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Fitness costs of resistance to Cry3Bb1 maize by western corn rootworm

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Abstract

Crops producing toxins derived from the bacterium *Bacillus thuringiensis* (Bt) are widely planted to manage insect pests including western corn rootworm, *Diabrotica virgifera virgifera* LeConte (Coleoptera: Chrysomelidae), which is a significant pest of maize in the United States and Europe. However, the widespread use of Bt maize places intense selection pressure on pest populations to evolve resistance, and field-evolved resistance to Bt maize by western corn rootworm has been documented in the United States. In conjunction with non-Bt refuges, fitness costs of Bt resistance can delay resistance evolution. Fitness costs arise in the absence of Bt toxin when individuals with resistance alleles have lower fitness than Bt-susceptible genotypes. We quantified the level of resistance and fitness costs of resistance for a strain of western corn rootworm with laboratory-selected resistance to transgenic maize (*Zea mays* L.) producing Bt toxin Cry3Bb1. Survival to adulthood on Cry3Bb1 maize was more than two-fold higher for resistant insects vs. susceptible insects, which is similar to the magnitude of resistance first observed in the field. Fitness costs were measured in two experiments; the first used maize hybrids and the second used inbred lines. The experiment with maize hybrids compared resistant and susceptible strains while the experiment with maize inbreds compared resistant, susceptible and heterozygous genotypes. The only non-recessive fitness cost detected (i.e. cost affecting heterozygotes) was for adult size. Recessive fitness costs (i.e. costs affecting the resistant strain) were observed for developmental rate, female survival and egg viability. However, when reared on non-Bt maize, the resistant strain also displayed higher fecundity, higher survival for males and greater adult longevity compared to the susceptible strain. These results suggest that resistance to Bt maize by western corn rootworm may not impose substantial fitness costs, and consequently, may evolve quickly and persist once present.

Introduction

In 2013, more than 75 million hectares were planted worldwide to genetically modified crops that produced insecticidal toxins derived from the bacterium *Bacillus thuringiensis* (Bt) (James 2013). The vast majority of this area was devoted to Bt maize and Bt cotton. In 2003, genetically modified maize producing the insecticidal Bt toxin Cry3Bb1 was commercially registered by the United States Environmental Protection Agency for management of larval western corn rootworm *Diabrotica virgifera virgifera* LeConte (Coleoptera: Chrysomelidae) (EPA 2010). Western corn rootworm is a key pest of maize in the United States and annually imposes yield losses and management costs in excess of one billion US dollars (Gray et al. 2009). Furthermore, this pest has developed resistance to several pest management strategies including conventional insecticides (Meinke et al. 1998), crop rotation (Levine and Oloumi-Sadeghi 1991) and Bt toxins Cry3Bb1 and mCry3A in transgenic maize (Gassmann et al. 2011, 2012, 2014; Gassmann 2012).

In the United States and elsewhere, the refuge strategy is used to delay the evolution of resistance to Bt crops. This strategy consists of growing non-Bt host plants in close proximity to Bt crops to permit the survival of Bt-susceptible insects that may then mate with resistant insects surviving on a Bt crop (Gould 1998; Carriére and Tabashnik 2001). Mating between susceptible individuals from refuges and Bt-resistant insects from Bt fields generates heterozygous progeny. To the extent that heterozygous genotypes have lower fitness on Bt plants than homozygous-resistant individuals, delays in resistance evolution may be achieved (Tabashnik et al. 2008). However, over time, the accumulation of resistance alleles within refuge populations disrupts this dynamic, leading to a rapid increase in the rate of resistance evolution (Comins 1977). A fitness cost of Bt resistance occurs, in the absence of Bt toxin, when individuals with Bt-resistance alleles have lower fitness than homozygous susceptible genotypes (Gassmann et al. 2009). Fitness costs of Bt resistance remove resistance alleles from refuge populations and, as a result, can delay the evolution of Bt resistance. Additionally, fitness costs directly affect how quickly resistance traits are lost from a population once selection is removed, with more costly resistance traits decreasing more rapidly in the absence of selection for resistance (Gassmann et al. 2009).

Fitness costs may affect a variety of life-history traits and may be affected by ecological factors such as larval host plants and exposure to entomopathogens, which may alter both the magnitude and dominance of fitness costs (Gassmann et al. 2009). Recessive fitness costs affect only homozygous-resistant genotypes while non-recessive fitness costs are manifest as a reduction in fitness for both homozygous resistant and heterozygous genotypes. Delays in resistance evolution caused by fitness costs increase as the magnitude and dominance of fitness costs increase (Carriére and Tabashnik 2001). Thus, understanding whether Bt-resistant traits may be accompanied by fitness costs, and the potential variation in the magnitude and dominance of costs, is important for assessing the risk of a pest developing resistance to a Bt crop.

In this study, we quantified the level of resistance and fitness costs of resistance for a laboratory-selected strain of western corn rootworm with resistance to Cry3Bb1 maize (Oswald et al. 2011). Resistance was quantified by measuring survival to adulthood with seedling mats of Cry3Bb1 maize and non-Bt maize held in a growth chamber, in a manner similar to other commonly applied seedling-mat-based assays to measure resistance of western corn rootworm to Bt maize (Nowatzki et al. 2002; Oswald et al. 2011; Frank et al. 2013). Fitness costs were evaluated by comparing the resistant strain to the susceptible strain in the absence of Bt maize, and this was done in two experiments, the first with three commercial non-Bt maize hybrids and the second with three non-Bt inbred lines of maize. In these fitness cost experiments, data on larval life-history variables were collected for larvae reared on single plants in the greenhouse and data on adult life-history variables were collected for adults held in environmental chambers. Data from these experiments are important for understanding the risks associated with the recent field-evolved resistance to Cry3Bb1 maize by western corn rootworm (Gassmann et al. 2014). The results of this work provide an understanding of the potential for additional cases of Bt resistance to evolve and for resistance traits to persist once selected in the field.

Materials and Methods

Insect strains

As detailed in Oswald et al. (2011), adult male western corn rootworm were collected from four locations in the United States and crossed with females from a non-diapausing strain. Two strains were then generated from this initial strain: (i) a susceptible strain that was not exposed to Bt maize and (ii) a resistant strain, the moderately selected strain in Oswald et al. (2011), that was fed Cry3Bb1 maize for an increasing duration over 11 generations (F0 to F10), resulting in significantly greater survival on Cry3Bb1 maize compared to the susceptible strain (Oswald et al. 2011).

These strains were sent to Iowa State University in the F13. Larvae were reared on maize seedling mats following Oswald et al. (2011), with strains maintained at a population size of 1200 adults per generation. Both strains were reared on non-Bt maize (Pioneer hybrid 34M94; DuPont-Pioneer, Johnston, IA). To increase the genetic similarity between strains, adults of the resistant strain were backcrossed to the susceptible strain in a 1 : 1 ratio, allowed to mate at random for one generation, and then selected on Cry3Bb1 maize (DeKalb hybrid DKC 61-69; Monsanto Company, St. Louis, MO). This occurred at the F13 and F15, for a total of two backcrosses followed by selection. The resistant strain was then selected a third and final time on Cry3Bb1 maize during the F17, after which it was used in experiments to measure fitness costs and to quantify resistance.

Assessing fitness costs on maize hybrids

This experiment was conducted from February to August of 2011 and used the F18 of the resistant and susceptible strains. The experiment was a fully crossed design with two western corn rootworm strains (resistant and susceptible) and three commercial maize hybrids: 36R19cn (Blue River Hybrids, Kelley, IA), 2T783 (Mycogen Seeds; Dow AgroSciences, Indianapolis, IN) and DKC 61-72 (DeKalb; Monsanto Company). None of the maize hybrids produced Bt toxins targeting rootworm, and any seed treatments were removed following Gassmann et al. (2011).

Maize was grown in a greenhouse (29°C; 16 : 8; L : D) with supplemental lighting (400 W high-pressure sodium bulbs; Ruud Lighting Inc., Racine, WI). Each plant was held in a 2-l plastic container (product number 61086; Encore Plastics Corporation, Pittsburg, PA) with all drainage holes covered by fine mesh to prevent escape of larvae. Containers were filled with 2 l of a soil mixture consisting of 20% field-collected soil, 40% SB300 potting soil (Sun Gro Horticulture Ltd., Vancouver, BC, Canada) and 40% LC1 potting soil (Sun Gro Horticulture Ltd.). Beginning 2 weeks after seeds were planted, plants were fertilized weekly with 250 ml of Peters Excel 15-5-15 Cal-Mag Special (Everris International, Geldermalsen, the Netherlands) at 4 mg per ml. Three weeks after planting, 25 neonate larvae were placed at the base of each plant. During the experiment, plants were watered as needed and containers were kept free from weeds. Sixteen containers were used for each combination of insect strain by maize hybrid, for a total of 96 containers.

One week after larvae were introduced, a covering of mesh fabric was fastened between the top of each container and the base of each plant, capturing all adult western corn rootworm that emerged from the soil. Plants were checked every other day and adults collected with a hand-held aspirator. Adults were then anesthetized with carbon dioxide, and sex determined following Hammack and French (2007). Plants were checked until no adults were collected for two consecutive weeks.

All adults, both males and females, collected on the same day, and from the same combination of insect strain and maize hybrid, were placed in a small screen cage (18 cm × 18 cm × 18 cm L × H × W; Megaview Science, Taichung, Taiwan) and held in a growth chamber (25°C; 16 : 8 L : D; 65% RH) for 6 days. This period of time allowed most males to reach sexual maturity (females are sexually mature upon emerging from soil) and begin mating (Spencer et al. 2009). After adults were held *en masse* in screen cages, mated pairs (i.e. one male and one female) were established. Mated pairs of western corn rootworm adults were held in a 0.5–l clear plastic container (product number RD 16; Johnson Paper and Supply Co. Minneapolis, MN) covered with a lid that provided ventilation through an opening (diameter = 7 cm) covered with fine mesh fabric. A 1.5% agar block was provided as a source of water, and food was provide with a complete adult diet (western corn rootworm diet; Bio-Serv, Frenchtown, NJ) that also was supplemented with young maize leaf. Each mated pair was given an oviposition substrate of sieved soil (particle size < 180 μm) held in a Petri dish (diameter = 4 cm) that was replaced every 7 days until the female died. For each combination of insect strain by maize hybrid, 30 mated pairs were established, for a total of 180 mated pairs in this experiment.

Eggs were washed from soil with a 250-μm sieve and counted using a microscope (MZ6; Leica Microsystems, Wetzlar, Germany). For each mated pair, egg viability was measured from a random sample of 25 eggs collected 2–3 weeks after a pair was established. To measure viability, eggs were placed on 1.5% agar in a Petri dish (diameter = 5 cm) and held in a growth chamber (25°C; 16 : 8 L : D; 65% RH). Petri dishes were checked daily for newly hatched neonate larvae, which were counted and then removed from the Petri dish.

Adult longevity was measured for all adults in mated pairs, and for a random sample of 16 males and 16 females in each combination of insect strain by maize hybrid, head capsule width was measured from eye to eye using a microscope with a digital camera (Moticam 2500; Motic, Richmond, BC, Canada) and image analysis software (Motic Images Plus 2.0; Motic).

Assessing fitness costs on maize inbreds

This experiment was conducted from June to November, 2011 and used the F20 of the resistant and susceptible strains. Three inbred maize lines were studied: CRW8-1, NGSDCRW-1 and PHZ51 (Nelson et al. 2008). CRW8-1 and NGSDCRW-1 have tolerance to larval feeding by western corn rootworm (Russell et al. 1976; Kahler et al. 1985). These maize lines were included in this experiment because they may be useful in future management of this pest. The experiment was a fully crossed design with three genotypes of western corn rootworm (resistant, susceptible and heterozygotes) and the three inbred maize lines (CRW8-1, NGSDCRW-1 and PHZ51). For each combination of insect genotype by maize inbred, 14 containers were established, for a total of 126 containers.

Four population cages were established to produce three insect genotypes: resistant (resistant ♂ × resistant ♀), susceptible (susceptible ♂ × susceptible ♀) and heterozygote (resistant ♂ × susceptible ♀) and (resistant ♀ × susceptible ♂). The three insect genotypes studied here were from the same population cages as those studied in Petzold-Maxwell et al. (2012). Virgin females and males were obtained by collecting newly emerged adults that were <5 h old (Ball 1957; Hill 1975) and sex was determined following Hammack and French (2007). A 1 : 1 ratio of males and females was placed in cages, with adults added over 10 weeks to maintain a population of ca. 1000.

Maize was grown in the greenhouse following the same methods used in the experiment with hybrids. Twenty-four neonate larvae were placed at the base of each plant with only one insect genotype placed on each plant. For plants receiving heterozygotes, 12 larvae from each of the two heterozygous crosses were placed on a plant. Adults were collected following the same methods used in the experiment with hybrids. Due to a low number of insects surviving to adulthood, insects were pooled within each week and held in cages for 4–8 days before establishing mated pairs. The number of mated pairs for each combination of insect genotype by maize inbred was as follows: CRW8-1, susceptible = 7, resistant = 5 and heterozygotes = 8; NGSDCRW-1, susceptible = 8, resistant = 1 and heterozygotes = 6; PHZ51, susceptible = 8, resistant = 7 and heterozygotes = 6. Mated pairs were established and data collected following the same methods used in the experiment with hybrids. However, egg viability was not measured because of fungal contamination.

Quantifying resistance to Cry3Bb1

This experiment was conducted from May to June of 2012 and used the F24 of resistant and susceptible strains. Bioassays were run using seedling mats of Cry3Bb1 maize (DeKalb hybrid DKC 61-69) and non-Bt maize (DeKalb hybrid DKC 61-72), which were near genetic isolines. Twelve small seedling mats were established for each combination of maize type (Bt vs. non-Bt) and insect strain (resistant vs. susceptible), for a total of 12 replicates per treatment and 48 seedling mats for the entire experiment. Small seedling mats were made in 0.95–l clear plastic containers (Pactiv Showcase®; Johnson Paper and Supply Co.) by combining 60 ml of water with 40 ml (~65 kernels) of maize seeds that had been pre-soaked in deionized water for 12 h. Seeds and water were then covered with 200 g of a soil mixture that was 40% field-collected soil and 60% LC1 potting soil. Containers were covered with mesh fabric and a ventilated lid, and placed in a growth chamber (25°C; 16 : 8 L : D; 65% RH) for 7 days, after which time, 50 neonate larvae were placed onto each seedling mat.

After 7 days, larvae were transferred to larger seedling mats. These larger seedling mats were produced by placing 150 ml of maize seeds, soaked in deionized water for 12 h, into a plastic container (21 cm × 27 cm × 10 cm; 1642N3; Rubbermaid, Fairlawn, OH) and then covering the seed with 1200 g of soil. Larger seedling mats were moistened with 400 ml of water and held in a growth chamber for 1 week, after which larvae were transferred by removing a small seedling mat from its container and then placing each small seedling mat on top of a larger seedling mat, where it remained for the duration of the bioassay. Each larger seedling mat contained the same maize type (i.e. Bt or non-Bt) used in the corresponding small seedling mat. When insects were 21 days old, bioassay containers were checked every 2–3 days for adults, until no adults were collected from any container for two consecutive weeks.

Adults were stored in ethanol, and data were collected on the number of adults surviving per seedling mat and the sex for each adult. Data on dry mass were collected for a random sample of 10 males and 10 females per seedling mat, for a total sample size of 240 individuals for each combination of strain by hybrid. In some cases, fewer adults were available: susceptible strain on Cry3Bb1 maize = 146 (57♀; 89♂), resistant strain on Cry3Bb1 maize = 235 (115♀; 120♂), susceptible strain on non-Bt maize = 238 (118♀;120♂).

Data analysis

For the experiments assessing fitness costs on maize hybrids and maize inbreds, data were analysed with a mixed-model analysis of variance (ANOVA) using PROC

MIXED in SAS (SAS 2012). When significant main effects or interactions were present, pairwise comparisons were made using the PDIFF statement based on LSMEANS. Significance levels in pairwise comparisons were adjusted with a Bonferroni correction (Sokal and Rohlf 1995). Random factors were tested for significance using the log-likelihood ratio statistic (−2 RES log-likelihood) based on a one-tailed χ^2 test with one degree of freedom (Littell et al. 1996). To increase statistical power, random factors were removed from the model when not significant at $P < 0.25$ following Quinn and Keough (2002). However, if higher order interactions were significant, then lower order terms were retained. To ensure normality of residuals, data were transformed as needed using log and square root functions.

For the experiment measuring fitness costs on maize hybrids, data on developmental rate and survival to adulthood were analysed with the fixed factors of sex, strain, hybrid and all possible interactions. Random factors were (i) container nested within strain by hybrid and (ii) sex crossed with container nested within strain by hybrid. For head capsule width and longevity of adults, fixed factors were sex, strain, hybrid and all possible interactions. Random factors were (i) mated pair nested within strain by hybrid and (ii) sex crossed with mated pair nested within strain by hybrid. In the analysis of egg viability, fixed effects were strain, hybrid and their interaction, and the random factor was mated pair nested within strain by hybrid. In cases where life-history traits differed among the three maize hybrids, all possible pairwise comparisons were made among hybrids with a significance level of $P < 0.017$ based on three pairwise comparisons. Data on fecundity were analysed with a repeated-measures ANOVA based on a split-plot design that included the fixed factors of hybrid, strain, week and all interactions. Random factors were (i) mated pair nested within hybrid by strain and (ii) week crossed with mated pair nested within hybrid by strain. Fecundity of resistant and susceptible strains was compared within each of the 15 one-week intervals, with a significance level set at $P < 0.003$ based on 15 pairwise comparisons.

For the experiment assessing fitness costs on maize inbreds, data were analysed in the same manner as the experiment with hybrids. Significant differences among genotypes were present for survival to adulthood and head capsule width (see Results), and linear contrasts were used to test for non-recessive costs (affecting both heterozygotes and resistant individuals) and recessive costs (present for resistant individuals only) following Gassmann et al. (2008).

When a statistically significant difference was detected between genotypes, the magnitude of the fitness cost was calculated following Gassmann et al. (2009) as $[(F_s - F_r)/F_s] \times 100\%$, except for developmental rate, which was calculated as $[(F_r - F_s)/F_s] \times 100\%$; where F_s = mean of a fitness component for the susceptible genotype and F_r = mean of a fitness component for the resistant or heterozygous genotype. Because both fitness costs and benefits were found for resistance to Cry3Bb1 maize (see Results), two meta-analyses were conducted to test whether there was an overall difference in fitness between the susceptible genotype and genotypes with resistance alleles (i.e. resistant and heterozygous genotypes). The first meta-analysis tested the null hypothesis that the mean value for all significant fitness costs and benefits did not differ from 0. The second meta-analysis tested the null hypothesis that the mean value for all significant fitness costs and benefits directly related to survival and reproduction (i.e. survival to adulthood, fecundity and egg viability) did not differ from 0. Both meta-analyses were based on a *t*-test (PROC TTEST in SAS) with the null hypothesis $H_o = 0$.

For the experiment quantifying resistance to Cry3Bb1 maize, data for survival to adulthood in each seedling mat, and average adult dry mass for males and females in each seedling mat, were analysed using a pure model I ANOVA (PROC GLM in SAS) with the factors of sex, strain, maize type and all possible interactions. For both variables, all possible pairwise comparisons were made among means for insect strain by maize type, with a significance level of $P < 0.008$ based on six pairwise comparisons. Corrected survival on Bt maize for the resistant and susceptible strains was calculated following Abbott (1925).

Results

Assessing fitness costs on maize hybrids

Both fitness costs (i.e. lower fitness for the resistant strain compared to the susceptible strain) and fitness benefits (i.e. higher fitness for the resistant vs. susceptible strain) were detected in this experiment. Fitness costs included significantly longer larval developmental rate for the resistant (32.02 ± 0.14 days) (mean ± SE) vs. susceptible strain (31.15 ± 0.14 days), which represents a 2.8% fitness cost (table 1; fig. 1a). Additionally, a significant reduction in egg viability was detected for the resistant strain (table 1; fig. 1c). Egg viability was 9.8% lower for the resistant strain

Table 1 Analysis of variance for fitness costs of *Bacillus thuringiensis* (Bt) resistance on maize hybrids

	Effect[1]	d.f.	F value	P value
Larval developmental rate[2]	Strain	1,90	6.42	0.01
	Hybrid	2,90	4.42	0.01
	Sex	1,90	232.14	<0.01
	Hybrid × Strain	2,90	0.09	0.91
	Hybrid × Sex	2,90	1.35	0.26
	Strain × Sex	1,90	0.01	0.94
	Hybrid × Strain × Sex	2,90	0.07	0.93
Survival to adulthood[3]	Strain	1,180	1.58	0.21
	Hybrid	2,180	9.04	<0.01
	Sex	1,180	15.25	<0.01
	Hybrid × Strain	2,180	0.24	0.78
	Hybrid × Sex	2,180	0.10	0.91
	Strain × Sex	1,180	3.06	0.08
	Hybrid × Strain × Sex	2,180	0.60	0.55
Adult head capsule width[3]	Strain	1,180	0.56	0.45
	Hybrid	2,180	11.46	<0.01
	Sex	1,180	20.77	<0.01
	Hybrid × Strain	2,180	0.51	0.60
	Hybrid × Sex	2,180	0.28	0.76
	Strain × Sex	1,180	0.05	0.82
	Hybrid × Strain × Sex	2,180	0.80	0.45
Adult longevity[4]	Strain	1,174	5.45	0.02
	Hybrid	2,174	2.29	0.10
	Sex	1,174	4.44	0.04
	Hybrid × Strain	2,174	0.32	0.73
	Hybrid × Sex	2,174	1.12	0.33
	Strain × Sex	1,174	3.38	0.08
	Hybrid × Strain × Sex	2,174	1.11	0.33
Egg viability[3]	Strain	1,119	8.37	<0.01
	Hybrid	2,119	0.16	0.86
	Strain × Hybrid	2,119	0.88	0.42

[1] Strain = Cry3Bb1-resistant strain vs. Cry3Bb1-susceptible strain; Hybrid = three non-Bt maize hybrids (see Materials and Methods).

[2] Random factors in the model were container (hybrid × strain) and sex × container (hybrid × strain).

[3] All random factors were pooled in the model (P > 0.25 see Materials and Methods).

[4] Random factor in the model was mated pair (hybrid × strain).

(0.83 ± 0.03) compared with the susceptible strain (0.92 ± 0.01). By contrast, fitness benefits were detected in both 20.1% greater longevity and 30.6% higher fecundity for the resistant strain compared to the susceptible strain. Longevity of adults from the resistant strain (49.69 ± 2.53 days) was significantly greater than the susceptible strain (41.37 ± 2.32 days) (table 1; fig. 1b), and resistant females produced significantly more eggs (538.00 ± 38.99) than susceptible females (412.06 ± 43.20) (table 2; fig. 2). Additionally, there was a significant strain by week interaction for fecundity with the resistant strain producing significantly more eggs than the susceptible strain during weeks 2 and 3 (fig. 2).

Overall, maize hybrids differed in their suitability as a larval host for western corn rootworm. Western corn rootworm larvae developed significantly faster on hybrid DKC 61-72 than hybrid 2T783 (fig. 1a). Significantly more insects per plant survived on hybrid DKC 61-72 than on hybrids 2T783 and 36R19cn (fig. 1d). Maize hybrids also significantly affected adult head capsule width, with insects from hybrid DKC 61-72 significantly larger (1234.9 ± 9.2 μm) than those from hybrid 2T783 (1169.8 ± 9.4 μm; P < 0.0001) and 36R19cn (1193.5 ± 11.6 μm; P = 0.0091) (table 1). Thus, insects had the highest fitness on hybrid DKC 61-72 because they were larger, developed more quickly and had higher survival.

In addition to effects of hybrids, some differences between male and female western corn rootworm were detected (table 1). Females took significantly longer to reach adulthood than males (fig. 1a), displayed higher survival to adulthood than males (fig. 1d), lived significantly longer than males (fig. 1b) and were significantly larger than males (head capsule width = 1225.0 ± 8.4 μm for females and 1173.8 ± 8.1 μm for males).

Assessing fitness costs on maize inbreds

Similar to the experiment conducted with refuge hybrids, both fitness costs and benefits were present when larvae were reared on non-Bt maize inbreds.

Resistance to Bt maize was found to have a nonrecessive fitness cost (i.e. affecting both resistant and heterozygous insects) for adult head capsule width (table 3). Head capsule width of resistant adults (1109.4 ± 8.5 μm) was significantly less (P < 0.0001), by 4.3%, than susceptible adults (1158.9 ± 7.3 μm;) and head capsule width of heterozygotes (1133. 2 ± 8.0 μm) was significantly less (P = 0.02) than susceptible adults by 2.2%. Because head capsule width of heterozygotes was significantly greater than resistant insects (P = 0.03) but smaller than susceptible insects, the fitness cost affecting head capsule width was non-recessive.

There was a significant genotype by sex interaction for survival (table 3; fig. 3). No significant differences were present between susceptible females vs. heterozygous females (P = 0.59) or between susceptible males vs. heterozygous males (P = 0.93). However, compared to heterozygotes and susceptible females,

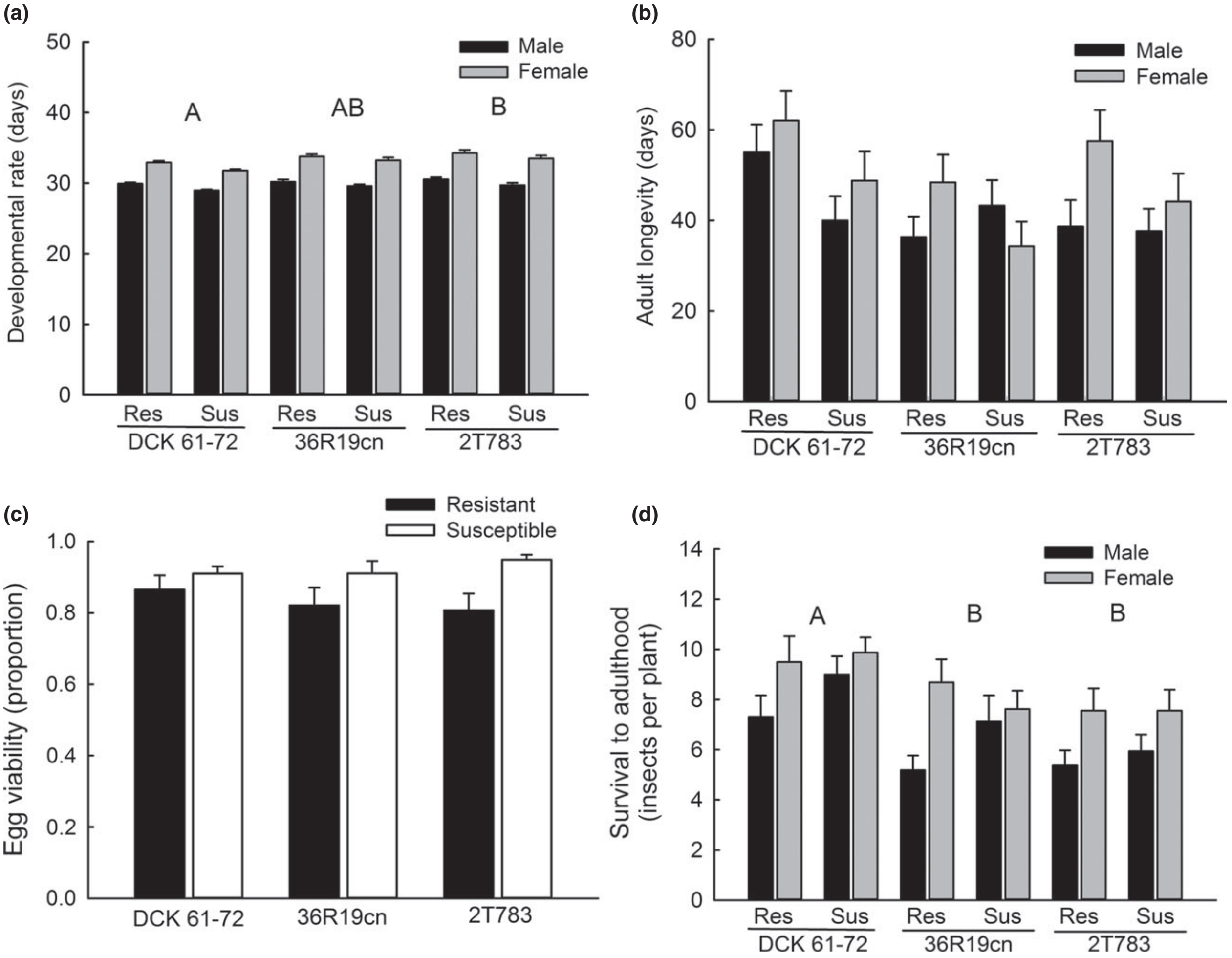

Fig. 1 Developmental rate (a), adult longevity (b), egg viability (c) and survival to adulthood (d) for Cry3Bb1-resistant (Res) and Cry3Bb1-susceptible (Sus) strains of western corn rootworm reared on maize hybrids lacking a rootworm active *Bacillus thuringiensis* toxin. Bars are sample means and error bars show the standard error of the mean. Letters indicate significant differences among hybrids. For survival to adulthood (d), 25 neonate larvae were placed in each container.

resistant females had significantly lower survival (P = 0.02), amounting to 42.9% recessive fitness cost (fig. 3). By contrast, resistant males had significantly higher survival (P = 0.04) than heterozygous and susceptible males, which was a 49.9% fitness benefit for resistant insects. There were no other significant effects of insect genotype (table 3).

No significant differences were present among maize inbreds, suggesting that the three inbred lines studied here were equally suitable hosts for larval western corn rootworm. Similar to the experiment with maize hybrids, females took significantly longer to reach adulthood than males (28.44 ± 0.41 days vs. 24.48 ± 0.20 days), and females lived significantly longer than males (51.11 ± 3.55 days vs. 34.45 ± 2.85 days) (table 3).

Meta-analysis of fitness costs

Averaging across all life-history characteristics that differed significantly among genotypes, there was a fitness benefit of 4.8 ± 10.0% (Mean ± SE) for resistance to Cry3Bb1 maize by western corn rootworm (table 4). Considering only the life-history traits directly related to survival and reproduction (i.e. survival to adulthood, fecundity and egg viability), a fitness benefit of 7.0 ± 20.8% was detected (table 4). However, the mean difference between genotypes was not significantly different from 0 when all traits were considered ($t = 0.48$; d.f. = 7; P > 0.50) or when only traits related directly to survival and reproduction were considered ($t = 0.33$; d.f. = 3; P > 0.75). This suggests that fitness costs associated with resistance to

Table 2 Repeated-measures analysis of variance for fecundity

	Effect	d.f.	F value	P value
Experiment with maize hybrids	Strain[1]	1,174	4.67	0.03
	Hybrid[2]	2,174	2.50	0.08
	Hybrid × Strain	2,174	0.44	0.64
	Week	14,2436	127.68	<0.01
	Hybrid × Week	28,2436	1.11	0.31
	Strain × Week	14,2436	2.16	<0.01
	Hybrid × Strain × Week	28,2436	1.41	0.08
Experiment with maize inbreds	Genotype[3]	2,47	0.30	0.74
	Inbred[4]	2,47	0.47	0.63
	Inbred × Genotype	2,47	0.59	0.67
	Week	10,470	25.98	<0.01
	Inbred × Week	20,470	0.31	1.00
	Strain × Week	20,470	0.97	0.49
	Inbred × Genotype × Week	40,470	0.63	0.97

[1] Cry3Bb1-resistant strain vs. Cry3Bb1-susceptible strain.

[2] Three non-*Bacillus thuringiensis* maize hybrids (see Materials and Methods).

[3] Genotypes were Cry3Bb1 resistant, Cry3Bb1 susceptible and heterozygotes.

[4] Inbred = three non-*Bacillus thuringiensis* maize inbred lines (see Materials and Methods).

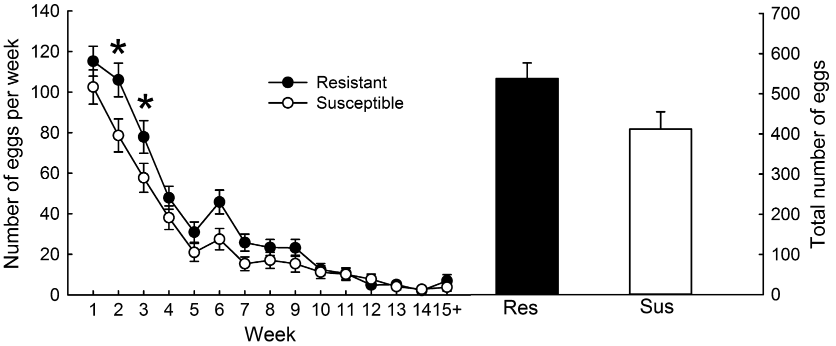

Fig. 2 Number of eggs per week and total number of eggs for Cry3Bb1-resistant (Res) and Cry3Bb1-susceptible (Sus) strains of western corn rootworm reared on maize hybrids lacking rootworm active *Bacillus thuringiensis* toxin. Circles represent mean number of eggs produced per female per week. Mean lifetime fecundity of resistant and susceptible western corn rootworm is indicated by the bar height. Error bars show the standard error of the mean. An asterisk indicates a significant difference between strains within a week.

Cry3Bb1 maize by western corn rootworm were either absent or negligible.

Quantifying resistance to Cry3Bb1 maize

Bioassays conducted during F24 (i.e. after the completion of all fitness cost experiments) revealed a significant interaction between strain (i.e. resistant vs. susceptible) and type of maize (i.e. Cry3Bb1 vs. non-Bt) for survival to adulthood (table 5; fig. 4a). Survival of resistant and susceptible strains did not differ on non-Bt maize, but was significantly higher for resistant insects compared to susceptible insects on Cry3Bb1 maize (fig. 4a). Additionally, resistant insects had lower survival on Cry3Bb1 maize than non-Bt maize (fig. 4a). The interaction of hybrid by sex also was significant (table 5). Although survival was significantly greater for males than females on Bt maize ($P = 0.04$) and non-Bt ($P < 0.0001$), this difference was more pronounced on non-Bt maize (fig. 4a). Corrected survival of the resistant and susceptible strains on Cry3Bb1 maize was 0.66 and 0.31, respectively, which equates to 2.13 (0.66/0.31) times higher survival for the resistant strain on Cry3Bb1 maize.

Dry mass of adults also was affected by a significant strain by hybrid interaction (table 5). Compared to

Table 3 Analysis of variance for fitness costs of *Bacillus thuringiensis* (Bt) resistance on maize inbreds

	Effect[1]	d.f.	F value	P value
Larval developmental rate[2]	Genotype	2,49	0.64	0.53
	Inbred	2,49	2.34	0.11
	Sex	1,49	62.04	<0.01
	Inbred × Genotype	4,49	2.20	0.08
	Inbred × Sex	2,49	2.54	0.09
	Genotype × Sex	2,49	2.05	0.14
	Inbred × Genotype × Sex	4,49	0.37	0.83
Survival to adulthood[3]	Genotype	2,49	0.11	0.90
	Inbred	2,49	2.85	0.07
	Sex	1,49	1.21	0.28
	Inbred × Genotype	4,49	0.35	0.84
	Inbred × Sex	2,49	0.64	0.53
	Genotype × Sex	2,49	6.32	<0.01
	Strain × Genotype × Sex	4,49	1.80	0.14
Adult head capsule width[4]	Genotype	2,254	10.17	<0.01
	Inbred	2,254	2.50	0.08
	Sex	1,254	0.87	0.35
	Inbred × Genotype	4,254	0.81	0.52
	Inbred × Sex	2,254	0.04	0.96
	Genotype × Sex	2,254	1.33	0.26
	Inbred × Genotype × Sex	4,254	1.30	0.27
Adult longevity[4]	Genotype	2,47	0.91	0.41
	Inbred	2,47	0.12	0.89
	Sex	1,47	9.70	<0.01
	Inbred × Genotype	4,47	0.47	0.76
	Inbred × Sex	2,47	0.83	0.44
	Genotype × Sex	2,47	0.50	0.61
	Inbred × Genotype × Sex	4,47	0.76	0.76

[1] Genotype = Cry3Bb1 resistant, Cry3Bb1 susceptible and heterozygote; Inbred = three non-Bt maize inbred lines (see Materials and Methods).

[2] Random factors in the model were container (inbred × genotype) and sex × container(inbred × genotype).

[3] Random factor in the model was container (inbred × genotype).

[4] All random factors were pooled in the model ($P > 0.25$ see Materials and Methods).

the susceptible strain, the resistant strain had numerically higher mass on Bt maize and numerically lower mass on non-Bt maize, which generated the overall significant interaction between strain and hybrid. However, both resistant and susceptible strains had significantly lower dry mass on Bt maize than non-Bt maize, and mass of resistant and susceptible strains did not differ statistically from each other on either Bt maize or non-Bt maize (table 5; fig. 4b). Females had significantly greater dry mass than males (table 5; fig. 4b).

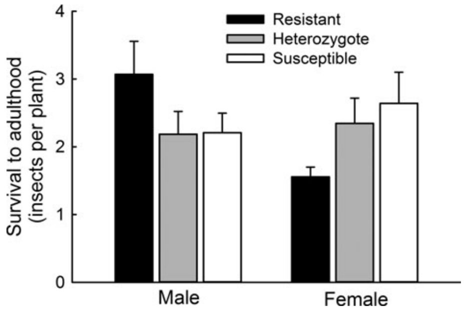

Fig. 3 Survival to adulthood for Cry3Bb1-resistant, Cry3Bb1-susceptible and heterozygous western corn rootworm reared on non-*Bacillus thuringiensis* maize inbreds. Twenty-four neonate larvae were placed in each container. Bars are sample means and error bars show the standard error of the mean.

Discussion

Field-evolved resistance by western corn rootworm to Cry3Bb1 maize has been documented in some fields and widespread resistance evolution could further diminish the effectiveness of this technology (Gassmann et al. 2011, 2012, 2014; Gassmann 2012). If present, fitness costs associated with Cry3Bb1 resistance in western corn rootworm may help to delay the evolution of resistance, or diminish the frequency of resistance alleles if farmers grow less Cry3Bb1 maize (Gassmann et al. 2009). In this study, fitness

Table 4 Meta-analysis of fitness costs[1]

Experiment[2]	Trait	Percentage difference between genotypes for all life-history traits	Percentage difference between genotypes for survival and reproduction
Maize hybrids	Developmental rate of larvae	−2.8	
Maize hybrids	Egg viability	−9.8	−9.8
Maize hybrids	Adult longevity	+20.1	
Maize hybrids	Fecundity	+30.6	+30.6
Maize inbreds	Head capsule width[3]	−4.3	
Maize inbreds	Head capsule width[4]	−2.2	
Maize inbreds	Survival to adulthood[5]	−42.9	−42.9
Maize inbreds	Survival to adulthood[6]	+49.9	+49.9
	Mean	+4.8	+7.0
	Standard Error	10.0	20.8

[1] Negative values indicate fitness costs (i.e. lower fitness for individuals with *Bacillus thuringiensis* (Bt)-resistant alleles compared to the susceptible strain) and positive values indicate a fitness benefit of resistance (i.e. higher fitness for individuals with Bt-resistant alleles compared to the susceptible strain).

[2] Data are compile from two experiments, one comparing strains on non-Bt maize hybrids and the other on non-Bt maize inbreds.

[3] Fitness cost for the resistant genotype.

[4] Fitness cost for the heterozygous genotype.

[5] Fitness cost for females.

[6] Fitness benefit for males.

Table 5 Analysis of variance for survival to adulthood and adult mass of resistant and susceptible strains reared on Cry3Bb1 maize and non-*Bacillus thuringiensis* maize

	Effect	d.f.	F value	P value
Survival	Strain[1]	1,88	36.61	<0.01
	Maize[2]	1,88	176.38	<0.01
	Sex	1,88	26.29	<0.01
	Maize × Strain	1,88	15.59	<0.01
	Maize × Sex	1,88	4.86	0.03
	Strain × Sex	1,88	2.37	0.13
	Maize × Strain × Sex	1,88	0.21	0.65
Dry mass	Strain[1]	1,87	0.38	0.54
	Maize[2]	1,87	1386.94	<0.01
	Sex	1,87	36.30	<0.01
	Maize × Strain	1,87	5.84	0.02
	Maize × Sex	1,87	0.75	0.39
	Strain × Sex	1,87	1.94	0.17
	Maize × Strain × Sex	1,87	0.34	0.56

[1] Cry3Bb1-resistant strain vs. Cry3Bb1-susceptible strain.

[2] Cry3Bb1 maize vs. non-*Bacillus thuringiensis* maize.

costs of Bt resistance were observed for egg viability, female survival, developmental rate and adult size. However, higher fitness for the resistant strain (i.e. a fitness benefit) was found for male survival, fecundity and adult longevity. Averaging across all traits where significant differences between genotypes were detected on non-Bt maize, we found 4.8 ± 10.0% higher fitness for individuals with alleles for Cry3Bb1 resistance; however, this did not differ significantly from 0. Similarly, when only survival and reproduction were considered, there was no overall difference between the susceptible strain and strains with resistance alleles. The results of this study suggest that, at least in some cases, resistance to Cry3Bb1 maize by western corn rootworm may not be accompanied by substantial fitness costs; an effect that may facilitate more rapid resistance evolution and enable the persistence of resistance alleles within a population in the absence of selection from Cry3Bb1 maize (Gassmann et al. 2009).

The resistant strain studied here displayed just over two times higher survival on Cry3Bb1 maize compared to the susceptible strain (fig. 4a). Additionally, resistance was incomplete with the resistant strain having significantly lower survival and mass on Cry3Bb1 maize compared to non-Bt maize (fig. 4). These results parallel patterns observed with the first cases of field-evolved resistance to Cry3Bb1 maize reported in Gassmann et al. (2011), in which resistance was incomplete and the resistant strain displayed three times higher survival on Cry3Bb1 maize compared to non-Bt maize. It is important to note that the populations studied in Gassmann et al. (2011) were from fields where farmers complained of poor performance of Cry3Bb1 maize and where high levels of feeding injury to Cry3Bb1 maize were observed. Additionally, corrected survival (i.e. survival on Bt maize adjusted for survival on non-Bt maize, see Materials and Methods) for the susceptible

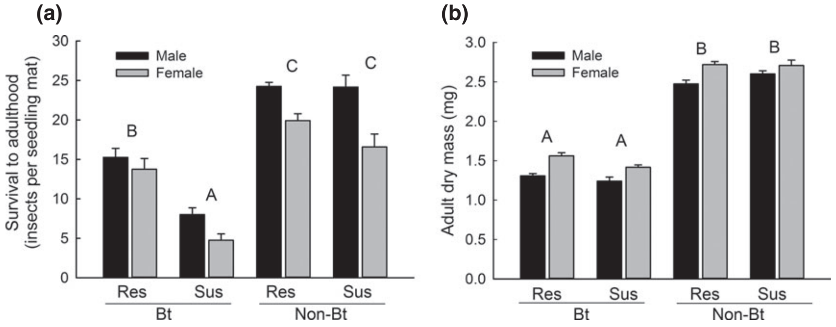

Fig. 4 Survival to adulthood (a) and dry mass of adults (b) for Cry3Bb1-resistant (Res) and Cry3Bb1-susceptible (Sus) strains of western corn rootworm reared on Cry3Bb1 maize and on its non-*Bacillus thuringiensis* near isogenic hybrid. Bars represent sample means and error bars show the standard error of the mean. Letters indicate significant differences among means for the four combinations of strain by hybrid. For (a) survival to adulthood, 50 neonate larvae were placed in each seedling mat.

strain on Cry3Bb1 maize in the seedling-mat bioassay was 31%, which is also within the range of observations from the field. For example, Petzold-Maxwell et al. (2013) observed mean corrected survival of 20% on Cry3Bb1 maize in a 2-year field study, although corrected survival on Cry3Bb1 maize ranged from 100% to 0% within individual plots of the field study. Taken together, these data indicate that the seedling-mat bioassay used here to measure resistance was consistent with observations of pest survival in the field and that the level of resistance studied here was relevant to levels of resistance that can cause substantial injury to Cry3Bb1 maize in the field.

In general, fitness costs of Bt resistance increase with the magnitude of resistance (Gassmann et al. 2009). The results of this study suggest that the initial evolution of Cry3Bb1 resistance may not impose fitness costs, which is consistent with the observation that Cry3Bb1 resistance by western corn rootworm can evolve after three generations of selection in the field (Gassmann et al. 2011). Over time, the magnitude of Cry3Bb1 resistance found in field populations has increased (Gassmann et al. 2014). Although populations sampled in 2009 displayed incomplete resistance and threefold higher survival on Cry3Bb1 maize, populations sampled in 2011 displayed sixfold resistance and no difference in survival between Cry3Bb1 maize and non-Bt maize (Gassmann et al. 2014). Additional research will be required to understand whether these higher levels of resistance have accompanying fitness costs. However, the lack of fitness costs found in this study suggests that once resistance is selected within a population it will persist, at least at some level, due to minimal accompanying fitness costs. For example, the S2 population in Gassmann et al. (2012) was found to be resistant to Cry3Bb1 maize even though the field from which it was collected had not been planted to Cry3Bb1 maize for 2 years. This observation is consistent with the hypothesis that, once Cry3Bb1 resistance evolves in a population, it may persist. It may be the case the resistance to Cry3Bb1 maize by western corn rootworm might parallel past observations for resistance to conventional insecticides. Western corn rootworm has evolved resistance to the insecticides aldrin and methyl parathion (Siegfried and Mullin 1989), and populations have retained this resistance for more than 20 years since these products were removed from the market (Parimi et al. 2006).

In the experiment with non-Bt maize hybrids, significant differences were found among hybrids in their suitability as hosts for larval western corn rootworm (table 1; fig. 1). However, there was no evidence that the magnitude of fitness costs differed as a result of this variation in host-plant quality, as evidenced by the lack of any significant strain by hybrid interactions (table 1). In some cases, fitness costs of Bt resistance have been found to change as a result of larvae feeding on more suitable vs. less suitable host plants (Janmaat and Myers 2006; Raymond et al. 2011). Additionally, past studies have found that host-plant quality can affect the dominance of fitness costs (Carrière et al. 2004; Bird and Akhurst 2007). In this study, we did not find any evidence that the dominance of fitness costs was affected when larvae fed on different maize inbreds. Rather in the experiment with maize inbreds, the only non-recessive cost detected was a 2% decrease in size of adults for heterozygotes compared to the susceptible strain, and this cost was not affected by maize inbred. The general lack of non-recessive costs found in this study is further evidence that resistance to Cry3Bb1 maize by western corn rootworm larvae may impose few fitness penalties.

An apparent lack of fitness costs found here is consistent with several other studies testing for costs of resistance to Cry3Bb1 maize by western corn rootworm, although some evidence for fitness costs also has been found (Meihls et al. 2012). Oswald et al. (2012) evaluated survivorship to adulthood, fecundity and egg viability for five Cry3Bb1-resistant strains of western corn rootworm (including the resistant strain here) and found no evidence of fitness costs; however, fitness benefits of resistance were observed in faster developmental rate and higher fecundity. Past studies have found that entomopathogenic nematodes and fungi did not impose fitness costs for the Cry3Bb1-resistant strain evaluated in this study (Petzold-Maxwell et al. 2012; Hoffmann et al. 2014). Minimal fitness costs of resistance coupled with a pattern of rapid evolution of Bt resistance found for western corn rootworm in both the laboratory (Meihls et al. 2008, 2011) and field (Gassmann et al. 2011, 2012, 2014) suggest that managing resistance by western corn rootworm to maize hybrids producing single Bt toxins will be difficult. Planting of pyramided hybrids (i.e. hybrids with multiple Bt toxins targeting western corn rootworm) may aid in managing resistance, although because selection for resistance to the same single toxins that compose these pyramids has already occurred, this will likely diminish the durability of pyramids (Roush 1998; Gassmann et al. 2014). Using Bt maize in a broader strategy of integrated pest management will likely be essential for delaying Bt resistance in western corn rootworm (Gassmann et al. 2011; Tabashnik and Gould 2012; Cullen et al. 2013). By applying a diversity of management approaches such as crop rotation and use of non-Bt maize with soil-applied insecticides, selection for Bt resistance will be reduced and the subsequent rate of resistance evolution should be diminished.

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