J. Insect Physiol., Vol. 28, No. 5, pp. 431–436, 1982
Printed in Great Britain.

0022-1910/82/050431-06 $03.00/0

ENVIRONMENTAL TRIGGERS TO CRYOPROTECTANT MODULATION IN SEPARATE POPULATIONS OF THE GALL FLY, *EUROSTA SOLIDAGINIS* (FITCH)

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(*Received* 6 *October* 1981)

Abstract—Northern and southern populations of the gall fly *Eurosta solidaginis* utilize quantitatively distinct adaptive strategies when exposed to a laboratory simulation of winter temperatures. Both populations are freezing tolerant and rely in part on the temperature-dependent accumulation of glycerol and sorbitol, and static but elevated levels of trehalose for protection. The accumulation triggers are time–temperature dependent. For northern and southern populations, exposure to 5°C for periods exceeding 24 hr, but less than 5 days or 5°C with a gradual reduction (1°C/day) results in the accumulation of sorbitol at 1.5 μg/mg/day. Glycerol levels remain essentially constant between 10° and −25°C in each population. However, the concentration of glycerol in the Minnesota population is 3–4 times greater that that of the Texas specimens. Haemolymph melting points varied quantitatively with changing cryoprotectant levels. No significant difference was noted between the supercooling points of each population. This suggests that ice-nucleator levels were comparable throughout the exposure period.

Key Word Index: Insect cold-hardiness, cryoprotectant, supercooling, *Eurosta solidaginis*

INTRODUCTION

THE DIVERSITY and complexity of the winter-hardening strategies utilized by insects is now apparent. BAUST and LEE (1981) have characterized hardening strategies. Type I hardening is characteristic of freezing-intolerant insects that supercool extensively and therefore avoid tissue freezing. Type II hardening characterizes freezing-tolerant stages which either extend super-cooling limits (II A) in a manner similar to Type I but may ultimately freeze (II B). With few exceptions (SOMME 1964; BAUST and MORRISSEY, 1975) most hardened species accumulate one or more types of cryoprotective agents (polyhydric alcohols and low molecular weight saccharides).

The goldenrod gall fly, *Eurosta solidaginis*, (Diptera: Tephritidae) serves as a model in winter-hardening studies. This species overwinters as third-instar larvae and is freezing tolerant. Second-instar larvae can be induced to overwinter but are not freezing tolerant (Type I) (MORRISSEY and BAUST, 1976). During late winter, third-instar larvae transform into freezing-susceptible pre-pupae (Type I) (UHLER, 1951; MORRISSEY and BAUST, 1976). This species ranges between central Canada (54° N) and the Gulf Coast region (27° N), accumulates glycerol, sorbitol and trehalose as major cryoprotective agents following near-freezing exposures (BAUST and LEE, 1981), contains ice-nucleating agents within its haemolymph (SOMME, 1978) and appears to vary nucleator activity during acclimation (Baust, 1981). Partial dehydration is not a component of the freeze-hardening process in either population. However, bulk water levels are reduced by enhanced binding. Dialyzable (polyol-sugar complexed) and non-dialyzable (protein-bound) fractions of soluble subcellular components increase water-binding capacity by 328% during cold acclimation of northern populations (22° to −30° C) (STOREY *et al.*, (1981a). This component of hardening results in a reduction in the level of free water available for ice formation. The enhanced binding phenomenon also yields a low-energy mechanism for concentrating cryoprotectants. Accompanying these changes is a reduction in glycogen levels, a 50% increase in the free amino acid pool due largely to proline, a relatively slight decrease in 'energy charge' (10%) and no alteration in either protein or glycoprotein composition (STOREY *et al.*, 1981b).

The environmental cue that triggers initiation of winter hardening is temperature (BAUST and MILLER, 1970; BAUST, *et al.*, 1979). For *E. solidaginis*, exposure to the first autumn frost results in the accumulation of glycerol and sorbitol and the distinction between types II A and B hardening (BAUST and LEE, 1981). The latter report only provided a coarse estimate of triggering in this species. The purpose of this study was to precisely identify the critical temperatures to cryoprotectant synthesis in separate populations of the gall fly.

MATERIALS AND METHODS

Approximately 20,000 ball galls of goldenrod, *Solidago canadensis*, were collected in mid September from sites in central Minnesota (45° N) and coastal Texas (29° 30′ N). Specimens were maintained in the galls in the dark throughout the experiment. Larvae were maintained for 2 weeks at 10°C and then exposed to a simulated 'winter cycle'. Simulation was accomplished by cooling the galls 1° C/day to −25°C.

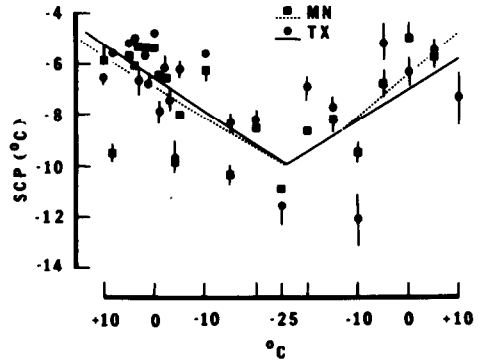

Fig. 1. Whole body supercooling points (SCP) of northern (Minnesota) and southern (Texas Gulf Coast) populations of the gall fly *Eurosta solidaginis* following laboratory acclimation. ($\bar{X} \pm$ S.E.M., $n = 6$).

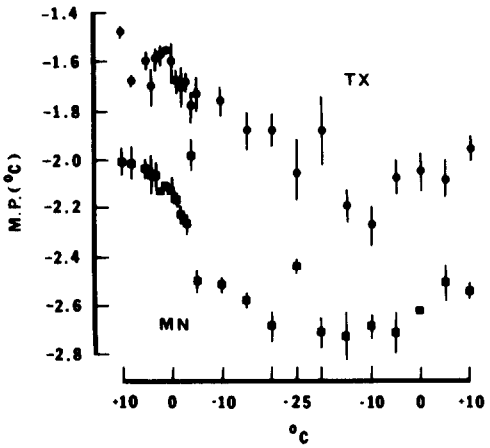

Fig. 2. Haemolymph melting points (M.P.) of northern and southern populations of the gall fly *Eurosta solidaginis* during laboratory acclimation. ($\bar{X} \pm$ S.E.M., $n = 6$).

Warm acclimation was accomplished by reversing the cooling pattern. In addition, specimens were maintained at various intermediate temperature for periods up to 75 days.

Supercooling plints were determined by attaching a 32-gauge copper-constantan thermocouple to the larvae. Each specimen was cooled at a rate of 1–2 C/min. The supercooling point was taken as the lowest temperature reached prior to the release of the latent heat of crystallization. Haemolymph melting points were observed in a modified Scholander apparatus (BAUST and MILLER, 1970). Melting points were determined by drawing 3–6 μl of haemolymph from a single larvae into a capillary tube. Each sample was covered rapidly with mineral oil and flash frozen. The melting point was defined as the point at which the crystal began to diminish in size upon gradual warming. No melting point–freezing hysteresis point greater than 0.2°C was observed. Values are expressed as $\bar{X} \pm$ SEM ($n = 9$–18 for supercooling and $n = 6$ for melting points).

At each collection temperature, a single 450-mg sample consisting of 12–18 third-instar larvae from each population was removed from the galls and freeze clamped in liquid nitrogen for later cryoprotectant determinations. Whole-body cryoprotectant levels were determined using high-performance liquid chromatography. The details of the extraction and analysis are given in BAUST and EDWARDS, (1979), HENDRIX *et al.* (1981) and BAUST and LEE (1981). This species does not vary water content annually (BAUST *et al.*, 1979). Therefore, all cryoprotectant levels are expressed on a wet weight basis. The standard error of analytical replication for each data point is less than the amplitude of each symbol (Figs. 3–8).

RESULTS

Supercooling and haemolymph melting points

The range of supercooling point variation was equivalent between populations. While variability was high during acclimation (Fig. 1), all specimens froze before −10°C and did not thaw until temperatures exceeded −2.8°C (Fig. 2). The changes in haemolymph melting points were related directly to changes in cryoprotectant levels.

Cryoprotectant accumulation patterns and trigger temperatures

The pattern of accumulation for sorbitol was similar between both populations during the hardening

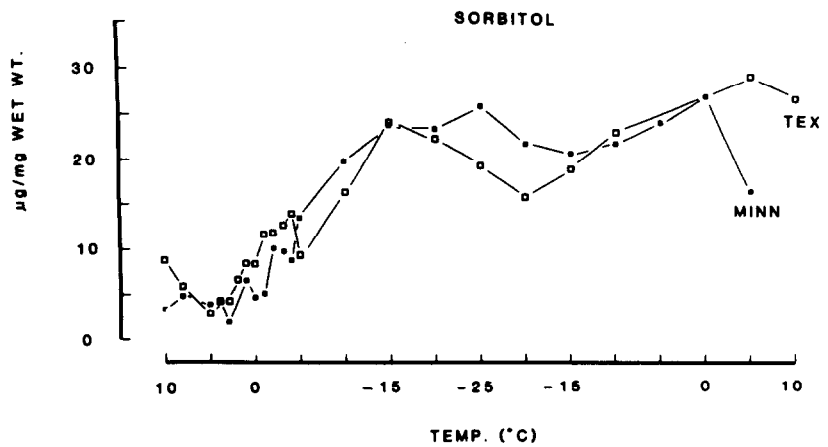

Fig. 3. Sorbitol content of northern and southern populations of *Eurosta solidaginis* following laboratory acclimation. (n = 12–18 larvae).

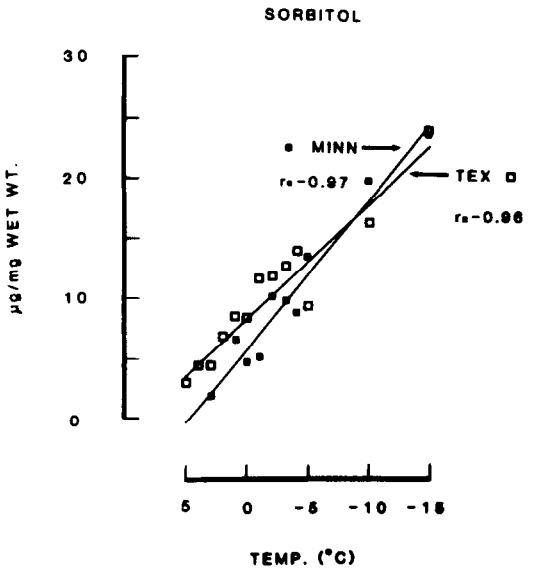

Fig. 4. Temperature trigger for sorbitol synthesis in northern and southern populations of *Eurosta solidaginis*. (n = 12–18 larvae) (r = correlation coefficient).

process (*ca.* +3 to −15 C) (Fig. 3). Sorbitol levels increased from pre-trigger levels of 3–4 to 24 µg/mg at −15 C. The data suggest that the actual trigger temperatures for sorbitol synthesis may differ by 2 –3 C between the two populations (Fig. 4). Most data points for sorbitol levels in the Texas population over the range of +5 to −5 lie above the corresponding Minnesota samples. Similarly, as indicated by the regression lines, the sorbitol levels double between 5 and 3 C for Texas larvae, while in Minnesota specimens, doubling does not occur until the 3 –1 C interval (Fig. 4). An examination of Fig. 5 suggests that one day at +5 C for Texas larvae results in a marked increase in sorbitol concentration for the southern population, while an increase in the corresponding northern sample is not observed until day 5.

Maintenance at temperature below 0 C resulted in continued sorbitol synthesis (Figs. 3 and 5). Since freezing occurred prior to −10 C, the continued accumulations and subsequent variations between −10 and −3 C occurred in 'frozen' larvae.

During the warming portion of the acclimation cycle, sorbitol levels remained high, except for a precipitous drop observed in the Minnesota specimens between 0 and 5 C. A corresponding decrease was not observed in Texas larvae (Fig. 3) over the same range of exposure.

Glycerol levels differed markedly between the two populations. Both populations have equivalent capabilities of synthesis under ambient conditions (Baust and Lee, 1981). However, northern residents produced 3–4 times more glycerol following equivalent acclimation (Fig. 6). Glycerol levels remained relatively constant throughout the acclimation schedule in each population.

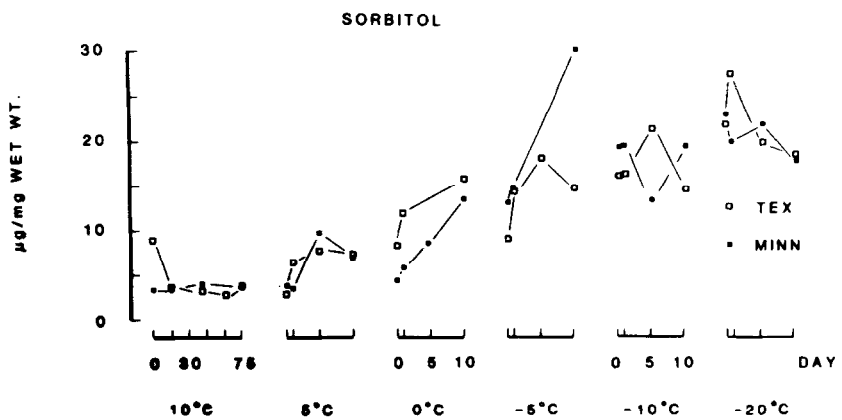

Fig. 5. Sorbitol content of northern and southern populations of *Eurosta solidaginis* following laboratory acclimation for varying exposure times. (n = 12–18 larvae).

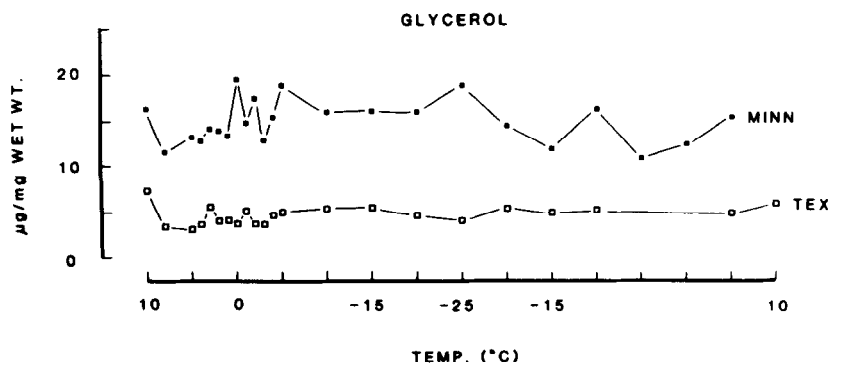

Fig. 6. Glycerol content of northern and southern populations of *Eurosta solidaginis* following laboratory acclimation. (n = 12–18 larvae).

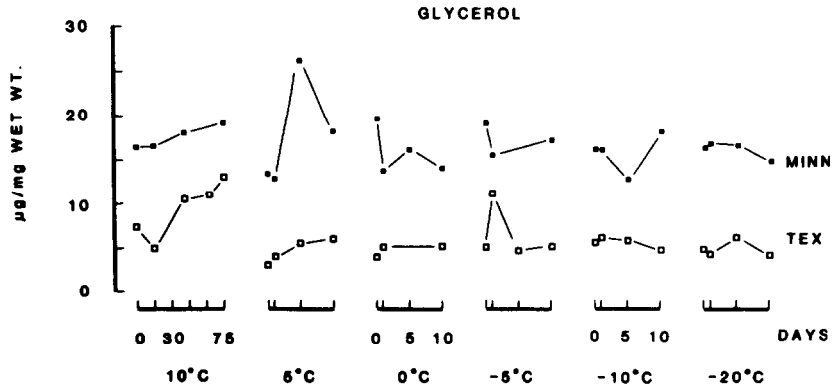

Fig. 7. Glycerol content of northern and southern populations of *Eurosta solidaginis* following laboratory acclimation for varying exposure times. (n = 12–18 larvae).

Maintenance at 10°C for 75 days resulted in increased glycerol levels in each population (Fig. 7). Northern populations increased levels by 18% as compared to 160% for the southern group. The period of 1–5 days at 5°C resulted in a pronounced overshoot, a 63% increase in glycerol levels as compared to 'winter' means. Approximate 'winter' mean levels were established after longer hold periods or at temperatures as low as −20°C (16.3 ± 0.7 µg/mg). Southern populations maintained approximately constant glycerol levels at hold temperatures between 5° and −20°C (5.3 ± 0.4 µg/mg) except for a single overshoot at −5°C. Warm acclimation (−25° to +10°C) was not characterized by large variations in glycerol levels; southern populations maintained constant levels while northern groups decreased levels slightly (Fig. 6).

Fructose and trehalose levels remained effectively constant through the acclimation period (Fig. 8). Each population contained equivalent levels of each carbohydrate (Northern: fructose, 2.9 ± 0.3, trehalose, 14.0 ± 0.5; Southern: fructose, 3.3 ± 0.4, trehalose, 11.9 ± 0.8 µg/mg). Slight diminutions were noted during warm acclimation.

DISCUSSION

BAUST and LEE (1981) have reported on the diversity of strategies utilized by separate populations of *E. solidaginis* during overwintering. Both populations are freezing tolerant as third-instar larvae and accumulate a variety of cryoprotective agents. These accumulations have been correlated with exposure to approx. 0°C.

Due to the different ambient-temperature regimes experienced by each population, it has been difficult to assess accurately thermal triggering events. Northern populations endure gradual, long-term chilling characterized by −40°C extremes, low-amplitude daily fluctuations and prolonged bouts of tissue freezing. Southern populations experience shorter and less-intense low temperatures. Winter ambient temperatures are rarely depressed below −5° to −10°C but daily fluctuations are great (20°–25°C).

Sorbitol is the principle cryoprotective agent in both populations. Sorbitol, trehalose and fructose levels were similar between populations. However, glycerol levels in Minnesota larvae were approx. three times greater than those from Texas. Thermal triggers for sorbitol production in both populations were identified in the +5 to +2°C range. The accumulation of sorbitol has both time and temperature components. Accumulation to peak levels required either progressive cooling to lower temperatures or increased duration at pre-trigger temperatures (Figs. 3 and 5).

Although glycerol was produced in concentrations three-fold greater in northern groups, the comparatively elevated glycerol levels may not have as great a protective value as this difference might imply. The survival benefit of multiple-type cryoprotectant systems can be estimated with the aid of a collective

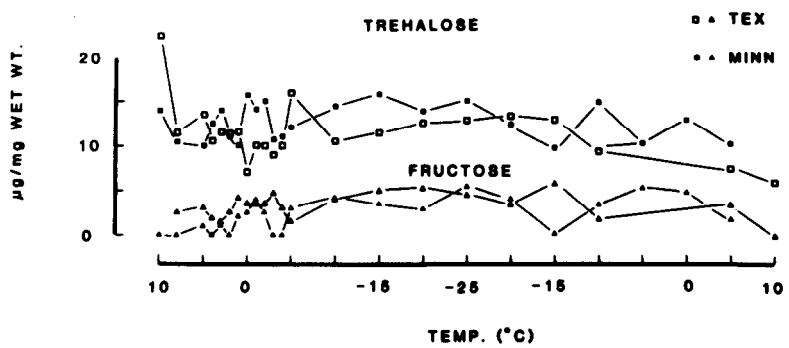

Fig. 8. Trehalose and fructose content of northern and southern populations of *Eurosta solidaginis* following laboratory acclimation. (n = 12–18 larvae).

Table 1. Acclimation characteristics of two populations of *Eurosta solidaginis*

Acclimation characteristics	Population Southern*	Northern†
Cryoprotectants:		
(1) Glycerol, Sorbitol, Trehalose	yes	yes
(2) Trigger (°C)	multiple set 1 = > 10 C	multiple set 1 = > 10 C
(a) Glycerol	set 2 ~ 4 C	set 2 ~ 0 C
(b) Sorbitol	5 (< 1 day)	3 (< 1 day) or 5 (> 1 day)
(c) Trehalose	—	—
(3) Hydroxyl Equivalents‡		
(a) pre-trigger	146.3	131.0
(b) post-trigger increase/day	5.67	10.9
(c) post-trigger, peak (−15 C)	259.3	338.5
(−25 C)	258.9	359.7
(d) $\bar{X}^{o}_{o}$ change (a–c)	77.2	166.5
(e) post-freeze variability (−15 to −25 to −10 C)	258.3 ± 4.1	311.3 ± 4.6
r =	−0.267	−0.434

* Texas Coastal Plain—16 km North of Galveston.

† Central Minnesota.

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

indicator of concentration, hydroxyl equivalent (BAUST and MORRISSEY, 1977; BAUST and LEE, 1981). Pre-trigger hydroxyl equivalent levels were nearly equivalent between populations (Table 1). The post-trigger increases per day were two-fold greater in northern populations (10.9 vs 5.7 μg/mg/day) suggesting that the potential for acclimation is greater in northern groups. Peak hydroxyl equivalent levels were 20% greater at −25°C in this group.

Since all larvae froze before −12°C (Fig. 1), the cryoprotectant levels found at −12°C would be expected to represent a maximum until thawing. However, cryoprotectant levels continued to vary. Sorbitol synthesis continued to −15°C followed by gradual changes until thawing. Between −20°, −25° and −20°C sorbitol levels diminished in southern populations. Between −15° and −5°C, levels again increased. It was not until temperatures exceeded 0°C that warm acclimation was triggered in northern populations and greater than 5°C in southern counterparts (Fig. 3). Despite the individual variations in cryoprotectants, the post-freeze (−15° to −25° to −10°C) hydroxyl equivalent levels were stable (Southern: 258.3 ± 4.1; Northern: 311.3 ± 4.6). The observation that the levels varied by 21% suggests that southern populations were only slightly less freezing tolerant following the acclimation protocol. In view of the extent of ambient differences in temperature, it is probable that southern populations of *E. solidaginis* have sufficient adaptive potential to endure a rigorous winter. In the present study most larvae of both populations survived the entire experimental regime (+10 to −25 to +10).

The supercooling points of whole bodies cycled in a typical seasonal fashion and demonstrated that all larvae were frozen by −12°C (Fig. 1). The continued decrease in larval supercooling points even though frozen, may reflect the continued elevation in hydroxyl equivalent (cryoprotectant synthesis) in the frozen state. It should be recalled that larvae must be thawed momentarily for supercooling point determinations at each acclimation temperature below −10°C. Paradoxically, the supercooling points elevated during the rewarming phase (−25° to −5°C) while hydroxyl equivalent levels were constant. It may be suggested tentatively that the activity or level of ice nucleators is under separate control and varied independently of cryoprotectants (ZACHARIASSEN *et al.*, unpublished observations).

The metabolic compensation that occurred upon exposure to and maintenance at equivalent thermal regimes was in part dissimilar from ambient collections of northern and southern populations (BAUST and LEE, 1981; BAUST, 1981). Both trigger temperatures varied for each cryoprotectant as well as the absolute levels of glycerol. The 21% elevation in hydroxyl levels would not seem to protect larvae from the formation of lethal intracellular ice if cooling proceeded at supraoptimal rates (MILLER, 1978). However, since increased cryoprotectants shift optimal cooling to high rates (LIEBO *et al.*, 1970; MAZUR, 1977), northern populations would be expected to have a survival advantage in an environment characterized by rapid temperature fluctuations. Once freezing occurred in the extracellular space, relative cryoprotectant levels would increase as bulk water was extracted as ice. Cells must therefore function in a concentrated (multimolar) cryoprotectant environment. This relative increase in cryoprotectant levels, and the problems attendant to toxicity may in part be ameliorated by changes in 'bound' water. Northern populations of *E. solidaginis* increased total bound water from summer lows of 20–52% upon acclimation to −30°C (STOREY *et al.*, 1981a). This would result in a 40% decrease in the potential volume of ice at any subfreezing temperature.

Acknowledgements—We thank Dr. WILLIAM D. SCHMID for providing the Minnesota specimens used in this study. This project was supported by grants PMC 78-09589 from the National Science Foundation and the UH Coastal Center to JGB.

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