject species. And insect cryobiology now lies on the threshold of another major development, the deciphering of the molecular structure of INAs and AFPs and their exploitation through transgenic technology in a range of applications, the scope of which is at this time impossible to imagine.

I am grateful to Jack Duman, Rick Lee, Richard Harrington, Claire Harris and Roger Worland for providing access to data, figures and unpublished information, and for comments on the manuscript.

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Discussion

C. Gerday (*Laboratory of Biochemistry, University of Liège, Liège, Belgium*). Is the synthesis of ice-nucleating proteins controlled by the photoperiodicity or temperature difference?

J. S. Bale. I think the dominant effect that seems to apply in most species is temperature and not photoperiod. I say dominant effect, in that in a number of species it has been demonstrated that when reared at lower temperatures or when they naturally experience lower temperatures, then the INA is synthesized. In some species, there is evidence that photoperiod is the dominant cue. In nature, lower temperature and decreasing photoperiod are occurring in tandem, whereas in the laboratory we can decouple the two factors and show which one is dominant.

A. Parker (*Department of Zoology, University of Oxford, Oxford, UK*). What is the cause of death in most of these insects? Is it always physiological or is there sometimes any large-scale anatomical damage?

J. S. Bale. This is a fascinating subject in which detailed information is lacking and I can honestly say in some areas of low-temperature mortality, we know little about the exact causes of death. There are lots of ideas and it is probable that there is not one single factor responsible for cold death. What I can say in relation to the second part of your question, is that there are a number of examples of exposure at certain low temperatures or rates of cooling which produce major morphological abnormalities. For instance, one of the most common is a malformation in the development of the wings or the ability to expand the wings at eclosion. Although the insect reaches the adult stage, it simply cannot emerge and then disperse.

M. A. Marahiel (*Department of Chemistry, Philipps-Universität Marburg, Marburg, Germany*). How general is this adaptation. You show us that adaptation is good for survival if you just freeze the insects to a reduced temperature but not to that temperature where they are dying. How general is this for insects living in cold climates and insects not living in cold climates? Is it a general effect or very specialized?

J. S. Bale. In polar environments, unless these insects were able to supercool and survive in the supercooled state, they would not be able to live there. You have to bear in mind that in most polar environments which we regard as among the most extreme on earth, throughout the winter season these insects are under several metres of snow, and so they are actually thermally buffered in many cases from the extremes of air temperature. When you put data loggers in some of these places you can show that it is -25 or -30 °C in the air, but it is probably -5 or -10 °C in the environment of the organism. So I think that the generality of being able to tolerate chilling at subzero temperatures is by far the most important attribute in polar species.

There are in fact relatively few freeze-tolerant species. To my knowledge, the experiments I described here on freeze–thaw cycles are the first to show that the freezing temperature of the insect is depressed following one or more freezing events. It would be interesting to apply the same experimental approach to freeze-tolerant insects from Alaska and freeze and thaw them repeatedly and see then whether they always freeze at the same temperature. In future, we may need to reassess our understanding of the freeze-tolerant state. I would be interested if anyone here can tell me why a nucleator should be less effective the second or the third time rather than the first time that it is required to come into play.

Anon. You were saying that insects accumulate large amounts of sugars during the cold acclimatization process

and I was wondering if the freezing tolerance in these insects may also be based on glass transitions or whether that does not play a role?

J. S. Bale. Over 10 years ago, there was a meeting at The Royal Society (I think in 1988) on low-temperature adaptation which concentrated on insects and there were the first reports then that insects might be able to enter a glassy vitreous state. John Baust and co-workers made a mock haemolymph of the composition found in an overwintering insect I referred to earlier (*Eurosta*) and they were able to show that at around −25 °C the fluid vitrified under certain conditions. To my knowledge, in the 10 years since then, no one has ever further investigated whether insects can naturally enter into glassy states.

I. A. Shanks (*Colworth Laboratory, Unilever Research, Bedford, UK*). I do not come from this area at all and there is always a chance that I am totally naive, but is there a significant metabolic load involved in this acclimatization process? If they have, in a matter of hours, to synthesize significant numbers of molecules and then get rid of them again as the night ends, is there heat generation associated with this process and is it likely that that plays some role in what you described?

J. S. Bale. If you look at the rapid cold-hardening data I showed you with insects transferred from 20 to 0 °C and then on to −8 °C, the interesting thing is that when those insects have been analysed there is little change in glycerol concentration or in heat-shock proteins. In other words, the sorts of mechanisms that enhance cold tolerance through seasonal acclimatization do not appear to be the same ones that produce this rapid cold-hardening ability. Also, note that the increases in cold tolerance through rapid cold hardening are quite small. If a population was rapidly cold hardened so most survived transfer to −7 °C, more would die at −8 °C and probably all would be dead at −11 °C. So there is about two to four degrees extra cold hardiness. Likewise, if the exposure time at −7 °C was 2 h, most of the sample would be dead if the duration was increased to 6 or 8 h. In other words, these are marginal increases but which may well be important for the individual. To my knowledge, the mechanisms of rapid cold hardening are not the same that produce the seasonal increases, where the metabolic costs as you have indicated are more clearly established.

P. L. Davies (*Department of Biochemistry, Queen's University, Ontario, Canada*). I just wanted to mention that I know of one environment at least where there is a very rapid temperature fluctuation in winter and that is on the eastern slopes of the Rocky Mountains. I think probably Charles Knight knows more about this than I do, but it seems to me that you can go through about 20 °C changes in temperature in a matter of hours. It might be worth looking at insect populations there.

J. S. Bale. I am sure that what you say is right. In fact, in Ohio at certain times of year there is a diurnal fluctuation in temperature of 13 °C, from a night-time low of 9 °C to a daytime high of 23 °C. Rick Lee has shown that during this natural fluctuation, both the lethal temperature and other thermal thresholds change. In future, we shall see more examples of insects that have the ability to rapidly shift biochemical pathways and to modify their basic metabolism to survive.

G. Warren (*School of Biological Sciences, Royal Holloway, University of London, Egham, UK*). You mentioned recrystallization inhibition as the most likely thing that antifreezes were doing in insects that actually freeze and survive the frozen state. Is that a theoretical implication or is there evidence that that is actually the mechanism?

J. S. Bale. I think Charles Knight and others have demonstrated that AFPs have the ability to inhibit secondary recrystallization. You are right it is a theory; I am not sure to what extent there is firm demonstration of this effect in insects. Maybe Charles could tell us.

G. Warren. Can I just say that I would certainly agree with Charles that antifreezes can inhibit recrystallization and I am more asking whether that is why insects have them?

J. S. Bale. If an insect freezes and cools to −40 or −50 °C by mid-winter, the ice in its body will 'warm' before melting occurs in spring. It is my understanding that there are changes in the physical structure of those ice crystals which might become mechanically damaging to cells if inhibition of this recrystallization did not occur.

G. Warren. I am interested obviously because antifreezes are there in plants too and I suppose I am professionally sceptical about exactly what they are doing. I meant that our best theory is recrystallization inhibition, but maybe there are some others that we have not thought of. I have heard of the formation of ice adhesions mooted, preventing or lessening that as another possibility. Perhaps there are others?

J. S. Bale. I agree that this inhibition of recrystallization is a theory which can be demonstrated *in vitro*, but is more difficult to prove in nature, at least for insects. The fact that insects contain these ice crystals which can alter their shape on warming, with the potential to cause cell damage, may have moved to us to this link without unequivocal evidence, as yet.

GLOSSARY

AFP: antifreeze protein
INA: ice-nucleating agent
SCP: supercooling point