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WINTER SURVIVAL OF THE GALL FLY LARVA, *EUROSTA SOLIDAGINIS*: PROFILES OF FUEL RESERVES AND CRYOPROTECTANTS IN A NATURAL POPULATION

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Abstract—Larvae of the freeze-tolerant gall fly *Eurosta solidaginis* were sampled from outdoor populations over the course of a winter season and levels of cryoprotectants (glycerol, sorbitol), sugars, fuel reserves (glycogen, glycerides, protein), and lactate were monitored and correlated with the ambient temperature profile. Glycerol production was first stimulated in response to average daily temperatures of 10–15°C with highest rates of synthesis during early October when average temperatures were cooler and minimal daily temperatures did not exceed 8°C. Sorbitol production was initiated in mid-November in response to minimal daily temperatures below 3°C. Winter accumulations of cryoprotectants averaged 360 ± 27 and 119 ± 18 μmol/g wet weight for glycerol and sorbitol, respectively. Loss of cryoprotectants began first with sorbitol whose levels started to decline in February as maximal daily temperatures began to exceed 0°C. Loss of glycerol began in mid-March when average daily temperatures were well above 0°C but significant amounts (125 ± 23 μmol/g) still remained in pupae sampled in mid-April. During early autumn, larvae increased glycogen and protein content by 9- and 2-fold, respectively and increased total glyceride content by 50%. Glycogen was then rapidly depleted and quantitatively converted into polyols as autumn progressed. However lipid and protein reserves remained stable over the winter. Glycogen also appeared to be the fuel reserve supporting basal metabolic rate throughout the winter. During the spring, sorbitol pools were reconverted to glycogen stores but glycerol carbon was not. Lactate levels were fairly constant at 1–2 μmol/g in larvae over the winter suggesting that periods of freezing, necessitating anaerobic carbohydrate fermentation, did not produce a cumulative stress on larvae.

Key Word Index: Winter cold hardiness, freeze tolerance, *Eurosta solidaginis*, cryoprotectants, insect overwintering

INTRODUCTION

Larvae of the golden rod gall fly *Eurosta solidaginis* are found in ball galls on the stems of golden rod plants. The third instar overwinters in the gall and is freeze tolerant. These larvae have been extensively used as a model system for studying freeze tolerance, biochemical adaptations by our laboratory (Storey, 1982, 1983, 1984; Storey and Storey, 1981, 1983a, b, 1985; Storey *et al.*, 1981a, b, c, 1984) and both physiological and biochemical adaptations by Baust and co-workers (Morrissey and Baust, 1976; Baust, 1981, 1983; Baust and Lee, 1981; 1982; Zachariassen *et al.*, 1982; Rojas *et al.*, 1983; Lee and Lewis, 1984). Briefly, 3rd-instar larvae supercool to about −8°C, survive freezing at temperatures at least as low as −25°C and ice contents of up to 65%, utilize haemolymph nucleating agents to induce ice formation, accumulate low molecular weight carbohydrates (glycerol, sorbitol, trehalose) as cryoprotectants, and increase the content of bound (versus free) water in cells to limit cellular dehydration and protein denaturation during freezing. Laboratory acclimations have found distinct triggers for glycerol versus sorbitol production (Baust, 1983; Storey and Storey, 1983a; Rojas *et al.*, 1983) resulting in accumulation of glycerol in an anticipatory manner during cooling while accumulation of sorbitol is in direct response to temperatures near 0°C (Storey and Storey, 1983a). In addition, sorbitol levels can be modulated in response to ambient temperature change but glycerol content, once synthesized, remains constant (Storey and Storey, 1983a).

Cryoprotectant accumulation and loss in outdoor populations over a winter season was first followed by Morrissey and Baust (1976) for animals from New York State. A later study compared the frost hardiness adaptations of Minnesota versus Texas populations including cryoprotectants, supercooling points and haemolymph melting points (Baust and Lee, 1981). The present study provides the first picture of the metabolic make-up of the larvae throughout the course of a winter season. Fuel reserves (glycogen, lipid, protein) were quantitated and evaluated both as precursors for polyol production and as energy sources for basal metabolism, temperature triggering of cryoprotectant synthesis in outdoor populations was examined and the role of anaerobic metabolism in winter survival of the larvae was evaluated.

MATERIALS AND METHODS

Animals and chemicals

Galls containing *E. solidaginis* larvae were collected at intervals from sites around the Carleton

University campus from August 4, 1982 to April 17, 1983. During winter only galls exposed above the snow were collected. Galls were opened immediately upon return to the lab and larvae were killed by freezing in liquid nitrogen and then stored at −80°C until analysis. All biochemicals and coupling enzymes were from Sigma Chemical Co. or Boehringer Mannheim Corp.

Outdoor temperatures

Daily temperatures (minimal, maximal and average) used were those recorded at the Ottawa International Airport by the Atmospheric Environment Service, Environment Canada.

Metabolite analyses

In most instances, 2 larvae (or pupae) were pooled per sample except during the first several collections when the small size of larvae required several to be pooled. Perchloric acid extracts of each sample of larvae were made as previously described (Storey *et al.*, 1981a) and were stored at −80°C until analysis. Glycogen, glucose, fructose, glycerol, sorbitol and lactate were determined enzymatically as previously described (Storey *et al.*, 1981a; Storey and Storey, 1983a). Total protein, pelleted in the perchloric acid extract, was dissolved in 0.2 N sodium hydroxide and then quantitated using the Coomassie blue dye-binding method with prepared reagent from Bio-Rad Laboratories and bovine gamma globulin as the standard. Total glycerides were extracted by homogenization in chloroform–methanol and quantitated as glycerol released after saponification (Storey *et al.*, 1981a).

RESULTS

Changes in the levels of selected metabolites in larvae over the course of the 1982–83 winter are shown in Fig. 1 for the cryoprotectants (glycerol, sorbitol), Fig. 2 for glycogen, glucose and fructose and Fig. 3 for lactate, total protein and total glycerides. Daily maximal and minimal temperatures are also plotted in Fig. 1 and animal weights are given in Fig. 3.

Synthesis of cryoprotectants in the larvae began in mid-September with production of glycerol. Sorbitol synthesis did not begin until mid-November. Conversely, sorbitol was the first cryoprotectant to be lost from the larvae beginning in mid-February while glycerol levels remained stable until mid-March. Glycerol, at levels of 100–150 μmol/g wet weight persisted in prepupae and pupae sampled in April. A number of correlates can be made between outdoor temperatures and cryoprotectant synthesis/degradation.

For glycerol, the first significant rise in content in the larvae was noted on September 1. The 6 days previous to this were the first extended period with cool nights, all having minimal temperatures below 10°C and 2 days below 5°C; average daily temperatures in this time ranged from 10 to 15°C. Glycerol content remained below about 50 μmol/g throughout September but then increased dramatically throughout early October. This correlated with a further drop in daily temperatures, all 10 days between September 29 and October 7 having minimal temperatures below 8°C and average temperatures in the range of 10°C. A decline in glycerol content in late October corresponded with a 6-day period of warmer weather at that time. Glycerol content had reached a plateau level by the January sampling with an average content of about 360 ± 27 (standard deviation) μmol/g over the winter. The first decrease in glycerol content was noted on March 23 correlated with a general warming and the first extended period of time with an average daily temperature above 0°C (5 of the previous 7 days) and maximal temperatures reaching as high as 10°C. Content stabilized at about 250 μmol/g when temperatures plunged again in late March and then continued to decline throughout April.

For sorbitol, the first significant rise in content was noted on November 11. The 6 days previous to this all had minimal temperatures below 3°C (2 days below 0°C) with average temperatures less than 5°C. Temperatures fell quite rapidly over the following weeks and sorbitol reached plateau levels during mid-winter at an average 119 ± 18 μmol/g. The first decrease in sorbitol content was noted on February 21 corresponding to a distinct warming period over the previous week; 6 out of 7 days had maximal temperatures above 0°C and on 2 days the average temperature was also above 0°C with minimal temperatures of about −5 to −8°C. Sorbitol content then declined rapidly over the next weeks reaching a low on March 16 correlated with average daily temperatures between 0 and 5°C. A renewed synthesis was then noted over the next 2 weeks corresponding to a sharp drop in environmental temperature with daily maxima not exceeding 0°C. Following this sorbitol content again rapidly declined as daily temperatures climbed above 0°C. No sorbitol was found in larvae or pupae on or after April 7.

Glycogen, the storage form of carbohydrate, accumulated rapidly in the larvae over the month of September reaching peak levels of 428 μmol/g wet weight [expressed as glucose units] (Fig. 2). Thereafter stored carbohydrate content declined in inverse proportion to the accumulation of glycerol and sorbitol. Total glycerol (360 μmol/g or 180 μmol/g in glucose units), sorbitol (119 μmol/g), glucose (12 μmol/g) and fructose (14 μmol/g) content accumulated by larvae was 325 μmol/g wet weight, an amount adequately accounted for by the loss of glycogen. By late winter, glycogen in the larvae was almost completely depleted (an average of 6 μmol/g remaining on February 14). Over the following weeks however, content increased by an average 113 μmol/g, an amount equal to the decrease in sorbitol content (an average loss of 117 μmol/g). Glycogen was lost again during the period of cold temperatures in March which stimulated a second synthesis of sorbitol. Content then rebounded as temperatures warmed and sorbitol was again lost. Final glycogen content in spring animals averaged 80 ± 15.4 μmol/g with no apparent differences between larvae and pupae in glycogen content. However, despite the fact that glycerol synthesis was obviously fueled from glycogen, there was no indi-

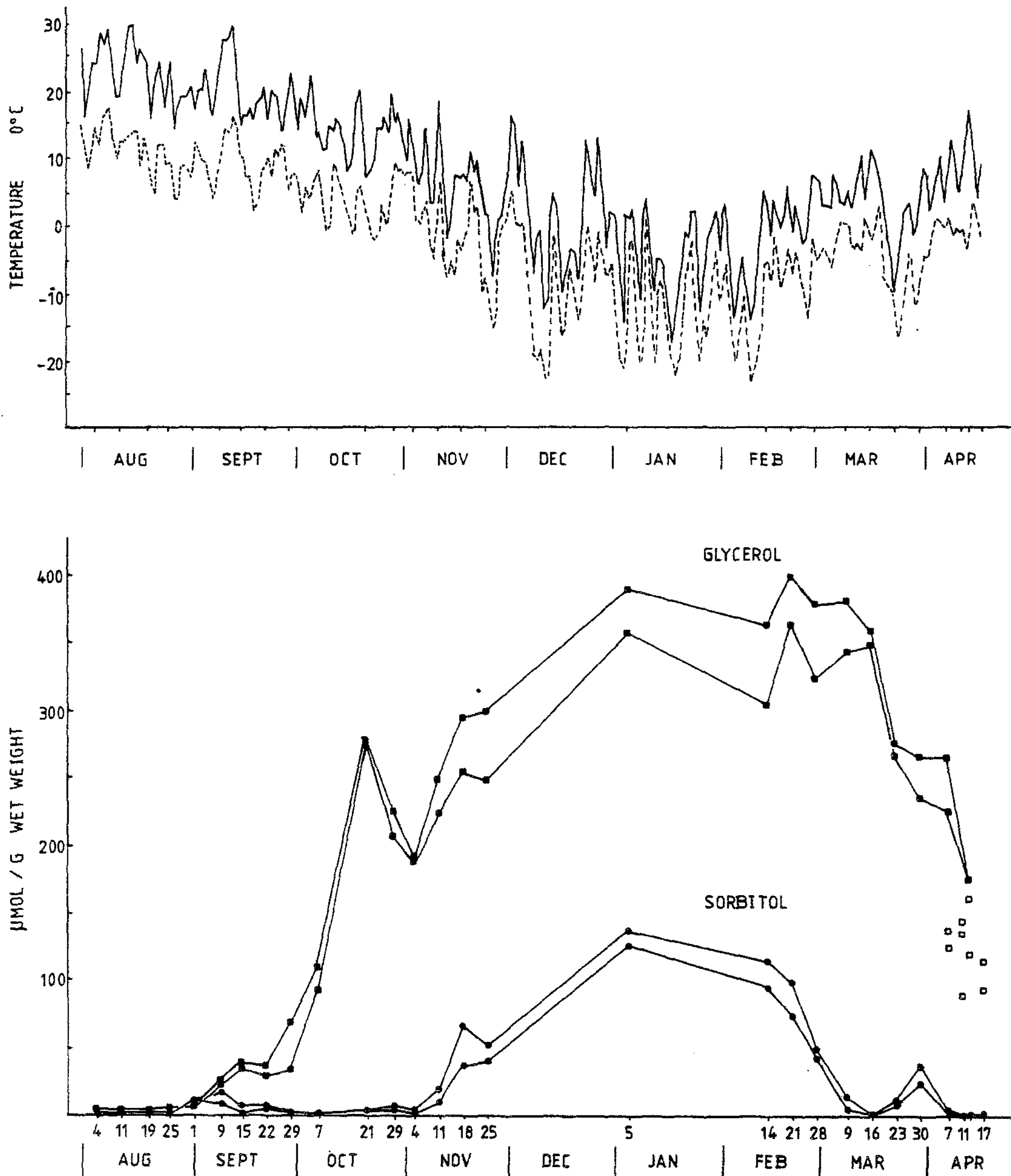

Fig. 1. A. Daily maximal (——) and minimal (---) temperatures for Ottawa over the autumn, winter and spring 1982–83. B. Concentrations of cryoprotectants in larvae of the gall fly, *Eurosta solidaginis*, over the autumn, winter and spring 1982–83. Larvae were sampled from outdoor populations at approximately weekly intervals over the autumn and spring. During the spring pupation, larvae (closed symbols) and prepupae or pupae (open symbols) were distinguished. Samples of both prepupae and pupae were separately analyzed but are not distinguished here as data for the 2 forms were found to be completely overlapping for all compounds analyzed (Figs 1–3); i.e. no trends were noted with the larva–prepupa–pupa transition. Data for the last sampling point, however, are for pupae only. Except for the first 3 sampling points the data shown are values from 2 samples, 2 or more animals pooled per sample. Experiments terminated in mid-April due to the birth of Jennifer E. Storey.

cation that glycerol carbon was reconverted to glycogen during the spring.

Glucose and fructose contents of the larvae are also shown in Fig. 2. Glucose is the direct precursor for sorbitol synthesis (Storey and Storey, 1983a). Glucose content had reached maximal levels on the first dates (November 11, March 23) that elevated sorbitol was found and remained elevated as long as sorbitol content remained high. Levels then dropped back to control amounts in early February ahead of the slower decline in sorbitol content. During the winter months fructose content of the larvae directly

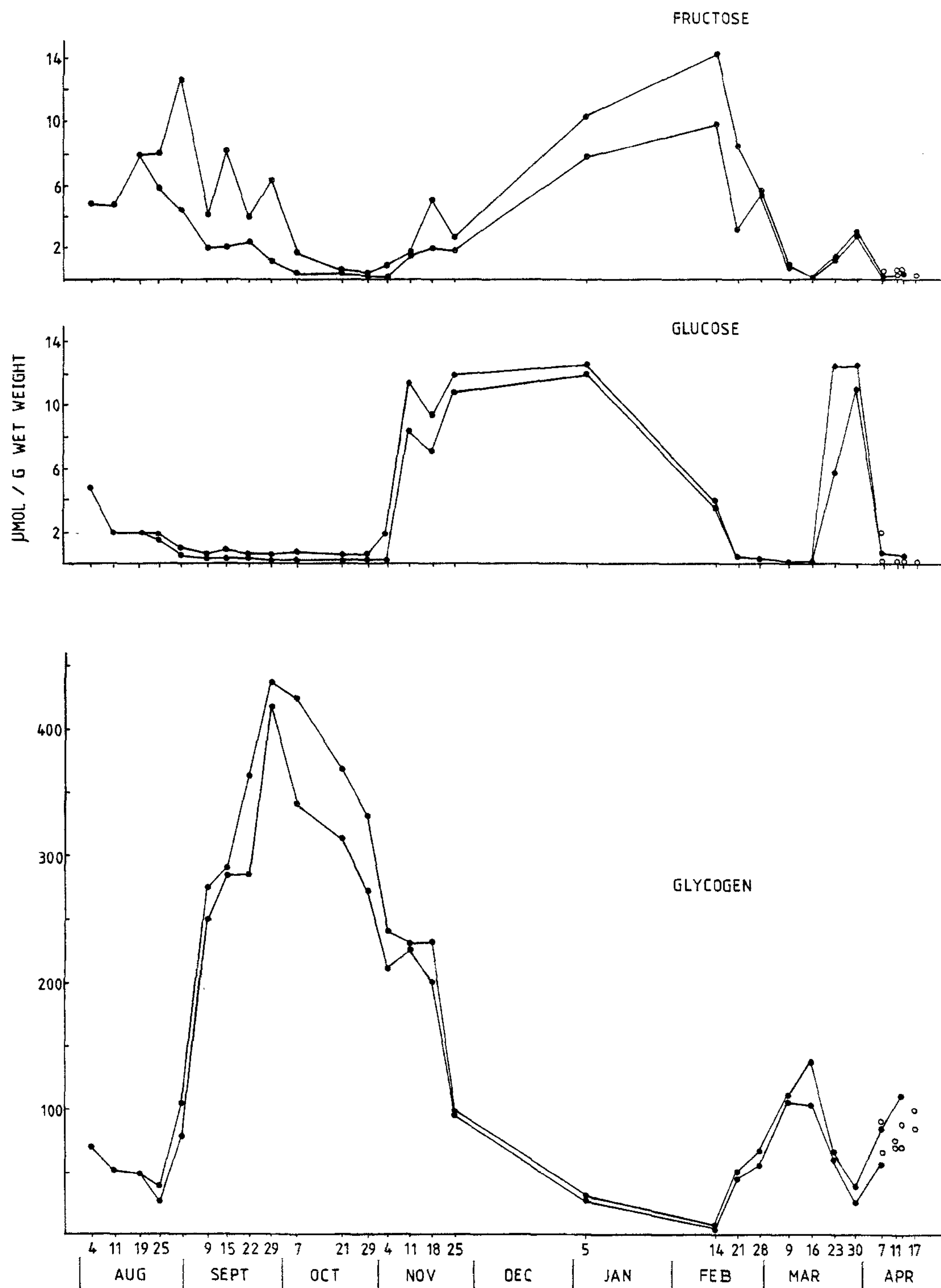

Fig. 2. Contents of glycogen, glucose and fructose in *E. solidaginis* larvae over the 1982–83 winter season. Glycogen is expressed in glucose units. Other details are as in Fig. 1.

paralleled sorbitol content during both synthesis and degradation of the polyol. As previously suggested (Storey and Storey, 1983a) this probably reflects the obligatory equilibrium between sorbitol and fructose created by the presence of sorbitol dehydrogenase in the larvae. Earlier in the autumn fructose content of the larvae was also elevated corresponding to the time of rapid glycogen accumulation. Although speculation, this might suggest that the larvae were ingesting fructose as a carbon source from the goldenrod plant.

Figure 3 shows lactate content in the larvae over

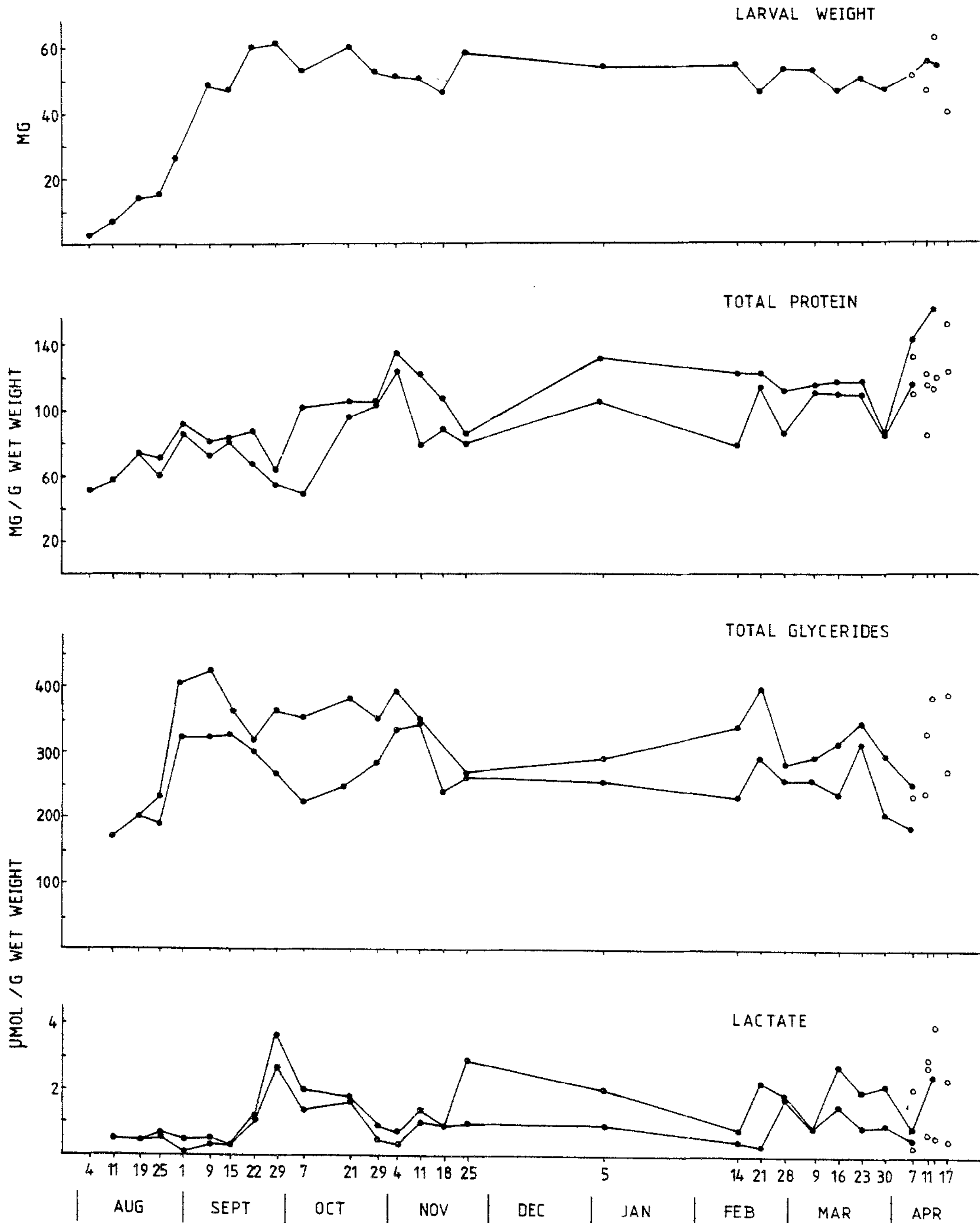

Fig. 3. Animal weights and contents of lactate, total glycerides and total protein in *E. solidaginis* larvae over the 1982–83 season. Total glycerides are expressed in glycerol units after saponification of lipids. Weights are an average of 6–13 animals. Other details are as in Fig. 1.

the course of the winter season. Lactate is the product of anaerobic glycolysis and is known to accumulate in the larvae in the frozen state (Storey and Storey, 1984). Although elevated compared to the early autumn average of 0.44 ± 0.11 μmol/g wet weight, lactate content throughout the winter and spring remained relatively constant between 1 and 2 μmol/g. Assuming a whole animal supercooling point of about -8°C and a melting point of -3°C there were only two 9-day periods (and several shorter periods) during the winter when daily temperatures were low enough to have kept larvae constantly in a frozen state. Since these were interspersed with warmer periods it is probable that animals were able to return to an aerobic state from time to time and avoid excessive lactate accumulation and anaerobic stress.

Total glycerides (mono-, di- and tri-glycerides) averaged about 200 ± 25 μmol/g wet weight (expressed as glycerol released) in the larvae during August but increased during early September. Over

the winter lipids remained constant at an average 312 ± 55 μmol/g. No correlations between lipid levels and the synthesis or degradation of polyols were seen suggesting that lipid reserves are not involved with the cryoprotectant pools. No significant change in content was seen when animals pupated.

Total protein content of the larvae is also shown in Fig. 3. Larvae at the earliest sampling date averaged 52 mg/g wet weight and this rose throughout the autumn to an average level of 102 ± 31 mg/g which was maintained over the winter months and into pupation. No difference in protein content was found between larvae and pupae.

Average weights of animals at each sampling time are also shown in Fig. 3. The animals increased in size dramatically throughout August and September with maximal weights achieved coincident with maximal contents of glycogen, lipid and protein. The average weight of larvae in autumn/winter (Sept 22–Nov 25) was 54.8 ± 5.4 mg and in winter/spring (Feb 14–Apr 17) was 50.7 ± 3.5 mg. Although not statistically significant, this suggests a slight weight loss over the winter.

DISCUSSION

Two studies by Baust and co-workers have examined cryoprotectants in natural populations of *E. solidaginis* larvae over winter seasons. The original study of a New York population showed an induction of glycerol accumulation in mid-September, rapid accumulation throughout November and stable levels of the polyol through until March with no glycerol remaining in pupae sampled in mid-April (Morrissey and Baust, 1976). Sorbitol was accumulated rapidly from mid to late November, was stable until mid-February and then declined until none remained in larvae in mid-March. A second study compared Minnesota and Texas populations of the larvae and provided some correlation with mean monthly temperatures (Baust and Lee, 1981). Minnesota populations appeared to trigger synthesis of both cryoprotectants after the first frost in early October although as in New York populations glycerol content rose much faster, reached a plateau earlier and was retained longer into the spring. Texas populations also accumulated both cryoprotectants, despite mean monthly temperatures which never fell below 10°C, but levels of both polyols were highly variable over the winter.

The present study reveals a pattern of cryoprotectant accumulation or loss very similar to that of the New York population (Morrissey and Baust, 1976) but adds detailed information on ambient temperatures to allow correlations to be made between outdoor temperatures and polyol synthesis. More importantly, the status of fuel reserves has, for the first time, been profiled over a winter. Compared with the Morrissey and Baust (1976) study a few differences in the pattern and timing of cryoprotectant accumulation or loss were noted; most of these however are probably the result of ambient temperatures which are overall somewhat cooler in the Ottawa area. Thus the Ottawa population showed its highest rates of glycerol accumulation throughout October while this was delayed until November in New York. Morrissey and Baust (1976) also found no glycerol in pupae, suggesting that cryoprotectants were lost at the larva–pupa transition. However prepupae and pupae sampled in the present study still had glycerol at levels of 100–150 μmol/g; this data suggests that loss of glycerol is keyed to the ambient temperature profile rather than to life stage. Morrissey and Baust (1976) found that pupae were not freeze tolerant and had very low (−23°C) supercooling points (compared to about −10°C for mid winter larvae); apparently then supercooling ability and freeze-tolerance capacity may not be dependent on or related to cryoprotectant content of the animals.

The present study suggests that temperature cues facilitate polyol synthesis in the natural environment. Although the enzyme machinery necessary for glycerol synthesis may be put in place by a developmental, photoperiod, desiccation or other trigger (Baust, 1983; Storey, 1984), glycerol accumulation is facilitated by the first prolonged autumn period with cool nights (6 days of minimal temperatures below 10°C) and moderate average temperatures (10–15°C). This agrees well with previous laboratory triggering studies; an abrupt temperature switch from 23 to 13°C stimulated glycerol production with a lag time of about 2 days (Storey and Storey, 1983a). Highest rates of glycerol synthesis occurred when overall temperatures were somewhat lower with daily average temperatures hovering around 10°C and minima below 8°C, maxima averaging about 15°C. In the laboratory glycerol synthesis is terminated when larvae are held at temperatures below 5°C (Storey *et al.*, 1981a); in the natural environment, then, the continued slower accumulation of glycerol throughout December may occur only on warmer days with the major polyol production at this time being sorbitol whose synthesis is favoured by temperatures in the 0°C range. Triggering of sorbitol synthesis occurred after 6 days of average temperatures of about 5°C and minimal temperatures below 3°C. Although this was not the first instance of low minimal temperatures, it was the first extended period of such a low average daily temperature. These findings again agree well with laboratory studies. When temperature is lowered 1°C per day, sorbitol production is triggered at 5°C (Storey *et al.*, 1981a) while an abrupt temperature switch from 13°C (where animals have no sorbitol) to 3°C stimulates sorbitol production with a lag time of about 24 h (Storey and Storey, 1983a). Baust and Lee (1982) and Rojas *et al.* (1983) have also elegantly pinpointed the trigger for sorbitol synthesis as chilling below 5°C. Laboratory triggering of sorbitol synthesis was preceded by elevated levels of glucose-6-P and glucose after 1 and 2 h, respectively, of the temperature switch to 3°C (Storey and Storey, 1983a). Similarly, the present results show that natural populations also precede sorbitol synthesis with a rapid rise in glucose, the substrate for polyol dehydrogenase. Sorbitol levels in the natural population were modulated with temperature change during late winter. Loss of the polyol began as average daily temperatures rose above 0°C (with maximal temperatures about 5°C) until all of the polyol was gone in early March. However, a renewed

cold spell in late March which plunged temperatures below 0°C produced a second synthesis of sorbitol; here we can also note that synthesis was accomplished on days in which not even the maximal temperature rose above 0°C. However we have previously established that sorbitol production can continue in the larvae at subzero temperatures down to the point at which extracellular freezing occurs (Storey *et al.*, 1981a).

Reserves of glycogen, lipid and protein were accumulated by the larvae from August through early October. Glyceride content rose by about 50% while protein content doubled and glycogen content increased almost 9-fold. This period of time was also one of rapid growth for the larvae which increased in weight from about 3 mg in early August to 55 mg in late September. Peak weight and peak accumulation of fuel reserves correlated with the natural cessation of feeding in the larvae. Throughout the summer larvae remain in the central cavity of the gall. Then just before entering the winter diapause, they eat out an exit tunnel to the surface of the gall (leaving only a thin skin) and then retire to the interior to pass the winter (Uhler, 1951). In the present study exit tunnels were first found on October 7 suggesting that this date was the approximate time at which food intake had ceased. Protein and lipid reserves remained virtually constant over the winter season suggesting that these are reserved for the pupal transformation and for life as an adult (including egg production in females). Glycogen reserves decreased dramatically over the winter however. Glycogen is apparently the sole source of carbon for the synthesis of cryoprotectants, the loss of glycogen mirroring both qualitatively and quantitatively the accumulation of polyols. Total glycogen loss (422 μmol/g) was somewhat greater than the total accumulation of polyols and sugars (326 μmol/g). This coupled with the observation that glycogen content continued to decrease over January/February despite constant polyol levels, suggests that carbohydrate is also utilized as the fuel for basal metabolism during the winter. This must be so at least in the frozen state where the animals rely on anaerobic glycolysis for energy production with lactate accumulating (Storey and Storey, 1985).

The two cryoprotectants, sorbitol and glycerol, have different fates during the spring loss of polyols from the larvae. Sorbitol is reconverted to glycogen. This appears to be stimulated solely by temperature change and is reversible; the same result occurs during rewarming of laboratory-acclimated animals (Storey and Storey, 1983; Rojas *et al.*, 1983). The metabolic basis for the ease with which these two carbon pools are interconverted may be the relatively simple pathways connecting them and a glycogen synthetase which is probably activated by high temperature just as glycogen phosphorylase is activated by low temperature (Ziegler *et al.*, 1979). The spring decline in glycerol content, however, does not result in a resynthesis of glycogen. Although the fate of glycerol carbon is not obvious from the present study two possibilities exist: (1) Glycerol is used as the carbohydrate substrate to support basal aerobic metabolism during the spring months, and/or (2) after partial oxidation to the level of acetyl-CoA, glycerol carbon may be shunted into lipid reserves (since synthesis of 1 mol of triolein requires 28 mol glycerol conversion of the total glycerol reserves of the larvae into triglycerides would raise the lipid pool of the larvae by less than 5%). Since sorbitol can be readily reconverted to glycogen, the metabolic block preventing gluconeogenesis from glycerol must be at the level of the enzyme fructose-1,6-diphosphatase. Previous reasons proposed for the maintenance of dual cryoprotectants in *E. solidaginis* included: (1) The constant nature of the glycerol pool versus the ability to modulate total polyols in response to winter temperatures using the sorbitol pool and (2) the anticipatory nature of glycerol accumulation versus the directly low temperature stimulated production of sorbitol (Storey and Storey, 1983). The differential behaviour of the glycerol and sorbitol pools during spring rewarming of the larvae may provide an additional reason for the maintenance of the dual cryoprotectant system. Thus only the winter sorbitol pool can provide the glycogen reserve for pupal and adult life suggesting that the larvae may have built-in mechanisms to maintain a set amount of carbon as glycogen/sorbitol while carbon, once committed to the glycerol pool, must take on a different fate in pupal and adult life.

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