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ORIGINAL CONTRIBUTION

Fitness costs associated with Cry1F resistance in the European corn borerJ. L. Petzold-Maxwell¹, B. D. Siegfried^{2,*}, R. L. Hellmich³, C. A. Abel³, B. S. Coates³, T. A. Spencer², R. J. Horikoshi^{1,4} & A. J. Gassmann¹

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Abstract

Crops producing insecticidal toxins derived from the bacterium *Bacillus thuringiensis* (Bt) are widely planted to manage insect pests. Bt crops can provide an effective tool for pest management; however, the evolution of Bt resistance can diminish this benefit. The European corn borer, *Ostrinia nubilalis* Hübner, is a significant pest of maize and is widely managed with Bt maize in the Midwest of the United States. When Bt crops are grown in conjunction with non-Bt refuges, fitness costs of Bt resistance can delay the evolution of resistance. Importantly, fitness costs often vary with ecological factors, including host-plant genotype and diapause. In this study, we examined fitness costs associated with Cry1F resistance in *O. nubilalis* when insects were reared on three maize lines. Fitness costs were tested in two experiments. One experiment assessed the fitness costs when Cry1F-resistant and Cry1F-susceptible insects were reared on plants as larvae and experienced diapause. The second experiment tested resistant, susceptible and F1 heterozygotes that were reared on plants but did not experience diapause. Despite some evidence of greater adult longevity for Cry1F-resistant insects, these insects produced fewer fertile eggs than Cry1F-susceptible insects, and this occurred independent of diapause. Reduced fecundity was not detected among heterozygous individuals, which indicated that this fitness cost was recessive. Additionally, maize lines did not affect the magnitude of this fitness cost. The lower fitness of Cry1F-resistant *O. nubilalis* may contribute to the maintenance of Cry1F susceptibility in field populations more than a decade after Cry1F maize was commercialized.

Introduction

The European corn borer, *Ostrinia nubilalis* Hübner (Lepidoptera: Crambidae), has been an economically important pest of maize in North America since accidental introduction to the eastern United States nearly a century ago (Mason et al. 1996; Siegfried and Hellmich 2012). Injury caused by *O. nubilalis* to whorl- and reproductive-stage maize can result in significant yield loss (Mason et al. 1996). In 1996 and 2001, respectively, maize producing insecticidal

toxins Cry1Ab and Cry1F, derived from the bacterium *Bacillus thuringiensis* (Bt), was commercialized for the management of *O. nubilalis* (EPA 2010). By 2015, Bt hybrids accounted for 81% of the total area planted to maize (ERS 2015). The extensive use of Bt maize targeting this pest has resulted in areawide suppression of *O. nubilalis* populations in several Midwest states (Hutchison et al. 2010). However, the widespread planting of Bt maize also has placed intense selective pressure on *O. nubilalis* to evolve Bt resistance.

The evolution of pest resistance to Bt crops is, arguably, the greatest threat to the success of this management tool. Several insect species have evolved resistance to Bt crops in the field (Tabashnik et al. 2013), although field populations of *O. nubilalis* currently remain susceptible to Bt maize (Siegfried and Hellmich 2012; Siegfried et al. 2014). The high-dose/refuge (HDR) strategy is an insect resistance management (IRM) strategy that aims to delay or prevent the increase in Bt-resistant phenotypes within target insect populations (Gould 1998; Tabashnik et al. 2003). Under this strategy, non-Bt plants serve as a refuge on which Bt-susceptible individuals can survive, thereby providing a large pool of homozygous susceptible individuals to mate with rare homozygous-resistant individuals that develop on Bt maize plants, resulting in heterozygous progeny (Gould 1998; Tabashnik et al. 2003). Bt crops that produce a high dose of Bt toxin kill these heterozygous individuals, as well as Bt-susceptible genotypes, thereby delaying the evolution of resistance (Gould 1998).

The HDR strategy assumes that the frequency of alleles conferring resistance to Bt toxins is low in target insect populations (Gould 1998; Bates et al. 2005). However, Siegfried et al. (2014) estimated the frequency of alleles conferring resistance to the Bt toxin Cry1F in *O. nubilalis* and reported a resistance allele frequency of 0.0268 during the initial 3 years following commercialization of Cry1F maize (2003–2005), and a frequency of 0.0253 for the following 3 years. By contrast, resistance allele frequency is typically assumed to be 0.001 or less in computer models used to simulate the evolution of Bt resistance for an insect population (Onstad and Guse 1999; Crowder et al. 2006; Onstad and Meinke 2010). Although the frequency of Cry1F resistance alleles in *O. nubilalis* populations is an order of magnitude higher than typically assumed, mathematical modelling suggests that the HDR strategy can still delay resistance if fitness costs are present (Carrière and Tabashnik 2001). Fitness costs often accompany resistance traits and occur when individuals with resistance alleles have lower fitness than homozygous susceptible individuals in the absence of Bt toxins (Gassmann et al. 2009). Fitness costs can delay resistance to Bt crops when refuges are present by decreasing the frequency of resistance alleles in a refuge population (Carrière and Tabashnik 2001; Pittendrigh et al. 2004; Gassmann et al. 2009).

Several studies have demonstrated fitness costs of Bt resistance in *O. nubilalis*. Bolin et al. (1999) showed a 10-fold decline in the resistance ratio of a Cry1Ac-resistant strain of *O. nubilalis* over nine generations in

the absence of selection, indicating the presence of fitness costs. Crespo et al. (2009, 2010) documented fitness costs associated with a Cry1Ab-resistance trait that included reduced larval survival and body mass, increased development time, a higher proportion of copulations resulting in unsuccessful matings and lower fertility. For *O. nubilalis* individuals with alleles conferring resistance to Cry1F toxin, Pereira et al. (2011) found that insects fed artificial diet without Cry1F toxin suffered fitness costs of reduced pupal mass and reduced larval growth rate for females. By contrast, no fitness costs were found for larval survival, growth rate or mass when Cry1F-resistant insects were tested on three different maize lines (Petzold-Maxwell et al. 2014). It is important to note that with the exception of Petzold-Maxwell et al. (2014), these studies were conducted with insects reared on artificial diets optimized for growth and development. In general, fitness costs of Bt resistance are observed more frequently when insects are fed plants compared to artificial diets (Gassmann et al. 2009).

For fitness costs, both the magnitude and the dominance (i.e. the extent to which costs affect homozygous-resistant versus heterozygous-resistant individuals) can vary with ecological conditions, including host-plant genotype and plant allelochemicals (Gassmann et al. 2009). For example, the presence of the cotton phytochemical gossypol in artificial diet increased the magnitude and dominance of some fitness costs in Cry1Ac-resistant *Pectinophora gossypiella* (Saunders) (Carrière et al. 2004), and a number of studies have shown that the magnitude of fitness costs can depend on host-plant species, genotype or quality (Carrière et al. 2005; Janmaat and Myers 2005; Bird and Akhurst 2007; Raymond et al. 2007). The dominance of fitness costs also may affect the evolution of Bt resistance, with non-recessive fitness costs imposing greater delays in resistance evolution compared to those that are recessive (Carrière and Tabashnik 2001; Gassmann et al. 2009). Additionally, as the magnitude of fitness costs increases, longer delays in resistance are expected (Carrière and Tabashnik 2001). Thus, understanding how host-plant genotypes used in refuges may affect fitness costs of Bt resistance could improve resistance management.

In this study, we examine whether fitness costs are present for larvae and adults of a Cry1F-resistant strain of *O. nubilalis*. Because plant genotype has previously been shown to affect fitness costs (Gassmann et al. 2009), and fitness costs can be influenced by stressful environments, such as diapause or overwintering (Foster et al. 1997; Carrière et al. 2001, 2007), we tested the hypothesis that either host-plant

genotype, diapause or both could affect fitness costs of Cry1F resistance in *O. nubilalis*. To test this hypothesis, we conducted two experiments using three different maize lines. One experiment used insects reared to adulthood on plants without experiencing diapause, and the other experiment used insects that were reared on plants but also experienced diapause. Results from this study will be useful in the future development of IRM strategies for *O. nubilalis* and other pest insects targeted by Bt crops.

Methods

Insect strains

The Cry1F-susceptible strain originated from field-collected insects near Geneva, New York, in 1985, with additional insects introduced to the strain in later years as described in Petzold-Maxwell et al. (2014). The Cry1F-resistant strain originated from insects collected throughout the United States Corn Belt in 1996. Both strains were maintained using standard rearing procedures (Lewis and Lynch 1969; Siqueira et al. 2004), as described in Pereira et al. (2008a). Selection for Cry1F resistance was initiated in 1998 and is described in detail by Pereira et al. (2008a). Insects were selected with increasing concentrations of Cry1F incorporated into rearing diet for 30 generations and maintained at 35 µg Cry1F per ml of diet for ten generations. Further selection was accomplished in 2001 and 2002 using Cry1F applied to the surface of artificial diet and exposing neonates for 7 days, after which surviving neonates were transferred to untreated diet. Neonates were exposed to 60 ng Cry1F per cm², corresponding to the upper limit of the 95% confidence interval of the LC99 for susceptible populations, once every three generations to maintain the Cry1F-selected strain (Pereira et al. 2008a). In diet bioassays, the Cry1F-resistant strain displayed a resistance ratio of >3 000 to Cry1F and larvae survived on reproductive-stage Cry1F-producing maize tissue (Pereira et al. 2008a,b).

Because differences in fitness between Bt-susceptible and Bt-resistant strains can be due to genetic differences unrelated to resistance, we repeatedly backcrossed the resistant strain to the susceptible strain (a total of five times), followed by selection on Cry1F, as described in Petzold-Maxwell et al. (2014). This method of backcrossing increased the genetic similarity between the resistant and susceptible strains and helped to increase the likelihood of finding differences in fitness between the two strains caused only by the presence of Bt resistance alleles. Diet bioassays

conducted following backcrossing and selection on Cry1F confirmed that the resistant strain was still highly resistant to Cry1F (Petzold-Maxwell et al. 2014).

On-plant experiments

Two experiments were conducted to examine life-history characteristics and survival of *O. nubilalis* strains on different maize lines. In the first experiment (hereafter referred to as experiment one), we measured pre-diapause and post-diapause survival and fitness characteristics of Cry1F-resistant and Cry1F-susceptible insects that developed as larvae on plants and completed pupation and eclosion in the laboratory. In the second experiment (hereafter referred to as experiment two), we measured survival and fitness characteristics of Cry1F-resistant, Cry1F-susceptible and heterozygous (i.e. F1) insects that completed all stages of development on plants and did not experience diapause. In both experiments, three different maize lines were used: the F1-hybrid B73 × Mo17 and two highly inbred lines, WF9 and B94. Both B73 × Mo17 and WF9 are susceptible to *O. nubilalis* (Manuwoto and Scriber 1985; Klenke et al. 1986). The maize line B94 has moderate 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA)-based resistance against *O. nubilalis* (Russell 1991; Abel et al. 2000). Maize plants were grown in a greenhouse in 9-l pots (Classic 1000, Hummert International, Earth City, Missouri) as described in Petzold-Maxwell et al. (2014). Greenhouse temperature throughout the experiment was 25.9°C ± 7.6°C (mean ± SD) for experiment one and 22.3°C ± 6.1°C for experiment two. In both experiments, larvae were placed on 8- to 9-week-old, reproductive-stage maize plants, corresponding to the second generation of the two generations per year that is typically observed for *O. nubilalis* in the northern Corn Belt (Munkvold et al. 1999). Plants contained 16–18 fully expanded leaves, and ≥90% had a visible tassel.

Experiment one – fitness characteristics of Cry1F-resistant and Cry1F-susceptible strains experiencing diapause

This experiment was conducted between June 2012 and March 2013. Maize line (B94, WF9 and B73 × Mo17) and insect strain (Cry1F-resistant and Cry1F-susceptible) were tested in a fully crossed design, yielding a total of six treatments. Eleven blocks were established, with two replicates per treatment in each block for a total of 12 plants per block (3 maize lines × 2 strains × 2 replicates), and a total of 132 plants in the experiment (11 blocks × 12 plants).

Larvae from either the susceptible or the resistant strain were placed on plants according to their respective treatment. Ten newly hatched larvae (<24 h old) each were placed in small microcentrifuge tubes using a fine paintbrush, and two microcentrifuge tubes containing larvae were placed in the whorl of each maize plant for a total of 20 larvae placed on each plant. To prevent movement of larvae among plants, each plant was enclosed in a cylindrical cage covered in mesh fabric (52 × 52 Amber Fabricated, Lumite, Baldwin, GA, USA). A wooden stake (height = 1.5 m) was placed in each pot, and cages were secured to the base of pots with Velcro (Industrial Webbing, Boynton Beach, FL) and closed with a twist tie at the top of the plant around the stake. Plants were watered as necessary during the experiments through the mesh fabric. Supplemental lighting was provided by 400-W high-pressure sodium bulbs (Ruud Lighting Inc., Racine, WI) set to 16/8 L/D. Due to high temperatures in the greenhouse bay, supplemental lighting was only used on cloudy days from the time plants were 3 weeks old until larvae were collected from plants. As a result of the decreasing day length between placement of neonates on plants and removal of larvae from plants (14 August 2012 to 18 October 2012), the larvae entered diapause.

Larvae were collected by carefully dissecting maize plants 8–9 weeks after neonates were placed on plants. At the time of collection, all larvae were fifth instars, based on head capsule width measurements following DeWitt and Stockdale (1980). Pre-diapause survival of larvae was calculated (number of larvae per plant ÷ 20), and larvae were weighed to the nearest 0.1 mg (XS205 analytical balance, Mettler-Toledo, Columbus, OH). Larvae were then placed individually into 22-ml plastic cups (Dart, Mason, MI) containing a 3 × 3 cm piece of slightly moistened paper towel (Envision 1-Ply, Georgia Pacific, Atlanta, Georgia) and held in pre-diapause conditions (15.5°C, 60% RH, 13.5 : 10.5 L : D). This was performed to provide a more gradual transition between larval rearing conditions and diapause conditions, as would be expected to occur in the field. After 4 weeks, all larvae were moved to a dark chamber at 8°C and 80% RH to induce diapause; paper towels were periodically moistened as needed. After 16 weeks of diapause conditions, all larvae were weighed and placed individually into small plastic cups containing an agar mixture (Andow and Stodola 2003) and a 2.5-cm-long section of a drinking straw, which had been tinted black using a marker to encourage pupation. Larvae were placed in a chamber held at 26.5°C, 80% RH and 24-h light to break diapause and checked daily for pupation.

Pupae were removed from straws and weighed and held at 26.5°C until eclosion. Survival to adulthood per plant was recorded as number of adults ÷ 20.

Concurrently, pupae from the Cry1F-susceptible and Cry1F-resistant laboratory strains, that had been reared on artificial diet, were separated by sex and placed individually in plastic containers for the purpose of mating with the plant-reared insects that experienced diapause. Within 24 h of eclosion, each plant-reared insect was mated singularly with a corresponding laboratory-reared insect of the same strain (i.e. resistant individuals were mated with resistant individuals). A total of 138 mated pairs were set up, with an average of 11.5 (SD = 4.4) pairs for each combination of strain by maize type by sex. Pairs were placed in small cages made of hardware cloth containing an adult diet of sucrose agar adapted from Andow and Stodola (2003) and held at 26.5°C, 80% RH and 16 : 8 L : D. Cages were placed on moistened sheets of cotton (U.S. Cotton, Cleveland, OH) that received water daily. Adults were checked for mortality daily. Wax paper sheets were placed on top of the cage to provide an oviposition substrate and changed daily after initiation of oviposition. Sheets were stored in plastic bags in a chamber held at 26.5°C, 80% RH and 16 : 8 L : D. The number of eggs, egg masses, and viable eggs were recorded daily for each pair using a dissecting microscope (MZ6, Leica Microsystems, Wetzlar, Germany). Viability was assessed by examining egg masses at the 'black-head' stage of development (approximately 2–8 h before hatching and when larvae are well formed and the head capsule is visible through the egg), and black-head eggs were considered viable. Collection of eggs was terminated once the female died, and within 24 h of death, females were dissected to determine whether a spermatophore was present, which confirmed that mating occurred (spermatophores were counted, but very few insects contained greater than one spermatophore).

Experiment two – fitness characteristics of Cry1F-resistant, Cry1F-susceptible and heterozygous insects

This experiment was conducted from February to June, 2013. Maize line (B94, WF9 and B73 × Mo17) and insect strain (Cry1F-resistant, Cry1F-susceptible and heterozygous larvae) were tested in a fully crossed design, yielding a total of nine treatments. Sixteen blocks were established, with one replicate per treatment in each block for a total of nine plants per block (3 maize lines × 3 strains × 1 replicate), and a total of 144 plants in the experiment (16 blocks × 9 plants).

To generate susceptible, resistant and heterozygous larvae for the experiment, four mass mating cages

were set up using the Cry1F-susceptible and Cry1F-resistant strains: (i) resistant males \times resistant females, (ii) susceptible males \times susceptible females, (iii) resistant females \times susceptible males and (iv) resistant males \times susceptible females. Prior to establishing these cages, pupae from each strain were separated by sex and individually placed in 22-ml plastic cups (Dart), and then transferred to mating cages within 24 h of eclosion in a 1 : 1 ratio (each cage contained 80–100 adults added over a period of 6 days). Mating cage cylinders (6.3 cm height \times 8 cm diameter, constructed from 27-gauge woven hardware cloth) were placed on a moistened sheet of cotton and covered with a sheet of wax paper. Adults were provided with a sucrose agar diet adapted from Andow and Stodola (2003). Each day, cotton sheets were moistened and diet and wax paper sheets were changed. Eggs from each of the four strains were placed in small cups containing a moist cotton ball and covered with vented lids. Upon peak hatch, 10 neonates (<24 h old) each were placed in microcentrifuge tubes as previously described, with two microcentrifuge tubes placed in the whorl of each plant, for a total of 20 neonates per plant. For plants that received heterozygotes, 10 neonates from one reciprocal cross were placed in a microcentrifuge tube and 10 neonates from the other reciprocal cross were placed in a second microcentrifuge tube, such that half of the larvae placed on each plant were from each of the two reciprocal crosses. Heterozygous larvae were pooled in this manner because past research found that Cry1F resistance is not a sex-linked trait in this strain (Pereira et al. 2008b). All plants were covered with mesh cages as described for experiment one and checked daily for adults 3 weeks after neonates were placed on plants.

Adults began emerging approximately 4 weeks after neonates were placed on plants, and data were collected on day of emergence by collecting adults daily and calculating the number of days that elapsed since emergence of the first adult in the experiment. Data were also collected on survival to adulthood as number of adults collected from a plant \div 20. As adults were collected, each was mated with an adult of the same strain that emerged from the same maize line (i.e. a Cry1F-resistant male that developed on a WF9 plant was mated with a Cry1F-resistant female that developed on a WF9 plant). All individuals were mated with an insect that emerged on the same day with the exception of five pairs, which emerged within a day of each other. Pairs were held in hardware-cloth cages containing food for adults and wax paper sheets for oviposition. Cages were maintained

as described for experiment one. Each pair was treated as an experimental replicate, and a total of 184 mated pairs were established for the entire experiment, with 18–25 pairs for each combination of maize line by insect strain. Cages were checked daily for mortality, eggs were collected daily and the number of eggs, egg masses and viable eggs were recorded daily for each pair.

Data analysis

For both experiments, analysis of variance (ANOVA) was performed in SAS Enterprise Guide 4.2 (SAS 2009). Random effects in models were tested using a log-likelihood ratio statistic (-2 RES log likelihood in PROC MIXED) based on a one-tailed chi-square test assuming one degree of freedom (Littell et al. 1996), with block and its interactions removed from the model to increase statistical power when these factors were not significant at a level of $\alpha < 0.25$ (Quinn and Keough 2002). Lower order terms were always retained if their higher order interactions were significant. When significant fixed factors were present, pairwise comparisons were conducted based on the Tukey–Kramer method (PDIFF option in PROC MIXED).

Experiment one

To analyse pre-diapause survival, survival to adulthood, pre- and post-diapause mass, proportion of pairs that mated, number of fertile eggs and number of egg masses, a mixed-model ANOVA (PROC MIXED) was used that included the fixed factors of maize line, insect strain and their interaction. Random factors included block and the interaction of block with all fixed factors. To ensure normality of the residuals, data on survival to adulthood were transformed by the log function ($X + 0.05$) and data on proportion of pairs that mated were arcsine square root-transformed. Sex of insect was not a factor in these models because, with the exception of survival to adulthood, sex was unknown or not applicable (e.g. proportion of pairs that mated). When survival to adulthood was analysed with sex as a factor, it was not possible to achieve normality of residuals. Because sex and all of its interactions were not significant in the analysis, sex was removed from the model because this produced residuals that followed a normal distribution. Additionally, data on number of fertile eggs and number of egg masses were analysed separately for females reared on plants and those reared on artificial diet.

For insects that were reared on plants and survived to adulthood, data were analysed on pupal mass, mass

lost during diapause and adult longevity with a mixed-model ANOVA that included the fixed factors of maize line, insect strain and sex, and all possible interactions among these three factors. Random factors included block and all of the interactions with fixed factors, with the exclusion of block by sex by maize line by insect strain, because it was not possible to achieve convergence of the model when that factor was included.

For the laboratory-reared insects (i.e. reared on artificial larval diet) that were mated with post-diapause insects reared on maize plants, adult longevity was analysed with a mixed-model ANOVA that included the fixed factors of sex, insect strain and the interaction between sex and strain. Random factors included block and the interaction of block with all fixed factors.

Experiment two

To analyse survival, day of emergence and adult longevity, a mixed-model ANOVA was used that included the fixed factors of maize line, insect strain, sex and all possible interactions of these three factors. Random factors included block and all of the interactions with fixed factors, with the exclusion of block by sex by maize line by insect strain for the analyses of survival and emergence because it was not possible to achieve convergence of the model when that factor was included. To ensure normality of the residuals, data on day of emergence were transformed with the log function ($X + 0.05$).

The proportion of pairs that mated, the number of fertile eggs per female and the number of egg masses per female were analysed with a mixed-model ANOVA that included fixed factors of maize line, insect strain and their interaction. Random factors included block (of the female insect) and the interaction of block with all fixed factors. To ensure normality of the residuals, data on number of egg masses per female were transformed with a square root function.

Results

Experiment one – fitness characteristics of Cry1F-resistant and Cry1F-susceptible strains experiencing diapause

Maize line, insect strain and the interaction of maize line and insect strain did not significantly affect survival of insects before diapause (on-plant survival to the fifth and final larval instar) or survival to adulthood following diapause (table 1; Table S1).

Pre-diapause larval mass was significantly affected by maize line (Table S1; $F_{2,20} = 8.07$; $P = 0.003$); mass

was significantly greater on both B73 × Mo17 and B94 compared to WF9 (adjusted $P < 0.029$ for both comparisons) (table 1). The decrease in mass during diapause (pre-diapause larval mass – pupal mass) was affected by a significant interaction between sex and maize line (table 1; Table S2 $F_{2,68} = 3.47$; $P = 0.037$). Females reared on B73 × Mo17 lost significantly more mass than males or females reared on WF9 and males reared on B73 × Mo17 (adjusted $P < 0.008$ in all cases), but no other significant differences were present among any combination of sex and maize line. Maize line ($F_{2,78} = 11.14$; $P < 0.0001$) and sex ($F_{1,78} = 55.56$; $P < 0.0001$) were significant factors affecting pupal mass (Table S2). Pupae were significantly larger on B73 × Mo17 compared to either WF9 or B94 (adjusted $P < 0.007$ in both cases), and females were significantly larger than males (fig. 1). No significant effects were detected for the proportion of mated pairs (Table S2).

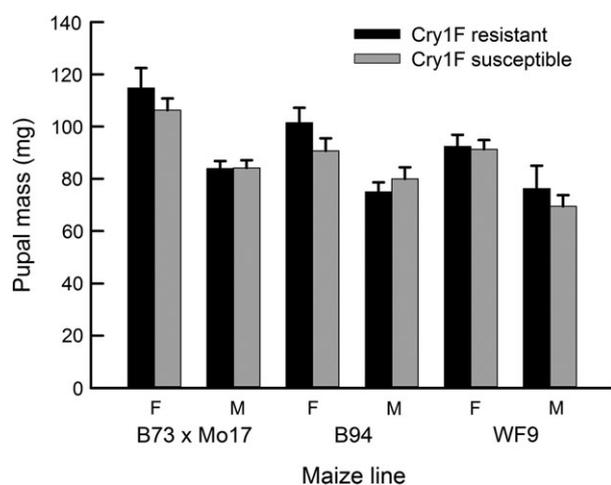
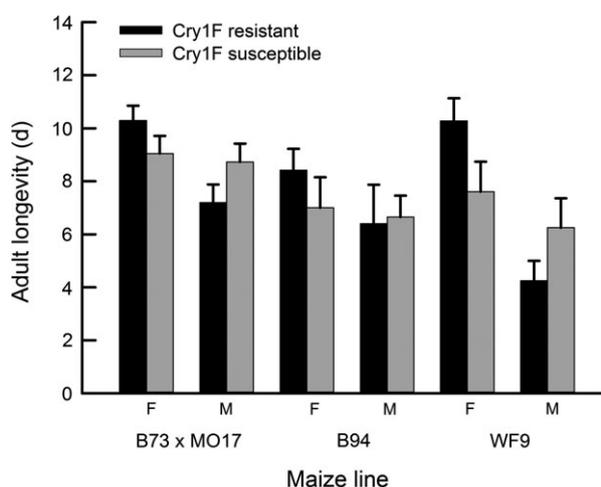
For insects in mated pairs that were reared on plants as larvae and experienced diapause (experimental insects), a significant strain by sex interaction was detected for adult longevity (Table S2; $F_{1,36} = 9.07$; $P = 0.005$), with resistant females living significantly longer than susceptible males and resistant males (adjusted $P < 0.009$ for both comparisons) (fig. 2). Additionally, there was a significant effect of maize line ($F_{2,13} = 4.56$; $P = 0.032$). Although no statistically significant pairwise differences were detected, adults from B73 × Mo17 lived the longest, and average survival was lower, and similar, on B94 and WF9 (fig. 2; Table S1). For laboratory-reared insects that were used to mate with insects that developed on plants and experienced diapause, adult longevity was not significantly affected by strain, sex or the interaction of sex and strain (mean ± SE: resistant: 13.11 ± 0.56 d, susceptible: 12.33 ± 0.57 d) (strain: $F_{1,120} = 1.21$, $P = 0.2740$; sex: $F_{1,120} = 0.08$, $P = 0.780$; sex by strain: $F_{1,120} = 0.00$, $P = 0.968$).

For laboratory-reared (diet-fed) females mated with males that developed on plants and experienced diapause, strain, maize line (of mate) and the interaction of maize line and strain did not significantly affect the number of egg masses produced by females (table 2; Table S1) or the number of fertile eggs produced (Table S1; fig. 3a). By contrast, for females that developed on plants and experienced diapause, resistant females produced significantly fewer fertile eggs ($F_{1,45} = 5.90$; $P = 0.019$) than susceptible females (Table S1; fig. 3b). However, maize line, strain and their interaction did not significantly affect the number of egg masses (table 2; Table S1).

Table 1 Mean pre-diapause survival, survival to adulthood, pre-diapause larval mass, post-diapause larval mass and total mass lost during diapause (standard error in parentheses) for Cry1F-resistant and Cry1F-susceptible strains of *Ostrinia nubilalis* developing on three maize lines and experiencing diapause (experiment one)

Maize line	Strain*	Pre-diapause survival (prop.)	Survival to adulthood (prop.)	Pre-diapause larval mass (mg)	Post-diapause larval mass (mg)	Total mass lost (mg) ^{†,‡}	
						M	F
B73 × Mo17	Res	0.15 (0.02)	0.08 (0.01)	117.14 (6.06)	100.75 (5.91)	18.20 (2.67)	34.86 (6.60)
B73 × Mo17	Sus	0.12 (0.02)	0.06 (0.01)	111.77 (5.98)	98.74 (6.34)	18.61 (2.63)	34.40 (4.23)
B94	Res	0.13 (0.02)	0.05 (0.01)	111.96 (6.29)	96.65 (8.41)	20.67 (3.57)	31.52 (3.60)
B94	Sus	0.10 (0.01)	0.04 (0.01)	103.82 (4.37)	92.00 (5.53)	23.07 (3.31)	19.91 (7.19)
WF9	Res	0.08 (0.01)	0.04 (0.01)	95.24 (6.01)	93.72 (5.35)	21.41 (4.23)	20.12 (3.61)
WF9	Sus	0.11 (0.02)	0.05 (0.01)	88.01 (4.50)	74.41 (5.58)	14.52 (2.19)	21.66 (4.21)

*Res: Cry1F-resistant; Sus: Cry1F-susceptible.

[†]Pre-diapause larval mass–post-diapause larval mass.[‡]Determined only for insects that survived to adulthood.**Fig. 1** Pupal mass for Cry1F-resistant and Cry1F-susceptible strains of *Ostrinia nubilalis* experiencing diapause following larval development on three maize lines (experiment one). Data are presented for insects that survived to adulthood. Bar heights represent sample means, and error bars are the standard error of the mean.**Fig. 2** Adult longevity for Cry1F-resistant and Cry1F-susceptible strains of *Ostrinia nubilalis* developing on three maize lines, experiencing diapause and subsequently mating with laboratory strains (experiment one). Bar heights represent sample means, and error bars are the standard error of the mean.

Experiment two – fitness characteristics of Cry1F-resistant, Cry1F-susceptible and heterozygous insects

In the second experiment, survival was significantly affected by maize line and sex (maize line: $F_{2,30} = 7.73$; $P = 0.002$; sex: $F_{1,15} = 6.13$; $P = 0.026$) (Table S3;). Male survival was higher than female survival, and survival was higher on the maize line WF9 compared with B73 × Mo17 and B94 (adjusted $P < 0.012$ for both comparisons) (table 3). Insects emerged significantly earlier on WF9 compared with B73 × Mo17 and B94 (adjusted $P < 0.0001$ for both comparisons) (table 3; Table S3). Sex also significantly affected day of emergence, with males

requiring fewer days to reach adulthood than females (table 3; Table S3). The proportion of insect pairs that mated and the number of egg masses produced per female were not significantly affected by strain, maize line or the interaction of strain and maize line (table 3; Table S3). The number of fertile eggs was significantly affected by strain ($F_{2,160} = 4.30$; $P = 0.015$), with resistant insects producing significantly fewer fertile eggs than susceptible insects (susceptible = 357 ± 35.6 (mean \pm SE) vs. resistant = 217 ± 36.2 ; adjusted $P = 0.0186$) (Table S3; fig. 3c). By contrast, the number of fertile eggs produced by heterozygous insects and susceptible insects was similar (susceptible = 357 ± 35.6 vs. heterozygotes = 334 ± 35.0)

Table 2 Mean proportion (standard error in parentheses) of pairs that mated and number of egg masses per female for Cry1F-resistant and Cry1F-susceptible strains of *Ostrinia nubilalis* (experiment one)

Maize line	Strain*	Prop. Mated	No. egg masses per female	
			Plant-reared diapausing females [†]	Laboratory-reared females [‡]
B73 × Mo17	Res	0.69 (0.09)	32.22 (6.86)	55.13 (10.73)
B73 × Mo17	Sus	0.79 (0.09)	32.09 (6.28)	49.13 (13.10)
B94	Res	0.75 (0.11)	23.74 (4.52)	59.80 (18.73)
B94	Sus	0.69 (0.11)	27.33 (7.21)	44.00 (8.94)
WF9	Res	0.72 (0.13)	28.35 (5.51)	32.00 (12.62)
WF9	Sus	0.71 (0.12)	25.94 (5.48)	47.63 (10.55)

*Res: Cry1F-resistant; Sus: Cry1F-susceptible.

[†]Data from females that were reared on one of three maize lines and experienced diapause.

[‡]Data from laboratory-reared females.

and did not differ statistically (adjusted $P = 0.883$). Taken together, these data suggest recessive inheritance of this fitness cost affecting the number of fertile eggs produced per female.

Discussion

We found that *O. nubilalis* from the Cry1F-resistant strain produced significantly fewer fertile eggs than insects from the Cry1F-susceptible strain when larvae were reared on maize plants (fig. 3), indicating a fitness cost of resistance. However, this fitness cost was absent for females reared on artificial diet in our experiments, indicating that this cost was induced when insects were reared on non-Bt maize plants. This plant-induced fitness cost arose regardless of whether or not insects experienced diapause. Although we found some evidence that resistant insects had greater adult longevity, this life-history difference apparently was not sufficient to ameliorate the fitness cost of lower fecundity experienced by the resistant strain. We did not observe an interaction between insect strain and maize line for fecundity, suggesting that the decreased production of fertile egg for the resistant strain was similar across the three maize lines tested in this study. Additionally, the fitness cost of reduced fecundity was present only for the resistant strain and not for heterozygous individuals, indicating that this fitness cost was recessive. No additional fitness costs were observed for the larval and adult life-history characteristics tested in this study.

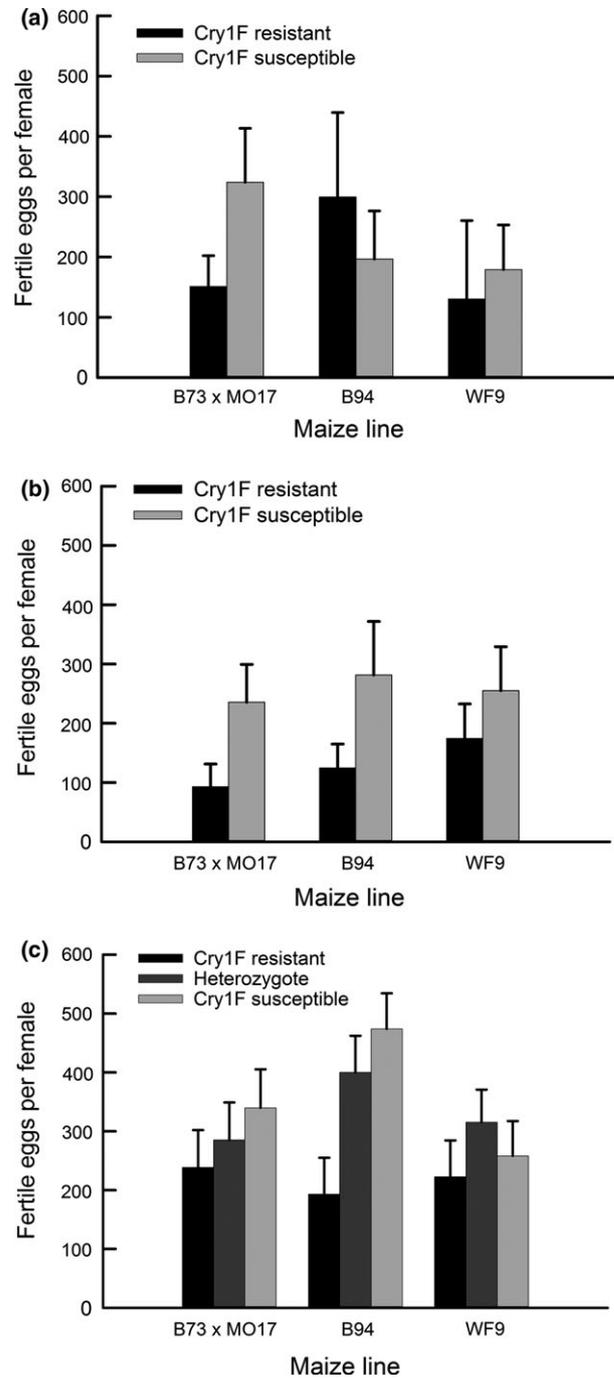


Fig. 3 Number of fertile eggs produced per *Ostrinia nubilalis* female for a) Cry1F-resistant and Cry1F-susceptible laboratory-reared females mated with experimental males experiencing diapause and completing larval development on three maize lines (experiment one), b) Cry1F-resistant and Cry1F-susceptible diapausing females completing larval development on three maize lines (experiment one) and c) Cry1F-resistant, Cry1F-susceptible and heterozygous strains completing development to adulthood on three maize lines but not experiencing diapause (experiment two). Bar heights represent sample means, and error bars are the standard error of the mean.

Table 3 Mean proportion survival, day of emergence, adult longevity, proportion of pairs that mated and number of egg masses per female (standard error in parentheses) for Cry1F-resistant, Cry1F-susceptible and heterozygous strains of *Ostrinia nubilalis* completing development on three maize lines (experiment two)

Maize line	Strain*	Sex	Prop. survival	Day of emergence	Adult longevity (d)	Proportion pairs mated	No. egg masses per female
B73 × Mo17	Het	F	0.23 (0.03)	6.66 (0.68)	11.21 (0.97)	0.84 (0.10)	32.74 (4.85)
B73 × Mo17	Res	F	0.22 (0.03)	7.65 (0.78)	11.54 (0.90)	0.71 (0.13)	36.84 (10.76)
B73 × Mo17	Sus	F	0.13 (0.01)	9.58 (1.44)	10.33 (0.98)	0.68 (0.15)	20.39 (3.81)
B94	Het	F	0.18 (0.03)	8.91 (1.26)	9.97 (1.15)	0.82 (0.12)	31.25 (4.96)
B94	Res	F	0.23 (0.02)	9.89 (1.29)	11.88 (1.37)	0.78 (0.11)	25.30 (7.34)
B94	Sus	F	0.17 (0.02)	10.62 (1.70)	13.20 (0.69)	0.94 (0.04)	26.48 (3.33)
WF9	Het	F	0.22 (0.02)	4.76 (0.64)	11.35 (0.54)	0.88 (0.08)	26.60 (3.99)
WF9	Res	F	0.25 (0.02)	5.34 (0.52)	11.48 (1.27)	0.62 (0.12)	27.10 (5.83)
WF9	Sus	F	0.26 (0.02)	5.60 (0.55)	9.98 (1.02)	0.74 (0.12)	20.68 (3.90)
B73 × Mo17	Het	M	0.23 (0.03)	5.34 (0.47)	12.65 (1.01)	–	–
B73 × Mo17	Res	M	0.23 (0.03)	6.53 (0.83)	11.21 (1.00)	–	–
B73 × Mo17	Sus	M	0.18 (0.03)	7.14 (0.84)	10.84 (1.22)	–	–
B94	Het	M	0.26 (0.03)	7.26 (0.59)	11.83 (0.84)	–	–
B94	Res	M	0.22 (0.03)	7.07 (0.91)	13.17 (1.22)	–	–
B94	Sus	M	0.22 (0.02)	7.33 (0.50)	13.68 (0.75)	–	–
WF9	Het	M	0.29 (0.02)	3.76 (0.33)	12.04 (1.33)	–	–
WF9	Res	M	0.28 (0.03)	4.83 (0.51)	11.97 (0.88)	–	–
WF9	Sus	M	0.29 (0.03)	5.11 (0.54)	11.47 (1.11)	–	–

*Het: heterozygotes; Res: Cry1F-resistant; Sus: Cry1F-susceptible.

For both experiments, females reared on maize plants experienced a fitness cost of decreased fecundity (i.e. reduced lifetime production of fertile eggs) and this cost occurred whether or not insects experienced diapause (fig. 3b,c). Apparently, this cost was driven by maternal rather than paternal effects, because when plant-reared males were mated to diet-reared females, no difference in fecundity was detected between resistant and susceptible strains (fig. 3a). While these results indicate that development on plants elicited a fitness cost of reduced fecundity, it is unclear to what extent diapause contributed to this fitness cost because plant-reared insects experienced a fitness cost regardless of whether or not they experienced diapause conditions in the laboratory, which were likely less stressful than overwintering conditions in the field. In some cases, fitness costs of resistance to Bt, and other factors, may be magnified by stressful conditions, such as the presence of natural enemies (Gassmann et al. 2006; Hannon et al. 2010), low nutrient availability (Bergelson 1994) and competition, quality of food resources, predation or herbivory (Gassmann and Futuyama 2005; Raymond et al. 2005). In addition, fitness costs of Bt resistance have been shown to arise when strains experience diapause (Alyokhin and Ferro 1999; Carrière et al. 2001). In general, fitness costs of Bt resistance are more common when insects are reared on plants

compared to artificial diet (Gassmann et al. 2009), and the reduced number of fertile eggs produced by Cry1F-resistant insects reared on maize plants, but not artificial diet, in this study is concordant with this general pattern.

Both the magnitude and dominance of fitness costs associated with Bt resistance may be affected by host-plant quality (Gassmann et al. 2009). However, in this study, we found neither to be the cause, as significant interactions were absent between maize line and insect genotype (or strain) for fecundity or for any of the other life-history traits measured. However, differences among maize lines were present for pre-diapause larval mass, mass lost during diapause, pupal mass, adult longevity, survival to adulthood and time to emergence, indicating that a difference in host-plant quality was present among the maize lines used in this study. Increases in the magnitude of fitness costs of resistance can delay resistance to Bt crops in insect populations, and in some cases, the magnitude of fitness costs can be affected by plant variety or plant species (reviewed by Gassmann et al. 2009). For example, Bird and Akhurst (2007) examined costs of Bt resistance for *Helicoverpa armigera* (Hübner) on three non-Bt host plants (cotton, pigeon pea and sorghum) and found that insects with resistance alleles exhibited increased developmental time on cotton and reduced pupal mass and fecundity on sorghum.

Other studies have shown that fitness costs are greatest when plant quality is poor, with the magnitude of fitness costs of Bt resistance increasing with declining host-plant suitability (Janmaat and Myers 2005) or presence of a plant defence compound (Carrière et al. 2004). From the perspective of resistance management, a plant variety or species that magnifies fitness costs of Bt resistance could enhance resistance management if used in non-Bt refuges, because it could act to decrease the frequency of Bt-resistance alleles, and consequently, delay the evolution of resistance. However, none of the maize lines used in this study imposed a significantly greater fitness cost than another, although the data do indicate that the greatest difference in egg production between resistant and susceptible insects occurred on maize line B94 (fig. 3b,c), which is expected to have the highest levels of DIMBOA (Russell 1991; Abel et al. 2000).

In the second experiment, wherein all insects were reared to adulthood on plants, resistant insects produced significantly fewer eggs than either susceptible or heterozygous insects, but fertile egg production did not differ between susceptible and heterozygous individuals, indicating that this fitness cost is inherited in a recessive manner. In general, recessive fitness costs impose smaller delays in resistance than non-recessive costs (Carrière and Tabashnik 2001; Gassmann et al. 2009). Fitness costs are non-recessive when fitness of Bt-susceptible individuals is higher than both resistant and heterozygous genotypes. Gassmann et al. (2009) reviewed fitness costs of Bt resistance and found that fitness costs were non-recessive in only 26% of cases. When resistance first evolves, resistance alleles are rare and the majority are expected to reside in heterozygous individuals. As such, non-recessive fitness costs will have a greater effect on heterozygote viability and will more effectively delay resistance compared to recessive fitness costs (Carrière et al. 2001; Gassmann et al. 2009). In some cases, larval host plants have been found to impose non-recessive fitness costs (Bird and Akhurst 2007). However, that was not the case in this study as no significant interaction was found between maize line and insect genotypes for production of fertile eggs (Tables S1, S3; fig. 3c).

The decreased fertility of Cry1F-resistant *O. nubilalis* found here is consistent with the results of Pereira et al. (2011), who reported fewer egg masses and fewer neonates produced per female for a Cry1F-resistant strain that was the progenitor of the Cry1F strain used in this study. Pereira et al. (2011) also reported recessive inheritance of fitness costs. In general, other studies have found fitness costs of Bt resistance for *O. nubilalis*. Bolin et al. (1999) detected a 22-fold decline

in Cry1Ac resistance after nine generations without selection on Cry1Ac, indicating the presence of fitness costs selecting against resistance. In a Cry1Ab-resistant strain of *O. nubilalis*, fitness costs were detected for larval survival and larval mass on reproductive-stage plants (Crespo et al. 2009). This same Cry1Ab-resistant strain exhibited fitness costs of Bt resistance for pupal mass, developmental time, mating success and fertility (Crespo et al. 2010). However, Huang et al. (2005) did not detect any fitness costs associated with Dipel resistance; and using the same strains as in the present study, Petzold-Maxwell et al. (2014) did not detect any fitness costs of Cry1F resistance for larval survival, mass or developmental rate. Past work has found that both the magnitude of Bt resistance and the number of traits measured are positively correlated with the number of fitness costs detected (Gassmann et al. 2009), and both of these factors may have contributed to the detection of fitness costs of Bt resistance in *O. nubilalis* in some past studies but not others.

Because fitness costs of Bt resistance impose selection against alleles for Bt resistance within refuge populations, they can act to delay the evolution of resistance to Bt crops (Gassmann et al. 2009). The fitness cost of Cry1F resistance found here, and elsewhere, may be one factor that has delayed the evolution of Cry1F resistance by European corn borer in the field (Siegfried and Hellmich 2012). Other factors that have likely been important in the successful management of resistance to Bt maize by European corn borer include the presence of non-Bt refuges, high-dose Bt events, dispersal of insects between refuges fields and Bt fields and use of maize hybrids that contain multiple Bt toxins targeting this pest (Siegfried and Hellmich 2012; Tabashnik et al. 2013).

The mechanism of Cry1F resistance in *O. nubilalis* remains unknown, but the resistant strain used in this study shows incomplete recessive inheritance and resistance has been linked to a single region of the genome (Coates et al. 2011). This genome region controlling Cry1F resistance recently was greatly narrowed to a segment of linkage group 12 that encodes an ABC transporter gene (Coates and Siegfried 2015), but it remains unknown to what extent other genes in this genome region may have contributed to the fitness costs observed in this study. Additionally, the number of genes constitutively differentially expressed between introgressed Cry1F-resistant and Cry1F-susceptible *O. nubilalis* fed on artificial diet are large in number, with 2 660 genes up-regulated and 2 795 genes down-regulated (Vellichirammal et al. 2015). Any analogous gene expression differences

between Cry1F-resistant and Cry1F-susceptible individuals reared on different maize lines also remains unknown, but may have influenced the fitness costs observed in the current study. Although it is unknown whether the resistance trait studied here would confer resistance to Cry1F maize in the field, Pereira et al. (2008b) showed similar survival on reproductive-stage Cry1F maize and non-Bt maize for a Cry1F-resistant laboratory-selected strain that was used to generate the Cry1F-resistant strain studied here. However, larval survival on vegetative-stage Cry1F maize was significantly lower compared with survival on non-Bt maize (Pereira et al. 2008b). Additional research on Cry1F-resistant strains with field-evolved resistance would be valuable for understanding the extent to which resistance to Cry1F maize in the field might have accompanying fitness costs.

The Cry1F-resistant strain used in this study was backcrossed to the susceptible strain five times, and then reselected on Cry1F following each backcross (Petzold-Maxwell et al. 2014). This was performed to minimize any genetic differences, not due to Cry1F resistance, that may have been present between the resistant and the susceptible strains. However, it is possible that genetic differences between the strains, that were also in physical linkage with resistance alleles (i.e. in close spatial proximity on the same chromosome), may have persisted despite backcrossing. To the extent that this occurred, such genetic difference also may have contributed to differences in fitness between the Cry1F-resistant and Cry1F-susceptible strains observed in this study.

The first experiment in this study used maize in the reproductive-stage and European corn borer that entered into diapause. The phenology of both the plant and insect in this experiment matches conditions that arise in the field (Mason et al. 1996). The second experiment also used reproductive-stage plants but European corn borer that did not enter diapause, which is less relevant to what occurs in the field. A follow-up experiment that would complement this work would use vegetative-stage maize and non-diapausing European corn borer. Not only may such an experiment better emulate field conditions but it may also result in a stronger effect of host-plant resistance, because levels of DIMBOA can be greater in earlier versus later phenological stages of maize (Klun and Robinson 1969).

Of the numerous fitness characteristics measured in this study, the only fitness cost of Cry1F resistance that was found was a decrease in fertile egg production, and this cost was recessive (fig. 3c). In general, fitness costs of Cry1F resistance in *O. nubilalis* have

been recessive (Huang et al. 2005; Crespo et al. 2010; Pereira et al. 2011). Although a relatively high frequency of Cry1F resistance alleles has been detected in field populations, no instances of unexpected injury to Cry1F maize by *O. nubilalis* in the field have been reported, suggesting that these populations remain susceptible to this toxin (Siegfried et al. 2014). The presence of high-dose Bt events, pyramided Bt traits, non-Bt refuges and fitness costs of resistance likely have contributed to the maintenance of susceptibility to Cry1F in the field. Further studies, particularly those examining fitness costs of Cry1F resistance in populations with field-evolved resistance, will be useful for better understanding the risks of Cry1F resistance evolving in *O. nubilalis*.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Analysis of variance for survival and fitness characteristics of Cry1F-resistant and Cry1F-susceptible strains of *Ostrinia nubilalis* developing on three maize lines, experiencing diapause and subsequently mating with laboratory strains (experiment one).

Table S2. Analysis of variance for survival and fitness characteristics for Cry1F-resistant and Cry1F-susceptible strains of *Ostrinia nubilalis* developing on three maize lines, experiencing diapause and subsequently mating with laboratory strains (experiment one).

Table S3. Analysis of variance for fitness characteristics of Cry1F-resistant, Cry1F-susceptible, and heterozygous strains of *Ostrinia nubilalis* completing development on three maize lines (experiment two).