Applying an Integrated Refuge to Manage Western Corn Rootworm (Coleoptera: Chrysomelidae): Effects on Survival, Fitness, and Selection Pressure

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Abstract
The refuge strategy can delay resistance of insect pests to transgenic maize producing toxins from *Bacillus thuringiensis* (Bt). This is important for the western corn rootworm, *Diabrotica virgifera virgifera* LeConte (Coleoptera: Chrysomelidae), because of its history of adaptation to several management practices. A 2-yr study across four locations was conducted to measure the effects of integrated refuge (i.e., blended refuge) on western corn rootworm survival to adulthood, fitness characteristics, and susceptibility to Bt maize in the subsequent generation. The treatments tested in this study were as follows: a pure stand of Bt maize (event DAS-59122-7, which produces Bt toxins Cry34Ab1/Cry35Ab1), a pure stand of refuge (non-Bt maize), and two variations on an integrated refuge consisting of 94.4% Bt maize and 5.6% non-Bt maize. Within the two integrated refuge treatments, refuge seeds received a neonicotinoid insecticidal seed treatment of either 1.25 mg clothianidin per kernel or 0.25 mg thiamethoxam per kernel. Insects in the pure stand refuge treatment had greater survival to adulthood and earlier emergence than in all other treatments. Although fecundity, longevity, and head capsule width were reduced in treatments containing Bt maize for some site by year combinations, Bt maize did not have a significant effect on these factors when testing data across all sites and years. We found no differences in susceptibility of larval progeny to Bt maize in bioassays using progeny of adults collected from the four treatments.

Keywords
event 59122, *Diabrotica virgifera virgifera*, refuge strategy, resistance management

Disciplines
Agronomy and Crop Sciences | Entomology | Systems Biology

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Applying an Integrated Refuge to Manage Western Corn Rootworm (Coleoptera: Chrysomelidae): Effects on Survival, Fitness, and Selection Pressure

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ABSTRACT The refuge strategy can delay resistance of insect pests to transgenic maize producing toxins from Bacillus thuringiensis (Bt). This is important for the western corn rootworm, Diabrotica virgifera virgifera LeConte (Coleoptera: Chrysomelidae), because of its history of adaptation to several management practices. A 2-yr study across four locations was conducted to measure the effects of integrated refuge (i.e., blended refuge) on western corn rootworm survival to adulthood, fitness characteristics, and susceptibility to Bt maize in the subsequent generation. The treatments tested in this study were as follows: a pure stand of Bt maize (event DAS-59122-7, which produces Bt toxins Cry34Ab1/Cry35Ab1), a pure stand of refuge (non-Bt maize), and two variations on an integrated refuge consisting of 94.4% Bt maize and 5.6% non-Bt maize. Within the two integrated refuge treatments, refuge seeds received a neonicotinoid insecticidal seed treatment of either 1.25 mg clothianidin per kernel or 0.25 mg thiamethoxam per kernel. Insects in the pure stand refuge treatment had greater survival to adulthood and earlier emergence than in all other treatments. Although fecundity, longevity, and head capsule width were reduced in treatments containing Bt maize for some site by year combinations, Bt maize did not have a significant effect on these factors when testing data across all sites and years. We found no differences in susceptibility of larval progeny to Bt maize in bioassays using progeny of adults collected from the four treatments.

KEY WORDS event 59122, Diabrotica virgifera virgifera, refuge strategy, resistance management

The western corn rootworm, Diabrotica virgifera virgifera LeConte (Coleoptera: Chrysomelidae), is a major pest of maize in the United States (Gray et al. 2009). Genetically modified maize producing insecticidal toxins derived from the bacterium Bacillus thuringiensis (Bt) was commercialized for management of western corn rootworm in 2003 (Environmental Protection Agency [EPA] 2003). Bt maize kills several insect pests, including western corn rootworm, and it has been widely adopted by growers, accounting for 67% of the total area planted to maize in 2012 (U.S. Department of Agriculture–Economic Research Service [USDA–ERS] 2012). The threat of widespread field-evolved resistance to Bt maize by western corn rootworm is significant, given its history of rapid adaptation to various management practices, including different classes of conventional insecticides and the cultural control practice of crop rotation (Meinke et al. 1998, Wright et al. 2000, Levine et al. 2002). In addition, field-evolved resistance to Cry3Bb1 maize has recently been documented in Iowa (Gassmann et al. 2011, 2012; Gassmann 2012). Thus, sustainable insect resistance management (IRM) strategies are essential for prolonging the effectiveness of Bt maize for management of western corn rootworm.

Currently, the U.S. EPA mandates the refuge strategy to delay development of insect resistance to Bt maize (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 homozygous resistant individuals that survive exposure to a Bt crop (Gould 1998), thus producing heterozygous offspring. On exposure to a high-dose event, these heterozygous individuals are expected to die, which can delay the onset of resistance. However, current Bt maize products targeting the western corn rootworm (Bt maize producing Cry3Bb1, mCry3a, and Cry34Ab1/Cry35Ab1) fail to meet the high-dose condition of imposing 99.99% mortality (Gassmann 2012, Devos et
In the case of nonhigh-dose events, the rate of resistance evolution becomes dependent on functional dominance, where a greater delay in resistance is expected as the recessiveness of the resistance trait increases (Tabashnik et al. 2008). Recently, the EPA approved integrated refuge, which allows Bt and refuge (non-Bt) seeds to be mixed in a preestablished percentage (EPA 2010).

The type of refuge deployed, whether integrated or in a separate block, can affect the evolution of resistance (Tabashnik 1994, Onstad 2006, Onstad et al. 2011). One benefit of an integrated refuge is complete compliance by farmers in planting of a refuge. This is important because there is evidence that some farmers do not comply with current refuge requirements (Jaffe 2009, Gray 2011). Simulation models show that pests evolve resistance more quickly as the percentage of the landscape planted to refuge decreases (Onstad 2006, Tabashnik et al. 2008, Gassmann et al. 2009, Pan et al. 2011). Another IRM benefit of integrated refuge is the potential for increased mating between adults from refuge plants and Bt plants (Onstad et al. 2011). In the case of western corn rootworm, adults generally emerge from Bt plants = 1 wk after insects emerge from refuge plants (Binning et al. 2010, Hibbard et al. 2010a, Petzold–Maxwell et al. 2012). Female western corn rootworm adults often mate soon after they emerge and likely before dispersal (Hill 1975, Quiring and Timmins 1990), making males the primary dispersers before mating. Proximity of refuge plants to Bt plants may promote mating between susceptible and resistant insects, a requirement necessary for success of the refuge strategy in delaying resistance to Bt crops (Gould 1994, 1998).

A concern of integrated refuge is the increased potential for larval movement between Bt and non-Bt plants, which may increase exposure of the individuals within the insect population to sublethal doses of the Bt toxin and potentially increase the rate of resistance evolution (Gould 1998, Onstad and Gould 1998). It is generally thought that if larvae with an allele for resistance move from a Bt plant to a non-Bt plant, this could result in ingestion of sublethal amounts of Bt toxin, leading to a fitness advantage for partially resistant insects that otherwise could not complete development on a Bt plant (Onstad and Gould 1998). However, because currently commercialized Bt maize targeting western corn rootworm is not considered high dose, survival of homozygous susceptible and heterozygous individuals will occur in even a pure stand of Bt maize (Siegfried et al. 2005, Meilhs et al. 2008, Hibbard et al. 2010a). Thus, it is possible that larval movement between refuge and Bt plants may not greatly affect the rate of resistance evolution for this species (Tabashnik 1994), although more studies are necessary to better understand how larval movement of western corn rootworm in integrated refuges can affect evolution of resistance.

Differences in effects of block vs. integrated refuges on fitness of insects also may affect resistance evolution. If insects emerging from Bt plants incur sublethal effects on fitness, the spread of resistance genes may be delayed compared with cases where sublethal effects are absent (Tabashnik et al. 2004). Past studies and this study considered the life history parameters of adult size, fecundity, and longevity to test whether sublethal effects on fitness are a general phenomenon associated with Bt maize (Al-Deeb and Wilde 2005, Murphy et al. 2011). Few field studies have examined fitness of western corn rootworm in a block vs. integrated refuge, or effects on susceptibility to Bt maize in the next generation (but see Hibbard et al. 2010a; Murphy et al. 2010, 2011; Zukoff et al. 2012). In this study, survival and fitness of western corn rootworm adults were measured after exposure in the field to pure stands of Bt maize (event 59122), pure stands of refuge (non-Bt maize), and an integrated refuge. In addition, the susceptibility of the offspring obtained from adult corn rootworm collected from each treatment was measured by exposing larvae to Bt and non-Bt maize seedling mats in the laboratory.

Materials and Methods

Experimental Design. Our goals were to 1) compare life history traits and survival of insects exposed to treatments that included a pure stand of Bt maize, a pure stand of refuge (non-Bt maize), and integrated refuges with two rates of neonicotinoid insecticidal seed treatments, and 2) determine susceptibility to Bt maize for progeny of insects that emerged from each of the treatments. Field studies were conducted in 2009 and 2010 at four locations (Ames, IA; Ithaca, NY; Ithaca, NE; and Urbana, IL). All locations relied on natural populations of western corn rootworm in both years. Bt maize used in this study contained the event DAS-59122-7, which produces the rootworm active Bt binary toxins Cry3Ab1/Cry35Ab1 (Pioneer brand hybrid 33W84, relative maturity: 111 d). The refuge maize used in this research was a near isoline to the Bt maize hybrid but lacked any rootworm-active Bt toxins (Pioneer brand hybrid 33W80, relative maturity: 111 d). In the pure stand Bt and pure stand refuge treatments, seeds were treated with Cruiser 250 (thiamethoxam, 0.25 mg/seed, Syngenta, Basel, Switzerland). Both integrated refuge treatments tested in this study contained 94.4% Bt maize and 5.6% non-Bt maize. All of the Bt seeds in the integrated refuge treatments were treated with Cruiser 250, while the refuge seeds were treated with either Cruiser 250, or Poncho 1250 (clothianidin, 1.25 mg/seed, Bayer Crop Science, Research Triangle Park, NC). Thus, a total of four treatments were measured in this study: 1) pure stand refuge, 2) pure stand Bt maize, 3) an integrated refuge in which refuge seeds were treated with Cruiser 250 seed treatment (hereafter referred to as integrated refuge 250), and 4) an integrated refuge in which refuge seeds were treated with Poncho 1250 seed treatment (hereafter referred to as integrated refuge 1250). For both years, each location consisted of 20 plots; three were planted with pure stand refuge, seven with pure stand Bt maize, and five with each integrated refuge 250, and integrated refuge 1250. The
number of plots differed among treatments because we anticipated greatest survival to adulthood in pure stand refuge plots and lowest survival to adulthood in pure stand Bt plots. Treatments were randomized within each location. Plots consisted of four rows 3 m long, with spacing of 0.76 m between rows, and were separated by 3.7 m of bare ground on all sides. Plots were kept free of weeds to ensure that rootworm larvae fed only on maize roots. For the integrated refuge plots, refuge (non-Bt) seeds were hand planted into rows within plots. Plots were thinned to 18 plants per row 2–3 wks after planting to achieve the desired percentage of refuge seeds, and covered with tents (3.7 by 3.7 m or 3.9 by 3.4 m) 6–7 wks after planting (Screenhouse 12 and Screenhouse L, Kelty, Boulder, CO). The sides of the tents were buried to prevent escape of rootworm adults. When tents were placed over plots, plants were trimmed to a height of 30–45 cm, with two to three green leaves left intact. Two plants were not trimmed so that the developing maize ears could serve as a food source for adults.

Survival to Adulthood and Life History Measurements. Adult western corn rootworm were collected from each tent twice per week at each location (every 3–4 d) with an aspirator, and within each location adults were then pooled by treatment. Western corn rootworm adults collected in all locations in both years were shipped to Iowa State University where they were separated by sex following Hammack and French (2007), and the number of males and females per treatment from each location at each collection date was recorded. In 2009, tents at the Illinois location suffered substantial damage from a storm on 4 August, and collection at this location was terminated after this date (71.2% [SD = 18.2] of all beetles collected per tent in Illinois had emerged by this date in 2010). For each combination of treatment by location by year, mated pairs were established during the period of peak adult emergence, and data were collected on adult life span, fecundity, and size (measured as width of the head capsule between the outer edges of the eyes). Collecting adults for mated pairs during peak emergence enabled a sample to be collected from the integrated refuge that coincided with emergence of adults from both the pure stand Bt and pure stand refuge treatments (Fig. 1). Thus, the adults used to generate mated pairs for the integrated refuge treatments provide a representative ratio of Bt-selected and refuge insects, and are not biased toward only refuge insects or only Bt-selected insects.

During each year, insects (within location and treatment) were held in screened cages (18 by 18 by 18 cm; Megaview Science, Taichung, Taiwan) in an environmental chamber (25°C and a photoperiod of 16:8 [L:D] h) for 1 wk after collection from the field, after which mated pairs were established. Insects were fed maize ear from the same hybrids they consumed as larvae (e.g., those insects emerging from pure stand Bt plots were fed only Bt maize ear), and 1.5% agar solid was provided as a source of moisture. Maize ear was obtained from small maize plots established close to Ames, IA. During each year, a maximum of 26 mated pairs for each treatment within location were established, with an average (±SD) per location of 20.3 (±2.9) pairs for the pure stand refuge treatment, 16.8 (±7.9) for the integrated refuge 250 treatment, 17.5 (±7.9) for the integrated refuge 1250 treatment, and 14.1 (±6.1) for the pure stand Bt treatment. The total number of mated pairs established across both years and all four locations was as follows: pure stand refuge maize = 163, integrated refuge 250 = 134, integrated refuge 1250 = 140, and pure stand Bt maize = 113.

Mated pairs were established by placing one male and one female in a plastic 475-ml container (Del-Pak, Reynolds, Richmond, VA) covered with a modified lid in which the center had been removed and replaced with a circular piece of mesh organza fabric (diameter = 6 cm) to allow for ventilation. Each container received maize ear (kernels, and several strands of silk ~2 cm long) corresponding to each respective treatment, 1.5% agar solid as a source of water, and an oviposition substrate consisting of moistened finely

![Fig. 1. Number of insects that emerged per tent over time for (A) 2009 and (B) 2010, averaged over the four locations. Box plots represent the time over which insects were collected from the integrated refuge treatments for use in mated pairs. Within box plots, the solid line shows median day of collection, boxes capture the 25th to 75th percentiles, whiskers indicate the 10th and 90th percentiles, and dots show outliers beyond the 5th and 95th percentile.](image-url)
sieved field soil (<180 μm) in a petri dish (diameter = 3.5 cm) covered with an arched piece of aluminum foil. Mated pairs were held in incubators (25°C and a photoperiod of 16:8 [L:D] h) until both individuals died. Maize and agar were changed every 3–4 d, and oviposition dishes were moistened and replaced as needed (every 3–10 d). Cups were checked every 3–4 d for dead insects, which were placed in 95% ethanol, and total life span was recorded. After the female from a mated pair died, eggs from that female were washed from the soil using a screen with 250-μm openings and enumerated using a dissecting microscope (MZ6, Leica Microsystems, Wetzlar, Germany) and a counting grid (grid size: 0.5 by 0.5 cm). Digital images of each dead insect were captured, and head capsule width was measured using a dissecting microscope and accompanying image analysis software (Motic Images Inc., British Columbia, Canada).

Susceptibility of Progeny to Bt Maize. Each year, any insects not used to produce mated pairs for life history measurements were placed in mass rearing cages according to treatment within location. The eggs collected from these adults were assayed for susceptibility to Bt maize with the exception of eggs collected from the Ames site in 2010, which experienced severe flooding and thus few larvae survived to adulthood and insufficient eggs were collected. Insects in mass rearing cages were given artificial diet (EU768B-M, Bio Serv, NJ), 1.5% agar solid, and maize leaves, as well as an ovposition dish filled with moistened field soil that was changed weekly. Eggs were washed from these dishes using a 60-μm mesh screen and pooled by treatment within location and year. Eggs were placed in 45-ml plastic cups (Solo Cup Company, Lake Forest, IL) filled with moistened field soil sieved to a particle size of <180 μm, and covered with lids containing four small holes. Cups were placed in sealed plastic bags, and held at 7°C for at least 5 mo to break diapause.

Eggs from each treatment within a location were used to measure offspring susceptibility to Bt maize event DAS-59122-7 (hereafter referred as 59122) following the sublethal seedling assay described in Nowatzki et al. (2008). Briefly, larvae were exposed to either Bt (59122) or non-Bt maize seedlings by placing eggs (∼5–7 d before hatch) in plastic deli containers (23 by 28 by 9 cm, Pactiv Corp., Lake Forest, IL) containing 150 kernels of the appropriate seed, 200 ml of a 1% thiophanate-methyl fungicide solution (42.25% ai) (3336 F Turf and Ornamental Systemic Fungicide; Cleary Chemical, Dayton, NJ), and 1,000 g Metro-Mix 200 plant growth media (Scotts–Sierra Horticultural Products Company, Marysville, OH). A replicate consisted of one container each of non-Bt maize and Bt (59122) maize, and based on availability of eggs, one to five replicates were established for each treatment within a location and year. Containers were placed in an incubator (25°C, 65% relative humidity [RH], and a photoperiod of 14:10 [L:D] h). For all treatments tested, a subsample of 50–200 eggs (depending on egg availability) was held in a petri dish under the same conditions as containers to monitor initial egg hatch, and to calculate egg viability.

Larvae were exposed to maize seedlings for 17 d after initial egg hatch. This period of time was chosen to maximize any developmental differences of larvae on Bt vs. non-Bt maize (Nowatzki et al. 2008). Seedling mats were then placed onto Berlese funnels for 4 d, and larvae were extracted into vials containing 85% ethanol. The total number of larvae extracted per container was counted. For the first year of the study, because egg hatch tests were unsuccessful, proportion survival in each replicate was calculated as the number of larvae extracted divided by the number of eggs placed in each container. During the second year of the study, the number of eggs placed in a container was first multiplied by the proportion of viable eggs to obtain number of viable eggs, and proportion survival was calculated as the number of larvae extracted divided by the number of viable eggs. A random sample of 50 larvae per container was scored for instar using a dissecting microscope (MZ6, Leica Microsystems) and accompanying image analysis software (Motic Images Inc.), according to the methods of Hammack et al. (2003). The percentage of first, second, and third instars was calculated for each replicate.

Data Analysis. All analyses were conducted in SAS Enterprise Guide 4.2 (SAS Institute 2009). Data from all locations and both years were tested in each analysis, except in the analysis of susceptibility of progeny to Bt maize, which excluded the Ames 2010 site because of lack of eggs (see above). For all data analyzed with a mixed-model analysis of variance (ANOVA), random effects were tested with a log-likelihood ratio statistic (−2 RES log likelihood in PROC MIXED and PROC GLIMMIX) based on a one-tailed χ² test assuming 1 df (Littell et al. 1996) and 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).

Survival to Adulthood and Life History Measurements. Data on the number of insects that emerged, the Julian day that insects emerged, insect size (head capsule width), and adult longevity (life span) were analyzed with a mixed-model ANOVA using PROC MIXED based on a split-plot design. Fixed effects in the model were sex, treatment, and their interaction, where treatment was analyzed as the whole plot factor and sex as the split-plot factor. Random factors included year, location, location crossed with year, the interaction of treatment with each of these three random factors, and sex crossed with treatment by year by location. When significant effects were present, pairwise comparisons were conducted based on the Tukey-Kramer method (PDIFF in PROC MIXED). Data on number of insects that emerged were log transformed to ensure normality of the residuals.

At each location during each year, the number of insects expected to emerge from the integrated refuge treatments was calculated based on the number of insects that emerged per plant in the pure stand treatments. These expected values were compared with actual emergence from the integrated refuge treat-
ments to test the hypothesis that emergence in the integrated refuge differed from the expected emergence. First, the number of insects that emerged per plant from the pure stand Bt and the pure stand refuge tents was calculated for each location within a year. Because our plots contained 72 plants, 5.6% of which were refuge, there were 68 Bt plants and four refuge plants in each integrated refuge plot. The number of insects expected to emerge per integrated refuge tent was calculated as: (number of insects that emerged per Bt plant in pure stands × 68) + (number of insects that emerged per refuge plant in pure stands × 4) for each location within a year. Expected and actual number of insects per tent for each location within a year were compared with a paired two-tailed t-test (PROC TTEST).

Fecundity was analyzed with a mixed-model ANOVA (PROC GLIMMIX), with a log link function and a Poisson distribution. Data were transformed with the log function to ensure normality of the residuals. Treatment was a fixed factor in the analysis. Random factors included year, location, location crossed with year, and the interaction of treatment with each of these random factors. To determine whether shipment of insects influenced fecundity, a mixed-model ANOVA (PROC GLIMMIX) was used, with a log link function and a Poisson distribution. Data were transformed with the log function to ensure normality of the residuals. Treatment, location, and the interaction of treatment and location were fixed factors in the analysis. Random factors included year, location crossed with year, and the interaction of treatment with each of these random factors. The CONTRAST statement was used to compare fecundity of adults collected in Iowa (study location) with those collected from the other locations. To determine whether insect density affected fecundity, we tested the correlation between the average number of adults per tent for the pure stand refuge treatment and the average egg production per female in the pure stand refuge treatment, using data from all locations and both years (PROC CORR).

Susceptibility of Progeny to Bt Maize. A partially nested mixed model ANOVA (PROC GLIMMIX) was used to analyze survival and the proportion of third instars. Fixed effects in the model were treatment (maize treatment in the field), maize hybrid in seed mats (Bt and non-Bt), and the interaction of treatment and hybrid. Random factors included year, location, location crossed with year, the interaction of treatment with each of these random effects, replicate nested within location by treatment by year, and the interaction of replicate by hybrid nested within location by treatment by year. Replicate was defined as a pair of Bt and non-Bt seedling mats that were established at the same time and used larvae from a single treatment in one location within a year.

Within each replicate, data on the proportion of third instar larvae were used to calculate an odds ratio and 95% confidence limits of obtaining a third instar larva on non-Bt vs. Bt maize. Odds ratios for each treatment were obtained using the ESTIMATE statement and transformed back to the original scale using the EXP option. The odds ratio for each of the four treatments was as follows: [odds of obtaining a third instar on non-Bt maize/odds of obtaining a first or second instar on non-Bt maize] / [odds of obtaining a third instar on Bt maize/odds of obtaining a first or second instar on Bt maize]. A value of 1 indicates no difference in development on Bt vs. non-Bt maize; a value >1 indicates faster development on non-Bt maize (i.e., higher odds of getting a third instar on non-Bt maize vs. Bt maize); and a value <1 indicates faster development on Bt maize. Treatments were significantly different if they had nonoverlapping 95% CIs.

Results

Survival to Adulthood. Across all locations and both years, there was a significant effect of treatment on survival to adulthood (Table 1); the number of adult beetles was significantly higher in the pure stand refuge than any other treatment (P < 0.01 for all comparisons) (Table 1; Figs. 1 and 2A). Relative to pure stand refuge, survival was reduced by 88.8% (±10.1) for integrated refuge 250, 88.5% (±9.0) for integrated refuge 1250, and 91.2% (±9.0) for pure stand Bt maize. The number of insects emerging from integrated refuge 250, integrated refuge 1250, and pure stand Bt maize plots did not differ (P > 0.35 for all comparisons). The number of insects expected to emerge from the integrated refuge tents (based on the number of insects that emerged per Bt and refuge plant in the pure stands) did not differ significantly from the actual number that emerged (IR 250: t = 0.09, P = 0.93; IR 1250: t = 0.61, P = 0.56) (Fig. 2B). Overall, significantly more females survived to adulthood. The number of adults that emerged per tent varied considerably among locations each year (Table 2). In 2009, survival to adulthood was greatest in Nebraska and lowest in Iowa (Table 2). In 2010, survival to adult-

### Table 1. ANOVA for western corn rootworm life history measurements

<table>
<thead>
<tr>
<th>Source</th>
<th>df*</th>
<th>F</th>
<th>P</th>
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<tr>
<td>Survival to adulthood</td>
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<tr>
<td>Treatment</td>
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<td>Sex</td>
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<td>0.8824</td>
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<tr>
<td>Timing of emergence</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Treatment</td>
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<td>13.40</td>
<td>0.0304</td>
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<tr>
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<td>&lt;0.0001</td>
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<tr>
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<td>Treatment</td>
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* df: numerator, denominator.
Both sex of beetles and maize treatment significantly affected time of adult emergence (Table 1; Fig. 2C). Average date of emergence was earlier for males compared with females for all treatments. Average date of emergence for insects from pure stand refuge plots was significantly earlier than insects from pure stand Bt plots ($P < 0.03$) and integrated refuge 1250 plots ($P < 0.05$), and marginally so for integrated refuge 250 plots ($P < 0.08$). However, time of emergence did not differ significantly among any treatment containing Bt plants (pure stand or with integrated refuge) ($P > 0.38$ for all comparisons) (Fig. 2C).

**Adult Life History Measurements.** Head capsule width varied among locations and years (Table 3), but across all locations and both years, treatment did not significantly affect head capsule width (Table 1). Overall, head capsule width was greater for females than males (Fig. 3A), and this effect was significant (Table 1). No other significant differences were detected (Table 1). Similarly, across all locations and both years, treatment did not significantly affect longevity (Table 1). However, females lived significantly longer than males (Fig. 3B). At some locations, longevity was greater for pure stand refuge compared with either integrated refuge or pure stand Bt treatments (e.g., IL in 2009), and at other locations the opposite was true (e.g., NE in 2009) (Table 3).

Over all locations and both years, females from the refuge treatment displayed the highest average egg production (Fig. 4), but the effect of treatment on fecundity was not significant (Table 1). At some locations, the average number of eggs produced per female was considerably greater for pure stand refuge compared with the other treatments (e.g., IL in 2009); however, fecundity was similar among treatments in other location by year combinations (e.g., NY in 2009) (Table 3). Fecundity did not differ between insects collected from Iowa and insects collected from other locations ($F_{1,3} = 0.13; P = 0.742$), indicating that ship-
ping insects did not influence fecundity. In addition, there was no significant correlation between insect density and fecundity in the refuge treatment ($r^2 = 0.60; P = 0.15$).

Susceptibility of Progeny to Bt Maize. Larval survival did not differ significantly among the four treatments (maize type from which parents emerged in the field), and there was no interaction between treatments.

### Table 3. Life history traits for western corn rootworm

<table>
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<th>Treatment</th>
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<th>Iowa</th>
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</table>

*Numbers in parentheses show SE.

Insects emerged from pure stand refuge, integrated refuge 250 (IR 250), integrated refuge 1250 (IR 1250), and pure stand Bt maize plots at four locations.

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**Fig. 3.** (A) Head capsule width and (B) longevity of insects emerging from pure stand refuge, integrated refuge 250 (IR 250), integrated refuge 1250 (IR 1250), and pure stand Bt maize plots at four locations. Bar heights are the sample mean and error bars the SEM. Asterisks indicate significant differences within a treatment ($P < 0.05$).

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**Fig. 4.** Fecundity of insects emerging from pure stand refuge, integrated refuge 250 (IR 250), integrated refuge 1250 (IR 1250), and pure stand Bt maize plots at four locations. Bar heights are the sample mean and error bars the SEM. Means with same letters are not significantly different ($P < 0.05$).
ment and the maize type to which larvae were exposed in containers (Bt or non-Bt) (Fig. 5A; Table 4). For all treatments, larval development was faster on non-Bt seedling mats, as indicated by a significantly higher proportion of third instar larvae recovered on non-Bt seedling mats compared with Bt seedling mats (Fig. 5B; Table 4), and by odds ratios for each treatment (Table 5). This difference in recovery of third instar larvae on the two types of maize did not differ significantly among treatments, as indicated by the lack of a significant interaction between treatment and maize type (Table 4). Furthermore, odds ratios did not differ significantly among treatments (Table 5). These results indicate that survivorship and development were not affected by the treatment from which adults emerged during the previous generation.

Discussion

In this study, Bt maize producing the Cry34/35Ab1 insecticidal protein significantly reduced survival of western corn rootworm to adulthood and significantly delayed the time of adult emergence. These effects were present for both the pure stand Bt maize and the integrated refuge treatments. In some location by year combinations, longevity, head capsule width, and fecundity were greater for pure stand refuge compared with the integrated refuge and pure stand Bt maize treatments. However, across all years and locations, exposure to Bt maize did not have significant effects on western corn rootworm adult longevity, size, and fecundity. Furthermore, the progeny of insects collected from pure stand refuge, pure stand Bt maize, and integrated refuge did not display significant differences in susceptibility to Bt maize in the next generation.

This study showed that the main effect of Bt maize producing the Cry34/35Ab1 insecticidal protein on western corn rootworm was a reduction in survival to adulthood (Figs. 1 and 2A). The reduction in survival observed for the integrated refuge and pure stand Bt treatments relative to the pure stand refuge treatment (88.5–91.2% relative to pure stand refuge; Fig. 2A) is similar to what has been reported in other studies. Storer et al. (2006) reported a 94.4–96% reduction in adult survival on maize producing Cry34/35Ab1 compared with non-Bt maize (see Hibbard et al. 2010b). Hibbard et al. (2010a) reported mortality values in the field from maize producing mCry3A (event MIR604) that ranged from 87.54 to 99.83%, and similar decreases

| Table 4. ANOVA for larvae exposed to seedling mats containing either Bt (event 59122) or non-Bt maize |
| Source | Proportion recovered | Proportion of third instars |
| Field treatment | df | $F$ | $P$ | df | $F$ | $P$ |
| Bt maize | 3, 3 | 0.31 | 0.8172 | 3, 3 | 1.48 | 0.3769 |
| non-Bt maize | 1, 42 | 3.70 | 0.0613 | 1, 42 | 71.32 | <0.0001 |
| Field treatment × maize type | 3, 42 | 1.18 | 0.3273 | 3, 42 | 0.21 | 0.8888 |

* df: numerator, denominator.
in survival have been reported for Bt maize producing Cry3Bb1 (event MON863), mCry3A (event MIR604), eCry3.1Ab (event 5307), and mCry3A pyramided with eCry3.1Ab (Al-Deeb and Wilde 2005; Hibbard et al. 2009, 2011; Frank et al. 2011). However, because this study relied on natural rootworm populations, we do not know whether density-dependent mortality occurred in the pure stand refuge treatment, which may result in mortality on Bt maize to be underestimated (Hibbard et al. 2010b).

Survival in the integrated refuge treatments was not significantly greater than survival in the pure stand Bt treatment, contradicting the expectation that the presence of refuge plants should result in increased survival in these treatments compared with pure stands of Bt. However, because refuge plants only represented 4 of the 72 plants in integrated refuge treatments, it is likely that a statistically significant difference in survival compared with pure Bt stands may be difficult to obtain. The actual number of rootworm that emerged from integrated refuge plots did not differ significantly from the expected number (Fig. 2B). It is unclear whether the relative proportion of insects that emerged from Bt and refuge plants in the integrated refuge treatments differed from those in pure stands because we did not measure emergence from individual plants. Murphy et al. (2010) found that the proportion of beetles emerging from refuge plants was significantly less in an integrated refuge than in a pure stand refuge; however, the proportion that emerged from Bt plants was greater. This finding is similar to a finding by Zukoff et al. (2012), who observed that significantly more western corn rootworm beetles could emerged from Bt maize plants (producing both Cry34/35Ab1 and Cry3Bb1) when these plants were surrounded by refuge plants on both sides as compared with Bt plants surrounded by Bt plants, or refuge plants surrounded by refuge plants. The authors postulated that movement from refuge plants to Bt plants by larvae could produce additional susceptible insects emerging from within the integrated refuge field, because a large proportion of initial larval development occurred on refuge plants (Zukoff et al. 2012).

To the extent that movement between refuge and Bt plants imposes selection for resistance, thereby decreasing the number of unselected refuge individuals, resistance may evolve more quickly. By contrast, increased survival of susceptible insects that move from non-Bt maize to Bt maize, compared with those that feed only on Bt maize, may delay resistance evolution (Pan et al. 2011). Meihls et al. (2008) found that progeny of larvae that were first fed Cry3Bb1-producing Bt maize but finished their development on non-Bt refuge plants did not have higher survival on Cry3Bb1 maize in a subsequent generation than progeny of insects fed only non-Bt maize. However, when larvae were fed non-Bt refuge maize for 1 wk and then fed Cry3Bb1 maize for the rest of larval development, the subsequent progeny did display increased survival on Cry3Bb1 (Meihls et al. 2008). Additional studies will be necessary to gain a more complete understanding of how larval movement between Bt and non-Bt maize plants affects survival to adulthood and resistance evolution.

In addition to imposing mortality, Cry34/35Ab1 maize also delayed adult emergence. Random mating of insects emerging from Bt and refuge plants is important for the success of the refuge strategy. Temporal asynchrony in emergence between insects from Bt and refuge plants may reduce random mating and diminish the extent to which refuges delay resistance (Gould 1998). Adult emergence of western corn rootworm on pure stands of Bt maize is generally later than on pure stands of non-Bt maize (Storer et al. 2006, Binning et al. 2010). Murphy et al. (2010) measured time of emergence for western corn rootworm from Bt maize plants (producing Cry3Bb1) and refuge plants in field plots planted as integrated refuges, block refuges, and strip refuges, and found that insects from refuge plants emerged more synchronously with insects from Bt plants for integrated refuges compared with blocks. Although we did not measure emergence timing from individual refuge plants in our integrated refuge treatments, our results also support more synchronous emergence of males and females in integrated refuges compared with pure stand refuge maize (Fig. 2C). An integrated refuge should enhance resistance management by increasing the probability of random mating because of greater spatial and temporal proximity of insects emerging from Bt and refuge plants (Gould 1998, Storer 2003).

Results from sublethal seedling assays indicate that the treatment from which adults were collected in the field did not significantly affect the susceptibility of their progeny to Bt maize (59122) in the next generation (Fig. 5; Table 4). Previous studies have shown that populations with decreased susceptibility to Bt maize producing Cry34/35Ab1 have faster larval development on maize producing Cry34/35Ab1 compared with susceptible populations (Nowatzki et al. 2008). The progeny of insects collected from all of our treatments (pure stand refuge, pure stand Bt maize, integrated refuge 250 and 1250 treatments) had odds ratios that were >1, and did not differ significantly from each other (Table 5). In addition, there was no interactive effect of treatment (maize type from which parents emerged in the field) and maize type used in the assay (Bt or non-Bt) on the proportion of third instars recovered (Table 4). This suggests that populations collected from treatments containing Bt maize producing Cry34/35Ab1, either in a pure stand or in an integrated refuge, did not exhibit a decrease in susceptibility to Bt maize (59122) after one generation of selection in the field. Hibbard et al. (2010a) tested whether western corn rootworm that experienced one generation of exposure to mCry3A maize in the field exhibited increased survivorship on mCry3A maize in the greenhouse during the next generation. Similar to our study, Hibbard et al. (2010a) found that there was no difference in survivorship on Bt maize for progeny of insects that emerged from either Bt or refuge maize in the field.

There were no differences in longevity or size among adults from any of the treatments tested (Table
years (Table 1; Fig. 3). There was also no significant difference in fecundity among treatments over locations and years (Table 1; Fig. 4). Pure stand refuge insects had higher fecundity than the other treatments in several year-by-location combinations, but in others, fecundity was higher for pure stand Bt insects or was similar among treatments (Table 3). The insecticidal seed treatments thiamethoxam (0.25 mg a.i. [active ingredient] per seed) and clothianidin (1.25 mg a.i. per seed) applied to refuge seeds in the integrated refuge did not differ in their effects on adult fecundity, longevity, size, emergence, or larval susceptibility to Bt maize (Figs. 1–5), possibly because they were applied to a small percentage of seeds (refuge seed equaled 5.6% of the total seed in the integrated refuge treatments).

Fecundity was lower in this study than what has been found in other studies, which report ranges in egg production from 125 to 441 eggs (Elliott et al. 1990), 372 to 418 eggs (Ball 1957), 385 to 516 eggs (Fisher et al. 1991), 440 to 671 eggs (Li et al. 2009), or an average of 353 eggs per female (Toepfer and Kuhlmann 2006). These studies use a variety of oviposition substrates, and many provide artificial western corn rootworm diet, silk, and leaves as a food source. It is possible that the small oviposition dishes used in this study were not optimal for egg collection, or that supplying artificial diet or other forms of maize in addition to the maize ear and small amounts of silk would have resulted in greater egg production.

The effects of several Bt proteins on various life history parameters of western corn rootworm, including head capsule width, weight, longevity, and fecundity, have been examined in a number of studies (Storer et al. 2006, Oyediran et al. 2007, El Khishen et al. 2009, Murphy et al. 2011, Zukoff et al. 2012). Studies examining fecundity and longevity have shown negative effects of Bt maize, but these effects were often weak and nonsignificant (Wilson 2003, Al-Deeb and Wilde 2005, Meissle et al. 2011). For example, in field studies comparing these life history traits for western corn rootworm emerging from Bt maize (producing Cry3Bb1) and non-Bt maize, Wilson (2003) found a reduction in fecundity but no difference in longevity. By contrast, Al-Deeb and Wilde (2005) found no effect on fecundity but some evidence for decreased longevity in males on Bt maize producing Cry3Bb1. In this study, there was a trend for smaller head capsule widths of males (but not females) in Bt treatments (Fig. 3A), although the interaction of sex and treatment was not significant. In some cases, adult head capsule width and weight are not affected by larval feeding on Bt maize (Storer et al. 2006, Frank et al. 2011, Meihls et al. 2011, Zukoff et al. 2012). In other cases, larval feeding on Bt maize can reduce the size of adults, as demonstrated by smaller head capsule widths or a reduction in adult mass (Binning et al. 2010, Murphy et al. 2011). Head capsule width is commonly used as a biological fitness parameter of rootworm adults (Murphy et al. 2011), but it can be influenced by density-dependent effects (Branson and Sutter 1985). Because adult abundance was significantly greater in the pure stand refuge treatment, density-dependent effects could have confounded differences in head capsule width, and other life history parameters, which may have otherwise arisen between the pure stand refuge treatment and the other treatments.

Effects of refuge configurations on resistance evolution can be influenced by the biology of the pest species. For example, structured refuges may be more appropriate than integrated refuges for European corn borer (Ostrinia nubilalis Hubner) (Davis and Onstad 2000, Siegfried and Hellmich 2012). European corn borer larvae tend to disperse within rows rather than between rows (Ross and Ostlie 1990), and thus will likely encounter the same type of plant in strip or block refuges. Larval movement of European corn borer among Bt and refuge plants is a primary concern in integrated refuges, especially because Bt maize is considered a high dose for this species. If larvae with an allele for resistance move from a Bt plant to a non-Bt plant and ingest sublethal amounts of Bt toxin, this could lead to a fitness advantage for partially resistant insects that otherwise could not complete development on a Bt plant, thereby underlining resistance management (Onstad and Gould 1998, Siegfried and Hellmich 2012). By contrast, for western corn rootworm, Bt events are not high dose and some heterozygous individuals, as well as susceptible genotypes, are expected to survive on Bt maize. For example, Binning et al. (2010) found that survival of susceptible western corn rootworm to adulthood on Bt maize producing Cry34/35Ab1 (59122) was as high as 5%, and for insects first exposed to non-Bt corn and then transferred to 59122 during the third instar, survival was 50%. The lack of a high dose for 59122 could diminish the effects of larval movement on resistance evolution (Tabashnik 1994). In addition, although integrated refuges increase random mating of adults compared with structured refuges, this benefit may depend on pest dispersal. In the case of European corn borer, which often disperse >0.8 km before mating (Hunt et al. 2001, Showers et al. 2001), a block refuge may be effective. By contrast, although long-distance dispersal is possible, western corn rootworm typically disperse <40 m per day (Spencer et al. 2003, 2009), and consequently, an integrated refuge may be most effective at facilitating random mating for this species (Pan et al. 2011).

To date, few studies have examined the effects of integrated refuge on western corn rootworm survival and life history traits in the field (Murphy et al. 2010, 2011). Results from simulation models suggest that integrated refuge can delay Bt resistance by corn rootworm longer than block refuges (Pan et al. 2011). Future work should examine the selection pressure of different refuge configurations on western corn rootworm over several generations. A more thorough understanding of how natural selection on western corn rootworm is affected by refuge deployment and insect behavior will aid in the development of better IRM strategies.
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