



# Effects of transgenic Bt cotton on overwintering characteristics and survival of *Helicoverpa armigera*

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## ARTICLE INFO

### Article history:

Received 15 September 2010

Received in revised form 20 October 2010

Accepted 20 October 2010

### Keywords:

Bt cotton

Cold-hardiness

Diapause

*Helicoverpa armigera*

Pupal survival

## ABSTRACT

The effects of transgenic Bt cotton on the overwintering generation of the cotton bollworm, *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae), are unknown. We hypothesized that a Bt cotton diet may adversely affect fitness of this generation and examined fresh weight, lipids, glycogens, low-molecular-weight sugars and SCPs (supercooling points) of pupae, as well as survival of larvae, diapausing pupae and adult emergence in comparison with controls. Field and laboratory experiments showed that larvae fed on Bt cotton had a decreased pupation rate, and fewer entered diapause and emerged as adults compared with larvae fed non-Bt cotton. Furthermore, larvae fed Bt cotton had reduced pupal weight, glycogen content and trehalose levels both in diapausing and in non-diapausing pupae, and only diapausing pupae had an increased SCP compared to controls. The SCPs of diapausing pupae reared on Bt cotton were significantly higher than those reared on non-Bt cotton. The trehalose levels of diapausing pupae reared on Bt cotton were significantly lower than those of larvae reared on non-Bt cotton. Thus, these results suggest that a Bt cotton diet weakens the preparedness of cotton bollworm for overwintering and reduces survival of the overwintering generation, which will in turn reduce the density of the first generation in the following year. Effects of transgenic Bt cotton on the overwintering generation of cotton bollworm appear to have significantly contributed to the suppression of cotton bollworm observed throughout northern China in the past decade.

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## 1. Introduction

The cotton bollworm, *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae), is a serious polyphagous pest throughout Asia (Wu et al., 2008) and has a wide range of host plants, including cotton (Zalucki et al., 1994). This species has four or five generations per year in northern China and overwinters as a diapausing pupa. The density of overwintering pupae of the cotton bollworm influences the densities of eggs and larvae of the first and succeeding generations of the following year (Ge et al., 2005). Thus, understanding the sources and sinks for the first generation each season is central to devising and delivering an integrated pest management program for cotton bollworm.

Diapause is a state of developmental arrest of insects, characterized by minima in both endergonic biosynthetic activities such as protein synthesis and exergonic energy-trapping activities such as respiration (Harvey, 1962). Diapause is generally regarded as an adaptation that has enabled insects to survive through periods unfavorable to development and/or reproduction (Pullin, 1996), or as an important strategy whereby insects can avoid adverse environmental conditions (Tauber et al., 1986; Danks,

1987). Insect diapause generally includes three different physiological processes, namely, pre-diapause, diapause and post-diapause, and each phase may include some sub-phases, the expression of which depends on both genotype-driven physiological changes and environmental conditions (Kostal, 2006). A physiological relationship between diapause and cold hardiness exists in some insects (Denlinger, 1991; Pullin, 1996). Cold hardiness is designated as the ability of an organism to survive low temperatures (Payne, 1927b). Some insects overwinter as a diapausing pupa (or at other life stage) (Danks, 1996). During the pre-diapause phase, insects must accumulate energy reserves such as carbohydrates to survive unfavorable environmental conditions before they enter winter diapause (Pullin, 1996).

Several environmental factors including photoperiod and temperature are involved in diapause induction (Nakai and Takeda, 1995; Hodkova and Socha, 1995). Additionally, the host plant has also been confirmed to be a major factor regulating insect diapause (Tauber et al., 1986) by influencing the accumulation of energy needed for overwintering (Zvereva, 2002). The host plant can affect larval growth rate, influence the sensitive stage for diapause induction, and cold hardiness of the diapausing insect (Hunter and McNeil, 1997). For example, Zvereva (2002) reported that *Salix borealis* could influence the overwintering success of the leaf beetle *Chrysomela lapponica*. Before entering winter diapause, insects

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accumulate energy reserves through feeding on host plants (Sakurai et al., 1992). These energy reserves, e.g. the low-molecular-weight sugars and/or sugar-alcohols during overwintering (Storey and Storey, 1991), are closely correlated with the nutritive quality of host plants (Zvereva, 2002). Our previous research found that the quality of six host plants including cotton, corn, kidney bean, tobacco and tomato not only affects the overwintering preparedness of *H. armigera* larvae but also influences the physiological dynamics of diapausing pupae; these factors are related to cold hardiness (Liu et al., 2007, 2009).

Bt crops, expressing the toxin protein from *Bacillus thuringiensis* (Bt), have become an important tool for insect pest management worldwide (Huang et al., 2002; Horner et al., 2003; James, 2007). Transgenic Bt cotton has recently been planted on a large scale in China, and it effectively protects plants from infestation by Lepidoptera and decreases usage of insecticides (Huang et al., 2002). Direct mortality to feeding larvae is the primary mechanism invoked to explain how Bt cotton may suppress *H. armigera* in northern China (Wu et al., 2008), and information on the effects of Bt cotton on the larvae and pupae of the overwintering generation are scarce. Such information is essential for the development of a theoretical foundation for managing overwintering populations and improving forecasts of the population dynamics of this pest.

We hypothesized that a Bt cotton diet may adversely affect fitness of the overwintering generation of *H. armigera*. Three queries were examined in this study: (1) How does Bt cotton affect the incidence of pupation and diapause incidence in *H. armigera*? (2) Does Bt cotton reduce the initial density and/or the ongoing survival of diapausing pupae, and subsequent adult emergence of *H. armigera* after a period of low temperature? (3) What are the physiological mechanisms whereby Bt cotton modifies the diapause of larvae and the overwintering preparedness of pupae?

## 2. Materials and methods

### 2.1. Insects and host plants

Cotton bollworm, *H. armigera* (Hübner) (Lepidoptera: Noctuidae) were obtained from a commercial insectary (Henan Jiyuan Baiyun Industry Co., Ltd., China). Neonate larvae were reared in groups until the 3rd instar, after which they were individually reared in glass tubes (2.0 cm diam. × 8.0 cm height) to prevent cannibalism. The larvae were reared on an artificial diet until the 3rd instar at 25 °C under a photoperiod of L:D = 14:10.

Two visible morphological characteristics of arrested development, the prolonged presence of pigmented eye spots and the condition of the fat body, were used to determine whether pupae were in diapause as cited (Liu et al., 2006).

Host plants used in this study were: (1) Bt cotton (GK12), a transgenic variety of cotton expressing the protein Cry1A, supplied by the Biotechnology Research Institute of Chinese Academy of Agricultural Sciences (Beijing); (2) non-Bt cotton variety (Simian3), obtained from the Institute of Plant Protection, Chinese Academy of Agricultural Sciences (Beijing). The plant seeds were obtained from the Chinese Academy of Agricultural Sciences. Host plants used in the experiments were planted on April 28, in 2009, consisting of ~45,000 plants/ha, and grown without pesticides in the field at Langfang Experiment Station (39.53° N, 116.70° E, Hebei Province, China). Plants were watered as needed and fertilized with a controlled release fertilizer. Total experimental area was ~0.5 ha equally divided between the two plant varieties.

### 2.2. Field studies

The 3rd instar larvae of the cotton bollworm were placed on two host plants of Bt cotton and non-Bt cotton on five dates in 2009,

respectively. About 200 larvae of the 2nd instar were inoculated on one of three plots of each cotton plant. Each plot was covered by a nylon net (5 m × 3 m × 2 m), containing 40–50 plants, to prevent larvae from escaping or being preyed upon by their natural enemies. Pupation was measured ~20 days after the inoculation in each plot. The incidence of pupation in each plot was calculated, and pupal survival was recorded. Ten days after pupation, diapause incidence was examined.

### 2.3. Diapause induction in the laboratory

In September 2009, larvae of the second instar of *H. armigera* (about 2000 individuals) were obtained from a commercial insectary (Henan Jiyuan Baiyun Industry Co., Ltd., China). The larvae were reared on an artificial diet until the third instar at 25 °C under a photoperiod of L:D = 14:10. Thereafter, the third instar larvae were transferred to the fruits of Bt and non-Bt cotton, respectively, at 20 °C under a photoperiod of L:D = 10:14 to induce winter diapause; the third to final instar larvae are most sensitive to diapause induction (Li and Xie, 1981). There were five replicates of each plant. Under these circumstances, the duration of larvae fed on the fruits varied from 15 to 25 days and the fruits were replaced with fresh ones every second day until pupation. Incidence of pupation ( $n/N\%$ ) of each replicate was calculated. Pupae were examined 10 days after pupation to determine their diapause status, and then their fresh mass (fw) weight was obtained. Diapause incidence ( $m/n\%$ ) was calculated. A sub-sample of pupae from each group was dried for 72 h at 60 °C, and their dry mass (dw) was determined for comparison with fw. Water content (% fw) and dry mass were (dw) calculated as  $[(fw - dw)/fw] \times 100$ ,  $fw \times (1 - \text{water content})$ , respectively.

### 2.4. Determination of pupal survival and adult emergence after a period of low temperature

To simulate the overwintering environment of the pest in nature, about 100 diapausing pupae of each treatment were embedded in moist soil in glass tubes (12 cm diam. × 7.0 cm height) and then placed outdoors in bare soil at a depth of 10 cm on 24 October 2009. Soil (at a depth of 10 cm) temperature was recorded every day. After two months, on 24 December, 100 overwintering pupae, respectively, from larvae reared on Bt and non-Bt cotton were disinterred to measure pupal survival ( $S\%$ ). These pupae were kept at 20 °C for 2 days to allow adults to emerge. Each pupa was then examined and labeled as dead or alive by the presence or absence of abdominal movement in response to stimulation. Adult emergence from alive pupa was also examined and the rate of adult emergence was defined as ( $E\%$ ).

### 2.5. Measurement of supercooling points (SCPs)

Approximately forty diapausing pupae were sampled randomly in each treatment (Bt and non-Bt cotton). Each individual pupa was fixed to a thermocouple linked to an automatic recorder (uR100, Model 4152, Yokogawa Electric Co., Seoul, Korea) via a bridge. Each pupa, with the thermocouple attached, was lowered into a freezing chamber held at –30 °C; the chamber temperature was lowered at a rate of 1 °C/min, and the declining body temperature of the pupa was measured. The SCP of each pupa was considered to be the temperature recorded by the thermocouple just before the thermocouple registered a rise in temperature caused by the emission of the latent heat of crystallization.

### 2.6. Measurement of lipid and glycogen levels

Measurements of lipid and glycogen levels were made using methods described in Liu et al. (2007). Twelve dried pupae from

each treatment were homogenized and their lipid content extracted with a chloroform–methanol (2:1) solution (Folch et al., 1957). After centrifugation at  $2600 \times g$  for 10 min, the supernatant was removed and the process repeated twice. The resulting pellet was dried at  $60^\circ\text{C}$  for 72 h and the lean dry weight (ldw) determined. Lipid content (mg/mg dw) was calculated with the formula:  $[(dw - ldw)/(dw/1000)]$ .

Twelve fresh pupae were homogenized with 2 ml 70% ethanol and centrifuged at  $2600 \times g$  for 10 min. Pooled supernatants from two replications of this process were discarded and the remaining pellet was used to isolate glycogen according to the method described in Ohtsu et al. (1992). Two milliliters 10% (v/v) trichloroacetic acid was added to the residue. The mixture was boiled in water for 15 min and then cooled and centrifuged at  $3000 \times g$  for 15 min. The supernatant was used to measure glycogen levels. Glycogen was determined by the phenol and sulfuric acid method (Dubois et al., 1956). Absorbance was determined at 490 nm on a spectrophotometer (DU650, Beckman, CO, USA). The results were expressed in mg glycogen/g dw using a calibration curve obtained by measuring glycogen standards in seven concentrations ranging from 0 to 0.6 mg/ml in decremental steps of 0.1 mg/ml.

### 2.7. Measurement of low-molecular-weight sugars and sugar-alcohols

Measurement of low-molecular-weight sugars and sugar-alcohols was made using methods described (Liu et al., 2007). Twelve diapausing pupae and 12 non-diapausing pupae were sampled randomly in each treatment (Bt cotton and cotton), and 10  $\mu\text{l}$  haemolymph was sampled per individual. Low-molecular-weight cryoprotectants were determined by capillary gas chromatography as their *o*-methyloxime trimethylsilyl derivatives, following the method described by Kostal and Simek (1996). In brief, 10  $\mu\text{l}$  haemolymph per individual was homogenized with 0.4 ml 70% (v/v) ethanol containing 10 mg of erythritol (an internal standard) in an Eppendorf tube that had been rinsed with 0.2 ml 70% ethanol. After centrifugation at  $10,000 \times g$  for 5 min, the supernatant was removed and the procedure repeated. The pooled supernatant was stored at  $-20^\circ\text{C}$ . Before analysis, the samples were evaporated to dryness under a stream of nitrogen at  $40^\circ\text{C}$  in a derivatization vial. Twenty-five microliters of dimethylformamide and 30  $\mu\text{l}$  *o*-methyl-hydroxylamine in pyridine (200 mg/ml) were added to the residue for oximation, and the sample was then heated at  $70^\circ\text{C}$  for 15 min. Silylation was accomplished by adding 75  $\mu\text{l}$  of dimethylformamide and 30  $\mu\text{l}$  trimethylsilylimidazol to the reaction mixture, which was further heated to  $80^\circ\text{C}$  for 15 min. After the desired derivatives were re-extracted into isooctane using  $2 \times 75 \mu\text{l}$  of the solvent, 1  $\mu\text{l}$  aliquot was injected into a chromatograph (Agilent 7890 GC) equipped with a splitless capillary injector, a flame ionization detector, and an HP 3394 integrator. Separation and quantification of sugars and polyols were achieved on a  $30 \times 250 \mu\text{m}$  i.d. DB-5 fused silica capillary column (J&W Scientific Inc.). The temperature program was held at 3 min at  $120^\circ\text{C}$ , and then increased to  $10^\circ\text{C}/\text{min}$  to  $280^\circ\text{C}$ , followed by a 40 min hold. Nitrogen (50 cm/s) was used as a carrier gas. Identification of the components was made by comparison with authentic standards.

### 2.8. Statistical analysis

All statistical analyses were conducted using SPSS software (SPSS.13, 2004). Incidence of pupation and diapause incidence was performed with Independent samples *t* Test. Fresh pupal weight (mg), water content % fw, SCPs, energy reserve (lipid and glycogen), low molecular weight substances were analyzed with

Independent samples *t* Test and GLM (SPSS); cotton variety and diapause status were the fixed factors. Pupal survival and adult emergence were tested by Chi-square. The percentage data in this paper were arcsine transformed before analysis, and untransformed data were presented.

## 3. Results

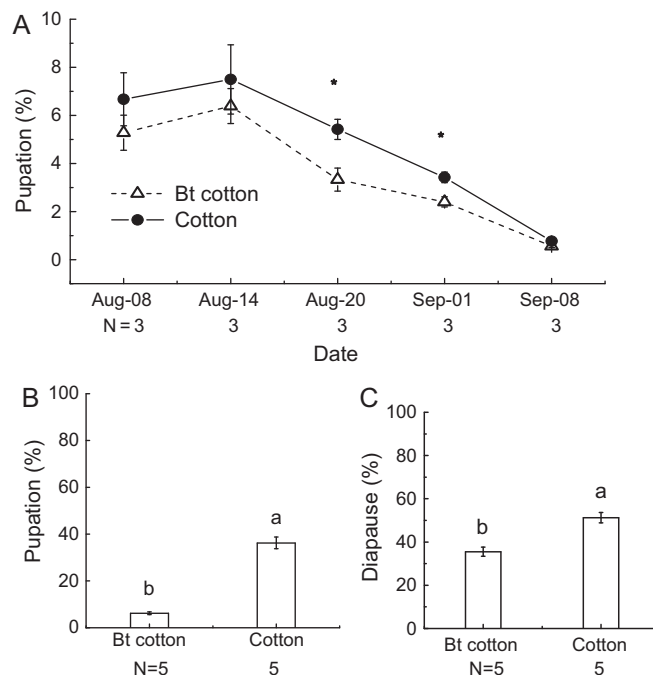
### 3.1. Pupation and diapause incidence of *H. armigera* fed on Bt and non-Bt cotton

The incidence of pupation for *H. armigera* larvae fed on Bt cotton was lower than those fed on non-Bt cotton in the field (Fig. 1A). The incidence of pupation for *H. armigera* fed on Bt cotton was significantly lower than for those fed on non-Bt cotton when the dates of artificial inoculation were August 20, 2009 ( $t = -3.273$ ,  $p = 0.031$ ) (Fig. 1A) and September 1 ( $t = -3.318$ ,  $p = 0.029$ ) (Fig. 1A). The diapause incidence was zero for *H. armigera* inoculated in the field on the five dates examined.

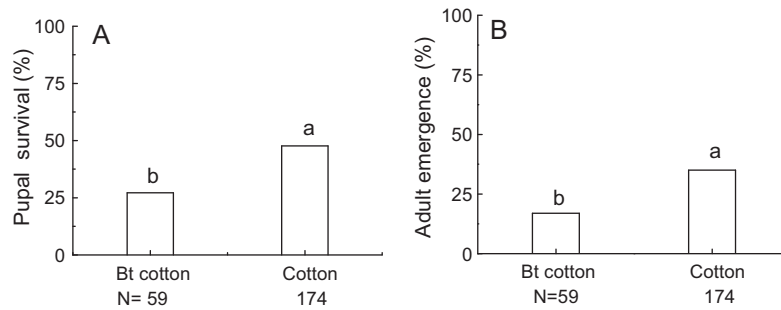
Similarly, *H. armigera* raised in the laboratory and fed on Bt-cotton had a significantly lower pupation incidence than those fed on non-Bt cotton ( $t = -11.261$ ,  $p < 0.001$ ) (Fig. 1B). Furthermore, the diapause incidence of larvae fed on Bt cotton was lower than that of larvae fed on non-Bt cotton in the laboratory ( $t = -4.864$ ,  $p = 0.001$ ) (Fig. 1C).

### 3.2. Pupal survival and adult emergence after a period of low temperature

Survival of overwintering pupae from larvae reared on Bt cotton (27.0%) was significantly lower than those of larvae reared on non-Bt cotton (48.0%) ( $\chi^2 = 5.880$ ,  $p = 0.015$ , Chi-square) (Fig. 2A). Soil



**Fig. 1.** Pupation (A) of *H. armigera* larvae fed on Bt cotton and non-Bt cotton (cotton) in the field from August 08 to September 08, 2009. Error bars indicate the SE. N indicates the number of samples tested. Each test was comprised of three replicates. \*Significant differences between rates of pupation of *H. armigera* larvae fed Bt cotton compared to non-Bt cotton  $p < 0.05$  (independent samples *t* Test). Pupation (B) and diapause incidence (C) of *H. armigera* larvae fed on Bt cotton and non-Bt cotton (cotton) in the laboratory. Each test was comprised of five replicates. Different lowercases above the bars indicate significant differences in larvae reared on Bt cotton vs non-Bt cotton at  $p < 0.05$  (independent samples *t* Test).



**Fig. 2.** Pupal survival (A) of diapausing pupae and adult emergence (B) of *H. armigera* reared on Bt and non-Bt cotton (cotton). *N* indicates the number of samples tested. Different letters above bars indicate significant differences at  $p < 0.05$  ( $\chi^2$  test).

temperatures at a depth of 10 cm were recorded daily from October 24 to December 24 in 2009. During this time, temperatures in the soil fluctuated within a range of  $-5$  to  $15$  °C.

Correspondingly, adult emergence of *H. armigera* fed on Bt cotton (17.0%) was significantly lower than those fed on non-Bt cotton (35.0%) ( $\chi^2 = 6.231$ ,  $p = 0.013$ , Chi-square) (Fig. 2B).

### 3.3. Characteristics of diapausing and non-diapausing pupae of *H. armigera*

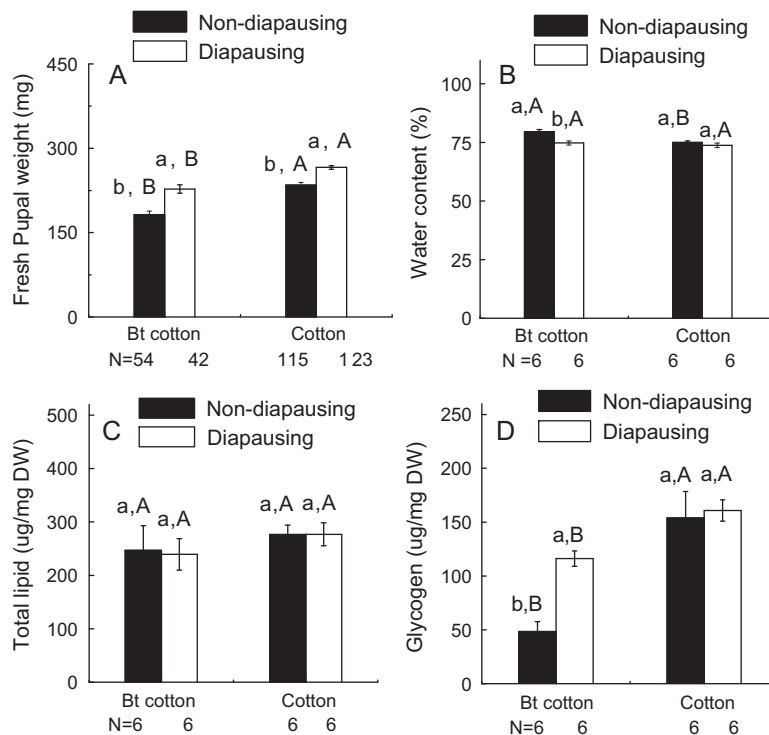
Fresh weights of diapausing pupae of *H. armigera* fed on Bt cotton and non-Bt cotton were heavier than those of non-diapausing pupae (Fig. 3A). Fresh mass of diapausing pupae from larvae fed on Bt cotton (227.6 mg) was significantly lower than for those fed on non-Bt cotton (265.9 mg) ( $t = -5.194$ ,  $p < 0.001$ ) (Fig. 3A). Similar results were found for fresh mass of the non-diapausing pupae. The fresh mass of non-diapausing pupae from larvae fed on Bt cotton (181.8 mg) was significantly lower than for

those fed on non-Bt cotton (234.5 mg) ( $t = -6.494$ ,  $p < 0.001$ ) (Fig. 3A). The effects of crop variety (Bt cotton and non-Bt cotton) and diapause status (diapausing and non-diapausing) on pupal weight were highly significant (Table 1 A).

Non-diapausing pupae from larvae fed on Bt cotton contained a significantly higher water content compared to pupae whose larvae had fed on non-Bt cotton ( $t = 3.889$ ,  $p = 0.003$ ) (Fig. 3B); similarly, water content of diapausing pupae from larvae fed on Bt cotton was significantly higher than in non-diapausing pupae from larvae fed on Bt cotton ( $t = -3.739$ ,  $p = 0.004$ ) (Fig. 3B). The effects of crop variety and diapause status on body water were significant (Table 1, B).

There were no significant differences in the total lipid of diapausing pupae from larvae fed on Bt cotton vs non-Bt cotton (Fig. 3C). Similar results were observed for the average total lipid contents of non-diapausing pupae (Fig. 3C) (Table 1, C).

Glycogen levels of diapausing pupae from larvae reared on Bt cotton (116.2  $\mu\text{g}/\text{mg dw}$ ), was significantly less than from larvae reared on non-Bt cotton (160.8  $\mu\text{g}/\text{mg dw}$ ) ( $t = -3.839$ ,  $p = 0.005$ )



**Fig. 3.** Fresh pupal weight (A), contents of body water (B), lipid (C), and glycogen (D) between diapausing and non-diapausing *H. armigera* larvae fed on Bt cotton and non-Bt cotton (cotton). Error bars indicate the SE. *N* indicates the number of samples tested. Different lowercases show significant difference between diapausing and non-diapausing pupae of larvae reared on the same crop, and different uppercases above the bars indicate significant differences in diapausing or non-diapausing pupae of larvae reared on Bt cotton vs non-Bt cotton (cotton) at  $p < 0.05$  (independent samples *t* Test).

**Table 1**Analysis of variance partitioning effects (GLM) of crop variety and diapause status on characteristics of pupae of *Helicoverpa armigera*.

No.	Variable	Source	df	MS	F	Sig.
A	Fresh pupal weight (mg)	Crop variety <sup>a</sup>	1	145,907.719	69.668	<0.0000 <sup>****</sup>
		Diapause status <sup>b</sup>	1	104,868.082	50.073	<0.0000 <sup>****</sup>
		Crop variety×diapause status	1	3648.791	1.742	0.1876
		Error	381	2094.321		
B	Water content % fw	Crop variety	1	29.300	6.188	0.0235 <sup>*</sup>
		Diapause status	1	39.421	8.326	0.0103 <sup>*</sup>
		Crop variety×diapause status	1	19.219	4.059	0.0600
		Error	17	4.735		
C	Total lipid (μg/mg dw)	Crop variety	1	3572.564	0.692	0.4169
		Diapause status	1	302.216	0.059	0.8117
		Crop variety×diapause status	1	0.060	0.000	0.9973
		Error	17	5159.329		
D	Glycogen (μg/mg dw)	Crop variety	1	24,309.909	20.557	0.0003 <sup>***</sup>
		Diapause status	1	4883.626	4.130	0.0581
		Crop variety×diapause status	1	6685.602	5.653	0.0294 <sup>*</sup>
		Error	17	1182.564		
E	Trehalose (μg/μl haemolymph)	Crop variety	1	2219.485	19.700	0.0013 <sup>**</sup>
		Diapause status	1	3782.715	33.575	0.0002 <sup>***</sup>
		Crop variety×diapause status	1	152.378	1.352	0.2718
		Error	10	112.664		
F	SCPs (°C)	Crop variety	1	165.251	16.066	0.0001 <sup>***</sup>
		Diapause status	1	9.086	0.883	0.3494
		Crop variety×diapause status	1	30.086	2.925	0.0901
		Error	106	10.286		

<sup>a</sup> Crop variety (Bt cotton and non-Bt cotton).<sup>b</sup> Diapause status (diapausing and non-diapausing).<sup>\*</sup>  $p < 0.05$ .<sup>\*\*</sup>  $p < 0.01$ .<sup>\*\*\*</sup>  $p < 0.001$ .<sup>\*\*\*\*</sup>  $p < 0.0001$ .

(Fig. 3D), and glycogen levels of non-diapausing pupae from larvae reared on Bt cotton (48.5 μg/mg dw), were also significantly less than those of larvae reared on non-Bt cotton (153.8 μg/mg dw) ( $t = -4.319$ ,  $p = 0.002$ ) (Fig. 3D). Glycogen content of non-diapausing pupae of larvae reared on Bt cotton (116.2 μg/mg dw), was significantly less than that of diapausing pupae reared on Bt cotton (48.5 μg/mg dw) ( $t = -5.879$ ,  $p < 0.001$ ) (Fig. 3D). The effects of crop variety on body water were highly significantly influenced (Table 1, D). The interaction between crop variety and development state had a significant effect on glycogen (μg/mg dw).

#### 3.4. SCPs of diapausing and non-diapausing pupae of *H. armigera*

The SCPs of diapausing pupae from larvae reared on Bt cotton ( $-16.8$  °C), were significantly higher than those reared on non-Bt cotton ( $-19.5$  °C), ( $t = -3.199$ ,  $p = 0.002$ ) (Fig. 4). There was no significant difference in the mean SCPs of non-diapausing pupae from larvae reared on Bt cotton vs non-Bt cotton.

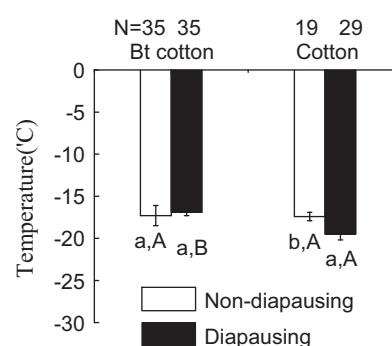
Non-diapausing pupae of *H. armigera* from larvae fed on non-Bt cotton exhibited significantly higher mean SCPs ( $-17.4$  °C) than did diapausing pupae ( $-19.5$  °C) ( $t = -2.385$ ,  $p = 0.020$ ) (Fig. 4). However, when larvae of *H. armigera* fed on Bt cotton, there was no significant difference in the mean SCPs between diapausing and non-diapausing pupae. The effects of crop variety on SCPs were highly significant (Table 1, F).

#### 3.5. Low-molecular-weight substances

Seven low-molecular-weight substances were identified as free sugars and sugar-alcohols in diapausing and non-diapausing pupae of *H. armigera*: glycerol, mannose, sorbitolum, glucose, galactose, myo-inositol, trehalose (Fig. 5A and B), and trehalose predominated.

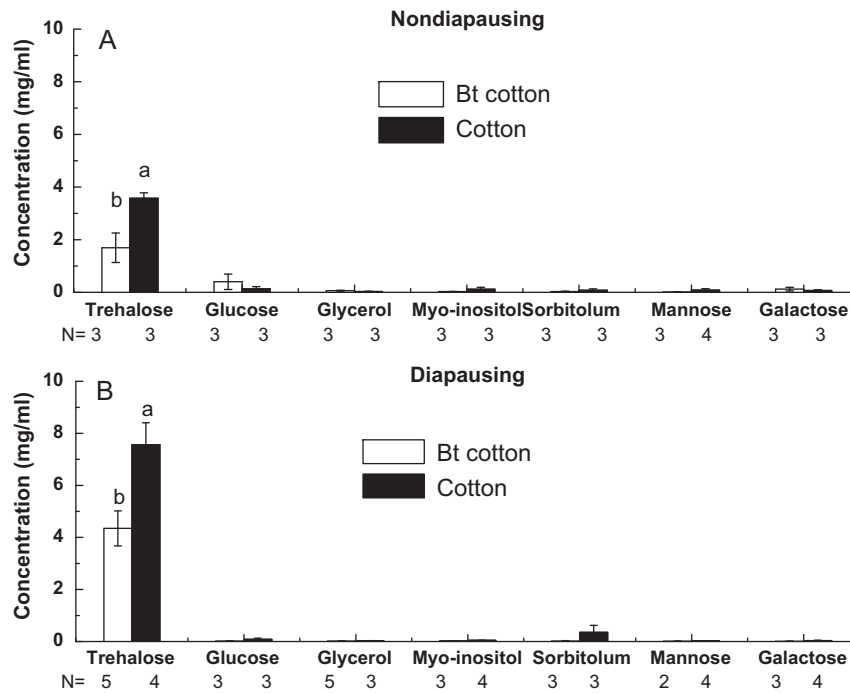
In non-diapausing pupae of *H. armigera*, only trehalose exhibited significant differences in pupae from larvae fed on Bt cotton vs non-Bt cotton. The trehalose levels of non-diapausing pupae reared on Bt cotton (1.70 μg/μl haemolymph) were significantly lower than those reared on non-Bt cotton (3.57 μg/μl haemolymph) ( $t = -3.141$ ,  $p = 0.020$ ) (Fig. 5A).

In diapausing pupae of *H. armigera*, trehalose exhibited significant differences in pupae from larvae fed on Bt cotton vs non-Bt cotton. Trehalose levels of diapausing pupae reared on Bt cotton (4.35 μg/μl haemolymph) were significantly lower than those reared on non-Bt cotton (7.56 μg/μl haemolymph) ( $t = -2.977$ ,  $p = 0.041$ ) (Fig. 5B).



**Fig. 4.** Supercooling points (SCPs) of diapausing and non-diapausing pupae of *H. armigera* reared on Bt and non-Bt cotton (cotton). Error bars indicate the SE. *N* indicates the number of samples tested. Different lowercases show significant differences between diapausing and non-diapausing pupae from larvae reared on the same crop, and different uppercases above the bars indicate significant differences in diapausing or non-diapausing pupae from larvae reared on Bt cotton vs non-Bt cotton at  $p < 0.05$  (independent samples *t* Test).





**Fig. 5.** The concentration (mg/ml haemolymph) of low molecular weight substances in non-diapausing (A) and diapausing (B) pupae from larvae fed on Bt cotton and non-Bt cotton. Error bars indicate the SE. *N* indicates the number of samples tested. Different lowercases above the bars indicate significant differences of diapausing and non-diapausing pupae of larvae reared on between Bt cotton and non-Bt cotton at  $p < 0.05$  (independent samples *t* Test).

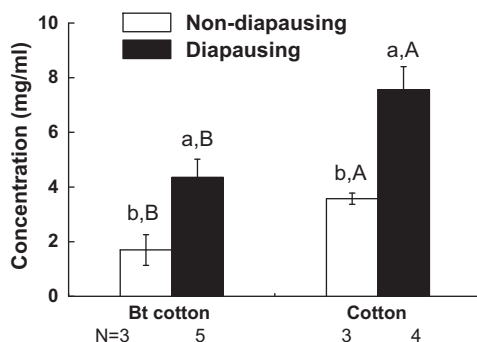
The concentration of trehalose in diapausing pupae (4.35  $\mu\text{g}/\mu\text{l}$  haemolymph) from Bt cotton was significantly higher, than that in non-diapausing pupae (1.70  $\mu\text{g}/\mu\text{l}$  haemolymph) ( $t = 3.052$ ,  $p = 0.028$ ) (Fig. 5). Pupae of *H. armigera* from non-Bt cotton had a significantly higher concentration of trehalose (7.56  $\mu\text{g}/\mu\text{l}$  haemolymph) compared to non-diapausing pupae (3.57  $\mu\text{g}/\mu\text{l}$  haemolymph) ( $t = 5.339$ ,  $p = 0.003$ ) (Fig. 6). The effects of crop variety and diapause status on the concentration of trehalose were highly significant (Table 1, E).

#### 4. Discussion

Bt cotton has been linked to decreased densities of eggs and larvae of cotton bollworm in the second and third generation throughout northern China in the past decade (Wu et al., 2008).

The moth densities observed for three generations of *H. armigera* that occurred between early June and late September were reduced with the introduction of Bt cotton in northern China (Gao et al., 2010). The overwintering generation of the cotton bollworm is very different with the summer generations in both endergonic biosynthetic activities and exergonic energy-trapping activities, and we show here that Bt cotton significantly decreased the pupation incidence and diapause incidence of *H. armigera* in the overwintering generation. Moreover, the Bt cotton caused higher mortality to diapausing pupae exposed to low temperatures. Survival of diapausing pupae and emergence of adults from larvae fed on Bt cotton were significantly lower than in those reared on non-Bt cotton. The inhibitory effect of Bt cotton on the overwintering generation of the cotton bollworm (*H. armigera*), especially on diapausing pupae, can reverberate through densities of the first and subsequent generations in the following year.

Overwintering success in a prolonged winter depends on both environmental factors and internal physiological processes (Han and Bauce, 1998). One of the major factors that determines the performance and physiology of herbivorous insects is the host plant (Scriber and Slansky, 1981; Naya et al., 2007). Our previous research indicated that different host plants could affect the nutritional preparedness of *H. armigera* pupae for overwintering and the insect's ability to overwinter (Liu et al., 2007, 2009). This research implies that Bt cotton can reduce cold hardiness of the overwintering pupae of *H. armigera*. Physiological characters determined by integrating indexes in physiology of *H. armigera* indicate that diapausing pupae from larvae fed on Bt-cotton are less cold hardy than those from larvae fed on non-Bt cotton. Overwintering mortality may result from the failure of diapause development, inadequate preparation to withstand the cold, or the premature exhaustion of energy reserves, and all of these could be significantly influenced by pre-winter circumstances (Han and Bauce, 1998). The supercooling capacity of overwintering pupa is significantly associated with host plants, pupal weight, water content, lipid and glycogen, and the concentration of glycerol (Liu



**Fig. 6.** Concentrations (mg/ml haemolymph) of trehalose in non-diapausing and diapausing pupae from larvae fed on Bt cotton and non-Bt cotton. Error bars indicate the SE. *N* indicates the number of samples tested. Different lowercases show significant difference between diapausing and non-diapausing pupae of larvae reared on the same crop, and different uppercases above the bars indicate significant differences in diapausing or non-diapausing pupae of larvae reared on between Bt cotton and non-Bt cotton at  $p < 0.05$  (independent samples *t* Test).

et al., 2007). Pupal survival also varies among host plants and is significantly related to pupal weight, lipid content, SCPs, and the concentration of trehalose. The higher the quality of the larval host plants, the better the insect's preparedness for overwintering, and the higher its chances for survival (Liu et al., 2007).

Cold hardiness can be monitored by measuring the supercooling point and water content (Payne, 1927a). Supercooling points have been considered an index of cold hardiness in many, but not all, insects (Worland, 2005). Low supercooling points can often be ascribed to the absence of ice-nucleating agents, an accumulation of cryoprotectant elements, or both (Milonas and Savopoulou-Soultani, 1999). Our results showed that SCPs of pupae from larvae reared on Bt cotton were much higher than those on non-Bt cotton, which implies that accumulation of cryoprotectant elements may be disturbed by Bt toxin. Overwintering pupae from larvae reared on Bt cotton may suffer from chilling damage during a prolonged cold winter due to having a high SCP. Simultaneously water content did not differ in overwintering pupae from larvae reared on Bt cotton vs non-Bt cotton.

Individuals accumulate large amounts of nutrients in pre-winter feeding (Hokkanen, 1993). Lipid and glycogen are two of the main forms of energy reserves whose patterns of use can differ during diapause (Adedokun and Denlinger, 1985). Large amounts of metabolic reserves, in the form of lipids and glycogen, are typically accumulated prior to diapause (Kostal et al., 1998; Ding et al., 2003). Lipids are the energy source for post-diapause development (Kostal et al., 1998) and probably directly affect post diapause pupal survival (Liu et al., 2007). In this study, pupal weights from larvae fed on Bt cotton were lower than those fed on non-Bt cotton for both diapausing and non-diapausing pupae based on fresh and dry pupal weights, which suggests that Bt toxin can disturb the weight gain processes in the cotton bollworm. The results of total lipid ( $\mu\text{g}/\text{mg}$  dw) in pupae from Bt cotton were lower than in those fed on non-Bt cotton in both diapausing and non-diapausing pupae, which implies that larvae fed on Bt cotton that produced the overwintering generation were not provided with enough energy to sustain life through diapause during a cold, long winter. Larvae that fed on Bt cotton may have compromised their development for overwintering by diverting scarce metabolic activity to degrade the Bt toxin. Glycogen, serving as the main metabolic fuel during the inactive diapause state, decreases substantially toward the end of diapause (Kostal et al., 1998). Metabolism of glycogen has been linked to the production of cryoprotectants (Li et al., 2002). Levels of accumulated glycogen reserve are highly linked to potential success in overwintering (Liu et al., 2007). In the present study, fewer glycogen reserves, measured as  $\mu\text{g}/\text{mg}$  dw, were found in the pupae from larvae fed on Bt cotton compared to those on non-Bt cotton in the overwintering generation at the onset of winter, indicating that Bt cotton can weaken the overwintering status of diapausing pupae.

In addition to accumulation of lipid and glycogen, many insect species accumulate low-molecular-weight substances such as cryoprotectants (Salt, 1961). Low-molecular-weight sugars and polyols, including glycerol, trehalose and inositol, have been reported as cryoprotective agents in many species of insects (Kostal and Simek, 1996). Trehalose, glycerol and inositol, were the main low-molecular-weight substances in diapausing pupae of the cotton bollworm, *H. armigera*, and trehalose and glycerol play important roles in both SCPs and pupal survival during overwintering (Liu et al., 2007). Trehalose is more involved in metabolism, and glycerol is the main supercooling reagent (Munyiri and Ishikawa, 2005). In this study, of all low-molecular-weight substances, only trehalose was kept at a high level in pupae of the cotton bollworm, which supports a previous study on diapausing pupae (Liu et al., 2007). Levels of trehalose in pupae reared on Bt cotton were significantly lower than that on non-Bt cotton for both diapausing and non-diapausing pupa, which

suggests that Bt cotton can influence metabolism of carbohydrates in pupae before overwintering. Glycerol levels were not significantly different among diapausing or non-diapausing pupae from either Bt cotton or non-Bt cotton.

This study indicates that Bt cotton suppressed the onset of diapause and of survival during diapause and success of adult emergence in the overwintering generation of the cotton bollworm. Hence, planting of transgenic Bt cotton can play an important role in regulating the population dynamics in the overwintering generation of the cotton bollworm that carry over to affect densities into the next year. A recent report demonstrated that a marked decrease in regional outbreaks of the cotton bollworm in multiple crops was associated with the planting of Bt cotton by analyzing egg and larval densities in the second and third generations of *H. armigera* on cotton and other crops from 1992 to 2007 in northern China (Wu et al., 2008). Effects of transgenic cotton on the overwintering generation may be one of the factors that explain a marked decrease in regional outbreaks of the cotton bollworm (*H. armigera*) observed throughout northern China in the past decade. Our present work indicates that in addition to the suppression effects Bt confers on cotton bollworm densities during the growing season, the effects on the overwintering physiological dynamics of the cotton bollworm during and after pupation also affect overwintering survival of this pest. Further work is needed to determine how transgenic cotton influences the occurrence of diapause before overwintering, emergence of the adult and fitness of the offspring after overwintering. This information will aid in devising better strategies to manage this pest.

#### Acknowledgements

We are grateful to Prof. Marvin Harris from Texas A&M University for reviewing the manuscript draft, and to Dr. Yucheng Sun from Institute of Zoology, Chinese Academy of Sciences for his constructive criticisms of earlier drafts. This project was supported by "National Basic Research Program of China" (973 Program) (No. 2006CB102006), National Nature Science Fund of China (Nos. 31030012 and 30621003) and the Special Program for new transgenic variety Breeding of the Ministry of Science and Technology, China (No. 2009ZX08012-005B).

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