

2014

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Petzold-Maxwell, Jennifer L.; Siegfried, Blair D.; Hellmich, Richard L.; Abel, Craig A.; Coates, Brad S.; Spencer, Terrence A.; and Gassmann, Aaron J., "Effect of Maize Lines on Larval Fitness Costs of Cry1F Resistance in the European Corn Borer (Lepidoptera: Crambidae)" (2014). *Faculty Publications: Department of Entomology*. 393.
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Effect of Maize Lines on Larval Fitness Costs of Cry1F Resistance in the European Corn Borer (Lepidoptera: Crambidae)

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J. Econ. Entomol. 107(2): 764–772 (2014); DOI: <http://dx.doi.org/10.1603/EC13359>

ABSTRACT Crops producing insecticidal toxins from the bacterium *Bacillus thuringiensis* (Bt) are widely planted and enable management of key insect pests while reducing the use of conventional insecticides. However, the evolution of Bt resistance could diminish these benefits. Fitness costs of Bt resistance occur in the absence of Bt toxin when individuals with resistance alleles show a reduction in fitness relative to susceptible individuals, and they can delay the evolution of resistance. Ecological factors including host-plant variety can affect the magnitude of fitness costs, and consequently, the degree to which fitness costs delay resistance. In this study, we measured fitness costs of resistance to Bt toxin Cry1F in the European corn borer *Ostrinia nubilalis* Hübner (Lepidoptera: Crambidae) using Cry1F-resistant and Cry1F-susceptible strains sharing a similar genetic background. Fitness costs were tested on three lines of maize, *Zea mays* L., by measuring larval survival and development in two greenhouse experiments with plants in either the vegetative or reproductive stage. Both experiments showed that maize line significantly affected larval survival and developmental rate. However, larval survival, mass, and developmental rate did not differ between the Cry1F-resistant and susceptible strains, indicating a lack of fitness costs of resistance to Cry1F for the larval fitness components measured in this experiment. Future experiments should test for fitness costs of Cry1F resistance affecting survival to adulthood and adult life-history parameters.

KEY WORDS *Bacillus thuringiensis*, Cry1F, ecological negative cross-resistance, *Ostrinia nubilalis*, resistance management

The European corn borer, *Ostrinia nubilalis* (Hübner) (Lepidoptera: Crambidae), is an economically important pest of maize in North America and Europe. Before the commercialization of transgenic maize producing insecticidal toxins derived from the bacterium *Bacillus thuringiensis* (Bt crops) in 1996, *O. nubilalis* was responsible for >US\$1 billion annually in yield losses and management costs in the United States (Mason et al. 1996). Bt maize is an effective method for management of *O. nubilalis*, and has been widely adopted in the United States, accounting for 67% of the total area planted to maize (*Zea mays* L.) in 2012 (Economic Research Service [ERS] 2012). Furthermore, widespread planting of Bt maize has resulted in area-wide suppression of *O. nubilalis* populations,

which is a benefit to both growers of Bt maize and non-Bt maize (Hutchison et al. 2010).

Several insect pests have evolved resistance to Bt crops in the field, which is defined as a genetically based increase in survival of a population on a Bt crop in response to selection in the field (van Rensburg 2007, Storer et al. 2010, Dhurua and Gujar 2011, Gassmann et al. 2011). Although field populations of *O. nubilalis* currently remain susceptible to Bt maize (Siegfried and Hellmich 2012), selection experiments in the laboratory have demonstrated the potential for development of resistance (Huang et al. 1997, Bolin et al. 1999a, Chaufaux et al. 2001, Siqueira et al. 2004). The high dose or refuge strategy is an insect resistance management tactic employed to delay resistance to transgenic crops (Gould 1998, Tabashnik et al. 2003). This strategy uses non-Bt plants as a refuge for Bt-susceptible genotypes, providing a pool of susceptible individuals to mate with resistant individuals that survive exposure to a Bt crop (Gould 1998), thus producing heterozygous offspring. The concentration of Bt toxin produced by a high dose-event in a crop should kill heterozygous individuals, ensuring that the resistance trait is functionally recessive, thereby removing resistance alleles from the population and delaying resistance evolution.

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Resistance to Bt is often associated with fitness costs, which occur in the absence of exposure to Bt toxins when individuals with resistance alleles have lower fitness compared with homozygous susceptible individuals (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). In addition, ecological factors including host-plant genotype (e.g., hybrid) can affect the dominance and magnitude of fitness costs (Gassmann et al. 2009). Thus, manipulating the host plants used as a refuge could reduce the selective advantage of individuals with resistance alleles and enhance any potential delays in resistance achieved through application of the high dose or refuge strategy (Carrière et al. 2004, Gassmann et al. 2009).

Several studies have examined whether fitness costs are associated with resistance to Bt toxins in *O. nubilalis*. Huang et al. (2005) were unable to detect any significant differences in survival or life-history characteristics between Dipel-susceptible and Dipel-resistant strains of *O. nubilalis*. However, the resistance ratio of a Cry1Ac-resistant strain of *O. nubilalis* declined by a factor of 10 over nine generations in the absence of selection, indicating the presence of fitness costs (Bolin et al. 1999b). Compared with susceptible insects, Cry1Ab-resistant *O. nubilalis* exhibited reduced survival and mass, increased development time, a higher proportion of unsuccessful matings, and lower fertility in the absence of Cry1Ab (Crespo et al. 2009, 2010). For individuals with alleles that confer resistance to Cry1F, there was evidence of reduced pupal mass and growth rate for females relative to a Cry 1 F-susceptible strain (Pereira et al. 2011). Mapping of quantitative trait loci for the Cry1F-resistant strain studied here indicated that resistance is controlled by a single recessive genetic locus (Coates et al. 2011).

It is important to note that past studies measuring fitness costs of Bt resistance in *O. nubilalis* were conducted under optimal rearing conditions using artificial diet, potentially masking fitness costs. Because plant genotype has previously been shown to affect fitness costs of Bt resistance (Gassmann et al. 2009), we tested whether fitness costs of Cry1F resistance were present on three non-Bt lines of maize that differed in the production of the plant allelochemical 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA), which confers antibiosis-based resistance to *O. nubilalis*.

Materials and Methods

Insect Strains. The Cry1F-susceptible strain originated from field-collected insects near Geneva, NY in 1985. Additional insects were introduced from two locations near Geneva in May 1996 (340 larvae and 60 pupae) and in October 1996 (966 adults). The Cry1F-resistant strain originated from insects collected throughout the U.S. corn belt in 1996 (115 females and 135 males). Both strains were maintained using standard rearing procedures (Lewis and Lynch 1969, Siqueira et al. 2004), as described in detail in Pereira

et al. (2008b). Briefly, larvae were reared on a wheat germ-based diet in containers with a corrugated cardboard ring that served as a pupation site. Pupal rings were transferred to mating cages, and egg masses were deposited by insects onto waxed paper lining the top of the cage. Egg masses were collected daily and incubated in petri dishes containing moistened filter paper until hatch.

Selection for Cry1F resistance was initiated in 1998 and is described in detail by Pereira et al. (2008b). 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 10 generations. Further selection was accomplished in 2001 and 2002 using Cry1F applied to the surface of artificial diet and exposing neonates for 7 d, after which surviving neonates were transferred to untreated diet. Maintenance of the Cry1F-selected strain was accomplished by exposing neonates to 60 ng Cry1F per cm^2 , corresponding to the upper limit of the 95% CI of the LC_{99} for susceptible populations, every three generations (Pereira et al. 2008b). This strain displayed a resistance ratio of >3,000-fold to Cry1F on diet assays (Pereira et al. 2008a,b). In addition, after 15 d of feeding on plants, larval survival and larval mass of the resistant strain did not differ between reproductive stage Cry1F maize and non-Bt maize (Pereira et al. 2008b). However, on vegetative stage maize, the resistant strain did display significantly lower larval survival and mass on Cry1F maize versus non-Bt maize after 15 d (Pereira et al. 2008b). No data have been collected on survival to adulthood on Cry1F maize or subsequent effects of Cry1F maize on reproductive success of the resistant strain.

Because differences in fitness between Bt-susceptible and Bt-resistant strains can be due to genetic differences not related to Bt resistance, we repeatedly backcrossed the resistant strain to the susceptible strain, followed by selection on Cry1F, resulting in strains with a common genetic background. Specifically, the selected strain was back-crossed to the susceptible strain, allowed to mate at random for an additional generation, and then selected by rearing on artificial diet with Cry1F applied to the surface of the diet. This process of crossing and selecting was repeated five times. The susceptible strain was maintained simultaneously (and separately). 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. It is unknown to what extent the backcrosses and reselection may have diminished the capacity of this strain to survive on Cry1F maize.

Plants. Maize plants were grown in a greenhouse in a soil mixture composed of equal volumes of two types of soil mixtures (Sunshine Mix #1/LC1 and Metro-Mix 900, Sun Gro Horticulture, Vancouver, BC, Canada) in 9-liter pots (Classic 1000, Hummert International, Earth City, MO). Supplemental lighting was supplied with 400 W high-pressure sodium bulbs (Ruud Lighting Inc., Racine, WI) with a photoperiod

of 16:8 (L:D) h. Seven days after germination, plants were treated with fungicide (Infuse, Bonide, Oriskany, NY) at a concentration of 3.18 ml/m². Plants were fertilized every 2 wks with 350 ml of Peters Excel 15-5-15 Cal-Mag Special (Everris, Geldermalsen, The Netherlands) at a concentration of 4 mg/ml. 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 levels of DIMBOA, which confers resistance against *O. nubilalis* (Russell 1991, Abel et al. 2000). These maize lines were selected to test the hypothesis that fitness costs of Cry1F resistance in *O. nubilalis* will be affected by the presence of host plant resistance factors, as has been found for other species of insects (Gassmann et al. 2009).

Diet Bioassays. The susceptible and resistant strains of *O. nubilalis* were tested for susceptibility to Cry1F using a standard diet bioassay described in detail by Marçon et al. (1999). Cry1F toxin was diluted in 0.1% Triton-X 100 nonionic detergent (Sigma-Aldrich, St. Louis, MO) and applied to the surface of artificial diet (King et al. 1985) adapted for *O. nubilalis* (Marçon et al. 1999) at a concentration of 60 ng/cm², which corresponds to the upper limit of the 95% CI of the LC₉₉ for susceptible populations (Pereira et al. 2008a). One neonate (<24 h after hatching) was placed in each well of a 128-well tray (CD international, Pitman, NJ) that contained the Cry1F toxin solution on the surface of the artificial diet. The control treatment consisted of diet to which only 0.1% Triton-X 100 was applied. Larvae were held for 7 d (27°C, 80% relative humidity), after which the proportion of larvae surviving was calculated, and surviving larvae were weighed to the nearest 0.1 mg (XS205 analytical balance, Mettler-Toledo, Columbus, OH). Larvae that had not grown beyond the first instar and weighed <0.1 mg were considered to be dead (Marçon et al. 1999). In total, 336 larvae were exposed to Cry1F-treated diet from each strain, and 48 larvae from each strain were exposed to control (nontreated) diet.

On-Plant Experiments. Two experiments were conducted to examine larval development, mass, and survival on the three different maize lines. 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 and 13 blocks were established for the first and second experiment, respectively. There were 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 first experiment (11 blocks × 12 plants) and 156 plants in the second experiment (13 blocks × 12 plants). Ten newly hatched larvae (<24 h old) 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 contain larvae, each plant was covered by a cylindrical cage that enclosed the whole plant and was made of mesh fabric (52 by 52 Amber

Fabricated, Lumite, Baldwin, GA). A 152-cm stake was placed in each pot, and cages were secured to the base of pots with elastic 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.

In the first experiment, larvae were placed on vegetatively growing plants with eight to nine fully expanded leaves (V8–V9 stage), and allowed to develop for 14 d, after which plants were carefully dissected and larvae were placed in 85% ethanol. In the second experiment, larvae were placed on early reproductive stage plants when plants had ≈18 expanded leaves or a visible primary ear shoot and tassel (VI8–R1), and allowed to develop for 21 d. In the northern Corn Belt *O. nubilalis* has two generations per year, one on vegetative stage corn and a second on reproductive stage corn (Munkvold et al. 1999). Thus, these experiments capture the phenological stages of maize on which *O. nubilalis* larvae are present. Greenhouse temperature throughout the experiment was 24.0°C ± 5.4 (mean ± SD) for the first experiment, and 26.1°C ± 6.5 for the second experiment. Percentage survival was calculated (number of larvae recovered divided by 20) for each plant. The larval instar of each recovered insect was recorded by measuring the head capsule width (DeWitt and Stockdale 1980). Average instar of larvae from each plant was calculated by multiplying the number of larvae in each stadium by the stadium number as follows: first instar = 1, second instar = 2, third instar = 3, fourth instar = 4, fifth instar = 5, pupa = 6, and then dividing this number by the total number of insects recovered from a plant. Larvae were grouped by plant and dried in an oven at 60°C for 48 h and weighed to the nearest 0.1 mg (XS205 analytical balance, Mettler-Toledo, Columbus, OH). Average mass per larva was calculated by dividing the mass of larvae from each plant by the number of larvae recovered from the plant.

Data Analysis. Analysis of variance (ANOVA) was performed in SAS Enterprise Guide 4.2 (SAS Institute 2009). For the diet bioassays, proportion mortality and larval mass were analyzed with a one-way ANOVA (PROC ANOVA), with the factor of treatment (resistant larvae on Cry1F-treated diet, susceptible larvae on Cry1F-treated diet, resistant larvae on control diet, susceptible larvae on control diet). Pairwise comparisons among means were made using Fisher LSD.

For experiments on plants, survival, mass, and instar were analyzed with a mixed-model ANOVA (PROC MIXED) that included maize line, insect strain, and the interaction between maize line and insect strain as fixed factors in the model. Random factors included block and the interaction of block with maize line, insect strain, and maize line by insect strain. Random effects 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

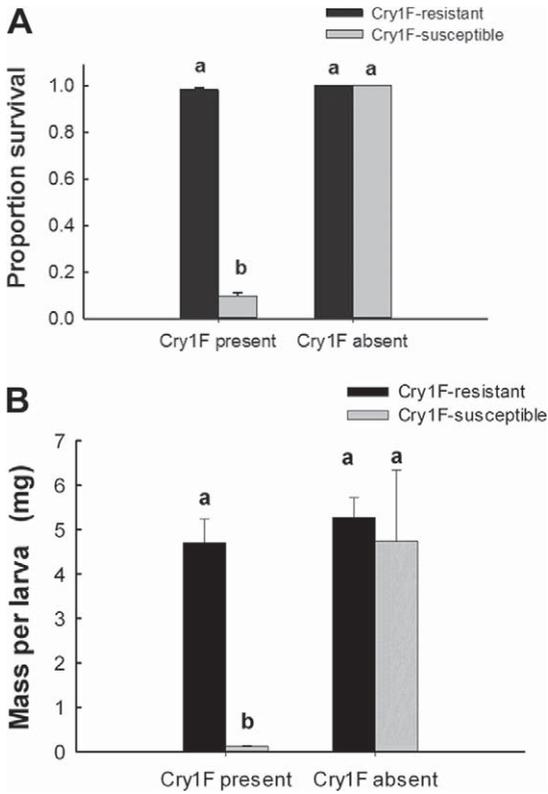


Fig. 1. (A) Survival and (B) mass for resistant and susceptible larvae in diet bioassays with diet treated or untreated with Cry1F. Scale bar heights represent sample means and error scale bars are the SE of the mean. Scale bars that do not share the same letter are significantly different ($P < 0.05$).

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 (PDIF option in PROC MIXED). To ensure normality of the residuals, data on survival and mass in the experiment using vegetative plants were transformed by the square-root function.

Results

The diet bioassay experiment showed that larvae from the resistant strain had a higher level of resis-

tance to Cry1F toxin compared with larvae from the susceptible strain. Survival was significantly different among the treatments ($F_{3,47} = 967.47, P < 0.0001$; Fig. 1A). Susceptible insects had significantly lower survival on Cry1F-treated diet compared with resistant insects on Cry1F-treated diet, and also compared with both strains on untreated diet (Fig. 1A). Mass was also significantly different among the treatments ($F_{3,47} = 99.65, P < 0.0001$; Fig. 1B). Susceptible insects on Cry1F-treated diet that survived were significantly smaller compared with resistant insects on Cry1F-treated diet and compared with both strains on untreated diet (Fig. 1B). No other significant differences were detected among treatments (Fig. 1).

In the two fitness cost experiments, where larvae developed on 1) vegetative plants or 2) reproductive stage plants, strain did not significantly affect survival, mass, or larval instar, and the strain by maize line interaction was not significant in any of the ANOVA models (Table 1). These results indicate that fitness costs of Cry1F resistance in *O. nubilalis* were not detected for larval survival, larval mass, or developmental rate. In contrast, maize line significantly affected larval survival and developmental rate (Table 1). When larvae developed on vegetative plants, survival was significantly greater on the maize line WF9 than on B94 or B73×Mo17 ($P < 0.0001$ for both), and on B94 compared with B73×Mo17 ($P = 0.048$; Fig. 2A). When larvae developed on reproductive plants, survival was significantly greater on WF9 than on B73×Mo17 ($P = 0.0014$; Fig. 3A). Mass was not significantly affected by any of the factors (Table 1; Figs. 2B and 3B). Average instar was significantly lower (thus developmental rate was significantly slower) for larvae on B94 compared with B73×Mo17 ($P = 0.045$; Fig. 2C) on vegetative plants. On reproductive plants, developmental rate also was significantly slower on B94 compared with both B73×Mo17 and WF9 ($P < 0.03$ for both), and on B73×Mo17 compared with WF9 ($P = 0.04$; Fig. 3C).

Discussion

The Cry1F-resistant strain used in this study displayed significantly greater survival and larval mass than the susceptible strain after 7-d exposure to Cry1F toxin in diet-based bioassays (Fig. 1). These results are consistent with prior results by Pereira et al. (2008a,b)

Table 1. ANOVA for survival, mass, and instar for *O. nubilalis*

Source	Survival			Mass			Instar		
	df ^a	F	P	df	F	P	df ^a	F	P
Vegetative plants									
Maize line	2,20	40.70	<0.0001	2,19	1.17	0.33	2,91	3.35	0.04
Strain	1,96	1.84	0.18	1,71	0.19	0.67	1,91	0.10	0.76
Maize line × strain	2,96	0.92	0.40	2,71	1.05	0.36	2,91	1.17	0.32
Reproductive plants									
Maize line	2,138	6.51	0.002	2,24	0.58	0.57	2,129	9.74	0.001
Strain	1,138	0.01	0.92	1,12	0.03	0.87	1,129	2.20	0.14
Maize line × strain	2,138	1.57	0.21	2,97	2.38	0.10	2,129	0.53	0.59

^a Numerator degrees of freedom, denominator degrees of freedom.

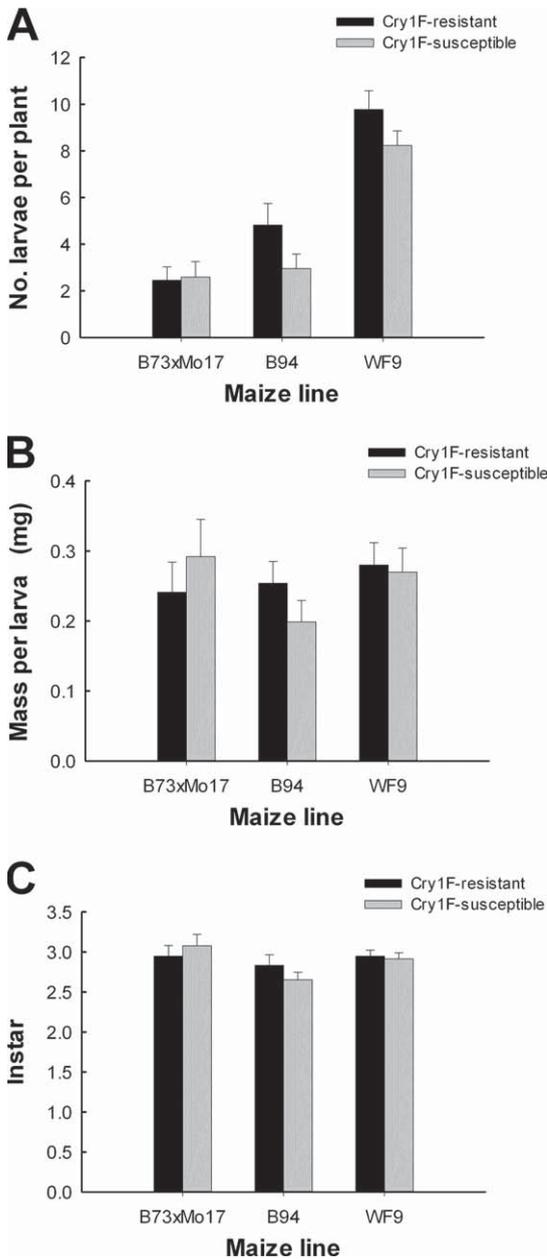


Fig. 2. (A) Survival, (B) mass, and (C) instar for resistant and susceptible larvae on three maize lines after 2 wk of development on vegetative-stage plants. Scale bar heights represent sample means and error bars are the SE of the mean.

that showed a high resistance ratio for this Cry1F resistance strain, and demonstrate that the resistant strain still harbored the resistance trait during the fitness cost experiments presented here. We did not detect significant differences between Cry1F-resistant and Cry1F-susceptible larvae for larval survival, developmental rate, or mass on any of the maize lines (Table 1; Figs. 2 and 3). Thus, we did not find evidence

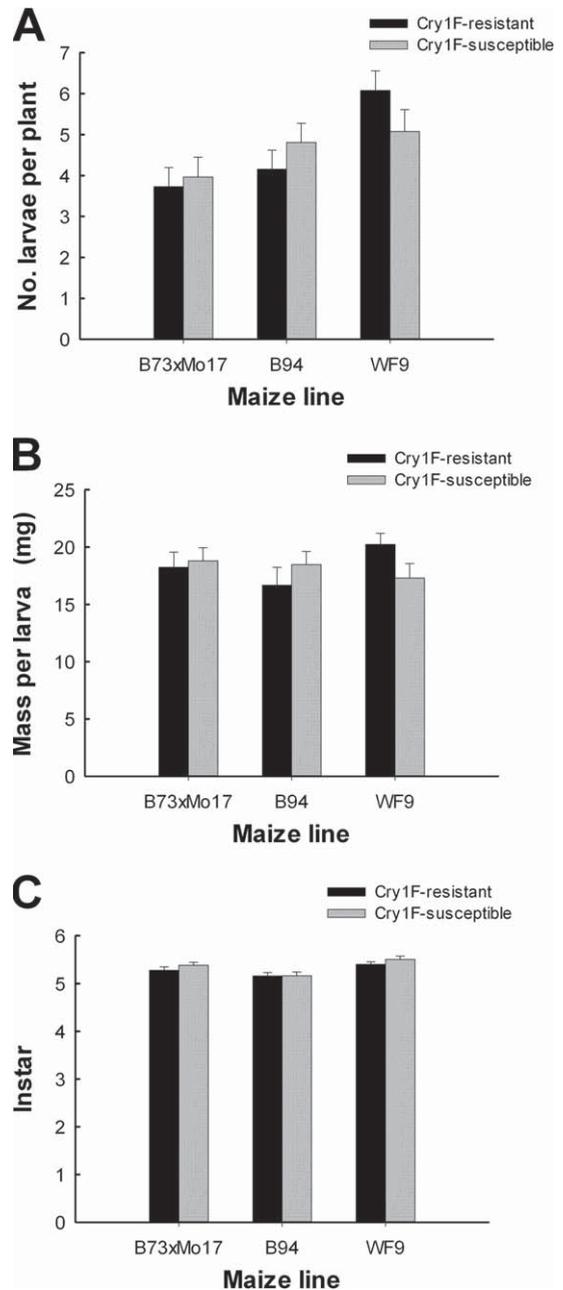


Fig. 3. (A) Survival, (B) mass, and (C) instar for resistant and susceptible larvae on three maize lines after 3 wk of development on reproductive-stage plants. Scale bar heights represent sample means and error bars are the SE of the mean.

for fitness costs associated with Cry1F resistance for larvae on the three maize lines used in this study.

In cases where *O. nubilalis* might evolve resistance to Cry1F maize in the field, the accompanying fitness costs may differ from the results reported in this study. This may arise in part because the mechanism of resistance may be different between our laboratory-

selected strain and field-selected strains (McKenzie and Batterham 1994). Resistance in the strain studied here was the result of laboratory selection at increasingly increasing concentrations of Cry1F in diet, which may differ from a field situation where larvae would need to survive on Cry1F maize. However, it is important to note that genetic analysis of field populations with an F_1 screen (Siegfried et al. 2013) confirmed that a relatively high frequency of individuals from these populations share the same genetic locus for Cry1F resistance as the laboratory-selected strain and that the frequency was already high before the introduction of Cry1F-expressing hybrids. Because the Cry1F-resistant strain used in our fitness comparisons was derived from this same laboratory-selected strain, it seems unlikely that the resistance is simply an artifact of laboratory selection.

In addition, it is unclear if the resistance trait studied here would confer resistance to Cry1F maize in the field. Although the strain studied here had similar larval survival on reproductive stage Cry1F maize and non-Bt maize, larval survival on vegetative stage Cry1F maize was significantly lower than on non-Bt maize (Pereira et al. 2008b). Furthermore, the potential survival to adulthood and the subsequent reproduction of the resistant strain on Cry1F maize is unknown, as is the effect of backcrossing and reselecting the resistant strain before conducting these experiments. Thus, higher levels of resistance may be required for survival on Cry1F than were present in the resistant strain studied here, and this higher level of resistance may have greater fitness costs, as has been observed for other species of insects (Gassmann et al. 2009).

Plant genotype or species can often affect a variety of fitness characteristics for insects, as was observed in this study for larval survival and development on the three maize lines (Table 1; Figs. 2 and 3). Two of the maize lines studied here were B94 and WF9. Abel et al. (2000) found that B94 produced 130 μg DIMBOA per gram of dried leaf tissue, while WF9 produced 22 μg DIMBOA per gram of dried leaf tissue. DIMBOA provides host plant resistance against *O. nubilalis* through antibiosis (Sullivan et al. 1974, Abel et al. 2000). In both experiments, survival was highest on WF9, which has low levels of DIMBOA and is often used as a susceptible control in experiments with *O. nubilalis* (Sullivan et al. 1974, Manuwoto and Scriber 1985). Developmental rate was slowest on B94, which may be explained by the moderate DIMBOA-mediated resistance to *O. nubilalis* for this maize line (Russell 1991, Abel et al. 2000). No differences existed among the three maize lines for larval mass (Figs. 2B and 3B). Because ecological factors, including host-plant genotype, can increase the dominance and magnitude of fitness costs, selection of some host plants as non-Bt refuges may enhance the effectiveness of the refuge strategy by imposing greater fitness costs on individuals that harbor alleles for Bt resistance (Pittendrigh et al. 2004, Carrière et al. 2005, Gassmann et al. 2009). However, we did not find any effect on larval fitness costs for the three maize lines tested here. The

use of diets to maintain laboratory strains of insects can diminish the capacity of the strains to survive on plants (Schoonhoven et al. 2005). Because the resistant and susceptible strains studied here were maintained for ≈ 180 generations on diet, the effects of maize plants on fitness and fitness costs may be different from that of strains maintained only on plants or for insects that live in maize fields.

Although other studies have shown fitness costs of Bt resistance in *O. nubilalis*, there are also examples where fitness costs were not detected. In a strain selected for Cry1Ac resistance, the resistance ratio declined from 22-fold to two-fold in the absence of selection for nine generations, indicating the presence of fitness costs selecting against resistance (Bolin et al. 1999b). In a Cry1Ab-resistant strain of *O. nubilalis*, larval survival and larval mass were lower on non-Bt maize than for a Cry1Ab-susceptible strain (Crespo et al. 2009). When reared on artificial diet lacking Cry1Ab, insects from this same Cry1Ab-resistant strain exhibited reduced pupal mass, increased development time, a higher proportion of unsuccessful matings, and lower fertility than was found for a susceptible strain (Crespo et al. 2010). In contrast, and similar to our results, Huang et al. (2005) did not find differences in developmental rate, larval mass, pupal mass, or survival between Dipel-susceptible and resistant strains. Using the same Cry1F-resistant strain that was used to initiate the resistant strain used in the current study, Pereira et al. (2011) compared life-history traits and population growth rates of resistant, susceptible, and heterozygous strains on artificial diet, and found reduced pupal mass and lower growth rates for resistant and heterozygous females compared with susceptible females. However, no fitness costs were detected for male growth rates or mass, and only slight but nonsignificant reductions in fecundity parameters were observed for Cry1F-resistant insects relative to susceptible insects (Pereira et al. 2011). In addition, no significant differences were found between the resistant and susceptible strains for five estimated population growth parameters (Pereira et al. 2011). It should be noted that, in contrast to the current study, these comparisons were made with insects reared on artificial diets optimized for growth and development such that fitness costs may not have been apparent. In addition, the resistant strain had not been backcrossed to the susceptible strain, which may have introduced some confounding genetic differences between strains that were not related to resistance.

Because fitness costs of Bt resistance are expected to select against resistance alleles and therefore decrease resistance allele frequency in a population when refuges are present (Carrière and Tabashnik 2001, Pittendrigh et al. 2004, Gassmann et al. 2009), a lack of fitness costs of Cry1F resistance could hasten the evolution of Bt resistance in *O. nubilalis*. A major assumption of the high-dose or refuge strategy is a low frequency of resistance alleles (Gould 1998, Bates et al. 2005); however, this may not be the case for *O. nubilalis*. Siegfried et al. (2013) measured the allele frequency for Cry1F resistance in *O. nubilalis*, and

reported a resistance allele frequency of 0.0268 (95% CI between 0.0116 and 0.0516) during the initial 3 yr of commercial availability of maize producing Cry1F (2003–2005), with a similar frequency for the next 3 yr (0.0253). In contrast, resistance allele frequency estimates of 0.001 or less have been commonly used in modeling the evolution of resistance to Bt toxins (Onstad and Guse 1999, Crowder et al. 2006, Onstad and Meinke 2010). In addition, annual monitoring for Cry1F resistance in 2008 and 2009 showed that resistance alleles were present in field populations (Siegfried et al. 2013). It is possible that the relatively high resistance allele frequency reported by Siegfried et al. (2013) results from a lack of strong fitness costs of resistance to Cry1F, as described here and elsewhere (Huang et al. 2005, Pereira et al. 2011). Despite the relatively high frequency of alleles for resistance to Cry1F detected in field populations of *O. nubilalis*, populations remain susceptible to Cry1F, indicating that the high-dose refuge strategy has been effective at delaying resistance evolution for *O. nubilalis* (Siegfried et al. 2013). Further studies are necessary to better understand the role that fitness costs of Bt resistance have in influencing resistance to Cry1F in populations of *O. nubilalis*.

Fitness costs of Bt resistance have been found in a number of important insect pests, including *Leptinotarsa decemlineata* Say (Colorado potato beetle [Coleoptera: Chrysomelidae]), *Plodia interpunctella* Hübner (Indian meal moth [Lepidoptera: Pyralidae]), *Trichoplusia ni* Hübner (cabbage looper [Lepidoptera: Noctuidae]), *Pectinophora gossypiella* Saunders (pink bollworm [Lepidoptera: Gelechiidae]), *Helicoverpa armigera* Hübner (cotton bollworm [Lepidoptera: Noctuidae]), and *Plutella xylostella* L. (diamondback moth [Lepidoptera: Plutellidae]), although cases exist in which fitness costs were absent for the same insect species (reviewed by Gassmann et al. 2009). For example, Bt-resistant strains of *P. xylostella* had reduced survival and fecundity in one study (Groeters et al. 1994), but no fitness costs of resistance in other studies (Tang et al. 1997, Ramachandran et al. 1998). For the western corn rootworm, *Diabrotica virgifera virgifera* LeConte (Coleoptera: Chrysomelidae), an important pest of maize, no fitness costs of Bt resistance were shown for a Cry3Bb1-resistant, nondiapausing strain in a study by Oswald et al. (2012) and Petzold-Maxwell et al. (2012); however, Meihls et al. (2012) found evidence of fitness costs. Gassmann et al. (2009) found that the likelihood of a study detecting fitness costs was positively correlated with the number of life-history characteristics measured. Although we did not observe any fitness costs of Bt resistance for larvae on the three lines of non-Bt maize in this study, it is possible that fitness costs could exist for survival to adulthood, or other adult life-history parameters such as fecundity and longevity. To better understand whether fitness costs may delay Bt resistance in *O. nubilalis*, future studies should test additional life-history characteristics for larvae that are reared on plants.

Acknowledgments

We thank Keith Bidne, Sean Bradley, Landon Momberg, and Hannah Owens for their assistance. This research was supported by Biotechnology Risk Assessment Grant Program competitive grant 2010-33522-21673 from the U.S. Department of Agriculture–National Institute of Food and Agriculture (USDA–NIFA). We gratefully acknowledge Dow AgroSciences for providing access to the resistant strain of *O. nubilalis*. Contributions were also made from the University of Nebraska, Department of Entomology, and the USDA, Agricultural Research Service (CRIS Project 3625-22000-017-00D).

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Received 14 August 2013; accepted 6 January 2014.
