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# DIVERGENT MECHANISMS OF FROST-HARDINESS IN TWO POPULATIONS OF THE GALL FLY, *EUROSTA SOLIDAGINSIS*

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**Abstract**—Two populations of the gall fly *Eurosta solidaginsis* utilize different strategies to endure seasonal exposure to temperatures below freezing. Both populations are freezing tolerant. In north temperate populations, supercooling points rise from −10.2°C to −6.2°C following exposures to temperatures below freezing. This level is maintained throughout winter and ensures frequent and prolonged periods of tissue freezing. South temperate populations depress the supercooling point to −14.2°C during autumn and early winter, and this depression precludes extracellular ice formation during periods of supra-optimal temperature fluctuations. During mid-winter, supercooling points rise to the same level as in northern groups.

Both populations accumulate three principal cryoprotective agents following first frost exposures (glycerol, sorbitol and trehalose). Cryoprotectants levels do not peak in northern populations until 4–6 weeks after first frost. In southern populations the accumulation profile is characterized by a high initial rate of synthesis, a protective overshoot and pronounced seasonal fluctuations. The relative survival advantages of each strategy are discussed.

## INTRODUCTION

THE DIVERSITY of strategies used by overwintering insects has been the subject of a number of recent reviews (DANKS, 1978; RING, 1980; BAUST, 1981). Insects survive sub-zero exposures either by extensive supercooling to avoid extracellular freezing (Type I) (SALT, 1959, 1962; SOMME, 1964; BAUST and MILLER, 1970, 1972; BAUST, 1973, 1980; BAUST and EDWARDS, 1979). Most freezing-tolerant species demonstrate a limited but concomitant extension of supercooling range as frost hardening progresses (Type IIA). These supercooling points are not, however, as low as those found in species capable of supercooling to the limits of homogeneous nucleation (MILLER and WERNER, 1981; RING, 1981). With minor exception, species demonstrating Type I or II hardening accumulate varying levels of cryoprotective polyhydric alcohols and low molecular weight saccharides.

BAUST *et al.* (1979) provided a preliminary report on the dichotomous strategies employed by latitudinally separate populations of the gall fly *Eurosta solidaginsis*. Populations from north temperate regions demonstrate a paradoxical elevation of supercooling points during the initial phases of cold hardening. This supercooling point elevation was maintained throughout the winter period (MORRISSEY and BAUST, 1976), and was categorized by BAUST *et al.* (1979) as Type IIB hardening. Southern populations following chilling and sub-zero exposure demonstrate Type IIA hardening. Not until acclimation temperatures reached −20°C did southern populations elevate supercooling points to levels equivalent to those of northern groups. SOMME (1978) has provided qualitative evidence suggesting the presence of proteinaceous nucleators in the haemolymph of northern populations. Preliminary studies by ZACHARIASSEN, LEE and BAUST (unpublished) suggest that northern populations rely on modulation of nucleators in response to low temperature acclimation while southern residents reduce overall nucleator potential (concentration and/or efficiency). Other aspects of the winter hardening response of northern populations include seasonal changes in 'bound water' and intermediary metabolism (STOREY *et al.*, 1981 a, b).

Both populations of *E. solidaginsis* are freezing tolerant; yet, each responds differently to laboratory acclimation procedures. Southern populations avoid freezing while northern populations ensure extracellular freezing at relatively high subfreezing temperatures. The probable adaptive advantages of these strategies have been suggested by BAUST (1981). Northern ambient temperatures fluctuate widely during autumn and winter (15°C to −30°C). Most daily cycles are characterized by sub-zero evening temperatures and near freezing days. Southern ambient temperatures are relatively stable during a comparable period. (20°C–0°C).

For third-instar *E. solidaginsis*, −18°C represents the approximate mean physical limit to supercooling. Accordingly, southern populations would not naturally experience tissue freezing. Northern residents would however experience frequent bouts of freezing at supra-optimal rates. Freezing at low temperatures would be accompanied by comparatively rapid ice growth, lack of adequate cell volume compensation and a higher probability of intracellular (lethal) ice formation (MAZUR, 1977). Presumably, to avoid these problems northern populations elevate supercooling points so that freezing occurs at warmer sub-zero temperatures. In this manner the frequency



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of freeze–thaw encounters is reduced, and when freezing occurs, the probability of intracellular ice formation is diminished.

The purpose of this study was to determine the relative similarities and differences between Type IIA and IIB hardening under ambient conditions, as characterized by changes in supercooling points, cryoprotectant levels and haemolymph freezing points.

## MATERIALS AND METHODS

Golden rod ball galls (*Solidago canadensis*) were collected between late September and March from sites in central Minnesota (45°) and coastal Texas (29° 30′N). Specimens were removed from the galls and freeze-clamped on dry ice and stored in liquid nitrogen. Supercooling points were determined by affixing a 28 gauge copper–constantan thermocouple to the cuticular surface. Variations in thawing procedures do not effect supercooling points. Specimens were cooled at approx. 1°C/min. The initiation of spontaneous freezing as indicated by the onset of loss of the latent heat of fusion is defined as the supercooling point.

Haemolymph melting points were observed in a modified Scholander apparatus as described by BAUST and MILLER (1970). Whole body cryoprotectant levels were determined by high pressure liquid chromatography (Waters Assoc.). Specimens were homogenized in distilled water (150 mg/ml) in a glass–teflon pedestal homogenizer. The homogenate was partitioned against an equal volume of chloroform–methanol (2:1), centrifuged to accelerate partitioning and the supernatant decanted. The organic partition was washed twice in distilled water, centrifuged and supernatants combined. Following 15 min aspiration at 50°C, the samples were deproteinized by the addition of 1.5 ml of 0.3 N barium hydroxide and 1.5 ml 0.3 N zinc sulphate (15 min). The protein precipitate was pelleted, washed twice, and the supernatants removed and evaporated to dryness at 50°C. The residue was re-suspended in 0.65 ml distilled water, deionized with a mixed bed resin (Bio Rad AG501-X8), filtered (0.22 μm pore) and degassed. Carbohydrates and polyhydric alcohols were separated on μm Bondapak carbohydrate columns (BAUST and EDWARDS, 1979) or on tetraethylenepentamine modified Radial-Pak B (Silica) columns. All values are expressed on wet weight basis. This species does not annually vary water content (BAUST *et al.*, 1979).

## RESULTS

### *Cryoprotectant accumulation patterns*

Both northern and southern populations accumulate the same array of cryoprotective agents (glycerol, sorbitol, trehalose and fructose). In 1979–1980, northern populations accumulated mean winter highs of 19.1 ± 0.7 μg/mg glycerol, 24.7 ± 1.4 μg/mg sorbitol, 9.9 ± 0.5 μg/mg trehalose and 4.0 ± 1.0 μg/mg fructose (Fig. 1). Southern populations experienced higher mean ambient temperatures (Table 1) and accumulated correspondingly lower levels of protective agents (mean winter highs were 12.2 ± 1.6 μg/mg glycerol, 5.9 ± 1.0 μg/mg sorbitol, 8.9 ± 1.4 μg/mg trehalose and 1 μg/mg fructose) (Fig. 2).

Cryoprotectants appear to accumulate in response to temperature cues. Following the first seasonal frost, the synthesis of each major cryoprotectant was initiated in northern populations. During the 2 days following first frost and sustained chilling, glycerol and sorbitol each increased two-fold. Sorbitol levels varied throughout winter in accordance with cycling

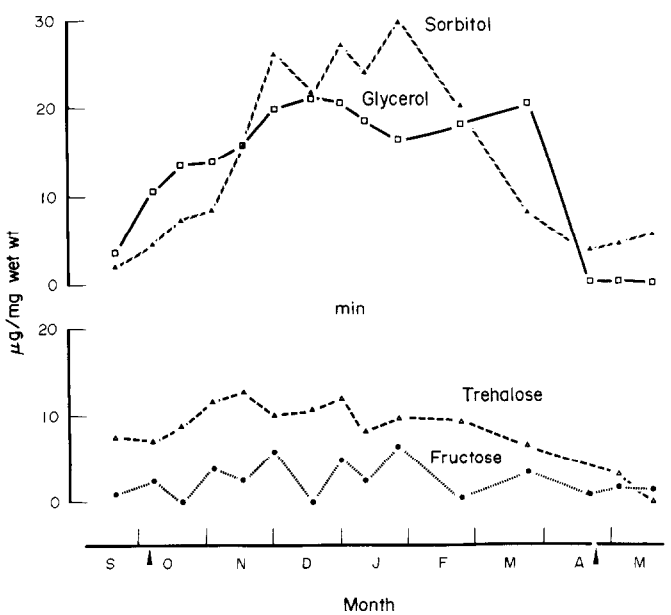


Fig. 1. Seasonal variations in cryoprotectant levels of northern (Minnesota) populations of the gall fly *Eurosta solidaginsis*.

Table 1. Mean monthly temperatures (°C) recorded at collection areas (1979–1980)

| | Month | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Sept. | Oct. | Nov. | Dec. | Jan. | Feb. | Mar. | Apr. |
| Minnesota (Central) | 17.4 | 8.1 | −0.2 | −3.3 | −9.3 | −9.3 | −2.6 | 9.6 |
| Texas (Coastal Plain | 24.7 | 22.4 | 14.7 | 12.1 | 13.9 | 12.6 | 16.6 | 18.8 |

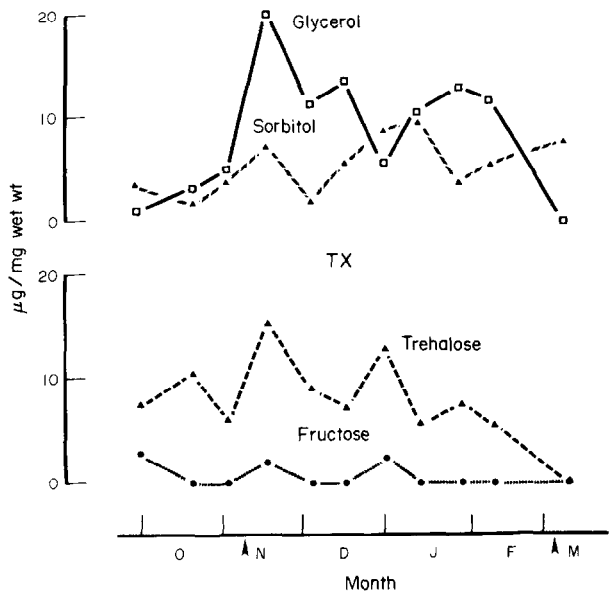


Fig. 2. Seasonal variations in cryoprotectant levels of southern (Texas Gulf Coast) populations of the gall fly *Eurosta solidaginis*.

ambient temperatures. Losses of glycerol and sorbitol do not appear to be correlated with similar seasonal cues. Southern populations show marked rises in glycerol and trehalose but not sorbitol following first frost. Each of the major cryoprotectants cycle throughout the winter but remain above preacclimatization levels in spite of relatively warm ambient temperatures. Glycerol and sorbitol do not vary in phase during winter.

Glycerol accumulation is southern populations is approximately twice that of northern populations following first frost. Glycerol and sorbitol increase four and two-fold, respectively. However, each of the major protective agents does not remain elevated during winter.

Haemolymph melting points vary directly with changes in the principal protective solutes (Fig. 3). The maximum difference in melting point (October–November) is 0.8°C for southern and 1.2°C for northern residents. Melting points are depressed 67% in southern groups while total cryoprotectant levels rose 65%. For northern residents, melting points rose 75% while total cryoprotectants increased 73%.

*Supercooling variations*

Seasonal patterns of supercooling points differ markedly between north and south temperate populations. Following first frost, both populations supercooling points are depressed approx. 4.5°C (Fig. 4). For both populations, this depression is transient but with a greater duration in southern populations. In each case this transient decrease in supercooling points occurs as cryoprotectant levels

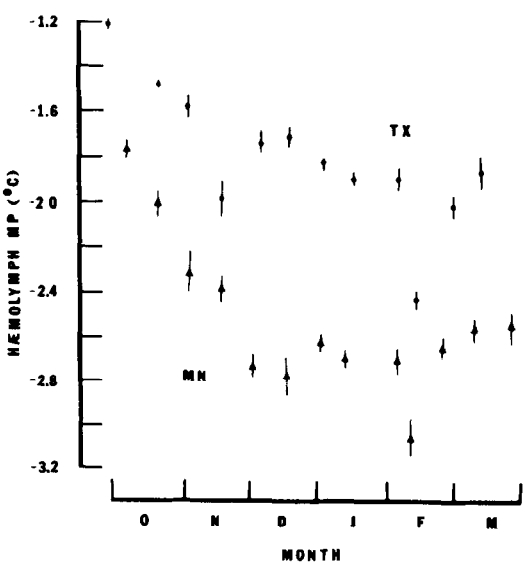


Fig. 3. Seasonal variations in haemolymph melting points of northern and southern populations of *Eurosta solidaginsis* ($\bar{x} \pm$ SEM).

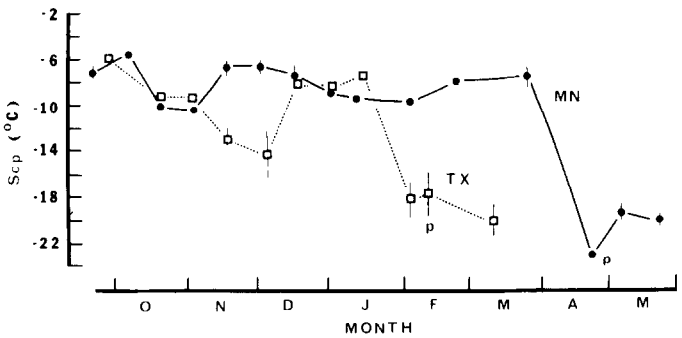


Fig. 4. Seasonal variations in whole body supercooling points of northern and southern populations of *Eurosta solidaginsis* ($\bar{x} \pm$ SEM).

increase. BAUST *et al.* (1979) reported a similar observation for this species. However, northern populations did not demonstrate a transient depression following laboratory acclimation to 0°C.

In the northern population, supercooling points return to pre-frost levels for the duration of the winter and remained constant until diapause termination in spring. In the southern population, supercooling points rise to pre-frost levels after a delay of 30–45 days. This pre-frost level (−8.0°C) is maintained until diapause is broken during spring.

## DISCUSSION

Both Texas and Minnesota populations of *Eurosta solidaginsis* are freezing tolerant but utilize apparently distinct adaptive strategies (Table 2). Southern laboratory (BAUST *et al.*, 1979) and field populations depress supercooling points following first frost, until the approximate species supercooling limit is attained. Simultaneously cryoprotectant content increases precipitously (Fig. 2).

This pattern of reponse, supercooling point depression accompanied by elevations in antifreeze/cryoprotective agents in a freezing tolerant species, is defined as Type IIA winter hardening. Southern populations are subject to rapid and extreme variations in temperature with autumn lows of −5° to −10°C. These chilling exposures are generally infrequent and of short duration. Northern populations are exposed to decidedly different climatic conditions. Temperature limits are lower (to −40°C), the amplitude of the low monthly means is greater and the daily range of temperature is damped compared to southern temperature conditions. The daily range of north temperate winter temperatures

Table 2. Acclimatization characteristics of two populations of *Eurosta solidaginsis*

| Acclimatization characteristics | Population | |
|---|---|---|
| | Southern* | Northern† |
| Adaptive strategy | Freezing avoidance (Type IIA) | Facilitates freezing (Type IIB) |
| Cryoprotectants | | |
| 1. Glycerol, Sorbitol, trehalose | yes | yes |
| 2. Frost 'trigger' | yes | yes |
| 3. Hydroxyl equivalent‡ | | |
| a. pre-frost | 85.5 | 86.7 |
| b. post-frost | 234.5 | 127.9 |
| c. peak | 234.5 | 340.5 |
| d. % change (a–b) | 174 | 48 |
| e. % change (a–c) | 174 | 293 |
| 4. Mid-winter modulation | | |
| a. glycerol | ++++ | + |
| b. sorbitol | +++ | ++ |
| c. trehalose | ++ | + |
| 5. Concomitant melting point depression (haemolymph) | + | + |
| Supercooling | | |
| 1. Change with first frost exposure | yes (transient) | no |
| 2. Mid-winter independence | yes | yes |
| 3. Post-diapause dependent development on last 'probable' freezing exposure | | |

* Texas Coastal plain—16 km north of Galveston.

† Central Minnesota.

‡ Calculated based upon number of 'free' hydroxyl groups per unit concentration of glycerol, sorbitol, fructose and trehalose.

rarely exceeds 20–25°C. Also, while the amplitute of low monthly means is reduced in south temperate regions, it is frequently multi-modal (Table 1).

Therefore, northern and southern populations of *Eurosta solidaginsis* experience different thermal regimes through the winter. One population must endure the rigors of a gradual, long term chilling characterized by low amplitude, daily extremes while the other population endures less extensive chilling but with high amplitude, daily temperature extremes.

Cooling at supra-optimal rates is known to be lethal (MAZUR, 1977). MILLER (1978) has provided the only estimate of optimal cooling rates in a freezing tolerant insect. For *Upis ceramboides*, a cooling rate below 0.3°C min was required to prevent mortality (optimal). Freezing resistance or tolerance must therefore be qualified with respect to rate of cooling. Southern populations survive prolonged extracellular freezing only after very slow cooling. High concentrations of cryoprotectants alone do not appear to afford protection (BAUST and MORRISSEY, 1977). Southern populations would not be expected to survive freezing following rapid cooling, as might occur under ambient conditions. Accordingly, this population supercools to levels that would preclude tissue freezing during autumn.

Winter temperatures in north temperate (inland) regions are characterized by progressive cooling, subzero monthly means and stable daily ambient temperatures. Chilling is gradual and predictable winter optimal limits. Also, the paradoxical elevation of supercooling points to autumn–winter means of $-10.4°$ to $-5.6°$C suggests that this population would remain 'frozen' for many months and thereby avoid the cyclic freeze–thaw stresses that might be experienced if this species' supercooling point was lower.

The elevation of supercooling point in the southern population corresponds with mid-winter and a period of reduced amplitude of temperature fluctuations. There appears to be no inherent survival in this elevation. The basis of these fluctuations in supercooling points is unknown. However, BAUST (1981) reported on the preliminary evidence of LEE, ZACHARIASSEN and BAUST (unpublished) which suggests that the levels of haemolymph ice nucleators vary along with changing supercooling point. During the course of cold acclimation, nucleator behaviour varied in both populations. Nucleator activity (concentration and/or effectiveness) was greater in northern populations.

Cryoprotectant levels varied directly with changes in haemolymph melting points. The three principal cryoprotectants account for the major melting point depression and variations between populations. Other solutes change markedly but do not significantly affect melting point depression. For example, northern residents, accumulate proline ($56.5 \pm \mu$mol/g) following acclimation to below $-5°$C (STOREY *et al.*, 1981). Proline accounts for 65% of the total free amino acid content but would not significantly affect colligative properties.

The trigger to cryoprotectant synthesis appears to be exposure to approx. 0°C. Glycerol concentration increases faster in southern residents during the accumulation phase. Sorbitol levels remain about 25% of those found in northern residents. Increases in sorbitol beyond first frost exposure appear to depend on continued sub-freezing exposure.

For species that accumulate multiple type cryoprotectants, the 'protective potential' can be represented by a combined indicator of concentration (activity). Polyhydric alcohols and low molecular weight saccharides are presumed to act in a number of ways associated with hydrogen bonding potential or hydroxyl equivalent ($E^{OH}$) (BAUST, 1973; BAUST and MORRISSEY, 1977). Prior to first frost, both populations have equivalent $E^{OH}$ levels (Table 2). Following frost exposures, southern populations increase $E^{OH}$ to a seasonal maximum. In this case the protective potential appears to be related to the extension of supercooling ability so that freezing is avoided. This accumulation of protective agents would probably not serve to protect *E. solidaginsis* from the lethal effects of intracellular ice that accompany the supra-optimal cooling rates. Increased cryoprotectant levels are known to shift cooling optima to slower rates (LEIBO *et al.*, 1970; MAZUR, 1977) in mammalian systems. Accordingly, southern populations would be more susceptible to freezing damage in spite of elevated glycerol and sorbitol levels.

Following an equivalent post-frost period, northern populations generate only one-third the $E^{OH}$ of southern populations. With comparatively lower cryoprotectant levels northern populations could endure more rapid cooling rates and therefore have a greater likelihood of surviving limited freezing exposures until peak protective levels were attained. Northern populations do not reach maximum $E^{OH}$ until a later winter period and after prolonged low temperature acclimatization. This group has maximum $E^{OH}$ levels approx. 1.5 times greater than in southern residents. Changes in both cryoprotectans and supercooling points appear to be independent of photoperiod. During laboratory acclimation experiments, supercooling points varied predictably with temperature while specimens were maintained in total darkness (BAUST *et al.*, 1979). During this study, changes in supercooling points between the two populations over the same 2 week period were not in phase (Fig. 4).

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