THE ROYAL SOCIETY

Received 11 March 2004
Accepted 25 March 2004
Published online 7 June 2004

Freezing induces a loss of freeze tolerance in an overwintering insect

C. L. Brown[1], J. S. Bale[1*] and K. F. A. Walters[2]

[1]*School of Biosciences, University of Birmingham, Edgbaston, Birmingham B15 2TT, UK*
[2]*Central Science Laboratory, Sand Hutton, York YO41 1LZ, UK*

Cold-hardy insects overwinter by one of two main strategies: freeze tolerance and freeze avoidance by supercooling. As a general model, many freeze-tolerant species overwinter in extreme climates, freeze above −10 °C via induction by ice-nucleating agents, and once frozen, can survive at temperatures of up to 40 °C or more below the initial freezing temperature or supercooling point (SCP). It has been assumed that the SCP of freeze-tolerant insects is unaffected by the freezing process and that the freeze-tolerant state is therefore retained in winter though successive freeze–thaw cycles of the body tissues and fluids. Studies on the freeze-tolerant larva of the hoverfly *Syrphus ribesii* reveal this assumption to be untrue. When a sample with a mean 'first freeze' SCP of −7.6 °C (range of −5 °C to −9.5 °C) were cooled, either to −10 °C or to their individual SCP, on five occasions, the mean SCP was significantly depressed, with some larvae subsequently freezing as low as −28 °C. Only larvae that froze at the same consistently high temperature above −10 °C were alive after being frozen five times. The wider occurrence of this phenomenon would require a fundamental reassessment of the dynamics and distinctions of the freeze-tolerant and freeze-avoiding strategies of insect overwintering.

Keywords: freezing; freeze tolerance; nucleator; strategy

1. INTRODUCTION

The ability of some insects to survive at temperatures as low as −30 °C to −70 °C is one of the most remarkable features of the natural world (Danks 1981; Miller 1982; Ring 1982; Kukal 1991). Most cold-hardy insects survive at low and sub-zero temperatures by one of two main strategies, freeze tolerance and freeze avoidance by supercooling (Salt 1961; Zachariassen 1985), though some invoke a desiccation-induced increase in winter cold hardiness (Holmstrup *et al.* 2002). The key difference between freeze tolerance and freeze avoidance is the activation or synthesis of ice-nucleating agents (INAs) in winter in freeze-tolerant species, and their inactivation or removal in freeze-avoiding species. Both freeze-tolerant and freeze-avoiding species contain polyols and antifreeze (thermal hysteresis) proteins, though their function differs between the two strategies (Duman 2001). In most freeze-tolerant species, INAs induce freezing above −10 °C in 'safe' extracellular areas. Once frozen, the insects survive further cooling to lower temperatures, although the difference between the freezing temperature and the lower lethal temperature varies between species from as little as 5 °C to more than 40 °C (Bale 2002).

The overwintering sites of many insects experience irregular environmental freeze–thaw cycles (Layne 1991; Sinclair 1997), although the frequency with which freeze-tolerant insects actually freeze, thaw and re-freeze in winter is unknown, as are the physiological consequences of these events. The effects of repeated freezing and thawing on overwintering freeze-tolerant insects have not, to our knowledge, been previously studied, though it seems to have been assumed that the insect will re-freeze at the same temperature each time, and that the freeze-tolerant condition will therefore be retained throughout winter. A recent study on summer collected larvae of the subantarctic beetle *Hydromedion sparsutum* (which is weakly freeze tolerant all year round) found that the supercooling point (SCP) was depressed in most larvae that had been frozen on more than one occasion, and that these individuals were no longer freeze tolerant (Bale *et al.* 2001).

We describe similar experiments to those conducted with *H. sparsutum*, the key difference being that, as far as we are aware, this is the first description of the effects of repeated freezing on the acclimated overwintering stage of a freeze-tolerant insect. *Syrphus ribesii* is a Holarctic species, distributed from Norway to Spain in Europe, east to China and Siberia, and Alaska to Mexico in America. In the UK, *S. ribesii* has two or three generations per year. At the end of summer, larval populations of the final generation feed mainly on sycamore aphids (*Drepanosiphum platanoidis* (Shrank)), and fall to the ground on senescing leaves, overwintering in the leaf litter at densities of 10–700 m^{-2} (F. S. Gilbert, personal communication). The soil surface–leaf litter interface is the coldest 'layer' in the vertical stratification of winter temperatures, and in the case of sycamore leaf litter in the UK, would be more or less continuously wet or frozen from October to March each year. The number of 'soil surface frosts' in northern England varies from 110 to 160 per year, with the minimum temperature ranging from −5 °C to below −15 °C (but usually above −20 °C) in different winters (Bale 1987). This study tested the propositions that: (i) overwintering freeze-tolerant larvae of *S. ribesii* freeze at the same temperature in successive freeze–thaw events; and (ii) survival of freezing and thawing is independent of the frequency of occurrence of these events.

* Author for correspondence (j.s.bale@bham.ac.uk).

2. MATERIAL AND METHODS

(a) *Hoverfly culture and acclimation*

Adult *S. ribesii* were collected from June to September while foraging on flowers from local sites around Birmingham. The laboratory culture was replenished each summer with a fresh supply of wild-caught flies. In the laboratory, adult flies oviposited onto bean plants (*Vicia fabae* cv Scirocco) infested with two aphid species, *Acyrthosiphum pisum* (Harris) and *Aphis fabae* Scopoli. Eggs were collected over a 48 h period to provide a larval cohort of similar age. Acclimated larvae were obtained by stepwise transfer of samples fed on aphid-infested bean stems from 15 °C, 18 L : 6 D (7 days) to 10 °C, 18 L : 6 D (7 days) to 5 °C, 24 L : 0 D, and then to 2 °C, 24 L : 0 D for 50 days. This acclimation regime had the highest larval survival in a preliminary study (more than 90%), and all larvae from this regime survived freezing at their SCP.

(b) *Supercooling points*

Larvae ($n = 24$) were cooled individually from their 'storage temperature' (2 °C) at 0.5 °C min^{-1} in contact with 35 SWG type T thermocouples in a programmable alcohol bath linked to a computer recording system (see Bale *et al.* (1984) and Hart & Bale (1997) for details). After the 'freezing rebound' at the SCP, larvae were allowed to return to the freezing temperature and then warmed slowly to 5 °C in test-tubes surrounded by ice water. Individual larvae were then placed in numbered wells in repli-dishes at 5 °C, 24 L : 0 D and survival assessed after 72 h.

(c) *Freeze–thaw cycles*

A sample of 50 acclimated larvae from the 2 °C storage group was cooled individually in glass test-tubes in a programmable alcohol bath from 2 °C to −10 °C at 0.5 °C min^{-1} on five occasions at hourly, daily and weekly intervals, and held at the minimum temperature for 15 min. All larval groups were warmed up to a temperature of 10 °C at 0.5 °C min^{-1} and survival assessed. The daily and weekly groups were then cooled at 0.5 °C min^{-1} to 2 °C and held at this temperature until the next exposure to −10 °C. The hourly group were immediately re-cooled from 10 °C to −10 °C at 0.5 °C min^{-1}. After the last exposure to −10 °C, all larvae were placed at 5 °C with a control group of 50 larvae that had been acclimated as previously described and then held at 2 °C for 50 days (preliminary experiments indicated that larvae were robust and could be moved between rearing environments at daily intervals without affecting survival, hence a handling control was considered unnecessary). Survival in the control and various treatment groups was assessed after 72 h. A sample of 24 larvae was then removed from the control and each treatment group and their SCP and post-freezing survival assessed as described above. These larvae, and the remainder from the hourly, daily and weekly groups that had not been used for SCP and freezing survival assessment, were then transferred to 10 °C, 12 L : 12 D for 10 days, and finally to 15 °C, 18 L : 6 D to assess subsequent pupation and emergence.

To ensure that all larvae were frozen in every sub-zero exposure, a sample of 24 larvae acclimated for 50 days at 2 °C were cooled individually to their SCP at 0.5 °C min^{-1} in a programmable alcohol bath on five occasions at daily and weekly intervals. Between exposures, larvae were warmed slowly in ice water to 5 °C, their survival assessed, and then held at 2 °C in numbered wells of a repli-dish. After the last freezing exposure, larvae were transferred to 10 °C and 15 °C as above, and pupation and emergence were subsequently assessed.

(d) *Analyses*

For all larvae, survival, pupation and emergence data were arcsine transformed before being analysed using ANOVA, followed by Tukey's multiple comparisons where appropriate. SCP data were also analysed using ANOVA. Where larvae were repeatedly cooled to the SCP, the first SCP value for each larva was compared to the final SCP for the same larva, using a two-way ANOVA.

3. RESULTS

The mean SCP of a winter-acclimated population of *S. ribesii* when frozen on a single occasion (the control) was −7.6 ± 0.4 °C with a range from −5.0 °C to −9.5 °C (table 1). All larvae were alive 72 h after freezing and thawing. Following exposure of similarly acclimated larvae to −10 °C on five occasions at hourly, daily or weekly intervals (with the expectation that all larvae would freeze in each exposure), in all cases, survival was greater than 70%, though significantly lower compared with the control in the daily and weekly interval exposures (figure 1*a*). Pupation and adult emergence were also both significantly reduced compared with the control (figure 1*b*,*c*). The mean SCP of the hourly treatment group after the five exposures to −10 °C was also significantly lower than the control ($p < 0.05$; table 1), and in each group, some larvae froze below −20 °C, a depression of the SCP in individual larvae of greater than 10 °C from their 'first freeze' value.

The survival of larvae cooled to −10 °C at hourly, daily or weekly intervals on five occasions, and then cooled to their individual SCP after the last exposure to −10 °C was significantly lower in larvae that were frozen and thawed at hourly intervals (figure 2*a*). Pupation was also significantly lower than the control in all treatment groups (figure 2*b*); adult emergence was significantly lower in larvae that were frozen and thawed at hourly and weekly intervals (figure 2*c*). In comparison with larvae that were similarly exposed on five occasions to −10 °C, but not cooled to their individual SCP after the last exposure (figure 1), survival was lower in the hourly and weekly exposures but similar in the daily group. However, no larvae pupated (and therefore none emerged), when the weekly exposed group were cooled to their SCP after the last exposure to −10 °C (figures 1*b*,*c* and 2*b*,*c*).

When similar samples of acclimated larvae were cooled to their individual SCP on five occasions at daily or weekly intervals, thus ensuring that every surviving larva was frozen in every exposure, the pattern of results was even more divergent from that of 'once frozen' larvae (table 2). The mean SCP was significantly lower between the first and last freeze, and the range of SCP increased with some larvae freezing as low as −28 °C. Also, survival and pupation of larvae cooled to their individual SCP on five occasions at daily or weekly intervals were significantly lower than that of larvae exposed to −10 °C (figure 1*a*) at the same intervals. The only larvae that survived repeated freezing on five occasions (12.5% and 8.3% for the daily and weekly groups, respectively) were those in which the SCP did not change between each freezing exposure (table 2). All larvae that showed a depression in the SCP (greater than 85% in each group) died soon afterwards, and no larva pupated successfully in either group, irrespective of the SCP.

Table 1. Mean (± s.e.m.) and range of SCP of acclimated third-instar *Syrphus ribesii* larvae subjected to five cycles of cooling to −10 °C and warming to 10 °C.
(Means followed by the same superscript letter are not significantly different at $p = 0.05$ (Tukey's pairwise comparisons).)

exposure interval	*n*	mean SCP (± s.e.m.) (°C)	range (°C)
none (control)	24	-7.6 ± 0.40^{a}	−5.0 to −9.5
hourly	24	-10.3 ± 0.94^{b}	−4.8 to −21.9
daily	24	$-8.5 \pm 0.69^{a,b}$	−5.4 to −21.5
weekly	24	$-9.9 \pm 0.80^{a,b}$	−5.6 to −20.2

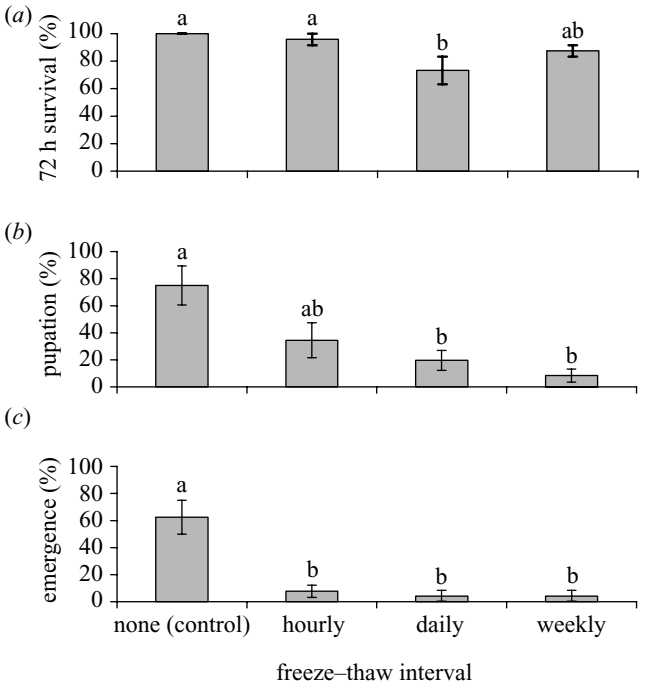

Figure 1. (*a*) Survival, (*b*) pupation and (*c*) emergence of acclimated third-instar larvae of *Syrphus ribesii* subjected to five cycles of cooling and warming from −10 °C to 10 °C at hourly, daily and weekly intervals. Columns labelled with the same letter represent means that are not significantly different at $p = 0.05$ (Tukey's pairwise comparisons).

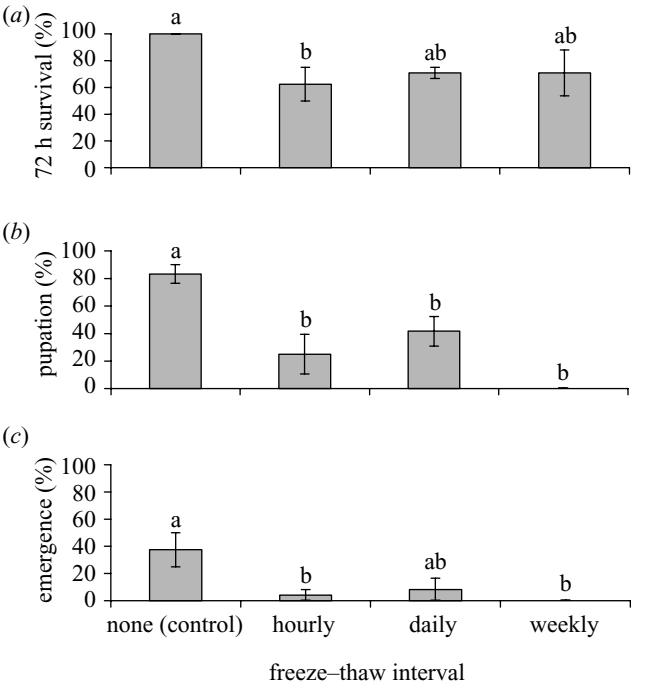

Figure 2. (*a*) Survival, (*b*) pupation and (*c*) emergence of acclimated third-instar larvae of *Syrphus ribesii* subjected to five cycles of cooling and warming from −10 °C to 10 °C at hourly, daily and weekly intervals and cooled to their individual SCP after the final exposure. Columns labelled with the same letter represent means that are not significantly different at $p = 0.05$ (Tukey's pairwise comparisons).

4. DISCUSSION

The classification of cold-hardy insects as freeze tolerant or freeze avoiding is well established (Salt 1961) and supported by an increasing understanding of the roles of INAs, antifreeze proteins and polyols in the two strategies (Duman 2001). It is known that many insects can avoid freezing to below −20 °C, yet die at higher temperatures, leading to recent 'reclassifications' of insects that die predominantly from non-freezing injuries (Bale 1993, 1996). By contrast, freeze-tolerant insects have been regarded as a more uniform group, largely because they all share the common ability to survive partial freezing of their body tissues and fluids, and all die at a temperature below that at which they freeze (Duman 2001; Bale 2002). There is, however, considerable variation between different species of freeze-tolerant insects in terms of the SCP, lethal temperature, type and location of the nucleating agent, and geographical distribution (Sinclair 1999; Duman 2001; Sinclair *et al.* 2003). Also, some species retain freeze tolerance in summer (Van der Laak 1982) and others alternate between freeze tolerance and freeze avoidance in different years (Horwath & Duman 1984; Kukal & Duman 1989). The results described here are, however, fundamentally different from previous studies because the loss of freeze tolerance occurs in the same individual in the same 'winter' within a single species.

Microclimatic monitoring has indicated that some freeze-tolerant insects experience environmental freeze–thaw cycles in their overwintering sites (Layne 1991; Sinclair 1997). It is clear, however, from the data reported here, that overwintering freeze-tolerant insects do not necessarily undergo internal freeze–thaw cycles with the same frequency as their external environment because the freezing event depresses the SCP, in some cases by up to 20 °C. In the case of *S. ribesii*, the depression of the SCP after only one freezing event is sufficient to prevent 're-freezing' of some larvae, even in the coldest of UK winters (Bale 1987).

This study is of interest because it highlights three features that, to our knowledge, have not been previously described for an overwintering freeze-tolerant insect. First,

Table 2. Initial and final SCP (mean ± s.e.m.) of *Syrphus ribesii* larvae frozen up to five times at daily or weekly intervals. (Means followed by the same superscript letter are not significantly different at $p = 0.05$ (Tukey's pairwise comparisons).)

freezing interval	*n*	initial SCP (mean ± s.e.m.) and range (°C)	final SCP (mean ± s.e.m.) and range (°C)	survival after 72 h (%)	pupation after 72 h (%)
daily	24	-7.5 ± 0.47^{a} -2.7 to -15.1	-10.2 ± 1.15^{b} -6.3 to -28.0	12.5 ± 8.0	0
weekly	24	-8.0 ± 0.41^{a} -6.7 to -13.3	-11.8 ± 1.12^{b} -5.6 to -21.1	8.3 ± 4.8	0

after the first freezing event with a consistent pattern of SCPs and all larvae surviving the freezing exposure, the sample becomes segregated into sub-populations in which some larvae freeze at temperatures between 15 °C and 20 °C below their 'first freeze' value. Second, after freezing once, the SCP is depressed in most of the population, which effectively become 'freeze-avoiding' insects because they are then killed by freezing. Third, insects that experience more than one freezing event are less likely to survive, pupate or emerge.

In turn, these observations give rise to two questions of fundamental importance to our understanding of insect overwintering strategies. First, why should a nucleator become less effective or totally inactivated after one freezing event, but only in certain individuals? It is known that in one freeze-tolerant insect (*Tipula trivittata*) high concentrations of a lipoprotein ice nucleator (1×10^{-9} M) are required to initiate freezing in 1 µl droplets (Neven *et al.* 1989; Duman *et al.* 1992), suggesting that either ice-nucleating ability is very rare in such molecules, or that aggregation and cooperation are required for effective nucleation (Duman 2001). Structural studies on the *Tipula* nucleator suggest that the size of embryo ice crystal formed depended on the orientation of the ice-nucleating surfaces of individual nucleator molecules (Duman *et al.* 1992). One explanation for the loss of nucleator efficiency in *S. ribesii* would be the destruction or distortion of this ice-nucleating surface after one freezing event, though this might be expected to be a random occurrence in successive freezing events, whereas the retention of a consistent nucleation temperature occurs in a small proportion of larvae.

Second, what are the advantages of changing strategy from freeze tolerance to freeze avoidance after one freezing exposure? In a recent study, Voituron *et al.* (2002) developed an optimized fitness model incorporating physiological parameters (energetic level, physiological stress, climatic variables) to determine the environmental conditions under which freeze tolerance, freeze avoidance or a mixed strategy would be the preferred overwintering option. Although the model identified combinations of initial energetic levels and relative sensitivities to climatic conditions under which freeze tolerance or freeze avoidance would be preferred, it was concluded that in variable environments, a 'mixed strategy' had energetic advantages, enabling animals to survive during periods of severe stress. It was further noted that very few mixed-strategy animals have so far been described, and importantly, none that shows the response reported here for *S. ribesii*.

The strategy switch from freeze tolerance to avoidance in *S. ribesii* larvae has some advantages, including a reduction in the deleterious effects associated with the 'freeze–thaw' process, such as the risk of secondary recrystallization (Knight *et al.* 1984; Bale 2002). There are also some disadvantages, the most obvious being that the larvae are killed by freezing; however, the depression of the SCP is so great in some individuals (to −28 °C) that the risk of instantaneous freezing would be very low in temperate climates and also in colder regions, where larvae would be thermally buffered under winter snow cover. In evolutionary terms, a dual overwintering strategy may be an effective 'bet hedging' response for species living in a variable climate where environmental freeze–thaw cycles are common. It might be expected therefore that this loss of freeze tolerance would be less common or absent in freeze-tolerant species inhabiting the most extreme climates, where individuals may remain frozen throughout the winter. This response has now been detected in both of the freeze-tolerant insects so far investigated (see Bale *et al.* 2001). The wider occurrence of this phenomenon would require a fundamental reassessment of the dynamics and distinctions of the freeze-tolerant and freeze-avoiding strategies of insect overwintering.

REFERENCES

Bale, J. S. 1987 Insect cold hardiness: freezing and supercooling—an ecophysiological perspective. *J. Insect. Physiol.* **33**, 899–908.

Bale, J. S. 1993 Classes of insect cold hardiness. *Funct. Ecol.* **7**, 751–753.

Bale, J. S. 1996 Insect cold hardiness: a matter of life and death. *Eur. J. Entomol.* **93**, 369–382.

Bale, J. S. 2002 Insects and low temperatures: from molecular biology to distributions and abundance. *Phil. Trans. R. Soc. Lond.* B **357**, 849–862. (DOI 10.1098/rstb.2002.1074.)

Bale, J. S., O'Doherty, R., Atkinson, H. J. & Stevenson, R. A. 1984 An automatic thermoelectric cooling method and computer-based recording system for supercooling studies on small invertebrates. *Cryobiology* **21**, 340–347.

Bale, J. S., Worland, M. R. & Block, W. 2001 Effects of summer frost exposure on the cold tolerance strategy of a sub-antarctic beetle. *J. Insect Physiol.* **47**, 1161–1167.

Danks, H. V. 1981 *Arctic arthropods*. Ottawa: Entomological Society of Canada.

Duman, J. G. 2001 Antifreeze and ice nucleator proteins in terrestrial arthropods. *A. Rev. Physiol.* **63**, 327–357.

Duman, J. G., Wu, D. W. & Yeung, K. L. 1992 Hemolymph proteins involved in the cold tolerance of terrestrial arthropods: antifreeze and ice nucleator proteins. In *Water and life: comparative analysis of water relationships at the organismic, cellular and molecular level* (ed. G. N. Somero, C. B. Osmond & L. Bolis), pp. 282–300. Berlin: Springer.

Hart, A. J. & Bale, J. S. 1997 Evidence for the first strongly freeze-tolerant insect found in the UK. *Ecol. Entomol.* **22**, 242–245.

Holmstrup, M., Bayley, M. & Ramlov, H. 2002 Supercool or dehydrate? An experimental analysis of overwintering strategies in small permeable arctic insects. *Proc. Natl Acad. Sci. USA* **99**, 5716–5720.

Horwath, K. L. & Duman, J. G. 1984 Yearly variations in the overwintering mechanisms of the cold hardy beetle *Dendroides canadensis*. *Physiol. Zool.* **57**, 40–45.

Knight, C. A., DeVries, A. L. & Oolman, L. D. 1984 Fish antifreeze protein and the freezing and recrystallisation of ice. *Nature* **308**, 295–296.

Kukal, O. 1991 Behavioral and physiological adaptations to cold in a freeze tolerant arctic insect. In *Insects at low temperature* (ed. R. E. Lee & D. L. Denlinger), pp. 276–300. New York: Chapman & Hall.

Kukal, O. & Duman, J. G. 1989 Switch in the overwintering strategy of two insect species and latitudinal differences in cold hardiness. *Can. J. Zool.* **67**, 825–827.

Layne, J. R. 1991 Microclimatic variability and the eurythmic nature of goldenrod gallfly (*Eurosta solidaginis*) larvae (Diptera: Syrphidae). *Can. J. Zool.* **69**, 614–617.

Miller, L. K. 1982 Cold hardiness strategies of some adult and immature insects overwintering in interior Alaska. *Comp. Biochem. Physiol.* A **73**, 595–604.

Neven, L. G., Duman, J. G., Low, M. G., Sehl, L. C. & Castellino, F. J. 1989 Purification and characterization of an insect hemolymph lipoprotein ice nucleator: evidence for the importance of phosphatidylinositol and apolipoprotein in the ice nucleator activity. *J. Comp. Physiol.* **159**, 71–82.

Ring, R. A. 1982 Freezing tolerant insects with low supercooling points. *Comp. Biochem. Physiol.* A **73**, 605–612.

Salt, R. W. 1961 Principles of insect cold hardiness. *A. Rev. Entomol.* **6**, 55–74.

Sinclair, B. J. 1997 Seasonal variation in freezing tolerance of the New Zealand alpine cockroach *Celatoblatta quinquemaculata*. *Ecol. Entomol.* **22**, 462–467.

Sinclair, B. J. 1999 How many kinds of frozen. *Eur. J. Entomol.* **96**, 157–164.

Sinclair, B. J., Addo-Bediako, A. & Chown, S. L. 2003 Climatic variability and evolution of insect freeze tolerance. *Biol. Rev.* **78**, 181–195.

Van der Laak, S. 1982 Physiological adaptations to low temperature in freezing-tolerant *Phyllodecta laticollis* beetles. *Comp. Biochem. Physiol.* A **73**, 613–620.

Voituron, Y., Mouquet, N., de Mazancourt, C. & Clobert, J. 2002 To freeze or not to freeze? An evolutionary perspective on the cold-hardiness strategies of overwintering ectotherms. *Am. Nat.* **160**, 255–270.

Zachariassen, K. E. 1985 Physiology of cold tolerance in insects. *Physiol. Rev.* **65**, 799–832.