

# Seasonal Changes in Glycerol Content and Enzyme Activities in Overwintering Larvae of the Shonai Ecotype of the Rice Stem Borer, *Chilo suppressalis* Walker

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Enzymes associated with glycogen metabolism and glycerol synthesis in larvae of the Shonai ecotype of the rice stem borer, *Chilo suppressalis*, were investigated over the winter in 2000–2001. Glycerol content was scarcely detected in September and October, rapidly increased in November and December, peaked in January, and then decreased. Glycogen was converted to glycerol over the winter until February, and glycerol was reconverted to glycogen in March. The trehalose content remained constant over the winter. The activities of enzymes associated with glycerol synthesis changed with the season. Glycerol accumulation was accomplished by activation of glycogen phosphorylase, inhibition of fructose-1,6-bisphosphatase and pyruvate kinase, and activation of enzymes associated with glycerol synthesis, mainly glyceraldehyde-3-phosphatase and polyol dehydrogenase with glycerol activity. These changes led to a diversion of triose phosphates into the pathway of glycerol synthesis. However, activities of the two initial enzymes of the hexose monophosphate shunt were not activated and remained relatively constant, but high during the period of active glycerol synthesis. Both decreasing temperature in the field and the transition from the diapause to the post-diapause state may be responsible for the changes in activities of enzymes associated with glycerol synthesis. Arch. Insect Biochem. Physiol. 50:53–61, 2002. © 2002 Wiley-Liss, Inc.

KEYWORDS: *Chilo suppressalis*; diapause; glycerol; enzymes; ecotype; cold hardiness; metabolism

## INTRODUCTION

Overwintering insects often accumulate polyols and sugars, such as glycerol and trehalose, as cryoprotectants to endure cold stress (Lee, 1991; Storey and Storey, 1991). Synthesis of these cryo-

protectants can be dependent on the development stage and diapause transition (Lee et al., 1987; Storey and Storey, 1991) and is also influenced by environmental factors, particularly temperature (Baust, 1982). Most studies have shown that cryoprotectant accumulation in overwintering insects

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Abbreviations used: 6PG = 6-phospho-D-gluconate; 6PGDH (EC 1.1.1.44) = 6-phosphogluconate dehydrogenase; ANOVA = analysis of variances; EDTA = ethylenediaminetetraacetic acid; EGTA = ethylene glycol-bis ( $\beta$ -aminoethyl ether)-*N*, *N*, *N'*, *N'*-tetraacetic acid; FBPase (EC 3.1.3.11) = fructose-1,6-bisphosphatase; G3P =  $\alpha$ -glycerophosphate; G3Pase = glycerol-3-phosphatase; G3PDH (EC 1.1.1.8) =  $\alpha$ -glycerophosphate dehydrogenase; G6P = D-glucose 6-phosphate; G6PDH (EC 1.1.1.49) = glucose-6-phosphate dehydrogenase; GAPase = glyceraldehyde-3-phosphatase; GPase (EC 2.4.1.1) = glycogen phosphorylase; GPase *a*% = the percentage of active form of glycogen phosphorylase; PDHald = polyol dehydrogenase, with glycerol activity; PFK (EC 2.7.1.11) = phosphofructokinase; PK (EC 2.7.1.40) = pyruvate kinase; PMSF = phenylmethylsulfonyl fluoride; PU = 1-phenyl-3-(2-thiazolyl)-2-thiourea. s.e.m. = standard error of the mean; ww = wet weight.

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is a function of the actual low temperature and the period of such an exposure (Baust and Lee, 1982; Lee, 1991; Han and Bauce, 1995; Li et al., 2000a). On the other hand, some studies have indicated that insects at different stages (i.e., at different diapause intensities) or during the period of diapause transition may respond differently to low temperature (Nordin et al., 1984; Lee et al., 1987; Pio and Baust, 1988; Denlinger, 1991; Han and Bauce, 1995). In other words, there exists an underlying developmental component that influences cryoprotectant synthesis.

The rice stem borer, *Chilo suppressalis* Walker, which is distributed throughout Japan, is one of its most important rice pests. The borer has a univoltine life cycle in Hokkaido and some parts of northern Honshu, a trivoltine life cycle in the southern parts of Shikoku and Kyushu islands, and a bivoltine life cycle in other parts of Japan (Ishikura, 1955; Fukaya and Mitsunashi, 1961; Kishino, 1974). The bivoltine borer in Japan is divided into at least two ecotypes, the Shonai ecotype, which is distributed in the northern part of its range in Japan, and the Saigoku ecotype, which is distributed in the southern part of its range (Fukaya and Mitsunashi, 1961). The overwintering mature larvae of both ecotypes undergo a facultative diapause, which is induced by photoperiods below 14 h in autumn (Inoue and Kamano, 1957; Kishino, 1974). The Shonai ecotype has a shorter diapause duration than the Saigoku ecotype. Larvae of the Shonai ecotype are in diapause till October and terminate diapause in November before winter, whereas larvae of the Saigoku ecotype terminate in February (Fukaya and Mitsunashi, 1961). The profiles of cold hardiness of natural populations of larvae of the two ecotypes are different. Cold hardiness in the Shonai ecotype is low at the diapause stage, but relatively high at the post-diapause stage, whereas it is high in the Saigoku ecotype at the diapause stage (Tsumuki, 1990; Goto et al., 2001). In *C. suppressalis* larvae, the major sugar is trehalose and the major polyol is glycerol, and the content of trehalose remains constant over the winter in both ecotypes (Tsumuki and Kanehisa, 1978; Goto et al., 2001). To eluci-

date the different bases of diapause termination and cold hardiness between these two ecotypes, studies on cryoprotectant accumulation and their enzymatic regulation are required. The metabolic rearrangement in the Saigoku ecotype has been partially elucidated (Tsumuki and Kanehisa, 1979, 1980a,b). In the Saigoku larvae, during the period of active glycerol synthesis, both the glycolytic pathway and the hexose monophosphate shunt are active, and glycerol accumulation is closely associated with a continuous rise in activity of glycerol-3-phosphate dehydrogenase and an inhibition of lactate dehydrogenase activity (Tsumuki and Kanehisa, 1980b). However, no systematic studies on seasonal profiles of glycerol and enzymatic activities associated with its accumulation in the Shonai ecotype have been conducted.

In the present study, seasonal changes in the levels of glycerol, trehalose, and glycogen in the Shonai ecotype of *C. suppressalis* were investigated. We found a rearrangement of enzyme activities related to glycerol synthesis.

## MATERIALS AND METHODS

### Chemicals and Insects

All biochemicals and coupling enzymes were purchased from Sigma Chemical Co. (St. Louis, MO) or Wako Chemical Co. (Osaka, Japan).

Larvae of *C. suppressalis* were collected from a paddy field in Takasaka in the Shonai district of Yamagata prefecture (38°5'N, 39°5'E) on the 22nd of every month from September 2000 to March 2001. The collected larvae (n = 6) were placed in long, uncapped tubes and transported to the lab within 10 min. Larvae were then transferred to -40°C for storage before use. Whole larvae were weighed and homogenized for chemical and enzyme analyses.

### Chemical Analysis

Glycerol and trehalose were measured by gas-liquid chromatography as described by Goto (1995). Glycogen was determined by the anthrone method as described by Li et al. (2000a).

## Enzyme Preparation

For enzymes G3Pase and GAPase, one larva was homogenized in 10 volumes of ice-cold 20 mM imidazole-HCl, pH 7.2, containing 0.1 mM PMSF and some PU with an ice-chilled teflon pestle-glass homogenizer. Homogenates were then centrifuged at 12,000g for 25 min at 0°C, and the supernatants were used for assays.

For the other enzymes, one larva was homogenized in 8 volumes of ice-cold 20 mM imidazole-HCl (pH 7.2) containing 15 mM  $\beta$ -mercaptoethanol, 5 mM EDTA, 5 mM EGTA, 50 mM NaF, 0.1 mM PMSF, and some PU using an ice-chilled teflon pestle-glass homogenizer. Homogenates were then centrifuged at 12,000g for 25 min at 0°C, and the supernatants were used for enzyme assays. Samples for GPase activity determinations were homogenized as above but were not centrifuged. Instead, the settled homogenate was used as the source of the enzyme (Joanisse and Storey, 1994).

## Enzyme Determination

All enzyme activities were measured at 25°C using a Hitachi U-2001 spectrophotometer. Activities were determined by continuous time scanning at 340 nm, except for G3Pase and GAPase, which were determined according to the amounts of  $P_i$  released in 30 min at 25°C by the method of Taussky and Shorr (1953). Controls for non-specific activity in the absence of substrate or enzyme were run for all assays. The final activity values were calculated by subtracting blank values. One unit of enzyme activity is defined as the amount of enzyme that will convert 1  $\mu$ mol of substrate per min at 25°C. Since larval wet weight did not change during the study period, activity was expressed as units per gram wet weight (units/g ww).

The conditions for the enzyme assays were modified according to Joanisse and Storey (1994) as follows:

GPase: 50 mM sodium phosphate buffer (pH 7.2), 6 mg/ml glycogen, 6  $\mu$ M glucose-1, 6-bisphosphate, 0.2 mM NADP<sup>+</sup>, 2 mM 5'-AMP, 15 mM Mg<sup>2+</sup>, 2 U phosphoglucomutase, 2 U

G6PDH (NADP<sup>+</sup> dependent). The active form of the enzyme (*a* form) was measured in the absence of 5'-AMP.

PFK: 20 mM imidazole-HCl (pH 7.4), 10 mM fructose-6-phosphate, 4 mM ATP, 0.15 mM NADH, 48 mM K<sup>+</sup>, 6 mM Mg<sup>2+</sup>, and 2 U aldolase, triosephosphate isomerase, and G3PDH.

FBPase: 20 mM imidazole-HCl (pH 7.4), 0.1 mM fructose-1,6-bisphosphate, 0.2 mM NADP<sup>+</sup>, 5 mM Mg<sup>2+</sup>, and 2 U phosphoglucose isomerase and G6PDH (NADP<sup>+</sup> dependent).

G6PDH: 20 mM imidazole-HCl (pH 7.4), 1.2 mM G6P, 0.2 mM NADP<sup>+</sup>, and 5 mM Mg<sup>2+</sup>.

6PGDH: 20 mM imidazole-HCl (pH 7.4), 1.2 mM 6PG, 0.2 mM NADP<sup>+</sup>.

G3PDH: 20 mM imidazole-HCl (pH 7.4), 1.0 mM dihydroxyacetone phosphate, 0.15 mM NADH.

PDHald: 20 mM imidazole-HCl (pH 7.4), 3.8 mM D-glyceraldehyde, and 0.1 mM NADPH.

PK: 20 mM imidazole-HCl (pH 7.4), 5 mM phosphoenolpyruvate, 2 mM ADP, 0.15 mM NADH, 48 mM K<sup>+</sup>, 6 mM Mg<sup>2+</sup> and 5 U lactate dehydrogenase.

G3Pase: 20 mM imidazole-HCl (pH 7.4), 10 mM D,L-G3P, 10 mM Mg<sup>2+</sup>.

GAPase: 20 mM imidazole-HCl (pH 7.4), 5.6 mM glyceraldehyde-3-phosphate, 10 mM Mg<sup>2+</sup>.

## Statistical Analysis

All data are reported as mean  $\pm$  s.e.m. Differences between treatments were compared either by *t*-test (for comparison of two means) or by one-way ANOVA, followed by a Tukey-test for multiple comparisons (SigmaStat 2.0, Jandel Scientific Co., Corte Madera, CA).

## RESULTS

### Seasonal Changes in Sugar and Glycogen Contents

The major sugar was trehalose and the major polyol was glycerol in overwintering larvae of *C. suppressalis*. Glycerol content differed significantly over the winter season ( $F = 75.8$ ,  $P < 0.001$ ). Glycerol content was barely detectable in September and October, 99.8  $\mu$ mol/g ww in November, 180.4

$\mu\text{mol/g ww}$  in December, peaked at  $360.0 \mu\text{mol/g ww}$  in January, and declined to  $300.7 \mu\text{mol/g ww}$  in February and  $183.4 \mu\text{mol/g ww}$  in March (Fig. 1). The trehalose content in the whole-body did not change significantly, having a mean value of  $30.3 \mu\text{mol/g ww}$  from September to March (Fig. 1).

A significant change in glycogen content was found from September to March ( $F = 29.8$ ,  $P < 0.001$ ). The glycogen content was  $162.7 \mu\text{mol/g ww}$  in September, and then decreased linearly till December to  $97.6 \mu\text{mol/g ww}$ . Afterward, the content was very low from January to February, and then dramatically increased to  $169.8 \mu\text{mol/g ww}$  in March, which was the same level as found in September (Fig. 1).

### Seasonal Changes in Enzyme Activities

Total activity of GPase remained at  $1.4 \text{ U/g ww}$  from September to December, but increased dramatically to  $4.0 \text{ U/g ww}$  in January and remained at that value in February. It then decreased to  $2.5 \text{ U/g ww}$  in March (Fig. 2A). The percentage of the *a* form of GPase remained below 50% from September to December, but increased to near 70% in larvae sampled from January to March (Fig. 2B). Activities of PFK decreased significantly from September to November, and then remained unchanged till February, but rebounded slightly in March (Fig. 3A). The activities of FBPase (Fig. 3A)

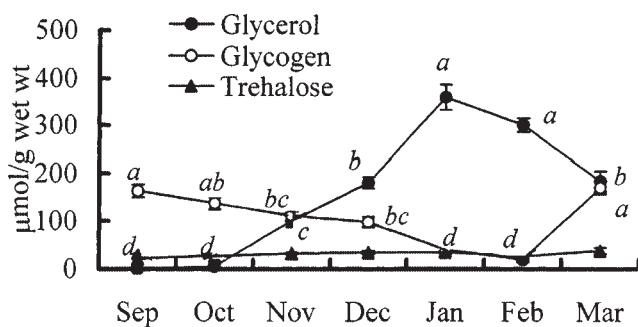


Fig. 1. Seasonal changes in the contents of glycerol, trehalose, and glycogen in whole-bodies of overwintering larvae of the rice stem borer *C. suppressalis*. Each symbol shows the mean value ( $\pm$  s.e.m.) of six replicates. Values labeled with the same letters on the same line are not significantly different at the 5% level by Tukey's test after ANOVA.

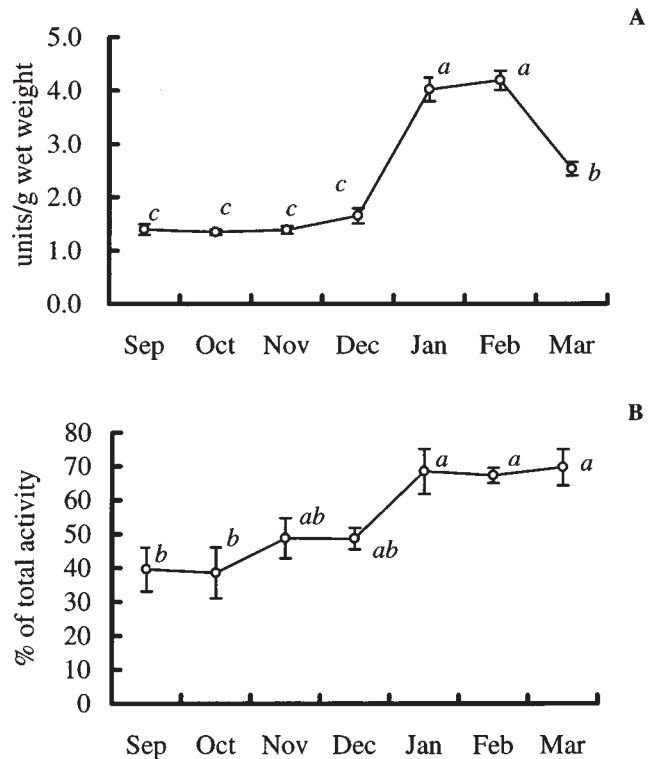


Fig. 2. Seasonal changes in total activity of GPase (glycogen phosphorylase) (A) and GPase *a*% (the percentage of active *a* form of glycogen phosphorylase) (B) in whole-bodies of overwintering larvae of the rice stem borer *C. suppressalis*. Each symbol shows the mean value ( $\pm$  s.e.m.) of six replicates. Values labeled with the same letters on the same line are not significantly different at the 5% level by Tukey's test after ANOVA.

and PK (Fig. 3B) changed significantly, and two obvious phases existed, i.e., the activities from September to December were higher than those from January to March.

Figure 4 shows the activities of enzymes associated with glycerol synthesis. The activity of G3PDH was  $2.14 \text{ U/g ww}$  in September, then increased to  $2.97 \text{ U/g ww}$  in October, remained at the same level till February, and then decreased to  $2.13 \text{ U/g ww}$  in March (Fig. 4A). The activity of G3Pase remained at nearly  $0.50 \text{ U/g ww}$  from September to December, increased to  $0.96 \text{ U/g ww}$  in January, and remained at that value till March (Fig. 4A). The activity of GAPase was about  $1.0 \text{ U/g ww}$  from September to November, increased to  $2.84 \text{ U/g ww}$  in December, peaked at  $5.16 \text{ U/g ww}$  in January, and then decreased to  $3.06 \text{ U/g ww}$  in February

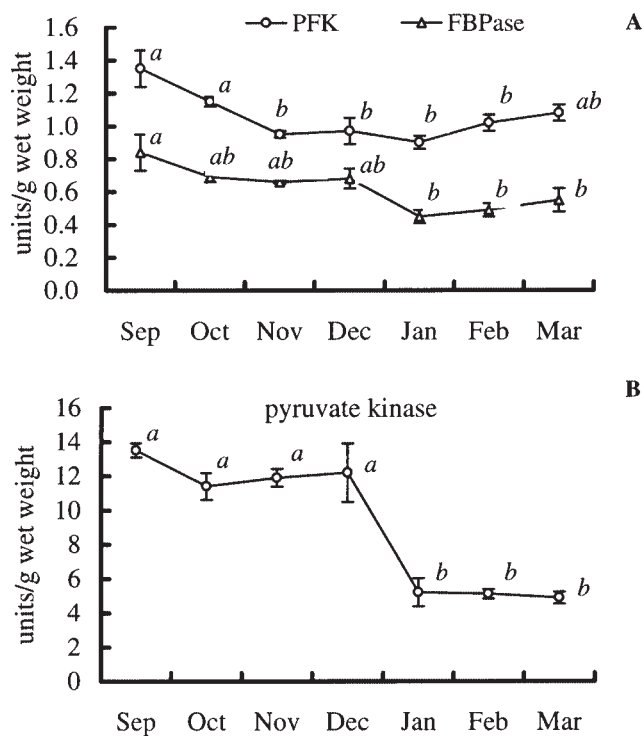


Fig. 3. Seasonal changes in activities of PFK (phosphofructokinase) and FBPase (fructose-1,6-bisphosphatase) (A) and pyruvate kinase (PK) (B) in whole-bodies of overwintering larvae of the rice stem borer *C. suppressalis*. Each symbol shows the mean value ( $\pm$  s.e.m.) of six replicates. Values labeled with the same letters on the same line are not significantly different at the 5% level by Tukey's test after ANOVA.

and to 2.36 U/g ww in March (Fig. 4B). The activity of PDHald remained at about 0.30 U/g ww from September to December, peaked at 0.64 U/g ww in January, and then decreased to 0.48 U/g ww in March (Fig. 4B).

The activities of G6PDH and 6PGDH, the enzymes in the first two steps of the hexose monophosphate shunt, were higher in September than in October, and then remained unchanged over the winter until March (Fig. 5).

## DISCUSSION

In the present study, *C. suppressalis* larvae of the Shonai ecotype accumulated glycerol from December to March. A substantial increase in glycerol content was found from October to January. This indicates that a developmental trigger (larval de-

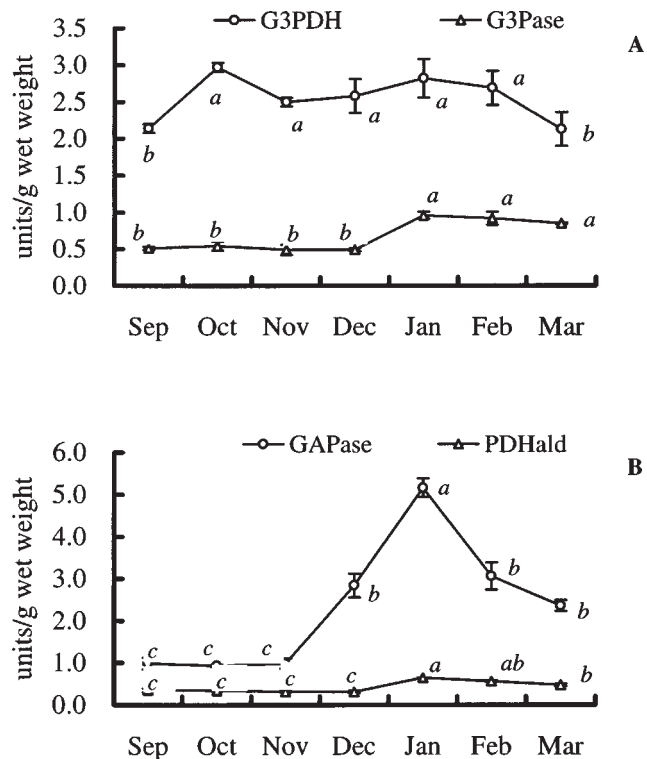


Fig. 4. Seasonal changes in activities of G3PDH ( $\alpha$ -glycerophosphate dehydrogenase) and G3Pase (glycerol-3-phosphatase) (A), and GAPase (glyceraldehyde-3-phosphatase) and PDHald (polyol dehydrogenase with glyceraldehyde activity) (B) in whole-bodies of overwintering larvae of the rice stem borer *C. suppressalis*. Each symbol shows the mean value ( $\pm$  s.e.m.) of six replicates. Values labeled with the same letters on the same line are not significantly different at the 5% level by Tukey's test after ANOVA.

velopment proceeds towards the end of diapause and to the post-diapause) is involved in the initiation of glycerol synthesis by the larvae. The average monthly ambient temperature in the Shonai district over the 2000–2001 winter ranged from 22.2°C in September to  $-0.3^{\circ}\text{C}$  in January to 3.6°C in March. The minimum daily temperature was  $-3$  to  $-4^{\circ}\text{C}$  in January and February (Fig. 6). Larvae collected in January and February accumulated high levels of glycerol, suggesting that low temperature may partially stimulate glycerol synthesis. The seasonal changes in glycerol content suggest that glycerol accumulation is dependent on low temperature exposure and diapause transition. Changes in the content of glycogen were opposite to those of glycerol from September to March,

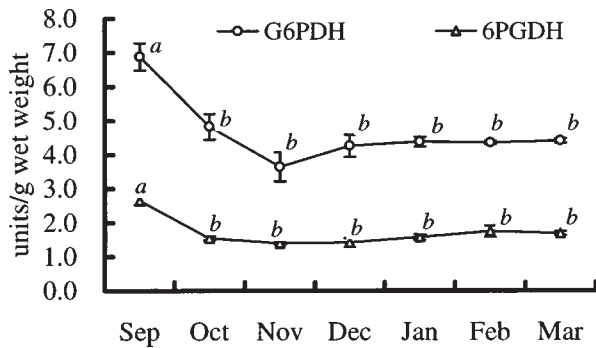


Fig. 5. Seasonal changes in activities of G6PDH (glucose-6-phosphate dehydrogenase) and 6PGDH (6-phosphogluconate dehydrogenase) in whole-bodies of overwintering larvae of the rice stem borer *C. suppressalis*. Each symbol shows the mean value ( $\pm$  s.e.m.) of six replicates. Values labeled with the same letters on the same line are not significantly different at the 5% level by Tukey's test after ANOVA.

suggesting that synthesis of glycerol occurs via the mobilization of glycogen reserves. However, on a carbon basis, the loss of glycogen was less than the gain of glycerol, indicating that other intermediates are also converted into glycerol. Glycogen rebounded transiently in March with the decrease of glycerol, suggesting that glycerol is reconverted to glycogen when the ambient temperature rises. In the Saigoku ecotype larvae of *C. suppressalis*, accumulation of glycerol was found from January to March and was associated with a decline of glycogen content from the pre-diapausing to the diapausing stage, while the quantity of glycerol in hemolymph de-

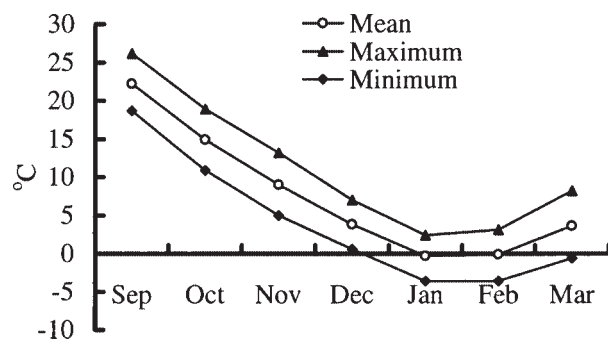


Fig. 6. Seasonal changes in average, minimum, and maximum ambient temperatures over the 2000–2001 winter at Shonai, Yamagata. Values were calculated from weather summaries obtained from a local meteorological station in Tsuruoka.

creased progressively with increasing glycogen in the fat body during the post-diapausing stage (from mid-February to June) (Tsumuki and Kanehisa, 1978; Tsumuki, 1990). Thus, glycerol accumulation in the Shonai ecotype larvae differed from that of the Saigoku larvae, but larvae of both ecotypes accumulated glycerol when the ambient temperature in the field was lowest.

The present study clearly demonstrates that the metabolic pathways of glycerol synthesis in the Shonai ecotype larvae of *C. suppressalis* change over the winter. The total activity of GPase increased significantly from December to January and subsequently remained high until March. The percentage of GPase in the active *a* form was also high during active glycerol synthesis. These changes could result in the consumption of more glycogen for glycolysis. Activation of GPase was also found in the Saigoku ecotype larvae during the diapause initiation stage (Tsumuki and Kanehisa, 1979). Here, the high GPase activity seemed to correlate with the breakdown of glycogen for glycerol synthesis. As reported in other overwintering insects that use glycerol as the main cryoprotectant, activation of PFK is essential (Storey and Storey, 1981, 1991). However, PFK activity tended to decrease during the period of active glycerol synthesis in the Shonai ecotype larvae. The decrease may result from the effects of low temperature and temperature-modifier interaction as found in *Eurosta solidaginis* (Joanisse and Storey, 1994). Further studies on the kinetic properties of this enzyme are needed to test this hypothesis. The activity of FBPase was higher from September to December than it was from January to March. It seems that gluconeogenesis is arrested during the period of glycerol formation. The increase in glycogen breakdown was also reflected in the ratio of PFK activity to FBPase activity. Because of the greater decrease in FBPase from January to March, the activity ratio increased from 1.4–1.6 to about 2.0 at the time of maximal glycerol synthesis from January to March. Moreover, PK activity decreased more than 50% from December to January. These results suggest that a greater number of carbon sources enter the glycolytic pathway for glycerol synthesis

in the Shonai ecotype larvae. Thus, the observed activation of GPase, the increase in the ratio of PFK activity to FBPase activity, and the inhibition of glycogenic flux at the level of PK would lead to more triose phosphates available for the glycerol synthesis pathway.

Previous studies found that there are two possible pathways for glycerol production from triose phosphates in some overwintering insects: a pathway via GAPase and PDHald, another pathway via G3PDH and G3Pase (Storey and Storey, 1981, 1983, 1988, 1991). In this study, GAPase activity was three times higher in December, February, and March and five times higher in January than during the period from September to November. Moreover, the activity of PDHald from January to March was 1.5–1.8 times higher than it was from September to December. The activity profiles of these two enzymes over the winter almost paralleled the glycerol content profile, indicating that this route appears to be closely related to glycerol accumulation. On the other hand, the activity of G3PDH remained constant over the winter until February. The activity of G3Pase was four- to sixfold less than that of G3PDH over the winter, and its activity increased 1.8 times and remained relatively constant during the period of maximal glycerol accumulation. This suggests that an alternative route of glycerol synthesis, via the enzymes G3PDH and G3Pase, probably is not important in the Shonai ecotype larvae. However, in the Saigoku ecotype larvae, G3PDH is one of the key enzymes responsible for glycerol formation (Tsumuki and Kanehisa, 1980b).

In this study, the activities of enzymes in the first two steps of the hexose monophosphate shunt (HMS), G6PDH and 6PGDH, decreased rather than increased during the winter. However, they are still higher than they are in other cold hardy insects that use glycerol as a cryoprotectant (Joanisse and Storey, 1994, 1995; Li et al., 2000b). This implies either that the amount of NADPH produced through HMS is enough for glycerol synthesis, or that NADPH is supplied from lipid by  $\beta$ -oxidation. In contrast, in the Saigoku ecotype larvae, G6PDH activity remained high during the diapausing stage from December to February.

In conclusion, the present results show that glycerol in the Shonai ecotype larvae of *C. suppressalis* accumulates during the post-diapause stage from January to February and that the activities of some enzymes undergo seasonal changes apparently to shift metabolism towards glycerol synthesis. Both the decreasing temperature in the field and the transition from diapause to post-diapause may be responsible for the changes in glycerol synthesis and activities of enzymes associated with its synthesis. However, because these two factors are interrelated in natural populations, the effect of low temperature on glycerol accumulation cannot be separated adequately from the effect of diapause intensity. Further acclimation experiments are needed to elucidate the mechanisms of glycerol synthesis in the Shonai ecotype of *C. suppressalis*. Further studies are also needed to elucidate the differences in the mechanisms regulating enzymes in larvae of the Shonai ecotype and the Saigoku ecotype.

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