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FAT BODY CELLS AND CALCIUM PHOSPHATE SPHERULES INDUCE ICE NUCLEATION IN THE FREEZE-TOLERANT LARVAE OF THE GALL FLY *EUROSTA SOLIDAGINIS* (DIPTERA, TEPHRITIDAE)

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Summary

During the autumn, the third-instar larvae of the gall fly *Eurosta solidaginis* acquire freeze tolerance and their crystallization temperatures increase into the −8 to −10 °C range. Despite conflicting reports, efficient endogenous ice nucleators have not been identified in this freeze-tolerant insect. We found large crystalloid spheres within the Malpighian tubules of overwintering larvae. Energy-dispersive X-ray microanalysis and infrared spectroscopy indicated that the spherules were a hydrate of tribasic calcium phosphate. To test for ice-nucleating activity, we placed the calcium phosphate spherules in 10 μl of Schneider's insect medium and cooled them in a refrigerated bath. The addition of spherules increased the crystallization temperature of Schneider's medium by approximately 8 °C, from −18.4±0.8 °C to −10.1±0.9 °C (mean ± S.E.M., *N*=20). Ice-nucleating activity (−10.9±0.9 °C) was also demonstrated in fat body cells suspended in 10 μl of Schneider's medium. Both calcium phosphate spherules and fat body cells have ice-nucleating activity sufficiently high to explain whole-body crystallization temperatures. Furthermore, other crystalloid deposits, commonly found in diapausing or overwintering insects, also exhibited significant ice-nucleating activity. These endogenous crystalloid deposits represent a new class of heterogeneous ice nucleators that potentially regulate supercooling and promote freeze tolerance in *E. solidaginis* and possibly in other overwintering insects.

Key words: gall fly, *Eurosta solidaginis*, freeze tolerance, fat body cell, calcium phosphate, Malpighian tubule.

Introduction

Most insects use one of two strategies for surviving low winter temperatures. Those species that cannot survive internal ice formation depress the temperature at which their body water freezes, termed the crystallization temperature (T_c) or supercooling point. This increase in supercooling capacity is achieved by synthesizing low molecular mass cryoprotectants and antifreeze proteins, and by removing internal heterogeneous ice nucleators. In contrast, freeze-tolerant insects typically increase the temperature at which spontaneous ice nucleation occurs. Some insects achieve this end by synthesizing hemolymph ice-nucleating proteins and lipoproteins; however, efficient internal heterogeneous nucleators have not been identified in all freeze-tolerant species (Lee, 1991).

Freezing at high sub-zero temperatures may confer several adaptive advantages. It has been suggested that the slow formation of extracellular ice allows cells to reach osmotic equilibrium with the extracellular space and thereby avoid the formation of potentially lethal intracellular ice (Zachariassen and Hammel, 1976). Freezing at high sub-zero temperatures lowers the metabolic rate and reduces the use of stored energy reserves (Storey *et al.* 1981). Lundheim and Zachariassen (1993) have recently demonstrated that unfrozen insects with higher vapor pressures lose water to frozen environments with lower vapor pressures; freezing equilibrates the vapor pressures and removes the driving force for water loss.

The gall fly *Eurosta solidaginis* has been studied extensively (in over 50 publications) as an insect cryobiological model (Baust and Nishino, 1991). *E. solidaginis* is a gall-forming dipteran (Tephritidae) that ranges from the Gulf of Mexico into Central Canada (for a review, see Uhler, 1951). The larvae begin to cold-harden in late September by synthesizing glycerol, part of a multicomponent cryoprotective system (Baust, 1986). Glycerol accumulation precedes exposure to environmental temperatures below 15 °C and is induced by the decreasing moisture content of the gall (Rojas *et al.* 1986). Sorbitol and trehalose are synthesized later in direct response to low temperatures (Baust and Lee, 1982). In the presence of

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these low molecular mass cryoprotectants, *E. solidaginis* is freeze-tolerant under natural conditions, and northern populations readily freeze at sub-zero temperatures as high as −8 °C (Morrissey and Baust, 1976). This high supercooling point in the absence of external ice suggests that *E. solidaginis* contains potent heterogeneous ice-nucleating agents. Early reports suggested that *E. solidaginis* contained hemolymph ice-nucleating proteins (Sømme, 1978). A later study challenged this claim and suggested that hemolymph nucleation was induced by the contamination of hemolymph samples with detrital frass (Bale *et al.* 1989). Although studied extensively for its freeze-tolerant properties, the exact mechanism of ice nucleation in *E. solidaginis* has not yet been determined. When we found large crystalloid spherules within the Malpighian tubules of the overwintering larvae of *E. solidaginis*, we hypothesized that these spherules might function as ice nucleators in the gall fly larvae. We report here a description of the endogenous spherules and crystallization temperatures from these spherules and fat body cells with ice-nucleating activity sufficient to explain the whole-body T_c of *E. solidaginis*.

Materials and methods

Approximately 5000 overwintering, third-instar larvae of *E. solidaginis* (Fitch) were collected from goldenrod fields in Butler County, Ohio, USA, between mid-January and mid-February 1994. Larvae were maintained at −20 °C until used. Before experimental manipulation, larvae were dissected from galls, thawed at room temperature, and washed by repeated vortexing in ultrapure water to remove frass and other surface contaminants. Crystallization temperatures were measured by placing larvae in 1.5 ml disposable microcentrifuge tubes with 36 gauge copper–constantan thermocouples, connected to an Omega multichannel temperature recorder, and held in place against the larvae with foam rubber plugs. The microcentrifuge tubes were inserted into glass test tubes and immersed in a Neslab CB-80 refrigerated ethanol-circulating bath and cooled at a rate of approximately 0.8 °C min^{-1}. The crystallization temperature was indicated by a rapid temperature increase due to the release of the latent heat of fusion.

Larvae were dissected with the aid of a dissecting microscope and spherule location and quantity were determined. Spherule composition was ascertained using energy-dispersive X-ray microanalysis (EDAX 9100) and infrared spectroscopy. Scanning electron microscopy, light microscopy and X-ray diffraction were used to investigate spherule structure. Pupariation was induced in some larvae by exposing them to room temperature. Crystallization temperatures were measured and spherule content was determined following pupariation.

After whole larval crystallization temperatures had been measured, the cuticle was opened with fine-tipped forceps. 10 μl hemolymph samples were drawn into glass capillary tubes and sealed with clay not in contact with the fluid. The larvae were then dissected in Schneider's insect medium, pH 6.9, 372 mosmol l^{-1} (Sigma Chemical Co.) (Schneider and Blumenthal, 1978). Fat body cells, Malpighian tubules, neural tissue, gut, muscle/epidermis, tracheae and spherules (contained in Malpighian tubules) were removed and washed in clean Schneider's medium. Tissues were suspended separately in fresh Schneider's medium and inspected with the aid of a dissecting microscope to ensure tissue homogeneity. Fat body cells were also isolated by enzymatic digestion. The viscera of 10 larvae were added to 10 ml of Schneider's medium containing 5 mg of collagenase and 10 mg of hyaluronidase. Visceral tissue was mechanically dissociated on a platform rotator for 5 min at 20 °C. The cell suspension was centrifuged at 87 ***g*** for 5 min and the pellet containing fat body cells was transferred to 10 ml of clean Schneider's medium. This technique allowed the rapid isolation of large quantities of individual fat body cells.

Isolated tissues and spherules, suspended in 10 μl of Schneider's medium, were placed in either 50 μl glass capillary tubes or 0.5 ml polypropylene microcentrifuge tubes. Copper–constantan thermocouples (36 gauge) were attached to the capillary and microcentrifuge tubes with adhesive tape and crystallization temperatures were measured following the cooling protocol for whole larvae. The ice-nucleating activity of other compounds known to form crystalloid deposits in other overwintering or diapausing insects was tested. These compounds included calcium phosphate, potassium phosphate, potassium urate, sodium urate and uric acid. For each compound, saturated solutions containing undissolved solute were prepared in Grace's insect medium at approximately 325 mosmol l^{-1} (Sigma Chemical Co.) (Grace, 1962). 35 μl samples were loaded into 50 μl glass capillary tubes and tested for ice-nucleating activity as described above.

Crystallization temperatures were compared among treatments using Kruskal–Wallis nonparametric analysis of variance (ANOVA), with means distinguished (P<0.05) using Dunn's comparison test.

Results

Identification of spherules

Like other cyclorrhaphid larvae, *E. solidaginis* have two pairs of Malpighian tubules that discharge into a larger common tube (Waterhouse, 1950). Large spherules were only found in the lumen of the anterior pair of Malpighian tubules. Each larva contained 25–45 spherules, evenly spaced in the distal half of the tubules that extend the length of the larva. Spherule size usually varied from 100 to 300 μm in diameter, but some spherules over 500 μm long were observed. The location of the spherules was evident as prominent swellings in the Malpighian tubules (Fig. 1A). Unlike the proximal Malpighian tubules, these tubules tightly surrounding the spherules were colorless and appeared to be composed of connective tissue. Scanning electron micrographs revealed that the spherules had a 'pebbled' surface composed of numerous individual round particles (Fig. 1B,C). The spherules had a concentric laminate appearance when viewed with the

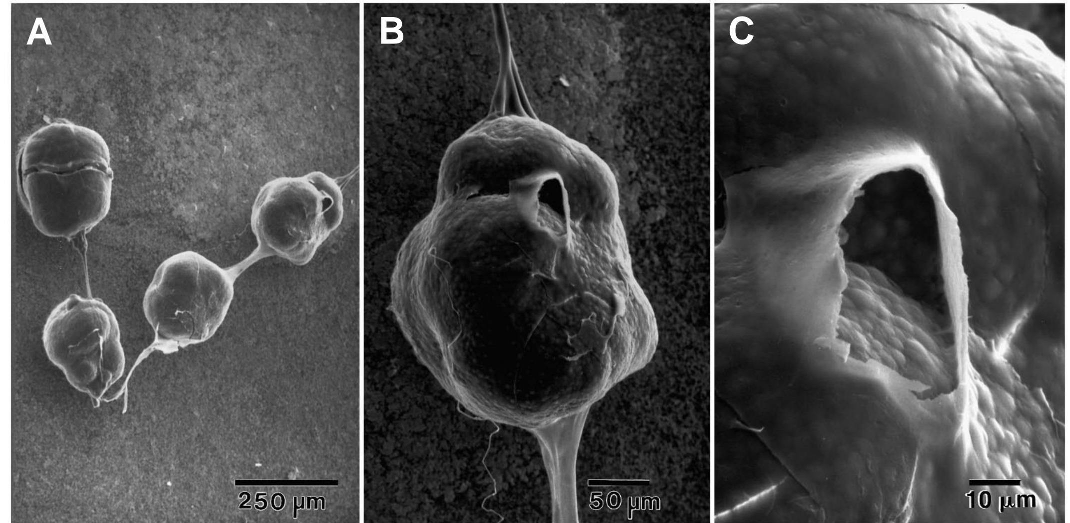

Fig. 1. Scanning electron micrographs of calcium phosphate spherules removed from *Eurosta solidaginis.* (A) String of four spherules enclosed in a Malpighian tubule. (B,C) Expanded views of the spherule on the far right side of A illustrating the conglomerate appearance and numerous round particles. Scale bars, A, 250 μm; B, 50 μm; C, 10 μm.

transmitted light microscope. Elemental analysis using energy-dispersive X-ray microanalysis (EDAX 9100) indicated that the spherules were primarily composed of calcium and phosphorus with a trace of magnesium. Infrared spectroscopy confirmed the identity of the spherules as the hydrate of tribasic calcium phosphate [$Ca_3(PO_4)_2 \cdot xH_2O$]. X-ray diffraction studies indicated that the spherules consist of an amorphous compound lacking crystalline structure (John Hughes, Department of Geology, Miami University, personal communication).

Observations of the proximal regions of both pairs of Malpighian tubules revealed the presence of three additional distinct, yet unidentified, mineral-like deposits. In the lumen, we observed several barrel-shaped deposits (5–7 μm in length) which were highly birefringent under polarized light. These deposits are most likely to be uric acid. Less frequently observed in the lumen were colorless, octahedron-shaped deposits. These deposits ranged in length from 20 to 30 μm and did not exhibit birefringence under polarized light. Numerous other needle-like, birefringent deposits appeared to be scattered throughout the cytoplasm of the epithelial cells in the Malpighian tubules.

Table 1. *Crystallization temperatures of various tissues removed from overwintering third-instar larvae of* Eurosta solidaginis

Tissue	Crystallization temperature (°C)
Whole larvae	–9.4±0.2 (38)
Fat body cells	–10.9±0.9 (19)
Fat body cells (after enzymatic digestion)	–8.9±0.2 (20)
Calcium phosphate spherules + Malpighian tubules	–10.1±0.9 (20)
Hemolymph	–17.8±0.5 (20) ***
Malpighian tubules	–21.4±1.2 (20) ***
Tracheae	–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 medium	–18.4±0.8 (20) ***

All temperatures are means ± S.E.M. (*N*).

Values followed by asterisks are significantly different from the value for whole larvae (Dunn's test): *$P<0.05$, **$P<0.01$, ***$P<0.001$.

Crystallization temperatures of isolated tissues and calcium phosphate spherules

Crystallization temperatures of whole larvae, spherules and isolated tissues were measured (Table 1). Hemolymph, gut, trachea, neural tissue, muscle/epidermis and Malpighian tubule crystallization temperatures were significantly lower than the T_c of whole larvae. Crystallization temperatures for fat body cells and $Ca_3(PO_4)_2$ spherules, surrounded by Malpighian tubule connective tissue, were not significantly different from the whole larva T_c. The cumulative freezing profiles for whole larvae and isolated tissues clearly demonstrate a wide range of ice-nucleating activities among individual tissues (Fig. 2). Some samples with spherules had ice-nucleating temperatures higher than those typically observed in whole larvae or in other isolated tissue treatments. Approximately 30 % of the samples containing spherules froze by −8 °C, with some freezing at temperatures as high as −6 °C. In contrast, 20 % of samples containing spherules and 25 % of samples with physically dissected fat body cells froze at temperatures below −12.1 °C, the lowest crystallization temperature for whole larvae.

During the larval–pupal metamorphosis, whole-body T_c decreased by approximately 9 °C from −9.2±0.1 to −18.3±1.1 °C (Table 2). This decrease suggests that a relatively efficient heterogeneous ice nucleator was removed during metamorphosis. During this transition, the number of spherules present decreased from 32.0±1.4 spherules per freeze-tolerant larva; no spherules were found in the freeze-susceptible pupae. The disappearance of the ice-nucleating calcium phosphate spherules during pupariation is consistent with the observed increase in the capacity of the pupae to supercool.

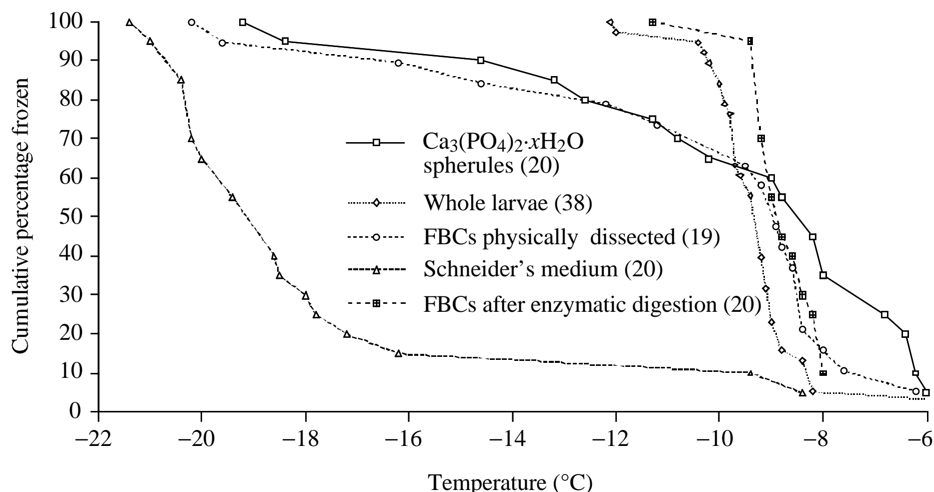

Fig. 2. Cumulative freezing profile based on individual crystallization temperatures for various tissues isolated from *Eurosta solidaginis*. The number of samples is given in parentheses. FBCs, fat body cells.

Table 2. *Crystallization temperatures and spherule contents of various developmental stages of* Eurosta solidaginis

Life stage	T_c (°C)	Number of spherules
Early larvae	−9.2±0.1	32.0±1.4
Late larvae	−11.3±0.9	31.3±2.0
Pupae	−18.3±1.1	0

Values are means ± S.E.M., N=21–23.

T_c, crystallization temperature.

Larvae exposed to room temperature for 8 days, which had not completed pupariation, were classified as 'late larvae'. Animals with completely tanned and hardened pupal casing were classified as 'pupae'.

Saturated solutions containing solid particles of chemical reagents commonly found in diapausing and overwintering insects demonstrated ice-nucleating activity (Table 3). Grace's medium saturated with calcium phosphate had a mean crystallization temperature (−9.7±0.5°C) similar to that observed in endogenous spherules (−10.1±0.9°C; Table 1) but significantly higher than that in Grace's medium alone (−16.2±0.6°C). Saturated solutions of potassium urate and sodium urate also had similar T_c values of −9.5±0.9°C and −9.2±1.0°C respectively.

Isolation techniques for the fat body cells appeared to influence ice-nucleating activity (Table 1). Fat body cells which were removed by dissection had a similar mean crystallization temperature (−10.9±0.9°C) to fat bodies isolated by collagenase/hyaluronidase enzymatic digestion (−8.9±0.2°C). However, cumulative freezing profiles (Fig. 2) showed that fat body cells isolated by enzymatic digestion had less variability in their ice-nucleating activity than cells isolated by dissection alone. All samples with enzymatically isolated cells froze by −11.3°C whereas less than 75% of the samples with physically dissected cells were frozen at this temperature. The freezing curve for enzymatically isolated fat body cells closely paralleled the freezing curve for whole larvae.

Table 3. *Ice-nucleating activity of Grace's insect cell medium saturated with undissolved chemical reagents analogous to compounds commonly found stored in insects*

Reagent solution	T_c (°C)
Grace's insect cell medium	−16.2±0.6
Calcium phosphate, tribasic	−9.7±0.5
Potassium phosphate, monobasic†	−11.7±0.2
Potassium urate	−9.5±0.9
Sodium urate	−9.2±1.0
Uric acid	−11.7±0.6

Values are means ± S.E.M.

T_c, crystallization temperature.

All samples were 35 μl with 12 samples per test.

Crystallization temperatures for solutions containing reagents were significantly different from those in the Grace's insect medium control (P<0.01, Dunnett's multiple comparisons test).

†With the exception of potassium phosphate, all reagents were insoluble or only slightly soluble and contributed insignificantly to the osmotic concentration of the media. Potassium phosphate was soluble in Grace's medium, increasing the osmotic concentration of the medium and contributing to a colligative decrease in T_c; this solution was excluded from the statistical analysis.

Discussion

These results indicate that both fat body cells and endogenous calcium phosphate spherules are heterogeneous ice nucleators with sufficient ice-nucleating activity to explain whole-body crystallization temperatures for the freeze-tolerant third-instar larvae of the gall fly *E. solidaginis*.

Heterogeneous ice nucleation occurs when a foreign substance acts as a nucleus onto which water molecules adsorb and assume an ice configuration. These foreign nuclei act as catalysts that increase the probability that embryonic ice crystals will form at higher sub-zero temperatures than found in homogeneous nucleation. Although the exact mechanism of ice nucleation is unknown, many heterogeneous nucleators appear to have a hexagonal physical structure similar to that of ice (Franks, 1985). Atmospheric scientists reported that

inorganic substrates, such as silver iodide (Vonnegut and Chessin, 1971), clay (Anderson and Tice, 1971), silicates (Angell, 1982) and a wide variety of organic compounds (Fukuta, 1966) have ice-nucleating activities between −4 and −20 °C. Aliphatic alcohols (Gavish *et al.* 1990), some amino acids (Gavish *et al.* 1992) and electrical charges may also nucleate ice at relatively warm sub-zero temperatures (Gavish *et al.* 1992; Salt, 1961). Biologists investigating naturally freeze-tolerant organisms have discovered new classes of ice-nucleating agents in insects and plants (see reviews in Lee *et al.* 1995). These ice-nucleating active agents include: bacteria (Schnell and Vali, 1972), fungi (Kieft, 1988; Pouleur *et al.* 1992), hemolymph proteins and lipoproteins (Duman *et al.* 1991; Zachariassen and Hammel, 1976) and unidentified nucleators found in gut contents (Shimada, 1989) and muscle tissue of insects (Tsumuki and Konno, 1991). The previous reports of the nucleating activity of some organic and inorganic crystals led some biological researchers to speculate that the uric acid crystals and metal-containing granules found in some invertebrates may have ice-nucleating activity (J. G. Duman, cited in Cannon and Block, 1988).

The calcium phosphate deposits found in *E. solidaginis* are common to many invertebrates which sequester waste products in the form of crystals and/or granules in cells (e.g. fat body cells) or organs (e.g. Malpighian tubules) (Wigglesworth, 1972). This sequestering of waste products is a form of ‘storage/excretion’ for it allows an organism to remove waste products from circulation without losing the valuable water required to dissolve and excrete the product (Brown, 1982). In addition to their storage/excretion role, we believe that these endogenous spherules represent a new class of heterogeneous ice nucleators that potentially regulate supercooling in overwintering insects.

Although it is improbable that these amorphous calcium phosphate spherules will induce nucleation as a result of similarities in ice crystal structure, surface cracks or other structural defects may initiate nucleation (Angell, 1982; Gavish *et al.* 1992). The hydration shell surrounding the calcium phosphate particles may also play a role in ice nucleation. The water in this layer may have an ‘ordered’ lattice structure similar to that of ice and, thereby, nucleate ice by a mechanism similar to that observed with alcohol (Gavish *et al.* 1990).

Our experiments indicated that isolated calcium phosphate spherules reduced the mean supercooling capacity of 10 μl samples of Schneider’s medium by approximately 8 °C. It should be noted here that the calcium phosphate spherules could not be completely removed from the connective tissues of the Malpighian tubules which surrounded them. However, since Malpighian tubules devoid of spherules did not induce nucleation, we attribute the observed nucleation to the presence of the spherules. The mean crystallization temperature for samples with spherules (−10.1±0.9 °C) was not significantly different from crystallization temperatures for whole larvae (−9.4±0.2 °C). Despite the similarity of the mean values, calcium phosphate spherules induced ice nucleation over a wider range of temperatures than that observed in whole larvae (Fig. 2). Fig. 2 also shows that not all samples with spherules exhibited ice-nucleating activity sufficiently high to explain all larval supercooling points. Within an intact organism, the actual T_c will be determined by the most active nucleator within its body fluids. It is possible that only a limited number of spherules in *E. solidaginis* have ice-nucleating activity in the range −8 to −10 °C. Some spherules with high ice-nucleating activity may have been overlooked or altered during removal in these experiments. The loss of even one or a few critical ice-nucleating particles could account for the variability of crystallization temperatures we observed.

Zachariassen (1980) suggested that freezing should be initiated in extracellular fluid to prevent damage to cells and closed structures (e.g. intestine) caused by intracellular ice formation or osmotic swelling and rupture. Despite this hypothesis, several reports implicate materials in the digestive tracts of freeze-tolerant insects as potential sources of protective ice nucleation (Fields and McNeil, 1988; Shimada, 1989). We believe that calcium phosphate spherules, contained in the Malpighian tubules, may induce ice formation which will propagate across the tubule and nucleate the extracellular fluid.

Other endogenous crystalloid deposits commonly found in diapausing and overwintering insects include ammonium, calcium, potassium and sodium urates, calcium carbonate, calcium oxalate and uric acid (Brown, 1982; Wigglesworth, 1972). We tested several commercially prepared reagents, chemically similar to compounds naturally found in insects, for ice-nucleating activity. Several reagents, including calcium phosphate, had ice-nucleating activity in the range −8 to −11 °C (Table 3). Although the ice-nucleating activity of these crystalline compounds is less than that of ice-nucleating proteins and lipoproteins, active in the range −6 to −9 °C, it is sufficient to explain the crystallization temperatures of insects that freeze at temperatures at −9 °C or lower. Like proteinaceous ice nucleators, these endogenous spherules may function to provide protective extracellular freezing at relatively high sub-zero temperatures. These spherules represent a novel class of heterogeneous ice nucleators that may regulate the temperature at which freezing occurs and promote freeze tolerance.

Another source of endogenous ice nucleators may be the ubiquitous fat body cells. Fat body cells isolated by physical dissection had a mean crystallization temperature of −10.9±0.9 °C. While this mean T_c value was not significantly different from that of whole larvae, the variability of nucleation was similar to that observed for calcium phosphate spherules (Fig. 2). Interestingly, when fat body cells were isolated by enzymatic digestion with collagenase and hyaluronidase, the mean crystallization temperature increased (−8.9±0.2 °C) while the variability decreased relative to that of the whole larvae (Table 1; Fig. 2). This increase in nucleating activity might be explained by two mechanisms. Baust and Zachariassen (1983) observed high ice-nucleating activity in homogenized tissues from a freeze-intolerant insect, although

this high activity was not observed in intact tissues. From this experiment, they concluded that the cell matrix had ice-nucleating activity which was usually masked by intact cells. It is possible that our enzymatic preparation weakened cell membranes and exposed normally masked cell-matrix-associated nucleators. A more probable mechanism takes into account the diversity of fat body cell structure and function. In general, the insect fat body is a dynamic tissue analogous to the vertebrate liver (Wigglesworth, 1972). Fat body cells function in protein, glycogen and lipid storage, protein synthesis and intermediary metabolism (Dean *et al.* 1984). The fat body tissue is composed of several cell types and these different cells may aggregate in different regions of the larva (Dean *et al.* 1984). If only a specific cell type or a cell with a specific function causes nucleation and is localized to a particular region, it is possible that these cells were not removed during physical dissection. This might account for the variability observed using this technique. In contrast, enzymatic digestion produced a heterogeneous suspension of fat body cells from random locations in the larvae. By removing a variety of cells from different locations, this non-specific isolation technique could account for the increased ice-nucleating activity and decreased variability. The loss of ice-nucleating activity during pupariation is also consistent with the massive structural changes (histolysis) that occur in dipteran fat body cells during the larval–pupal metamorphosis (Dean *et al.* 1984).

The generally accepted belief regarding the role of ice-nucleating agents is that these agents limit lethal intracellular freezing by initiating ice formation in the extracellular space at high sub-zero temperatures. However, owing to the remarkable ability of fat body cells from *E. solidaginis* to survive intracellular ice formation and temperatures as low as $-80\,^{\circ}\mathrm{C}$ (Lee *et al.* 1993; Salt, 1962), ice nucleation occurring on or within these cells should not affect their survival. Earlier cryomicroscopic investigations of fat body cell suspensions covered in oil and cooled at $2\,^{\circ}\mathrm{C\,min^{-1}}$ did not demonstrate ice-nucleating activity (Lee *et al.* 1993). Since the mechanism of nucleating activity and the specific site within the fat body are not known, we believe that the oil coating may have masked nucleating activity. If nucleation is localized in specific regions or specific cells, it is also possible that ice-nucleating active fat body cells were not chosen during these previous observations.

The presence of ice-nucleating agents in fat body cells could explain reports of hemolymph ice nucleators in *E. solidaginis* (Sømme, 1978; Zachariassen *et al.* 1982). Ice nucleation in the hemolymph may have been caused by contamination from the ubiquitous fat body cells ruptured during hemolymph sampling.

In summary, both calcium phosphate spherules within the Malpighian tubules and fat body cells exhibit sufficient ice-nucleating activity to explain the T_c of intact overwintering larvae of *E. solidaginis*. Calcium phosphate and other naturally occurring crystalloid deposits in overwintering insects may function as endogenous ice nucleators in insects to regulate the temperature at which they freeze and, thereby, to promote winter survival.

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References

ANDERSON, D. M. AND TICE, A. R. (1971). Low-temperature phases of interfacial water in clay–water systems. *Soil Sci. Am. Proc.* **35**, 47–54.

ANGELL, C. A. (1982). Supercooled water. In *Water – A Comprehensive Treatise*, vol. 7 (ed. F. Franks), pp. 1–82. New York: Plenum Press.

BALE, J. S., HANSEN, T. N. AND BAUST, J. G. (1989). Nucleators and sites of nucleation in the freeze tolerant larvae of the gall fly *Eurosta solidaginis* (Fitch). *J. Insect Physiol.* **35**, 291–298.

BAUST, J. G. (1986). Insect cold hardiness: freezing tolerance and avoidance – the *Eurosta* model. In *Living in the Cold: Physiological and Biochemical Adaptations* (ed. H. C. Heller, X. J. Musacchia and L. Wang), pp. 125–130. New York: Elsevier.

BAUST, J. G. AND LEE, R. E. (1982). Environmental triggers to cryoprotectant modulation in separate populations of the gall fly, *Eurosta solidaginis* (Fitch). *J. Insect Physiol.* **28**, 431–436.

BAUST, J. G. AND NISHINO, M. (1991). Freezing tolerance in the goldenrod gall fly (*Eurosta solidaginis*). In *Insects at Low Temperature* (ed. R. E. Lee and D. L. Denlinger), pp. 260–275. New York: Chapman & Hall.

BAUST, J. G. AND ZACHARIASSEN, K. E. (1983). Seasonally active cell matrix associated ice nucleators in an insect. *Cryo. Lett.* **4**, 65–71.

BROWN, B. E. (1982). The form and function of metal-containing 'granules' in invertebrates. *Biol. Rev.* **57**, 621–667.

CANNON, R. J. C. AND BLOCK, W. (1988). Cold tolerance of microarthropods. *Biol. Rev.* **63**, 23–77.

DEAN, R. L., LOCKE, M. AND COLLINS, J. V. (1984). Structure of the fat body. In *Comprehensive Insect Physiology, Biochemistry and Pharmacology*, vol. 3 (ed. G. A. Kerkut and L. I. Gilbert), pp. 155–210. Elmsford, NY: Pergamon Press.

DUMAN, J. G., XU, L., NEVEN, L. G., TURSMAN, D. AND WU, D. W. (1991). Hemolymph proteins involved in insect subzero-temperature tolerance: Ice nucleators and antifreeze proteins. In *Insects at Low Temperature* (ed. R. E. Lee and D. L. Denlinger), pp. 94–127. New York: Chapman & Hall.

FIELDS, P. G. AND MCNEIL, J. N. (1988). The cold-hardiness of *Ctenucha virginica* (Lepidoptera: Arctiidae) larvae, a freezing-tolerant species. *J. Insect Physiol.* **34**, 269–277.

FRANKS, F. (1985). *Biophysics and Biochemistry at Low Temperatures.* Cambridge: Cambridge University Press. 210pp.

FUKUTA, N. (1966). Experimental studies of organic ice nuclei. *J. atmos. Sci.* **23**, 191–196.

GAVISH, M., POPOVITZ-BIRO, R., LAHAV, M. AND LEISEROWITZ, L. (1990). Ice nucleation by alcohol arranged in monolayers at the surface of water drops. *Science* **250**, 973–975.

GAVISH, M., WANG, J. L., EISENSTEIN, M., LAHAV, M. AND LEISEROWITZ, L. (1992). The role of crystal polarity in α-amino acid crystals for induced nucleation of ice. *Science* **256**, 815–818.

GRACE, T. D. C. (1962). Establishment of four strains of cells from insect tissues grown in *vitro*. *Nature* **195**, 788–789.

KIEFT, T. L. (1988). Ice nucleation activity in lichens. *Appl. env. Microbiol.* **54**, 1678–1681.

LEE, R. E. (1991). Principles of insect low temperature tolerance. In *Insects at Low Temperature* (ed. R. E. Lee and D. L. Denlinger), pp. 17–46. New York: Chapman & Hall.

LEE, R. E., MCGRATH, J. J., MORASON, R. T. AND TADDEO, R. M. (1993). Survival of intracellular freezing, lipid coalescence and osmotic fragility in fat body cells of the freeze-tolerant gall fly *Eurosta solidaginis*. *J. Insect Physiol.* **39**, 445–450.

LEE, R. E., WARREN, G. J. AND GUSTA, L. V. (1995). *Biological Ice Nucleation and Its Applications*. St Paul, MN: The American Phytopathological Society. 370pp.

LUNDHEIM, R. AND ZACHARIASSEN, K. E. (1993). Water balance of over-wintering beetles in relation to strategies for cold tolerance. *J. comp. Physiol.* B **163**, 1–4.

MORRISSEY, R. E. AND BAUST, J. G. (1976). The ontogeny of cold tolerance in the gall fly, *Eurosta solidaginis*. *J. Insect Physiol.* **22**, 431–437.

POULEUR, S., RICHARD, C., MARTIN, J.-G. AND ANTOUN, H. (1992). Ice nucleation activity in *Fusarium acuminatum* and *Fusarium avenaceum*. *Appl. env. Microbiol.* **58**, 2960–2964.

ROJAS, R. R., LEE, R. E. AND BAUST, J. G. (1986). Relationship of environmental water content to glycerol accumulation in the freezing tolerant larvae of *Eurosta solidaginis* (Fitch). *Cryo. Lett.* **7**, 234–245.

SALT, R. (1961). Effect of electrostatic field on freezing of supercooled water and insects. *Science* **133**, 458–459.

SALT, R. W. (1962). Intracellular freezing in insects. *Nature* **193**, 1207–1208.

SCHNEIDER, I. AND BLUMENTHAL, A. (1978). *Drosophila* cell and tissue culture. In *Biology and Genetics of Drosophila*, vol. 2A (ed. M. Ashburner and T. R. F. Wright), pp. 266–315. New York: Academic Press.

SCHNELL, R. C. AND VALI, G. (1972). Atmospheric ice nuclei from decomposing vegetation. *Nature* **236**, 163–165.

SHIMADA, K. (1989). Ice-nucleating activity in the alimentary canal of the freezing-tolerant prepupae of *Trichiocampus populi* (Hymenoptera: Tenthredinidae). *J. Insect Physiol.* **35**, 113–120.

SØMME, L. (1978). Nucleating agents in the haemolymph of third instar larvae of *Eurosta solidaginis* (Fitch) (Dipt., Tephritidae). *Norw. J. Ent.* **25**, 187–188.

STOREY, K. B., BAUST, J. G. AND STOREY, J. M. (1981). Intermediary metabolism during low temperature acclimation in the overwintering gall fly larva, *Eurosta solidaginis*. *J. comp. Physiol.* B **144**, 183–190.

TSUMUKI, H. AND KONNO, H. (1991). Tissue distribution of the ice-nucleating agents in larvae of the rice stem borer, *Chilo suppressalis* Walker (Lepidoptera: Pyralidae). *Cryobiology* **28**, 376–381.

UHLER, L. D. (1951). Biology and ecology of the goldenrod gall fly, *Eurosta solidaginis* (Fitch). *Cornell Univ. agric. exp. Stat. Mem.* **300**, 1–51.

VONNEGUT, B. AND CHESSIN, H. (1971). Ice nucleation by coprecipitated silver iodide and silver bromide. *Science* **174**, 945–946.

WATERHOUSE, D. F. (1950). Studies of the physiology and toxicology of blowflies XIV. The composition, formation and fate of the granules in the Malpighian tubules of *Lucilia cuprina* larvae. *Austr. J. Sci. Res. Series B* **3**, 76–112.

WIGGLESWORTH, V. B. (1972). *The Principles of Insect Physiology*. New York: Chapman & Hall. 827pp.

ZACHARIASSEN, K. E. (1980). The role of polyols and nucleating agents in cold-hardy beetles. *J. comp. Physiol.* B **140**, 227–234.

ZACHARIASSEN, K. E., BAUST, J. G. AND LEE, R. E. (1982). A method for quantitative determination of ice-nucleating agents in insect hemolymph. *Cryobiology* **19**, 180–184.

ZACHARIASSEN, K. E. AND HAMMEL, H. T. (1976). Nucleating agents in the haemolymph of insects tolerant to freezing. *Nature* **262**, 285–287.