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Enzyme activity profiles in an overwintering population of freeze-tolerant larvae of the gall fly, *Eurosta solidaginis*

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Abstract. The activity of some enzymes of intermediary metabolism, including enzymes of glycolysis, the hexose monophosphate shunt, and polyol cryoprotectant synthesis, were measured in freeze-tolerant *Eurosta solidaginis* larvae over a winter season and upon entry into pupation. Flexible metabolic rearrangement was observed concurrently with acclimatization and development. Profiles of enzyme activities related to the metabolism of the cryoprotectant glycerol indicated that fall biosynthesis may occur from two possible pathways: 1. glyceraldehyde-phosphate → glyceraldehyde → glycerol, using glyceraldehyde phosphatase and NADPH-linked polyol dehydrogenase, or 2. dihydroxyacetone-phosphate → glycerol-3-phosphate → glycerol, using glycerol-3-phosphate dehydrogenase and glycerol-3-phosphatase. Clearance of glycerol in the spring appeared to occur by a novel route through the action of polyol dehydrogenase and glyceraldehyde kinase. Profiles of enzyme activities associated with sorbitol metabolism suggested that this polyol cryoprotectant was synthesized from glucose-6-phosphate through the action of glucose-6-phosphatase and NADPH-linked polyol dehydrogenase. Removal of sorbitol in the spring appeared to occur through the action of sorbitol dehydrogenase and hexokinase. Glycogen phosphorylase activation ensured the required flow of carbon into the synthesis of both glycerol and sorbitol. Little change was seen in the activity of glycolytic or hexose monophosphate shunt enzymes over the winter. Increased activity of the α-glycerophosphate shuttle in the spring, indicated by greatly increased glycerol-3-phosphate dehydrogenase activity, may be key to removal and oxidation of reducing equivalents generated from polyol cryoprotectant catabolism.

Key words: Insect freeze tolerance – Enzymes – Metabolism – Cryoprotectant synthesis – *Eurosta solidaginis*

Introduction

Freeze tolerance is used by numerous types of animals to survive the winter cold, and is quite common in the insect world (Duman et al. 1991; Storey and Storey 1992). Freeze-tolerant larvae of the gall fly *Eurosta solidaginis* (Fitch) have been used as a model insect to study many aspects of the biochemical adaptations required for successful freezing survival (Baust and Nishino 1991; Storey and Storey 1992). The third-instar larvae of this dipteran overwinter in ball-shaped stem galls on goldenrod plants, where they must endure temperatures well below the freezing point of their body fluids throughout several winter months. Ice nucleation limits supercooling to about −7 to −10 °C in this species, and glycerol and sorbitol are accumulated as colligative cryoprotectants. These polyols provide protection against a freeze-induced concentration of cellular contents produced as the result of osmotic stresses created by extracellular freezing. Survival of the larvae is limited to a maximum of 65% of extracellular water as ice (Storey and Storey 1992).

Synthesis of polyol cryoprotectants by *E. solidaginis* shows a marked temperature dependence (Baust 1983; Storey and Storey 1983; Rojas et al. 1983). Glycerol accumulates to levels reaching 0.5–0.6 mol·l^{-1} (Morrissey

Abbreviations: 6PGDH, 6-Phosphogluconate dehydrogenase; DHAP, dihydroxy acetone phosphate; F6P, fructose-6-phosphate; F6Pase, fructose-6-phospha-tase; FBPase, fructose-bisphosphatase; G3P, glycerol-3-phosphate; G3Pase, glycerol-3-phosphate phosphatase; G3PDH, glycerol-3-phosphate dehydrogenase; G6P, glucose-6-phosphate; G6Pase, glucose-6-phosphatase; G6PDH, glucose-6-phosphate dehydrogenase; GAK, glyceraldehyde kinase; GAP, glyceraldehyde-3-phosphate; GAPase, glyceraldehyde-3-phosphatase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GDH, glycerol dehydrogenase; GPase, glycogen phosphorylase; HMS, hexose monophosphate shunt; LDH, lactate dehydrogenase; NADP-IDH, $NADP^+$-dependent isocitrate dehydrogenase; PDHald, polyol dehydrogenase, glyceraldehyde activity; PDHgluc, polyol dehydrogenase, glucose activity; PFK, phosphofructokinase; PGI, phosphoglucoisomerase; PGK, phosphoglycerate kinase; PGM, phosphoglucomutase; PK, pyruvate kinase; PMSF, phenylmethylsulfonylfluoride; SoDH, sorbitol dehydrogenase; V_{max}, maximal enzyme activity; ww, wet weight

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and Baust 1976; Storey et al. 1981), its synthesis initiated around 15 °C, slowing below 10 °C and essentially stopping below 5 °C (Storey et al. 1981). Below 5 °C, sorbitol synthesis begins as the result of a temperature-dependent metabolic switch (Storey et al. 1981; Baust and Lee 1982; Rojas et al. 1983; Storey and Storey 1983). Sorbitol can accumulate to 0.2 mol·l^{-1} in haemolymph (Morrissey and Baust 1976). The control of and differential temperature effects on GPase and PFK reactions have been linked to the distinct profiles of biosynthesis of the two polyols, and the hexose monophosphate shunt is believed to be essential as the source of NADPH for the enzymes of cryoprotectant synthesis (Storey 1982; Storey and Storey 1988). Glycogen is known to be the major precursor for polyol synthesis in *E. solidaginis* (Storey et al. 1981), as in other insects (Wyatt 1967).

Although much is known about various aspects of metabolism and specific enzyme characteristics of *E. solidaginis* larvae, an overall picture of how metabolic make-up changes or reorganizes over the winter months is missing. In this study we investigated seasonal and developmental changes in metabolic organization by assessing changes in enzyme complements including glycolysis, the hexose monophosphate shunt, cryoprotectant synthesis and other key enzymes of intermediary metabolism. This was achieved by measuring the maximal (V_{max}) activities of enzymes, which relates directly to the potential throughput at these metabolic loci (Newsholme and Crabtree 1986). The results were correlated with previous data in order to establish the relative contribution of the various metabolic pathways to overall metabolism during overwintering and early pupation in *E. solidaginis*.

Materials and methods

Chemicals and animals. All biochemicals and coupling enzymes were purchased from Sigma Chemical Co., St. Louis, Miss., or Boehringer Mannheim, Montréal, Québec.

Galls containing larvae of *E. solidaginis* were collected from goldenrod plants in fields around Ottawa during the fall of 1989 and kept outdoors in cloth sacs. At each sampling date galls were brought indoors and placed in incubators set to the outdoor temperature for that day. Table 1 shows mean outdoor temperatures for the month (30–31 days) and week (7 days) preceding the sampling dates as an indicator of larval acclimatization. Galls were quickly opened and larvae were removed and killed by dropping into a container of liquid N_2. Larvae were then transferred to −80 °C for storage before use.

Sample preparation for enzyme assays. Whole larvae were homogenized 1:5 (w/v) in ice-cold 20 mmol·l^{-1} imidazole pH 7.2, 5 mmol·l^{-1} EGTA, 5 mmol·l^{-1} EDTA, 10 mmol·l^{-1} 2-mercaptoethanol, and 50 mmol·l^{-1} NaF with a few crystals (~0.1 mmol·l^{-1}) of PMSF using either a glass/glass homogenizer for volumes smaller than 1.5 ml or a Tekmar Ultra-Turrax homogenizer for larger volumes. Buffer additions (EDTA, EGTA, NaF) prevented any changes in enzyme activities in vitro as a result of protein kinase or protein phosphatase action. Homogenates were then centrifuged at 20000 *g* for 20 min, and the supernatant was removed and desalted by centrifugation through a Sephadex G-25 column (1 × 5 cm) pre-equilibrated in homogenization buffer. Samples for GPase activity determinations were homogenized as above but were not subject to centrifugation or a spun column. Instead, settled homogenate was used as the source of enzyme. One unit of enzyme activity is defined as the amount of enzyme that will convert 1 µmol of substrate per min at 25 °C.

Coupled enzyme assays. All enzyme activities were measured at 25 °C using a Gilford 240 recording spectrophotometer. Controls for non-specific activity were run for all assays and any blank value subtracted to yield final activity values. Optimal conditions for maximal activities were determined to be:

Glycogen phosphorylase (GPase; EC 2.4.1.1) (total *a*+*b*): 50 mmol·l^{-1} potassium phosphate buffer (pH 7.0), 4 mg·ml^{-1} glycogen, 5 µmol·l^{-1} glucose-1,6-P_2, 0.2 mmol·l^{-1} $NADP^+$, 2 mmol·l^{-1} AMP, 15 mmol·l^{-1} $MgSO_4$, and excess phosphoglucomutase and $NADP^+$-dependent G6PDH. The active form of the enzyme (*a*) was measured in the absence of AMP.

Hexokinase (EC 2.7.1.1): 20 mmol·l^{-1} imidazole-HCl buffer (pH 7.2), 2 mmol·l^{-1} glucose, 2 mmol·l^{-1} ATP, 0.5 mmol·l^{-1} $NADP^+$, 10 mmol·l^{-1} $MgSO_4$, and excess $NADP^+$-dependent G6PDH.

Glucokinase (EC 2.7.1.2): same conditions as for hexokinase except for 100 mmol·l^{-1} glucose. Hexokinase activity was subtracted as the blank.

Phosphofructokinase (PFK; EC 2.7.1.11): 20 mmol·l^{-1} imidazole-HCl buffer (pH 7.2), 10 mmol·l^{-1} fructose-6-phosphate, 4 mmol·l^{-1} ATP, 0.15 mmol·l^{-1} NADH, 50 mmol·l^{-1} KCl, 5 mmol·l^{-1} $MgSO_4$, and excess aldolase, triosephosphate isomerase, and G3PDH.

Glycerol-3-phosphate dehydrogenase (G3PDH; EC 1.1.1.8): 20 mmol·l^{-1} imidazole-HCl buffer (pH 7.2), 1 mmol·l^{-1} DHAP, and 0.15 mmol·l^{-1} NADH.

Pyruvate kinase (PK; EC 2.7.1.40): 20 mmol·l^{-1} imidazole-HCl buffer (pH 7.2), 5 mmol·l^{-1} phosphoenolpyruvate, 2 mmol·l^{-1} ADP, 0.15 mmol·l^{-1} NADH, 50 mmol·l^{-1} KCl, 10 mmol·l^{-1} $MgSO_4$, and excess LDH.

Lactate dehydrogenase (LDH; EC 1.1.1.27): 20 mmol·l^{-1} imidazole-HCl buffer (pH 7.2), 2 mmol·l^{-1} pyruvate, 0.15 mmol·l^{-1} NADH.

$NADP^+$-dependent isocitrate dehydrogenase (NADP-IDH; EC 1.1.1.42): 20 mmol·l^{-1} TRIS-HCl (pH 8.0), 5 mmol·l^{-1} D,L-isocitrate, 0.2 mmol·l^{-1} $NADP^+$, and 5 mmol·l^{-1} $MgSO_4$.

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH; EC 1.2.1.12): 20 mmol·l^{-1} imidazole-HCl (pH 7.2) 20 mmol·l^{-1} 3-phosphoglycerate, 1 mmol·l^{-1} ATP, 0.15 mmol·l^{-1} NADH, 5 mmol·l^{-1} $MgSO_4$, and excess phosphoglycerate kinase.

Phosphoglycerate kinase (PGK; EC 2.7.2.3): 20 mmol·l^{-1} imidazole-HCl (pH 7.2), 10 mmol·l^{-1} 3-phosphoglycerate, 1 mmol·l^{-1} ATP, 0.15 mmol·l^{-1} NADH, 5 mmol·l^{-1} $MgSO_4$, and excess GADPH.

Aldolase (EC 4.1.2.13): 20 mmol·l^{-1} imidazole-HCl (pH 7.2), 0.1 mmol·l^{-1} fructose-1,6-P_2, 0.15 mmol·l^{-1} NADH, and excess triosephosphate isomerase and G3PDH.

Glucose-6-phosphate dehydrogenase (G6PDH; EC 1.1.1.49): 20 mmol·l^{-1} imidazole-HCl (pH 7.2), 1 mmol·l^{-1} G6P, 0.2 mmol·l^{-1} $NADP^+$, and 5 mmol·l^{-1} $MgSO_4$.

6-Phosphogluconate dehydrogenase (6PGDH; EC 1.1.1.44): 20 mmol·l^{-1} imidazole-HCl (pH 7.2), 1 mmol·l^{-1} 6-phospho-D-gluconate, and 0.2 mmol·l^{-1} $NADP^+$.

Phosphoglucomutase (PGM; EC 2.7.5.1): 20 mmol·l^{-1} imidazole-HCl (pH 7.2), 1.5 mmol·l^{-1} glucose-1-phosphate, 0.02 mmol·l^{-1} glucose-1,6-P_2, 0.2 mmol·l^{-1} $MgSO_4$, 0.2 mmol·l^{-1} $NADP^+$, and excess G6PDH.

Phosphoglucoisomerase (PGI; EC 5.3.1.9): 20 mmol·l^{-1} imidazole-HCl (pH 7.2), 2 mmol·l^{-1} fructose-6-phosphate, 0.2 mmol·l^{-1} $NADP^+$, 5 mmol·l^{-1} $MgSO_4$, and excess G6PDH.

Fructose-1,6-bisphosphatase (FBPase; EC 3.1.3.11): 20 mmol·l^{-1} imidazole-HCl (pH 7.2), 0.05 mmol·l^{-1} fructose-1,6-P_2, 5 mmol·l^{-1} $MgSO_4$, 0.2 mmol·l^{-1} $NADP^+$, and excess PGI and G6PDH.

Glyceraldehyde kinase (GAK; EC 2.7.1.28, triokinase): 20 mmol·l^{-1} imidazole-HCl (pH 7.2), 10 mmol·l^{-1} D-glyceraldehyde, 1 mmol·l^{-1} ATP, 1 mmol·l^{-1} PEP, 0.2 mmol·l^{-1} NADH, 5 mmol·l^{-1} $MgSO_4$, and excess PK and LDH.

Sorbitol dehydrogenase (SoDH; EC 1.1.1.14): 20 mmol·l^{-1} imidazole-HCl (pH 7.2), 500 mmol·l^{-1} D-fructose, and 0.15 mmol·l^{-1} NADH.

Polyol dehydrogenase, glucose activity (PDHgluc): 20 mmol·l^{-1} imidazole-HCl (pH 7.2), 250 mmol·l^{-1} D-glucose, and 0.1 mmol·l^{-1} NADPH.

Polyol dehydrogenase, glyceraldehyde activity (PDHald): 20 mmol·l^{-1} imidazole-HCl (pH 7.2), 4 mmol·l^{-1} D-glyceraldehyde, and 0.1 mmol·l^{-1} NADPH.

Phosphatase assays. Larvae were homogenized 1:5 (w/v) in imidazole-HCl buffer (pH 7.2) with 0.1 mmol·l^{-1} PMSF in a glass/glass homogenizer. Non-specific background was decreased by desalting homogenates by passage through Sephadex G-25 columns (1 × 5 cm) equilibrated in homogenization buffer.

Solutions of homogenate and appropriate reagents were incubated for 30 min at 25 °C. The enzyme reactions were then stopped by the addition of 2 volumes of acid molybdate reagent (10:5:65 mix of 5% Lubrol wax: 2% ammonium molybdate in 1.8 mol·l^{-1} H_2SO_4: distilled water) (Atkinson et al. 1973). Precipitated protein was removed by a 1-min spin in an Eppendorf centrifuge at full speed. Blanks contained all components of the assay mixture, but were stopped at time zero. Standard curves of phosphate solutions (0–0.8 mmol·l^{-1}) containing the reaction substrate were run simultaneously. Colour development was monitored as absorbance at 390 nm on a Pye-Unicam SP8-100 spectrophotometer. Assay conditions used were:

Glucose-6-phosphatase (G6Pase; EC 3.1.3.9): 20 mmol·l^{-1} imidazole-HCl (pH 7.2), 5 mmol·l^{-1} G6P, and 10 mmol·l^{-1} $MgSO_4$.

Fructose-6-phosphatase (F6Pase): 20 mmol·l^{-1} imidazole-HCl (pH 7.2), 5 mmol·l^{-1} F6P, and 10 mmol·l^{-1} $MgSO_4$.

Glycerol-3-phosphatase (G3Pase): 20 mmol·l^{-1} imidazole-HCl (pH 7.2), 10 mmol·l^{-1} D,L-G3P, and 10 mmol·l^{-1} $MgSO_4$.

Glyceraldehyde-3-phosphatase (GAPase): 20 mmol·l^{-1} imidazole-HCl (pH 7.2), 5.6 mmol·l^{-1} GAP, and 10 mmol·l^{-1} $MgSO_4$.

Metabolite assays. Individual pre-weighed larvae were extracted in 500 µl 6% perchloric acid containing 1 mmol·l^{-1} EDTA (Storey et al. 1981); an aliquot of the homogenate was retained for glycogen determination (Keppler and Decker 1974). Homogenates were centrifuged at 18000 *g* at 4 °C and then the supernatant was removed and neutralized with 3 mol·l^{-1} KOH/0.4 mol·l^{-1} TRIS/0.3 mol·l^{-1} KCl and recentrifuged. Neutralized supernatants were frozen at −70 °C until use. Metabolites were quantified by coupled enzyme assay using a Pye-Unicam SP8-100 spectrophotometer and the methods of: glycerol (Eggstein and Kuhlman 1974), sorbitol (Bergmeyer et al. 1974), glucose and fructose (Lowry and Passonneau 1972).

Statistical analysis. All enzyme data are reported as mean ± SEM for $n = 4$ samples with 1 or 2 larvae per sample. Metabolite levels are mean ± SEM for $n = 4$–6 samples, 1 larva per sample. Data were analyzed with a one-way analysis of variance. When a significant F ratio was found a Student-Neuman-Keuls test was performed to examine the difference between means; $P < 0.05$ was considered statistically significant.

Results

Figure 1 shows cryoprotectant and associated metabolite levels in *E. solidaginis* over the 1989–1990 winter season. Data are for third-instar larvae except for the final (28 April) sampling point when larvae had pupated. Glycerol and sorbitol accumulated during the fall to reach maximal levels during the mid-winter months (190–230 µmol·g ww^{-1} for glycerol, 140 µmol·g ww^{-1} for sorbitol). Glycerol levels remained high and constant throughout the winter into April, at which point they began to drop. Levels decreased to 55.5 ± 5.1 µmol·g ww^{-1} by 12 April and to 41.3 ± 8.5 µmol·g ww^{-1} by 21 April. Sorbitol levels decreased by mid-March to 9.5 ± 0.9 µmol·g ww^{-1},

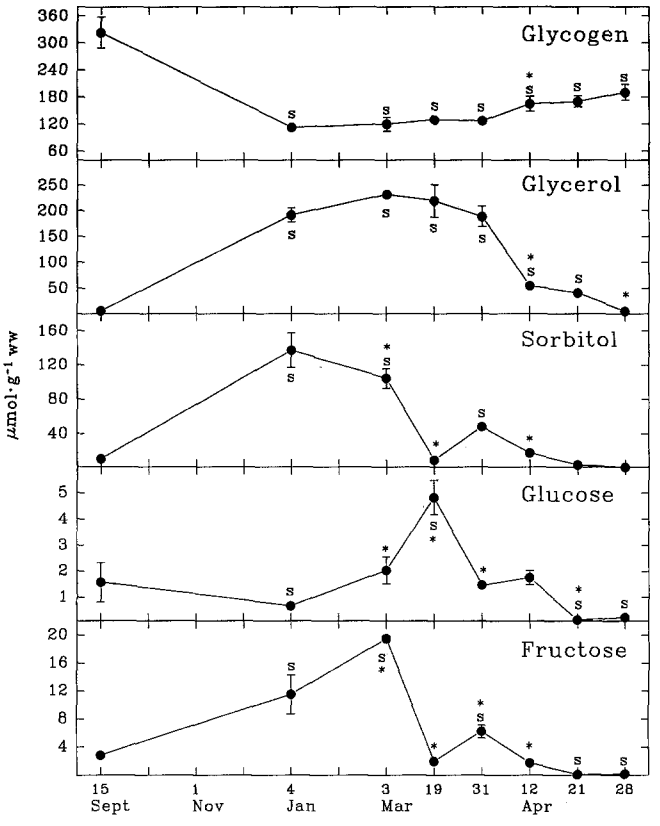

Fig. 1. Metabolite levels in *E. solidaginis* over the 1989–90 winter season. Animals were larvae throughout except for the final sampling date (28 April) when all had pupated. Metabolites were measured enzymatically as described in Materials and methods. Glycogen values are expressed as µmol glucose after enzymatic hydrolysis. Results are mean ± SEM for $n = 4$–6 samples (1 larva per sample) at each point. The date is presented as day/month. *s*, significantly different from Sept 15 values. *, significantly different from previous sampling date

Table 1. Mean Ottawa monthly (30–31 days) and weekly (7 days) temperatures immediately preceding *Eurosta solidaginis* collection dates.

Collection date	Preceding month (°C ± SD)		Preceding week (°C ± SD)	
	max	min	max	min
Sept 15	23.5 ± 3.1	13.1 ± 3.9	22.7 ± 4.6	14.7 ± 4.1
Nov 1	14.4 ± 6.2	4.1 ± 3.4	21.1 ± 0.6	6.0 ± 3.1
Jan 4	−10.8 ± 6.6	−20.0 ± 6.3	−6.0 ± 9.2	−17.4 ± 9.3
Mar 3	−1.9 ± 6.2	−11.6 ± 6.5	−4.4 ± 6.7	−14.3 ± 7.1
Mar 19	−0.1 ± 7.4	−9.7 ± 8.5	7.4 ± 2.4	1.4 ± 1.2
Mar 31	2.2 ± 6.1	−6.4 ± 7.1	0.3 ± 3.1	−8.0 ± 2.2
Apr 12	4.8 ± 3.7	−1.9 ± 4.0	5.2 ± 2.3	−1.9 ± 2.3
Apr 21	5.3 ± 4.3	−2.3 ± 4.2	9.5 ± 3.3	0.6 ± 3.6
Apr 28	9.9 ± 8.2	0.7 ± 5.3	22.9 ± 6.0	7.6 ± 4.1

Numbers were calculated from weather summaries obtained by the Land Resource Research Center (Agriculture Canada)

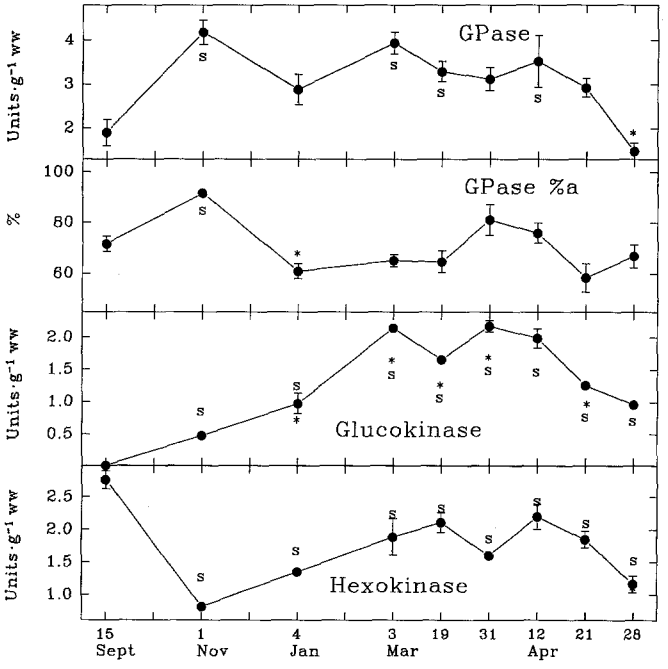

Fig. 2. Activities of enzymes responsible for substrate entry into glycolysis in *E. solidaginis* over the 1989–90 winter season. Animals were larvae throughout except for the final sampling date (28 April) when all had pupated. Activities are U·g ww^{-1}, mean ± SEM for $n = 4$–6 samples (1 or 2 larvae per sample). The date is presented as day/month. *s*, significantly different from Sept 15 values. *, significantly different from previous sampling date. For glycogen phosphorylase (GPase) both total (*upper panel*) and active (%*a*) are shown

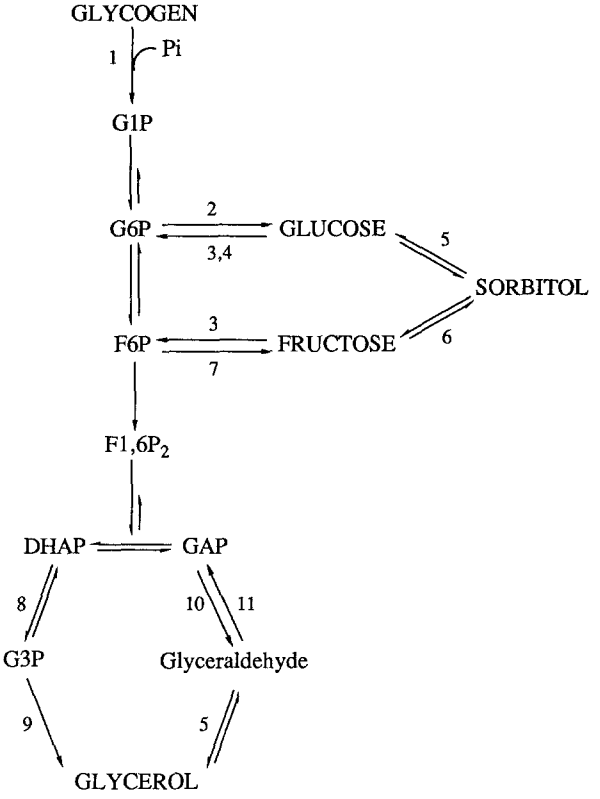

Fig. 3. Proposed pathways for glycerol and sorbitol metabolism in overwintering *E. solidaginis* larvae. Enzymes are: *1*, GPase; *2*, G6Pase; *3*, hexokinase; *4*, glucokinase; *5*, polyol dehydrogenase; *6*, SoDH; *7*, F6Pase; *8*, G3PDH; *9*, G3Pase; *10*, GAPase; *11*, GAK

but rebounded transiently in late March to 48.4 ± 4.5 μmol·g ww^{-1}. The temperatures corresponding to these periods show that the preceding 7-day average minimum and maximum were substantially higher in mid- than late-March (Table 1). Sorbitol levels resumed their decline in April upon return of warmer temperatures such that by late April pupae contained little of the polyol. Changes in fructose levels paralleled those of sorbitol, whereas glucose levels followed a pattern opposite to that of sorbitol. Pupae of *E. solidaginis* (28 April) contained little glycerol (5.9 ± 1.7 μmol·g ww^{-1}) or sorbitol (0.57 ± 0.16 μmol·g ww^{-1}).

Synthesis of polyol cryoprotectants occurs via the mobilization of glycogen reserves in *E. solidaginis* (Storey et al. 1981) and Fig. 1 confirms this. Glycogen decreased by ~210 μmol·g ww^{-1} as glucose equivalents over the course of cryoprotectant synthesis in the fall. The use of glycogen is dependent on the activity of glycogen phosphorylase. Figure 2 shows, as expected, that total glycogen phosphorylase activity increased significantly from September to November. Activity subsequently remained high through to 21 April but had decreased by half when larvae pupated. The percentage of glycogen phosphorylase in the active *a* form was also highest (>90%) during

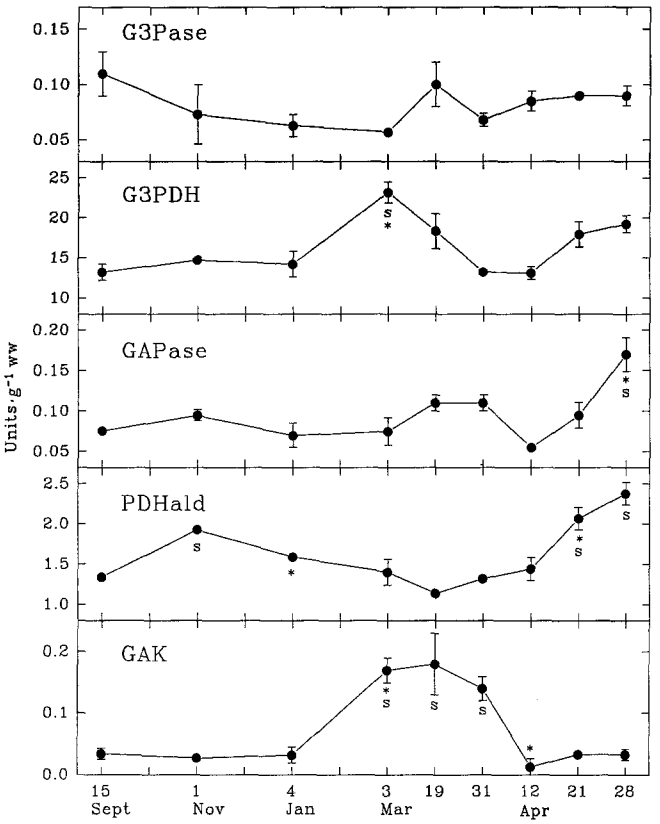

Fig. 4. Activities of enzymes of glycerol metabolism in overwintering *E. solidaginis* larvae. Details as in Fig. 2

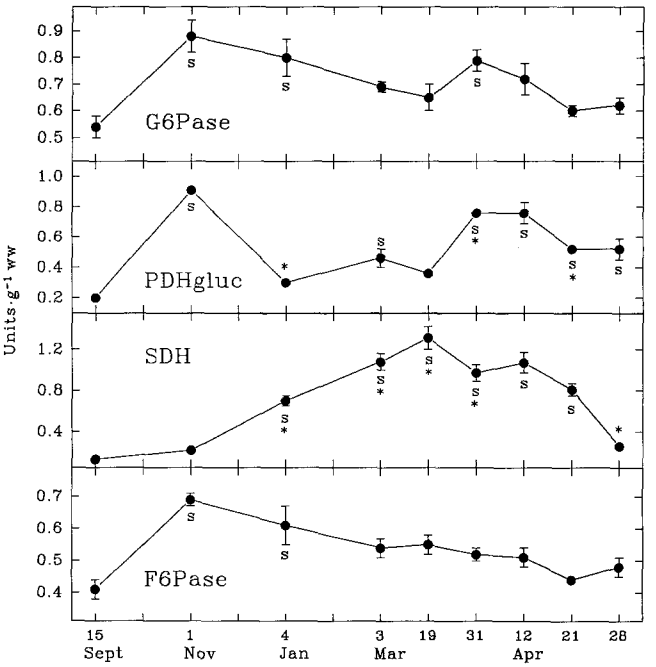

Fig. 5. Activities of enzymes of sorbitol metabolism in overwintering *E. solidaginis* larvae. Details as in Fig. 2

the period of autumn cryoprotectant synthesis (November) but remained at >60% *a* over the remainder of the time-course.

Two possible pathways for glycerol biosynthesis have been proposed, leading from either DHAP or GAP at the triosephosphate isomerase branch point of glycolysis (Fig. 3; Storey and Storey 1981). From DHAP, the enzymes involved are G3PDH and G3Pase, whereas from GAP the route is via GAPase and PDHald. Figure 4 shows the status of these enzyme activities over the winter months. In overwintering *E. solidaginis*, G3Pase activity did not change significantly over the study period (Fig. 4). However, G3PDH activity increased from January to a peak in March (a twofold increase), and then declined to reach initial levels again by 31 March. An increasing trend was seen for G3PDH activity from this date into the pupal stage. The activity of GAPase, required for glycerol synthesis from GAP, remained low and constant throughout the larval stage but increased significantly upon puparium formation (Fig. 4). The activity of PDHald increased during the fall, and subsequently decreased to September values by 19 March. The activity then increased significantly into the pupal stage. Figure 4 also shows the activity of GAK, an enzyme required for the reentry of glycerol into central metabolism. GAK activity increased sevenfold in March, preceding the decrease in glycerol levels, and subsequently declined to September levels by 4 April and remained low into puparium formation. The alternative route of glycerol catabolism requires glycerol kinase. However, we failed to detect activity of this enzyme at any time over the fall, winter, or spring. Similarly, GDH, another enzyme that might participate in glycerol metabolism, was not detected at any time.

Sorbitol synthesis has been proposed to occur via G6P, involving the dephosphorylation of the sugar phosphate and then reduction of glucose via PDH (Fig. 3; Storey and Storey 1981). Changes in the activities of G6Pase and PDHgluc (Fig. 5), needed for the synthesis of sorbitol from G6P, paralleled the profile of sorbitol synthesis almost exactly. Activities of both enzymes increased significantly in the fall, by 4.5-fold for PDHgluc and 2-fold for G6Pase, during the period of active sorbitol synthesis. G6Pase activity decreased slightly during the winter, whereas PDHgluc activity decreased significantly from November into spring. Both activities rose again in late March, corresponding to the transient increase in sorbitol synthesis in response to an early spring cold spell (Fig. 1, Table 1). The activities of F6Pase and SoDH (Fig. 5), representing sorbitol synthesis from F6P, did not follow the pattern of sorbitol accumulation in *E. solidaginis*. Although F6Pase activity was highest during sorbitol synthesis in the fall, changes in SoDH activity paralleled the course of sorbitol removal upon spring warming. SoDH activity remained low and constant into November, after which it began to increase to peak by 19 March, a tenfold increase. The activity of SoDH decreased during late March when a cold spell induced a transient sorbitol synthesis, but then remained high until larvae entered the pupal stage. F6Pase activity largely paralleled G6Pase activity, possibly suggesting that both activities originated from a single broadly specific hexose-phosphate phosphatase.

Hexokinase (Fig. 2), which is required for the uptake of hexose sugars into glycolysis, is a key enzyme needed

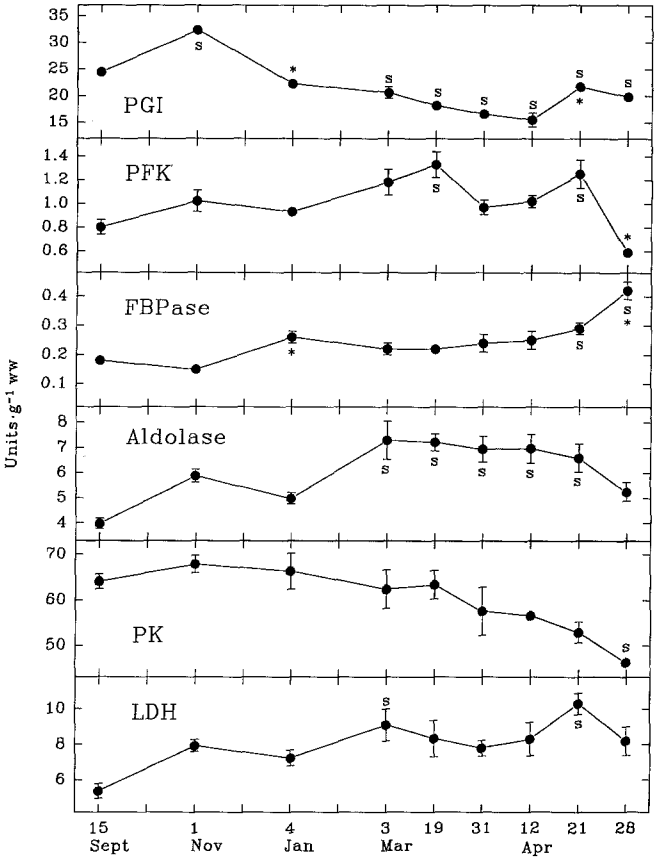

Fig. 6. Glycolytic enzyme activities in overwintering *E. solidaginis* larvae. Details as in Fig. 2

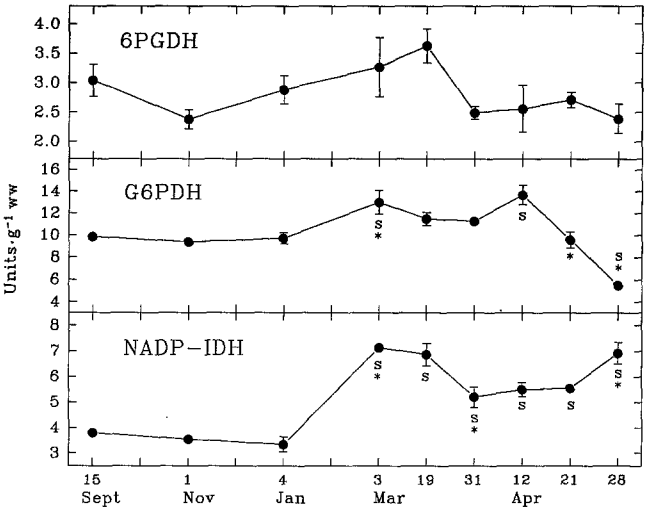

Fig. 7. Activities of enzymes of the hexose monophosphate shunt and NADP-IDH in overwintering *E. solidaginis* larvae. Details as in Fig. 2

for the return of sorbitol equivalents into intermediary metabolism. The activity of hexokinase decreased approximately fivefold from September to November during maximal sorbitol synthesis; however, hexokinase activity then increased steadily from November into the spring. The activity of glucokinase, a high-K_m glucose-specific kinase, was also lowest in the fall and increased steadily from September to 3 March, with a slight decline in activity during the mid-March cold spell-induced sorbitol accumulation. Taken together, the activities of these two kinases were maximal during sorbitol removal in the spring, and minimal during sorbitol synthesis in the fall. Entry into the pupal stage was preceded by significant decreases in the activities of both enzymes.

Figure 6 shows the activities of several enzymes of glycolysis over the winter months. The flow of carbon from glycogen into cryoprotectant synthesis is dependent upon key enzymes of glycolysis. The maximal activity of the two irreversible enzymes which regulate the metabolism of hexose phosphates, PFK and FBPase, were relatively constant during the fall (Fig. 6). FBPase activity was lowest during maximal cryoprotectant synthesis (November), fivefold lower than that of PFK, reflecting the glycolytic (versus gluconeogenic) metabolic state of the larvae. FBPase activity increased slightly in January, and subsequently remained constant until 21 April when the activity increased upon entry into the pupal stage. PFK activity peaked in early March but then decreased twofold upon entry into the pupal stage, such that the ratio of PFK to FBPase activities approached 0.8 in pupae compared to a ratio of 0.25 in September. Aldolase activity increased in March and remained elevated until entry into the pupal stage at which point activity decreased to values equivalent to those seen in the fall. PGI activity was elevated in November, as seen for other enzymes related to sorbitol synthesis, but decreased to reach a minimum by April. PK activity was constant throughout the winter but decreased in the spring to reach a minimal value upon entry into the pupal stage (28 April). LDH activity remained constant from September through January but had increased by March. Except for a transient increase on 19 March (30.0 ± 1.3 U·g ww^{-1}), PGK activity was not significantly different from 15 September activity (21.1 ± 1.0 U·g ww^{-1}) throughout the winter (data not shown). GAPDH and PGM activities did not change over the time-course from 15 September values, 47.3 ± 2.1 and 25.0 ± 0.4 U·g ww^{-1}, respectively (data not shown).

The activity of the two initial enzymes of the hexose monophosphate shunt, G6PDH and 6PGDH, remained relatively constant during the overwintering period (Fig. 7). Cryoprotectant synthesis must rely on the activity of the HMS to provide NADPH for polyol dehydrogenase activity. The activity of 6PGDH was three- to fourfold less than that of G6PDH during overwintering, the difference in activities narrowing to twofold upon entry into pupation due to a decrease in G6PDH activity. This suggests a regulatory function for 6PGDH in the flux of carbon through the HMS. NADP-IDH activity, key to providing NADPH for fat synthesis and potentially also for cryoprotectant synthesis, nearly doubled from 4 January to 3 March, and remained high even after a slight decrease by 31 March. A slight increase in NADP-IDH activity was also seen upon puparium formation.

Discussion

Overall, the activities of many enzymes in *Eurosta solidaginis* larvae changed substantially over the course of the winter season. Many of the changes observed correlated with the synthesis and/or degradation of polyol cryoprotectants. Others appeared to be developmentally related since significant changes in the activities of eight enzymes occurred with the larval to pupal transition in late April. Clearly, metabolic reorganizations take place throughout the winter in the larvae with several different patterns seen. In the fall, activities of many enzymes associated with cryoprotectant synthesis increased significantly. During the winter diapause, corresponding to November to January activities in this study, relatively few changes were seen; however, some enzymatic activities were changing during this time, reflecting the dynamic nature of metabolism even in dormant larvae at low ambient temperatures. Between January and March, numerous changes in enzyme activities appeared to be preparatory for the spring warming and resumption of development. Thus, the activities of enzymes associated with polyol catabolism increased to allow cryoprotectant catabolism as ambient temperatures warmed. Entry into the pupal stage was also characterized by specific changes in enzyme activities. Taken together, these results show a complex and flexible system of enzymatic rearrangement in this species in response to acclimatization or developmental needs.

The seasonal pattern of cryoprotectant accumulation and clearance by overwintering *E. solidaginis* seen in this study was essentially the same as that previously reported for this species (Morrissey and Baust 1976; Storey and Storey 1986). Glycerol accumulated early in the fall, followed by sorbitol upon arrival of colder temperatures. Both cryoprotectants were synthesized at the expense of glycogen. As such, the activity of glycogen phosphorylase in both total units ($a+b$) and the percent active (%a), was found to be maximal in the fall during active cryoprotectant synthesis. The importance of GPase activity to the synthesis of cryoprotectants was further illustrated by the correlation between high GPase %a and periods of active sorbitol synthesis. The increase in sorbitol levels on 31 March, resulting from a late winter cold spell, was correlated with an increase in the active (%a) form of GPase. The minimum and maximum temperature averages of the week preceding 31 March were well below the 5 °C trigger temperature believed to be key for the initiation of sorbitol synthesis (Baust 1983; Storey and Storey 1983; Rojas et al. 1983). This also reinforces the importance of this polyol as an easily reusable cryoprotectant, one that is readily interconverted with glycogen and that is energetically inexpensive to synthesize.

Glycerol synthesis by cold-hardy insects has been proposed to occur by two possible pathways, shown in Fig. 3 (Storey and Storey 1988). In the first pathway GAP would be dephosphorylated to glyceraldehyde which would then be reduced to glycerol by PDHald using NADPH, presumably produced by the hexose monophosphate shunt. The second pathway relies on the activity of G3PDH to make G3P from DHAP (with NADH), which would then be dephosphorylated by G3Pase. Evidence supporting glycerol synthesis via the first pathway includes the continuous presence of GAPase over the winter and an increase, during the period of maximal cryoprotectant synthesis in the fall, in PDHald activity. However, the concurrent presence of G3PDH and G3Pase activities could also allow the second pathway to operate in the synthesis of glycerol. In fact, the lowest activity enzymes in both pathways, G3Pase and GAPase, were present in nearly identical levels during the period of cryoprotectant synthesis indicating near equal metabolic potential of both pathways.

The accumulation of G3P upon the cessation of glycerol biosynthesis (Storey and Storey 1981; Storey et al. 1981) has previously been used to argue for the routing of synthesis through G3PDH and G3Pase. However, the relatively high potential activity of the HMS in both *E. solidaginis* and other cold-hardy insects (Storey et al. 1991) has suggested that polyol synthesis is NADPH-linked and therefore implicates PDHald in glycerol synthesis. Indeed, of the various sugar substrates that are recognized by insect polyol dehydrogenases, relative maximal activity and affinity for glyceraldehyde is the highest by far (Faulkner 1957; Chino 1960; Sømme and Velle 1968; Takahashi et al. 1974). Furthermore, radiolabelling studies have shown that carbon flow through the HMS increases relative to glycolytic flux as temperature decreases (Tsumuki et al. 1987). The V_{max} activity of 6PGDH was considerably lower than that of G6PDH (Fig. 6), suggesting a regulatory function for this enzyme in the HMS. Indeed, the kinetic properties of 6PGDH from *E. solidaginis* have been shown to be geared towards maximal HMS and NADPH synthesis during cryoprotectant synthesis (Holden and Storey 1994). Carbon shuttling through the HMS also leads to GAP and F6P outputs from the cycle, suggesting that both GAP and NADPH products of the cycle may be channeled into glycerol synthesis. However, it has been calculated that although most of the G6P from glycogenolysis must pass through the HMS to produce sufficient reducing equivalents for glycerol synthesis, only 20% of the carbon skeletons are retrieved as triose sugars, GAP and DHAP (Storey and Storey 1988). The remaining 80% exits as F6P, and must be converted to fructose-1,6-bisphosphate (by PFK) to be used for glycerol production. Glycerol biosynthesis is then dependent on ATP supply.

The present study also changes previous concepts of how glycerol is catabolized by cold-hardy insects. The failure to detect glycerol kinase activity in the larvae at any time indicates that the catabolism of glycerol cannot follow the path glycerol to G3P to DHAP as previously suggested (Storey and Storey 1992, 1988, 1981). Instead, the increase in GAK activity in early March correlated well with the decrease in glycerol levels, suggesting that the removal of glycerol undoubtedly occurs via its reconversion to glyceraldehyde (via PDHald) followed by phosphorylation to produce GAP (via glyceraldehyde kinase). To our knowledge, this is the first report of the use of this pathway in cryoprotectant catabolism. The spring decline in glycerol does not occur with resynthesis of glycogen pools (Fig. 1, Storey and Storey 1986). One pos-

sible explanation for the fate of glycerol carbon is the conversion of glycerol to acetyl-CoA for energy production in the TCA cycle or for the production of lipid reserves. Increasing mitochondrial enzyme activity (Joanisse and Storey 1994) and increased NADP-IDH activity in the spring (Fig. 6) could correspond to such metabolic arrangements (NADPH produced from NADP-IDH is utilized for fat synthesis). Further studies on lipid metabolism enzymes are currently underway in our laboratory. Elevated G3PDH activity in the spring might also suggest an increase in the activity of the α-glycerophosphate shuttle, used by insects to transfer reducing equivalents into the mitochondria (Sacktor 1976). Indeed, G3PDH from *E. solidaginis* has properties geared towards the operation of this cycle (Storey and Storey 1982). NADPH produced from PDHald conversion of glycerol to glyceraldehyde could be converted to NADH by cellular transhydrogenases and utilized by the α-glycerophosphate shuttle. GAP from glycerol catabolism could also be converted to DHAP by triosephosphate isomerase, and utilized directly in the shuttle. This shuttle could be key to the removal of reducing equivalents liberated by the catabolism of polyols. The spring increase in G3PDH also correlates with the general increase in mitochondrial enzyme activity (Joanisse and Storey 1994); the α-glycerophosphate shuttle, required for aerobic carbohydrate oxidation in insects, is then closely linked with the increased aerobic scope of the larvae in the spring.

An absence of change in the maximum activity of PFK over the winter suggests that coarse control cannot be a factor in creating the required shift in metabolism from glycerol to sorbitol production at low temperatures. Instead, the shut down at PFK appears to result from the effects of temperature and temperature-modifier interactions, the enzyme having a Q_{10} of 3.64 between 10 and 0 °C, and in the cold a decreased affinity for F6P, decreased activation by AMP, and increased inhibition by ATP (Storey 1982).

Sorbitol synthesis from glycogen could occur through the glycolytic intermediates G6P or F6P in *E. solidaginis* (Fig. 3) but the data from this study support the routing G6P. This is evidenced by the activity profiles of PDHgluc and SoDH. PDHgluc activity peaked in the fall, and was eightfold higher than SoDH activity. The predominance of carbon shuttling through the HMS and not glycolysis during sorbitol synthesis (Tsumuki et al. 1987) also couples the NADPH produced in this pathway to PDHgluc, which requires this nucleotide as a specific cofactor for the reduction of glucose. The removal of sorbitol in the spring might also proceed by either of two routes. Both glucokinase and the nonspecific (glucose or fructose utilizing) hexokinase activities increased in the spring, suggesting that the catabolism of sorbitol could be channeled into either G6P or F6P. However, by springtime the activity of SoDH had become greater than that of PDHgluc, suggesting this is probably the pathway for sorbitol degradation. In addition, equilibrium constants and K_m's for both PDHgluc and SoDH support the use of PDHgluc for anabolism and SoDH for catabolism of sorbitol (Goil and Harpur 1978; Yaginuma and Yamashita 1979; Leissing and McGuinness 1983; O'Brien et al. 1983; Jeffrey and Jörnvall 1988). These interpretations also agree with previous precursor-product studies that indicated sorbitol synthesis from G6P via PDHgluc versus the clearance of sorbitol via SoDH to fructose and then F6P (Storey and Storey 1981, 1983). Increased SoDH activity correlated with sorbitol catabolism has also been seen in other insect species (Yaginuma and Yamashita 1979; Yamashita et al. 1988).

Gluconeogenesis would be expected to contribute little to metabolism in overwintering *E. solidaginis*, as metabolic flux is primarily directed to carbohydrate conversion into polyol cryoprotectants. This is reflected in the PFK/FBPase activity ratio. Throughout the winter PFK activity was fivefold greater than that of FBPase. However, pupation was characterized by an increase in FBPase activity and a decrease in PFK, so that the activity ratio approached unity (~0.8). This suggests a greater gluconeogenic flux as a part of pupal reorganization.

The constant activities of PK indicate that the total potential shuttling of carbon into mitochondria from glycolysis through this enzyme remains unchanged over the winter. However, as with PFK, temperature effects have been shown to affect strongly the activity of the enzyme. Thus, cold temperatures decrease the catalytic activity of PK, shutting down flow from glycolysis to the TCA cycle (Storey and Storey 1983). Mitochondrial enzyme activities have been shown to decrease dramatically by midwinter and to subsequently recover in the spring (Joanisse and Storey 1994), reflecting the reduced dependence of the larvae on aerobic metabolism during dormancy and freezing.

An overall metabolic picture in overwintering *E. solidaginis* takes shape from the present and previous studies. As fall progresses, enzymes of glycolysis and the HMS channel glucose equivalents down to DHAP and GAP, where they are used for the massive production of the cryoprotectant glycerol, which probably occurs primarily via GAPase and PDHald but also via G3PDH and G3Pase (Fig. 3). Lower temperatures shut down PFK, carbon flow shifting to the HMS which supplies NADPH for sorbitol synthesis which occurs via G6Pase and PDHgluc (Fig. 3). Upon the return to higher temperatures in the spring, SoDH and hexokinase activities lead to sorbitol removal (back to glycogen). Flexible activities of G6Pase and PDHgluc ensure the resynthesis of sorbitol in the spring in the event of a cold spell. As spring temperatures increase and the chance of freezing decreases, glycerol removal begins through PDHald and GAK. Increased α-glycerophosphate shuttle enzyme activity ensures the transfer of reducing equivalents from cryoprotectant catabolism into mitochondria for oxidation, mitochondrial enzyme activity increasing concurrently (Joanisse and Storey 1994). Gluconeogenic enzyme activities (FBPase, hexokinase and glucokinase) remain low during cryoprotectant synthesis, but increase in the spring for the return of glucose equivalents to glycogen or other metabolic fates.

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