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Diapausing pupae of the flesh fly *Sarcophaga crassipalpis* (Diptera: Sarcophagidae) are more resistant to inoculative freezing than non-diapausing pupae

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Abstract. The resistance of diapausing (overwintering) and non-diapausing (summer) *Sarcophaga crassipalpis* (Diptera: Sarcophagidae) pupae to inoculative freezing was examined. Although both types of pupae resisted inoculative freezing after 24-h submergence in water, diapausing pupae were overall significantly more resistant than non-diapausing pupae. Exposing the thin pupal cuticle by removing the ends of the puparial case eliminated the capacity of both pupal types to resist inoculative freezing, indicating that resistance to inoculative freezing resides with the puparium. Pupae submerged in surfactant solution were significantly less resistant to inoculative freezing than those submerged in water. Furthermore, the puparial water content of pupae submerged in surfactant solution was significantly greater than that of puparia from pupae submerged in water. Surfactant may have promoted inoculative freezing by facilitating the spread of water over the surface of and into the puparium, thereby creating bridges between external ice and pupal body fluids. Extracting puparial surface lipids with chloroform/methanol (2 : 1, v:v) decreased the resistance of non-diapausing pupae to inoculative freezing but did not significantly affect that of diapausing pupae. This finding indicates that the puparium of diapausing pupae contains protection against inoculative freezing separate from its surface lipids. This barrier may be important in protecting the freezing-intolerant overwintering pupae against inoculative freezing within their soil hibernaculum.

Key words. Cuticular lipids, inoculative freezing, puparium, *Sarcophaga crassipalpis*, temperature of crystallization.

Introduction

A number of factors influence the capacity of organisms to supercool, that is, remain unfrozen below the equilibrium freezing point of their body fluids (see reviews by Lee, 1991; Bale, 1993; Block, 1995). The probability of ice formation within a sample of supercooled water increases with time and decreasing temperature. Furthermore, the capacity of such a sample to remain in the metastable supercooled state is limited by its volume; that is, larger volumes of water tend to supercool less. For this reason, the small size of most insects facilitates extensive supercooling (Lee, 1991). This capacity can be further enhanced by the production of thermal-hysteresis (sometimes called antifreeze) proteins or low molecular mass cryoprotectants such as glycerol (Duman *et al.*, 1995). By contrast, certain proteins, ingested food particles, a few species of microorganisms and inorganic crystals may catalyse ice formation at temperatures well above those at which the body fluids of an organism would otherwise spontaneously freeze (Lee *et al.*, 1996). In this paper, the temperature at which ice forms within the body fluid of an organism, whether spontaneously or as a result of inoculation by external ice, is referred to as the temperature of crystallization or T_C.

Whereas insects isolated from their natural environment often supercool extensively, the supercooling capacity of those

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cooled in contact with environmental ice may be limited by a phenomenon termed inoculative freezing (see review by Lee & Costanzo, 1998). In this case, crystals on the surface of the insect grow through its integument and seed freezing of haemolymph. Because it ensures that ice formation will begin at high subzero temperatures, inoculative freezing is critical for the survival of some freeze-tolerant arthropods including the centipede, *Lithobius forficatus* (Tursman *et al.*, 1994). By contrast, inoculative freezing presents a potential danger to freeze-intolerant insects.

The freeze-intolerant flesh fly (*Sarcophaga crassipalpis*) has been well studied as a model of insect cold hardiness (Lee & Denlinger, 1985; Chen *et al.*, 1987; Denlinger, 1991). Larvae of this species burrow a few centimetres beneath the soil surface before pupariating. At the initiation of metamorphosis, third-instar larvae undergo an event in which their cuticle becomes sclerotized and tanned to form a puparium, the case in which the soft-cuticled pupa and pharate adult develop (Denlinger & Zdarek, 1994). Diapausing pupae overwinter for up to 180 days, during which time they are likely to be subjected to subzero temperatures as well as significant variation in soil moisture and ice content. By contrast, non-diapausing pupae found during the late spring and summer, probably never encounter subzero temperatures and emerge as adults ≈ 14 days after pupariation (Denlinger, 1972). Both types of pupae supercool extensively, typically exhibiting $T_C < -22°C$ (Lee & Denlinger, 1985). However, whereas diapausing pupae can survive prolonged chilling at temperatures as low as −17°C, the non-diapausing form dies due to non-freezing injury when cooled to −10°C for as little as 1 h (Chen *et al.*, 1987). Although differences in the cold tolerance of the two types of pupae (Lee & Denlinger, 1985; Chen *et al.*, 1987) have been well documented, little is known of whether, or how, either resists inoculative freezing and water exchange in wet soil.

In this study we examined the mechanism by which *S. crassipalpis* pupae resist inoculative freezing. We first tested the hypothesis that diapausing individuals are better able to resist inoculative freezing than their non-diapausing counterparts. To examine cuticular mechanisms of resistance, we determined whether lowering the surface tension of water with a surfactant and/or extracting lipids on the surface of the puparium with organic solvents decreased the resistance of pupae within their puparia to inoculative freezing. Next, we removed part of the puparium to determine whether the thin pupal cuticle could prevent inoculative freezing. Because water uptake by pupae, and by the puparia in which they reside, could potentially affect their capacity to supercool, and thereby affect T_C, we also examined the relationship between pupal resistance to inoculative freezing and the water content of intact pupae and their isolated puparia.

Materials and Methods

Insect rearing

Sarcophaga crassipalpis were reared according to the methods described by Lee & Denlinger (1985). Diapausing pupae were obtained by rearing flies at 25°C under a short-day photoperiod (LD 12 : 12 h), whereas non-diapausing groups were reared at 25°C under long-day conditions (LD 15 : 9 h). Sugar and water were provided to adults *ad libitum*. During the first 6 days post-emergence, flies were provided with beef liver as a protein source to allow normal oogenesis and embryonic development. On the eleventh day following emergence, a 50-g packet of liver was provided as a substrate for larviposition. On the day following larviposition, larvae were transferred to fresh packets of liver at a density of approximately one larva per g of liver. These packets were placed in plastic tubs lined with a 2-cm layer of sawdust into which the larvae were allowed to wander and pupate. Following pupariation, pupae were sifted from the sawdust and stored in Petri dishes until their emergence or use in experiments.

Effect of water and surfactant solution on pupal resistance to inoculative freezing

Diapausing and non-diapausing pupae encased within their puparia were individually submerged in 250 µl of water or surfactant solution (a suspension of 0.1% w/v Tegopren 5878, Goldschmidt Industrial Chemical Corp., McDonald, Pennsylvania, U.S.A.) within 1.8-ml microcentrifuge tubes for 24 or 48 h at 22°C. For both water and surfactant solution experiments, a wet-control (0 h) group was submerged and immediately cooled until they froze at their T_C. The T_Cs of dry diapausing and non-diapausing controls were determined by cooling otherwise untreated pupae in the absence of external moisture. Following 24 or 48 h of submergence, with the pupa and water or surfactant solution still in the tube, a 36-ga copper–constantan thermocouple was positioned against the surface of the puparium to measure pupal temperature. The microcentrifuge tubes were stoppered with foam rubber and placed individually in glass test tubes immersed in a refrigerated bath (Neslab RTE-8) set at 0°C. Bath temperature was decreased at 0.3°C/min, and the T_C of each individual was identified as the beginning of the exotherm produced by the release of the latent heat of fusion as its body fluids froze. In >90% of the determinations, pupal T_C was distinguishable from that of the surrounding fluid. Those few samples in which the T_C of the pupa was masked by the exotherm of the surrounding fluid were omitted from data analysis.

Effect of extracting puparial surface lipids on T_C

To assess their role in preventing inoculative freezing, lipids on the surface of the puparium were extracted by washing puparia containing pupae for 2 min in chloroform/methanol (2 : 1, v:v) prior to the water or surfactant treatments outlined above. This solvent mixture is known to remove effectively surface lipids from the puparium (Yoder *et al.*, 1992). Immediately following treatment, pupal T_C was determined as described above.

Capacity of the pupal cuticle to prevent inoculative freezing

To assess the capacity of the thin pupal cuticle to prevent the propagation of ice into the pupa, we examined the effect of exposing this delicate membrane to air or water. Pupal cuticles were exposed by removing the puparium at each end of the pupa. Half of these pupae were individually placed into 1.8-ml centrifuge tubes, then covered with 250 µl of water containing a potent ice nucleating agent (a killed preparation of *Pseudomonas syringae*, kindly provided by Genencor International, Rochester, NY). The remainder were left dry, to control for the effects of removing the ends of the puparium. The T_Cs of all of pupae were determined according to the methods described for assessing the effects of water or surfactant treatment on T_Cs of intact pupae.

Determination of whole pupa and puparial case water content

The amount of moisture that diapausing and non-diapausing pupae, including their puparia, gained over the course of each 24-h and 48-h treatment was determined as the change in their mass during water or surfactant submersion. So that only the water absorbed by each individual during treatment would be included in our measurements, their surfaces were blotted dry with tissue paper prior to weighing.

Gravimetric assessments were used to determine the water contents of puparia removed from pupae in each control (denoted as 0 h treatment, because they were wetted and immediately blotted dry before determination of their water content), 24-h or 48-h treatment. Following treatment, isolated puparia were obtained by removing the pupae which they contained. Each isolated puparium was cut into four approximately flat strips, which were blotted three times with tissue paper to remove surface moisture. The strips from each puparium were weighed, then dried at 60°C until no further weight loss occurred. Puparial water content was calculated as the change in mass divided by the initial mass and the resulting proportion multiplied by 100.

Data analysis

The effects of water and surfactant solution, both without and following solvent extraction of puparial surface lipids, were analysed by multifactor analysis of variance. Because changes in puparial water content and body water content were expressed as percentages, these data were subjected to arcsine-square root transformations prior to analysis. The effect of exposing the pupal cuticle by removing portions of the puparium was assessed using an unpaired *t*-test. Data were considered significant at a level of $\alpha < 0.05$ and are reported as mean ± SEM.

Results

Effect of water, surfactant and solvent pre-treatment on pupal T_C.

Comparing across all treatments, diapausing pupae ($T_C = -18.3 \pm 0.5$°C) were significantly more resistant to inoculative freezing than their non-diapausing counterparts ($T_C = -14.5 \pm 0.5$°C, $F = 29.56$, $P < 0.0001$; Fig. 1A–D). Although the resistance of both pupal types to inoculative freezing decreased as the duration of their submergence increased ($F = 54.15$, $P < 0.0001$), the relative difference in resistance between diapause and non-diapause pupae remained the same over time (Fig. 1A–D). Therefore, we did not extend our comparison of diapausing and non-diapausing pupae to include specific comparisons between each time interval.

Submergence in surfactant solution significantly reduced the resistance of pupae to inoculative freezing relative to those submerged in water alone (compare Figs 1A to C, 1B to D, $F = 84.94$, $P < 0.0001$). Because submergence in surfactant solution caused parallel increases in the susceptibility of diapausing and non-diapausing pupae to inoculative freezing we did not expand the water- vs. surfactant-solution comparison to distinguish between the two types of pupae. However, relative to water, surfactant solution significantly diminished pupal resistance to inoculative freezing ($F = 5.32$, $P = 0.0055$). For example, although pupae submerged in surfactant solution for 0 h were significantly ($P = 0.035$) more susceptible to inoculative freezing than their counterparts submerged in water (difference in $T_C = 3.1$°C), those submerged in surfactant for 48 h experienced an even greater increase ($P < 0.0001$) in susceptibility relative to their water-treated counterparts (difference in $T_C = 8.5$°C, Fig. 1A–D).

Extracting puparial-surface lipids with a mixture of chloroform and methanol increased the overall susceptibility of pupae to inoculative freezing (compare Figs 1A to B, 1C to D). However, this increase resulted almost exclusively from a highly significant increase in the susceptibility of non-diapausing pupae (difference in $T_C = 4.5$°C, $P < 0.0001$). Extracting lipids from the puparial surface had no significant effect on the susceptibility of diapausing pupae to inoculative freezing.

Capacity of the pupal cuticle to prevent inoculative freezing.

On its own, the pupal cuticle afforded diapausing and non-diapausing pupae little, if any, resistance to inoculative freezing (Table 1). For instance, whereas diapausing pupae encased within their puparia and cooled in water containing the ice nucleator *P. syringae* supercooled to -22.5 ± 0.2°C, those in which the pupal cuticle had been partially exposed froze as ice formed in the water around them ($T_C = -3.2 \pm 0.2$°C). Because these pupae froze while the surrounding fluid released its latent heat of crystallization, they did so at a higher temperature ($T_C = -2.7 \pm 1.2$°C) than the surrounding medium.

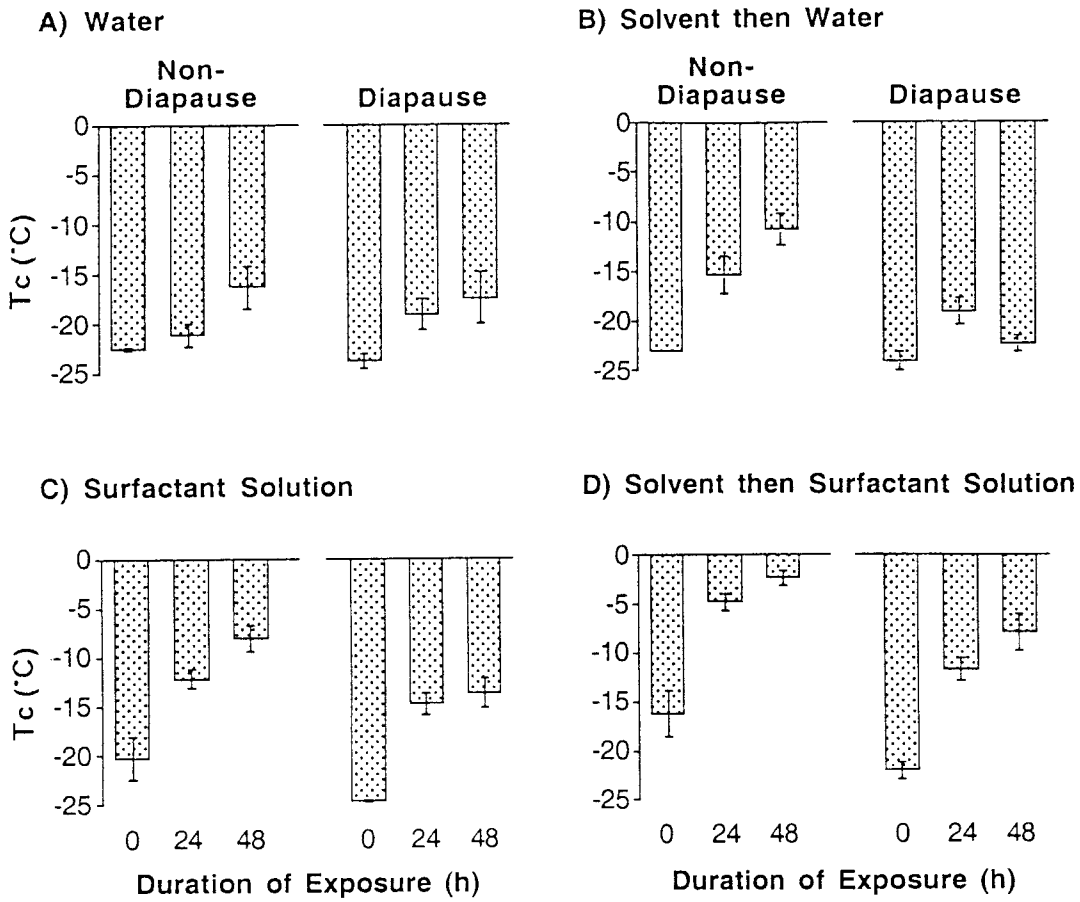

Fig. 1. Effects of various treatments on the temperature of crystallization (T_C) of diapausing and non-diapausing *Sarcophaga crassipalpis* pupae: (A) submersion in water; (B) extraction of puparial surface lipids with a mixture of chloroform and methanol (2 : 1, v:v), then submersion in water; (C) submersion in surfactant solution; (D) Extraction of puparial surface lipids, then submersion in surfactant solution. Each column represents the mean $T_C \pm$ SEM ($n = 8$–33).

Table 1. Effect of partial puparium removal on the susceptibility of *Sarcophaga crassipalpis* pupae to inoculative freezing. Values listed represent the mean $T_C \pm$ SEM of water in which pupae were submerged ($n = 40$) and pupae ($n = 5$). Exposing the pupal cuticle significantly ($P < 0.001$) decreased the resistance of both types of pupae to inoculative freezing.

		Temperature of crystallization (°C)			
		Intact pupae		Partially exposed pupae	
Pupal state	Water	Dry	Wet	Dry	Wet
Non-diapausing	−3.2 ± 0.2	−22.8 ± 0.6	−22.5 ± 0.4	−23.9 ± 0.3	−5.0 ± 0.4
Diapausing	−3.2 ± 0.2	−22.8 ± 0.2	−23.8 ± 0.3	−23.3 ± 0.6	−2.7 ± 0.5

Effect of water, surfactant and solvent pre-treatment on whole pupa and puparial water contents

To determine whether the treatment-induced increases in T_C were related to dilution of pupal body fluids resulting from water uptake, we examined the effect of all four treatment regimes on the water content of intact pupae within and including their puparia. Although extraction of puparial surface lipids with chloroform and methanol significantly ($F = 92.117$, $P < 0.0001$) increased the amount of water or surfactant solution individuals gained over 24 and 48 h of treatment, the most substantial elevation was only 7.6 ± 0.7% (Fig. 2). Assuming that all the water entered the pupae, this increase would correspond to approximately a 10% dilution of the body fluids. Colligatively, this amount of dilution would be expected to decrease pupal supercooling capacity by only

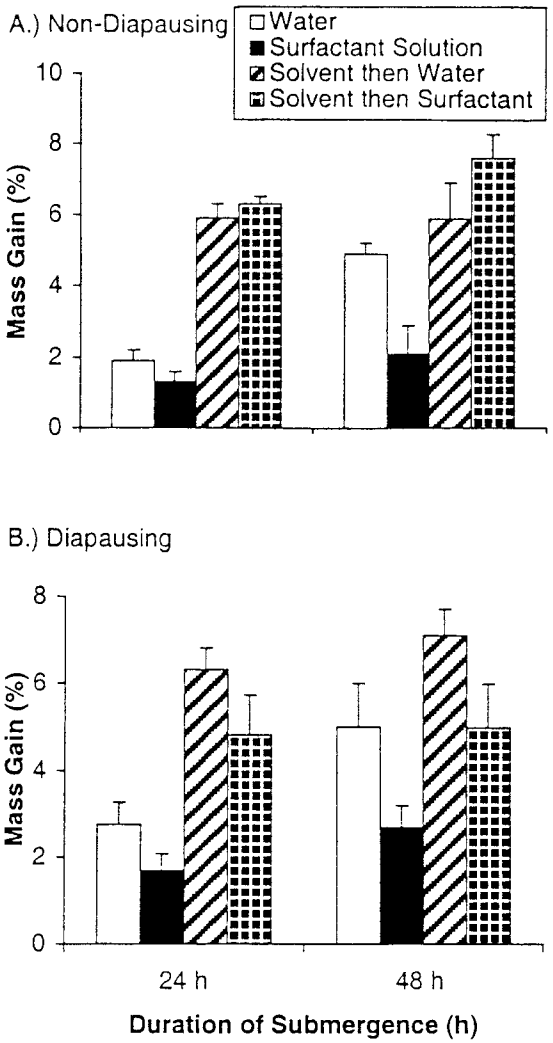

Fig. 2. Effect of extraction of *Sarcophaga crassipalpis* puparial surface lipids with chloroform and methanol and 24 or 48 h of water or surfactant treatment on the mean percent mass gain ± SEM of: (A) non-diapausing and (B) diapausing *S. crassipalpis* pupae. For each column, $n = 9$ or 10.

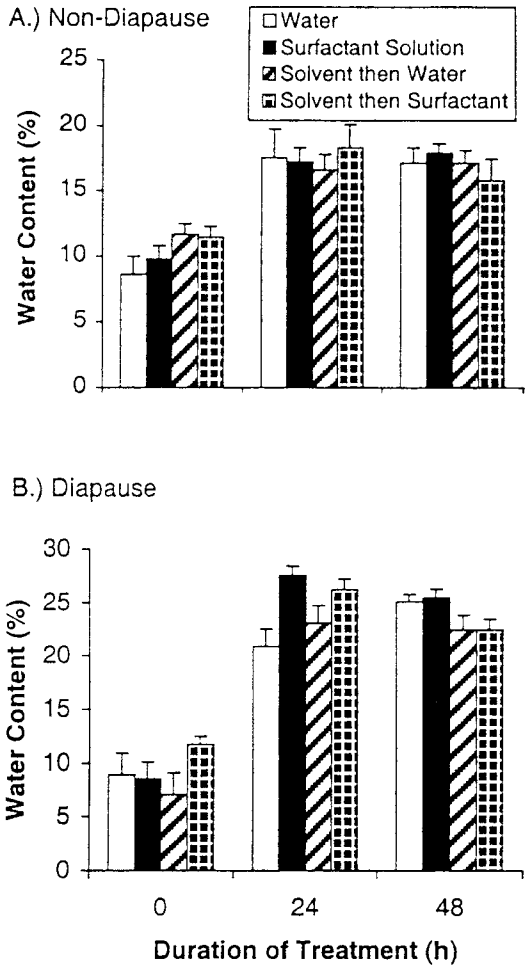

Fig. 3. Effect of prior extraction of *Sarcophaga crassipalpis* puparial surface lipids with chloroform and methanol on the puparial water content of: (A) non-diapausing and (B) diapausing pupae following submersion in water or surfactant solution for 24 or 48 h. After 24 and 48 h, puparial water content increased significantly relative to controls ($P < 0.0001$). However, no significant differences were found between 24 and 48 h treatments. Each column represents mean percent water content ± SEM of 5–10 puparia.

0.1°C (Zachariassen, 1991). Even the most substantial elevation in water content that we observed would have been insufficient to dilute the pupal body fluids sufficiently to account for the elevated T_Cs that we observed.

To test whether increases in T_C were correlated with increased hydration of the puparium following submersion in water or surfactant solution, we determined the effects of these manipulations on puparial water content (Fig. 3). Overall, the water contents of puparia from pupae submerged for 24 (24.4 ± 0.8%) and 48 h (23.9 ± 0.5%) were indistinguishable from each other, but were both significantly greater than that of puparia from 0 h controls (9.5 ± 0.8%, $P < 0.0001$). Although extraction of puparial surface lipids prior to submergence significantly increased the susceptibility of pupae to inoculative freezing, it did not significantly affect the water content of puparia. By contrast, puparia of pupae submerged in surfactant solution (21.3 ± 1.0%) contained significantly more water than did those of pupae submerged in water alone (18.9 ± 1.1%, $F = 9.772$, $P = 0.0023$).

Discussion

Because overwintering (diapausing) *S. crassipalpis* pupae spend prolonged periods in shallow soil hibernaculae, where they are likely to experience both severe low temperature and contact with environmental ice, we hypothesized that these pupae would be more resistant to inoculative freezing than their non-diapausing (summer) counterparts. Our data support this hypothesis. Both types of pupae supercooled extensively when dry, and resisted inoculative freezing after prolonged (48 h) submergence in water. However, prior extraction of

puparial surface lipids and/or submergence in water containing surfactant increased the susceptibility of non-diapausing pupae to inoculative freezing significantly more than diapausing pupae. Our data indicate that the greater resistance of the diapausing pupa to inoculative freezing is associated with differences between its puparium and that of the non-diapausing pupa.

Having determined that while encased in their puparia both types of pupae resist inoculative freezing, we examined possible mechanisms which underlie this capacity. Pupae are potentially protected by two barriers between external ice and their body fluids, the puparium and the thin pupal cuticle. We found that the pupal cuticle alone did not prevent inoculative freezing; dry pupae whose cuticle had been exposed by removal of portions of the puparium supercooled extensively, but when cooled in contact with water froze soon after ice formed in the surrounding liquid.

As the pupal cuticle did not prevent inoculative freezing, we next examined the mechanisms by which the puparium impedes the inward propagation of ice. By decreasing the surface tension of water, surfactants increase its capacity to spread over the surface of and penetrate the puparial cuticle. The surfactant we chose facilitates pesticide uptake into a variety of plant leaves by enhancing the passage of water through narrow stomata (Knoche *et al.*, 1991). Similarly, Lee *et al.* (1998) found that treating the lady beetle, *Hippodamia convergens*, with water containing surfactant increased their susceptibility to inoculative freezing, presumably by facilitating the growth of the ice lattice through cuticular pores or other openings. We found that submergence in water containing surfactant decreased the resistance of both types of pupae to inoculative freezing. Additionally, we found that the addition of surfactant increased the amount of water gained by puparia. Although lacking easily visible pores, the dipteran puparium allows the efflux (Bursell, 1958) and influx (Yoder *et al.*, 1992) of water, perhaps via pore canals retained after its transformation from the larval cuticle (Neville, 1975; Noble-Nesbitt, 1991). By enhancing the penetration of water through the puparium, surfactant probably facilitated the creation of bridges between external ice and the body fluids of the pupa, thereby promoting inoculative freezing.

The importance of cuticular lipids in restricting water loss from a variety of insects has been extensively documented (Wigglesworth, 1945; Noble-Nesbitt, 1991). We extracted puparial surface lipids with solvent to determine whether they contribute to the capacity of the puparium to prevent inoculative freezing. Although this manipulation significantly increased the susceptibility of non-diapausing pupae to inoculative freezing ($P < 0.0001$), it had no discernible effect on the resistance of those in diapause (compare Figs 1A to B, 1C to D). This finding indicates that the puparia of diapausing pupae contain a barrier to inoculative freezing separate from their puparial surface lipids and resistant to solvent treatment.

At this point, the nature of the solvent-resistant barrier in diapausing pupae remains unclear. The inner surface of the puparium of the diapausing pupa is lined with three times more lipid than that of the non-diapausing pupa (Yoder *et al.*, 1992). This enhanced layer functions to restrict water loss under desiccating conditions (Yoder *et al.*, 1992; Yoder & Moreau, 1994). We hypothesize that this layer constitutes an enhanced barrier to inoculative freezing in diapausing pupae. Located on the inner surface of the puparium, these lipids are likely to be protected against the solvent extraction of surface lipids that we employed.

The fact that the diapausing pupa, the overwintering stage of *S. crassipalpis*, is much more cold tolerant than the non-diapausing summer pupa is well documented (Lee & Denlinger, 1985; Lee *et al.*, 1987; Denlinger, 1991). Whereas non-diapausing pupae are killed by non-freezing injury caused by brief exposure to subzero temperatures well above their T_C, diapausing pupae withstand prolonged exposure to much lower temperatures (Lee & Denlinger, 1985). By demonstrating that diapausing pupae are significantly more resistant to inoculative freezing than non-diapausing ones in this study, we provide evidence for an additional seasonal adaptation of diapausing pupae. Similar seasonal increases in resistance to inoculative freezing have been found in taxonomically diverse insects including *Dendroides canadensis* (Olsen *et al.*, 1998), *Cisseps fulvicollis* (Fields & McNeil, 1986) and *Smicronyx fulvus* (Rojas *et al.*, 1992). However, the specific mechanisms underlying resistance to inoculative freezing differ between species. For example, whereas epicuticular lipids are an important factor contributing to the resistance of *D. canadensis* (Olsen *et al.*, 1998), the resistance of diapausing *S. crassipalpis* was not affected by extraction of puparial surface lipids. Regardless of the underlying mechanism, an increased capacity of freeze-intolerant overwintering insects, such as *S. crassipalpis*, to resist inoculative freezing would enhance their chances of survival during periods when microenvironmental temperature falls below the equilibrium freezing point of their tissue fluids and ice forms in the soil around them.

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