For submission to Journal of Economic Entomology

Running title: Wang *et al*., Effects of Bt toxin on Adults of *Pectinophora gossypiella*

Adult Exposure to Bt Toxin Cry1Ac Reduces Lifespan and Reproduction of Resistant and Susceptible Pink Bollworm

LING WANG[1], PENG WAN[2], SHENGBO CONG[2], JINTAO WANG[3], MINSONG HUANG[2], BRUCE E. TABASHNIK[4], XIANCHUN LI[1,4], AND KONGMING WU[1*]

1. State Key Laboratory for Biology of Plant Diseases and Insect Pests, Institute of Plant Protection, Chinese Academy of Agricultural Sciences, Beijing 100193, P.R. China.

2. Institute of Plant Protection and Soil Fertility, Hubei Academy of Agricultural Sciences, Wuhan 430064, China.

3. Hubei Insect Resources Utilization and Sustainable Pest Management Key Laboratory, Huazhong Agricultural University, Wuhan 430070, China.

4. Department of Entomology, University of Arizona, Tucson, AZ 85721, USA.

* Corresponding author, E-mail: kmwu@ippcaas.cn.

ABSTRACT Insecticidal proteins from *Bacillus thuringiensis* (Bt) are used widely in sprays and transgenic plants to control insect pests. Although much research has elucidated the effects of Bt toxins on larvae, relatively little is known about their effects on adults. Here we evaluated the effects of exposing adults to Bt toxin Cry1Ac on the lifespan and reproduction of two strains of pink bollworm (*Pectinophora gossypiella*). In larval diet bioassays, the concentration of Cry1Ac killing 50% of larvae (LC_{50}) was 640 times higher for the laboratory-selected resistant strain (AZP-R) than the susceptible strain (APHIS-S). In experiments with adults, the highest concentrations of Cry1Ac tested (160 and 640 μg Cry1Ac per ml of 5% honey water) reduced lifespan for both strains. Treatments with 10, 40 and 160 μg Cry1Ac per ml reduced the duration of the oviposition period as well as the number of eggs laid by both strains, but did not affect the percentage of pairs producing eggs, the duration of the pre-oviposition period, or the percentage of eggs hatching for either strain. Adult lifespan did not differ between strains at low to moderate concentrations of Cry1Ac, but it was significantly greater for the resistant strain than the susceptible strain at the two highest concentrations of Cry1Ac tested. The reduced susceptibility to high concentrations of Cry1Ac in adults of the AZP-R strain relative to the APHIS-S strain provides the first evidence of expression of resistance to a Bt toxin in adult Lepidoptera.

KEY WORDS *Pectinophora gossypiella*, *Bacillus thuringiensis*, adult lifespan, reproduction

Introduction

Insecticidal crystalline (Cry) proteins from the bacterium *Bacillus thuringiensis* (Bt) are used widely in sprays and transgenic crops to kill larvae of some lepidopteran, coleopteran and dipteran pests (Bravo et al. 2011, Sanahuja et al. 2011). These Cry toxins have provided substantial economic and environmental benefits (Hutchison et al. 2010, Tabashnik et al. 2010, Downes and Mahon 2012, Lu et al. 2012), but several pests have evolved resistance to the Cry toxins in sprays and transgenic crops (Tabashnik et al. 2013, 2014, Farias et al. 2014). To kill larvae, Cry toxins must bind to midgut receptors (Pardo-López et al. 2013, Adang et al. 2014) and reduction of this binding is the most common and most potent mechanism of resistance to Cry toxins (Ferré and Van Rie 2002, Wu 2014).

Although the larval stage of pests is the primary target of Cry toxins, considerable evidence indicates that ingestion of Cry toxins by adults is harmful to some species from several orders of insects (e.g., Wierenga et al. 1996, Alyokhin and Ferro 1999a, b, Ashouri et al. 2001, Grove et al. 2001, Meissle et al. 2011, Qi et al. 2013, Yamaguchi et al. 2013, Zhang et al. 2013). Zhang et al. (2013) proposed that the harm to adults caused by Bt toxins could have two implications for pest management: adults might be potential targets for control by Bt toxins and toxicity of Bt toxins to adults could interfere with the refuge strategy for delaying evolution of resistance to Bt cotton. In particular, refuge effectiveness could be reduced if exposure to Bt toxins in the nectar of Bt cotton reduces the lifespan or reproduction of susceptible adults that move into Bt cotton fields after emerging from non-Bt cotton refuges (Zhang et al. 2013).

We also note that if adults are susceptible to Bt toxins, then they may have the ability to evolve resistance. In principle, under some conditions, resistance of both

adults and larvae to Cry1Ac could accelerate evolution of resistance relative to larval resistance alone. Two previous studies compared effects of transgenic potato foliage producing Bt toxin Cry3A on adults between strains of Colorado potato beetle (*Leptinotarsa decemlineata*) in which the larvae were either resistant or susceptible to Cry3A (Wierenga et al. 1996, Alyokhin and Ferro 1999a). However, as far as we know, previous research has not compared effects of adult exposure to Bt toxins between conspecific strains of Lepidoptera that differ in larval susceptibility to Bt toxins.

Here we addressed this issue with pink bollworm (*Pectinophora gossypiella*), one of the world's most destructive pests of cotton (Henneberry and Naranjo 1998). For nearly two decades, this widespread pest has been targeted by transgenic cotton producing Bt toxin Cry1Ac, either alone or with other Bt toxins (Tabashnik et al. 2012). In India, pink bollworm evolved practical resistance to Bt cotton producing Cry1Ac in less than 10 years, which means that larval resistance to Cry1Ac was severe enough to have practical consequences for controlling this pest in the field (Dhurua and Gujar 2011, Fabrick et al. 2014, Ojha et al. 2014, Tabashnik et al. 2014). In addition, field failures of two-toxin Bt cotton producing both Cry1Ac and Cry2Ab have been reported in India (Kurmanath 2015). By contrast, larvae in field populations of pink bollworm have remained susceptible to Cry1Ac for more than 17 years in the United States (Tabashnik et al. 2010, 2012), and have shown significant, but small increases in the frequency of resistance to Cry1Ac in China (Wan et al. 2012). In laboratory-selected strains from Arizona and field-selected populations from India, resistance of pink bollworm larvae to Cry1Ac is associated with mutations disrupting a cadherin protein that binds Cry1Ac in the larval midgut (Morin et al. 2003, Fabrick and Tabashnik 2007, 2012, Fabrick et al. 2014). Previous evidence suggests that pink bollworm adults ingest nectar from cotton (Lukefahr and Griffin 1956, Naranjo and Martin 1973) and thus they

could be exposed to Cry1Ac via this route in Bt cotton fields.

In this study, we compared adult responses to Cry1Ac between two laboratory strains of pink bollworm from Arizona: the AZP-R strain with larvae resistant to Cry1Ac and the APHIS-S strain with larvae susceptible to Cry1Ac (Morin et al. 2003). Our primary goals were to determine if adult exposure to Cry1Ac affected adult survival and reproduction, and if the effects of Cry1Ac on adults differed between the two strains. As a positive control, based on the known toxicity of pyrethroids to pink bollworm adults (Schouest and Miller 1988, Osman et al. 1991), we also tested adults against a pyrethroid (cypermethrin).

Materials and Methods

Insects. We used two strains of *P. gossypiella* from Arizona: APHIS-S (susceptible to Cry1Ac) and AZP-R (resistant to Cry1Ac). APHIS-S had been reared without exposure to Bt toxins or other insecticides for more than 30 years (Liu et al. 2001). AZP-R was started by pooling individuals sampled from 10 field populations in 1997, and had been selected repeatedly with Cry1Ac in the laboratory (Tabashnik et al. 2000, Morin et al. 2003). These strains were derived independently and are unrelated aside from their common origin in Arizona.

Both strains (referred to hereafter as the susceptible and resistant strains, respectively) were obtained from the University of Arizona in August 2011 and were reared after that at the Institute of Plant Protection in Beijing, China using methods similar to those used in Arizona (Liu et al. 2001). In China, AZP-R was selected every fifth generation by exposing neonates to diet containing 10 μg Cry1Ac protoxin per ml diet and rearing the survivors to continue the strain. We bought Cry1Ac protoxin from Zhongbao Biotechnology Company, Beijing, China.

123 **Insecticides**. For bioassays, we used trypsin-activated Cry1Ac (Pusztai-Carey et
124 al. 1994) purchased from Marianne Pusztai-Carey (Case Western Reserve University,
125 Cleveland, OH), and ß-cypermethrin 4.5% E.C. purchased from Anhui Fengle
126 Agrochemical Co. Ltd., Hefei City, China. For brevity we refer to these insecticides
127 hereafter as Cry1Ac and cypermethrin.

128 **Larval Bioassay.** To determine if the previously reported resistance to Cry1Ac of
129 AZP-R relative to APHIS-S based on bioassays in Arizona (e.g., Tabashnik et al. 2002;
130 Morin et al. 2003) persisted at the time of this study in China, we evaluated larval
131 susceptibility to Cry1Ac using diet incorporation bioassays. We added sodium
132 carbonate (50 mmol/L, pH = 10) to produce a stock dilution of Cry1Ac. The stock
133 dilution was added to liquid wheat germ diet in amounts necessary to create final
134 concentrations of 0 (control), 0.05, 0.1, 0.15, 0.2, 0.25 and 10 μg Cry1Ac per ml
135 solution for APHIS-S, and 0, 10, 40, 60, 80, 100 and 120 μg Cry1Ac per ml for AZP-R
136 (Tabashnik et al. 2002). Diet was made in 1 liter batches of each concentration, cooled,
137 shredded into pieces (ca. 2 by 1 by 1 cm) and dispensed into 24-well culture plates
138 (Haimeng Shengbang Laboratory Equipment Co., China) with 3 g diet per well.
139 Neonates were placed individually in each well. For each strain, each concentration
140 was replicated three to four times, with 24 larvae (one plate) per replicate. Plates were
141 placed in an environmental chamber at 29±1℃ with a photoperiod of 16:8 (L:D) h.
142 After 21 days, live fourth instars and pupae were scored as survivors.

143 **Adult Lifespan Bioassay.** For adult bioassays, the larvae from both strains were
144 reared on artificial diet (Wu et al. 2008) without exposure to Bt toxin. We transferred
145 one neonate per well onto diet in wells of 24-well plates. We determined the sex of
146 pupae under a microscope, weighed them, and chose female pupae of 14-18 mg and
147 male pupae of 10-14 mg to yield adults for bioassays. Both pupae and adults were held

148 in glass tubes (10 cm long × 2.5 cm diameter) with one individual per tube. Larvae and
149 adults were held at 28 ± 1°C and 14 light:10 dark. Relative humidity was 40 to 60%
150 for larvae and 60 to 80% for adults. We used the lower humidity for larvae to avoid
151 problems with mold.

152 We confirmed the sex of each newly emerged virgin adult and transferred it to new
153 glass tube that had a 200-µl centrifugal tube cap. We filled the caps with a mixture of
154 sodium carbonate (50 mmol/L, pH = 10) and 5% honey water with one of six
155 concentrations of Cry1Ac (0, 2.5, 10, 40, 160, 640 µg Cry1Ac per ml). We chose this
156 range of concentrations to extend beyond the concentrations used for larval bioassays
157 (see above). To increase solubilization of Cry1Ac in 5% honey water, we first dissolved
158 10 mg of Cry1Ac in 2.5mL of 50 mmol / L sodium carbonate solution (pH = 10.0).
159 When Cry1Ac was completely mixed into the liquid, we used it as the stock solution.
160 Then 5% honey water was used to dilute the stock solution to the six concentrations of
161 Cry1Ac and kept each treatment to be the same content of sodium carbonate solution.
162 We also tested 9 µg cypermethrin per ml in a mixture of sodium carbonate and 5%
163 honey water without Cry1Ac. For each strain, sex, and concentration of Cry1Ac or
164 cypermethrin, we tested three to four replicates with 10 adults per replicate. We refilled
165 the caps daily with the honey water containing the appropriate mixture and recorded
166 mortality daily until all adults died.

167 **Female Fecundity Bioassay.** We transferred one pair of newly emerged virgin
168 adults of APHIS-S or AZP-R to a plastic cup (6 cm diameter × 6 cm height) that
169 contained a 200-µl centrifugal tube cap. We placed a piece of white art paper (Wenzhou
170 Snow Mountain Paper Co., Wenzhou City, Zhejiang Province, China) on top of each
171 cup to prevent escape and to collect eggs. We filled the caps daily with a mixture of
172 sodium carbonate and 5% honey water (negative control), any of 3 concentrations of

Cry1Ac dissolved in a mixture of sodium carbonate (50 mmol / L, pH=10) and 5% honey water (10, 40, 160, μg/ml), with 0.6 mmol/L sodium carbonate in each concentration. For each concentration or control, we set up at least 30 single pair mating cups. We monitored each cup and replaced its white paper daily until the death of the female to record the first time of oviposition and the number of eggs laid each day. These raw data were used to calculate the percentage of pairs producing eggs, the duration of pre-oviposition and oviposition of each female and total eggs per female. We also selected approximately 15 to 20 egg-containing white papers with 50 to 100 newly laid eggs each from each concentration or control and put each paper in a 5 cm petri dish for 7 days. We recorded the total number of eggs on each paper on Day 1 and the remaining unhatched eggs on Day 7 to calculate hatching rate as: (total eggs minus unhatched eggs) / total eggs multiplied by 100%.

Data Analysis. We analyzed larval diet bioassay data with probit regression using IBM SPSS (Jia 2006) Statistics 22.0 to determine LC_{50} values and their 95% fiducial limits, and slopes of the concentration-mortality lines and their standard errors (SE). We calculated corrected mortality (%) of larvae and adults after 14 d of exposure to 10 μg Cry1Ac per ml (in artificial diet for larvae and in honey water for adults) as: ((mortality % in treatment - mortality % in control)/(100% - mortality % in control)) X 100%. We calculated corrected mortality for each strain and life stage using the corresponding observed means (with range in parentheses) for percentage control mortality: 6.2% (0.0-12.5%) for APHIS-S larvae, 3.1% (0.0-8.3%) for AZP-R larvae, 10% (10-10%) for APHIS-S adults, and 3.3% (0.0-10%) for AZP-R adults. We applied the arcsine-square root transformation (Milligan 1987) to the percentage corrected

196 mortality and used t-tests to make comparisons between strains separately for larvae,
197 adult females, and adult males.

198 We used two complementary approaches to analyze effects of Cry1Ac and
199 cypermethrin on adult lifespan. First, we used analysis of covariance (ANCOVA) by
200 IBM SPSS (Jia 2006) with adult lifespan as the dependent variable, concentration of
201 Cry1Ac as the covariate, and sex and strain as the categorical variables. This approach
202 enabled testing of the overall main effects on adult lifespan of concentration of Cry1Ac,
203 sex, strain, and their interactions. Second, to include effects of cypermethrin and to
204 determine thresholds for the concentrations of Cry1Ac that affected adult lifespan, we
205 used one-way ANOVA and Tukey's HSD test. Finally, we also used one-way ANOVA
206 and Tukey's HSD test to evaluate effects of Cry1Ac on the percentage of pairs
207 producing eggs, the duration of pre-oviposition and oviposition periods, the number of
208 eggs laid and percentage of eggs hatching.
209

210 **Results**

211 **Effects of Cry1Ac on Larval Mortality.** For pink bollworm larvae, the LC_{50} of
212 Cry1Ac was 640 times higher for the resistant AZP-R strain than the susceptible
213 APHIS-S strain (Table 1). Larval mortality at 10 μg Cry1Ac per ml diet was 100% for
214 APHIS-S versus 10% for AZP-R ($P < 0.01$, Fig. 1).

215 **Effects of Exposing Adults to Cry1Ac on Adult Mortality and Lifespan.** When
216 adults were given honey water containing 10 μg Cry1Ac per ml, mortality at 14 d did
217 not differ significantly between strains (Fig. 1).

218 ANCOVA shows that adult lifespan was significantly affected by sex, strain, and
219 the concentration of Cry1Ac in honey water (0 to 640 μg Cry1Ac/ml) provided as adult

220 food (Table 2). These significant main effects reflect that lifespan (mean in days) was
221 generally greater for females (14.8) than males (13.9), greater for AZP-R (15.0) than
222 APHIS-S (13.7), and greater at lower concentrations of Cry1Ac (Table 3).

223 Interactions were not significant, except for the strain X concentration interaction
224 indicating the difference in lifespan between strains was affected by the concentration
225 of Cry1Ac ($P = 0.048$, Table 2). For both sexes, AZP-R adults lived significantly longer
226 than APHIS-S adults at the two highest concentrations (160 and 640 μg Cry1Ac/ml),
227 but adult lifespan did not differ significantly between strains at lower concentrations (0
228 to 40 μg Cry1Ac/ml) (Table 3). These results indicate that at the two highest
229 concentrations, AZP-R adults were resistant to Cry1Ac relative to APHIS-S adults.

230 For both sexes and both strains, the two highest concentrations of Cry1Ac
231 significantly reduced lifespan relative to the control without Cry1Ac (Table 3). For
232 both sexes, the intermediate concentration of 40 μg Cry1Ac/ml significantly reduced
233 lifespan relative to the control for APHIS-S, but not AZP-R (Table 3). The maximum
234 reduction in adult lifespan caused by Cry1Ac (which occurred at 160 μg Cry1Ac/ml for
235 both sexes and both strains) was 46% for APHIS-S females, 45% for APHIS-S males,
236 32% for AZP-R females, and 38% for AZP-R males (Table 3).

237 **Effects of Exposing Adults to Cypermethrin on Adult Lifespan.** Exposure to
238 cypermethrin reduced adult lifespan by 79 to 86%, yielding significantly shorter adult
239 lifespan than any of the treatments with Cry1Ac (Table 3). For adults of both sexes
240 exposed to cypermethrin, adult lifespan was significantly longer for AZP-R than
241 APHIS-S (Table 3). These results imply that, relative to APHIS-S, AZP-R adults were
242 resistant to cypermethrin.

243 **Effects of Exposing Adults to Cry1Ac on Reproduction.** Compared with feeding
244 pairs of adults untreated honey water (control), feeding pairs of adults honey water with

either 10, 40, or 160 µg Cry1Ac/ml did not affect the percentage of pairs producing eggs, duration of the pre-oviposition period, or the egg hatching rate; but significantly reduced the oviposition period and number of eggs laid for both strains (Table 4). With adult pairs fed either treated or untreated honey water, no significant differences occurred between strains in any of the five reproduction traits evaluated (Table 4).

Discussion

The results here from larval diet bioassays confirm previous data showing resistance to Cry1Ac in the laboratory-selected AZP-R strain of pink bollworm relative to the unselected APHIS-S strain (Morin et al. 2003, Tabashnik et al. 2002). The Cry1Ac resistance ratio of 640 for AZP-R relative to APHIS-S seen here (Table 1) is somewhat lower than some previous estimates, including 1500 (Tabashnik et al. 2009), 3,100 (Tabashnik et al. 2002) and >3,700 (Soberón et al. 2007). This difference could reflect changes in AZP-R over time, differences in the bioassay methods between studies, or both. We used activated Cry1Ac toxin in this study, whereas Soberón et al. (2007) tested Cry1Ac protoxin, and Tabashnik et al. (2002 and 2009) tested MVPII, which is a formulation containing a hybrid protoxin nearly identical to Cry1Ac (Welch et al. 2015). Simultaneous testing of Cry1Ac protoxin and activated toxin against resistant and susceptible strains of pink bollworm is needed to determine if the form of the toxin affects the resistance ratio, as observed in many other species of Lepidoptera (Tabashnik et al. 2015).

The results here demonstrate that adult lifespan of adults from pink bollworm strains AZP-R and APHIS-S was reduced by providing adults with honey water containing Cry1Ac at high concentrations (160 and 640 µg Cry1Ac/ml), but not lower

concentrations (2.5, 10 and 40 µg Cry1Ac/ml) (Table 3). In addition, for both strains, 10, 40, or 160 µg Cry1Ac/ml reduced the oviposition period and number of eggs laid, but did not affect the percentage of pairs producing eggs, the duration of the pre-oviposition period, or hatching of eggs (Table 4). In a previous study, Henneberry and Jech (2006) reported that providing adults with a sucrose solution containing MVPII, with concentrations of 25 and 100 µg Cry1Ac/ml, did not affect survival of adults after 7 days. Similar to the results here showing greater adult lifespan for females than males, they found mortality after 7 days was lower for females than males.

Consistent with previous studies of pink bollworm (Henneberry and Jech 2006) and other lepidopterans (Zhang et al. 2013), the results here imply that adults are likely to be affected much less than larvae by the Bt toxins in transgenic plants. The results here show that 10 µg Cry1Ac/ml killed 90% of susceptible pink bollworm larvae from APHIS-S, but did not significantly reduce survival of susceptible APHIS-S adults (Fig. 1). Thus, pink bollworm adults are not a promising target for control with Cry1Ac.

Henneberry and Jech (2006) reported that Cry1Ac in nectar from Bt cotton grown in a greenhouse was below the detection level of their ELISA tests in 13 of the 15 samples they analyzed, but they did not report the detection threshold for their tests, or the concentration of Cry1Ac in the two positive samples. Also, they tested only one Bt cotton cultivar and apparently sampled only from extrafloral nectaries (Henneberry and Jech 2006). Therefore, more extensive quantification of Cry1Ac in Bt cotton nectar is required for definitive conclusions. Meanwhile, we hypothesize that the negative effects of Cry1Ac from Bt cotton on pink bollworm adults in the field are minimal and not likely to substantially interfere with the refuge strategy. This hypothesis is supported by analyses indicating non-Bt cotton refuges effectively delayed resistance to Bt cotton in Arizona (Tabashnik et al. 2005, 2012). Based on the relatively low

concentrations of Cry1Ac in floral organs of cotton compared with concentrations that harmed adults, Zhang et al. (2013) suggested that the concentration of Cry1Ac in cotton nectar is too low to poison adults of *Spodoptera frugiperda* and *Helicoverpa armigera*.

In the AZP-R strain, as in other resistant strains of pink bollworm, resistance to Cry1Ac is conferred by mutations disrupting a cadherin protein that binds Cry1Ac in the midgut of susceptible larvae (Morin et al. 2003, Fabrick and Tabashnik 2007, 2012; Fabrick et al. 2014). Analysis of the APHIS-S strain shows that cadherin is transcribed in adults as well as in larvae, with the abundance of cadherin mRNA about three times higher in adult males than females (Carrière et al. 2009). However, susceptibility of APHIS-S adults was not higher for males than females (Table 3). For example, with no Cry1Ac, APHIS-S females lived an average of 1.1 days longer than APHIS-S males, but at each of the two highest concentrations of Cry1Ac tested, females outlived males by only 0.5 days (Table 3). These results imply that the abundance of cadherin mRNA was not the sole factor determining the relative susceptibility to Cry1Ac between the two sexes in this strain. The role of cadherin and other potential receptors in mediating the effects of Cry1Ac on adults of pink bollworm and other insects remains to be determined.

Because resistance to Cry1Ac usually does not cross-resistance to pyrethroids (e.g., Wu and Guo 2004), we suspect that the resistance to cypermethrin of AZP-R adults relative to APHIS-S adults (Table 3) probably does not reflect cross-resistance. Instead, we hypothesize that this resistance to cypermethrin was caused by greater exposure to pyrethroids in the field for the populations sampled to start the AZP-R strain relative to those sampled to start the APHIS-S strain. Whereas APHIS-S was started more than 30 years ago (Liu et al. 2001), before substantial pyrethroid resistance was widespread in Arizona populations of pink bollworm, AZP-R was started more than a decade later

(Tabashnik et al. 2000), after more extensive exposure to pyrethroids had occurred and resistance to pyrethroids was more likely in the field (Osman et al. 1991).

The significantly greater lifespan of AZP-R relative to APHIS-S at the two highest concentrations of Cry1Ac (Table 3) provides the first evidence of expression of resistance to a Bt toxin in adult Lepidoptera. This result is consistent with previous studies showing decreased susceptibility to Cry3A in adults of Colorado potato beetle from a strain with larval resistance to Cry3A (Wierenga et al. 1996, Alyokhin and Ferro 1999a). Although larval and adult resistance are positively associated in both cases where this relationship has been examined, similar studies in other species are needed to determine if this pattern is widespread.

Acknowledgments

The research was funded by research grants from National Natural Science Foundation of China (30625028 and 31321004).

References Cited

Adang, M.J., N. Crickmore, and J.L. Jurat-Fuentes. 2014. Diversity of *Bacillus thuringiensis* crystal toxins and mechanism of action. Adv. Insect Physiol. 47: 39-87.

Alyokhin, A. V., and D. N. Ferro. 1999a. Modifications in dispersal and oviposition of Bt-resistant and Bt-susceptible Colorado potato beetles as a result of exposure to *Bacillus thuringiensis* subsp. *tenebrionis* Cry3A toxin. Entomol. Exp. Appl. 90: 93-101.

Alyokhin, A. V., and D. N. Ferro. 1999b. Mating ability of Colorado potato beetles fed transgenic potato (Solanaceae) foliage. Can. Entomol. 131: 539-540.

Ashouri, A., D. Michaud, and C. Cloutier. 2001. Unexpected effects of different potato resistance factors to the Colorado potato beetle (Coleoptera: Chrysomelidae) on the potato aphid (Homoptera: Aphididae). Environ. Entomol. 30: 524-532.

Bravo, A., S. Likitvivatanavong, S.S. Gill, and M. Soberón. 2011. *Bacillus thuringiensis*: A story of a successful bioinsecticide. Insect Biochem. Mol. Biol. 41: 423-431.

Carrière, Y., A.M. Showalter, J.A. Fabrick, J. Sollome, C. Ellers-Kirk, B.E. Tabashnik. 2009. Cadherin gene expression and effects of Bt resistance on sperm transfer in pink bollworm. J. Insect. Physiol. 55: 1058-1064.

Dhurua, S., and G.T. Gujar. 2011. Field-evolved resistance to Bt toxin Cry1Ac in the pink bollworm, *Pectinophora gossypiella* (Saunders) (Lepidoptera: Gelechiidae), from India. Pest Manag. Sci. 67: 898-903.

Downes, S. and R. Mahon. 2012. Successes and challenges of managing resistance in *Helicoverpa armigera* to Bt cotton in Australia. GM Crops and Food 3: 228–234.

Fabrick, J.A., and B.E. Tabashnik. 2007. Binding of *Bacillus thuringiensis* toxin Cry1Ac to multiple sites of cadherin in pink bollworm. Insect Biochem. Mol. Biol. 37: 97-106.

Fabrick, J.A., and B.E. Tabashnik. 2012. Similar genetic basis of resistance to Bt toxin Cry1Ac in boll-selected and diet-selected strains of pink bollworm. PLoS ONE. 7: e35658.

Fabrick, J.A., J. Ponnuraj, A. Singh, R.K. Tanwar, G.C. Unnithan, A.J. Yelich, X.C. Li, Y. Carrière, and B.E. Tabashnik. 2014. Alternative splicing and highly variable cadherin transcripts associated with field-evolved resistance of pink bollworm to Bt cotton in India. PLoS ONE. 9: e97900.

Farias, J.R., D.A. Andow, R.J. Horikoshi, R.J. Sorgatto, P. Fresia, A.C. dos Santos, and C. Omoto. 2014. Field-evolved resistance to Cry1F maize by *Spodoptera frugiperda* (Lepidoptera: Noctuidae) in Brazil. Crop Prot. 64: 150-158.

Ferré, J., and J. Van Rie. 2002. Biochemistry and genetics of insect resistance to *Bacillus thuringiensis*. Annu. Rev. Entomol. 47: 501-533.

Grove, M., W. Kimble, and W. J. McCarthy. 2001. Effects of individual *Bacillus thuringiensis* insecticidal crystal proteins on adult *Heliothis virescens* (F.) and *Spodoptera exigua* (Hubner) (Lepidoptera: Noctuidae). BioControl. 46: 321-335.

Henneberry, T.J., L. Jech, and J. Maurer. 2006. Pink bollworm (Lepidoptera: gelechiidae) and tobacco budworm, cabbage looper and beet armyworm (Lepidoptera: Noctuidae) larval mortalities fed on *Bacillus thuringiensis* (BG) cotton pollens and pink bollworm adult mortality fed on sucrose solutions containing Cry1Ac toxin. Southwest. Entomol. 31: 1-12.

Henneberry, T.J., and S.E. Naranjo. 1998. Integrated management approaches for pink bollworm in the southwestern United States. Integ. Pest Manag. Rev. 3: 31-52.

Hutchison, W. D., E. C. Burkness, P. D. Mitchell, R. D. Moon, T. W. Leslie, S. J. Fleischer, M. Abrahamson, K. L. Hamilton, K. L. Steffey, M. E. Gray, et al. 2010. Areawide suppression of European corn borer with Bt maize reaps savings to non-Bt maize growers. Science 330: 222-225.

Jia, C.S. 2006. Calculating the LC_{50} of insecticides with software SPSS. Chin. Bull. Entomol. 43: 414-417.

Kurmanath KV, Wily Pink Bollworm Survives Monsanto's Bollgard-II. The Hindu Business Line. 28 October 2015. http://www.thehindubusinessline.com/economy/agri-business/wily-pink-bollworm-survives-monsantos-bollgardii/article7814810.ece. Accessed 31 October 2015.

Liu, Y. B., B. E. Tabashnik, S. K. Meyer, Y. Carrière and A. C. Bartlett. 2001. Genetics of pink bollworm resistance to *Bacillus thuringiensis* toxin Cry1Ac. J. Econ. Entomol. 94: 248-252.

Lu, Y., K. Wu, Y. Jiang, Y. Guo, and N. Desneux. 2012. Widespread adoption of Bt cotton and insecticide decrease promotes biocontrol services. Nature 487: 362–365.

Lukefahr, M. and J. A. Griffin. 1956. The effects of food on the longevity and fecundity of pink bollworm moths. J. Econ. Entomol. 49: 876~877.

Meissle, M., R. L. Hellmich, and J. Romeis. 2011. Impact of Cry3Bb1-expressing *Bt* maize on adults of the western corn rootworm, *Diabrotica virgifera virgifera* (Coleoptera: Chrysomelidae). Pest Manag. Sci. 67: 807-814.

Milligan, G.W. 1987. The use of the arc-sine transformation in the analysis of variance. Educ. Psychol. Meas. 47: 563-573.

Morin, S., R.W. Biggs, M.S. Sisterson, L. Shriver, C. Ellers-Kirk, D. Higginson, D. Holley, L.J. Gahan, D.G. Heckel, Y. Carrière, T.J. Dennehy, J.K. Brown, and B.E. Tabashnik. 2003. Three cadherin alleles associated with resistance to *Bacillus thuringiensis* in pink bollworm. Proc. Natl. Acad. Sci. USA 100: 5004-5009.

Naranjo, S.E. and J. M. Martin. 1993. Comparative development, reproduction and oviposition of pink bollworm (Lepidoptera: Gelechiidae) on a resistant okra-leaf cotton, and commercial upland and Pima cultivars. J. Econ. Entomol. 86, 1094-1103.

Ojha, A., K. S. Sree, B. Sachdev, M.A. Rashmi, K.C. Ravi, P.J. Suresh, K. S. Mohan, and R. K. Bhatnagar. 2014. Analysis of resistance to Cry1Ac in field-collected pink bollworm, *Pectinophora gossypiella* (Lepidoptera: Gelechiidae), populations. GM Crops Food 5: 280-286.

Osman, A. A., T. F. Watson, and S. Sivasupramaniam. 1991. Susceptibility of field populations of pink bollworm (Lepidoptera: Gelechiidae) to azinphosmehtyl and permethrin and synergism of permethrin. J. Econ. Entomol. 84: 358-362.

Pardo-López, L., M. Soberón, and A. Bravo. 2013. *Bacillus thuringiensis* insecticidal three-domain Cry toxins: mode of action, insect resistance and consequences for crop protection. FEMS Microbiol. Rev. 37: 3-22.

Pendleton, O.J., M. Tress, and R. Bremer. 1986. Interpretation of the four types of analysis of variance tables in sas. Commun. Stat-Theor. M. 15: 2785-2808.

Pusztai-Carey, M., P. R. Carey, T. Lessard, and M. Yaguchi, M. 1994. Isolation, quantitation, and purification of insecticidal proteins from *Bacillus thuringiensis*. U.S. patent 5,356,788.

Qi, G., J. Lu, P. Zhang, J. Li, F. Zhu, J. Chen, Y. Liu, Z. Yu, and X. Zhao. 2013. The Cry1Ac gene of *Bacillus thuringiensis* ZQ-89 encodes a toxin against long-horned beetle adult. J. Appl. Microbiol. 110: 1224-1234.

Sanahuja, G., R. Banakar, R. Twyman, T. Capell, and P. Christou. 2011. *Bacillus thuringiensis*: a century of research, development and commercial applications. Plant Biotechnol. J. 9: 283–300.

Schouest, L. P., and T. A. Miller. 1988. Factors influencing pyrethroid toxicity in pink bollworm (Lepidoptera: Gelechiidae): Implications for resistance management. J. Econ. Entomol. 81: 431-436.

Soberón, M., L. Pardo-López, I. López, I. Gómez, B.E. Tabashnik, A. Bravo. 2007. Engineering modified Bt toxins to counter insect resistance. Science. 318: 1640-1642.

Tabashnik, B. E., A. L. Patin, T. J. Dennehy, Y. B. Liu, Y. Carrière, M. A. Sims, and L. Antilla. 2000. Frequency of resistance to *Bacillus thuringiensis* in field populations of pink bollworm. Proc. Natl. Acad. Sci. USA 97: 12,980-12,984.

Tabashnik, B.E., Y.B. Liu, T.J. Dennehy, M. Sims, M. Sisterson, R.W. Biggs, and Y. Carrière. 2002. Inheritance of resistance to Bt toxin Cry1Ac in a field-derived strain of pink bollworm (Lepidoptera: Gelechiidae). J. Econ. Entomol. 95: 1018-1026.

Tabashnik, B. E., T. J. Dennehy and Y. Carrière. 2005. Delayed resistance to Bt cotton in pink bollworm. Proc. Natl. Acad. Sci. USA 102: 15,389-15,393.

Tabashnik, B.E., G.C. Unnithan, L. Masson, D.W. Crowder, X.C. Li, and Y. Carrière. 2009. Asymmetrical cross-resistance between *Bacillus thuringiensis* toxins Cry1Ac and Cry2Ab in pink bollworm. Proc Natl. Acad. Sci. USA 106: 11,889-11,894.

Tabashnik, B.E., M.S. Sisterson, P.C. Ellsworth, T.J. Dennehy, L. Antilla, L. Liesner, M. Whitlow, R.T. Staten, J.A. Fabrick, G.C. Unnithan, A.J. Yelich, C. Ellers-Kirk, V.S. Harpold, X. Li, and Y. Carrière. 2010. Suppressing resistance to Bt cotton with sterile insect releases. Nat. Biotechnol. 28: 1304-1307.

Tabashnik, B.E., S. Morin, G.C. Unnithan, A.J. Yelich, C. Ellers-Kirk, V.S. Harpold, M.S. Sisterson, P.C. Ellsworth, T.J. Dennehy, L. Antilla, L. Liesner, M. Whitlow, R.T. Staten, J.A. Fabrick, X. Li, and Y. Carrière. 2012. Sustained susceptibility of pink bollworm to Bt cotton in the United States. GM Crops Food 3: 194-200.

Tabashnik, B.E., T. Brévault, and Y. Carrière. 2013. Insect resistance to Bt crops: lessons from the first billion acres. Nat. Biotechnol. 31: 510-521.

Tabashnik, B.E., D. Mota-Sanchez, M.E. Whalon, R.M. Hollingworth, Y. Carrière. 2014. Defining terms for proactive management of resistance to Bt crops and pesticides. J. Econ. Entomol. 107: 496-507.

Tabashnik, B.E., M. Zhang, J.A. Fabrick, Y. Wu, M.J. Gao, F.N. Huang, J.Z. Wei, J. Zhang, A. Yelich, G.C. Unnithan, A. Bravo, M. Soberón, Y. Carrière, and X.C. Li. 2015. Dual mode of action of Bt proteins: protoxin efficacy against resistant insects. Sci. Rep. 5: 15107.

Wan, P., Y.X. Huang, H.H. Wu, M.S. Huang, S.B. Cong, B.E. Tabashnik, and K.M. Wu. 2012. Increased frequency of pink bollworm resistance to Bt toxin Cry1Ac in China. PloS ONE 7: e29975.

Welch, K.L., G.C. Unnithan, B.A. Degain, J.Z. Wei, J. Zhang, X.C. Li, B.E. Tabashnik, and Y. Carrière. 2015. Cross-resistance to toxins used in pyramided Bt crops and resistance to Bt sprays in *Helicoverpa zea*. J. Invertebr. Pathol. 132: 149-156.

Wierenga, J.M., D.L. Norris, and M.E. Whalon. 1996. Stage-specific mortality of Colorado potato beetle feeding on transgenic potatoes. J. Econ. Entomol. 89: 1047-1052.

Wu, K. M. and Y. Guo. 2004. Changes in susceptibility to conventional insecticides of a Cry1Ac-selected population of *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae). Pest. Manag. Sci. 60: 680 – 684.

Wu, J.P., H.H. Wu, P. Wan, M.S. Huang. 2008. Technique for rearing the pink bollworm (*Pectinophora gossypiella*) with artificial diet. Hubei Agricul. Sci. 47: 7.

Wu, Y.D. 2014. Detection and mechanisms of resistance evolved in insects to Cry toxins from *Bacillus thuringiensis*. Adv. Insect Physiol. 47: 297-342.

Yamaguchi, T., H. Bando, and S. Asano. 2013. Identification of a *Bacillus thuringiensis* Cry8Da toxin-binding glucosidase from the adult Japanese beetle, *Popillia japonica*. J. Invertebr. Pathol. 113: 123-128.

Zhang, Y., Y. Ma, P.J. Wan, L.L. Mu, and G.Q. Li. 2013. *Bacillus thuringiensis* insecticidal crystal proteins affect lifespan and reproductive performance of *Helicoverpa armigera* and *Spodoptera exigua* adults. J. Econ. Entomol. 106: 614-621.

Table 1. Responses to Cry1Ac of pink bollworm larvae from a resistant strain (AZP-R) and a susceptible strain (APHIS-S).

Strain	Slope (SE)[a]	LC_{50} (95% FL)[b]	RR[c]
APHIS-S	2.6 (0.3)	0.153 (0.11-0.22)	
AZP-R	2.8 (0.4)	98.2 (88-110)	640

a. Slope of the concentration-mortality line with its standard error in parentheses

b. Concentration killing 50% with 95% fiducial limits in parentheses, in μg Cry1Ac per ml diet.

c. Resistance ratio, the LC_{50} for AZP-R divided by the LC_{50} for APHIS-S.

511 **Table 2. Analysis of covariance (ANCOVA) for adult lifespan of pink bollworm[a]**

Source	Type III sum of squares	df	Mean square	F	P
Model	2920.1	7	417.2	26.6	<0.001
Intercept	124,626.1	1	124,626.1	7952.8	<0.001
Sex	116.3	1	116.3	7.424	0.007
Strain	103.2	1	103.2	6.586	0.010
Concentration	2418.3	1	2418.3	154.3	<0.001
Sex * Strain	9.259	1	9.259	0.591	0.442
Sex * Concentration	1.100	1	1.100	0.070	0.791
Strain * Concentration	61.521	1	61.521	3.926	0.048
Sex * Strain * Concentration	8.708	1	8.708	0.556	0.456
Error	11,157.5	712	15.671		
Total	162,141.0	720			
Corrected Total	14,077.6	719			

512 [a] The categorical variables were sex and strain (susceptible or resistant), with the
513 covariate of concentration (0, 2.5, 10, 40, 160 or 640 μg Cry1Ac per ml).

514

515

516 **Table 3. Effects of Cry1Ac and cypermethrin on adult lifespan of resistant (AZP-**

517 **R) and susceptible (APHIS-S) strains of pink bollworm.**

Treatment (concn. in µg/ml)	Female		Male	
	AZP-R	APHIS-S	AZP-R	APHIS-S
Control	17.7±0.5 a A	18.0±0.6 a A	18.2±0.7 a A	16.9±0.5 a A
Cry1Ac (2.5)	17.7±0.6 a A	17.1±0.8 ab A	15.8±0.7 ab A	14.9±0.6 ab A
Cry1Ac (10)	15.5±0.8 a A	15.2±0.9 ab A	15.1±0.8 b A	14.4±0.5 ab A
Cry1Ac (40)	16.3±0.8 a A	14.9±0.9 b A	15.3±0.7 b A	13.9±0.6 b A
Cry1Ac (160)	12.0±0.5 b A	9.8±0.6 c B	11.2±0.5 c A	9.3±0.5 c B
Cry1Ac (640)	13.0±0.5 b A	10.4±0.7 c B	11.9±0.5 c A	9.9±0.4 c B
Cypermethrin (9)	3.7±0.3 c A	2.6±0.3 d B	3.4±0.3 d A	2.4±0.3 d B

518 Values are means ± SE. Data were analyzed with ANOVA followed by Tukey's HSD
519 test. Different lower case letters indicate significant differences between treatments
520 within each strain. Different upper case letters indicate significant differences between
521 strains for a given treatment.

522

523 **Table 4. Effects of Cry1Ac on reproduction of resistant (AZP-R) and susceptible (APHIS-S) strains of pink bollworm.**

Cry1Ac (µg/ml)	Pairs producing eggs (%)		Pre-oviposition period (days)		Oviposition period (days)		Eggs		Hatch (%)	
	AZP-R	APHIS-S	AZP-R	APHIS-S	AZP-R	APHIS-S	AZP-R	APHIS-S	AZP-R	APHIS-S
0	94.1±3.4a	98.1±1.9a	3.1±0.2a	2.8±0.1a	8.4±0.5a	8.2±0.4a	154±15a	151±12a	82.5±1.4a	81.7±1.9a
10	96.1±2.0a	95.7±2.2a	2.9±0.2a	2.8±0.1a	4.7±0.4b	4.8±0.3b	109±11b	91.0±8.3bc	84.0±1.9a	83.6±1.5a
40	92.3±5.2a	91.5±2.0a	3.1±0.2a	3.0±0.1a	5.0±0.4b	5.0±0.2b	80.5±8.4bc	76.2±7.0bc	84.2±1.6a	83.3±1.4a
160	86.3±2.0a	90.7±1.9a	3.4±0.2a	3.2±0.2a	4.4±0.4b	4.2±0.3b	79.4±11.2bc	61.8±6.2c	81.3±2.5a	80.9±2.7a

524 Values are means ±SE. Sample size ranged from 48 to 52 pairs for each strain at each concentration of Cry1Ac (total = 406 pairs). Data were
525 analyzed with ANOVA followed by Tukey's HSD test. Different lower case letters indicate significant differences between treatments within
526 each strain. For each treatment, no significant differences occurred between strains.
527

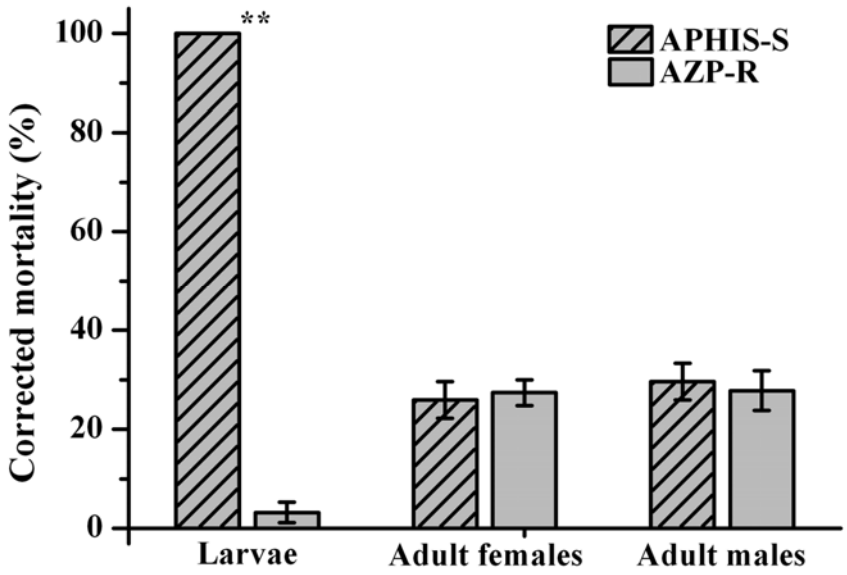

Fig. 1. Mortality of larvae and adults of the AZP-R (Cry1Ac-resistant) and APHIS-S (susceptible) strains of pink bollworm when exposed to Cry1Ac (10 μg Cry1Ac per ml artificial diet for larvae and 10 μg Cry1Ac per ml honey water for adults). The asterisks show a significant difference between strains in larval mortality (t-test, $t = 46.7$, $df = 6$, $P < 0.001$). The difference between strains in adult mortality was not significant for either females (t-test, $t = -0.33$, $df = 4$, $P = 0.76$) or males (t-test, $t = 0.34$, $df = 4$, $P = 0.75$).