# Diapause, cold hardiness and flight ability of Cry1Ac-resistant and -susceptible strains of *Helicoverpa armigera* (Lepidoptera: Noctuidae)

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**Abstract.** The diapause inducement condition, cold hardiness, and flight ability in Cry1Ac-resistant (BtR) and Cry1Ac-susceptible (96S) strains of *Helicoverpa armigera* (Hübner) were compared in the laboratory. The BtR strain was derived from the 96S strain and shows 1375-fold resistance to the Cry1Ac toxin after having been selected for 52 generations. Compared with the 96S strain, the Bt-resistant strain was more likely to go into diapause under a short-photoperiod environment. At 11L: 13D, 12L: 12D and 13L: 11D photoperiods, the percentages of BtR insects entering diapause were 72.7%, 82.9% and 68.7%, respectively, which were significantly higher than those in the 96S strain (58.6%, 67.4% and 46.3%, respectively) under the same conditions. The supercooling points (SCP) and freezing points (FP) were not significantly different between the BtR and 96S strains. The LT<sub>50</sub> (50% lethal time) and LT<sub>90</sub> (90% lethal time) of BtR pupae were also not significantly different from those of the 96S strain at –15°C. The moths from both strains had similar flight ability when their larvae were fed with nontoxic control diet. However, the total flight distance of these BtR moths was 56.2 km whose larvae fed on normal diet, which was more than twice as much as for those feeding on Bt diet (26.2 km). Flight duration for these BtR moths was longer after feeding on normal diet (11.6 h) than after feeding on Bt diet (7.3 h).

### INTRODUCTION

Helicoverpa armigera (Hübner) is one of the most serious pests of cotton, maize, wheat, sorghum and many other crops in the old world (Luttrell et al., 1994; Guo, 1997) and its high field-resistance to chemical insecticides is a major threat to cotton production in many countries (Forrester et al., 1993; Wu et al., 1997a). Currently, Bacillus thuringiensis Berliner transgenic cotton (Bt cotton) engineered to express a  $\delta$ -endotoxin from Bt has become a major means for control of this pest in China, India and Australia (Shelton et al., 2002; Wu & Guo, 2005).

One of the main concerns associated with the widespread adoption of Bt cotton is the evolution of H. armigera resistance to Cry1Ac. In fact, the capacity of H. armigera for resistance development to Cry1Ac has been demonstrated in several laboratory-selected strains from India, China and Australia (Kranthi et al., 2000; Liang et al., 2000; Akhurst et al., 2003). The "high-dose/refuge" strategy, has been in practice to delay the development of field resistance to Bt crops by insect pests (Bates et al., 2005). In this strategy, the Bt crop was designed to produce enough toxin to kill nearly all heterozygous insects, while a nearby area of non-Bt plants would allow susceptible insects to survive so that the moths from both populations could mate, thus diluting the resistance gene in the offspring (Liu & Tabashnik, 1997; Peck et al., 1999; Shelton et al., 2000; Tang et al., 2001; Caprio et al., 2004; Tabashnik et al., 2004).

Current theory dictates that the evolution of resistance to Bt involves the interaction of many genetic and biological factors (Tabashnik, 1994; Gahan et al., 2005; Tabashnik et al., 2005), in which fitness costs are incurred that are the result of negative pleiotropic effects of genes that confer resistance and result in the fitness of resistant individuals being lower than that of susceptible individuals in the absence of toxin. These effects play an important role in the development of resistance in natural environments (Groeters et al., 1994). In general, fitness costs associated with Bt-resistance are well-documented and can substantially affect survival and development (Tang et al., 1997; Ramachandran et al., 1998; Akhurst et al., 2003; Tabashnik et al., 2003; Tabashnik & Carrière, 2004), pupation rate and pupal weight (Huang et al., 2005), diapause (Carrière et al., 2001) and mating success (Groeters et al., 1994; Alyokhin & Ferro, 1999; Higginson et al., 2005). There have also been two reports on reduced fitness in Bt-resistant H. armigera on transgenic cotton affecting survival, development and diapause (Bird & Akhurst, 2004, 2005). However, other fitness differences between the Bt-resistant and wild-type H. armigera have been unclear until now. Here we report research results on differences in cold hardiness, diapause and flight ability between Cry1Ac-resistant and wild-type H. armigera.

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### MATERIAL AND METHODS

#### Insect cultures and bioassays

A field population of H. armigera was collected from Xinxiang County, in Henan Province, China in 1996 and cultured on artificial diets in the laboratory (the artificial diets were mixed as described by Liang et al., 1999). As a control treatment (96S), the original field population was reared on artificial diet without exposure to any chemical insecticide or Bt toxin. For selecting a resistant strain, increasing amounts of Cry1Ac protoxin (extracted from B. thuringiensis HD73, cordially provided by the Biotechnology Group in Institute of Plant Protection, Chinese Academy of Agricultural Sciences) were added into the artificial diet with a selective pressure at which about twenty percent of the selected neonates could develop successfully to pupation (Liang et al., 2000). The Cry1Ac-resistant H. armigera strain (BtR) in this study has been selected with Cry1Ac protoxin for 52 generations. In order to minimize genetic differences other than the resistance character between the 96S and BtR strain, the selected strain was backcrossed with 96S strain in both the 27th and 49th generations. After these backcrosses, the offspring were reselected with a lower concentration Bt-diet followed by continually increasing doses of Cry1Ac in the artifical diet generation by generation. The insect cultures and bioassays in this study were maintained at 27  $\pm$  $2^{\circ}$ C,  $75 \pm 10\%$  RH and a photoperiod of 14L : 10D, unless otherwise noted.

In bioassays different amounts of Cry1Ac protein (MVP endotoxin protein, Mycogen, San Diego, CA) were homogeneously mixed with the artificial diet. After the artificial diet solidified, 0.3–0.4 g portions were transferred to 24-well trays. One *H. armigera* neonate was transferred into each well and mortality of the larvae was recorded after 7 days. A total of 12 neonates were used for each concentration of Bt protein in the diet (2700, 900, 300, 100, 33.33  $\mu$ g/g for resistant strain; 3.7, 1.23, 0.41, 0.14, 0.05  $\mu$ g/g for susceptible strain) and for the control treatment (with only distilled water added). Each treatment had four replicates.

# Diapause inducement condition and critical photoperiod test

H. armigera neonates were placed on the artificial diet in glass-tubes and maintained in growth chambers at 22°C with photoperiods of either 11L: 13D, 12L: 12D, 13L: 11D or 14L: 10D until pupation. The diapause and non-diapause pupae were distinguished by observing the movement and eventual disappearance of the pigmented eyespots during H. armigera pupal development (Phillips & Newsom, 1966). Two hundred neonates were tested for each treatment. Each experiment was repeated three times. After seven days, diapause and non-diapause pupae were recorded and the data were compared to the normal time for diapause for this species (Wu & Guo, 1995). The percentages of diapause pupae under different photoperiods between the susceptible and resistant H. armigera strains were calculated.

# The cold-hardiness test

Pupal temperature gradually decreases along with the temperature in the freezer until the pupal temperature reaches the supercooling point (SCP), when the body fluids begin to freeze. At this time, the pupal temperature rapidly increases due to the heat released from the freezing pupae. Pupal temperature increases and then decreases again, forming a small peak, and the peak temperature is determined to be the freezing point (FP).

The SCPs and FPs of diapause and non-diapause pupae in the BtR and 96S strains were measured as described by Zhu et al. (1994). In brief, a heat-sensitive probe was fixed to a pupa and the pupa was placed in a low-temperature refrigerator (0°C)

with the temperature reduced by 0.1°C/min. Pupal temperature was automatically monitored using a computer. Seven days after the larvae pupated, the largest and healthiest individuals of diapause and non-diapause pupae from the BtR and 96S strain were selected for testing. A total of 15 diapause or non-diapause pupae were tested each time. The experiment was repeated three times.

The survivability of diapause and non-diapause pupae between the BtR and 96S strains was determined as described by Wu et al. (1997b). The pupae of both strains were kept for 5 d at 4°C, and then were embedded in a container under 6–8 cm of soil at –15°C. The diapause and non-diapause pupae were taken out from the freezer at different time intervals, maintained under laboratory conditions (25°C), and pupal survival was recorded after 72 h. Twelve diapause pupae each were taken out after 3, 4, 5, 6, 8, and 12 h had elapsed. Fifty non-diapause pupae each were taken out after 4, 6, 8, 12, 24, 48, and 72 h had elapsed. Each experiment was repeated three times.

## Flight ability test

The test apparatus was a 32-channel computer-monitored flight-mill system made by the Institute of Plant Protection, Chinese Academy of Agricultural Sciences, Beijing. The 32 flight mills, positioned on wire shelves, were operated in a controlled room. The system automatically recorded the main flight parameters of 32 adults, including flight speed, duration and distance. Approximately 2 h before the onset of testing, tethers were attached to individual moths that were slightly anesthetized using ether, on the dorsal surface of the thorax, from which scales had been removed of. The moths were attached by placing a drop of 502 Glue (a cyanoacrylate glue, Youxing Adhesive Co., Guangdong, China) on the small loop of the tether electrical wire (the moth tether was fabricated with a 20-mm length of 0.64-mm-diameter wire and a 13-mm length of plastic insulation that had been removed from electrical wire of 1 mm diameter) and holding the wire loop and glue in contact with the dorsal surface of the anesthetized moth's thorax with the aid of forceps and a magnifying glass. After drying the adhered portion, the anesthetized adult was put into a test tube. After recovery from anesthesia, the adult was allowed to fly. At the time of moth attachment to the flight mill, the shape of the tether wire was modified by bending as necessary to position the moth in a horizontal flight attitude (Beerwinkle et al., 1995). The flight ability of 15 males and 15 females reared on Bt-diet (the dose of Cry1Ac was 14 µg/g) or normal diet in the 96S and BtR strains was measured in the laboratory (23°C, 60% RH and photoperiod of 0L: 24D). The effects of strain, diet and sex on flight potential in 24 h of unmated 1-d-old moths were recorded. Each experiment was repeated three times.

# Data analysis

The 50%-lethal concentration (LC<sub>50</sub>) values were calculated by probit regression using POLO Software (Russell et al., 1977). Samples for which the 95% confidence intervals did not overlap were considered to be significantly different. The resistance ratio of the BtR strain was expressed as the LC<sub>50</sub> of the resistant strain divided by the LC<sub>50</sub> of the susceptible strain.

Survival of *H. armigera* at low temperature ( $-15^{\circ}$ C) was analyzed by time-mortality regressions using the probit analysis (POLO, Russell et al., 1977) as described by Eger et al. (1982). A two-sample *t*-test procedure for comparing the percentage of diapause pupae between the two strains at each photoperiod was conducted (SAS Institute, 1996). When p < 0.05 the results were considered to be significant. The percentage of diapause pupae, supercooling points, freezing points, flight distance, flight speed and flight time in the two strains were analyzed by two-way

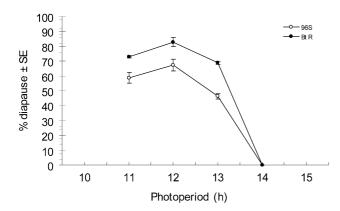


Fig. 1. The critical photoperiods of Bt susceptible and resistant strains of *Helicoverpa armigera*.

analysis of variance (ANOVA) with means separated using the least significant difference test (LSD) (SAS Institute, 1996).

## RESULTS

#### Resistance ratio of BtR strain

The LC<sub>50</sub> values for the 96S strain and the BtR strain were 0.3  $\mu$ g/g (95% confidence interval = 0.2–0.5) and 412.5  $\mu$ g/g (95% confidence interval = 114.1–1420.7), respectively. Thus the relative resistance ratio of the BtR strain was 1375-fold that of the 96S strain.

# Diapause inducement condition

The mean percentage of diapause pupae in the 96S strain under a 12L : 12D h photoperiod was  $67.4 \pm 3.9\%$ , which was significantly higher than under 11L: 13D  $(58.6 \pm 3.4\%)$ , 13L : 11D  $(46.3 \pm 1.7\%)$ , or 14L : 10D (0%) photoperiods (F = 128.87; df = 3,8; P = 0.0001) (Fig. 1). The pupal diapause rates were not significantly different between the treatments of 11L: 13D and 13L: 11D photoperiods. No diapause pupae were found when H. armigera were under the photoperiod of 14L: 10D. Similar results were obtained for the BtR strain. The percentage of diapause pupae in the BtR strain under a 12L: 12D photoperiod was  $82.9 \pm 3.0\%$  which was significantly higher than under 11L : 13D (72.7  $\pm$  0.8%), 13L : 11D (68.7  $\pm$  1.1%), or 14L : 10D (0%) photoperiods (F =413.11; df = 3.8; P = 0.0001) (Fig. 1). The percentage of diapause under the 11L: 13D photoperiod in the BtR strain was significantly higher than that in the 96S strain (t = 4.01, P = 0.036), which was also the case for the 12L : 12D photoperiod (t = 3.01, P = 0.040) and the 13L : 11D photoperiod (t = 10.84, P = 0.0004). There were no differences at 14L: 10D photoperiod between the two strains, as no diapause pupae were found in either strain.

TABLE 1. The comparison of supercooling points and freezing points of *Helicoverpa armigera* between diapause and non-diapause pupae of Bt-resistant and susceptible strains.

Strain	Pupal diapause status	$Mean \ SCP \pm SE*$	Mean FP $\pm$ SE*
BtR	Diapause pupae	$-19.1\pm0.7$	$-7.4 \pm 1.7$
	Non-diapause pupae	$-18.7\pm1.2$	$-6.7 \pm 1.8$
96S	Diapause pupae	$-19.1\pm0.2$	$-7.3 \pm 2.0$
	Non-diapause pupae	$-18.3\pm1.6$	$-6.2 \pm 2.3$

<sup>\*</sup>SCP is the supercooling point. FP is the freezing point.

# Cold hardiness of H. armigera

SCPs were not different between diapause and nondiapause pupae within either the BtR strain (F = 0.45; df = 1,8; P = 0.7219) or the 96S strain (F = 2.01; df = 1,8; P= 0.1648) (Table 1). The differences of FPs were also not significant between the diapause and non-diapause pupae in either the BtR (F = 0.66; df = 1,8; P = 0.5819) or 96S (F = 2.20; df = 1.8; P = 0.1473) strain. When comparing the BtR strain to the 96S strain, there were no significant differences in SCPs or FPs in the diapause (F = 0.73; df = 3,8; P = 0.3958) or non-diapause (F = 0.40; df = 3,8; P =0.5275) pupae. However, the diapause pupae had stronger cold hardiness than the non-diapause pupae within both the BtR and 96S strains. The combined data for SCP of diapause pupae from both strains was -19.1°C which was significantly lower than the combined SCP of nondiapause pupae (-18.5°C) (F = 6.02; df = 3,8; P =0.0165). There also was a significant difference between combined data for freezing points of diapause pupae  $(-7.4^{\circ}\text{C})$  and those of non-diapause pupae  $(-6.4^{\circ}\text{C})$  (F =4.23; df = 3.8; P = 0.0434).

The survival times at  $-15^{\circ}$ C of diapause or nondiapause *H. armigera* pupae were not significantly different between the BtR and 96S strains, as the 95% CI of LT<sub>50</sub> values in these two strains overlapped (Table 2). The LT<sub>90</sub> values between the diapause pupae and nondiapause pupae in both the BtR (95% CI: 11.2–46.7 vs. 7.1–11.3) and 96S (11.6–32.5 vs. 6.4–11.8) strains were marginally significant based on slight overlapping of CI.

### Flight ability

The larvae of the 96S strain that fed on Bt diet all died before pupation. Most of BtR strain larvae feeding on Bt diet completed their life cycle and the emerged moths could fly naturally but their flight ability was weaker than moths reared on normal diet (Table 3). The moths of the BtR strain reared on normal diet had similar flight ability to those of the 96S strain moths that were also reared on normal diet. The BtR *H. armigera* reared on normal diet

Table 2. Survival of diapause and non-diapause pupae of *Helicoverpa armigera* at -15°C between the Bt-resistant and susceptible strains.

Strain	Diapause/non-diapause	$Slope \pm SE$	$LT_{50}(h)$	95% CI*	$LT_{90}(h)$	95% CI*
96S	Diapause	$3.2\pm0.6$	6.7	4.0-10.6	16.8	11.2-46.7
BtR	Diapause	$3.4 \pm 0.8$	6.7	4.8-9.3	16.0	11.6-32.5
96S	Non-diapause	$9.9 \pm 2.2$	6.1	5.2-7.7	8.3	7.1–11.3
BtR	Non-diapause	$6.5 \pm 1.5$	4.9	3.9-6.2	7.7	6.4–11.8

<sup>\*</sup>CI is the confidence interval.

TABLE 3. Flight ability of *Helicoverpa armigera* adults in different strains reared on different diets.

Strain / Diet / Gender	Mean total flight distance ±SE (km)	Mean total flight time ±SE (h)	
96S, Normal diet, Female	$63.6 \pm 16.0$	$9.4 \pm 2.8$	
96S, Normal diet, Male	$50.7 \pm 11.5$	$9.3 \pm 1.8$	
BtR, Normal diet, Female	$62.3\pm14.0$	$12.6\pm2.0$	
BtR, Normal diet, Male	$48.7 \pm 8.9$	$10.4\pm1.9$	
BtR, Bt diet, Female	$34.0 \pm 5.9$	$9.6 \pm 1.7$	
BtR, Bt diet, Male	$21.0 \pm 9.7$	$5.7 \pm 2.7$	

had significantly higher flight ability than those reared on Bt diet. The total flight distance of these BtR moths was  $56.2 \pm 11.5$  km for larvae fed on normal diet, which was more than twice as far than those feeding on Bt diet (26.2  $\pm$  6.4 km) and this difference was significant (F = 4.62; df = 1,10; P = 0.0408). Flight duration for these BtR moths after feeding on normal diet was also significantly longer (11.6  $\pm$  2.0 h) than those fed on Bt diet (7.3  $\pm$  1.6 h) (F = 5.65; df = 1,10; P = 0.0284).

The flight distances and flight times of females were higher than for males in both the 96S and BtR strains after the larvae fed on normal diet (F = 2.46; df = 5,12; P = 0.1252). A similar result was also observed in the BtR strain after their larvae were fed with Bt diet (F = 0.61; df = 5,12; P = 0.4400), however none of these differences were significant. The differences of flight distance (F = 1.44; df = 5,12; P = 0.2329) and flight time (F = 1.13; df = 5,12; P = 0.3617) in strain × diet × gender interaction were not significant.

## DISCUSSION

H. armigera generally has four generations on cotton in northern China. The fourth generation larvae undergo diapause under a short photoperiod, overwintering as diapause pupae. The pupation percentage and the cold hardiness of pupae directly affect the size of populations in the coming year (Wu et al., 1997b). The research reported here shows that compared with a Cry1Ac-susceptible strain, a Cry1Ac-resistant strain had a higher percentage of diapause pupation at the same photoperiod, which could affect subsequent years' populations (in terms of both population genetics and population size). Similar results have been reported in the literature. Carrière & Roff (1995) demonstrated that insecticide-induced mortality could produce evolutionary change in diapause propensity of insects. In contrast, Hu et al. (1999) reported that the pupae of H. armigera with high levels of fenvalerate resistance had a lower diapause rate and reduced winter hardiness compared to pupae with lower levels of fenvalerate resistance. Bird & Akhurst (2004) found the proportion of larvae that pupated under diapausing conditions was similar for Cry 1A-resistant and -susceptible populations of *H. armigera*.

The  $LT_{50}$ , SCPs and FPs of the BtR strain were not significantly different from those of the 96S strain. However, the  $LT_{90}$  of diapause pupae at  $-15^{\circ}$ C was marginally significantly higher than that of non-diapause

pupae and the SCP and FP of diapause pupae were also significantly lower than those of non-diapause pupae. In other words, the diapause pupae were more cold-hardy than the non-diapause pupae. Because the percentage of diapause in the BtR strain was higher than in the 96S strain under the same conditions, the BtR strain may ultimately have more endurance in a cold environment. In contrast, Carrière et al. (2001) investigated overwintering costs in pink bollworm, Pectinophora gosypiella (Saunders) strains with different degrees of resistance to Bt cotton and found that emergence from diapause in the spring was 71% lower in three highly resistant strains than in two heterogeneous strains from which the resistant strains were derived. Their data also underestimate the overwintering cost because the frequency of the resistance allele was relatively high in the heterogeneous strains. Our experiments were conducted in the laboratory, not in the field, and the survival time of pupae were determined at -15°C. Field conditions are more complex, with the soil temperature fluctuating regularly and many other biotic and abiotic factors affecting pupal survival. A comparison of pupal survival in the field between BtR and 96S strains should provide more insight into this phenomenon.

There were no significant differences in either flight distance or time between the 96S and BtR strains (Table 3). However, the BtR individuals reared on Bt diet were not as robust as those reared on normal diet, as shown by their reduced flight. In addition, the pupae and emerged moths reared on Bt diet (the mean weights of these pupae and moths were 289.2 mg and 188.1 mg, respectively) were significantly smaller than pupae and moths reared on normal diet, whose mean weights of were 318.8 mg (F = 20.26; df = 1,4; P = 0.0108) and 197.7 mg (F = 9.40; df = 1,4; P = 0.0374), respectively.

The BtR strain was highly resistant to Cry1Ac in artificial diets, BtR larvae survived for a longer time on Bt cotton, and more BtR larvae developed to the 5th instar on Bt cotton, whereas susceptible (96S) larvae had high mortality on Bt cotton. However, only a few individuals of the resistant H. armigera (BtR) strain could pupate on Bt cotton, while most BtR individuals could complete their life cycle on artificial diet with a high concentration of Cry1Ac protoxin (50  $\mu$ g/g) (Liang et al., unpubl. data). Reasons for this observed difference may include interactions between plant chemistry and Bt toxins or nutritional benefits from the artificial diet.

In northern China, the cotton bollworm passes through the cold winter as a diapause pupae. It has been suggested that the overwintering limit for cotton bollworm is –15°C for the mean low temperature in January (Wu et al., 1997b). Changes to the diapause inducement condition and cold hardiness should affect the overwintering survival percentage of *H. armigera*. Flight ability directly affects the *H. armigera* moth mating percentage and gene exchange in the field. Although Bt-cotton resistance has not become a problem in China (Wu & Guo, 2005), more research on Bt-resistance management is needed.

The results of the current study give insight into physiological and life-cycle changes in *H. armigera* associated with Bt resistance. We hope that these results will contribute to the further establishment of efficacious Bt-resistance management strategies in China and other countries where *H. armigera* are exposed to Bt crops.

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