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Source: Journal of Economic Entomology, 113(6) : 2873-2882

Published By: Entomological Society of America

URL: https://doi.org/10.1093/jee/toaa213

Journal of Economic Entomology, 113(6), 2020, 2873–2882
doi: 10.1093/jee/toaa213
Advance Access Publication Date: 29 September 2020
Research

Insecticide Resistance and Resistance Management

Inheritance and Fitness Costs of Cry3Bb1 Resistance in Diapausing Field Strains of Western Corn Rootworm (Coleoptera: Chrysomelidae)

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Subject Editor: Lisa Bird

Received 20 June 2020; Editorial decision 15 August 2020

Abstract

Field-evolved resistance to Cry3Bb1 corn by western corn rootworm, *Diabrotica virgifera virgifera* LeConte (Colleoptera: Chrysomellidae), has been reported in field populations in Iowa, Illinois, Nebraska, and Minnesota. Inheritance and fitness costs associated with Cry3Bb1 resistance have been determined for non-diapausing laboratory strains of western corn rootworm with either laboratory-selected resistance or field-derived resistance. However, information on inheritance and fitness costs of Cry3Bb1 resistance in the diapausing field populations is lacking. In this study, we determined the inheritance of Cry3Bb1 resistance for four diapausing field strains of western corn rootworm using plant-based bioassays. We also determined the fitness costs for eight diapausing field populations in a greenhouse experiment. We found that Cry3Bb1 resistance was an autosomal trait and that the inheritance of resistance was mostly non-recessive; however, there was some variation in the dominance of Cry3Bb1 resistance. We did not find evidence of fitness costs affecting survival to adulthood, developmental rate, or adult dry mass. However, we did detect a fitness cost affecting adult size. The results of this study will add to the current understanding of field-evolved resistance to Cry3Bb1 corn by western corn rootworm and help in developing better strategies to manage resistance.

Key words: Bt resistance, corn, *Diabrotica virgifera virgifera*, diapause, fitness cost

Western corn rootworm, *Diabrotica virgifera virgifera* LeConte (Colleoptera: Chrysomellidae) is a key pest of corn in the United States and Europe (Kiss et al. 2005, Gray et al. 2009). Western corn rootworm adults feed on corn silk and leaves, and lay eggs in the soil of cornfields. Upon hatching, larvae feed on corn roots and can cause severe root injury (Godfrey et al. 1993). Larval feeding on corn roots causes injury to the root tissue, and as a result, water and nutrient uptake can be reduced, in addition to rendering corn plants unstable and more vulnerable to lodging (Spike and Tollefson 1991a, b; Godfrey et al. 1993). Heavy infestations of western corn rootworm larvae in a cornfield causes substantial yield loss (Dun et al. 2010, Tinsley et al. 2013).

The western corn rootworm is univoltine, diapausing species. Female adults lay eggs in the soil of cornfield in summer, and eggs undergo diapause during the winter. Diapause is induced by exposure of eggs to sub-freezing temperatures and terminated by exposure to higher temperatures and moisture (Krysan 1978). It takes 426 degree days (base 11.2°C) for 50% of the eggs hatch following diapause (Levine et al. 1992). Diapausing as eggs allows western corn rootworm to survive in a temperate climate, where food in unavailable for much of the year (Krysan et al. 1977). A non-diapausing strain of western corn rootworm has been developed through laboratory selection, and has facilitated research by allowing the study of multiple generations per year (Branson 1976). However, diapausing and non-diapausing strains differ in their genetics, biology and behavior, and the field populations of western corn rootworm possess the diapause trait (Branson 1976, Branson et al. 1981, Kim et al. 2007). Planting of transgenic Bt corn is a common practice for managing rootworm in the United States. Currently available corn hybrids for management of western corn rootworm larvae produce Bt toxins Cry3Bb1, mCry3A, Cry34/35Ab1, and eCry3.1Ab, either singly or as a pyramid, which is a combination of two or more Bt toxins that target the same insect pest (Gassmann 2016, Ludwick and Hibbard 2016). However, some populations of western corn rootworm have developed resistance to all Bt traits that are currently available (Gassmann et al. 2016, 2020). Field-evolved resistance to a Bt crop is defined as a genetically based decrease in the susceptibility of a population to a Bt toxin due to the exposure of the population to the toxin in the field (Tabashnik et al. 2009). Field-evolved resistance to multiple Bt traits and cross-resistance among traits has complicated management of western corn rootworm (Gassmann et al. 2014, 2020; Jakka et al. 2016).

For Bt crops grown in the United States and elsewhere, insect resistance management (IRM) strategies are used to delay field-evolved Bt resistance (Bates et al. 2005, Tabashnik and Gould 2012). Current IRM approaches are based on high-dose/refuge strategy (Head and Greenplate 2012, Tabashnik and Carrière. 2017). In theory, high-dose Bt plants kill 99.99% of the pest population or produce 25 times the amount of Bt protein required to kill Bt-susceptible individuals (US EPA 1998). Production of Bt toxin at this high dose is designed to kill individuals that are heterozygous for resistance alleles of large effect and renders Bt resistance a functionally recessive trait (Gould 1998). A large number of Bt-susceptible insects produced in the non-Bt refuges increases the probability of mating between Bt-susceptible and Bt-resistant individuals, thus reducing the proportion of homozygous resistant individuals in a population and delaying the evolution of Bt resistance (Gould 1998).

The high-dose-refuge approach has been successful for some lepidopteran pests (Huang et al. 2011). However, Bt corn hybrids developed for western corn rootworm are not high dose cultivar, which reduces the effectiveness of refuges at delaying resistance and may alter the effective dominance of resistance (Gassmann 2012, 2016; Tabashnik and Gould 2012). Corn hybrids producing Bt toxin Cry3Bb1 were first planted commercially in 2003; after 6 yr of commercialization, the first case of field-evolved Cry3Bb1 resistance was found in Iowa in 2009 (Gassmann et al. 2011). Western corn rootworm has developed resistance to all Bt toxins within eight, or fewer, years after commercialization (Gassmann et al. 2011, 2014, 2016; Jakka et al. 2016; Tabashnik and Carrière 2017). Simulation studies have shown that the rate of resistance evolution depends on the genetic dominance of resistance and whether fitness costs of resistance are present (Carrière and Tabashnik 2001, Onstad et al. 2001). Recessive inheritance of resistance coupled with fitness costs will delay the evolution of resistance. In contrast, non-recessive inheritance of resistance and minimal fitness costs will cause resistance to evolve more quickly. Therefore, a clear understanding of the inheritance and the fitness cost for Bt resistance is required to predict the durability of Bt traits and to develop resistance management strategies.

The dominance of insecticide resistance is quantified in terms of a dominance value, which is the mortality of heterozygotes relative to the mortality of two homozygotes (Bourguet et al. 2000). The dominance value determined based on the arbitrary dose of insecticide such as an LC_{50} values. Use of these values may be of less relevant to resistance management, because this may or may not represent the insecticide exposure experience by the insect in the field (Liu and Tabashnik 1997). In contrast, a more meaningful approach is to calculate dominance values based on the mortality at some practical dose that represents what insects will experience in a field setting. The dominance value based on the mortality of heterozygotes at a practical dose is also known as effective dominance (Bourguet et al. 2000).

In addition to inheritance, fitness costs suffered by individuals carrying resistance-conferring alleles are an important factor affecting the rate of evolution of Bt resistance. Fitness costs of Bt resistance represent a trade-off in which Bt-resistant insect have reduced fitness on a non-Bt crop, compared with Bt susceptible insects (Gassmann et al. 2009). Fitness costs can be measured for various life-history parameters such as survival, growth rate, size, or fecundity. The presence of fitness costs for Bt-resistant insects can delay the evolution of Bt resistance when non-Bt refuges are present because resistance-allele carrying individuals are relatively less fit in the refuge (Gassmann et al. 2009).

Past research on the inheritance and fitness costs of Cry3Bb1 resistance in western corn rootworm have used either laboratory-selected strains (Meihls et al. 2012, Oswald et al. 2012, Petzold-Maxwell et al. 2012, Hoffmann et al. 2015) or strains where insects with field-evolved resistance were crossed with a non-diapausing laboratory strain (DLS) to generate a Cry3Bb1-resistant non-DLS (Ingber and Gassmann 2015, Paolino and Gassmann 2017). However, results obtained with these laboratory strains may differ from field strains. Here, we present results from the laboratory and greenhouse experiments characterizing the inheritance and fitness costs for diapausing field strains of western corn rootworm with field-evolved Cry3Bb1 resistance. Results obtained from this research will provide a more complete understanding of why Bt resistance evolved rapidly in this key agricultural pest, and provide insights into steps that may be taken to delay pest resistance to other Bt traits.

Materials and Methods

Inheritance Study

Four Cry3Bb1-resistant diapausing field strains were crossed to a Bt-susceptible DLS, and resistance to Cry3Bb1 was then quantified in parental and F1 hybrid strains with plant-based bioassays (Fig. 1a). Diapause of rootworm eggs was induced by storing them in cold room at 6°C for at least 5 mo and diapause was broken by incubating the eggs at 25°C.

Cry3Bb1-Resistant Field Strains

During the summer of 2012, western corn rootworm adults ($n = 219 \pm 227$, Mean ± SD) were sampled from nine cornfields with high levels of root injury (1.9 ± 0.8 node of root injury (Oleson et al. 2005), Mean ± SD) to either Cry3Bb1 corn or mCry3A corn in Iowa (Fig. 2a). Adults were collected using a handheld aspirator (1135A, BioQuip, Rancho Dominguez, CA) from 10 to 31 July 2012. Adults collected from each cornfield were kept in separate cages (17.5 × 17.5 × 17.5 cm Catalog # 4S1515, BugDorm Store, Taiwan) and fed a complete adult diet (product AG-F9768B-M, Frontier Agricultural Sciences, Newark, DE). Eggs were collected in Petri dishes filled with fine sieved soil (particle size <180 µm) and then stored in a cold room at 6°C to break diapause. One of the field populations produced over 22,000 eggs, and this population was used as an individual strain, Northern Iowa strain. However, adults from the other eight cornfields produced less than 20,000 eggs each (10150 ± 3916, Mean ± SD). Consequently, subsets of these populations with less than 20,000 eggs, from the same geographical region, were combined into three composite strains named: Central Iowa, Eastern Iowa, and Western Iowa. In total, four Cry3Bb1-resistant strains of western corn rootworm were used for this study: Central Iowa, Eastern Iowa, Northern Iowa, and Western Iowa.

Those four field strains of western corn rootworm were reared on untreated Cry3Bb1 corn (DKC 61–69 or DKC62-63, Monsanto Co., St. Louis, MO) for one generation in the laboratory to select out susceptible individuals that may have been collected in the field sample. Selection on Cry3Bb1 corn was performed from December 2013 to February 2014. Two types of seedling mats were used for rearing rootworm larvae; hereafter referred as small seedling mats and large seedling mats. Small seedling mats were prepared by adding 30 ml of corn seed, 80 ml of water, and 200 g of a soil mixture in a 946 ml plastic container (Dart C32DE, Dart Container Corporation, Mason, MI). The soil mixture was prepared by combining field soil (dried, crushed, and sieved) with LC1 potting medium (SunGro

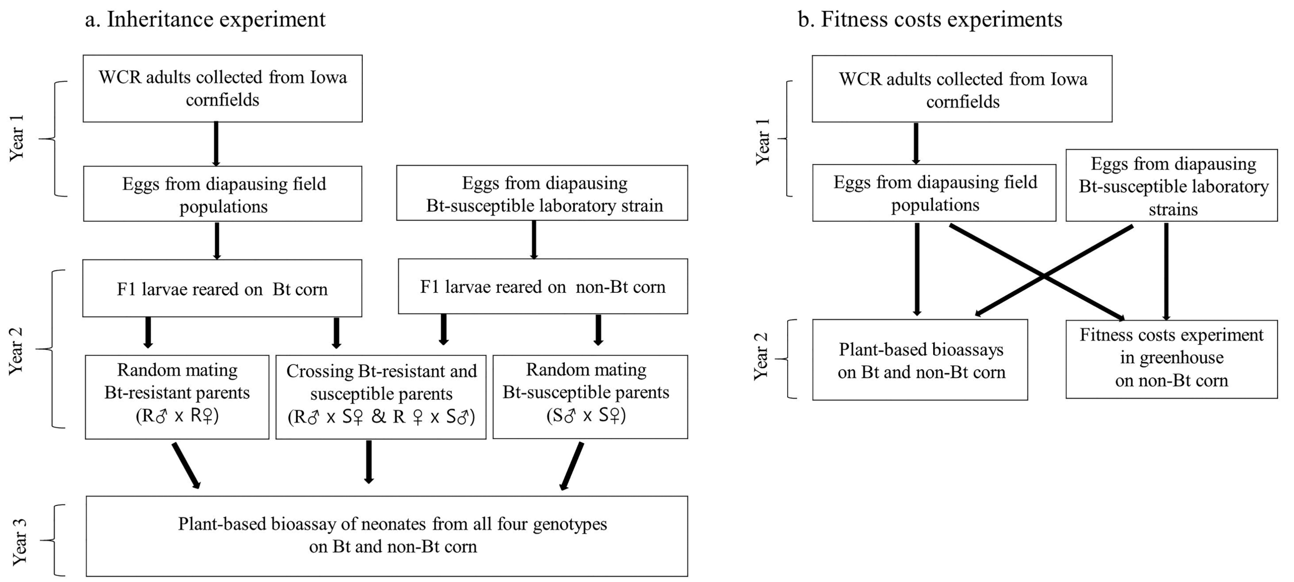

Fig. 1. Schematic for experiments. (a) Inheritance experiment and (b) Fitness cost experiment. WCR denotes western corn rootworm, R denotes Cry3Bb1-resistant, and S denotes Bt-susceptible.

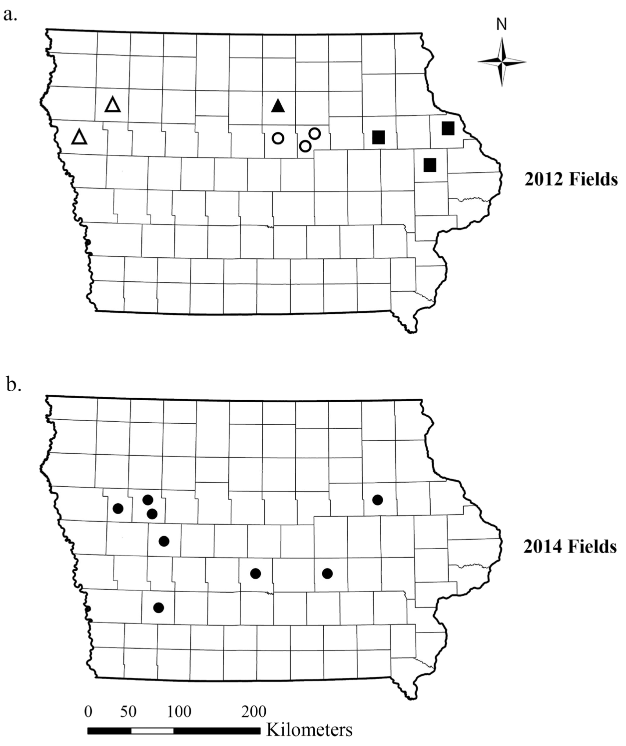

Fig. 2. Maps showing locations of western corn rootworm adult collection. (a) Collection sites for adults used for the testing inheritance of resistance to Cry3Bb1 corn. Symbols show which fields were sampled to generate each of the strains: Central Iowa (○), Eastern Iowa (■), Northern Iowa (▲), Western Iowa (Δ). (b) Collection sites for adults used for testing the fitness cost of resistance to Cry3Bb1 corn.

Horticulture, Vancouver, British Columbia, Canada) in a 2:3 ratio. Eggs mixed in 0.15% agar solution were added to small seedling mats at the rate of 950–2,000 eggs per small seedling mat. Twenty small seedling mats were prepared for each strain. Four days after preparing small seedling mats, 10 large seedling mats were prepared by adding 125 ml of corn seed, 500 ml of water, and 2,000 g of soil mix to a 5.7-liter flatbed plastic storage box with lid (Sterlite 1642, Sterlite Corporation, Townsend, MA). All seedling mats were stored in an incubator at 25°C and 16:8 (L:D) h cycle. The contents of two small seedling mats were transferred to one large seedling mat 14 d after the small seedling mats were prepared. Adults were collected three times a week, in 2–3 d interval, as they emerged from the large seedling mats.

Laboratory Crosses

Eggs from a Bt-susceptible DLS were received from the USDA-ARS North Central Agricultural Research Laboratory in Brookings, SD. The DLS originated from adults collected from cornfields in Moody County, South Dakota in 1987. This strain has never been exposed to Bt corn (Kim et al. 2007). The Bt-susceptible DLS was reared on non-Bt corn at the same time the field strains were reared on Cry3Bb1 corn. Forty small seedling mats and twenty large seedling mats were prepared weekly for 5 wk using a non-Bt corn hybrid that was the genetic isoline of the Cry3Bb1 hybrid used with the field strains (DKC 61–72 or DKC 62-61, Monsanto Co., St. Louis, MO).

Virgin adults from four Cry3Bb1-selected field strains were crossed with virgin adults of Bt-susceptible DLS strain from 29 January to 27 February 2014. Virgin females can mate within 24 h after emergence, males are unable to mate until 2–3 d after emerging from the soil (Quiring and Timmins 1990). Thus, newly emerged adults were collected within 2 h after adult emergence to ensure that insects had not mated. To accomplish this, all adults present inside the large seedling mats container were removed early in the morning and added into a parental cage, then newly emerged adults were collected every 2 h over a 6–8 h period. Each virgin adult was kept in a small plastic petri dish, and sex was determined by examining basitarsi of front leg under a stereo-microscope (MZ6, Leica, Microsystems, Wetzlar, Germany) (Hammack and French 2007). After the determination of sex, virgin males and females of each strain were kept in separate rearing cages.

Reciprocal crosses between four Cry3Bb1-resistant field strains and Bt-susceptible DLS were made by combining virgin males and females in a 1:1 ratio (25–132 male–female pairs per cross). Parental strains of four Cry3Bb1-resistant field strains and Bt-susceptible DLS strains were made by adding, to individual cages, all first-generation adults (366 to 19,663 adults per parental strain) that were not used in reciprocal crossing experiments. Rootworm adults were fed a complete adult diet and provided with a 2% agar solid in a petri dish as a water source. Eggs from each cross and parental strain (700 to 15,270 eggs per strain) were collected using an oviposition cup, which were prepared by adding a thick layer (2.5 cm) of finely sieved soil (<80 mesh particle size) in 0.5 l plastic cup (DeliPRO, TD40016, TRiPAK Industrial USA, LLC, White Plains, NY) and adding ~5 cm thick layer of soil clods (2–4 cm) on top. Eggs were stored in a cold room at 6°C to break diapause and the resulting larvae then used in plant-based bioassay.

Plant-Based Bioassays

The level of Cry3Bb1 resistance of four Cry3Bb1-resistant parental strains, one Bt-susceptible parental strain, and eight F1 hybrid strains was determined in plant-based bioassay from February to March in 2015. Rootworm strains were grouped into four sets based on the Cry3Bb1 resistant parental strain (Eastern Iowa, Central Iowa, Northern Iowa, and Western Iowa) and each set contained four strains (a resistant parental strain, a susceptible parental strain, and the two F1 hybrid strains generated by the reciprocal crossing of the parental strains (i.e., resistant ♂ × susceptible ♀; susceptible ♂ × resistant ♀). A set, with four strains, was evaluated every 2 wk. We tested all four strains per set except for Eastern Iowa set (one of the F1 hybrid strains, i.e., Eastern Iowa ♀ × DLS ♂, did not produce enough viable eggs for plant bioassays). Each strain was tested on Cry3Bb1 corn (DKC 6169, Monsanto Co., St. Louis, MO) and the non-Bt isoline to Cry3Bb1 corn (DKC 6172). Sixteen corn plants per corn hybrid were used for each strain when possible; however, for four of the genotypes within sets, fewer assays were conducted due to the limited number of neonates available for bioassay. The resistant parental strains for Northern Iowa and Central Iowa were tested only on nine plants per corn hybrid. The Eastern Iowa parental strain was tested on 13 corn plants per corn hybrid and a F1 hybrid strain, Central Iowa ♀ × DLS ♂, was tested on five Cry3Bb1 corn and four non-Bt isoline corn plants.

Plant-based bioassays were conducted following Gassmann et al. (2014). Corn plants were grown in a 1-liter clear plastic cup in the greenhouse for about a month to the V4–V5 stage (Abendroth et al. 2011). Twelve newly hatched neonate (<24 h olds) larvae were placed on the roots at the base of each plants, and plants with larvae were then held in a biological incubator (Percival, Perry, IA) at 24°C, 60% relative humidity, and 16:8 (L:D) h cycle for 17 d. After 17 d of feeding on corn roots, larvae were extracted using Berlese funnel in 15 ml glass vial, half-filled with 85% ethanol. Rootworm larvae were counted under a stereomicroscope (MZ6, Leica Microsystems, Wetzlar, Germany). The proportion of larval survival on each corn plant was calculated by dividing the number of larvae recovered by the number of larvae initially placed on that plant.

Fitness Cost Study

Fitness costs of Cry3Bb1 resistance in eight field populations of western corn rootworm were determined in a greenhouse experiment at Iowa State University from June to August 2015 (Fig.1b). Fitness costs were measured for four life-history traits: (1) survival to adulthood; (2) adult emergence time; (3) head capsule width; and (4) adult dry mass. These traits were selected because of their direct implication to field-evolved resistance. Low survival to adulthood, longer developmental time, and poor growth on non-Bt corn would decrease the overall fitness of resistant individuals in non-Bt refuges, thereby delaying resistance evolution.

Rootworm adults (*n* = 242.4 ± 116.1, Mean ± SD) were collected from eight randomly selected cornfields in seven counties in Iowa from 1 to 26 August 2014 (Fig. 2b). Adults from each field were kept in separate cages and fed on an artificial diet and fresh corn leaves. Rootworm eggs (13,312 ± 5,444, Mean ± SD) were collected from each population in a small petri dish filled with fine soil and stored in a cold room at 6°C. In addition, eggs of six Bt-susceptible DLSs were received from the USDA laboratory at Brookings, SD. The location and year of collection for each USDA strain was a follows: Moody Co. SD in 1987, York Co. NE in 1996, Butler Co. NE in 1999, Center Co. PA in 2000, Phelp Co. NE in 1995, and Potter Co. SD in 1995. These Bt-susceptible strains were collected from the field before Bt corn was commercialized (i.e., 2003) and were never exposed to Bt corn in the laboratory. The level of Cry3Bb1 resistance in each of these rootworm populations was determined with plant-based bioassays following Gassmann et al. (2014), as described previously. The bioassays were performed from 14 March to 22 August 2015. Each rootworm population was evaluated on 12 Cry3Bb1 corn plants and 12 non-Bt isoline corn plant. The proportion of larval survival on each corn plant was calculated by dividing the number of larvae recovered by the number of larvae initially placed on that plant.

A greenhouse experiment was performed in a completely randomized design with 14 western corn rootworm populations and 11 replications. Each population was tested on 11 non-Bt corn plants in the greenhouse. Non-Bt corn (Mycogen 2K591, Dow AgroSciences, Indianapolis, IN) plants were grown in a greenhouse to evaluate the rootworm populations. Non-Bt corn seeds were originally treated with thiamethoxam insecticide (CruiserMaxx 250, Syngenta Cop Protection Inc., Greensboro, NC), therefore, corn seeds were washed following Gassmann et al. (2011) before planting, to remove the insecticide from the seeds. A total of 165 plastic pots (diameter = 25 cm, height = 23 cm) were each filled with ca. 10 liter of crushed clay-loam soil collected from a soybean field in the summer of 2014. Seeds were planted on 5 June 2015. Plants were grown in a greenhouse for 89 d at 16:8 (L:D) h photoperiod. Plants were examined every day and irrigated with 250–600 ml of water as needed. Plants were fertilized with 200–400 ml of fertilizer solution once a week. Fertilizer solution was prepared by mixing 4 g of fertilizer (Peters Excel 15-5-15 cal-Mag, Scotts Company, Marysville, OH) per liter of water. Plants were randomized once a week before applying irrigation and fertilizer.

Thirty neonate larvae (<24 h old) were placed on each plant at the V6–V7 stage (31–34 d after planting). Eleven corn plants received larvae from each rootworm population. Immediately after larvae were placed on plants, pots were covered with fabric sleeves (46 cm height and 86 cm circumference, white chiffon fabric) which was secured around the pot with a rubber band, and around the plant stem with a thin plastic coated wire.

Rootworm adults emerged from each plant were collected three times a week in 2–3 d intervals and placed in microfuge tubes filled with 85% ethanol, and plants were checked for adults until no adults were found for 14 consecutive days. Survival to adulthood was calculated by dividing the total number of adults that emerged from a plant by 30, a total number of larvae infested in that plant. The sex of each adult was determined using a stereomicroscope (MZ6, Leica Microsystems, Wetzlar, Germany) based on basitarsi morphology (Hammack and French 2007). For each insect, the head capsule width (the outer most boundary of one compound eye to another) was measured under a stereomicroscope using a digital camera (Motic North America, British Columbia, Canada) with image analysis software (Motic Images Advanced V3.0 software; Motic North America, British Columbia, Canada). Males and females collected from each corn plant were kept in separate 2 ml microfuge tubes and dried in a convection oven (Model# 6530, Thermo Scientific, Waltham, MA) at 60°C for 48 h, with dry mass, to the nearest 0.001 mg, determined using an analytical balance (XS205 DU, Mettler-Toledo, LLC, Columbus, OH). Average mass of males and females per plant was calculated by dividing the total dry mass by the number of adults weighed.

Data Analysis

Inheritance Study

Data on larval survivorship in single-plant bioassays were transformed with the square root function to improve homoscedasticity in the data. Then, an mixed-model analysis of variance (ANOVA) was performed (PROC MIX) using SAS V9.4 (SAS Institute 2015). Genotypes of the rootworm, corn hybrids, and their interaction were fixed-effects, while rootworm strains nested within genotypes and the interaction between corn hybrids and rootworm strain nested within genotypes were random effects (Table 1). The significance of each random factor in the model was determined based on −2 Res loglikelihood statistic following Littell (et al. 1996). Random factors with a significance of $P > 0.25$ were pooled to increase statistical power (Quinn and Keough 2002). However, random factors were retained in the model if their higher-order interactions were significant.

Analysis of variance revealed a significant interaction between the corn hybrid and rootworm genotype; therefore, several linear contrasts were conducted to understand the nature of this interaction. First, we compared survival of the four genotypes on non-Bt corn to determine whether differences in survival in bioassays, not related to the presence of Bt, were present. Second, survivorship of F1 hybrids on Cry3Bb1 corn was compared with test for sex-linked effects, and since no significant difference ($P = 0.52$) was found, F1 hybrids were pooled in subsequent analyses. Third, we compared larval survival on Cry3Bb1 corn between susceptible parent versus F1 hybrid, with significantly greater survival for F1 hybrids versus susceptible insects indicating non-recessive inheritance of resistance. Fourth, we compared larval survival on Cry3Bb1 corn for the resistant parent versus F1 hybrids, with significantly great larval survival for the resistant parent indicating non-dominant inheritance of resistance.

For each set of strains, corrected larval survivorship was calculated as the complement of corrected mortality based on Abbott (1925), with proportion survival on Cry3Bb1 plant divided by the average survival on the non-Bt near isoline. Within each set of strains, corrected survival data were transformed using square root function and analyzed with an ANOVA (PROC GLM). Because the same susceptible strain was tested with each set of rootworm strains, bioassay data for the susceptible strain were pooled into a single dataset to increase the statistical power. For each set of rootworm strains, three comparisons were made (CONTRAST statements in PROC GLM) with a critical value of $P > 0.017$ based on a Bonferroni correction for three pairwise comparisons. Comparisons, which were the same as those made for the overall analysis of survival described previously, were as follows: (1) between two F1 hybrid strains; (2) susceptible parent versus pooled F1 hybrids; and (3) resistant parent versus pooled F1 hybrids, with F1 hybrid strains pooled because no significant differences were detected ($P > 0.30$ in all cases).

Table 1. Analysis of variance of the larval survivorship in plant-based bioassays

Fixed effects	df	*f*	*P*
Genotypes	3, 11	3.54	0.05
Corn hybrids	1, 11	64.77	<0.01
Genotypes × Corn hybrids	3, 11	3.76	0.04
Random effects	df	χ^2	*P*
Strains (Genotypes)	1	0.00	0.50
Strains × Corn hybrids (Genotypes)	1	14.50	<0.01

The dominance of Cry3Bb1 resistance (h) was calculated based on corrected larval survival on Cry3Bb1 corn with each set of strains as: h = (F1 hybrids-susceptible) ÷ (resistant-susceptible), where dominance value = 0 means recessive, 1 means dominant, and values between 0 and 1 means non-recessive inheritance (Liu and Tabashnik 1997, Tabashnik et al. 2004). We performed a *t*-test to evaluate the hypothesis that the average dominance of Cry3Bb1 resistance among field strains was equal to 0 or 1 (Sokal and Rohlf 1995). Resistance ratios were calculated by dividing corrected survival of each resistant strain by the overall corrected survival of the susceptible strain.

Fitness Cost Study

The level of Cry3Bb1 resistance in each rootworm population was determined by calculating corrected larval survivorship on Cry3Bb1 corn then one-tailed *t*-tests (PROC TTEST) were performed to determine whether the corrected larval survival of field populations were higher than Bt-susceptible laboratory strains. Fitness costs of Cry3Bb1 resistance were evaluated by regressing values for the various life-history traits (i.e., survival to adulthood, adult emergence time, dry mass, and head capsule width of adults) onto corrected survival (PROC REG). A regression analysis was performed separately for males and females for each life-history parameters. The null hypothesis in all cases was that slope of a regression line was equal to zero and the alternative hypothesis was that there was a negative relationship for survival, head-capsule width and mass and a positive relationship with developmental rate ($P < 0.05$ based on a one-tailed test).

Results

Inheritance of Resistance to Cry3Bb1 Corn

Analysis of variance for larval survivorship of rootworm strains provided evidence for non-recessive inheritance of Cry3Bb1 resistance. The ANOVA showed a significant interaction between rootworm genotype and corn hybrid (Table 1). Larval survival on non-Bt corn was similar among four rootworm genotypes suggesting all four genotypes performed similarly in the plant-based bioassay ($P > 0.67$ in all cases). There was no significant difference between larval survival of two F1 hybrids on Cry3Bb1 corn (df =1, 11; $F = 0.44$; $P = 0.52$) (Fig. 3). The similar larval survival on Cry3Bb1 corn for both reciprocal crosses indicated that Cry3Bb1 resistance is an autosomal trait (i.e., not a sex-linked trait). Therefore, we pooled the larval survival data from two types of F1 hybrids for further comparisons. Larval survival of susceptible parents was significantly lower than the F1 hybrids (df = 1, 9; $F = 5.27$; $P = 0.05$) suggesting non-recessive inheritance of resistance. We also found significantly higher larval survival on Cry3Bb1 corn for a resistant parent than F1 hybrids (df = 1, 9; $F = 6.81$; $P = 0.03$) indicating the inheritance of Cry3Bb1 resistance was not dominant.

For the analysis of variance with each set of rootworm strains, there were no significant differences between corrected larval survival of two F1 hybrid strains for all sets of rootworm strains ($P >$ 0.31 in all cases). Therefore, corrected survivorship of both F1 hybrid strains was combined into a single F1 strain to estimate the dominance of resistance to Cry3Bb1 corn (Table 2). In all but one instance (i.e., Western Iowa strain), we found that survival of the F1 hybrids was significantly greater than the susceptible parent, indicating non-recessive inheritance. In the case of the Northern Iowa strain, survival of the F1 hybrid did not differ from the resistant strain, but this was likely due to the large standard error associated with corrected survival of the resistant strain (Table 2). A one-tailed *t*-test showed that average dominance value for field strains (0.34 ± 0.22, Mean ± SD) was significantly greater than zero (df = 3, t = 3.07, P = 0.05) and significantly smaller than one (df = 3, t = −6.02, P < 0.01) suggesting, that in general, inheritance of Cry3Bb1 resistance in western corn rootworm was non-recessive. The resistance ratio of field strains ranged from 11.0 to 19.2 (Table 2).

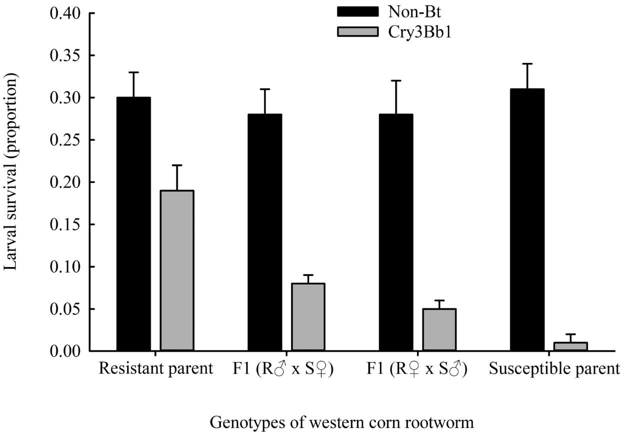

Fig. 3. Survivorship of larvae on Cry3Bb1 corn and non-Bt isoline corn in a plant-based bioassay. Bar heights represent sample means and error bars the standard error of the mean. Larval survival on non-Bt corn did not differ among genotypes (P > 0.67 in all cases), and there was no significant difference between F1 hybrids on Cry3Bb1 corn (df =1, 11; F = 0.44; P = 0.52). F1 hybrids displayed significantly higher survival on Cry3Bb1 corn compared with susceptible insects (df = 1, 9; F = 5.27; P = 0.05) but significantly lower survival than resistant insects (df = 1, 9; F = 6.81; P = 0.03).

Fitness Costs of Resistance to Cry3Bb1 Corn

Rootworm populations tested for fitness costs revealed a wide range of resistance to Cry3Bb1 corn. The average corrected survival of larva on Cry3Bb1 corn for field populations ranged from 0.56 to 1.28; however, it ranged from 0.00 to 0.05 in laboratory populations (Fig. 4). The one-tailed *t*-tests of corrected larval survival on Cry3Bb1 corn revealed that corrected larval survival of field populations on Cry3Bb1 corn were significantly higher (P < 0.01 in all cases) than the average corrected larval survival (0.02) of susceptible laboratory populations, suggesting all field populations tested in this experiments were resistant to Cry3Bb1 corn. Regression between corrected larval survival on Cry3Bb1 corn (i.e., the indicator of level of Cry3Bb1 resistance) and survival to adulthood, adult emergence time and dry mass of adults revealed no significant relationship for either males or females (P > 0.15 for all cases) suggesting there were no fitness costs of resistance to Cry3Bb1 for these life-history traits (Fig. 5). However, we found the significant negative relationship between corrected larval survival on Cry3Bb1 corn and head capsule width of females (slope = −0.041, df = 1, t = −4.99, P < 0.01) (Fig. 5g) and males (slope = −0.020, df = 1, t = −2.05, P = 0.03) (Fig. 5h). This indicates the presence of fitness cost of Cry3Bb1 resistance affecting adult size.

Discussions

In this study, we determined the inheritance and fitness cost of Cry3Bb1 resistance for field strains of western corn rootworm in Iowa. The reciprocal crossing experiment showed that the inheritance of Cry3Bb1 resistance in the field strains was mostly non-recessive and autosomal; however, there was some variation in the level of dominance (0.08 to 0.54). We did not find fitness costs of Cry3Bb1-resistance by western corn rootworm affecting survival to adulthood, adult emergence time or dry mass of adults. However, there was a fitness costs of Cry3Bb1 resistance affecting the head capsule width of adults. The presence of non-recessive inheritance of resistance and minimal fitness costs accompanying resistance is consistent with past research, and both of these factors have likely contributed to the rapid evolution of resistance in the field (Ingber and Gassmann 2015, Gassmann 2016, Paolino and Gassmann 2017).

Many biological and environmental factors contribute to the rate of field-evolved resistance to Bt crops. However, the survival of F1 hybrids on Bt crops, which correspond to the dominance of resistance, is a key factor (Tabashnik et al. 2004). Our results on the inheritance of Cry3Bb1 resistance were similar to the findings of past studies, which reported non-recessive inheritance

Table 2. Dominance of Cry3Bb1 resistance in field strains of western corn rootworm

	Corrected larval survival on Cry3Bb1 corn[a]					
Sets of rootworm strains	Res.[b]	R♂×S♀	R♀×S♂	Sus.[c]	h[d]	RR[e]
Central Iowa	0.66* (0.24)	0.19 (0.05)	0.17 (0.12)	0.05* (0.02)	0.23	13.2
Eastern Iowa	0.55* (0.10)	0.30 (0.07)		0.05* (0.02)	0.50	11.0
Northern Iowa	0.96 (0.42)	0.59 (0.11)	0.48 (0.13)	0.05* (0.02)	0.54	19.2
Western Iowa	0.68* (0.16)	0.12 (0.08)	0.08 (0.03)	0.05 (0.02)	0.08	13.6

[a] Mean value with standard error given in parentheses.

[b] Larval survival of resistant parents followed by an asterisk were significantly higher than F1 hybrids.

[c] Larval survival of susceptible parents followed by an asterisk were significantly lower than F1 hybrids.

[d] Dominance of Cry3Bb1 resistance, h = (Pooled F1 hybrids-Susceptible) ÷ (Resistant-Susceptible).

[e] RR denotes resistant ratio, which is the larval survivorship of resistant parents divided by average larval survivorship for susceptible parents.

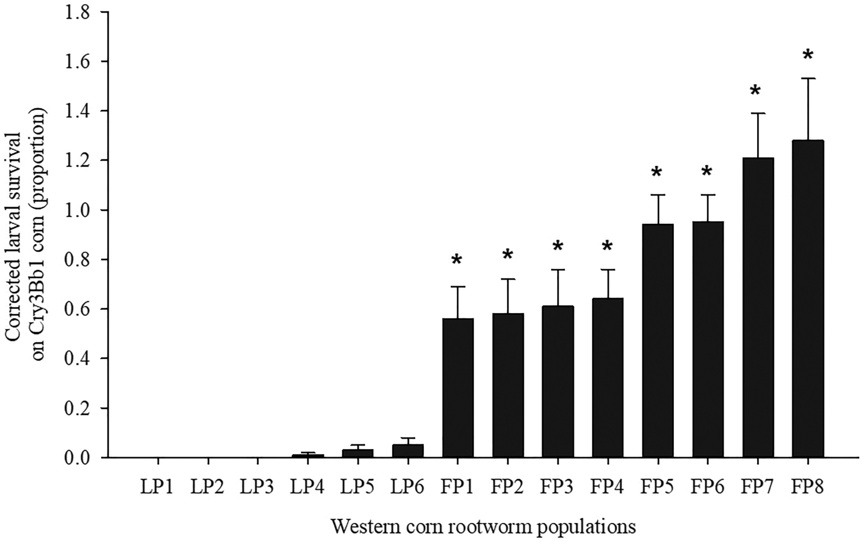

Fig. 4. Corrected larval survival on Cry3Bb1 corn for western corn rootworm populations evaluated for fitness costs of resistance to Cry3Bb1 corn. LP denotes laboratory populations and FP denotes field populations. Bar heights represent sample means and error bars represent the standard error of the mean. The asterisk above a bar indicates significantly higher corrected larval survival on Cry3Bb1 corn for a field population compared to the average corrected larval survival of a laboratory control population (i.e., > 0.02).

of Cry3Bb1 resistance in strains of western corn rootworm with laboratory-selected resistance (Meihls et al. 2008, Petzold-Maxwell et al. 2012, Geisert et al. 2016) and field-evolved resistance (Ingber and Gassmann 2015, Paolino and Gassmann 2017). Survival of F1 hybrids on a Bt crop is directly related to the number of generations needed for a population to evolve resistance, with the rate of resistance evolution displaying a positive relationship with dominance (Roush 1997). In a computer-simulation study of six lepidopteran pest species, Tabashnik et al. (2008) found that the dominance of resistance was the primary factor determining the rate of resistance evolution. Thus, the presence of non-recessive inheritance of resistance to Cry3Bb1 corn was likely an important factor facilitating field-evolved resistance by western corn rootworm.

When fitness costs of resistance occur, this can delay the evolution of Bt resistance when non-Bt refuges are present (Gassmann et al. 2009). Among the several life-history characteristics measured, a fitness cost was only detected for adult size (Fig. 5). This suggests that fitness costs of Cry3Bb1 resistance in western corn rootworm are present but may be minimal, which is consistent with studies of laboratory strains with either laboratory-selected resistance or field-evolved resistance. In some cases, studies with western corn rootworm stains with laboratory-selected resistance did not detect fitness costs (Oswald et al. 2012, Petzold-Maxwell et al. 2012, Hoffmann et al. 2014, Geisert and Hibbard 2016). However, in other cases, evidence of costs was detected (Meihls et al. 2012, Hoffmann et al. 2015). Similarly, in studies of laboratory strains with field-evolved Cry3Bb1 resistance, fitness costs were absent for two strains (Hopkinton and Monona) but were present in two other strains (Cresco and Elma) (Ingber and Gassmann 2015, Paolino and Gassmann 2017). The fitness cost affecting size in this study may translate to differences in fecundity because larger females rootworm tend to have higher fecundity (French et al. 2015). To the extent that fitness costs of Cry3Bb1 resistance may be present, it appears that the effect of fitness costs on delaying resistance was outweighed by factors favoring the evolution of resistance including non-resessive inheritance of resistance, wide-spread planting of Bt crops, and a lack of compliance in planting of refuges (Gassmann et al. 2011, Dunbar et al. 2016).

Comparison of F1 hybrids in this study (i.e., resistant males crossed with susceptible females versus susceptible males crossed with resistant females), did not detect a difference in survival on Cry3Bb1 corn, indicating an absence of maternal effects or sex-linked resistance. Studies of western corn rootworm resistance with laboratory-selected resistance to eCry3.1Ab and Cry3Bb1 have also reported an absence of sex linkage (Meihls et al. 2008, Geisert et al. 2016). However, Petzold-Maxwell et al. (2012), also studying a strain with laboratory-selected Cry3Bb1, found evidence of sex linkage, with a paternal effect of higher Cry3Bb1 resistance. Similarly, a paternal influence of Cry3Bb1 resistance was found for one laboratory strain with field-evolved Cry3Bb1 resistance, although no evidence of sex linkage was found for three other cases (Ingber and Gassmann 2015, Paolino and Gassmann 2017). The presence of a paternal influence on Cry3Bb1 resistance in some strains but not others, suggests the presence of multiple mechanisms of resistance among strains.

Resistance ratios of field populations tested in this study ranged from 11 to 19 (Table 2) and plant-based bioassays of field populations with Cry3Bb1 resistance have reported similar resistance ratios (Gassmann et al. 2011, 2012, 2014). Because Bt corn targeting corn rootworm lacks a high dose, and susceptible western corn rootworm can survive on Bt corn, the modest resistance ratios observed here and elsewhere are sufficient for pest to achieve equivalent survival on Bt corn compared with non-Bt corn, and to impose substantial injury to Bt corn in the field (Gassmann 2012, 2016). This is in contrast to high-dose Bt crops where survival of susceptible individuals is absent or rare (Tabashnik and Carriere 2017). Resistance to high-dose Bt events is often accompanied by loss of functions mutations, for example, truncation of cadherin proteins, which in turn results in traits that are recessive (Bortoli and Jurat-Fuentes 2019). For western corn rootworm, the lack of high dose may enable gain of function traits (such as toxin sequestration) or down-regulation of genes that produce toxin binding sites to confer resistance (Xiao and Wu 2019). The modest resistance ratios accompanying field-evolved resistance in western corn rootworm likely also influence the minimal fitness costs observed here and elsewhere, because magnitude of fitness costs tend to be

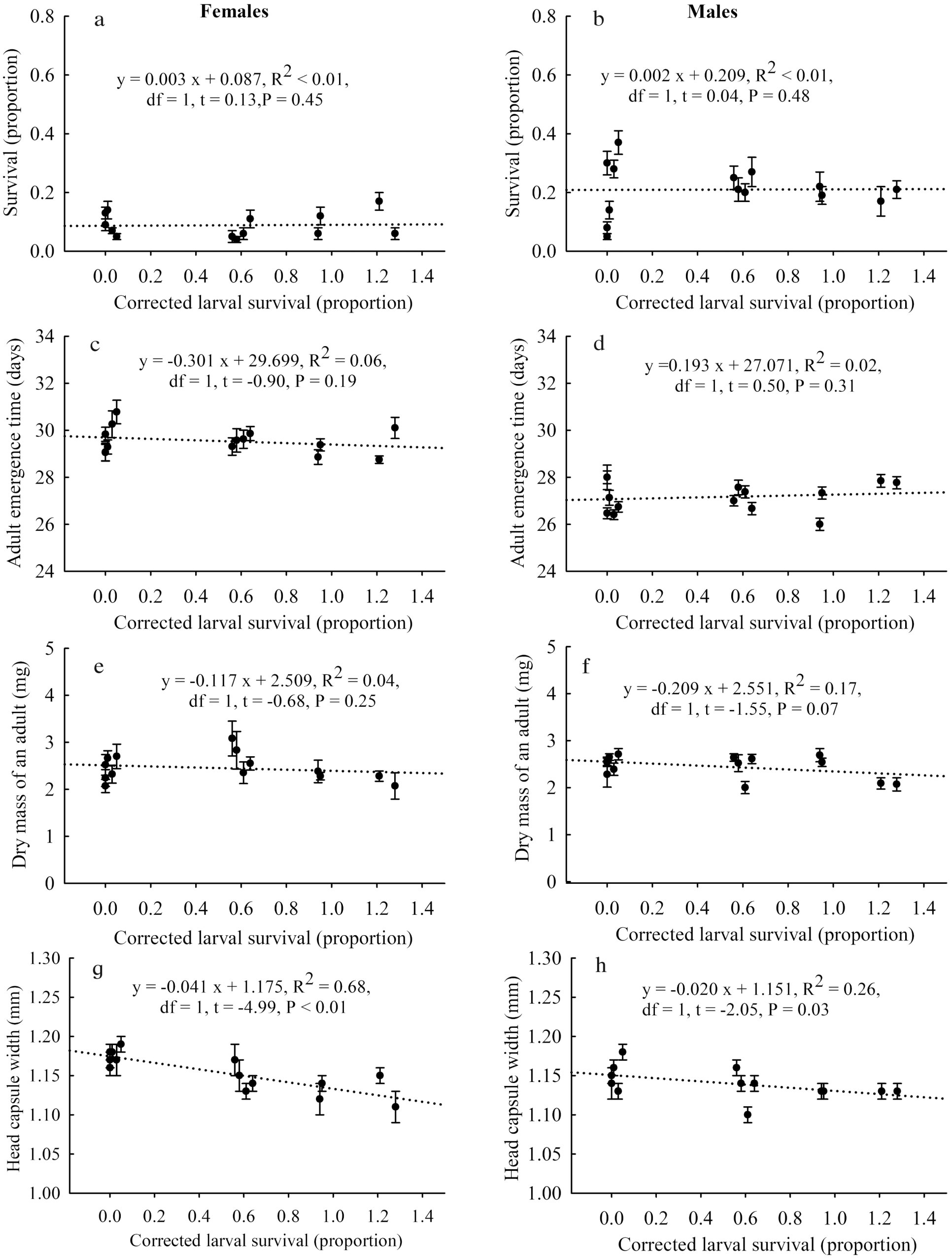

Fig. 5. Relationships between the corrected larval survival on Cry3Bb1 corn and various life-history traits: (a) survival to adulthood for females, (b) survival to adulthood for males, (c) emergence time for females, (d) emergence time for males, (e) dry mass of females, (f) dry mass of males, (g) head capsule width of females, and (g) head capsule width of males.

positively associated with the magnitude of resistance (Gassmann et al. 2009). In sum, the lack of a high dose by Cry3Bb1 corn, and other types of Bt corn, targeting western corn rootworm likely facilitated the evolution of resistance by enabling small increases in resistance ratios to substantially increase survival and crop injury in the field, and by leading to the evolution of resistance traits that had minimal accompanying fitness costs.

Both non-recessive inheritance of resistance and minimal fitness costs highlight the vulnerability of Cry3Bb1 corn to the evolution of resistance by western corn rootworm. Currently, Cry3Bb1-resistant western corn rootworm are found throughout the US Corn Belt (Gassmann et al. 2011, 2012, 2014; Wangila 2015; Zukoff 2016; Schrader 2017), although the prevalence within the agricultural landscape varies from state to state (Reinders et al. 2018, Shrestha et al. 2018). Because of Cry3Bb1 resistance and cross-resistance with other Bt traits, Cry34/35Ab1 now plays a key role in the management of corn rootworm; however, recent cases of Cry34/35Ab1 resistance raise concerns about the long-term efficacy of this trait (Jakka et al. 2016, Gassmann et al. 2020). The use of more diversified management will be important for preserving the utility of Cry34/35Ab1 and future transgenic technologies for the management of western corn rootworm (Cullen et al. 2013). Additionally, more proactive resistance management in response to suspected resistance should help to delay resistance (Andow et al. 2016).

Acknowledgments

We thank Wade French, USDA ARS, Brookings, SD, for providing eggs of Bt-susceptible western corn rootworm strains. We also thank

Mike Dunbar, Siva Jakka, and John Doudna for helping with the collection of adults from field populations and Patrick Weber, Benjamin Brenizer, Joseph Metz, Aaron Johnson, Basanta Woli, and Selah Zaldarriaga for their help in running experiments.

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