INTRODUCTION

An earlier review in this journal (Baust and Rojas, 1985) encouraged investigators to "critically reassess" much of the "generally accepted dogma" which characterises research on insect cold hardiness and to undertake an assessment of the "founding hypotheses" of the subject. In their contribution Baust and Rojas considered the factors which may influence the classification of a species as freezing tolerant or intolerant (supercooling point, optimal cooling/warming rates, state of adaptation and methods of determining survival) and summarized the observations which challenge the consensus view which identifies the gut as the probable prime site for ice nucleation in freezing-intolerant species.

The likelihood of death for an individual insect from the effects of cold depends on (i) the cold hardiness of the specimen and (ii) the temperatures and periods of exposure experienced in the overwintering site. The interaction between these two factors will determine the proportion of a population that lives or dies. It is important to recognise that the term cold hardiness refers to the combined attributes required by an insect to overcome the various deleterious effects of low temperature. Viewed from an ecological perspective insect cryobiology is therefore concerned with all the events and processes governed by low temperature which influence and ultimately determine survival or mortality in the natural environment. In practise, research over 50 years has concentrated on the physiological and biochemical mechanisms of surviving or avoiding freezing while largely disregarding the possibility that for some or many species (studied or unstudied) other injurious effects of cold may be a more important threat to life. Additionally much of this work on cold hardiness has been based on laboratory temperature regimes which take no account of ecological aspects such as behaviour, overwintering site microclimate and the interaction of mortality factors in nature (Danks, 1978).

The aim of this review is to focus attention on one of the fundamental principles of insect cold hardiness which has largely determined the pattern of research for the last half-century, namely that freezing is the most important lethal effect of low temperature and must be tolerated or avoided to ensure survival of the species. In a review of the principles of insect cold hardiness, Salt (1961) states, "Insects hibernating in cold regions are generally able to withstand fairly low temperatures for long periods of time. Under natural to freezing intolerant (supercooling point, optimal cooling/ warming rates, state of adaptation and methods of determining survival) and summarized the observations which challenge the consensus view which identifies the gut as the probable prime site for ice nucleation in freezing-intolerant species."

REVIEW

INSECT COLD HARDINESS: FREEZING AND SUPERCOOLING—AN ECOPHYSIOLOGICAL PERSPECTIVE

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and polyhydroxy alcohols which function to limit freeze damage (Duman and Horwath, 1983). Freezing is lethal to freezing-intolerant species; this event is avoided by supercooling in which the body tissues and fluids are maintained in the liquid state below their equilibrium freezing point (Salt, 1936). Seasonal increases occur in the concentration of one or more polyols which extend the inherent ability to supercool (Baust, 1981, Somme, 1982), and the activity of antifreeze proteins (Duman, 1977) which lower the freezing point of the haemolymph relative to its melting point and may act to stabilise the supercooled state (Zachariassen and Husby, 1982). Similar cryoprotective antifreezes and antifreeze proteins are found in both freezing-tolerant and intolerant insects; the characteristic difference between the strategies is the winter loss or masking of the nucleators in supercooling—dependent species and the synthesis or unmasking of nucleating agents in freezing-tolerant species (Duman, 1982; Baust and Rojas, 1983).

FREEZING INTOLERANCE

"Ability to supercool is the only protection against freezing that most hibernating insects possess; it is a direct measure of their cold hardiness. Supercooling, therefore, is the dominant factor in the winter survival of such insects, and anything that influences it becomes of importance" (Salt, 1958). Many possible influences on supercooling have been investigated by Salt, including time (1920, 1966), feeding (1953), moisture content and temperature (1956), glycerol (1957), cooling rate (1966a) and the action of nucleators (1966b, 1968). The general characteristics of freezing-intolerant insects based on these pioneering studies and many similar reports have been reviewed by Somme (1982). It should be noted that these features of cold hardiness are expressed only in terms of an ability to supercool:

1. There is a seasonal variation in cold hardness which is at a maximum in winter.
2. Increased cold hardness is related to the accumulation of cryoprotective substances.
3. Production of cryoprotectant antifreezes may be induced by low temperature.
4. Contact with surface moisture can reduce supercooling by insulative ice formation through the cuticle.
5. Feeding reduces supercooling through the action of gut nucleators.

If this albeit simplified account is accepted by an investigator and experiments on previously unstudied species are designed according to the same methods, then the results obtained can indeed "reiterate and intensify" (Baust and Rojas, 1985) the established view. For instance there are clear changes in the cold hardness of the beet leaf mining weevil *Rhyynchus foja* with mean supercooling points of 

<table>
<thead>
<tr>
<th>Species</th>
<th>First instar</th>
<th>Adult</th>
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<tr>
<td><em>Myzus persicae</em></td>
<td>-27.4 ± 0.2</td>
<td>-26.0 ± 0.2</td>
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<tr>
<td><em>Aphis fabae</em></td>
<td>-26.3 ± 0.6</td>
<td>-25.3 ± 0.2</td>
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<tr>
<td><em>Sitobion avenae</em></td>
<td>-26.9 ± 0.7</td>
<td>-25.5 ± 0.2</td>
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<tr>
<td><em>Brexicoryne brassicae</em></td>
<td>-26.9 ± 0.3</td>
<td>-23.0 ± 0.3</td>
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and Bale, 1985). So, when studied in terms of supercooling responses, the features of aphid cold hardiness can be summarized in these terms:

1. There is no seasonal increase in cold hardiness and some aphids lose supercooling ability in winter.
2. There is no acclimation response at low temperature.
3. Surface moisture inoculates a variable proportion of a population.
4. Feeding (on sap) does not reduce supercooling.

These features largely contrast the "typical" characteristics of other so-called "freezing-intolerant" insects and while it is of scientific interest to identify a group of insects which are apparently atypical in their cold hardiness it also suggests that some caution is required in the use of supercooling date to predict survival or death at low temperature, particularly under field conditions.

**ECOLOGICAL VALIDITY OF SUPERCOOLING MEASUREMENTS**

The primary use of supercooling points is to provide a comparative index of cold hardiness between different stages of the life cycle, species or seasons. Somme (1982) has compiled a detailed list of the lowest mean supercooling points for freezing-intolerant eggs, larvae, pupae and adults in different orders of insects and Block (1982) provides similar data for invertebrate poikilotherms across many phyla. In theory all of these "freezing intolerant" species will die from the effects of freezing following a momentary exposure below their supercooling point. However, the established view that the supercooling point is a direct measure of the lower lethal limit (instantaneous low temperature death point) is only tenable for those species (or individuals) which (i) die from the effects of freezing, and (ii) remain alive (and capable of recovery) down to the temperature at which freezing occurs. These are critical requirements which must be satisfied to validate the use of the supercooling point as a direct measure of cold hardiness and of the lower lethal limit of freezing-intolerant insects. **For most species currently classified as freezing intolerant, survival in the supercooled state is an untested assumption both during the time-course of a supercooling experiment and in longer-term exposures; and far from being the starting point of an integrated study of cold hardiness, supercooling is often the only aspect that is investigated.** It is therefore not surprising that the literature provides few answers to these basic questions, even for "model" species which have been studied over many years by different investigators: What temperatures do insects experience in overwintering sites? What proportion of the population dies in winter from the effects of cold? How does low temperature kill insects? Is the supercooling point the true lower lethal limit? Clearly from an ecological viewpoint these questions are of fundamental importance. The lack of information in key areas arises because insect cryobiology is primarily a laboratory science which utilises physiological and biochemical methods to investigate an ecological relationship between insects and low temperature. The incorporation of "field studies" is usually only a sampling exercise in which insects are taken back to the laboratory for supercooling or cryoprotectant analyses. As Storey (1984) observed with reference to the dominant areas of cold-hardiness research, physiological ecologists have examined hundreds of species determining freeze tolerance/intolerance, supercooling/freezing/melting points of body fluids, and type/amount of cryoprotectant, while biochemists and biophysicists have concentrated on problems such as the structure of water in cells, cryo-enzymology and the protein chemistry of antifreeze molecules. Immediately apparent from Storey's accurate summary is the absence of comparative studies which relate laboratory estimates of cold hardiness (e.g. supercooling) to environmental temperatures and some measure of survival/mortality during the winter season; and unless this is done there is no basis for assessing the requirement or efficacy of the proposed cold-hardiness strategy in nature, or making the transition from hypothesis to fact. Paradoxically many studies by population ecologists have identified winter cold stress as an important density-independent regulating mechanism of insects, without investigating the freezing-tolerant/intolerant nature of the species or considering the value of indices of cold hardiness such as the supercooling point in predictive models. Against this background a more comprehensive research protocol is required to integrate knowledge on the physiology, biochemistry and ecology of insects at low temperature, allow laboratory assumptions and hypotheses to be examined and tested in the field, and expand our conception of insect cold hardiness beyond the process and effects of freezing.

**INTEGRATED STUDIES ON COLD HARDINESS**

An attempt is made here to describe a research protocol which combines field and laboratory methods with particular reference to the determination of the freezing status of insects and the ecological interpretation of supercooling data in apparently freezing-intolerant species. The areas of study outlined below have been applied to the grain aphid Sitobion avenue and appropriate results are included in the relevant sections.

**Determination of freezing status**

Cooling of specimens at 1°C min⁻¹ to the supercooling point followed by an assessment of survival after warming (or thawing) will provide a preliminary indication of the freezing status of the species and allow comparisons with much of the existing literature. The influence of optimal and sub-optimal cooling and warming rates on the designation of a species as freezing tolerant or intolerant together with the difficulty of selecting an appropriate time interval after exposure to assess survival has been discussed by Baust and Rojas (1985). In an ecological context survival *per se* is unimportant unless the individual can develop normally, and as an adult, reproduce and so contribute to the next generation; although individuals which recover but are incapable of reproduction may still contribute to the predator–prey balance in the ecosystem.
Supercooling data is normally presented as the mean value ± the standard error. The range of supercooling points is also relevant information since a proportion of a population may be at risk of a freezing death even though the mean supercooling point exceeds the lowest winter temperatures (Semme, 1982); additionally the distribution of supercooling points may be skewed or bimodal (Block, 1982a).

The mean supercooling point of laboratory populations of *S. avenae* varied from \(-27.0 ± 0.2°C\) in first instars, to \(-24.2 ± 0.3°C\) as adults. There was no acclimation in supercooling at 0 or 5°C and the mean supercooling point of the majority of a field population remained below \(-20°C\) throughout the winter (Knight and Bale, 1986). There were no survivors below the supercooling point and *S. avenae* in common with other aphids studied so far was classified as freezing intolerant.

**Annual, seasonal and microclimate temperatures**

The winter season imposes physiological stress on insects but the level of this effect varies temporally from year to year, month to month, and day to day, and spatially at any one moment in time. Variations are seen in both the total number of frost days and the minimum temperatures of winter.

**Annual variations in frost days and minimal temperatures.** In nine successive winters (1975-76 to 1983-84) in northern England the total number of air-frost days per winter varied from 50 to 100 with a mean of 67 ± 6. Equivalent figures for ground level frosts (grass minimal temperatures) ranged from 110 to 160 with a mean of 140 ± 5. When compared on the basis of lowest temperatures, one winter (1975-76) could be described as “mild” when there was no air or ground frost below \(-10°C\), two winters (1978-79 and 1981-82) were “very cold” with 6 days with a ground-level frost below \(-15°C\) (but above \(-20°C\)) and the remaining six winters were “average” with the coldest days between \(-10°C\) and \(-15°C\). Descriptions of winters as “mild”, “average” and “very cold” are relative and will vary in usage with different investigators and climates; additionally the most deleterious effect of cold on one species may be the frequency of exposure in the supercooled state (total number of frosts) whereas for others the critical factor may be the lowest temperature experienced throughout the winter. In the nine year records there was considerable variation in both these variables although on no occasion did the temperature fall below the mean supercooling point of the overwintering population of *Sitobion avenae*.

**Microclimate of overwintering sites.** Winter habitats of insects are often described in terms of some single standard measurement of air temperature whereas the microclimate temperatures of the overwintering site may be markedly different, above or below the air value. For instance beech weevils (*Rhynchaenus fagi*) overwinter in the leaf litter of the forest floor and the aerial canopy of conifers (Bale, 1981); the grain aphid overwinters on young wheat and barley plants 0-10 cm above the soil surface. The vertical stratification of temperature on the coldest night in the years 1975-1984 in northern England and on a normal cold night that would occur many times in most winters is shown in Fig. 1. On both nights the grass minimal temperatures were 3-5°C colder than would occur at the top of a 1.5 m high tree, although a frost of \(-18.4°C\) at ground level did not penetrate 10 cm into the soil. How relevant are the air temperatures of \(-13.1°\) and \(-1.5°C\) to the level of cold experienced by the weevil under the leaf litter or the aphid on a cereal seedling? This meso- or microscale inversion of temperature such that the ground is colder than the air above it, is caused by nocturnal radiant cooling and accompanying cold-air drainage (Wellington and Trimble, 1984). In hillside forests,
the temperature variation between the vertical strata can be so large that the spring abundance and damage potential of some pest species varies at different elevations according to the differential mortality of the overwintering stages (Tenow, 1975).

Clearly, the vertical distribution of overwintering sites together with the insulating properties of host plants and leaf cover at the soil surface are important considerations when assessing the level of cold experienced by insects in winter.

Population dynamics of insects in winter

The most important requirement for the study of population changes of insects in winter is an accurate sampling method taking into consideration the diversity of overwintering sites occupied by the species and the distribution of the insect (random or aggregated) in each site. A detailed study of the overwintering biology of the grain aphid *Sitobion avenae* (McGrath and Bale, in Knight *et al.*, 1986) monitored an aphid population on 100 randomly selected plants of winter barley, from the early stages of colonisation of the crop by alates (winged migrants) in autumn, through the phases of population increase, peak, and decline (Fig. 2). The peak density equivalent to 1,000,000 aphids ha$^{-1}$ occurred in mid-winter and declined to less than 30,000 ha$^{-1}$, a 97% mortality over 2 weeks, when the lowest grass minimal temperature was $-8.1^\circ C$ in the first week (approx 50% kill) and $-9.7^\circ C$ in the second week. The mean supercooling point of the same field population was below $-20^\circ C$. A similar study in an adjacent field indicated an abrupt increase in mortality when the temperature decreased to $-8^\circ C$ (Knight and Bale, 1987). In nature, most aphids are killed in winter at temperatures above their inherent ability to supercool. Extensive supercooling alone is not necessarily an indicator of a cold-hardy insect, nor a guarantee of winter survival.

Mortality in relation to supercooling ability

Integration of the information on *S. avenae* showed that (i) the mean supercooling point throughout the winter was below $-20^\circ C$, (ii) temperatures close to $-20^\circ C$ were extremely rare in the cool temperate winters of Britain and did not occur during the study period, but (iii) aphid mortality was very high in the field at temperatures much above the known supercooling point. When the field mortality of a species in winter is much higher than would be expected from a knowledge of supercooling capacity and environmental temperatures, the influence of low temperature can be examined in more detail under experimental conditions. By varying the cooling rate, minimal temperature and periods of exposure and prior acclimation, in the absence of interactions with wind or precipitation and with the exclusion of natural enemies, it is possible to estimate the direct effect of low temperature on the species and separate time-dependent deaths by freezing above the supercooling point from pre-freeze mortality.

When large samples of *S. avenae* taken from a $20^\circ C$ maintained laboratory population were cooled at 1°C min$^{-1}$ to a range of minimal temperatures and mortality assessed at daily intervals after exposure it was found that (i) mortality commenced at $-5^\circ C$ and there were no survivors at $-15^\circ C$, (ii) most aphids were fatally injured rather than killed instantaneously and died progressively within 24 h of exposure, (iii) aphids which died up to 96 h after exposure neither moulted nor reproduced before death, and (iv) the adult LT$\alpha$ 24 h after exposure was $-8.1^\circ C$ whereas the mean supercooling point was $24.2^\circ C$ (Knight *et al.*, 1986). In similar experiments with *Myzus persicae* (mean supercooling point $-25^\circ C$), the LT$\alpha$ of adult aphids maintained at 20, 10 and 5°C throughout their nymphal development and examined 3 days after exposure was $-6.9$, $-11.1$ and $-11.6^\circ C$ respectively (Bale *et al.*, 1987 and unpublished data). Acclimation therefore depresses the LT$\alpha$ although in all cases
there was 100% mortality above the mean supercooling point.

However, the relationship between the pre-freeze mortality of aphids observed in the laboratory and the decline in winter of field populations of the same species at temperatures above their known supercooling point, do not necessarily have a common origin. Supercooling points and LT$_{90}$ values are derived from aphids which may have recently fed (on plant sap) but are removed from their host plants for the experiments. In nature, unlike most overwintering insects, aphids normally remain in feeding contact with their host plants throughout winter, but may have to move to new leaves or plants a number of times to survive (Harrington and Cheng, 1984); additionally, the inhibition of plant sap is not a continuous process although the styles are not usually withdrawn between periods of feeding at the same site. Powell (1974) observed that green spruce aphids (Elatobium abietinum) in feeding contact with their host, were killed at temperatures above their inherent supercooling level when the spruce needles froze, whereas detached aphids survived when cooled to the same temperature. It was suggested that during feeding, a ‘bridge’ of phloem sap is formed between the plant vascular tissues via the styles to the aphid gut resulting in inoculative freezing of the insect. This phenomenon is a possible alternative explanation for the high mortality in winter populations of S. avenae (Knight et al., 1986) and M. persicae (Harrington and Cheng, 1984), but would have to operate at temperatures above the pre-freeze event to have any importance under field conditions.

Crop plants on which aphids feed in winter must be sufficiently cold hardy to survive at low temperature and continue their growth in spring; such plants would freeze many times in a normal temperate winter. The process of extracellular freezing tolerance in plants has been reviewed by Levitt (1980). Ice normally crystallizes first at a few nucleation sites in the main xylem vessels because of their large diameter and dilute sap. Ice from the vessels spreads throughout the plant via the intercellular spaces but is prevented from inoculating the contents of living cells by the lipid plasma membrane. If the temperature continues to fall at a gradual rate, the intercellular ice masses increase in size as cell water diffuses progressively through the semipermeable lipid plasma membrane resulting in a “freeze concentration” of the cell sap including the phloem. Intracellular freezing of the phloem is lethal.

The extent of ice formation in a plant and the likelihood of intracellular freezing is governed largely by the rate of cooling relative to the rate of diffusion of water from within the cell to ice loci in the intercellular spaces which is limited by the permeability of the cell membrane. In nature, the freeze is likely to be so slow that the ice front will not spread throughout the plant body. At the upper limit of “slow” cooling, the ice front spreads throughout the plant and contacts all the cells; at faster rates the diffusion of cell water to extracellular ice cannot occur with sufficient speed to increase the concentration of the cell contents as the temperature falls so that eventually there will be spontaneous intracellular freezing.

From this information it appears that under normal conditions the phloem food source of aphids in cold-hardy plants remains unfrozen throughout winter. If inoculative freezing by a “phloem bridge” is important in aphids it would seem to require the phloem sap to freeze (or be nucleated) within a phloem cell or in the styles, at an elevated temperature compared to that at which it freezes in the aphid gut, since feeding aphids removed from plant tissue consistently supercool to below $-20^\circ$C. Inoculation may occur from intercellular ice formation around the stylet bundle since the styles of aphids are thought to penetrate the plant mainly via intercellular routes in order to reach individual phloem cells [sieve tubes] (Pollard, 1973). However the intercellular path is lined by a secreted salivary sheath and this may act as a barrier to inoculative freezing.

To investigate the potential for inoculative freezing from feeding, a culture of M. persicae was reared at $10^\circ$C throughout their nymphal development to produce an acclimated population. Newly moulted adults were allowed to establish feeding sites on leaf discs of oil seed rape (a winter host plant) at the same temperature. When cooled at $1^\circ$C min$^{-1}$ the leaf discs froze consistently between $-5$ and $-6^\circ$C. The LT$_{90}$ values for adult aphids cooled during feeding on leaf discs were $-16.6$, $-13.6$ and $-12.5^\circ$C, when assessed 24, 48 and 72 h after exposure; the equivalent values for acclimated aphids cooled in isolation from plant tissue were $-15.6$, $-11.4$ and $-11.1^\circ$C respectively (Bale, Harrington and Clough, unpublished data). In combination with previous experiments, these results (i) confirm the occurrence of pre-freeze mortality processes in aphids, including acclimated populations (ii) indicate that inoculative freezing from feeding does not increase mortality above that resulting from the direct effect of cold and (iii) suggests a largely artefactual irrelevance for the supercooling ability of aphids in nature.

**Life and death at low temperature: physiology and biochemistry**

The inclusion of physiological and biochemical analyses at this stage in a study reflects the opinion of the author that until the causes of low-temperature death (pre-freezing, freezing or post-freezing) and their relative importance have been established, it is not possible to plan experiments on a rational basis to identify mechanisms and strategies of survival. Intuitively most investigators would screen insects for cryoprotectants, antifreeze and nucleator proteins, or bound-water content, whereas for some species, particularly those which suffer pre-freeze injury, it would be more informative to target research at the membrane, enzyme or mitochondrial level. Storey (1984) recognises this distinction as adaptations for low-temperature preservation and low-temperature function and gives a comprehensive account of appropriate methodologies including new technologies such as nuclear magnetic resonance. It is important to recognise that death can occur through a failure in any one process and that species inhabiting very cold climates have probably evolved a complex array of adaptations for both function and preservation, only one of which, the tolerance and avoidance of freezing, has yet been studied in any detail.
During cooling of aphids at 1°C min⁻¹ in a system capable of resolving temperature changes to 0.1°C (Bale et al., 1984), no "physiological" event was observed in the cooling curve in the mortality range from −5 to −15°C, only the supercooling point rebound at around −24°C. In the first use of a differential scanning calorimeter to study the cold hardiness of whole insects, Knight et al. (1986) detected a pre-freeze exotherm occurring consistently within the mortality range of S. avenue (usually between −7 and −11°C), corresponding to less than 1% of the heat released during freezing. The exotherm appears to be of direct biological origin, irreversible and non-repeatable, since there was no endotherm on warming and the exotherm was never observed in repeat cooling of the same specimen. At this stage it is not known whether the exotherm is related to the cause of pre-freeze cold death in aphids or is a coincidental event occurring at the same temperatures, although recently, individuals of M. persicae have been observed to survive through the exotherm and moul and reproduce when returned to normal culture conditions (Bale et al., 1987). There is no doubt however that aphids die (or are fatal injured) before they freeze and the possible causes could include a thermotropic phase transition in a membrane, a cold inactivation of proteins, or the decoupling of normal metabolic processes.

**DISCUSSION**

The classification of insects as freeze tolerant or intolerant is not redundant; nor is it suggested that many species currently regarded as freezing intolerant will be found by future research to die in large numbers above their supercooling point. It is unlikely that alpine and polar insects could survive winter microhabitat temperatures between −20 and −30°C without extensive and effective supercooling and the absence of any significant pre-freeze mortality or long-term chill injury. It is equally unlikely that species from such very cold regions should have evolved complex triggering mechanisms for the synthesis of ice-nucleating agents or antifreeze cryoprotectants, only then to die in large numbers from thermal processes unrelated to the tolerance or avoidance of freezing. Nevertheless the tolerance/intolerance classification, originally based on strategies apparent in insects from extreme climates (for which it may be essentially correct) has gained a general acceptance among investigators as a descriptive code, and a basis for experimentation on insects from many taxa which inhabit widely differing climates ranging from the cool temperate to the maritime Antarctic or the northern tundra. The heterogeneity of form and function in insects combined with the diversity of their winter habitats predisposes any common theory of cold hardiness to be oversimplified and restrictive. The lethal effect of low temperature on insects cannot be described in terms of a single process, freezing, and to do so places a rigid conceptual framework around experimental protocols and allows untested hypotheses and generalisations to prevail; none more so than the assumption of "survival by supercooling" in species known to be dead when frozen.

Clearly the tolerance/intolerance categories are ecologically meaningless for tropical insects which die at temperatures above 0°C because they are unable to adjust their metabolism to enter a dormant state, and in nature never experience sub-zero temperatures. The classification is also inappropriate for species from colder climates, if some or all of an overwintering population are killed by cold, above the supercooling point and without freezing.

The integrated, ecophysiological approach to the study of insect cold hardiness recommended in this review relates a laboratory determined index of cold tolerance (supercooling) to known winter microclimate temperatures, and assessments of survival (which ideally should include different combinations of cooling/warming rates, exposure periods and minimal temperatures in the laboratory), and an accurate sampling procedure to monitor population density in the field. By this approach it becomes apparent whether (a) the species is genuinely freezing intolerant and survives to the supercooling point, or (b) a proportion of the population is killed or fatally injured before freezing, and (c) at what temperatures and under what conditions such mortality occurs and varies. Additionally, a comparison of laboratory and field survival, provides an estimate of the winter mortality which is attributable to factors other than cold. With this information it is also possible to plan a more rational sequence of physiological and biochemical experiments. For instance, it is logical to analyse the spectrum and concentration of antifreezes in an insect before assessing the ability of the species to survive above the supercooling point?

Some recent studies on insect cold hardiness have combined physiological and biochemical methods in the laboratory with field ecology to assess cold tolerance and mortality and the examples selected below exemplify the benefits of this interdisciplinary approach. The goldenrod gall moth *Epiblema scudderiana* overwinters as mature larvae in stem galls on the goldenrod plant in North America. Mean supercooling points of the larvae decreased from −13.9 in the early autumn to stabilise between −35 to −40°C throughout the winter; the lowest winter temperature was −26°C. There was a 90% successful pupation and emergence of a field population in spring and 100% survival of winter larvae maintained at −18°C for 197 days (Rickards et al., in press). Clearly this species survives at low temperatures by virtue of extensive supercooling which is accompanied by a winter decrease in body water content and an increase in glycerol concentration to 18.7% of the fresh weight.

The collembolan *Cryptopygus antarcticus* and the cryptostigmatid mite *Alaskozetes antarcticus* are relatively abundant species in the very limited arthropod fauna of the Antarctic. The mean supercooling point of *Cryptopygus* decreases seasonally from −5 to −10°C in summer to about −25°C in winter, associated with the evacuation of gut contents and its partly dehydrated condition in winter, and an increase in total potential cryoprotectant concentration. In a standard test exposure to −15°C for 24 h, the low survival of summer field samples (<50%) increased to more than 80% in winter populations (Block, 1987, in press). Similar and more consistent increases
in supercooling were found in adult Alaskozetes, and winter collected mites with a mean supercooling point of $-30^\circ C$ showed a 52% survival after 250 days at $-15^\circ C$ and 73% survival after 100 days at $-20^\circ C$ (Cannon, 1987). Again these studies provide evidence for the adequacy of the supercooling strategy even after prolonged exposures to low temperatures. However, in the light of an 80% survival of Crypto- pygus in the laboratory in a 24 h exposure to $-15^\circ C$, it is interesting to note that Block (1982b) has recorded overwintering population declines of this species of approx 22% in a moss turf (where the density was higher) and 79% in a moss carpet. These substantial mortalities may be related to freezing in the supercooled state, chill injury, action of predators, or other physiological stresses which occur with the changing season, and also show how population density and mortality can vary in different overwintering sites.

A contrasting pattern of cold hardiness and strategy for survival has been found in the temperate Collembola Tomocerus minor and Orchesella cincta. The mean supercooling point of the two species in winter was $-11$ and $-14^\circ C$ respectively; however in a 24 h exposure to a range of minimal temperatures, although there was a seasonal increase in “cold hardiness”, the LT$_{50}$ of winter collected samples was only $-6.5$ and $-8.7^\circ C$, or $4.5^\circ$ above the mean supercooling point of each species (van der Woude and Verhoeef, 1986). These differences have been attributed to the time-dependent nature of freezing during extended exposure (24 h) in the supercooled state, but in common with many “time” experiments, there is no evidence that freezing was the cause of death. In such a short exposure it is possible that pre-freeze processes acting at relatively high temperatures may be an important mortality factor in these species. Their limited tolerance of cold compared to other temperate insects is in any case compensated by the insulation afforded by snow and leaf-litter cover in winter such that the overwintering mortalities of adults or juveniles of either species sampled from the soil did not exceed 65%.

The peach potato aphid Myzus persicae shows a very similar pattern of cold hardiness and winter decline to that described for Sitobion avenae. The mean supercooling point of the field population remains below $-20^\circ C$ throughout the year (O'Doherty and Bale, 1985). In successive winters there were population declines of approx 99.9 and 98% when lowest grass minimal temperatures were $-18.8$ and $-11.1^\circ C$ respectively, and the under-leaf minimal temperatures of a preferred overwintering site (winter cabbage) were only $-9.4$ and $-7.2^\circ C$. The LT$_{50}$ of a laboratory population maintained at $20^\circ C$ was $-7.4^\circ C$ whereas the mean supercooling point was $-25^\circ C$ (Harrington and Cheng, 1984; Bale et al., 1987). There is a clear indication from these results of a large-scale pre-freeze mortality in such species.

In order of description these studies reveal (a) a species which undergoes a winter increase in supercooling capacity of more than $20^\circ$ with a high survival after prolonged exposure in the supercooled state in both the field and laboratory (Epiblema), (b) a species with a seasonal increase in supercooling of 15–20°, high survival in short exposures to sub-zero temperatures, but considerable mortality overwinter in the field for reasons as yet unknown (Crypto- pygus), (c) species in which the lower lethal limit is consistently above the supercooling point, increases seasonally by only $2^\circ$ and overlaps soil surface temperatures such that behavioural avoidance may be more important than physiological tolerance of cold as a mechanism for survival (Orchesella and Tomocerus), and (d) species in which there is no seasonal increase in supercooling, the lower lethal limit is markedly above the supercooling point, population declines overwinter of 90–99% are common, and survival depends on a diversity of favourable overwintering sites (e.g. glasshouses) and an additional and supposedly “more cold hardy” overwintering state, the egg (Sitobion and Myzus). Interestingly, although aphid eggs supercool to $-35^\circ C$ in winter (Sømme, 1969; James and Luff, 1982), and are regarded as the most cold-hardy stage of the aphid life cycle, Leather (1980, 1981) has recorded winter egg mortalities in the absence of predators, of 35–50%, although temperatures were always 10–25° above the supercooling points. Mortalities in the absence of predators, of 35–50%, although temperatures were always 10–25° above the supercooling points. The variation seen between these species in seasonal increases in supercooling, minimal supercooling points, lower lethal limits, field and laboratory mortalities, environments and overwintering sites, argues strongly against the proposition of any common theory of cold tolerance in insects.

Studies on the temperate Collembola and aphids should not be viewed in isolation on the grounds that the insects are not “typical cold-hardy species”. Typ- ical and atypical are relative terms used to describe species which do or do not conform to particular criteria, such as the supercooling characteristics of “freezing-intolerant” insects; if the criteria are changed so do the relative proportions of typical and atypical species. And what is an acceptable definition of a “cold-hardy” insect? Does a species have to undergo a process of cold hardening or acclimation such as the seasonal increase in supercooling capacity and correlated increases in cryoprotectant concentration, to be classed as cold-hardy? If then, 90% of the overwintering population is killed, is the species still cold-hardy, or is it partially cold-hardy? In the final analysis, only the survivors are sufficiently cold hardy. Clearly, what is required at this time is a critical reassessment of current theories and an integration of knowledge from different disciplines, taxa and climates.

Temperate aphids and Antarctic Collembola share at least one common character of cold hardiness; in winter both groups supercool and therefore avoid ice formation in short exposures to $-20^\circ C$. However, exposure to temperatures between $-10$ and $-15^\circ C$ kills large numbers of aphids whereas most of the Collembola survive. Either the Collembola can avoid or tolerate the process which kills the aphids, or they are physiologically so different that the process does not affect them. Identification of the processes which kill less cold-hardy species, which may inhabit cool rather than cold climates, will focus attention on the adaptive mechanisms which allow species (though sometimes few individuals) to survive winter in the most extreme environments. Freezing-intolerant in-
sects do not survive at low temperature only by virtue of their supercooling ability.

The observation that cold can lead to injury and death in the absence of freezing is not new although some of the earlier reports described mortality at temperatures well above 0°C (e.g. Blattella germanica, Colhoun, 1954) and may be more relevant to lethal processes in tropical species than those affecting insects below 0°C in cool and colder climates. More recently pre-freeze cold death has been observed in species and life cycle stages which experience sub-zero temperatures overwinter (e.g. Mamestra configurata, Turnock et al., 1983; Sarcophaga crassipalpis, Lee and Denlinger, 1985; Sitobion avenae, Knight et al., 1986; Myzus persicae, Bale et al., 1987). The causes of pre-freeze mortality are still a matter of speculation rather than a distillation of evidence from purposeful research. Prosser (1973) states that the causes of death at either high or low temperature are "not well understood" and are "certainly multiple", including increased cell membrane permeability, failure to maintain ionic gradients and pumps, insufficient energy liberation, decoupling of enzyme reactions and malfunction of the central nervous system. Similar hypotheses have been advanced by Hochachka and Somero (1973) who also discussed biochemical adaptations to the effects of temperature changes on biological systems (1984).

Danks (1978), and Knight et al. (1986), who suggested that the lethal cooling injury to aphids may be attributable to changes in membranes, proteins or normal metabolic processes. Apparently, our knowledge and understanding of the basal causes of low-temperature death in insects has not advanced greatly over the last quarter-century, although over the same time period large numbers of species have been added to the categories of freezing tolerance and intolerance, based on studies which have frequently measured nothing other than supercooling, and usually excluded any assessment of mortality, particularly under field conditions.

In nature as the temperature decrease seasonally and daily, insects may pass through a sequence of potentially injurious or lethal states. The proportion of the population that is injured or killed in each state will depend on factors such as inherent levels of cold hardiness, acclimatisation and rate-based effects. Eventually as temperature continues to fall, the only remaining threat to the survivors (which may be a high or low proportion of the original population) will be the process of freezing. The full return on the metabolic investment in supercooling will be realised only by individuals which have survived at higher temperatures, and only those individuals are in the strictest sense, freezing intolerant. If a species with these properties is cooled under laboratory conditions, mortality will be observed to increase with decreasing temperature and probably with increasing time of exposure at a constant temperature and this pattern of results has been described for many "freezing-intolerant" insects.

Identification of the range and complexity of low-temperature mortality processes is fundamental to the development of insect cryobiology, irrespective of whether the primary interest is biochemical, physiological or ecological. Such information is also vitally important to the development of ecological models of insect populations, prediction of pest outbreaks and storage of biomedical materials at low temperature, whether supercooled, frozen or vitrified. In conclusion it is hoped that this review will encourage investigators to consider that the study of low-temperature mortality will provide a clearer understanding of the mechanisms and strategies of survival, that physiological and biochemical studies on individuals in the laboratory must be related to the ecology and performance of populations in the field, and finally, that the tolerance or avoidance of freezing by a frequently small number of individuals of a relatively limited number of species inhabiting extreme environments, is only one of the principles of insect cold hardiness.

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