Evaluating Western Corn Rootworm (Coleoptera: Chrysomelidae) Emergence and Root Damage in a Seed Mix Refuge

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ABSTRACT   Resistance management is essential for maintaining the efficacy and long-term durability of transgenic corn engineered to control western corn rootworm (*Diabrotica virgifera virgifera* Le Conte). Theoretically, a refuge can be provided by growing susceptible (refuge) plants in either a separate section of the field adjacent to resistant (transgenic) plants, or as a seed mixture. We examined the effects of varying the structure of a 10 and 20% refuge between currently approved structured refuges (block or strip plantings), as well as deploying the refuge within a seed mix, on adult emergence timing and magnitude, root damage and yield. Our 2-yr field study used naturally occurring western corn rootworm populations and included seven treatments: 10 and 20% block refuge, 10 and 20% strip refuge, 10 and 20% seed mix refuge, and 100% refuge. Beetles emerging from refuge corn emerged more synchronously with those emerging from transgenic (*Bacillus thuringiensis* [Berliner] *Bt*-RW) corn in seed mix refuges when compared with block refuges. The proportion of beetles emerging from refuge plants was significantly greater in a block and strip refuge structure than in a seed mix refuge. More beetles emerged from *Bt*-RW corn plants when they were grown as part of a seed mix. We discuss the potential benefits and drawbacks of a seed mix refuge structure in light of these findings.

KEY WORDS   resistance management, seed mix refuge, refuge-in-a-bag, western corn rootworm, *Bt* corn

Western corn rootworm (*Diabrotica virgifera virgifera* Le Conte), one of the most damaging and widespread pests of corn (*Zea mays* L.), results in the "single largest use of conventional pesticides in the United States" (EPA 2005a). Recently introduced transgenic *Bt* (*Bacillus thuringiensis* Berliner) corn provides an alternative control tactic for western corn rootworm and is being rapidly adopted by growers (James 2006). Broad acceptance and use of transgenic crops can reduce pesticide applications while improving yields and returning economic benefits (Brooks and Barfoot 2005, Carpenter and Gianessi 2001, Shelton et al. 2002). However, as with any control tactic, the potential for long-term use of transgenic crops may be limited without implementation of appropriate and sustainable resistance management strategies.

When transgenic corn for rootworm control was first registered in February 2003, an insect resistance management (IRM) plan, based on mandatory refuges, was required and included with the purchase of the seed (EPA 2005b). This plan is based on the refuge strategy where the resistant crop plants are planted with a refuge of susceptible plants (EPA 1998). The refuge strategy seeks to maintain a susceptible population of insect pests through environmental manip-

ulation (Roush and Daly 1990, Gould 1998, Bourguet et al. 2000, Tabashnik et al. 2004). Nevertheless, there is concern that growers may not comply with refuge requirements (i.e., currently, 20% of the crop must be nontransgenic corn) because of the additional effort associated with planting a refuge and lower returns on refuge crops compared with transgenic (Mallet and Porter 1992, EPA 2005b, Mendelsohn 2006, Hurley et al. 2006).

For the refuge strategy to be successful at managing resistance to *Bt*, susceptible and resistant adults must disperse and mate randomly in the field (Gould 1986, 1994). However, in the field females often mate within several hours after emergence and are unlikely to disperse before mating (Hill 1975, Quiring and Timmins 1990, Marquardt and Krupke 2009), meaning that males are the primary dispersers in this system before mating. Moreover, resistant and susceptible beetles are less likely to mate because of a temporal shift in emergence from their natal plants (Ferro 1993). Adults emerge from refuge areas approximately a week before their counterparts in the transgenic area of the same field (Crowder et al. 2005, Storer et al. 2006). Though males normally emerge before females and are capable of mating multiple times, females generally mate only once (Cates 1968, Hill 1975, Branson et al. 1977, Hein et al. 1988, Quiring and Timmins

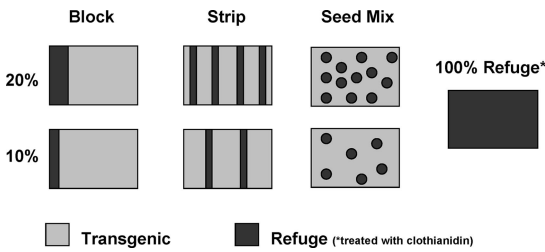[1] Corresponding author, e-mail: amckinni@purdue.edu.

Fig. 1. Diagrammatic representation of the seven refuge planting treatments. Not drawn to scale.

1990) and males are less likely to mate as they age (Kang and Krupke 2009). This suggests that the proximity of refuge and transgenic plants to one another may be a significant factor in determining the mating frequency between beetles emerging from these respective host plants. Presently growers must plant a refuge in strips at least six rows wide across the field (strip), or in a separate section within or adjacent to the transgenic (*Bt*-RW) portion of the field (block) (EPA 2005b). Although EPA guidelines currently do not allow transgenic and refuge seed to be planted as a mix, seed mixes have been discussed as an option and may be available to growers soon (EPA 2009). However, there is little published data evaluating different refuge structures empirically under field conditions. In this study, we compare the timing of adult western corn rootworm emergence and quantify the root damage and yield of corn planted in fields where the refuge is either a seed mix or one of the currently approved refuge structures. We test the hypothesis that western corn rootworm emergence, root damage and yield are the same, regardless of refuge structure.

Method

Experimental Design. Field studies using a randomized complete block design were conducted in 2007 and 2008 at the Throckmorton Purdue Agricultural Center (TPAC) and the Agronomy Center for Research and Education (ACRE), both in Tippecanoe Co., IN. These two locations have historically high levels of rootworm pressure. There were three replications at each location during each year. The seven treatments consisted of: (1) 20% block design, (2) 20% strip design, (3) 20% seed mix design, (4) 10% block design, (5) 10% strip design, (6) 10% seed mix design, (7) 100% refuge (control) (Fig. 1). The width of the strips was one row of corn. All of the *Bt*-RW hybrids contained the MON88017 event and all hybrids used in both refuge and *Bt*-RW areas contained the MON810 event (i.e., European corn borer resistant) and the NK603 event (i.e., glyphosate-tolerant, Roundup Ready). The hybrids used for refuge and *Bt*-RW plantings were DKC61–73 and DKC61–69, respectively (Dekalb, St. Louis, MO). The *Bt*-RW seed was treated with an insecticidal seed treatment, clothianidin at 0.25 mg/kernel (Poncho 250 Bayer Crop Science, Research Triangle Park, NC), which is not effective at reducing rootworm damage. However,

the refuge seed was treated with clothianidin at 1.25 mg/kernel (Poncho 1250) to avoid excessive rootworm damage, as these fields have historically high pressure levels; this product is labeled for rootworm control and is sold commercially for this purpose (Bayer 2005, Gray et al. 2006). The appropriate percentage of refuge seed for the seed mix refuge treatments was determined by weight (grams), because the individual seeds were similar in size.

Each treatment consisted of three 15.3×15.3 m (50×50 feet), 20-row plots. Planting occurred during conventional planting times in northwestern Indiana: on 2 and 3 May 2007 and on 1 and 2 May 2008. All fields had been planted with a delayed planting of corn (i.e., a trap crop) the previous year, to promote oviposition by gravid, pollen-seeking western corn rootworm females. The row spacing and plant density were 0.76 m and 68,448 kernels/ha (30 in and 27,700 seeds/acre), respectively. The individual plots were planted with 18 m (60 feet) rows and then trimmed to 15 m (50 feet) by removing 1.5 m from each end to form alleys between the plots. Each treatment was bordered by one guard row of refuge corn on two sides, and refuge corn surrounded the experimental field as well. The individual plots were divided in half so that one portion could be used for destructive sampling techniques whereas the other was reserved for yield studies. Postemergence glyphosate was applied for weed control at all locations, excluding ACRE in 2007.

Testing for Protein Expression. We tested for the expression of the *Bt*-RW protein in plants from every row of the structured refuge plots. A single plant was randomly selected from each row and QuickStix for Cry3B YieldGard Rootworm Corn (Product No. AS 015; Envirologix Inc., Portland, ME) test strips were used according to the manufacturer's instructions to assay for the presence of the Bt-RW protein. In the seed mix plots, all plants in a 6×6 m subsection were assayed for the presence of Cry3Bb1. To determine the location of the subsection in the plot, we randomly selected the first row (between rows 2–12) and then randomly selected the starting plant (between plants 3–5). If needed, additional 6 m rows were sampled to obtain at least thirteen refuge plants for sampling in the subsection. The areas of each plot dedicated to yield measurements were also checked using QuickStix for Cry3B YieldGard Rootworm Corn test strips at this time. The yield rows consisted of ten randomly selected 2.7 m long rows for each seed mix plot. Each refuge plant within the yield rows and subsection of a seed mix treatment was marked at the base. Field maps of the sampled areas were constructed.

Emergence Studies. We measured the emergence of beetles in the field by caging three refuge plants and three *Bt*-RW plants (with the exception of the 100% refuge plot, where six refuge plants were selected) that were randomly selected within each plot. The randomization was stratified across the inner 18 rows so that there were only two cages in a section of six rows, when refuge structure permitted, and cages were not clustered in one portion of the plot. All of these plants were assayed for presence or absence of
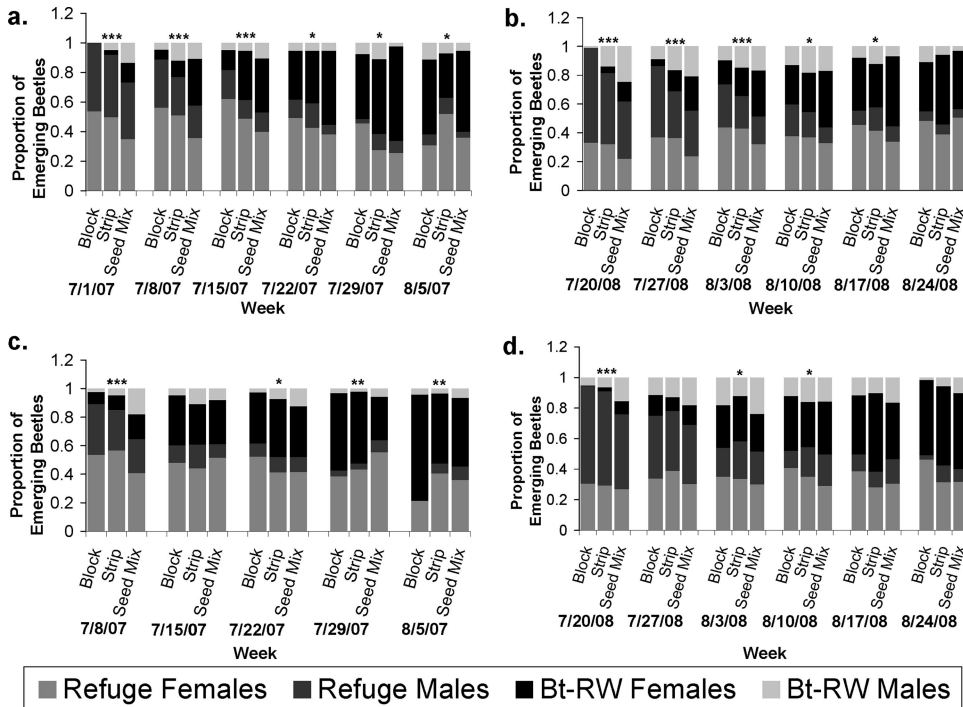
Fig. 2. Proportion of male or female western corn rootworm beetles emerging from refuge or *Bt*-RW plants for different refuge structures over time; (a) 20% refuges 2007, (b) 20% refuges 2008, (c) 10% refuges 2007, and (d) 10% refuges 2008. Analyzed using a χ^2 test for homogeneity for each week. Weeks that had significantly different proportions than expected are noted above using a single set of symbols centered over the treatments in that date. ***P value <0.001, **P value <0.01, *P value ≤0.05.

the Cry3Bb1 protein as described above. The ground surrounding individually selected plants was covered with emergence cages in mid-June. The emergence cages measured 0.8×0.5 m and were constructed of fine wire mesh (3 mm mesh size) covering wood frames. The upper portion of the trap was fitted with a single funnel trap, which served as the collection container (Fig. 2). The funnel trap was secured and maintained upright using a wooden stake. Cage bases were sealed by embedding the wooden frame in the ground and securing it with two tent pegs. Strips of foam were wrapped around the stalk to seal the opening around the corn plant. The emergence cages were checked twice each week beginning 25 June 2007 and 26 June 2008. In 2007, beetles were transferred to labeled containers in the field. To prevent escape during collection, the beetles were slowed using dry ice. Traps were monitored throughout the season and emerged beetles were collected, stored in individually in labeled vials and frozen at −10°C. All beetles were counted and sexed using visual characters and a microscope when necessary (Kuhar and Youngman 1995).

Root Ratings. We assessed the feeding damage by western corn rootworm larvae to the roots of 10 refuge and 10 *Bt*-RW plants per plot on 24 July 2007 and 22 and 23 July 2008. Roots were randomly selected and stratified across rows when refuge structure permitted. Stratified randomization was accomplished by

dividing the 18 inner rows of a plot into three sections of six rows each, then randomly selecting three or four rows from each section for sampling. Each row was sampled by digging a single root system from that row. In the seed mix plots, the mapped subsample encompassed a limited number of rows and stratification was not necessary. Instead, refuge plants were numbered, randomly selected, and paired with a corresponding *Bt*-RW plant. Ratings were performed using the Oleson (0–3) quarter-node injury rating scale (Oleson et al. 2005), which correlates root damage linearly with root rating. Roots were excavated individually by hand using shovels, labeled on the stalk, and then washed with a power sprayer before rating.

Yield. Ten randomly selected 2.7 m long rows from the yield portion of each plot were hand-harvested in October of each year. Yield was scaled to match the refuge and *Bt*-RW proportions in each plot. We measured the weight of the harvested corn in each row to within 45 g and later adjusted for moisture content. The yield for refuge and *Bt*-RW corn was measured separately in each row of the seed mix plots.

Data Analyses. Emergence data collected over the period of a single week (two sampling dates) were combined, providing 36 samples from either refuge or *Bt*-RW plants for each treatment per week. The 2007 and 2008 weekly emergence data were analyzed individually using multiple χ^2 tests for homogeneity (each week was tested separately) to compare the

Table 1. Magnitude and timing of average peak adult emergence by treatment for 2007 and 2008

Treatment	Refuge females		Refuge males		Bt-RW females		Bt-RW males	
	Peak	Date	Peak	Date	Peak	Date	Peak	Date
2007								
20% Block	3.61 ± 0.58a	7/8	2.17 ± 0.43a	7/1	1.25 ± 0.25a	7/22	0.28 ± 0.15a	7/8
20% Strip	3.14 ± 0.72ab	7/8	2.54 ± 0.75ab	7/1	1.18 ± 0.34a	7/15	0.68 ± 0.16a	7/8
20% Seed mix	2.06 ± 0.45ab	7/15	1.19 ± 0.22ab	7/8	2.11 ± 0.35a	7/22	0.58 ± 0.20a	7/8
10% Block	2.64 ± 0.68ab	7/8	1.75 ± 0.55ab	7/1	1.94 ± 0.55a	8/5	0.17 ± 0.07a	7/15
10% Strip	2.11 ± 0.40ab	7/8	1.11 ± 0.21ab	7/1	1.42 ± 0.24a	7/29	0.31 ± 0.12a	7/15
10% Seed mix	1.81 ± 0.54b	7/15	0.69 ± 0.25b	7/8	1.89 ± 0.60a	8/5	0.53 ± 0.15a	7/8
100% Refuge	3.24 ± 0.43ab	7/8	1.85 ± 0.26a	7/8				
2008								
20% Block	3.74 ± 0.56a	8/3	5.50 ± 1.14a	7/20	1.92 ± 0.34a	8/10	0.86 ± 0.28a	8/3
20% Strip	4.25 ± 0.64a	8/3	3.47 ± 0.74a	7/20	1.94 ± 0.43a	8/3	1.44 ± 0.36ab	8/3
20% Seed mix	2.36 ± 0.44a	8/3	2.36 ± 0.52a	7/27	2.36 ± 0.35a	8/3	1.53 ± 0.38ab	7/27
10% Block	2.97 ± 0.57a	7/27	4.25 ± 0.87a	7/20	2.14 ± 0.41a	8/10	1.39 ± 0.28ab	8/3
10% Strip	2.36 ± 0.40a	7/27	2.44 ± 0.46a	7/20	1.75 ± 0.38a	8/3	0.78 ± 0.23a	7/27
10% Seed mix	2.64 ± 0.35a	8/3	2.61 ± 0.42a	7/27	2.58 ± 0.46a	8/10	2.08 ± 0.45b	8/3
100% Refuge	4.31 ± 0.44a	8/3	5.46 ± 0.67a	7/20				

Mean beetles emerging/plant ±SEM for all the samples taken in a single week for a given treatment, $n = 36$. The letters represent the grouping for significance at a level of 0.05. Values followed by the same letter within each column are not significantly different. Data were analyzed using a Kruskal-Wallis followed by using pair-wise Mann-Whitney tests (with a Bonferroni adjustment).

emergence of males and females from refuge and Bt-RW corn plants 20% treatments and then 10% treatments. Only the weeks with sufficient numbers of beetles to perform a χ^2 test were analyzed. Following a χ^2 test, pairwise comparisons were made by performing z-tests for two proportions (De Veaux et al. 2005, Minitab Inc. 2007). The peak emergence data could not be transformed to satisfy the assumptions of normality and constant variance required of ANOVA, and were analyzed using a Kruskal-Wallis (Minitab Inc. 2007). Post hoc testing of emergence data was performed using multiple Mann-Whitney tests followed by a Bonferroni adjustment of the P value (Minitab Inc. 2007). The total emergence data for 2007 (converted to the total number of beetles per plot and adjusted for either a refuge size of 10 or 20%) did not meet the normality and constant variance assumptions, so males, females and total western corn rootworm data were-transformed using Johnson transformations and analyzed using ANOVA followed by a Dunnett's test. The total number of beetles per plot data for 2008 (also adjusted for either a refuge size of 10 or 20%) were first transformed using Johnson transformations and then analyzed using ANOVA followed by a Dunnett's test (Minitab Inc. 2007). The mean number of beetles emerging per plant for 2007 and 2008 was transformed using either a natural log function (2007 males), Johnson transformations (2007 and 2008 females and total western corn rootworm), or a Box-Cox transformation (2008 males) and then analyzed using ANOVA followed by a Tukey's multiple comparison test (Minitab Inc. 2007).

The mean root rating per plot for 2007 and 2008 (combined) data were analyzed separately based on plant and refuge size. The mean root ratings from refuge roots in 10 and 20% refuge structures, as well as the mean root rating from transgenic plants in 10% refuge structures, were transformed using Johnson transformations. The mean root ratings for transgenic roots from for 20% refuge treatments met the assump-

tions of normality and constant variance and were not transformed. These data were analyzed individually using ANOVA followed by Tukey's multiple comparisons (Minitab Inc. 2007). The total yield data for 2007 and 2008 combined was transformed using a Johnson transformation and then analyzed with ANOVA, followed by a Tukey's multiple comparison test (Minitab Inc. 2007).

The root rating data were also categorized based on the presence or absence of damage and proximity to the alternative hybrid (refuge or Bt-RW). A refuge root was defined as "not adjacent to a Bt-RW root" if there were less than two Bt-RW plants within a radius of 19 cm within row and 76 cm across rows, which is within the range of potential western corn rootworm larval movement reported by Hibbard et al. (2003). A Bt-RW root was classified as "far from a refuge plant" if there were no refuge plants within a 38 cm radius within row and a 76 cm radius across rows (Hibbard et al. 2003). Bt-RW roots with ratings of 0.05 or greater were defined as "visibly fed upon." A z-test for two proportions was used to compare the proportion of damaged refuge plants near Bt-RW plants to the proportion of damaged refuge plants located far from Bt-RW plants. A similar comparison was performed for Bt-RW plants, also using a z-test for two proportions (Minitab Inc. 2007). All analyses and Johnson transformations were performed using Minitab 15.1.20.0 Statistical Software (Minitab Inc. 2007).

Results

Emergence Timing. In 2007 and 2008, the number of males and females emerging from refuge plants peaked consistently later in the seed mix plots than in block or strip refuge structures (Table 1). In 2007, the number of males emerging from refuge and the Bt-RW portions of the 20% seed mix and 10% seed mix treatment peaked synchronously during the week of 8 July (Table 1). Conversely, males emerged from the refuge

Table 2. Summary of adult western corn rootworm emergence in 2007 and 2008

Treatment	Refuge males			Refuge females			Bt-RW males			Bt-RW females		
	Start	End	Days	Start	End	Days	Start	End	Days	Start	End	Days
2007												
20% Block	6/24	8/12	50	6/24	8/26	64	7/8	8/12	36	7/8	8/26	50
20% Strip	6/24	8/12	50	6/24	8/26	64	7/1	8/12	43	6/24	8/26	64
20% Mix	6/24	8/5	43	6/24	8/26	64	7/1	8/12	43	7/1	8/26	57
10% Block	6/24	7/29	36	6/24	8/19	57	7/1	8/12	43	7/1	8/26	57
10% Strip	6/24	8/12	50	6/24	8/26	64	7/8	8/12	43	7/1	8/26	57
10% Mix	6/24	8/19	57	7/1	8/26	57	6/24	8/19	57	6/24	8/26	64
100% Refuge	6/24	8/12	50	6/24	8/26	64						
2008												
20% Block	7/6	9/7	64	7/13	9/28	78	7/20	8/31	43	7/13	9/21	71
20% Strip	7/6	8/31	57	7/13	9/21	71	7/13	9/7	57	7/6	9/21	78
20% Mix	7/6	8/24	50	7/13	9/14	64	7/13	8/24	43	7/20	8/31	43
10% Block	7/13	8/31	50	7/13	9/28	78	7/20	8/31	43	7/20	9/28	71
10% Strip	7/6	8/31	57	7/6	9/21	78	7/6	8/31	57	7/13	9/28	78
10% Mix	7/13	8/24	43	7/13	9/28	78	7/20	8/31	43	7/20	9/21	64
100% Refuge	7/6	8/24	50	7/13	9/28	78						

Ending dates were determined based upon the last week before a full week of no emergence.

portion of plots with the block and strip refuge structures at least a week earlier than from the Bt-RW plants. In 2008, the same trend was seen only in the 20% seed mix treatment.

Emergence Timing, Duration, and Relative Abundance. There were distinct differences in the timing and duration of emergence between the two growing seasons (Table 2). In 2007, adult emergence spanned approximately two months from 25 June to 30 August, whereas in 2008 emergence lasted about 3 mo, from 10 July until 2 October.

A greater proportion of males and females emerged from Bt-RW plants in seed mix treatments compared with the block or strip refuge structures for 10 or 20% refuge treatments in 2007 and 2008 for the first few weeks of each season (Fig. 2). However, the proportion of males and/or females emerging from refuge plants in the seed mix plots was significantly less than the proportion emerging from block refuges for 10 or 20% refuge treatments in 2007 and 2008 (Fig. 2). The mean number of cumulative beetles emerging per plot per week demonstrate the emergence dynamics of each refuge structure for both years (Figs. 3–6). These cumulative curves provide further support for the trends demonstrated in Fig. 2, particularly when block refuges are compared with seed mix refuge treatments
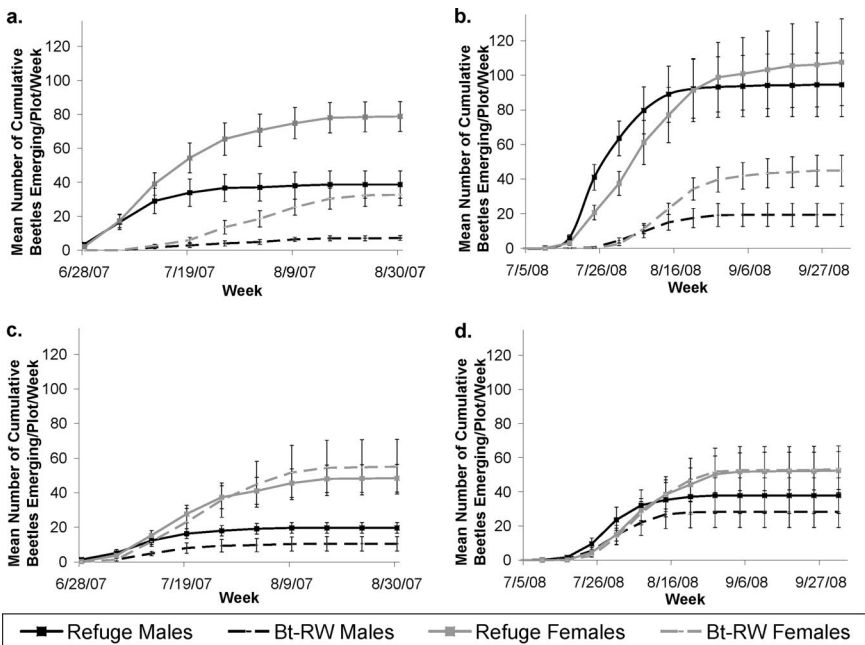

Fig. 3. Cumulative mean beetles emerging per plot per week through 2007 and 2008 for the 20% block and 20% seed mix treatments ±SEM; (a) 2007 20% block, (b) 2008 20% block, (c) 2007 20% seed mix, and (d) 2008 20% seed mix.
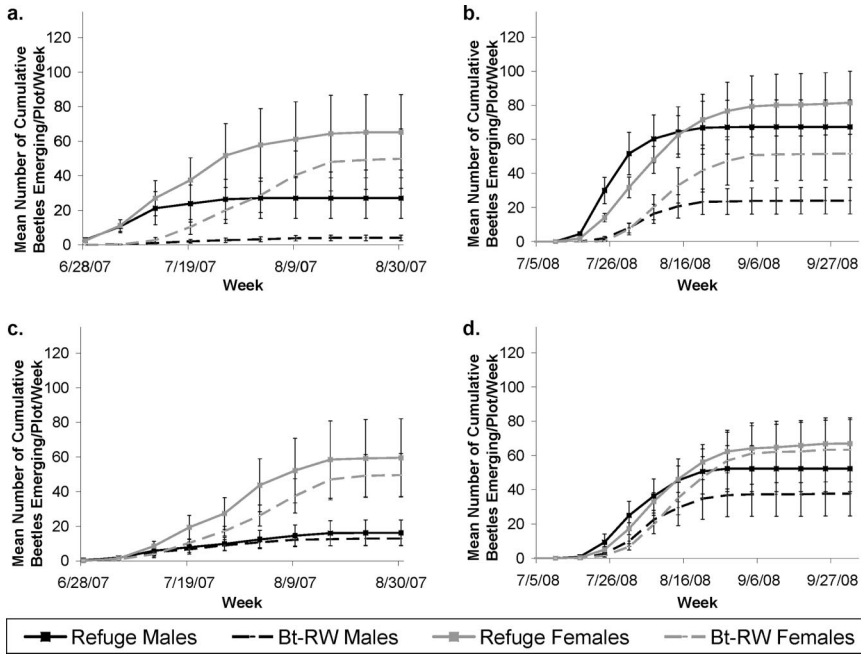
Fig. 4. Cumulative mean beetles emerging per plot per week through 2007 and 2008 for the 10% block and 10% seed mix treatments ±SEM; (a) 2007 10% block, (b) 2008 10% block, (c) 2007 10% seed mix, and (d) 2008 10% seed mix.

(Figs. 3 and 4). Furthermore, there were no significant differences between treatments in the mean number of male, female or total beetles emerging per plot (adjusted to more accurately represent the refuge proportions) in 2007 or 2008, though the numbers of males in 2007 ($F = 6.57$; df = 6, 30; $P = <0.001$) and 2008 ($F = 4.87$; df = 6, 30; $P = 0.001$) as well as the total beetles in 2008 ($F = 3.16$; df = 6, 30; $P = 0.016$) emerging per plot for several treatments were significantly lower than the control (Table 3). Similar dif-
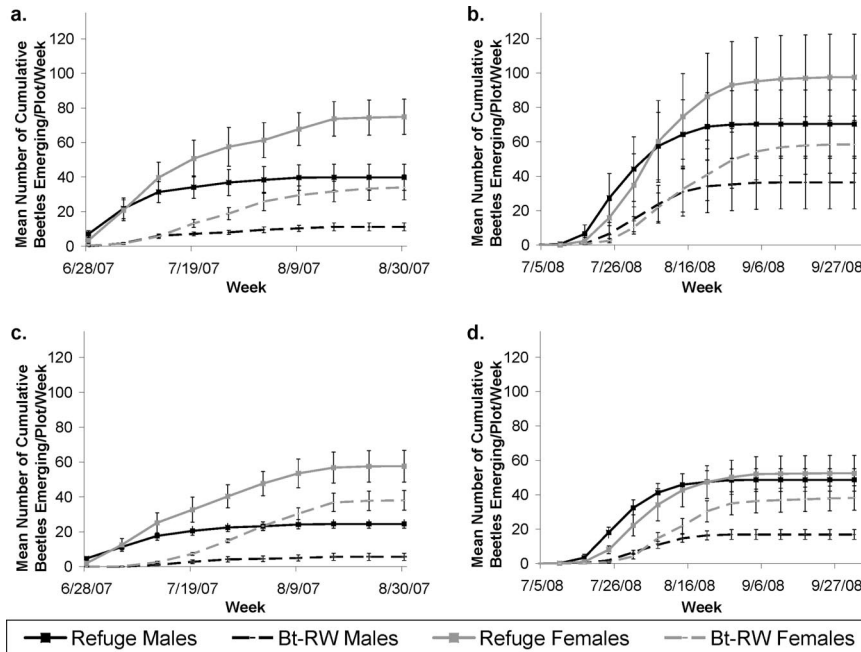

Fig. 5. Cumulative mean beetles emerging per plot in a week through 2007 and 2008 for the 20 and 10% strip refuge treatments ±SEM; (a) 2007 20% strip, (b) 2008 20% strip, (c) 2007 10% strip, and d) 2008 10% strip.
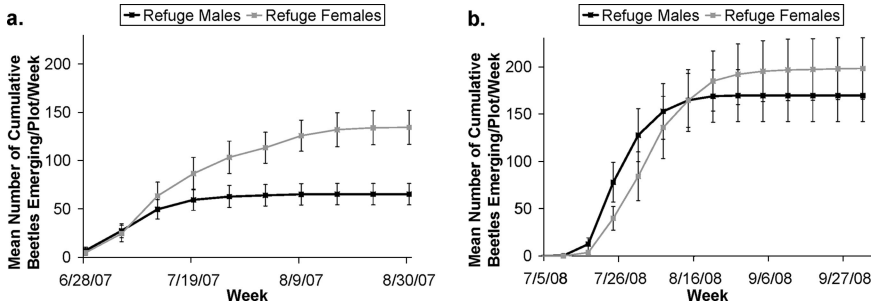
Fig. 6. Cumulative mean beetles emerging per plot per week through 2007 and 2008 seasons for the 100% refuge treatments ±SEM; (a) 2007 100% refuge and (b) 2008 100% refuge.

ferences existed between the mean number of beetles emerging per plant in treatments versus the control for males in 2007 ($F = 4.82$, df = 6, 241; $P < 0.001$) and 2008 ($F = 6.34$, df = 6, 241; $P < 0.001$), 2008 females ($F = 4.38$, df = 6, 241; $P < 0.001$) and 2008 total beetles ($F = 5.23$, df = 6, 241; $P < 0.001$) (Table 4).

Root Ratings. Refuge structure influenced the mean amount of larval feeding damage on the roots of refuge and *Bt*-RW plants. In 20% treatments, the mean root damage on refuge plants in a seed mix was significantly lower compared with a block or strip structure ($F = 8.90$; df = 2, 22; $P = 0.001$), while the 10% refuge plants demonstrated a similar trend (Fig. 5). Conversely, *Bt*-RW plants in a 10% seed mix had significantly greater root damage per plot compared with a strip refuge structure ($F = 5.74$; df = 2, 22; $P = 0.010$). The 20% refuge structures demonstrated a similar trend (Fig. 7). Refuge plants adjacent to a *Bt*-RW plant suffered significantly less damage than those that were not adjacent to a *Bt*-RW plant ($z = -2.63$, $P = 0.008$) (Fig. 8). Additionally, *Bt*-RW plants located near a refuge plant had a significantly greater proportion of

roots that were visibly fed upon compared with those located farther away ($z = 4.29$, $P < 0.001$) (Fig. 8).

Yield. There were no significant differences in yield between treatments in 2007 and 2008 combined (ANOVA), although the variation within treatments was high.

Discussion

The results of our study reveal some of the strengths and weaknesses of providing a refuge as a seed mix when compared with structured refuges. The synchronous emergence of adults from refuge and *Bt*-RW plants that results from mixing the seed may facilitate random mating because of increased proximity of adult beetles in both space and time, theoretically enhancing resistance management (Gould 1986, Storer 2003). However, this benefit may be minor because of movement of adult beetles in block and strip refuge structures. For example, there is considerable intrafield movement of male beetles in current block and strip refuge structures, which may result in sufficient mixing of the populations (Spencer et al. 2005, Marquardt and Krupke 2009). Additionally, ad-

Table 3. Mean western corn rootworm emergence per plot for 2007 and 2008 ±SEM

	Males	Females	Total
2007			
20% Block	26.9 ± 3.4a	83.8 ± 7.8a	110.7 ± 9.7a
20% Strip	33.8 ± 3.4ab	84.3 ± 10.6a	118.1 ± 11.9a
20% Seed Mix	24.7 ± 6.9a	107.3 ± 26.1a	132.0 ± 30.6a
10% Block	12.6 ± 4.4a	102.7 ± 31.9a	115.3 ± 33.4a
10% Strip	15.1 ± 3.9a	79.9 ± 10.7a	95.0 ± 12.9a
10% Seed Mix	26.3 ± 8.5a	101.0 ± 26.4a	127.3 ± 33.5a
100% Refuge	65.3 ± 11.0b	134.5 ± 17.5a	199.8 ± 26.2a
2008			
20% Block	66.9 ± 17.9a	114.7 ± 23.9a	181.6 ± 40.7ab
20% Strip	86.3 ± 28.3ab	132.3 ± 31.7a	218.6 ± 59.8ab
20% Seed Mix	60.5 ± 17.5a	106.3 ± 24.1a	166.8 ± 41.1a
10% Block	56.7 ± 15.9a	109.3 ± 30.9a	166.0 ± 46.3a
10% Strip	40.0 ± 5.4a	79.2 ± 13.9a	119.2 ± 17.8a
10% Seed Mix	78.3 ± 25.5a	127.4 ± 36.1a	205.7 ± 61.3ab
100% Refuge	169.5 ± 27.4b	198.2 ± 32.5a	367.7 ± 56.9b

Numbers have been adjusted to account for the 10 and 20% refuge structures by weighting the refuge and *Bt*-RW data accordingly. Data were analyzed (following Johnson transformations) separately using ANOVA followed by a Dunnett's test. The letters represent the grouping for significance at a level of 0.05. Values followed by the same letter within each column are not significantly different.

Table 4. Mean western corn rootworm emergence per plant for 2007 and 2008 ±SEM

	Males	Females	Total
2007			
20% Block	7.6 ± 1.4ab	18.6 ± 2.2a	26.2 ± 3.4a
20% Strip	8.3 ± 1.6ab	17.6 ± 2.6a	25.9 ± 4.1a
20% Seed mix	5.0 ± 0.8a	17.2 ± 2.2a	22.3 ± 2.7a
10% Block	5.2 ± 1.5a	19.2 ± 3.7a	24.3 ± 4.8a
10% Strip	5.0 ± 0.9a	15.9 ± 1.8a	21.0 ± 2.4a
10% Seed mix	4.8 ± 1.0a	18.2 ± 2.9a	23.0 ± 3.7a
100% Refuge	10.9 ± 1.5b	22.4 ± 3.0a	33.3 ± 4.2a
2008			
20% Block	18.5 ± 3.3a	24.7 ± 3.3ab	43.2 ± 6.3ab
20% Strip	17.8 ± 2.9a	26.0 ± 3.4ab	43.8 ± 6.2ab
20% Seed mix	11.0 ± 1.6a	17.6 ± 2.2a	28.7 ± 3.7a
10% Block	15.2 ± 2.7a	22.2 ± 3.1ab	37.4 ± 5.6a
10% Strip	10.9 ± 1.7a	15.1 ± 2.0a	26.0 ± 3.5a
10% Seed mix	15.0 ± 2.0a	21.7 ± 2.7ab	36.7 ± 4.5a
100% Refuge	28.3 ± 2.8b	33.0 ± 3.5b	61.3 ± 6.0b

Data were analyzed, after transformation, using one-way ANOVA followed by a Tukey's multiple comparison test. The letters represent the grouping for significance at a level of 0.05. Values followed by the same letter within each column are not significantly different.
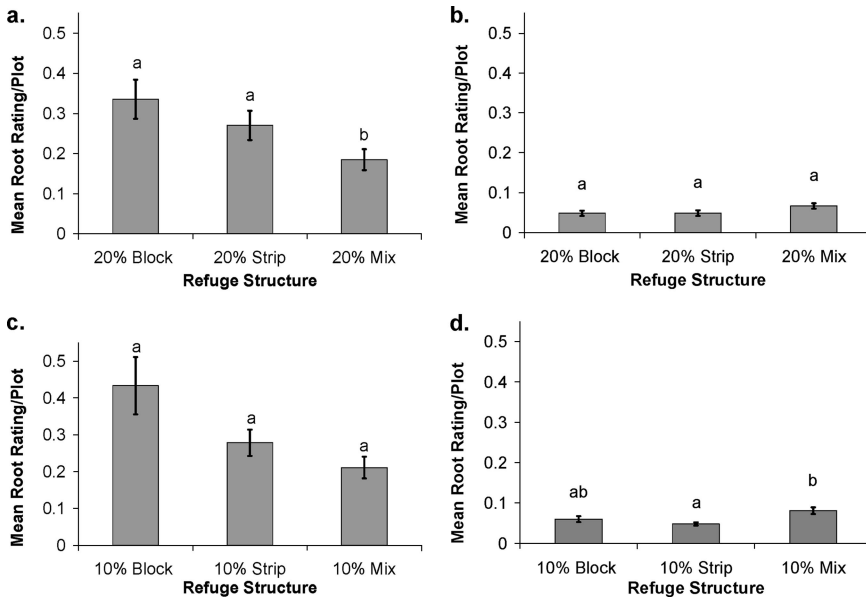
Fig. 7. Mean root rating per plot for 2007 and 2008 combined; (a) 20% refuge roots, (b) 20% *Bt*-RW roots, (c) 10% refuge roots, and (d) 10% *Bt*-RW roots. Mean rating/plot for 2007 and 2008 ±SEM. Analyzed using ANOVA followed by a Tukey's multiple comparison test.

vanced emergence from the susceptible plants is common to traditional block refuge structures and may provide males emerging from the refuge an extended

Fig. 8. The proportion of damaged roots based on proximity to the alternative hybrid (refuge or *Bt*-RW roots). (a) The proportions of damaged refuge roots, and (b) the proportions of *Bt*-RW roots that were visibly fed upon. Analyzed using a *z*-test for two proportions.

period of dispersal before females emerging from the *Bt*-RW portion of the field (Storer 2003).

The results of this study and others (Crowder et al. 2005, Storer et al. 2006) suggest that males emerging from refuges structured as blocks and strips may initially mate with females from the refuge. Moreover, males are more likely to mate early in their adult lives (Kang and Krupke 2009). In fields where structured refuges are used, females emerge from *Bt*-RW portions of the field up to 18 d after males have emerged from the refuge (Crowder et al. 2005, Storer et al. 2006). A seed mix refuge structure may maximize the likelihood that comparatively young, unmated refuge males are available to mate with females emerging from *Bt*-RW corn.

The synchronous emergence of adults from the seed mix refuge structures is likely the result of larval movement between plants (Hibbard et al. 2003, 2005). In a high-resolution study using controlled numbers of larvae, Hibbard et al. (2005) describe results that support the hypothesis that *Bt*-RW corn repelled western corn rootworm larvae. Additional support for the hypothesis that some western corn rootworm larvae may exhibit nonpreference for transgenic corn was provided by a study done by Clark et al. (2006). In this study, larvae exhibited two different behavioral responses to transgenic Cry3Bb1 corn. Some of the neonate larvae fed in a single location while others sampled root hairs as they moved around the root system. This apparent aversion described in the second behavioral response to Bt-RW corn may explain the delayed emergence of beetles from the seed mix treatment when compared with traditional refuges. We speculate that neonate larvae may move from *Bt*-RW plants to refuge plants because of nonpreference for
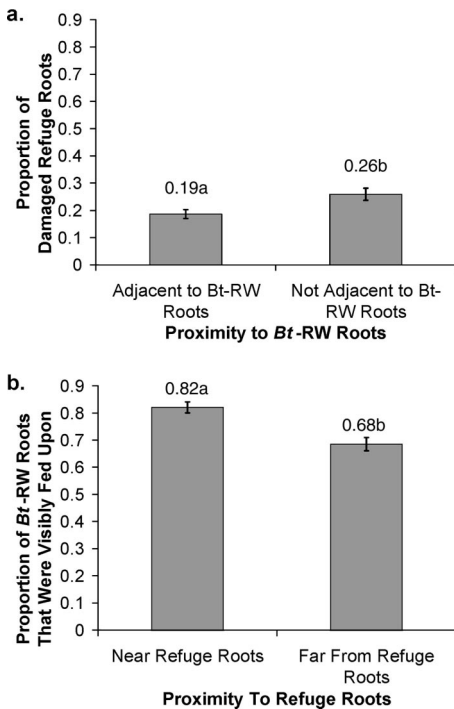
Bt-RW corn, rather than directed movement toward the refuge plants. In turn, this may have led to delayed development because of density-dependent effects and/or sublethal effects of feeding on toxic *Bt*-RW plants (Woodson 1994, Hibbard et al. 2004, Clark et al. 2006, Lefko et al. 2008). It is also possible that late-instar larvae may move from highly-damaged refuge plants to *Bt*-RW plants because the Cry3B1 toxin is less toxic to second and third instar larvae (Hibbard et al. 2005).

Concern over the movement of larvae between plants has limited the deployment of a seed mix refuge structure using *Bt* toxins (Mallet and Porter 1992, Onstad and Gould 1998, Tabashnik 1994). These concerns have historically surrounded high-dose toxins, and the toxins expressed by currently available *Bt* corn hybrids targeting western corn rootworm are not considered high-dose. A recent study by Meihls et al. (2008) revealed that forced movement from isoline to *Bt* corn (i.e., late exposure to *Bt*) could result in the more rapid evolution of resistance. In a seed mix environment, this situation may be realized when larvae that initially colonize refuge plants later move to *Bt*-expressing hybrids.

Larval movement in a seed mix environment could expose western corn rootworm larvae to sublethal doses of the *Bt* toxin at later larval instars; providing a fitness advantage to individuals with tolerant phenotypes (Mallet and Porter 1992, Gould 1998, Onstad and Gould 1998). However, more recent models by Onstad (2006) predict that larval movement may not significantly alter the estimated time required for resistance to develop in a seed mix refuge structure.

We argue that the larval movement inferred from our results (namely, the synchrony of emergence in seed mix refuge structures and the increased damage levels on *Bt*-expressing plants located adjacent to refuge plants) may threaten resistance management if 100% *Bt*-RW stands provide high levels of control of heterozygotes. Conversely, this may not be as much of a concern if 100% *Bt*-RW stands do not provide high levels of control of heterozygotes. From a resistance management perspective, the disadvantage of potential larval movement in seed mixes needs to be balanced against the advantage resulting from improving the probability of random mating between adults.

Larger refuge sizes in seed mixes lead to a larger population of susceptible individuals, providing a greater potential for movement between *Bt*-RW and refuge plants, and greater heterozygote production (Tang et al. 2001, Storer 2003, Carrière et al. 2004, Hibbard et al. 2005, Onstad 2006). However, yield is an important consideration when designing realistic refuge planting strategies. In the short-term, the economic drawbacks of a larger refuge (i.e., loss of yield because of greater feeding damage) may negate any potential long-term IRM benefits. In our study, the yield from plots planted as a seed mixture was comparable to those planted with other refuge structures. This may be because of the limitations of our study; to focus upon insect damage and emergence we used small plots that necessitated hand harvesting, which in

turn leads to large amounts of variation in the data. Future studies should use larger plots that are machine-harvested to allow for more powerful tests of this critical parameter.

There is another important limitation in extrapolating our small-plot data to a production field setting where the strip refuge is implemented. In our study the strip and block refuge structures were scaled to fit plot size, so that a strip refuge was reduced to one row (in both the 20 and 10% treatments) when it would normally be a minimum of six rows (EPA 2005b). This is important because a single-row refuge allows for a far greater proportion of larvae to encounter *Bt*-expressing plants.

Our results, obtained using *Bt*-RW corn containing the Cry3Bb1 toxin, may be applicable to Cry 34/35Ab1 toxins currently available in commercial corn hybrids, Cry 34/35Ab1, because of their similar modes of action and effects on western corn rootworm emergence and biology (Gill et al. 1992, Moellenbeck et al. 2001, Lefko et al. 2008, Meihls et al. 2008). There are also delays in emergence of western corn rootworm in response to Cry34/35Ab1 (Crowder et al. 2005, Storer et al. 2006). Additionally, both Cry3Bb1 and Cry34/35Ab1 result in female-biased sex ratios (Al-Deeb and Wilde 2005, Storer et al. 2006, Meinke et al. 2009). Despite these similarities, we recommend that empirical data be generated with these other commercial hybrids to verify if similar trends truly occur.

Our adult emergence and root rating data lend empirical support to current models for the development of resistance in western corn rootworm (Storer 2003, Onstad 2006). Our results also indicate that deployment of a seed mix refuge with the currently available toxins may present some risks for *Diabrotica* spp. resistance management (Mallet and Porter 1992, Onstad and Gould 1998). The advantages of a seed mix refuge are convenience to growers (ease of planting, compliance) and increased adult proximity of adults upon emergence. These are offset by the two main disadvantages of a seed mix refuge, namely the potential for larval movement between refuge and *Bt*-RW plants that can reduce the number of susceptible beetles while increasing the number of potential heterozygotes, and exposure of later-instar larvae to sublethal doses of *Bt* toxin. Future research is needed to quantify the sublethal dose received by larvae that move between refuge and *Bt* plants and to determine when and where during the larval life stages sublethal exposures are likely to occur.

Acknowledgments

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