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Diapause development and acclimation regulating enzymes associated with glycerol synthesis in the Shonai ecotype of the rice stem borer larva, *Chilo suppressalis* walker

Yi-Ping Li ^a, Michiyo Goto ^{a, *}, Lei Ding ^a, Hisaaki Tsumuki ^b

^a Department of Bioprocess Engineering, Faculty of Agriculture, Yamagata University, Tsuruoka 997-8555, Japan

^b Research Institute for Bioresources, Okayama University, Kurashiki 710, Japan

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Abstract

Overwintering larvae of the Shonai ecotype of the rice stem borer, *Chilo suppressalis*, enter diapause in early September and terminate diapause at the end of October. Cold acclimation at 0°C did not influence glycerol, trehalose or glycogen content in larvae collected on 22 September. Acclimation at 0°C increased the glycerol content and reduced the glycogen content significantly in larvae collected on 2 October and 22 November compared with acclimation at 15°C. These results indicate that overwintering larvae at different phases of diapause development respond differently to the low temperature stimulus for glycerol synthesis. Thus, we evaluated the metabolic rearrangements associated with glycerol synthesis during diapause development and after temperature acclimation. Larvae collected on 2 October were acclimated at 15°C for 15 and 60 days. Some of those acclimated at 15°C were then moved to 0°C for 15 days. The larvae acclimated at 15°C for 15 days were in deep diapause and accumulated little glycerol, while larvae acclimated at 15°C for 60 days were nearly ready to emerge from diapause and accumulated glycerol at 155.5 μmol/g. When larvae acclimated to 15°C for 15 days were transferred to 0°C, glycerol accumulation was stimulated to the same extent (ca 140 μmol/g) as it was in larvae that were acclimated to 15°C for 60 days and then transferred to 0°C. These results indicate that low temperature has a cumulative effect on glycerol production in larvae at different phases of diapause development. Glycerol accumulation was accomplished by activation of glycogen phosphorylase and inhibition of fructose-1,6-bisphosphatase, and activation of enzymes associated with glycerol synthesis, mainly glyceraldehyde-3-phosphatase and polyol dehydrogenase with glyceraldehyde activity. © 2002 Elsevier Science Ltd. All rights reserved

Keywords: Shonai ecotype; Diapause development; Cold acclimation; Glycerol synthesis; Enzymes

1. Introduction

The rice stem borer, *Chilo suppressalis* Walker, is distributed throughout the islands of Japan from Hokkaido to Kyushu. The borer has a bivoltine life cycle in most parts of Japan (Ishikura, 1955; Fukaya and Mitsuhashi, 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 Japan, and the Saigoku ecotype, which is distributed in the southwestern region (Fukaya and Mitsuhashi, 1961). In au-

umn, mature larvae of both ecotypes respond to decreasing photoperiods and enter facultative diapause under short day-length (Inoue and Kamano, 1957; Kishino, 1974). The coldest monthly temperatures experienced by the rice stem borers in Japan occur in January (about 0.8°C in distribution areas of the Saigoku ecotype) and February (about -4.1°C in distribution areas of Shonai ecotype). The Shonai ecotype has a shorter diapause duration than the Saigoku ecotype. Larvae of the Shonai ecotype enter diapause in early September and require only a short period of low temperature for diapause termination, which occurs at the end of October before the coldest months. However, larvae of the Saigoku ecotype require a longer duration of cold exposure (about 4 months or more), and remain in diapause during the coldest months, until February (Fukaya and Mitsuhashi, 1961; Tsumuki and Kanehisa, 1978; Goto et al., 2001).

* Corresponding author. Tel./fax: +81-235-28-2847.

E-mail addresses: ypli13@hotmail.com (Y.-P. Li); mgoto@tds1.tr.yamagata-u.ac.jp (M. Goto).

Cold hardiness in the Shonai ecotype is low during diapause, but is relatively high during the post-diapause stage, whereas it is high in the Saigoku ecotype during diapause stage and relatively low during the post-diapause stage (Tsumuki and Kanehisa, 1978; Goto et al., 2001). Larvae of both ecotypes accumulate glycerol during the coldest months over the winter to increase cold hardiness. Glycerol in the Shonai ecotype peaked in January during the post-diapause stage, whereas glycerol accumulation in the Saigoku ecotype was found in February during the diapause stage (Tsumuki and Kanehisa, 1978; Tsumuki, 1990; Li et al., in press). In other words, glycerol accumulation in the Shonai ecotype occurs at low temperatures and at a low diapause intensity, while in the Saigoku ecotype it occurs at low temperatures and at a high diapause intensity. In the field, the mechanisms regulating enzymes associated with glycerol formation are different between larvae of the Shonai and Saigoku ecotypes (Tsumuki and Kanehisa, 1980b; Li et al., in press). In the Shonai ecotype larvae, the activity profiles of glyceraldehydes-3-phosphatase (GAPase) and polyol dehydrogenase, with glyceraldehyde activity (PDHald) over the winter were found to almost parallel the glycerol content profile, suggesting that this route is closely related to glycerol accumulation. On the other hand, in the Saigoku larvae, α -glycerophosphate dehydrogenase (G3PDH) is one of the key enzymes responsible for glycerol production. These results suggest that both progressively decreasing temperature in the field and diapause development (from diapause to post-diapause) may be responsible for the changes in activities of enzymes associated with glycerol synthesis (Li et al., in press). Because decreasing temperature and diapause development overlap during the period of maximal glycerol production in overwintering *C. suppressalis* larvae, their separate effects are not readily apparent.

In the present study, we used acclimation experiments to distinguish the effects of these two factors on larvae of the Shonai ecotype of *C. suppressalis*, focusing on the mechanisms regulating the activities of enzymes related to glycerol synthesis.

2. Materials and methods

2.1. Chemicals

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

2.2. Effects of sampling date on glycerol, trehalose and glycogen content in overwintering larvae after temperature acclimation

Stems containing *C. suppressalis* larvae (the Shonai ecotype) were collected from the paddy field in Takasaka

at Yamagata prefecture on 22 September, 2 October and 22 November 2000 and acclimated at 0 or 15°C in the dark; the former temperature was chosen to stimulate rapid glycerol synthesis whereas at 15°C glycerol would remain low (Goto et al., 2001). After 15 days, larvae were rapidly removed from the stems and frozen quickly and stored at –40°C for chemical analysis.

2.3. Effect of diapause intensity and temperature acclimation on glycerol, trehalose and glycogen content

Stems containing *C. suppressalis* larvae (the Shonai ecotype) were collected from the paddy field in Takasaka on 2 October 2000 and acclimated at 0 or 15°C in the dark according to the following temperature regimes:

- (i) 15°C for 15 days;
- (ii) 15°C for 15 days, followed by a 15 day exposure to 0°C;
- (iii) 15°C for 60 days;
- (iv) 15°C for 60 days, followed by a 15 day exposure to 0°C.

Sub-samples from (i) and (ii) were collected for determination of diapause intensity as indicated by the number of days required for adult emergence and respiration rates. All acclimated samples were collected for chemical analysis and enzyme determination.

2.4. Adult emergence

Larvae from (i) and (ii) ($n = 7$) were incubated at 25°C under a photoperiod of 12 h light and 12 h dark (12L–12D), and adult emergence was observed daily for 90 days.

2.5. Respiration ratio

For each replicate, a single larva from (i) and (ii) ($n = 4$) was placed in a 10-ml vial and the vial was kept at 15°C in darkness. After 24 h, a gas sample (1 ml) was withdrawn with a needle and syringe through the cap of the vial. The volumes of CO₂ released and O₂ consumed by the individuals were analyzed by a gas-solid chromatograph equipped with a thermal conductivity detector (GL Sciences GC-320) as described by Tanaka and Saito (1984). The respiration quotient (*RQ*) was expressed as a ratio (volume of CO₂ released/volume of O₂ consumed).

2.6. 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).

2.7. Crude enzyme preparation

One larva was homogenized in 8 volumes of ice-cold 20 mM imidazole-HCl (pH 7.2) containing 15 mM β -mercaptoethanol, 5 mM ethylenediaminetetraacetic acid, 5 mM ethylene glycol-bis(β -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid, 50 mM NaF, 0.1 mM phenylmethylsulfonyl fluoride (PMSF) and some 1-phenyl-3-(2-thiazolyl)-2-thiourea (PU) using an ice-chilled Teflon pestle-glass homogenizer. Homogenates were then centrifuged at 12,000 *g* for 25 min at 0°C, and the supernatants were used for enzyme assays. Samples for glycogen phosphorylase (GPase) activity determinations were homogenized as above but were not centrifuged. Instead, the settled homogenate was used as the enzyme source (Joanisse and Storey, 1994).

For enzymes of glycerol-3-phosphatase (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 12,000 *g* for 25 min at 0°C, and the supernatants were used for assays.

2.8. Enzyme activity assays

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 μ mol of substrate per min at 25°C. Since larval wet weight did not change significantly during acclimation, activity was expressed as units per gram wet weight (units/g ww).

Conditions for enzyme assay were modified according to Joanisse and Storey (1994) as follows:

GPase: 50 mM sodium phosphate buffer (pH 7.2), 6 mg/ml glycogen, 6 μ M glucose-1, 6-disphosphate, 0.2 mM NADP⁺, 2 mM 5'-AMP, 15 mM Mg²⁺, 2 units phosphoglucomutase, 2 units G6PDH (NADP⁺ dependent). The active form of the enzyme (*a* form) was measured in the absence of 5'-AMP.

Phosphofructokinase (PFK): 20 mM imidazole-HCl (pH 7.4), 10 mM fructose-6-phosphate, 4 mM ATP, 0.15 mM NADH, 48 mM K⁺, 6 mM Mg²⁺, and 2 units aldolase, G3PDH and triosephosphate isomerase.

Fructose-1,6-bisphosphatase (FBPase): 20 mM imidazole-HCl (pH 7.4), 0.1 mM fructose-1,6-

bisphosphate, 0.2 mM NADP⁺, 5 mM Mg²⁺, and 2 units phosphoglucose isomerase and glucose-6-phosphate dehydrogenase (NADP⁺ dependent).

Glucose-6-phosphate dehydrogenase (G6PDH): 20 mM imidazole-HCl (pH 7.4), 1.2 mM D-glucose-6-phosphate, 0.2 mM NADP⁺, and 5 mM Mg²⁺.

6-phosphogluconate dehydrogenase (6PGDH): 20 mM imidazole-HCl (pH 7.4), 1.2 mM 6-phospho-D-gluconate, 0.2 mM NADP⁺.

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.

Pyruvate kinase (PK): 20 mM imidazole-HCl (pH 7.4), 5 mM phosphoenolpyruvate, 2 mM ADP, 0.15 mM NADH, 48 mM K⁺, 6 mM Mg²⁺ and 5 units lactate dehydrogenase.

G3Pase: 20 mM imidazole-HCl (pH 7.4), 10 mM D,L- α -glycerophosphate, 10 mM Mg²⁺.

GAPase: 20 mM imidazole-HCl (pH 7.4), 5.6 mM glyceraldehyde-3-phosphate, 10 mM Mg²⁺.

2.9. Statistical analyses

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

3. Results

3.1. Effects of sampling date on contents of glycerol, trehalose and glycogen after temperature acclimation

Glycerol was scarcely detected in larvae collected in September, and in larvae acclimated at 15°C for 15 days, but it appeared in three of six larvae acclimated at 0°C for 15 days. However, no significant differences among these groups were detected ($F = 1.720$; $P = 0.213$). Temperature acclimation also resulted in no significant changes in contents of glycogen in larvae collected in September.

Acclimation to 0°C increased the glycerol contents of larvae collected on 2 October and 22 November to 140.3 and 162.7 μ mol/g ww, respectively. This increase was significantly greater than the increase observed in larvae acclimated at 15° (Fig. 1). On the other hand, the glycogen content was significantly lower in larvae acclimated to 0°C than in larvae acclimated to 15°C. However, the glycerol and glycogen contents of larvae acclimated to 15°C were not significantly different from those of lar-

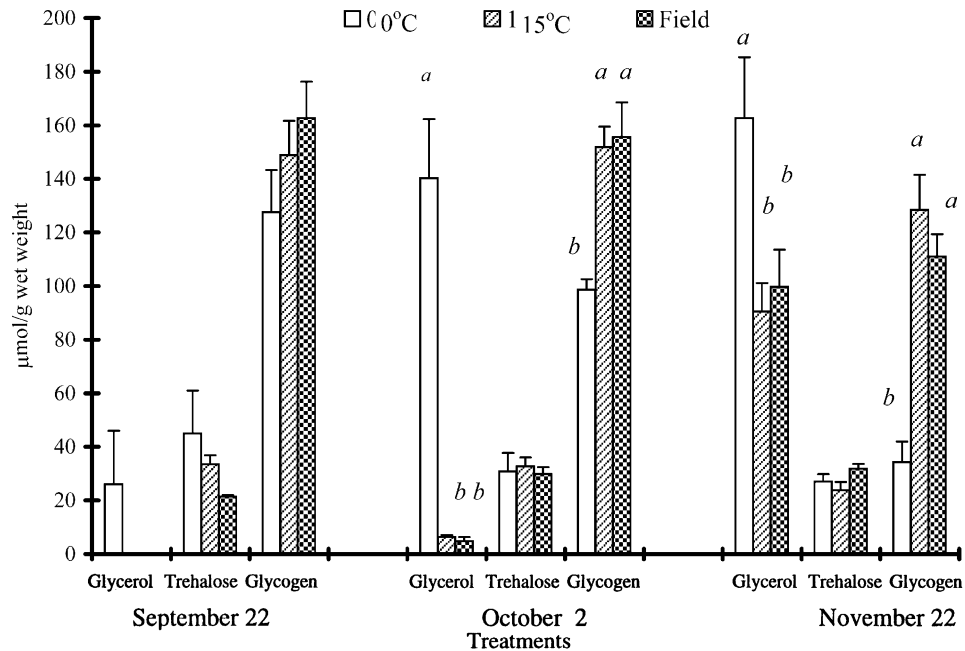


Fig. 1. Effect of temperature acclimation on the levels of glycerol, trehalose and glycogen in whole bodies of the rice stem borer of the Shonai ecotype of *C. suppressalis*. Larvae were collected on 22 September, 2 October and 22 November 2000 and then acclimated at 0 or 15°C for 15 days. Each symbol shows the mean value (\pm s.e.m.) of six replicates (one larva per replicate). Values labeled with the same letters in each group are not significantly different at the 5% level by Tukey's test after ANOVA.

vae collected in the field on 2 October 2 and 22 November (Fig. 1).

No significant differences in trehalose contents were detected among the larvae acclimated to the acclimation temperatures (Fig. 1).

Thus, in the next series of experiments, larvae collected on 2 October were used to evaluate the metabolic rearrangements during diapause development and after temperature acclimation.

3.2. Changes in diapause intensity and respiration during acclimation

Larvae acclimated at 15°C for 15 and 60 days were transferred to 25°C, and the period required for adult emergence was determined. The time for emergence of the 60-day-acclimated larvae (16.3 days) was significantly shorter than the time for the 15-day-acclimated larvae (61.3 days) ($P < 0.05$, Table 1). The period was

similar to that of larvae after diapause termination (15.8 days), suggesting that the larvae acclimated to 15°C for 60 days were only slightly into diapause or just came out of diapause.

The O_2 uptake in larvae acclimated at 15°C for 60 days was 2.1 times greater than that of larvae acclimated at 15°C for 15 days (Table 1). Similarly, the CO_2 release in the 60-day acclimated larvae was 1.6 times greater than that of the 15-day acclimated larvae. As a result of these changes, the RQ decreased from 1.02 in the 60-day-acclimated larvae to 0.79 in the 15-day-acclimated larvae (Table 1).

3.3. Effect of diapause intensity by temperature acclimation

3.3.1. Glycerol, trehalose and glycogen content during acclimation

Fig. 2 shows the contents of the major sugar, trehalose, and the major polyol, glycerol, in the Shonai eco-

Table 1

Effect of length of acclimation at 15°C on the period required for adult emergence ($n = 7$), respiration (O_2 uptake and CO_2 release, $n = 4$) and respiratory quotient (RQ) ($n = 4$) in the rice stem borer of the Shonai ecotype of *C. suppressalis*. Larvae were collected on 2 October 2000 and acclimated at 15°C for 15 days (15°C, 15 d), or acclimated at 15°C for 60 days (15°C, 15 d)^a

	Period (days)	O_2 uptake (μ l/g/h)	CO_2 release (μ l/g/h)	RQ value
15°C, 15 d	63.1 \pm 7.3 **	143.2 \pm 8.57*	145.4 \pm 11.9*	1.018 \pm 0.085
15°C, 60 d	16.3 \pm 2.8	304.0 \pm 33.7	233.6 \pm 19.7	0.788 \pm 0.096

^a Significantly different from the corresponding values in larvae acclimated at 15°C for 60 days at ** $P < 0.001$ or * $P < 0.05$ by *t*-test.

type of *C. suppressalis* larvae. Glycerol was scarcely detected in larvae acclimated to 15°C for 15 days, but increased to 148.1 $\mu\text{mol/g ww}$ when 15°C-acclimated larvae were moved to 0°C for 15 days. Glycerol content was 155.5 $\mu\text{mol/g ww}$ in larvae acclimated to 15°C for 60 days. When these larvae were acclimated for another 15 days at 0°C, glycerol content rose nearly two-fold to 300.8 $\mu\text{mol/g ww}$. The trehalose content in the whole body did not change significantly at different treatments ($F = 3.03, P > 0.05$; Fig. 2).

Significant differences in glycogen contents were detected among the various treatments ($F = 32.4, P < 0.001$; Fig. 2). The glycogen content of larvae acclimated at 15°C for 15 days (151.9 $\mu\text{mol/g ww}$) was not significantly different from that of larvae acclimated

at 15°C for 60 days (130.5 $\mu\text{mol/g ww}$). However, the glycogen content decreased to 91.5 and 78.0 $\mu\text{mol/g ww}$, respectively, when larvae acclimated to 15°C for 15 and 60 days were chilled to 0°C for 15 days (Fig. 2).

3.3.2. Enzyme activities during acclimation

Total activity of GPase (Table 2) did not change significantly among the treatments ($F = 1.70, P = 0.198$), but the percentage in the active form (the *a* form) increased from 38.8% in larvae acclimated at 15°C for 15 days to 71.0% following 0°C-acclimation for 15 days. A high percentage of the *a* form was also found in larvae acclimated at 15°C for 60 days (81.6%), and in larvae acclimated at 15°C for 60 days after acclimation at 0°C for 15 days (89.5%). Temperature acclimation resulted in no significant changes in activities of PFK ($F = 1.80, P = 0.181$) or PK ($F = 0.70, P = 0.560$), two key enzymes for glycolysis. However, activity of FBPase in larvae acclimated to 15°C for 15 days was higher than in larvae subjected to the other treatments ($P < 0.05$). Temperature acclimation influenced activities of enzymes associated with glycerol synthesis significantly, except for that of G3Pase ($F = 1.20, P = 0.335$; Table 2). Activity of G3PDH was 2.14 units/g ww in larvae acclimated at 15°C for 15 days, and remained at the same level (2.48 units/g ww) when larvae acclimated 15°C for 15 days were moved to 0°C for 15 days (Table 2). However, G3PDH activity increased significantly to 3.28 units/g ww in larvae acclimated at 15°C for 60 days. When the latter larvae were chilled to 0°C for an additional 15 days, G3PDH activity remained at almost the same level, 3.12 units/g ww (Table 2). The activities of GAPase did not change significantly in larvae acclimated to 15°C for 15 and 60 days and in larvae acclimated at 15°C for 15 days following a 15-day chill to 0°C, but reached 5.01 units/g ww when larvae acclimated to 15°C for 60 days were moved to 0°C for 15 days, representing a 3.6-fold increase over other treatments (Table 2). PDHald activity in larvae acclimated at 15°C for 15 days (0.27 units/g ww) doubled after these larvae were transferred to 0°C for 15 days. PDHald activity also rose significantly to 0.54 units/g ww in the larvae acclimated to 15°C for 60 days. When the latter larvae were transferred to 0°C for 15 days, the PDHald activity increased significantly again to 0.86 units/g ww (Table 2).

Temperature acclimation resulted in no significant changes in activity of G6PDH ($F = 1.3, P = 0.297$). However, 6PGDH activity was significantly lower in the larvae acclimated to 0°C than in the larvae acclimated to 15°C ($F = 49.8, P < 0.001$; Table 2).

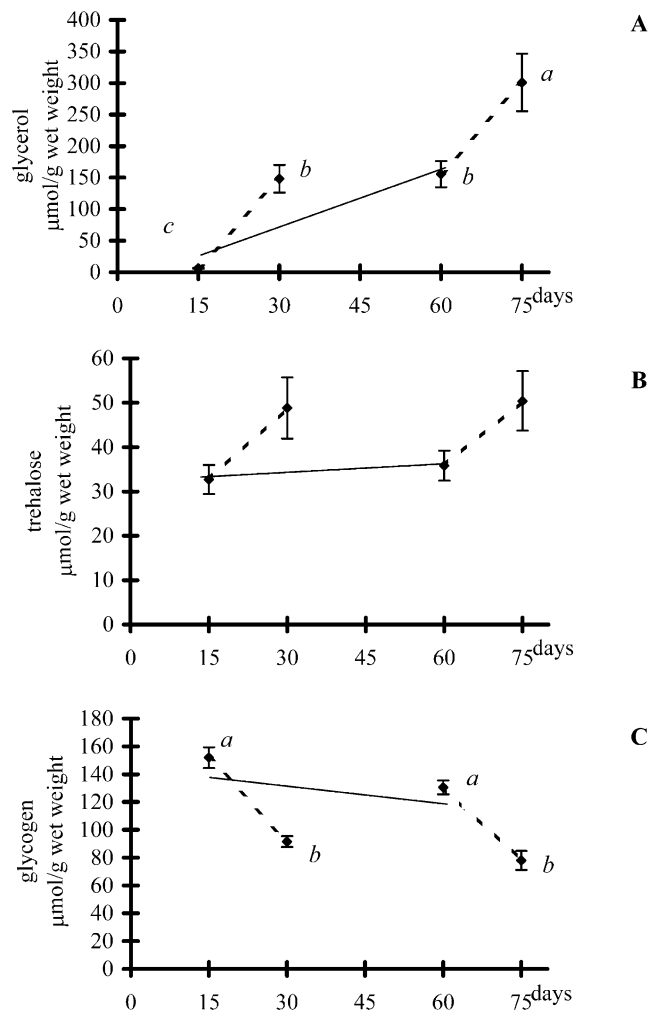


Fig. 2. Effect of temperature acclimation on the levels of glycerol (A), trehalose (B) and glycogen (C) in whole bodies of the rice stem borer of the Shonai ecotype of *C. suppressalis*. Larvae were acclimated at 15°C for 15 or 60 days, and then moved to 0°C for 15 days. The dashed line shows the effect of transferring larvae to 0°C for 15 days after 15- and 60-day acclimations at 15°C. Each symbol shows the mean value (\pm s.e.m.) of six replicates (one larva per replicate). Values labeled with the same letters are not significantly different at the 5% level by Tukey's test after ANOVA.

4. Discussion

Overwintering larvae of the Shonai ecotype of *C. suppressalis* were sampled from September to November to

Table 2

Effect of temperature acclimation on enzyme activities in larvae of *C. suppressalis*. Larvae were collected on 2 October 2000 and acclimated at 15°C for 15 days (15°C, 15 d), or acclimated at 15°C for 15 days, then transferred to 0°C for 15 days (15°C, 15d→0°C, 15 d), or acclimated at 15°C for 60 days (15°C, 60 d), or acclimated at 15°C for 60 days, then transferred to 0°C for 15 days (15°C, 60 d→0°C, 15 d). Data are units/g fresh weight, except for glycogen phosphorylase a%, and are expressed as mean±s.e.m. ($n = 6$). Values labeled with the same letters at the same row are not significantly different at the 5% level by Tukey's test after ANOVA

	15°C, 15 d	15°C, 15d→0°C, 15 d	15°C, 60 d	15°C, 60 d→0°C, 15 d
Glycogen phosphorylase (total)	1.79±0.11	1.76±0.09	1.80±0.09	2.03±0.09
Glycogen phosphorylase (a%)	38.8±2.58 ^c	71.0±2.41 ^b	81.6±5.29 ^{ab}	89.5±4.37 ^a
Phosphofructokinase	0.75±0.01	0.84±0.04	0.87±0.06	0.88±0.05
Fructose-1,6-biphosphatase	0.91±0.09 ^a	0.61±0.03 ^b	0.66±0.06 ^b	0.60±0.05 ^b
Pyruvate kinase	14.9±0.56	15.8±0.55	14.5±1.19	14.0±1.13
Glycerol-3-phosphate dehydrogenase	2.14±0.08 ^b	2.48±0.11 ^b	3.28±0.18 ^a	3.12±0.11 ^a
Glycerol-3-phosphatase	0.74±0.10	0.71±0.07	0.68±0.03	0.57±0.05
Glyceraldehyde-3-phosphatase	1.38±0.15 ^b	1.49±0.60 ^b	1.41±0.34 ^b	5.01±0.58 ^a
Polyol dehydrogenase, glyceraldehyde activity	0.27±0.03 ^c	0.53±0.05 ^b	0.54±0.05 ^b	0.86±0.04 ^a
Glucose-6-phosphate dehydrogenase	5.82±0.48	5.20±0.21	5.51±0.13	5.03±0.28
6-Phosphogluconate dehydrogenase	2.22±0.08 ^a	1.55±0.09 ^b	1.77±0.06 ^b	1.05±0.03 ^c

investigate whether an underlying developmental component influences cryoprotectant synthesis. The present results indicate that diapausing larvae collected on 22 September do not have an inherent ability to accumulate glycerol even after acclimation at low temperature (0°C). However, larvae sampled on 2 October 2 or 22 November did have the ability to accumulate high levels of glycerol as a result of the breakdown of glycogen as previously reported (Goto et al., 2001; Li et al., in press). These results indicate that overwintering larvae of *C. suppressalis* at different phases of diapause development respond differently to low temperature with respect to glycerol synthesis. To test this hypothesis, larvae collected on 2 October were used to evaluate the metabolic rearrangements associated with glycerol synthesis as a result of diapause intensity and acclimation temperature.

Our previous studies showed that in *C. suppressalis* overwintering larvae glycerol accumulation peaked at the post-diapause stage in January after the onset of cold weather (Goto et al., 2001; Li et al., in press). This suggests that glycerol accumulation in *C. suppressalis* larvae is associated with low temperature exposure and diapause intensity. Because decreasing temperature and diapause development overlap during the period of maximal glycerol production in overwintering *C. suppressalis* larvae, their separate effects are not readily apparent. Thus, diapausing larvae on 2 October, which have not begun glycerol accumulation, were collected to acclimate under different conditions. According to the period required for adult emergence at 25°C and the res-

piration rates, larvae acclimated at 15°C for 15 days were in deep diapause, while larvae acclimated at 15°C for 60 days were nearly ready to emerge from diapause. This suggests that diapause development in *C. suppressalis* does not require cold temperature exposure.

A substantial increase in the glycerol level was found in *C. suppressalis* larvae after a 60-day period (at 15°C) of diapause development. This indicates that a developmental trigger (larval development proceeds towards the end of diapause) was involved in the initiation of glycerol synthesis by the larvae, and low temperature exposure is not a prerequisite for glycerol accumulation. However, in *Choristoneura fumiferana* larvae, only negligible amounts of glycerol were detected during a 25-week period (at 18°C) in diapause larvae, and a moderate increase in glycerol level could be induced in the larvae only under low temperature (2°C) exposure (Han and Bauce, 1995). When larvae acclimated at 15°C for 15 days were transferred immediately to low temperature (0°C for 15 days), glycerol accumulation was stimulated. These data suggest that a low temperature stimulation of glycerol synthesis can probably occur at a fairly high diapause intensity, even though it is impossible for natural populations of the larvae sampled in September. The same magnitude of increase in glycerol content occurred when the transfer was delayed for 45 days, even when very high levels of glycerol (155.5 µmol/g ww) were already present. These results indicate that low temperature exposure could trigger glycerol accumulation at certain levels of diapause intensity, and that the ability to respond to low temperature cues are sustained at the

same levels while larval development proceeded towards the end of diapause. This situation was different from that of diapause larvae of *C. fumiferana*. In early- and mid-diapause larvae of this species, low temperature was favorable for glycerol synthesis, but the ability diminished as the larvae developed to the end of diapause (Han and Bause, 1995). The present results also indicate that the effects of low temperature on glycerol accumulation can be alternatively obtained by transferring larvae to relatively high temperature (15°C) to hasten the end of diapause. Furthermore, maximal glycerol accumulation could be achieved by transferring larvae at a low level of diapause intensity (i.e., larvae that are near the end of diapause) to low temperature (0°C).

During a 45 day period at 15°C, the glycogen content of *C. suppressalis* larvae decreased negligibly, whereas glycerol content increased significantly. This suggests that other intermediate substances are also converted into glycerol. These substances may be from lipid catabolism, which is suggested by an *RQ* value near 0.8 during diapause development. However, a maximal decrease in glycogen was associated with a maximal accumulation of glycerol after 0°C acclimation. Moreover, on a carbon basis, the loss of glycogen was nearly equal to the gain of glycerol. This implies that glycogen was converted to glycerol in larvae after 0°C acclimation.

The data in the present study clearly demonstrated that the metabolic pathways of glycerol synthesis in *C. suppressalis* larvae are rearranged during diapause development and cold acclimation. Although the total activity of GPase remained at the same level during the treatments, a substantial rise in the percentage of active GPase (the *a* form) after 0°C exposure and diapause development could channel more carbon sources from glycogen into the pathway of glycolysis. Cold activation of GPase (conversion of phosphorylase *b* to phosphorylase *a*) has been well documented from many insects (Ziegler et al., 1979). As reported in other overwintering insects using glycerol as the main cryoprotectant, activation of PFK is essential (Storey and Storey, 1981, 1991). However, no differences in activities of PFK among the treatments were found in *C. suppressalis* larvae (only slight increases) after 0°C exposure and diapause development at 15°C. The activity of FBPase decreased slightly after 0°C exposure and diapause development, but this change was not significant. Thus, the observed activation of glycogen breakdown coupled with an inhibition of glycogenic flux at the level of FBPase would lead to a diversion of more carbon flow into the pathway of glycerol synthesis. However, this does not imply that glycerol synthesis will be stimulated, because the pathway of glycolysis via PK also consumes some carbon sources (Storey and Storey, 1991). In this study, no significant changes in activity of PK were detected among the treatments. This means that more

triose phosphates produced by the glycolytic pathway would enter into the pathways for glycerol formation. Previous studies found two pathways for glycerol formation. Glycerol can be produced from triose phosphates either via G3PDH and G3Pase or via GAPase and PDHald (Storey and Storey, 1991). In this study, the activity of PDHald increased 1.6–2.0 times when larvae acclimated to 15°C were moved to 0°C, or when the incubation period at 15°C was increased to 60 days. The activity of GAPase increased 3.6 times when larvae acclimated at 15°C for 60 days were chilled to 0°C. However, the activity of G3PDH increased 50% after the larvae were acclimated at 15°C for 60 days, and it remained at the same level when larvae acclimated at 15°C were moved to 0°C. The present data conclusively demonstrate, therefore, that the route of glycerol synthesis from glycogen in the larvae is via the enzymes GAPase and PDHald, and an increasing pattern of activity of PDHald seems to be closely related to accumulation of glycerol. Thus, more glycolytic carbon will flow into the synthesis of glycerol after 0°C exposure and diapause development. An alternative route of glycerol synthesis, via the enzymes G3PDH and G3Pase, postulated previously by Storey and Storey (1981, 1983, 1991), probably does not function in those larvae. This is different from the case in the Saigoku ecotype larvae, where G3PDH is one of the key enzymes responsible for glycerol formation (Tsumuki and Kanehisa, 1980a, b).

Enzymes of the hexose monophosphate shunt in some overwintering insects are active in providing NADPH for polyol synthesis (Storey and Storey, 1991; Li et al., 2000b). In this study, the first two enzymes of this pathway were not stimulated, while the activity of 6PGDH tended to decrease. However, the activities of G6PDH and 6PGDH were higher than the values reported for other cold hardy insects that produce glycerol as a cryoprotectant (Joanisse and Storey, 1995). This implies either that the amounts of reducing equivalent produced by the hexose monophosphate shunt are enough for glycerol synthesis, or that the reducing power is supplied through lipid by β -oxidation. Probably β -oxidation makes at least some contribution because the *RQ* of larvae acclimated at 15°C decreased from 1.02 to 0.79, but this change in *RQ* was not significant. Analyses of the intermediates and enzymes of this pathway will need to be tested to evaluate this suggestion.

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