

Regulation of supercooling and ice nucleation in insects

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Abstract. Since most insects are unable to survive internal ice formation a key factor in their overwintering survival is the regulation of the temperature at which they spontaneously freeze, termed the supercooling point or temperature of crystallization. Most insects enhance their supercooling capacity during the winter by eliminating endogenous ice nucleators, accumulating low-molecular-weight polyols and sugars, and synthesizing hemolymph antifreeze proteins. In the absence of heterogeneous ice nucleators, small body size promotes supercooling to temperatures 25°C or more below the freezing point of their body fluids. Although susceptibility to inoculative freezing is clearly detrimental for most species, in some insects it is required for freeze tolerance. Some freeze-tolerant species produce ice-nucleating proteins or lipoproteins that initiate freezing at relatively high subzero temperatures. Insoluble crystalloid deposits, such as calcium phosphate, present in a variety of insects, represent a newly-discovered class of endogenous ice nucleators. During the last few years, a number of research groups have reported ice-nucleating-active bacteria and fungi as normal flora in the gut of overwintering insects. Since these ice-nucleating-active microorganisms markedly reduce the supercooling capacity of insects when ingested or applied topically, they may offer a novel means for the biological control of insect pests during the winter.

INTRODUCTION

Interest in the adaptations of insects to low temperature have proliferated over the last decade. Recent reviews and papers have considered various aspects of insect adaptations to low temperature ranging from cellular, endocrine and metabolic functions to organismal and ecological considerations, as well as the implications for pest management (Bale, 1987; Block, 1990; Cannon & Block, 1988; Danks, 1987; Duman et al., 1992, 1995; Leather et al., 1993; Lee, 1989; Lee R.E. et al., 1993b, 1995a; Lee & Denlinger, 1991; Sømme, 1989; Storey & Storey, 1988; Tauber et al., 1986; Zachariassen, 1985, 1992; Zachariassen & Lundheim, 1992). The purpose of this brief review is to summarize factors regulating supercooling and ice nucleation in insects. Particular emphasis is given to the site of ice formation, the significance of inoculative freezing, a novel class of endogenous crystalloid ice nucleators, and the regulation of insect supercooling using ice-nucleating-active microorganisms for biological control.

In contrast to the case with freeze-intolerant insects, species that tolerate extensive ice formation within their body fluids often increase their supercooling point as they cold-harden for winter. It is generally believed that freezing at high subzero temperatures is adaptive because it slows the rate of ice formation, allowing insects to gradually adjust to the osmotic and mechanical stresses associated with freezing. This response may also promote conservation of metabolic reserves since the metabolic rate is significantly depressed

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in the frozen state. Zachariassen (1985) postulated that another benefit of freezing is water conservation. He proposed that insects overwintering in a supercooled state would lose more water than frozen insects since the vapor pressure of their body water would be higher than that of ice within their hibernaculum. Thus overwintering insects should desiccate less while frozen. Support for this concept was provided by Lundheim & Zachariassen (1993) when they empirically demonstrated that supercooled insects experience greater water loss than insects frozen at the same temperature.

REGULATION OF SUPERCOOLING

Supercooling refers to the maintenance of body water in the liquid state at temperatures below its equilibrium freezing/melting point. The supercooling point refers to the lowest temperature to which an insect may be cooled before spontaneous ice nucleation occurs within body fluids. Typically insects exhibit seasonal patterns of their supercooling capacities. Freeze-intolerant insects typically enhance their supercooling capacity as they prepare for winter and thereby decrease the chance that lethal freezing of their body fluids will occur.

Although a number of factors contribute to the capacity of insects to supercool, perhaps the most important one is a consequence of their small size and accordingly their small water volume. For example, the relatively larger vertebrate ectotherms have only a limited capacity to supercool that is strongly correlated with body mass (Costanzo & Lee, 1995). Similarly the supercooling capacity of terrestrial arthropods increases with decreasing body mass (Block & Young, 1979). In a recent study of terrestrial mites, Pugh (1994) found that low water content was a pre-adaptive characteristic promoting supercooling. Similarly, supercooling capacity in the Colorado potato beetle (*Leptinotarsa decemlineata*) is strongly influenced by body water content (Fig. 1). This trend closely matches physical studies in which small volumes of pure water readily remain unfrozen at temperatures 40°C below their equilibrium freezing point (Angell, 1982). Within living systems it is believed that the onset of ice nucleation occurs via a heterogeneous mechanism that requires a non-water substance to serve as the nucleus upon which the ice lattice may organize and grow (Franks, 1985; Lee, 1991).

Cessation of feeding and voiding of the gut, often associated with preparation for overwintering, appears to be another major factor promoting supercooling. Gut evacuation may increase supercooling by removing efficient ice nucleating agents, which may include inorganic or organic nuclei as well as ice-nucleating-active microorganisms. Evidence supporting this function is particularly strong for polar microarthropods in which starvation causes major shifts in the distribution of supercooling points (for a review see Block, 1990).

Hydration state of the body also exerts an important influence on supercooling capacity, presumably due to changes in total water content. In eggs of the stonefly, *Arcynopteryx compacta*, dehydration increases supercooling capacity and cold tolerance without the accumulation of cryoprotectants (Gehrken & Sømme, 1987). In a recent review, Ring & Danks (1994) emphasize that resistance to desiccation and cold-hardiness are closely linked and that cryoprotectant accumulation may have originally evolved as an adaptation to other factors such as water conservation.

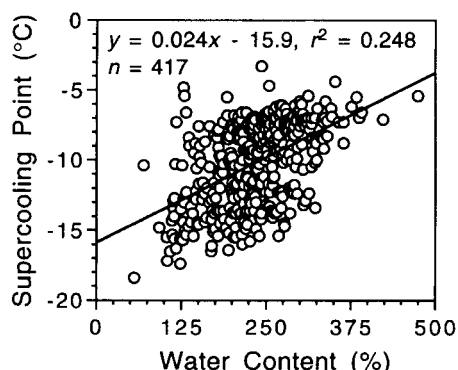


Fig. 1. Correlation of body water content (% dry mass) and supercooling point of adult Colorado potato beetles (*Leptinotarsa decemlineata*). Beetles were acclimated to 4°C in darkness and tested during the winter. (J.P. Costanzo, J.B. Moore, R.E. Lee, P.E. Kaufman & J.A. Wyman, unpublished data.)

et al., 1992, 1995). These proteins cause a thermal differential between the melting point (i.e., temperature at which the last ice crystal melts with slow warming) and the freezing point (i.e., the temperature at which an ice crystal will begin to grow in a solution). In freeze-intolerant insects such antifreeze proteins function to stabilize the supercooled state and prevent internal ice formation. Also, the correlation between increased levels of antifreeze proteins and resistance to inoculative freezing suggests that, like the antifreeze proteins of polar fishes, these agents may serve to prevent the propagation of ice across the insect integument (Duman et al., 1993). Finally, antifreeze proteins may prevent freezing injury in freeze-tolerant species by inhibiting damaging ice recrystallization (Duman et al., 1993).

In summary, enhanced supercooling capacity in insects is strongly linked to their small size, and consequently small water volume, and the removal of efficient nucleating agents within the gut or elsewhere within the body. Water balance, cryoprotectant accumulation, and antifreeze proteins also may influence supercooling capacity.

SITE OF ICE NUCLEATION AND FREEZING: INTRA VERSUS EXTRACELLULAR

Under naturally experienced environmental temperatures and cooling rates it is generally believed that survival of freezing is only possible if ice formation is restricted to the extracellular space (Mazur, 1984). As ice forms in the extracellular fluids only water molecules join the ice lattice, causing an increase in the solute concentration of the unfrozen water fraction surrounding cells. This hypertonic stress results in the gradual removal of cell water and promotes supercooling of cytoplasmic water. As the body temperature decreases more ice is formed and cells become progressively more dehydrated.

In 1959 and 1962, R.W. Salt published two papers in *Nature* that challenged the dogma that intracellular freezing was lethal by reporting that fat body cells of the freeze-tolerant goldenrod gall fly larvae, *Eurosta solidaginis*, survived intracellular freezing. Lee et al.

The well-known accumulation of low molecular mass polyols and sugars such as glycerol, sorbitol and trehalose by overwintering insects may also increase supercooling capacity in insects (for reviews see Zachariassen, 1985; Duman et al., 1995). These cryoprotectants generally depress the supercooling point about twice as much as the melting point is decreased (Block & Young, 1979). Since melting points decrease by 1.86°C per osmol, extremely high concentrations of cryoprotectants must be synthesized by insects to significantly enhance supercooling capacity based on this colligative effect.

Lastly, the supercooling capacity of overwintering insects also may be increased by the production of antifreeze proteins and glycoproteins (Zachariassen, 1985; Duman

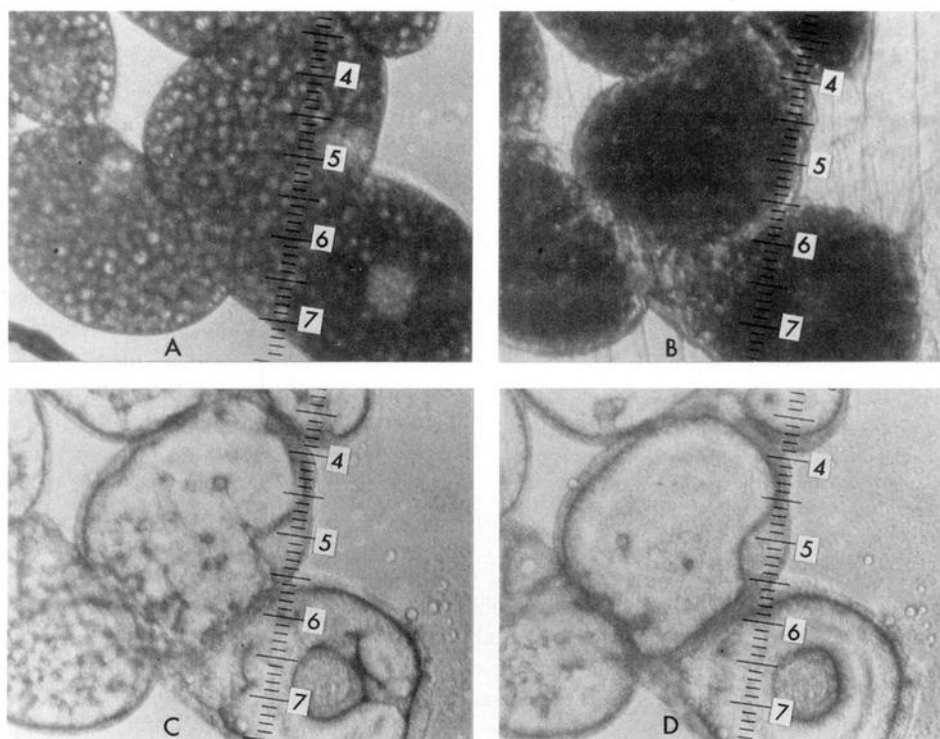


Fig. 2. Representative cryomicroscopic photographs of sequential freezing and thawing in fat body cells of the freeze-tolerant gall fly larvae, *Eurosta solidaginis*. A – unfrozen fat body cells at 5°C; B – intracellular freezing of fat body cells; C – beginning of lipid coalescence immediately after thawing; D – extensive lipid coalescence within one minute after thawing. Each minor scale division = 10 μ m. (From Lee et al., 1993a.)

(1993a) confirmed and extended Salt's observations using cryomicroscopy and fluorescent vital dyes. Intracellular freezing in larval *E. solidaginis* was evidenced by an abrupt darkening of the cytoplasm, termed "flashing", shortly after ice in the surrounding medium contacted the cell membrane (Fig. 2). Intracellular freezing occurred at the relatively high temperature of -4.6°C . This result is markedly different than the case in most mammalian cells where, with slow cooling, inoculative freezing of the cytoplasm is blocked by the cell membrane at temperatures above -15°C (Mazur, 1984). The relative ease with which ice inoculates the cytoplasm of fat body cells of *E. solidaginis* suggests that this may be a cellular adaptation for freeze tolerance in this species. Although no larvae survive freezing to -80°C , 60% of the fat body cells survive freezing to this temperature. Fat body cells survive intracellular freezing even though lipid droplets coalesce and displace the nucleus and other organelles to the periphery of the cell (Morason et al., 1994).

The idea that intracellular freezing is lethal is based on cryobiological investigations using mammalian and human tissues that obviously would never naturally experience freezing temperatures. The results with *E. solidaginis* fat body raise the possibility that

intracellular freeze tolerance may occur in other cell types and perhaps in other species. It is possible that intracellular freeze tolerance may be common in organisms that are naturally freeze-tolerant. Cryomicroscopy and fluorescent vital dyes provide useful tools for investigating this possibility.

INOCULATIVE FREEZING

Two general mechanisms for initiating freezing in insects are known: inoculative freezing by external ice, and heterogeneous ice formation within body fluids promoted by non-water nuclei, such as ice nucleating proteins, crystalloid compounds and ice-nucleating-active microorganisms. Inoculative freezing is the process whereby contact with external ice initiates freezing within the body fluids. The term "crystallization temperature", a synonym for supercooling point, seems particularly appropriate when used to describe the onset of ice nucleation caused by inoculative freezing since there may be little or essentially no supercooling of body fluids prior to ice formation.

Inoculative freezing has received considerable attention in recent years and appears to be an important factor in the cold-hardiness of many insects. A number of terrestrial arthropods require inoculative freezing in order to survive internal ice formation (Table 1), since if contact with external ice is prevented these species supercool several degrees or more and die when spontaneous freezing occurs. For example, in larvae of a freeze-tolerant crane fly (*Tipula* sp.), that overwinter terrestrially, inoculative freezing of rectal fluids on the cuticle surrounding the anus leads to growth of the ice lattice into the larval gut (Gehrken & Southon, 1992).

TABLE 1. Terrestrial arthropods for which inoculative freezing at high subzero temperatures is required for freeze tolerance.

Stage and species	Reference
fly prepupa, <i>Sciara</i> sp. (Sciaridae, Diptera)	Tanno, 1977
larva, <i>Cisseps fulvicollis</i> (Arctiidae, Lepidoptera)	Fields & McNeil, 1986
larva, <i>Chymomyza costae</i> (Drosophilidae, Diptera)	Shimada & Riihimaa, 1988
adult, <i>Bolitophagus reticulatus</i> (Tenebrionidae, Coleoptera)	Gehrken et al., 1991
larva, <i>Tipula</i> sp. (Tipulidae, Diptera)	Gehrken & Southon, 1992
centipede, <i>Lithobius forficatus</i> (Chilopoda)	Tursman et al., 1994

Inoculative freezing appears to play a detrimental role in the cold-hardiness of migrating and overwintering monarch butterflies, *Danaus plexippus*. At overwintering sites in Mexico, adult monarchs incur increased freezing mortality when they are exposed to dew or rain (Calvert et al., 1983; Alonso-Mejia et al., 1992). In the laboratory, adult monarchs misted with water froze and died when exposed to -4°C for 24 h, whereas all dry individuals remained supercooled and survived this treatment (Fig. 3) (Larson & Lee, 1994). Furthermore, wetted monarchs had significantly higher supercooling points that were correlated with their water content (Fig. 3). These results indicate that inoculative freezing may play a significant role in the low temperature survival of monarchs during migration and overwintering.

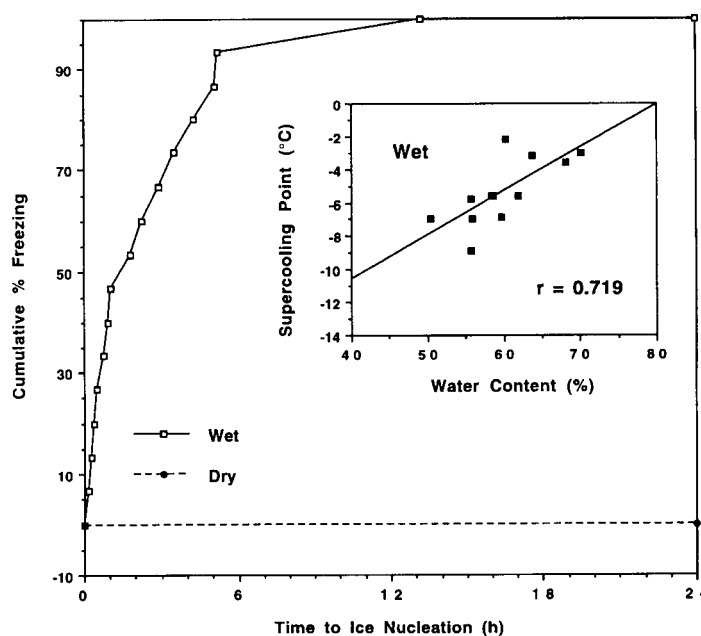


Fig. 3. Duration of supercooling at -4°C as measured by the cumulative percent frozen and the time to ice nucleation of misted (surface-wetted) monarch butterflies ($n = 16$) acclimated at 4°C . Control (dry) acclimated monarchs ($n = 16$) remained supercooled, at -4°C , and did not freeze within 24 h. Insert: Correlation of supercooling point and water content of misted monarch butterflies after acclimation over a 6 to 8 week period to simulated winter conditions of 4°C . (Adapted from Larson & Lee, 1994.)

ICE NUCLEATING PROTEINS

A number of ice nucleating proteins and lipoproteins have been identified in freeze tolerant insects. The ice-nucleating activity of these proteins is typically in the range of -6 to -10°C . The reader is referred to Duman et al. (1992, 1995) and Zachariassen (1992) for recent reviews of the structure and function of this well-known class of ice nucleators.

CRYSTALLOID COMPOUNDS WITH ICE-NUCLEATING ACTIVITY

Recent studies with the freeze-tolerant larvae of *E. solidaginis* suggest that endogenous crystalloid deposits may represent a new class of compounds with heterogeneous ice-nucleating activity in insects. A variety of inorganic and organic crystals are known to have ice-nucleating activity (Fukuta, 1966; Gavish et al., 1992). Although some investigators have speculated that endogenous crystals in insects might also have ice-nucleating activity, until recently this idea had not been confirmed (J.G. Duman as cited in Cannon & Block, 1988).

Third instar larvae of *E. solidaginis* are intolerant of freezing during the summer, but in autumn acquire freeze tolerance and their supercooling points increase from below -15°C to approximately -10°C (Morrissey & Baust, 1976). The hemolymph of overwintering larvae typically supercools to -18°C or lower indicating the absence of efficient ice

nucleators (Bale et al., 1989). Our experiments with large crystalloid spherules found within the Malpighian tubules of overwintering larvae indicate that these spherules function as ice nucleators with sufficient activity to regulate the supercooling point of the gall fly larvae (Lee et al., 1992a; Mugnano et al., in press).

Like other cyclorrhaphid larvae, *E. solidaginis* has two pairs of Malpighian tubules (Waterhouse, 1950). Each larva contains 25–45 crystalloid spherules spaced at regular intervals in the anterior pair of tubules as evidenced by prominent swellings. Scanning electron micrographs showed that the 100–300 µm in diameter crystals were conglomerates of numerous round particles. Energy dispersive X-ray microanalysis and infrared spectroscopy indicated that the spherules were a hydrate of tribasic calcium phosphate $[\text{Ca}_3(\text{PO}_4)_2 \cdot x \text{H}_2\text{O}]$. X-ray diffraction studies revealed that the spherules were an amorphous compound lacking crystalline structure.

Calcium phosphate spherules significantly reduced the supercooling capacity of the suspension medium (-18°C) as indicated by the $\sim 8^\circ\text{C}$ increase in the mean supercooling point (Table 2). Of particular interest is the fact that some samples with spherules had particularly high nucleating activity and froze at temperatures as high as -6.0°C . The ice-nucleating activity of calcium phosphate spherules corresponded closely to the whole body supercooling point of overwintering larvae (Table 2). The spherule supercooling point was significantly higher than those from isolated larval tissues with the exception of fat body cells, which appear to induce ice nucleation through an as yet unidentified mechanism (Table 2). In contrast, Tsumuki & Konno (1991) found highest levels of ice-nucleating activity in muscle and epidermis of the rice stem borer.

TABLE 2. Supercooling points of various tissues removed from overwintering, third-instar larvae of *Eurosta solidaginis* (from Mugnano et al., 1996). All calcium phosphate spherules and tissues removed from a single larva were placed in glass capillary tubes containing 10 µl Schneider's insect media and tested for ice-nucleating activity using previously described methods (Lee et al., 1981).

Tissue	Supercooling point ($^\circ\text{C}$) \pm SEM
whole larvae	-9.4 ± 0.2 (38)
fat body cells	-10.9 ± 0.9 (19)
fat body cells (enzymatic digestion)	-8.9 ± 0.2 (20)
calcium phosphate spherules	-10.1 ± 0.9 (20)
hemolymph	-17.8 ± 0.5 (20) ***
Malpighian tubules	-21.4 ± 1.2 (20) ***
trachea	-17.0 ± 1.0 (20) **
neural tissue	-18.3 ± 1.1 (20) ***
gut	-18.6 ± 1.2 (20) ***
muscle/epidermis	-15.5 ± 1.0 (19) *
Schneider's solution (suspension media)	-18.4 ± 0.8 (20) ***

All temperatures are means \pm standard error. Number of samples shown in parenthesis. Values followed by asterisks are significantly different from the whole larvae value (Dunn's test), * $P < 0.05$, ** $P < 0.01$, *** $P < 0.0001$.

During the larval-pupal metamorphosis, the whole body supercooling point decreased by $\sim 10^\circ\text{C}$, from -10.0 to -20.3°C , while the spherule content decreased to zero. The disappearance of the ice-nucleating-active calcium phosphate spherules in pupae is consistent

with their increased capacity to supercool, and further supports the hypothesis that at least one function of these endogenous spherules is to regulate freezing.

Other endogenous crystalloid deposits occur in insects including ammonium, calcium, potassium and sodium urates, calcium carbonate, calcium oxalate, potassium phosphate and uric acid (Brown, 1982; Wigglesworth, 1972). Saturated solutions of undissolved potassium phosphate, uric acid reagents, potassium urate, and sodium urate had mean supercooling points between -9.2°C and -11.7°C . Although the ice-nucleating activity of these crystalloid compounds is less than that of ice nucleating proteins and lipoproteins that are active in the range of -6 to -9°C , they are sufficient to explain the supercooling points of insects that freeze at slightly lower temperatures. Like proteinaceous ice nucleators these endogenous spherules may function to provide protective extracellular freezing at relatively high subzero temperatures (Zachariassen & Hammel, 1976). Although additional experiments are needed to investigate the role of endogenous compounds in other insects, we believe these spherules represent a novel class of heterogeneous ice nucleators in invertebrates.

ICE-NUCLEATING-ACTIVE MICROORGANISMS

A little more than 20 years ago a unique group of bacteria with ice-nucleating activity was discovered (see review by Upper & Vali, 1995). These asporogenous, Gram-negative, facultative anaerobic rods have greater ice-nucleating activity than any known substance other than ice itself, with the capacity to catalyze ice formation at temperatures as high as -2°C . Ice-nucleating activity in these bacteria derives from the aggregation of membrane-bound proteins in the outer cell wall. Most of these bacteria are epiphytic plant pathogens that promote freezing injury by nucleating water on the plant surface that causes freezing of the underlying tissues. These unusual bacteria are responsible for substantial amounts of frost-related crop losses. More recently, ice-nucleating activity has been described in the fungal component of lichens and free-living fungi. The molecular biology, biochemistry and ecology, as well as technological applications, of these microorganisms are reviewed in a recent book on biological ice nucleation (Lee R.E. et al., 1995b).

ICE NUCLEATING MICROORGANISMS IN THE INSECT GUT

Previous reports have provided direct and indirect evidence that efficient ice nucleators are found in the insect gut, although the specific type of nucleator remained unidentified. Recently, however, several reports described ice-nucleating-active bacteria or fungi as normal flora in the insect gut. Several strains of ice-nucleating-active *Enterobacter taylorae* and *Enterobacter agglomerans* were isolated from adult beetles of *Hippodamia convergens* and *Cerotoma trifurcata* (Strong-Gunderson et al., 1990a; Lee et al., 1991). *E. taylorae* had not previously been reported to have ice-nucleating activity. Both *Enterobacter* species were efficient ice nucleators with maximal activity of -2°C as determined using the droplet freezing assay of Vali (1971). This level of activity was only slightly less than the highly efficient epiphytic bacterium *Pseudomonas syringae*. The activity of *E. taylorae* and *E. agglomerans* was confirmed in vivo by feeding them to overwintering adults of *H. convergens* whose supercooling points increased markedly (Strong-Gunderson et al., 1990b).

Kaneko et al. (1991a,b) isolated *Erwinia herbicola* from the gut of the diamondback moth, *Plutella xylostella*. Larvae that had been reared on aseptic radish seeds produced pupae with low supercooling points. In contrast, ones reared on non-aseptic seeds had elevated supercooling points.

An ice nucleating fungus (*Fusarium* sp.) is known from the gut of the rice stem borer, *Chilo suppressalis* (Tsumuki et al., 1992). This discovery was made after determining that the gut and frass had relatively high supercooling points similar to the temperature at which the whole body spontaneously froze (-8.3°C), while other body compartments supercooled considerably more.

Considering the relative ease with which our research group has identified ice-nucleating-active microorganisms from insects, as well as the recent isolation of a ice-nucleating-active strain of *Pseudomonas putida* from a freeze-tolerant frog (Lee M.R. et al., 1995), and the apparent fact that few research groups have searched for these organisms, it is possible that they are commonly found in the gut of insects and other animals. For example, Worland et al. (1993) reported ice-nucleating activity in the frass of two beetles that suggested the presence of ice-nucleating-active bacteria. For freeze-tolerant insects such as *C. suppressalis*, these microorganisms apparently function as another class of biological ice nucleator that insures protective freezing at high subzero temperatures (Lee et al., 1993b). However, for species such as *H. convergens* that do not tolerate internal ice formation, the function of ice nucleating microorganisms is unclear. Presumably these microorganisms would have to be removed from the gut or their ice nucleating suppressed to successfully overwinter.

BIOLOGICAL CONTROL OF INSECT PESTS USING ICE-NUCLEATING-ACTIVE MICROORGANISMS

The use of ice nucleating microorganisms for biological control is based on several premises: 1) the insect pest is intolerant of freezing as is true for most species, 2) the supercooling point of the pest can be elevated by applying ice-nucleating-active microorganisms, and 3) environmental temperatures are sufficiently low so that insects whose supercooling points have been elevated will freeze (Lee et al., 1993b, 1995b). In our initial study, *H. convergens* adults fed water or a non-ice-nucleating-active bacterial control (*Escherichia coli*) supercooled to about -16°C before freezing. In contrast, ingestion of ice-nucleating-active *P. syringae* markedly increased the supercooling point by as much as 14°C (Strong-Gunderson et al., 1990b).

Since that initial report the supercooling point of more than 15 species representing four insect orders has been elevated by ingestion or surface application of ice-nucleating-active bacteria and fungi (Strong-Gunderson et al., 1992, 1994; Fields et al., 1993; Lee et al., 1993b, 1995b). A surprising outcome was the ease with which the supercooling point could be elevated using surface misting of the bacterial solution on insects whose mouths had been sealed to prevent ingestion. Steigerwald et al. (1995) recently investigated the possibility that the spiracles might serve as the route by which surface application of bacteria made contact with and nucleated body water. Untreated adults of *H. convergens* had a mean supercooling point of -14.9°C . Application of suspensions of *P. syringae* to the thoracic spiracle elevated the supercooling point to -5.6°C , a value that was significantly higher than the other sites tested (Fig. 4). Application of the ice nucleating fungus

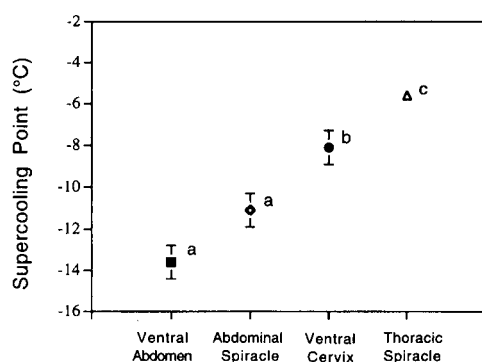


Fig. 4. Supercooling points (mean + SEM, $n = 24$) of adult *Hippodamia convergens* after surface application of an aqueous suspension of 0.5 μ l of 20,000 ppm of *Pseudomonas syringae* preparation to four anatomic sites. Untreated adults had a mean supercooling point of -14.9°C . Mean values identified by different letters are statistically distinguishable. (Data from Steigerwald et al., 1995.)

product pests and found that treatment with *P. syringae* significantly elevated mean supercooling points and decreased survival during 24-hour exposures at -5° and -10°C (Lee et al., 1992b). Fields (1993) extended his studies with *C. ferrugineus* to field conditions in Manitoba granaries. Survival was markedly reduced in treated beetles during the first three weeks when granary temperatures first cooled to temperatures below 0°C .

One concern for the use of *P. syringae* for the control of stored-product pests was its stability at high temperature. Since it would be simplest to add the control agent as the granaries were filled in the late summer or autumn, Fields et al. (1995) examined the stability of *P. syringae* at 30°C over a 16-week period. During this time the capacity of the bacterial treatment to reduce beetle cold-hardiness was only slightly reduced, a result that supports the use of this approach for biological control.

The Colorado potato beetle is the primary pest of potatoes in North America. The species has developed resistance to a wide range of insecticides including synthetic pyrethroids. One approach for the integrated pest management of this species relies on cultural methods of control to expose overwintering adults to lethal low temperatures (Kung et al., 1992; Milner et al., 1992). These cultural manipulations aggregate beetles into areas where insulating mulch can be removed during the winter to suddenly expose the overwintering beetles to low temperature. Freeze-intolerant adults burrow into the soil to overwinter. Application of *P. syringae* to overwintering adults significantly increased their supercooling points from -7.6°C for the untreated group to -3.7°C for beetles treated with 1,000 ppm (Lee et al., 1994). The cumulative supercooling point distributions for beetles treated with 100 ppm suggest that 80% of the beetles would die if exposed to -5°C , while essentially none of the untreated beetles would be expected to die at this temperature. These results suggest that ice-nucleating-active bacteria may be used as a biological insecticide in

(*Fusarium* sp.) to the thoracic spiracle also significantly decreased supercooling capacity. These data support the hypothesis that ice nucleating microorganisms may elevate the supercooling point using non-oral routes.

Stored-product pests are likely candidates for the use of ice nucleating microorganisms for biological control (Fields, 1992). Fields (1990) first suggested this idea when he demonstrated that the cold-hardiness of the rusty grain beetle, *Cryptolestes ferrugineus*, could be significantly diminished using *P. syringae*. At the highest concentration tested (1,000 ppm) supercooling points were elevated 11°C above that of controls. Correspondingly survival at -10°C was significantly reduced following this treatment. Our laboratory tested seven additional stored-

conjunction with cultural manipulations against overwintering adults of this species (Lee et al., 1995a).

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