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Adaptation by Western Corn Rootworm (Coleoptera: Chrysomelidae) to Bt Maize: Inheritance, Fitness Costs, and Feeding Preference

JENNIFER L. PETZOLD-MAXWELL,1,2 XIMENA CIBILS-STEWART,1,3 B. WADE FRENCH,4 AND AARON J. GASSMANN1

ABSTRACT

We examined inheritance of resistance, feeding behavior, and fitness costs for a laboratory-selected strain of western corn rootworm, Diabrotica virgifera virgifera LeConte (Coleoptera: Chrysomelidae), with resistance to maize (Zea mays L.) producing the Bacillus thuringiensis Berliner (Bt) toxin Cry3Bb1. The resistant strain developed faster and had increased survival on Bt maize relative to a susceptible strain. Results from reciprocal crosses of the resistant and susceptible strains indicated that inheritance of resistance was nonrecessive. No fitness costs were associated with resistance alleles in the presence of two entomopathogenic nematode species, Steinernema carpocapsae Weiser and Heterorhabditis bacteriophora Poinar. Larval feeding studies indicated that the susceptible and resistant strains did not differ in preference for Bt and non-Bt root tissue in choice assays.

KEY WORDS Diabrotica virgifera virgifera, feeding behavior, pest resistance, transgenic maize

The western corn rootworm, Diabrotica virgifera virgifera LeConte (Coleoptera: Chrysomelidae), is one of the most economically important pests of maize (Zea mays L.) in the United States (Gray et al. 2009). In 2003, genetically modified maize producing insecticidal toxins derived from the bacterium Bacillus thuringiensis Berliner (Bt) was commercialized for management of western corn rootworm (EPA 2003). Bt maize kills western corn rootworm, greatly reducing root injury, and it is widely planted by farmers, with 39 million ha planted globally in 2010 (Vaughn et al. 2005, James 2010). Recently, field-evolved resistance to Bt maize producing the Cry3Bb1 toxin was documented in eastern Iowa (Gassmann et al. 2011, Gassmann 2012). Widespread field-evolved resistance to Bt maize by western corn rootworm is a potential threat, given that this insect has repeatedly adapted to numerous management approaches, including conventional insecticides and the cultural practice of crop rotation (Meinke et al. 1998, Wright et al. 2000, Levine et al. 2002).

Resistance management strategies aim to delay resistance, and among the most important is the refuge strategy, which has been widely adopted for Bt crops (Gould 1998, Tabashnik et al. 2003). Under the refuge strategy, non-Bt plants serve as a refuge for Bt-susceptible genotypes, providing a pool of homozygous susceptible individuals to mate with rare homozygous resistant individuals, resulting in heterozygous progeny (Gould 1998). Many factors can influence insect adaptation to Bt toxins, including inheritance of resistance, fitness costs associated with resistance, multitrophic interactions, and behavioral responses to toxins (Gould 1998, Tabashnik et al. 2003, Bowling et al. 2007, Gassmann et al. 2009a). Understanding characteristics associated with Bt resistance in western corn rootworm is important for implementing effective resistance management strategies.

Currently, little is known about how resistance alleles are inherited in western corn rootworm. In a study by Meihls et al. (2008), reciprocal crosses of resistant and susceptible strains were produced to examine inheritance of resistance alleles. This study found that resistance was inherited as a nonrecessive trait (Meihls et al. 2008). As the dominance of resistance decreases, the delay in evolution of resistance is expected to increase, becoming greatest when resistance alleles are completely recessive (Tabashnik et al. 2004). In simulation models, assuming a single toxin and at least 5% refuge, evolution of resistance for western corn rootworm never occurred within 50 yr if resistance was recessive (Onstad and Meinke 2010). However, when the resistance allele was dominant, resistance evolved in 6–7 yr for a low-dose toxin, and in ≤5 yr with a medium- or high-dose toxin (Onstad and Meinke 2010).

Another important factor affecting the evolution of Bt resistance is the presence of fitness costs (Gassmann et al. 2009a, and references therein). Fitness costs occur when individuals with resistance alleles have lower fitness than homozygous susceptible individuals in the absence of Bt toxins. Fitness costs can delay resistance to Bt crops when refuges are...
present (Carrière and Tabashnik 2001, Pittendrigh et al. 2004, Gassmann et al. 2009a) and can vary with ecological conditions, with the magnitude and dominance of costs becoming greater under some ecological conditions than others. Thus, it may be possible to enhance the refuge strategy by incorporating ecological conditions that impose the greatest fitness costs (Carrière et al. 2001, 2004; Pittendrigh et al. 2004; Gassmann et al. 2009a). Host plants and entomopathogens can affect the magnitude and dominance of fitness costs and could therefore be manipulated to bolster resistance management (Gassmann et al. 2009a). Two species of entomopathogenic nematodes have been shown to magnify fitness costs of resistance to Bt for the pink bollworm, Pectinophora gossypiella (Saunders) (Gassmann et al. 2009b). In addition, entomopathogenic nematodes can serve as biological control agents (Shapiro-Ilan et al. 2006, and references therein); thus, it could be useful to consider treating non-Bt refuges with entomopathogens for some pest species. In a study examining survivorship, fecundity and viability in laboratory-selected Cry3Bb1-resistant western corn rootworm lines, one of which was used in the current study, no fitness costs were observed for any of the lines (Oswald et al. 2012). Few additional data are available on fitness costs of Bt resistance for the western corn rootworm, and whether natural enemies increase fitness costs of Bt resistance for this species has yet to be tested.

Finally, understanding behavioral responses of insects to toxins is an important aspect of resistance management, because behavioral responses can affect the rate of resistance evolution (Gould and Anderson 1991, Frutos et al. 1999, Peck et al. 1999). Specifically, behavioral responses can influence the amount of exposure to toxins, which in turn can influence selection pressure (Gould 1984, Onstad 2008). For example, physiologically mediated resistance is expected to develop more slowly in the presence of behavioral avoidance of toxins (Jallow and Hoy 2007). Thus, feeding behavior resulting in preference or nonpreference for Bt maize could have important implications for the evolution of resistance. Clark et al. (2006) observed feeding behavior of Bt-susceptible western corn rootworm on Cry3Bb1 maize and non-Bt maize. Results from this study suggested that larvae presented with Bt maize roots consumed significantly less tissue than larvae on non-Bt maize, and that neonate larvae may alter feeding behavior to reduce exposure to Bt toxins (Clark et al. 2006). Currently, there are no data on feeding behavior of Bt-resistant western corn rootworm larvae with respect to preference for Bt or non-Bt maize.

Here, we used a laboratory-selected Cry3Bb1-resistant strain of western corn rootworm to test the inheritance of resistance and to determine whether there were fitness costs in the presence of two entomopathogenic nematode species, Steinernema carpocapsae Weiser (Rhabditida: Steinernematidae) and Heterorhabditis bacteriophora Poinar (Rhabditida: Heterorhabditidae). In addition, we characterized feeding preference of resistant and susceptible strains by examining preference of larvae for Cry3Bb1 maize versus non-Bt maize.

**Materials and Methods**

**Resistant and Susceptible Strains.** Insects used in all experiments were from two strains sharing a common genetic background: a strain selected in the laboratory for resistance to Cry3Bb1 Bt maize (resistant strain) and an unsselected strain (susceptible strain). Both strains originated from the same nondiapausing genetically diverse base population and were originally obtained from the U.S. Department of Agriculture’s North Central Agricultural Research Laboratory (USDA–ARS) in Brookings, SD. To produce the base population, nondiapausing females from a strain maintained at the USDA–ARS laboratory for >30 yr (>150 generations) were crossed to field-collected males from four different geographic regions across the Corn Belt, as described in Oswald et al. (2011). Selection for resistance to the Bt toxin Cry3Bb1 was achieved by incrementally increasing the duration of larval exposure to Cry3Bb1 maize roots over 11 generations (Oswald et al. 2011). Initially, the resistant strain was selected for a duration of 24 h. The duration of selection increased in 12-h increments through the fourth generation and then increased in 24-h increments through generation 9, with one final 12-h increment at generation 10 (Oswald et al. 2011). This resulted in a strain that displayed significantly increased survivorship on Bt maize (average survival to adulthood on Bt maize increased six-fold over the final six generations) (Oswald et al. 2011), thus demonstrating that the strain was Bt resistant (Tabashnik 1994). This is comparable to other resistant strains selected in the laboratory. For example Meihls et al. (2008, 2011) found a similarly rapid response to selection by western corn rootworm strains on Bt maize producing Cry3Bb1 and mCry3A, respectively.

Once sent to Iowa State University, the resistant and susceptible strains were maintained at a population size of >1,200 adults, using standard western corn rootworm rearing procedures (Jackson 1986). Briefly, rootworm were reared on seedling mats produced by placing 40 ml (~65 kernels) of pregerminated maize seeds in 0.95-liter plastic deli trays (Pactiv Showcase, Johnson Paper and Supply Co., Minneapolis, MN), followed by 60 ml of deionized water and 200 g of soil. Soil consisted of a 1:1 mixture of potting soil (Sunshine Mix #1, Sungro, Bellevue, WA) and thoroughly dried field soil. Six hundred rootworm eggs were placed on each seedling mat. Seven days after eggs hatched, rootworm were moved to fresh seedling mats by inverting two seedling mats containing larvae over a larger fresh seedling mat held in a 21- by 27- by 10-cm (length by width by height) plastic container (Rubbermaid, Fairlawn, OH) covered with mesh fabric. Adults were collected from these containers using a vacuum aspirator, and placed in cages (30 by 30 by 30 cm, Megaview Science, Taichung, Taiwan) in a growth chamber (25°C and a photoperiod of 16/8 [LD] h). Cages contained maize leaf tissue, an arti-
ficial diet (western corn rootworm diet, Bio-Serv, Frenchtown, NJ) and a water source provided by a 1.5% agar solid. Adults were provided with an oviposition substrate that consisted of moist, finely sieved soil (<180 μm) placed in a 10-cm petri dish. Eggs from these oviposition dishes were used to infest new seedling mats.

To generate strains with a similar genetic background, the resistant strain was back-crossed to the susceptible strain, allowed to mate at random for an additional generation, and then selected by rearing on Cry3Bb1 maize seedling mats to adulthood for one generation. This process of crossing and selecting was repeated twice. The susceptible strain was maintained simultaneously (and separately) on non-Bt maize. Although backcrossing introduces susceptible genes into the resistant strain, the subsequent selection on Cry3Bb1 maize removes genes for Bt susceptibility. Importantly, 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.

Characterizing Resistant and Susceptible Strains. The Bt maize used in this study produced Cry3Bb1 (hybrid DKC 61–69), whereas the non-Bt maize was the near isolate to the Bt hybrid and lacked Cry3Bb1, and any other rootworm active Bt toxin (DKC 61–72) (Monsanto Co., St. Louis, MO). We placed neonate larvae from the resistant and susceptible strains on seedling mats to measure resistance to Bt maize. Seedling mats were produced by placing 40 ml (~65 kernels) of either Bt maize or non-Bt maize pregerminated seeds in 0.95-liter plastic deli trays, followed by 60 ml of deionized water and 200 g of soil. Soil consisted of a 1:1 mixture of potting soil (Sunshine Mix #1) and thoroughly dried field soil collected from the top 5 cm of ground in agricultural fields at an Iowa State University Research Farm in Ames, IA. These fields were fallow, had been planted to soybean [Glycine max (L.) Merr.] the previous year, and they did not receive insecticides for >1 yr before collection of soil. Containers were covered with plastic lids that had six holes (diameter, 1 cm). One week thereafter, a paintbrush was used to transfer 30 neonates (<1 d old) onto the germinated maize by moving soil to expose the seedling mat. Neonates were from petri dishes that contained moistened sieved soil (<180 μm) and eggs collected from the appropriate strain. Fabric (20 by 15 cm) was placed under the plastic lid to prevent larvae from escaping. Containers were held in a growth chamber (25°C, 65% RH, and a photoperiod of 16:8 [L:D] h) for 14 d. The seedling mat and soil from each container were then individually placed on Berlese funnels for 4 d to extract larvae, and the number of larvae in each instar and the total number of larvae extracted per container were recorded.

A period of 14 d of development was used to assess larval survival because most larvae on non-Bt corn in these seedling mats have reached the third instar at this point (see Results), and would have begun to pupate soon after. Head capsule width was measured using a Lieca MZ6 dissecting microscope and accompanying image analysis software (Motic Images Inc., Richmond, BC, Canada), and larval instar was determined following Hammack et al. (2003). Nine replicates in total were tested for each strain, with a replicate consisting of one Bt seedling mat and one non-Bt seedling mat.

Inheritance of Resistance. We measured survival and development on Bt and non-Bt seedling mats for the resistant and susceptible strains, and for the reciprocal crosses of these two strains. For the generation used to produce the crosses, insects were reared on non-Bt corn. Reciprocal crosses were made by collecting virgin adults every 3–4 h from plastic rearing trays. Virgin adults from each strain were immediately separated by sex (Hammack and French 2007), and added to populations cages in a 1:1 ratio on a daily basis to produce the two reciprocal crosses: 1) resistant ♀ × susceptible ♀ and 2) resistant ♀ × susceptible ♂. Susceptible and resistant crosses used in this experiment were also produced in this manner (resistant ♀ × resistant ♂ and susceptible ♀ × susceptible ♂). The same number of resistant and susceptible insects was added to all crosses daily, and insects from both strains were reared continuously, with new larval rearing containers set up on a twice-weekly basis, thus yielding a constant supply of adult insects for the crosses. Adults were randomly assigned to each respective cross, and continually added to these four genotypic classes for 10 wk. We added ~1,750 insects per sex in total to each of the four genotypic classes and maintained each of these crosses at a population size of ~1,000 adults. Each genotypic class was held in a 28- by 28- by 28-cm screen cage containing artificial diet, 1.5% agar solid for moisture, fresh maize leaves, and an oviposition substrate of a petri dish (diameter, 10 cm) with moistened finely sieved field soil (<180 μm). Oviposition dishes were replaced every 3–4 d.

Eggs were collected from the four genotypic classes, and assays were conducted with neonates that hatched from these eggs as in the experiment characterizing the resistant and susceptible strains (described under Characterizing Resistant and Susceptible Strains). In total, six blocks were completed, with two to four pairs of Bt and non-Bt seedling mats per genotypic class per block (total of 14 seedling mats per maize type per genotypic class in the entire experiment). For each of the genotypic classes, the same number of pairs for Bt and non-Bt seedling mats were completed per block. We counted the total number of larvae recovered from each seedling mat and determined larval instar for each larva based on head capsule width (Hammack et al. 2003).

Fitness Cost. We tested whether fitness costs were imposed by the entomopathogenic nematodes S. carpocapsae (strain BU) and H. bacteriophora (strain BU), both of which can kill western corn rootworm larvae and have been found in maize fields (Pilz et al. 2008). We measured mortality imposed by these two nematode species on the four genotypic classes described above (Bt-resistant, Bt-susceptible, resistant ♀ crossed with susceptible ♂, and resistant ♂ crossed...
with susceptible ). Nematodes were originally obtained from Becker Underwood (Ames, IA) and were maintained in the laboratory at Iowa State University through culturing in Galleria mellonella (L.) (Lepidoptera: Pyralidae) larvae (Kaya and Stock 1997). Infective juvenile nematodes were used for assays and were no >2 wk of age. Nematode concentrations were determined using a compound microscope (Eclipse E200, Nikon, Melville, NY) set at 40× magnification and a Sedgewick-Rafter counting cell (Pyser-SCI, Edenbridge, Kent, United Kingdom). Only live nematodes were counted, and solutions were diluted to the desired concentration with deionized water. Each nematode species was tested separately.

We conducted bioassays using 45-ml cups with lids (Translucent Plastic Soufflé Cup, Solo Cup Company, Highland Park, IL). Each cup contained three non-Bt corn seedlings 4 d postgermination that were covered with 30 g of soil moistened to 25% water holding capacity. Treatment cups contained nematodes mixed into the soil at concentrations of 25, 50, 75, and 100 nematodes per ml soil, for a total of four treatments per nematode species. Soil in control cups lacked nematodes. Six neonates (<1 d old) were transferred to the maize seedlings in each cup. We made three small holes in each lid for ventilation and placed fine mesh cloth under lids to prevent larvae from escaping. Cups were placed in a growth chamber (25°C, 65% RH, and a photoperiod of 16:8 [LD] h) and covered with moist paper towels. After 10 d, each cup was placed on a Berlese funnel for 24 h to extract larvae. In total, eight blocks were run for each nematode species. Each block consisted of two cups for each of the four genotypic concentrations (32 treatment cups per block for each nematode species), and four control cups per genotypic class (16 control cups per block for each nematode species). Thus, there were 48 bioassay cups per block and a total of 384 cups used to evaluate each nematode species. Feeding Behavior. We tested preference of resistant and susceptible larvae for Bt and non-Bt maize by conducting choice tests in petri dishes. Filter paper (Whatman grade 2, 90 mm, Thermo Fisher Scientific, Waltham, MA) was marked with four equal quadrants, placed inside a petri dish (diameter, 10 cm) and moistened with 1 ml of deionized water. Bt roots (DKC 61-69, producing Cry3Bb1) and non-Bt roots (DKC 61-72) were placed onto the filter paper in opposite quadrants of the petri dish. The distal 2.5-cm portion of each root was removed with a razor blade and placed in the middle of each quadrant. Roots were from maize seedlings (age, 7 d) that were germinated in moistened paper towels (24- by 25-cm Brown Singlefold Towels 23504, Georgia Pacific, Atlanta, GA). A single larva was placed in the center of each dish using a fine paintbrush. Dishes were sealed with parafilm, placed on trays held in a growth chamber (25°C, 65% RH, and a photoperiod of 16:8 [LD] h), and checked with a compound microscope. Larvae were checked at 12, 14, 16, and 18 h after initially placed in petri dishes. We quantified larval behavior (feeding or not feeding) and location (quadrant with Bt or non-Bt maize). At each time point, a value of 1 or 0 was given to larvae feeding on non-Bt maize or Bt maize, respectively. A preference score was calculated for each larva by dividing the number of times feeding on a non-Bt root by the total number of times feeding. A score of 0.5 indicates no preference, a score >0.5 indicates a preference for non-Bt maize, and a score <0.5 indicates a preference for Bt maize. There were eight larval treatments tested in preference experiment, and each treatment evaluated both the susceptible strain and the resistant strain, for a total of 16 combinations of treatment by strain. For each of the 16 combinations of treatment by strain, 31–36 individual preference tests in total were conducted. Treatments 1, 2, and 3 consisted of first-, second-, and third-instar larvae, respectively. Two other treatments consisted of larvae that were fed either Bt maize (treatment 4) or non-Bt maize (treatment 5) for 48 h preceding the experiment. Treatments 6 and 7 consisted of naïve neonates (<1 d old). In treatment 6, the cut end of each root was covered with a small dot of hot glue (Craftsticks, Surebonder, Wauconda, IL) to prevent neonates from directly entering the cut portion of the root, whereas no glue was used for treatment 7. Treatment 8 consisted of larvae that were fed only Bt maize for 7 d before the preference test. Larvae for treatments 1–3 were raised to the appropriate instar by placing eggs in 0.95-liter trays that contained a mat of 40 ml of germinated non-Bt maize seedlings (of a different genetic background than the non-Bt maize used in the preference tests [Pioneer 34M94]) covered with 200 g of a 1:1 mixture of potting soil (Sunshine Mix #1) and field soil. Upon reaching the appropriate instar, larvae were transferred from seedling mats and transferred to preference tests. Larvae in treatments 4 and 5 were first placed as neonates into petri dishes (50 per dish) containing 20 freshly germinated non-Bt maize seedlings on top of a moistened filter paper for 3–5 d and then moved into preexposure petri dishes (12 larvae and 12 pregerminated seedlings per dish) with either Bt (DKC 61-69) or non-Bt maize (DKC 61-72) seedlings for 48 h. From these preexposure dishes, larvae were transferred to preference tests. Larvae in treatment eight were neonates (<1 d old) that were transferred directly from a petri dish with soil but no maize to a preexposure petri dish with Bt maize for 7 d preceding the experiment; most larvae from the resistant strain were second instars when placed into preferences tests, whereas larvae from the susceptible strain were mostly first instars.

Data Analysis. Analysis of variance (ANOVA) was performed in SAS Enterprise Guide 4.2 (SAS Institute 2009). In all mixed models, random effects were tested with a log-likelihood ratio statistic (−2 RES log-likelihood in PROC MIXED) based on a one-tailed chi-square test assuming 1 df (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 α < 0.25 (Quinn and Keough 2002).
Characterizing Resistant and Susceptible Strains. Survival of western corn rootworm larvae in bioassays was analyzed with a mixed model ANOVA (PROC MIXED). Fixed factors in the analysis included maize type (Bt and non-Bt) and strain (resistant and susceptible), and the interaction of maize type and strain. Replicate was a random factor. There was a significant interaction between maize type and strain, thus pairwise comparisons were made using the PDIFF option in PROC MIXED with a Tukey adjustment. To determine whether there were differences in the proportion of each of the larval instars recovered between the resistant and susceptible strains on each type of maize, a Student’s t-test was used (Sokal and Rohlf 1995).

Inheritance of Resistance. Survival of western corn rootworm larvae was analyzed with a mixed model ANOVA. Fixed factors in the analysis were maize type (Bt and non-Bt) and genotypic class (resistant, susceptible, S R S and S S R), and the interaction between maize type and genotypic class. Random effects were block and all possible interactions with the fixed factors. Because there was a significant interaction between maize type and genotypic class, pairwise comparisons were made using the PDIFF option in PROC MIXED with a Tukey adjustment. The proportion of third-instar larvae recovered was analyzed with the same statistical model as survival, except that genotypic type was coded as a continuous variable rather than a categorical variable. Data were transformed by the arcsine of the square root to ensure normality of the residuals. Between maize type and genotypic class, pairwise comparisons were made using the PDIFF option in PROC MIXED with a Tukey adjustment. To determine whether there were differences in the proportion of each of the larval instars recovered between the resistant and susceptible strains on each type of maize, a Student’s t-test was used (Sokal and Rohlf 1995).

Characterizing Resistant and Susceptible Strains. Survival of western corn rootworm larvae in bioassays was analyzed with a mixed model ANOVA (PROC MIXED). Fixed factors in the analysis included maize type (Bt and non-Bt) and strain (resistant and susceptible), and the interaction of maize type and strain. Replicate was a random factor. There was a significant interaction between maize type and strain, thus pairwise comparisons were made using the PDIFF option in PROC MIXED with a Tukey adjustment. To determine whether there were differences in the proportion of each of the larval instars recovered between the resistant and susceptible strains on each type of maize, a Student’s t-test was used (Sokal and Rohlf 1995).

Results

Characterizing Resistant and Susceptible Strains. For larval survival at 14 d on seedling mats, there was a significant interaction between strain and maize type (F15,15 = 7.36; P = 0.016). Survival of western corn rootworm larvae on Bt maize was significantly lower for the susceptible strain compared with the resistant strain; however, survival did not differ between the two strains on non-Bt maize, or between Bt and non-Bt maize for the resistant strain (Fig. 1A). Larvae from the resistant strain developed faster on Bt maize compared with the susceptible strain, as indicated by a significantly lower proportion of second instars (F15 = 2.64; P = 0.009) and a higher proportion of third instars (F15 = 2.83; P = 0.006) for the resistant strain on Bt maize compared with the susceptible strain (Fig. 1B). Development did not differ between the two strains on non-Bt maize, as every larva recovered was a third instar (Fig. 1B). Thus, the resistant strain is characterized by significantly greater larval survival and significantly faster development on Bt maize.

Inheritance of Resistance. There was a significant interaction between genotypic class and maize type for survival (Table 1). For the resistant genotypic class and for the S S R class, survival on the two types of maize did not differ (P > 0.943 for both comparisons) (Fig. 2A). However, there was significantly lower survival on Bt maize compared with non-Bt maize for the
susceptible genotypic class and for the $\varphi R \delta S$ class ($P < 0.0006$ for both comparisons) (Fig. 2A).

Corrected survival on Bt maize did not differ significantly between the resistant and $\varphi S \delta R$ genotypic classes ($t_{15} = 1.12, P = 0.684$) or between the susceptible and $\varphi R \delta S$ genotypic classes ($t_{15} = 0.95, P = 0.780$) (Fig. 2B). There was a significant difference between the $\varphi R \delta S$ and $\varphi S \delta R$ genotypic classes ($t_{15} = 5.40, P = 0.0004$), indicating evidence of sex linkage. The $h$ value for corrected survival on Bt maize was $0.19 \pm 0.09$ for $\varphi R \delta S$, significantly $>0$ ($t_{12} = 2.01$, $P = 0.034$) and significantly $<0$ ($t_{12} = 3.25$, $P = 0.003$). The $h$ value was $1.22 \pm 0.15$ for $\varphi S \delta R$ (although $h$ ranges from 0 to 1, survival on Bt maize relative to non-Bt maize was numerically greater for $\varphi S \delta R$ compared with the resistant strain, resulting in a value $>1$ for $h$). This value is significantly $>0.5$ ($t_{13} = 4.75, P = 0.0002$) but is not significantly different from 1 ($t_{13} = 1.47, P = 0.083$).

There was a significant interaction between genotypic class and maize type for proportion of third instars recovered from seedling mats (Table 1). Development on non-Bt maize was faster than development on Bt maize for all genotypic classes, as indicated by a significantly higher proportion of third instars on non-Bt maize compared with Bt maize for each class ($P < 0.0001$ for all comparisons) (Fig. 3; Table 1). Proportion of third instars from Bt maize seedling mats was lowest for the susceptible strain, intermediate for the crosses, and highest for the resistant strain (Fig. 3). The $h$ value using proportion of third instars on Bt maize adjusted for the proportion on non-Bt maize was $0.51 \pm 0.13$, significantly $<1$ and significantly $>0$ ($P < 0.0004$ for both) but not significantly different from 0.5 ($t_{26} = 0.111, P = 0.913$).

Fitness Costs. Based on ANCOVA, we did not detect a significant difference in corrected mortality among genotypic classes when exposed to either of the nematode species, indicating that nematodes did not affect fitness costs of Bt resistance (Table 2). For each nematode species, western corn rootworm mortality significantly increased as nematode concentration increased (Table 2; Fig. 4). In assays with $H. bacteriophora$, average mortality of experimental controls not exposed to nematodes was 0.25 (resistant), 0.19 (susceptible), 0.16 ($\varphi R \delta S$), and 0.19 ($\varphi S \delta R$). Control mortality did not differ significantly among genotypic classes ($F_{3,22} = 0.75, P = 0.5320$). In assays with $S. carpocapsae$, average mortality of experimental controls not exposed to nematodes was 0.25 (resistant), 0.20 (susceptible), 0.27 ($\varphi R \delta S$), and 0.28 ($\varphi S \delta R$), which did not differ significantly among genotypic classes ($F_{3,22} = 0.65; P = 0.5888$).

Feeding Behavior. The only case in which a strain showed a preference for a maize type was in treatment 6, where resistant neonates given roots that were sealed at the cut end showed a preference for non-Bt maize ($t_{31} = 3.05, P = 0.005$) (Fig. 5). However, there was no difference between the susceptible and resistant strains in this treatment ($t_{32} = 1.06, P = 0.294$) or in any other treatment ($P > 0.110$ for all comparisons). Neither susceptible nor resistant larvae in any other treatment showed preference for a maize type, as indicated by a score that did not differ significantly from 0.5 ($P > 0.05$) (Fig. 5).

Table 1. Mixed model analysis of variance for survival and proportion of third instars recovered for western corn rootworm among the genotypic classes (resistant, susceptible, $\varphi R \delta S$ and $\varphi S \delta R$) on Bt and non-Bt maize seedling mats (maize type)

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Discussion

Widespread field-evolved resistance of western corn rootworm to Bt maize could cut short the benefits of this technology. The evolution of resistance by insects to insecticidal toxins is influenced by many
factors, including the dominance of resistance, fitness costs associated with resistance, multitrophic interactions, and behavioral responses to toxins (Gould 1998, Tabashnik et al. 2003, Bowling et al. 2007, Gassmann et al. 2009a). In this laboratory-selected strain, we found that inheritance of resistance to Cry3Bb1 maize was nonrecessive and that there was evidence of sex linkage (Figs. 2 and 3). The entomopathogenic nematodes S. carpocapsae and H. bacteriophora did not impose any fitness costs associated with Bt resistance (Fig. 4). In addition, there were no differences in feeding preferences between Bt-resistant and Bt-susceptible larvae (Fig. 5).

Bt-resistant strains of western corn rootworm show increased survival, faster growth, or both on Bt maize relative to susceptible strains (Meihls et al. 2008, 2011; Nowatzki et al. 2008; Gassmann et al. 2011; Petzold-Maxwell et al. 2012). In this study, we found no difference in larval survival (survival over 14 d) on Bt and non-Bt maize for the resistant strain and decreased survival on Bt maize compared with the susceptible strain (Fig. 1A). The resistant strain also developed faster on Bt maize compared with the susceptible strain (Fig. 1B). For western corn rootworm, a number of studies have shown that larval survival is an accurate predictor of adult survival in both the laboratory (Meihls et al. 2008, 2011; Binning et al. 2010) and the field (Hibbard et al. 2010, Frank et al. 2011). For example, Meihls et al. (2011) found that both larval and adult survival of an unselected strain was higher on non-Bt maize than on mCry3a-producing maize but did not differ between the two maize types for a mCry3a-selected strain. Similarly, for field-selected populations that showed increased larval survival relative to control populations on Cry3Bb1 maize but not on Cry34/35Ab1 maize in laboratory bioassays (Gassmann et al. 2011), increased adult survival on Cry3Bb1 maize (but not on Cry34/35Ab1 maize) also was shown the following year in fields from which these populations were collected (Gassmann 2012).

The inheritance of resistance can affect how quickly resistance will evolve, with resistance expected to evolve faster as the degree of dominance increases (Tabashnik et al. 2004). Larval survival data from the reciprocal crosses revealed that Cry3Bb1 resistance in this strain is inherited in a nonrecessive manner (Fig. 2), with $h$ values of 0.19 ± 0.09 for the $H/R S$ genotypic class, and 1.22 ± 0.15 for the $S/S R$ genotypic class. Dominance values for survival suggest that the resistance trait is sex-linked, with resistance alleles inherited from the male (Fig. 2), although we do not know how this could arise. It is possible that males from the resistant strain may have had enhanced contributions to offspring fitness by male provisioning via the sper-

**Table 2.** Mixed model analysis of covariance for survival of genotypic classes of western corn rootworm (resistant, susceptible, $H/R S$ and $S/S R$) exposed to H. bacteriophora and S. carpocapsae (treatment)

<table>
<thead>
<tr>
<th>Effect</th>
<th>df</th>
<th>$F$</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>H. bacteriophora</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Genotypic class</td>
<td>3, 21</td>
<td>0.43</td>
<td>0.7369</td>
</tr>
<tr>
<td>Treatment</td>
<td>1, 7</td>
<td>29.00</td>
<td>0.0010</td>
</tr>
<tr>
<td>S. carpocapsae</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Genotypic class</td>
<td>3, 21</td>
<td>0.41</td>
<td>0.7508</td>
</tr>
<tr>
<td>Treatment</td>
<td>1, 7</td>
<td>62.97</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

Fig. 2. Western corn rootworm larval survival over 14 d (A) and corrected survival (B). The four genotypic classes of rootworm tested were resistant, susceptible, $H/R S$ and $S/S R$. Bars are least squares means and error bars are SEM. Asterisks in A indicate a significant difference between non-Bt and Bt maize, within a genotypic class; different letters in B indicate significant differences among the treatments ($P < 0.05$).

Fig. 3. Average proportion of third-instar western corn rootworm larvae from Bt maize and non-Bt maize seedling mats, for susceptible, resistant, and heterozygote ($F_1$) larvae (combined data from $H/R S$ and $S/S R$). Points are sample means and error bars show 1 SEM.
matophore (Tallamy et al. 2000). Evidence of sex linkage for inheritance of methyl-parathion resistance in Nebraska populations of western corn rootworm was found in a study by Parimi et al. (2003); however, this was only detected in one of the strains tested and results were inconclusive with regard to the precise nature of inheritance. In contrast to the survival data, the heterozygous genotypic classes in this study displayed similar developmental rates on Bt maize, and this trait seemed to be additive ($h = 0.51 \pm 0.13$) (Fig. 3). Future experiments with additional strains will be needed to gain a better understanding of the inheritance of resistance in western corn rootworm.

Oswald et al. (2011) measured the realized heritability values ($h^2$) for the resistant strain used in the current study at generations 6, 8, and 10 and found low heritability ($h^2 = 0.16$), indicating that survival on Bt maize is heavily influenced by environmentally based variation relative to genetically based variation. Few studies have examined the inheritance ($h$) of resistance alleles in western corn rootworm, although evidence of nonrecessive inheritance of Cry3Bb1 resistance in western corn rootworm has been found previously (Meihls et al. 2008). By contrast, resistance is functionally recessive for P. gossypiella on cotton (Gossypium hirsutum L.) producing Cry1Ac (Liu et al. 1999), Plutella xylostella (L.) on broccoli (Brassica oleracea L.) producing Cry1C (Zhao et al. 2000), and for Ostrinia nubilalis (Hübner) on maize producing Cry1F (Pereira et al. 2008). For O. nubilalis and for
sugarcane borer, *Diatraea saccharalis* (F.), on maize producing Cry1Ab, inheritance of resistance was not always recessive and depended on plant stage (Wu et al. 2007, Crespo et al. 2010). Comparisons across pest species indicate that recessive inheritance of resistance is one key factor in delaying the evolution of resistance (Tabashnik et al. 2008, 2009; Carrière et al. 2010). Nonrecessive inheritance of resistance for western corn rootworm, as indicated in this study and in the study by Meihls et al. (2008), increases the risk of Bt resistance in western corn rootworm. Simulation models predict that the western corn rootworm will develop resistance in the field far more quickly when inheritance is nonrecessive (Onstad and Meinke 2010).

In addition to recessive inheritance of resistance, the presence of fitness costs associated with Bt resistance can act to delay pest resistance (Carrière and Tabashnik 2001). Fitness costs of resistance to Bt can be influenced by several factors, including interspecific interactions, and they can vary with ecological conditions (Gassmann et al. 2009a). Thus, understanding ecological factors that can alter fitness costs may help improve resistance management. In this study, we did not observe any fitness costs of resistance to Cry3Bb1 maize in the presence of the entomopathogenic nematodes *S. carpocapsae* and *H. bacteriophora*, as indicated by no differences in survival among the four genotypic classes (Fig. 4). Both entomopathogenic nematodes and viruses can increase fitness costs of Bt resistance (Gassmann et al. 2006, Raymond et al. 2007). The entomopathogenic nematode *Steinernema riobrave* Cabanillas, Poinar & Raulston imposes fitness costs associated with resistance to Cry1Ac in *P. gossypiella* (Gassmann et al. 2006, 2009b; Hannon et al. 2010), and *H. bacteriophora* has been shown to impose a fitness cost when larvae are reared on cotton bolls (Gassmann et al. 2009b). However, fitness costs were not found in this insect for the presence of the entomopathogenic nematodes *Steinernema sp.* (ML18 strain), *S. carpocapsae*, or *Heterorhabditis sonorensis* Stock, Rivera-Orduno & Flores-Lara (Hannon et al. 2010). The results of this study indicate that application of *S. carpocapsae* or *H. bacteriophora* to refuges probably would not aid in delaying Bt resistance in western corn rootworm. The only other study examining fitness costs of Cry3Bb1 resistance in western corn rootworm, which included the strains used in this study, found no evidence for costs affecting survivorship, fecundity, or egg viability (Oswald et al. 2012).

Behavioral responses of insects to toxins can affect resistance evolution by influencing exposure to toxins (Gould 1984, Frutos et al. 1999, Peck et al. 1999, Onstad 2005). In this study, we did not find a significant difference in preference for Bt or non-Bt maize between the resistant and susceptible strains, although resistant neonates did show a preference for non-Bt maize over Bt maize in one treatment (Fig. 5). This occurred for the treatment in which the cut end of the root tissue was sealed with glue. The preference for non-Bt maize exhibited by Bt-resistant neonate larvae when forced to enter the root through the epidermis could indicate behavioral avoidance of areas containing higher amounts of Bt toxins. Root growing points are more metabolically active and higher in soluble protein compared with older root tissue (Raven et al. 1999). There is evidence that the quantity of Bt toxins in maize roots follows this pattern (Lefko et al. 2008; Meissle et al. 2009; T. Vaughn, personal communication). Although later instar larvae were seen either feeding within the interior of the root or feeding on the root from the exterior, all neonate larvae in the preference experiment fed inside the root (i.e., tunneling), but it is unclear whether neonates that were not exposed to the glued roots entered the root through the cut end or through the root epidermis.

The few studies examining feeding behavior of both Bt-susceptible and Bt-resistant insect strains when presented with Bt and non-Bt substrates report little or no differences between the strains in feeding behavior or preference (Schwartz et al. 1991, Whalon et al. 1993, Harris et al. 2006, Heuberger et al. 2008). In general, physiological resistance to toxins is expected to evolve more slowly if an insect avoids toxic plants or plant parts because this will reduce selection pressure (Gould and Anderson 1991). Results from this study indicate that western corn rootworm larvae do not exhibit behavior that leads to either reduced or increased exposure to toxins. Bernklaus et al. (2010) found no differences in feeding behavior of Bt-susceptible western corn rootworm neonates on *mCry3A* maize and non-Bt maize. Results from a study of larval feeding behavior of susceptible western corn rootworm on non-Bt and Bt maize roots producing Cry3Bb1 suggest that susceptible neonate larvae may alter feeding behavior to reduce exposure to Bt proteins (Clark et al. 2006). However, it is difficult to compare the results of the current study, in which behavior was observed in a choice test over 18 h, with the study by Clark et al. (2006), where behavior was observed over 12 d in a no-choice test with intact root systems growing in a gel medium.

Laboratory-selected strains are important for understanding Bt resistance, but the selection regime used to develop the strains tested here can differ from how insects experience selection in the field. Future studies using strains with field-evolved resistance, such as those identified in Gassmann et al. (2011), will be useful models for gaining further insight to Bt resistance for the western corn rootworm. Here, we found evidence of nonrecessive inheritance of resistance, a lack of fitness costs associated with Bt resistance in the presence of two entomopathogenic nematode species, and no difference in preference for non-Bt versus Bt maize between susceptible and resistant larvae. Data from this study and from others showing nonrecessive inheritance of resistance (Meihls et al. 2008) and lack of fitness costs (Oswald et al. 2012) suggest the western corn rootworm may have the potential to adapt quickly to Cry3Bb1 maize. Given the risk of widespread, field-evolved resistance of this pest species to Bt maize, and the likelihood of Bt maize hybrids as a fixture in the agricultural land-
scape, more studies characterizing Bt resistance will critical for improving resistance management.

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References Cited


Boeing, R. D., R. A. Higgins, A. Ahmad, and G. Wilde. 2007. Feeding behavior and growth of corn earworm (Lepidoptera: Noctuidae) larvae on Bacillus thuringiensis-treated (Dipel 4L) and untreated medicid diet. J. Econ. Entomol. 100: 1221–1228.


Harris, M. O., N. Markwick, and M. Sandanayake. 2006. Is resistance to Bacillus thuringiensis endotoxin Cry1Ac associated with a change in the behavior of light brown apple moth larvae (Lepidoptera: Tortricidae)? J. Econ. Entomol. 99: 508–518.


Meinke, L. J., B. D. Siegfried, R. J. Wright, and L. D. Chandler. 1998. Adult susceptibility of Nebraska western corn rootworm (Coleoptera: Chrysomelidae) populations to selected insecticides. J. Econ. Entomol. 91: 594–600.


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