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FACTORS CONTRIBUTING TO SEASONAL INCREASES IN INOCULATIVE FREEZING RESISTANCE IN OVERWINTERING FIRE-COLORED BEETLE LARVAE *DENDROIDES CANADENSIS* (PYROCHROIDAE)

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Summary

The insects and microarthropods that vary seasonally in susceptibility to cross-cuticular inoculation by external ice (inoculative freezing) represent a phylogenetically diverse group; however, few studies have explored possible mechanisms experimentally. This study documents seasonally variable inoculative freezing resistance in *Dendroides canadensis* beetle larvae and combines immunofluorescence, *in vivo* removal of epicuticular lipids and *in vitro* chamber studies to explore the roles of seasonal modification in the cuticle and in epidermal and hemolymph antifreeze proteins (AFPs). Seasonal cuticular modifications contribute to the inhibition of inoculative freezing since more cold-hardy larvae froze inoculatively when epicuticular waxes were removed with hexane and, in *in vitro* chamber experiments, cuticle patches (with the underlying epidermis removed) from winter larvae provided greater protection from inoculative freezing than did cuticle patches from summer larvae. The results indicate that seasonal modifications in epidermal and hemolymph AFPs contribute most strongly to the inhibition of inoculative freezing. Subcuticular epidermal AFPs were present in immunocytochemically labeled transverse sections of winter larvae but were absent in summer ones. Winter integument patches (cuticle with epidermis) were more resistant to inoculative freezing than were summer integument patches. Integument patches resisted inoculative freezing as well as live winter-collected larvae only when hemolymph AFP was added. The results also suggest that some integumentary ice nucleators are removed in cold-hardy larvae and that AFP promotes supercooling by inhibiting the activity of these nucleators.

Key words: freeze avoidance, inoculative freezing resistance, cuticle modification, haemolymph, season, antifreeze protein, epidermis, beetle, *Dendroides canadensis*.

Introduction

Overwintering freeze-avoiding insects (those that die when frozen) must adapt behaviorally, physiologically and biochemically to survive potentially lethal subzero temperatures (Danks, 1978; Zachariassen, 1985; Block, 1990; Duman *et al.* 1991*a*, 1993, 1995). Behavioral adaptations include avoidance of subzero temperatures *via* migration (Lee, 1989) or by seeking sheltered microhabitats where temperature minima and diurnal fluctuation are moderated (Danks, 1978, 1991). If a sheltered microhabitat provides insufficient thermal buffering, freeze-avoiding insects must lower their nucleation temperature or supercooling point (SCP). However, if hibernaculum temperatures are colder than hemolymph freezing points, freeze-avoiding insects that overwinter in moist hibernaculae must prevent cross-cuticular, or inoculative, freezing as a prerequisite for maintaining and promoting the supercooled state (Duman *et al.* 1991*a*).

Although it is generally believed that the waxy cuticle of insects serves as a protective barrier to inoculative freezing, contact moisture (Salt, 1963; Somme and Conradi-Larsen, 1977; Tanno, 1977; Somme, 1982; Humble and Ring, 1985; Fields and McNeil, 1986; Shimada and Riihimaa, 1988; Bale *et al.* 1989; Duman *et al.* 1991*a*; Gehrken *et al.* 1991; Gehrken, 1992; Rojas *et al.* 1992) and topical applications (aerosol mist) of ice-nucleating active bacterial suspensions (Strong-Gunderson *et al.* 1992; Lee *et al.* 1993) can severely limit the ability of insects to supercool. Resistance to inoculative freezing increases seasonally as part of the natural cold-hardening process for *Smicronyx fulvus*, the red sunflower seed weevil (Rojas *et al.* 1992), and for *Cisseps fulvicollis*, the tenuchid scape moth (Fields and McNeil, 1986). The insects and microarthropods that are known to be susceptible to inoculative freezing represent a phylogenetically diverse group consisting of at least one collembolan, two ticks and an oribatid mite, one centipede, nine coleopterans, one lepidopteran, two hymenopterans and four dipterans. The significance of inoculative freezing varies among insect species. For some freeze-tolerant species, it is necessary for freeze tolerance (Fields and McNeil, 1986; Shimada and Riihimaa, 1988; Tursman *et al.* 1994), while for freeze-avoiding species it is deleterious (Popham *et al.* 1991; Rojas *et al.* 1992).

Various avenues of entry for external ice have been suggested: mouth, anus, spiracles, sensory receptors, cuticular channels and pore canals (Salt, 1953, 1961, 1963; Frazier, 1985; Humble and Ring, 1985; Layne *et al.* 1990; Gehrken, 1992; Strong-Gunderson *et al.* 1992). However, the mechanisms behind inoculative freezing or seasonal changes in inoculative freezing resistance are largely unknown – probably because few studies have explored these topics experimentally.

In pioneering work, Salt (1963) demonstrated experimentally that the rate of inoculative freezing in larval blowflies (*Calliphora* sp.), mealworms (*Tenebrio molitor*), goldenrod gall flies (*Eurosta solidaginis*) and wheat stem sawflies (*Cephus cinctus*) was (1) proportional to the area in contact with ice and inversely proportional to temperature down to −10 °C, (2) increased after a previous inoculative freeze and (3) exhibited ontogenetic effects (e.g. larval blowflies, *Calliphora* sp., were susceptible while adults were resistant to inoculative freezing). After manipulating photic and temperature cues to alter the activity of colligative- and noncolligative-acting antifreezes in the hemolymph of two overwintering beetle species (*Rhagium inquisitor* and *Ips acuminatus*), Gehrken (1992) found that hemolymph antifreeze proteins (AFPs) inhibited, to varying degrees, inoculative freezing in both species. Also, hemolymph melting point depression was positively correlated with supercooling ability in the presence of contact moisture for *I. acuminatus*, but not for *R. inquisitor*, suggesting that colligative-acting hemolymph antifreezes (i.e. polyols) serve a functionally significant role in the latter (Gehrken, 1992).

Dendroides canadensis (Pyrochroidae) overwinter as non-diapausing larvae (Horwath and Duman, 1983) beneath the bark of decaying deciduous trees. Their hemolymph contains polyols and AFPs that increase seasonally in activity and concentration (Duman, 1980; Olsen and Duman, 1997*a*) cued by seasonal photo- and thermoperiodic cycles (Horwath and Duman, 1983, 1986). Their hemolymph thermal hysteresis activity increases from 0.5 °C in summer to 5 °C or greater during midwinter (Duman, 1980; Olsen and Duman, 1997*a*). Thermal hysteresis activity is absent from the midgut fluid during summer but increases seasonally to a level commensurate with that of the hemolymph (Duman, 1984; Olsen and Duman, 1997*b*). Hemolymph AFPs have been characterized (Wu *et al.* 1991) and are produced in the fat body (Xu and Duman, 1991), with induction being under the control of juvenile hormone (Horwath and Duman, 1983; Xu *et al.* 1992). Immunofluorescent staining of transverse sections of cold-hardy *D. canadensis* larvae indicates that AFPs are found in the subcuticular epidermal layer (Duman *et al.* 1993); however, the role of these epidermal AFPs in inoculative freezing resistance is unknown.

D. canadensis can overwinter as either freeze-avoiding or freeze-tolerant insects; however, most individuals in the population are either one or the other at any given time (Horwath and Duman, 1984; Olsen and Duman, 1990). When freeze-avoiding (as during the current study), *D. canadensis* promote and maintain the metastable supercooled state – larval SCPs decrease from a summer maximum of −2 to −7 °C to a winter minimum of −20 °C or lower *via* the removal of highly active hemolymph and gut fluid ice nucleators combined with the inhibition of residual nucleators by AFP (Olsen and Duman, 1997*a*,*b*). However, biochemical adaptations that promote supercooling will be ineffectual unless inoculative freezing is prevented. The present study explores the functional roles of *D. canadensis* cuticle and epidermal and hemolymph AFPs in effecting seasonal changes in inoculative freezing resistance using immunofluorescence microscopy and *in vivo* and *in vitro* (chamber) experiments.

Materials and methods

Collection of insects and determination of hemolymph thermal hysteresis activity

Dendroides canadensis Latreille were collected from beneath the bark of partially decayed deciduous trees located on woodlots within a 50 km radius of South Bend, Indiana, USA. Field-collected *D. canadensis* were dipped repeatedly in distilled water and blotted dry. The region to be punctured was swabbed with 95 % ethanol (to reduce the risk of contaminating hemolymph with exogenous nucleators) and allowed to dry. Using 10 µl capillary tubes, hemolymph was collected by puncturing the dorsolateral region of the first segment caudal to the head with a 28 gauge needle. The capillary tubes, containing 3–5 µl of hemolymph, were flame-sealed at one end, centrifuged briefly at 4 °C, and then the other end was sealed with alternating layers of air and mineral oil, leaving a 2 µl air space between the oil and hemolymph.

In the absence of AFPs, the equilibrium freezing point equals the melting point (MP). Thus, when viewed through a microscope, a small (approximately 0.25 mm diameter) ice crystal about to melt will begin to grow noticeably when the temperature is lowered by as little as 0.01–0.02 °C. In contrast, when AFPs are present, the temperature may be lowered by as much as 5 °C or more below the MP before a similar-sized ice crystal begins to grow. This difference between the non-equilibrium freezing point and the MP is termed thermal hysteresis (DeVries, 1971, 1986; Duman *et al.* 1991*b*, 1993). The AFP activity (thermal hysteresis) of the sample was determined as described by DeVries (1986). A small seed crystal (0.25 mm in diameter) was initiated in the sample by spraying with an aerosol refrigerant. The sample was then placed in a temperature-controlled (±0.02 °C) ethanol bath and the temperature was slowly raised (by 0.02 °C min^{-1}). The crystal was observed through a viewing port using a dissecting microscope. The disappearance of the crystal indicates the MP. The temperature was then lowered 0.05 °C below the MP, another crystal was sprayed, the sample was placed in the bath and the temperature was lowered by 0.02 °C min^{-1} until 0.1 °C below the MP. If the sample does not contain AFPs, the crystal will immediately begin to grow. If the crystal did not start growing, the cooling rate was increased to 0.1 °C min^{-1} and the temperature lowered until the sample froze, thus identifying

the hysteretic freezing point, or HFP (Duman *et al.* 1991*b*), indicating the presence of AFPs.

Testing for inoculative freezing in field-collected larvae

The larval supercooling point (SCP) (the spontaneous nucleation temperature with desiccant present) and the inoculative freezing point or IFP (the temperature at which larvae froze when surrounded by ice) were determined as follows. Individual larvae were squeezed gently, and only those that did not exude hemolymph were used for determination of IFPs. Exuding hemolymph indicated damaged cuticle through which the hemolymph would be rapidly seeded when the external ice reached temperatures colder than the hemolymph freezing point. Using petroleum jelly, insects were attached to a Yellow Springs Instruments no. 427 thermistor suspended in a 250 ml Erlenmeyer flask that contained either ice at −0.5 °C or $CaSO_4$ desiccant. Desiccant served to absorb moisture on the insect's surface or in the surrounding air that could condense on the insect's surface and potentially cause inoculative freezing as the temperature was decreased. The flask was placed in a refrigerated ethanol bath and, after 15 min, the temperature was lowered by 0.5 °C min^{-1} until an exotherm detected by the thermistor was recorded using a Fisher Recordall series 5000 pen recorder. If SCPs of groups of larvae were significantly lower than the IFPs, insects were said to have frozen inoculatively.

Immunofluorescence: preparation of frozen tissue frozen sections

Using immunofluorescence, the present study assessed whether epidermal AFPs (known to be found in cold-hardy larvae; Duman *et al.* 1993) were present in summer-collected larvae.

D. canadensis larvae were fixed [4 % paraformaldehyde in 10 mmol l^{-1} phosphate-buffered saline (PBS), pH 7.3, for 12 h under vacuum at 4 °C] and cleared under vacuum at 4 °C with PBS and increasing concentrations of embedding medium (OCT) for frozen tissue sections (Miles Laboratories) in PBS, culminating with a 12 h infiltration in undiluted OCT. Larvae were then frozen in 0.6 ml of OCT-containing microfuge tubes and stored at −20 °C until sectioned. Larvae were sectioned transversely at 10 µm using a microtome cryostat (International Equipment Company) and then mounted on gel-coated slides.

Indirect immunofluorescence staining

The following procedure was modified from that of Wu (1991). After two rinses in PBS, approximately 50 µl of anti-*D. canadensis* AFP antiserum (Xu and Duman, 1991) at 1:16 dilution in PBS with 0.25 % bovine serum albumin (BSA) (w/v) was used to cover transversely cut *D. canadensis* sections for 1 h at 25 °C. After a 20 min immersion in PBS, sections were incubated in the dark for 1 h at 25 °C with 50 µl of fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG antiserum (Calbiochem) diluted 1:16 in PBS with 0.25 % BSA (w/v). After three rinses in PBS, sections were mounted in a 1:2 (v/v) dilution of glycerol:PBS, and coverslips were sealed with isobutylmethacrylate. Slides were stored in the dark at 4 °C until viewed (12–24 h later) on a Zeiss transmission fluorescence microscope with a darkfield condenser, a FITC filter and a no. 53 barrier filter. Controls consisted of transverse sections of *D. canadensis* larvae (as described above) incubated in anti-*D. canadensis* AFP antiserum only, FITC-conjugated goat anti-rabbit IgG antiserum only (non-specific binding control) or PBS only (to observe autofluorescence). Non-immune rabbit serum was also tested in place of anti-*D. canadensis* AFP antiserum.

In vivo *tests of the epicuticular and subepicuticular inoculative freezing barriers*

Hexane was used to remove epicuticular waxes from larvae (Hadley *et al.* 1986) that remained supercooled when in contact with ice at a temperature colder than their hemolymph HFP. Removing the epicuticular lipids with hexane and then placing larvae on ice that was colder than their hemolymph HFP tested the effectiveness of the epicuticular lipid barrier *versus* the combined effectiveness of the subepicuticlar layers, including the epidermis. The epidermis is the final barrier through which propagating ice must travel before inoculating the hemolymph (Salt, 1963).

At the end of a 10 week acclimation period (photoperiod 9 h:15 h L:D, 10 °C during the light phase, 4 °C during the dark phase), autumn-collected (4 November), cold-acclimated larvae had an HFP of −4.2±0.5 °C (mean ± 1 S.E.M., N=6); however, several larvae remained supercooled at −8 °C while in contact with ice for at least 18 h. Larvae were screened to find those resistant to inoculative freezing by using 250 ml beakers stoppered with foam rubber and half-filled with ice at −2 °C, each one containing 20–25 larvae. An inserted thermistor was used to record the temperature in each beaker. The temperature was lowered (0.2 °C min^{-1}) to −8 °C, and those larvae that were still supercooled after 18 h at −8 °C were removed and arbitrarily divided into two groups. Since larvae change from translucent to opaque and lighten in color immediately after freezing, frozen larvae can readily be distinguished visually from supercooled ones. Larvae in one group were swabbed with hexane while those in the other were not. Larvae (6–15) from each group were put on ice in six beakers (as above), the temperature was lowered to −8 °C (rate as above) and the proportion of larvae frozen in each beaker after 18 h was determined. To ensure the proportion data met stable variance and normality assumptions, data were transformed (arcsin $p^{1/2}$) (Box *et al.* 1978) before statistical testing by analysis of variance (ANOVA).

In vitro *chamber experiments*

The roles of cuticle, epidermal AFPs and high-performance liquid chromatography (HPLC)-purified AFP (from *D. canadensis* hemolymph) in effecting seasonal changes in inoculative freezing resistance were explored using a Plexiglas chamber (Fig. 1) modified from Hadley *et al.* (1986). In the experimental design, a patch of integument (cuticle with epidermis intact) or cuticle only (integument with epidermal

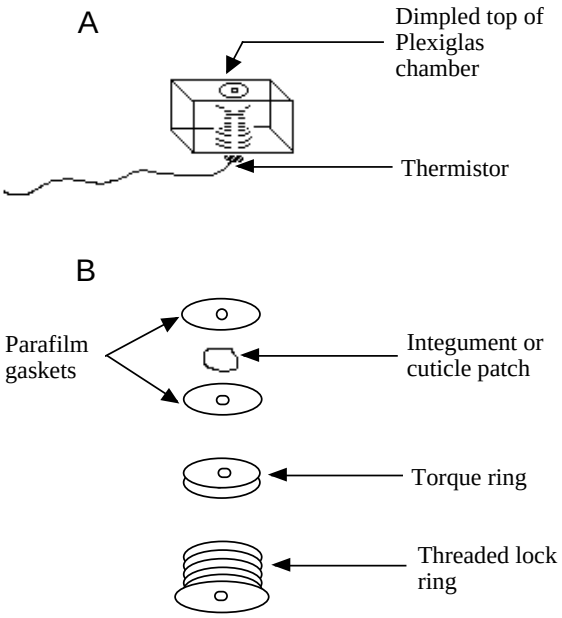

Fig. 1. Plexiglas chamber (modified from Hadley *et al.* 1986) used in testing the role of cuticle and of epidermal and hemolymph antifreeze proteins in seasonal increases in inoculative freezing resistance in *Dendroides canadensis* larvae. (A) The chamber measured 12 mm×12 mm×6 mm (width × length × height) and contained a central aperture (7 mm outer diameter) into which was inserted the sandwiched integument or cuticle, nylon torque ring and threaded Plexiglas lock ring. (B) When assembled, the apparatus had a continuous 1 mm center hole (occluded only by the integument or cuticle patch) running from the dimpled top of the chamber to the exterior of the lock ring. To determine the inoculative freezing point (IFP), a drop of distilled water contained in the dimpled top of the chamber was intentionally seeded and the temperature at which this external ice propagated across the integument or cuticle patch was measured. To determine the supercooling point (SCP), no water droplet was used in the dimpled top of the chamber. A thermistor affixed to the exterior of the lock ring recorded the temperature, including the exotherm which marked freezing in the fluid-filled interior of the chamber. See text for further details.

layer removed; Machin *et al.* 1985) was situated between ice (on the exterior side of the patch) and either (a) AFP-free Hanks solution (Gibco no. 1576 tissue culture medium; balanced salt solution with glucose) or (b) HPLC-purified *D. canadensis* AFP dissolved in 50 mmol l^{-1} Tris–HCl (AFP solution) on the interior. The AFP used was one of the four antifreeze proteins (AFP-4) purified from *D. Canadensis* hemolymph of winter larvae (Wu *et al.* 1991; Li *et al.* 1998). The exotherm of the Hanks or AFP solution defined the IFP (the temperature at which ice crossed the cuticle or integument membrane) and was recorded using a Yellow Springs Instrument no. 427 thermistor affixed to the lower part of the chamber (Fig. 1). The SCP – the nucleation temperature without ice contact – was also determined. The patch was taken from a site devoid of spiracles and sensory receptors which might serve as channels for cross-cuticular ice propagation (see below).

The roles of the cuticular and epidermal components of the integument were assessed by first using Hanks solution and comparing the abilities of integument (cuticle plus underlying epidermis) *versus* cuticle-only patches to resist cross-cuticular ice progagation. This was important since immunofluorescence studies (Duman *et al.* 1993) indicate that the epidermal layer in cold-hardy larvae contains AFPs, suggesting that the ice propagation temperature of the integument patch might be lower than that of cuticle only.

Replacement of the Hanks solution (no thermal hysteresis, MP −0.45 °C) with a solution of HPLC-purified (AFP-4) from *D. canadensis* hemolymph (1.82 °C thermal hysteresis, HFP −2.27 °C) assessed the functional role of AFP in inoculative freezing resistance.

Only Hanks and AFP solution with low SCPs (approximately −20 °C) were used in the chamber. Before each run of the experiment, the chamber was washed in detergent, rinsed in distilled water then dipped in 95 % ethanol. Once the ethanol had dried, the chamber was immersed in 5 % PROSIL 28 coating (an organo-silane concentrate which, when diluted and applied, provides a surface film that is water-repellent and has improved lubricity; PCR Incorporated, Gainesville, FL, USA). The chamber in this current experiment was PROSIL-coated to ensure that any heterogeneous ice nucleators remaining on the interior surface of the Plexiglas chamber were prevented from nucleating the Hanks or AFP solution.

During the experiment, the chamber rested inside a 250 ml Erlenmeyer flask that had a perforated false floor containing $CaSO_4$ desiccant beneath the floor. The flask was suspended in a refrigerated ethanol bath (Frigomix 1496 with a Thermomix 1480 temperature control).

Integument patches were prepared and inserted into the chamber as follows. *D. canadensis* larvae were dipped repeatedly in distilled water and blotted dry. The gut was removed aseptically, and larvae were rinsed in sterile 0.9 % saline (Duman, 1984). Using microdissection scissors, larvae were cut from posterior to anterior along the lateral edges, and Dumont forceps were used to separate the dorsal from the ventral side. A 3 mm×3 mm patch of integument was snipped from the dorsal thoracic region (known to be devoid of spiracles and sensory receptors) and dipped repeatedly in sterile 0.9 % saline. For experiments using an integument patch (cuticle plus epidermis), adhering muscle and fat body were gently scraped from the integument using whittled wooden applicator sticks while the patch (immersed in saline) was viewed under a dissecting microscope. In experiments using a cuticle patch, the thin layer of epidermis was peeled from the cuticle, by scraping more vigorously with applicator sticks (Machin *et al.* 1985) while the immersed patch was viewed under a dissecting microscope. Gaskets made of Parafilm-wax-coated laboratory film with 1 mm diameter center holes were used to form the outer layers of a sandwich such that the integument or cuticle was sandwiched between the Parafilm gaskets – one above and one below the patch. After the sandwich had been examined under the dissecting microscope to check the alignment of the holes, the sample was placed in

a Plexiglas chamber and the torque ring set in place (Fig. 1). Using a 10 µl micropipette with bulb (Drummond Scientific), a 1 µl droplet of Hanks or AFP solution was squeezed into the interior side of the center hole of the torque ring (to ensure that the fluid contacted the interior side of the integument patch), and the lock ring was then screwed in place (Fig. 1). Using the micropipette and bulb, approximately 3 µl of AFP or Hanks solution was squeezed into the center hole of the lock ring so that the fluid dimpled slightly on the exterior of the lock ring. The entire chamber was checked under a dissecting microscope for alignment of holes and to ensure that no air bubbles were present in the fluid-filled chamber. Mineral oil was applied to the exterior of the lock ring, and the thermistor was attached to the center hole of the lock ring with tape, thus forcing the dimpled fluid into the chamber.

Next, a Pasteur pipette was used to place a droplet of distilled water in the well (dimpled top) in the center of the chamber so that the droplet covered the hole in the center of the well (Fig. 1). The temperature was lowered (by 0.2 °C min^{-1}) until the attached thermistor read −0.5 °C, and the distilled water on top of the chamber was then seeded by touching the supercooled drop with an ice-filled 100 µl Corning microsampling pipette. The chamber was allowed to equilibrate (5–10 min) before gradually lowering the temperature (by 0.2 °C min^{-1}). The eventual exotherm, signaling freezing of the fluid in the inner chamber (the IFP), was detected by the thermistor and recorded using a Fisher Recordall 5000 series pen recorder. The SCP was determined as above except that no water droplet was placed on the dimpled top of the Plexiglas chamber.

For experiments requiring larvae with low SCPs, groups of 15–20 *D. canadensis* were placed onto ice (−0.5 °C) in beakers suspended in a refrigerated ethanol bath. The temperature was lowered (by 0.5 °C min^{-1}), and larvae that did not freeze after 3 h at −20 °C were removed for use in experiments.

Hanks solution and a Parafilm patch (in place of a cuticle or integument patch) were used as a barrier control (to test whether the spread of ice from the top of the chamber to the interior could be prevented) and to determine whether the PROSIL 28 coating was effective in preventing heterogeneous nucleation of the Hanks solution. Under these conditions, the SCP was −21.3±0.4 °C (N=3) and the IFP was −19.8±0.4 °C (N=7) (means ± S.E.M.; P>0.05, t-test), affirming both tests. Additionally, the *in vitro* inoculation temperatures of integument patches were compared with the *in vivo* inoculation temperatures of larvae (IFP of larvae surrounded by ice).

Results

Evidence for seasonal changes in inoculative freezing resistance in field-collected larvae

During summer and early autumn, larvae were readily seeded across the cuticle by ice, but they became resistant to inoculative freezing during late autumn and winter (Fig. 2). On average, summer larvae inoculated at 0.6 °C below the hemolymph HFP (P>0.05, Fig. 2); however, winter larvae, on average, remained supercooled in contact with ice to 11.9 °C below their hemolymph HFP (P<0.001, Fig. 2). During summer, the larval IFPs (freezing while in contact with ice) were higher than the larval SCPs (heterogeneous nucleation temperatures in the presence of desiccant) (P<0.001, Fig. 2). In contrast, winter larval IFPs and larval SCPs did not differ (P>0.05, Fig. 2). Also, winter IFPs were much lower than those in summer (P<0.001), indicating that adaptations that inhibited inoculative freezing had occurred.

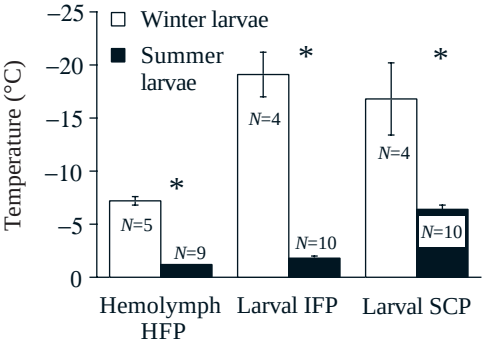

Fig. 2. Hemolymph hysteretic freezing point (HFP), larval inoculative freezing point (IFP) (larvae in contact with ice to promote potential cross-cuticular seeding by external ice) and larval supercooling point (SCP) (larvae in flask with desiccant to prevent condensation of water on the exterior of the insect) of *Dendroides canadensis* larvae collected in mid-September (summer) and early January (winter). Asterisks indicate significant differences (t-test) between winter and summer means (P<0.001). For summer larvae, IFP>SCP (P<0.0001); however, for winter larvae, IFP and SCP did not differ (P>0.05), indicating an absence of inoculative freezing in the winter larvae. Hemolymph HFP>larval IFP for winter larvae (P<0.001) but HFP and IFP did not differ for summer larvae (P>0.005). Values are means ± 1 S.E.M.; N=number of larvae.

Role of seasonal modification of the cuticle in winter- versus summer-collected larvae

Transverse sections of larvae viewed under a transmission fluorescence microscope indicated that the degree of autofluorescence in the cuticle from winter-collected larvae (single arrow in Fig. 3C) was greater than that in summer-collected larvae (single arrow in Fig. 3B), suggesting that modification of the cuticle (e.g. epicuticular lipids) plays a role in seasonal increases in inoculative freezing resistance.

When hexane was used to remove surface waxes from the cuticle of cold-acclimated *D. canadensis* larvae, more hexane-treated larvae (45 of 55 or 82 %) froze while in contact with ice at −8 °C over an 18 h period than did untreated larvae (11 of 55 or 20 %) over the same temperature and duration of exposure to ice (P<0.0001).

During *in vitro* chamber experiments using cuticle patches (epidermis removed) with Hanks solution, cuticle from winter larvae was more effective in preventing inoculative freezing than that from summer larvae (P<0.0001, Fig. 4A).

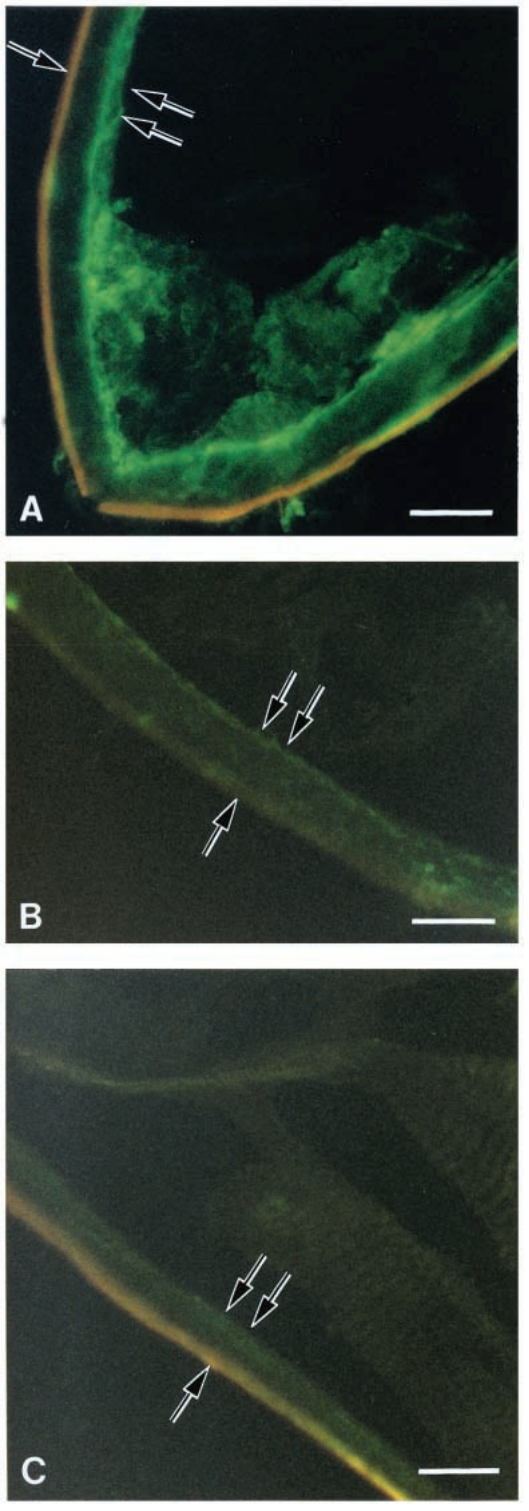

Fig. 3. Indirect immunofluorescence staining of 10 µm thick transverse sections of *Dendroides canadensis* larval integument and attached underlying tissue from winter (February) (A) and summer (July) (B) field-collected larvae that had been incubated in anti-*D. canadensis* antifreeze protein antiserum plus fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG antiserum or (C) winter larvae incubated in FITC-conjugated goat anti-rabbit IgG antiserum only (controls). Single arrows indicate the epicuticle (with yellow autofluorescence) and double arrows indicate the epidermis (with green immunofluorescence). Additional tissue with green immunofluorescence in A is fat body. Scale bars, 48 µm. All photographs were taken using the same light intensity and exposure time.

Role of epidermal and hemolymph AFPs in winter- versus *summer-collected larvae*

Whereas the epidermal region in transverse sections of winter larvae incubated in anti-*D. canadensis* antifreeze antiserum (Xu and Duman, 1991) plus FITC-conjugated goat anti-rabbit IgG antiserum (Calbiochem) immunofluoresced brightly (double arrow in Fig. 3A), this was not the case with summer-collected larvae treated in the same manner (double arrow in Fig. 3B). The epidermal layer of both summer-collected larvae treated with anti-*D. canadensis* antifreeze antiserum plus FITC-conjugated goat anti-rabbit IgG antiserum and winter negative controls treated with FITC-conjugated goat anti-rabbit IgG antiserum only (no anti-*D. canadensis* antifreeze antiserum) did not show immunofluorescence (i.e. the epidermal layer was not immunofluorescent; double arrows in Fig. 3B and C respectively). The additional tissue with green immunofluorescence in Fig. 3A is the fat body, a site of AFP production in cold-hardy *D. canadensis* (Xu and Duman, 1991; Xu *et al.* 1992).

During *in vitro* chamber experiments using integument patches (cuticle plus underlying epidermis) with Hanks solution, integument from winter larvae was more effective in preventing inoculative freezing than integument from summer larvae ($P<0.0001$, Fig. 4B).

Integument patches resisted inoculative freezing better than did cuticle patches for both summer- and winter-collected larvae only when hemolymph AFP was present ($P<0.0001$, Fig. 5). When Hanks solution was used, IFPs of integument did not differ ($P>0.05$) from those of cuticle for either summer- or winter-collected larvae (Fig. 5).

In all cases but one (winter cuticle), the replacement of Hanks solution with AFP solution resulted in a significantly higher ($P<0.001$) degree of resistance to inoculative freezing (Fig. 5).

Comparison of nucleation temperatures of integument with and without AFP

The SCPs of both summer and winter integument patches with Hanks solution were significantly higher ($P<0.00001$) than that of the Parafilm patch (control) with Hanks solution, and the SCP of summer integument with Hanks solution was significantly higher ($P<0.01$) than that of winter integument with Hanks solution (Fig. 6). In contrast, when AFP replaced Hanks solution, the SCPs of summer and winter integument patches (1) did not differ from each other ($P>0.05$), (2) were significantly lower than the SCP of similar patches with Hanks solution ($P<0.00001$) and (3) did not differ from the SCP of the barrier control ($P>0.05$, Fig. 6).

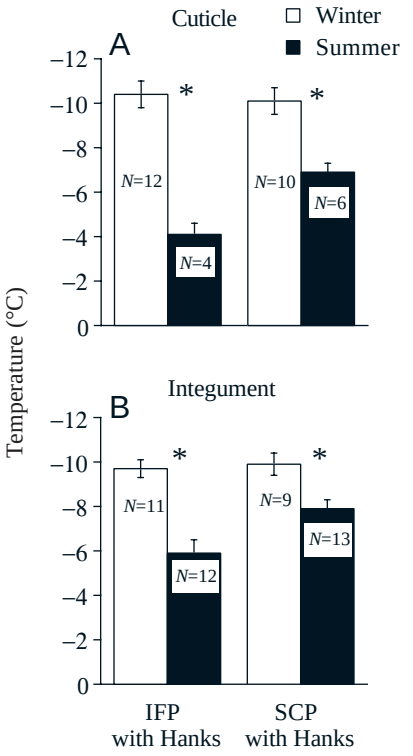

Fig. 4. Results of *in vitro* chamber experiments comparing the inoculative freezing point (IFP) – a measure of resistance to ice propagation – across summer- or winter-collected *Dendroides canadensis* cuticle (epidermis removed) (A) or integument (cuticle with intact epidermis) patches (B). The supercooling point (SCP) – the nucleation temperature without contact with external ice – is also depicted. The fluid in the chamber was antifreeze-protein-free Hanks solution (no thermal hysteresis, melting point –0.45 °C). Asterisks indicate significant differences between summer and winter means (*t*-test) (P<0.0001 for IFP; P<0.01 for SCP). IFP>SCP for both cuticle and integument of summer-collected larvae (P<0.01); however, IFP did not differ from SCP for either cuticle or integument of winter-collected larvae (P>0.05), indicating an absence of inoculative freezing in winter larvae. Values are means ± 1 S.E.M.; N=number of larvae.

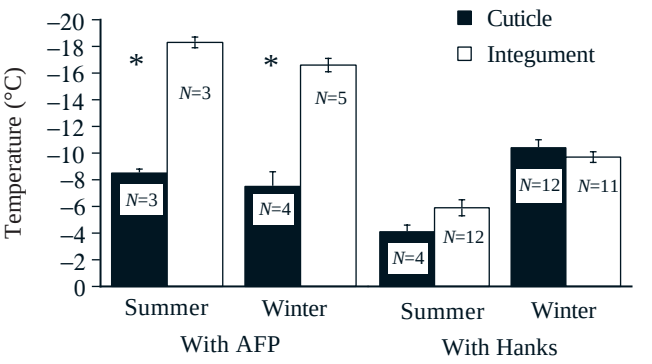

Fig. 5. Results of *in vitro* chamber experiments comparing the inoculative freezing point (IFP) – a measure of the degree of resistance to ice propagation – across summer- or winter-collected *Dendroides canadensis* cuticle (epidermis removed) or integument (cuticle with intact epidermis) patches when the chamber contained a solution of *D. canadensis* antifreeze protein (AFP) (1.82 °C thermal hysteresis, hysteretic freezing point –2.27 °C) or AFP-free Hanks solution (no thermal hysteresis, melting point –0.45 °C). Integument IFP differed from cuticle IFP for both summer- and winter-collected larvae, but only when an AFP solution was used. Asterisks indicate a significant difference between treatments, P<0.0001, *t*-test. Values are means ± 1 S.E.M.; N=number of larvae.

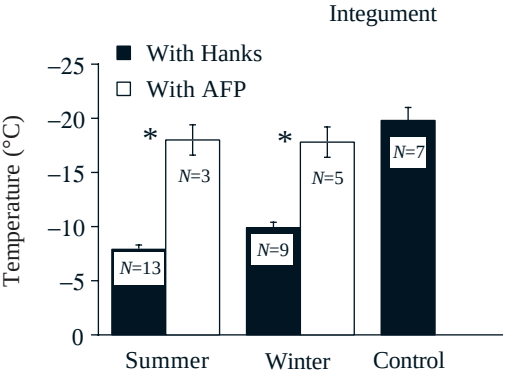

Fig. 6. Results of *in vitro* chamber experiments comparing the supercooling point (SCP) – the nucleation temperature without contact with external ice – of summer- or winter-collected *Dendroides canadensis* integument (cuticle with intact epidermis) patches when the chamber contained a solution of AFP-free Hanks (no thermal hysteresis, melting point –0.45 °C) or *D. canadensis* antifreeze protein (AFP) (1.82 °C thermal hysteresis, hysteretic freezing point –2.27 °C). Parafilm, a wax-coated membrane with very low ice-nucleating activity, replaced the integument in the control SCP treatment. Asterisks indicate significant differences in SCP between treatments for both summer- and winter-collected larvae (*t*-test) (P<0.00001). The SCPs of both summer and winter integument patches with Hanks solution were significantly higher (P<0.00001) than that of the Parafilm patch (control) with Hanks solution, and the SCP of summer integument with Hanks solution was significantly higher (P<0.01) than that of winter integument with Hanks solution. In contrast, when AFP replaced Hanks solution, the SCPs of summer and winter integument patches were both significantly lower than the SCP of similar patches with Hanks solution (P<0.00001) and did not differ from the SCP of the control (P>0.05). Values are means ± 1 S.E.M.; N=number of larvae.

Comparison of inoculation temperatures in field-collected larvae versus in vitro *chamber studies*

The *in vitro* inoculation temperatures for summer cuticle integument patches (–5.9±0.6 °C, N=12) (mean ± S.E.M.) combined with Hanks solution did not differ (*t*-test, P>0.05) from the *in vivo* inoculation temperatures of the summer-collected larvae used in chamber experiments (the IFP of larvae surrounded by ice) (–4.6±0.3 °C, N=8). Likewise, the mean winter *in vitro* temperatures for winter integument patches (–16.6±0.5 °C, N=5) combined with AFP solution did not differ (*t*-test, P>0.05) from the *in vivo* IFP of the winter-collected larvae used in chamber experiments (–17.3±1.1 °C, N=9).

Discussion

This study documented seasonally variable resistance to

inoculative freezing in field-collected *Dendroides canadensis* beetle larvae and used immunofluorescence, *in vivo* removal of epicuticular lipids and *in vitro* chamber studies to explore possible mechanisms driving these seasonal changes. In summer, larvae were highly susceptible to cross-cuticular inoculation since inoculation was initiated at less than 1 °C below the hemolymph HFP. However, by winter, larvae were highly resistant to inoculation by external ice, remaining supercooled to −20 °C or below (10–15 °C below the hemolymph HFP) (Fig. 2). The data suggest that the increase in inoculative freezing resistance results from an interaction between seasonal modifications in the cuticle and the addition of epidermal and hemolymph AFPs in winter.

Role of the cuticular component of the integument

The results suggest that seasonal changes in the cuticle (perhaps in epicuticular lipids) contribute to the increased impermeability of winter cuticle to ice propagation (relative to that of summer cuticle). Surface waxes may play a pivotal role in inoculative freezing resistance since, in most terrestrial arthropods, the epicuticular wax layer is the principal barrier to water efflux (Hadley, 1981). The present results indicated a brighter autofluorescence in the epicuticle of winter- *versus* summer-collected *D. canadensis* (Fig. 3) that may arise from seasonal changes in the surface density or composition of epicuticular lipids (Hadley, 1982). Although no other studies have explored the effects of ultrastructural changes in the epicuticle on cross-cuticular ice propagation, there is a large body of literature indicating that arthropods do alter the density, architecture and molecular composition of their epicuticular waxes in response to seasonal variations in temperature and relative humidity and that these changes can greatly alter cuticular permeability (for reviews, see Hadley, 1980, 1981). In the present study, the results of *in vivo* experiments using hexane to remove epicuticular waxes suggest that, in cold-hardy *D. canadensis*, the cuticular layer of the integument is a functionally important component of inoculative freezing resistance. While only 20 % of the untreated larvae froze inoculatively at −8 °C or below over an 18 h period, 82 % of the hexane-treated larvae froze over the same period.

The results of chamber experiments also support a role for seasonal cuticular modification. When AFP-free Hanks solution was used to simulate hemolymph, both summer and winter cuticle patches retarded cross-cuticular ice propagation; however, winter cuticle was significantly more effective (10.0 °C *versus* 3.7 °C below the MP of the Hanks solution) (Fig. 4A). Few studies have utilized chambers to explore mechanisms of inoculative freezing resistance, and only Yingst (1978) addressed the role of arthropod integument in resisting inoculative freezing. Yingst (1978) found that a cuticle patch (integument with epidermis and underlying tissue removed) from the marine benthic Arctic isopod *Mesidotea entomon* prevented inoculative freezing to at least −5 °C (3 °C below the hemolymph MP). This value is in close agreement with that obtained in the present study using (summer) *D. canadensis* cuticle susceptible to inoculative freezing; however, winter *D. canadensis* cuticle prevented inoculative freezing to −10 °C.

Role of the epidermal layer and hemolymph AFPs

Although Valerio *et al.* (1992) addressed the role of the epidermal layer in the prevention of ice propagation in Arctic marine fishes (flounder *Pseudopleuronectes americanus* and ocean pout *Macrozoarces americanus*), and AFP activity has been detected in the skin of an arctic marine fish (cunner *Tautogolabrus adspersus*) (Valerio *et al.* 1990), the present study is the first to explore the role of insect epidermis and AFP in inoculative freezing resistance. Micrographs of immunocytochemically labeled transverse sections of *D. canadensis* larvae clearly indicated that epidermal AFPs are present in winter-collected larvae but absent in summer-collected ones (Fig. 3). If the epidermal layer is a functionally important component of the inoculative freezing barrier, then, in chamber experiments, the ice propagation temperature of the integument patch (cuticle with underlying epidermis) should be lower than that of the cuticle patch (underlying epidermis removed). Our results support this prediction for summer- and winter-collected larvae, but only when purified AFP solution was present inside the chamber (Fig. 5). When Hanks solution (AFP-free) was used, IFPs for integument patches did not differ from those of cuticle patches for either summer- or winter-collected larvae (Fig. 5). The lack of difference in inoculative freezing resistance for cuticle *versus* integument patches when AFP was absent from the solution may result, in part, from the procedure used for preparing the integument patch. To maintain the integrity and viability of the epidermal cells, larvae were floated in sterilized 0.9 % saline solution during dissection and removal of the integument patch. Diffusion of the epidermal AFPs during this immersion in saline solution may have resulted in a subthreshold level of epidermal AFP. Addition of hemolymph AFP solution to the chamber may have restored threshold levels of AFPs in the epidermis. Further evidence for a threshold comes from preliminary experiments (data not shown) in which HPLC-purified AFP solution with 1.4 °C thermal hysteresis produced no significant increase compared with Hanks solution in preventing ice propagation across integument patches, whereas AFP with 1.8 °C thermal hysteresis activity (as was used in the current study) did.

The increased inoculative freezing resistance in response to the addition of AFP seen in the present chamber experiments is probably conservative relative to that occurring *in vivo*. The present study used one of the four antifreeze proteins (AFP-4) purified from *D. canadensis* hemolymph at a concentration that produced 1.82 °C thermal hysteresis. *In vivo*, winter thermal hysteresis activity of hemolymph is 5–6 °C (Olsen and Duman, 1997*a*), resulting from a synergism among the multiple forms of AFPs, increased concentration (Wu *et al.* 1991) and activation by an endogenous hemolymph activator protein (Wu and Duman, 1991).

In all but the case of winter cuticle, the replacement of

Hanks solution with an AFP solution resulted in a strongly significant increase in resistance to inoculative freezing ($P<0.001$, Fig. 5). Our results suggest that, in the winter cuticle plus AFP treatment, highly active ice nucleators caused ice formation (nucleation) in the AFP solution before cross-cuticular seeding by external ice could occur, thereby confounding the test of inoculative freezing resistance for this treatment. The IFP for winter cuticle plus AFP (−7.5±1.1 °C, $N=4$) was very high and did not differ (*t*-test, $P=0.56$) from the SCP of the winter cuticle + AFP treatment (−6.8±7 °C, $N=4$) (means ± S.E.M.).

Olsen and Duman (1997*a*) found that, as winter approaches, *D. canadensis* can lower larval SCP by removing highly active ice nucleators from the hemolymph and that hemolymph AFPs could inhibit the activity of residual hemolymph ice nucleators. The results of the present study suggest that, besides preventing the cross-cuticular spread of ice, hemolymph AFP plays an important role in inhibiting heterogeneous ice nucleator activity associated with the integument. Muscle and epidermis were found to have significantly higher nucleation temperatures than any of the other tissues assayed in the overwintering diapausing larvae of the rice stem borer *Chilo suppressalis* (Lepidoptera: Pyralidae) (Tsumuki and Konno, 1991). Although the nucleation temperature of *D. canadensis* epidermis was not determined in this current study, mean SCPs of both summer and winter integument patches with Hanks solution were significantly higher than that of the Parafilm patch (control) with Hanks solution, suggesting the presence of ice nucleators on the integument (Fig. 6). In contrast, when an AFP solution replaced Hanks solution, the SCPs of summer and winter integument patches did not differ from each other or from that of the Parafilm control patch and were significantly lower than the SCP of similar patches with Hanks solution (Fig. 6). The results also suggest the seasonal removal of integumentary ice nucleators since the SCP of summer integument with Hanks solution was significantly higher than that of winter integument (Fig. 6).

The IFP depression afforded by combining AFP-4 (Wu *et al.* 1991; N. Li, C. A. Andorfer and J. G. Duman, manuscript in preparation) purified from the hemolymph of winter larvae with winter integument far exceeds the HFP (−2.27 °C). In contrast to the 0.25 mm diameter ice crystal used to measure thermal hysteresis in the method of DeVries (1986), the ice propagating through a cuticular pore in *D. canadensis* would be orders of magnitude smaller. Epicuticular pores range in diameter from 6.5×10^{-6} mm for the beetle *Tenebrio molitor* (Locke, 1974) to 3×10^{-3} mm for the butterfly *Epipyrops anomale* (Marshall *et al.* 1974). Since the magnitude of thermal hysteresis is inversely proportional to crystal size (Zachariassen and Husby, 1982), such a reduction in crystal size may account for the amplification of the non-equilibrium freezing point depression by *D. canadensis* integument seen in the present chamber experiments.

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