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Effect of Seed Blends and Soil-Insecticide on Western and Northern Corn Rootworm Emergence from mCry3A + eCry3.1Ab Bt Maize

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ABSTRACT Seed blends containing various ratios of transgenic Bt maize (*Zea mays* L.) expressing the mCry3A + eCry3.1Ab proteins and non-Bt maize (near-isoline maize) were deployed alone and in combination with a soil applied pyrethroid insecticide (Force CS) to evaluate the emergence of the western corn rootworm, *Diabrotica virgifera virgifera* LeConte, in a total of nine field environments across the Midwestern United States in 2010 and 2011. Northern corn rootworm, *Diabrotica barberi* Smith & Lawrence emergence was also evaluated in four of these environments. Both western and northern corn rootworm beetle emergence from all Bt treatments was significantly reduced when compared with beetle emergence from near-isoline treatments. Averaged across all environments, western corn rootworm beetle emergence from 95:5, 90:10, and 80:20 seed blend ratios of mCry3A + eCry3.1Ab: near-isoline were 2.6-, 4.2-, and 6.7-fold greater than that from the 100:0 ratio treatment. Northern corn rootworm emergence from the same seed blend treatments resulted in 2.8-, 3.2-, and 4.2-fold more beetles than from the 100:0 treatment. The addition of Force CS (tefluthrin) significantly reduced western corn rootworm beetle emergence for each of the three treatments to which it was applied. Force CS also significantly delayed the number of days to 50% beetle emergence in western corn rootworms. Time to 50% beetle emergence in the 100% mCry3A + eCry3.1Ab treatment with Force CS was delayed 13.7 d when compared with western corn rootworm beetle emergence on near-isoline corn. These data are discussed in terms of rootworm resistance management.

KEY WORDS *Diabrotica virgifera virgifera*, *Diabrotica barberi*, refuge-in-a-bag, MIR604, 5307, insect resistance management, seed mix refuge

The western corn rootworm, *Diabrotica virgifera virgifera* LeConte, is one of the most serious pests of maize (*Zea mays* L.) in the United States and Europe. Although adults can injure maize plants under some circumstances (Ball 1957, Culy et al. 1992), larvae are the most economically damaging life stage. Larval feeding injury to maize roots can reduce the uptake of water and nutrients by plants (Kahler et al. 1985, Godfrey et al. 1993), increase the susceptibility of plants to lodging due to reduced brace root support (Levine and Oloumi-Sadeghi 1991), and reduce yields (Dun et al. 2010, Tinsley et al. 2013). The northern corn rootworm, *Diabrotica barberi* Smith & Lawrence, can also be a very severe pest of maize and has sometimes been the predominant pest in certain pockets of the Corn Belt such as parts of southern Minnesota. Ratios of these two species change over time due to many factors likely including weather, as northern corn rootworm eggs are more tolerant to supercooling than western corn rootworm (Ellsbury and Lee 2004).

Management options for corn rootworms in the United States have historically included rotation with a nonhost crop, the application of granular and liquid soil insecticides, and adult control measures to prevent egg-laying. Transgenic maize that expresses insecticidal Cry proteins derived from the soil bacterium *Bacillus thuringiensis* Berliner (Bt) has been developed by several seed companies as an additional management tactic to mitigate damage caused by corn rootworm larvae (Moellenbeck et al. 2001; Ellis et al. 2002; Vaughn

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et al. 2005; Walters et al. 2008, 2010). In the marketplace, there are three competing Bt maize proteins (Cry3Bb1, Cry34/35Ab1, and mCry3A) targeting corn rootworms that were originally registered for commercial sale as single events. In 2013, an additional Bt protein, eCry3.1Ab (event 5307), was registered, but only as a pyramid with mCry3A (MIR604) under the trade name Agrisure Duracade. Hibbard et al. (2011) showed that eCry3.1Ab controlled ∼99.79% of western corn rootworm larvae when averaged across five Missouri field environments, a greater efficacy than has been reported for the other rootworm-active proteins (Storer et al. 2006, Hibbard et al. 2010a, Clark et al. 2012), but still not quite achieving a "high dose" (a dose that kills 99.99% of the susceptible insects in the field) as defined by the environment protection agency (EPA, Scientific Advisory Panel 1998) for single trait events.

Given the history of adaptation by the western corn rootworm and northern corn rootworm to various management tactics such as crop rotation (Krysan et al. 1986, Levine et al. 2002, Gray et al. 2009) and some conventional insecticides (Ball and Weekman 1962, Hamilton 1965, Meinke et al. 1998), the mandate of the United States EPA that all registered Bt crops have an insect resistance management (IRM) plan was likely warranted at that time. Under the implemented strategy, a refuge of susceptible plants is maintained near the Bt crop. It is expected that susceptible insects emerging from the refuge will mate with any resistant individuals emerging from the Bt crop to produce heterozygous susceptible offspring and thus delay the evolution of pest resistance. The first IRM plans for rootworm-active Bt maize required at least a 20% block or strips (structured refuges) of non-Bt maize planted within or adjacent to the 80% of the field planted to Bt maize (Tabashnik and Gould 2012). Seed companies have now begun registering Bt products as seed blends where specific percentages of Bt and non-Bt seed are preblended in the bag sold to growers. This ensures that growers comply with refuge requirements and ensures that refuge plants (and the beetles that emerge from them) are distributed throughout the stand of Bt plants.

Various simulation models have shown that pyramiding of multiple Bt proteins in a plant may delay the evolution of pest resistance when compared with plants expressing a single protein (Zhao et al. 2003, Onstad and Meinke 2010, Ives et al. 2011). Given modeling data, pyramided products for corn rootworm control have been registered with a smaller refuge (5%). Initially, the pyramid of Cry3Bb1 and Cry34/35Ab1 (SmartStax) was registered with a 5% block or strip refuge (EPA 2009) and later as a seed blend containing 5% non-Bt (EPA 2011a). In addition, the pyramid of mCry3A and Cry34/35Ab1was registered with a 5% seed blend refuge (EPA 2011b) and most recently, the pyramid of mCry3A and eCry3.1Ab was registered with a 5% seed blend (EPA 2013).

Although simulation models have shown that combining Bt maize with an insecticide may help prolong the durability of the transgenic trait (Pan et al. 2011), only Petzold-Maxwell et al. (2013a) have evaluated the effects of insecticides combined with Bt maize on insect mortality in the field. The goal of this study was to determine the emergence of western corn rootworm and northern corn rootworm on transgenic maize expressing the mCry3A and eCry3.1Ab proteins used in various seed blends with and without the application of a soil insecticide at planting.

Materials and Methods

Study Sites and Planting. Field studies were conducted in five environments in 2010 and 2011 across the Midwestern United States (Table 1). Seed blends consisted of a ratio of percentage (%) mCry3A plus eCry3.1Ab: % near-isoline. Treatments included: (1) 80:20 seed blend, (2) 90:10 seed blend, (3) 95:5 seed blend, (4) 95:5 seed blend with insecticide treatment, (5) 100:0 seed blend, (6) 100:0 seed blend with insecticide treatment, (7) eCry3.1Ab, (8) mCry3A, (9) near-isoline (hereafter isoline), and (10) isoline with insecticide treatment. Cultural practices (tillage, fertilization, herbicide application, etc.) were typical of that recommended for agricultural procedures for each area; however, soil insecticides were only applied as part of a specific treatment. Each field site consisted of 10 treatments replicated three times (four times in Champaign Co., IL in 2010) in a randomized complete block design.

Each plot consisted of four 3.05 m long rows (3.05 m long by 3.05 m wide) planted with 20 seeds per row (80 seeds per plot) with a 76.2 cm row spacing. To ensure that corn rootworm larvae would not move between plots, a 3.05 m buffer containing no vegetation was maintained between each plot within rows and between rows of plots (at the Missouri location buffer rows between plot rows were planted to maize). Maize was hand or machine planted at various locations.

Insecticide Application. Insecticide treatments consisted of the pyrethroid soil insecticide Force CS (Tefluthrin, Syngenta Crop Protection, Greensboro, NC) applied at the time of planting (Table 1). Equipment varied between locations, but the recommended rate of 11.8 ml of liquid Force CS was applied per 304.8 m (0.46 fluid ounces per 1,000 row ft).

Table 1. Important dates associated with the 2010 and 2011 field study

Environment	Planting	Tent set-up	First beetle collected
2010			
Tippecanoe Co., IN	April 30	June 18	June 25
Champaign Co., IL	May 28	July 1	July 5
Boone Co., MO	April 21	June 29	July 6
Story Co., IA	May 5	June 29	July 3
Kossuth Co., IA	May 18	July 16	July 23
2011			
Tippecanoe Co., IN	May 13	June 24	June 27
Champaign Co., IL	May 12	June 24	July 5
Boone Co., MO	May 4	June 29	July 11
Story Co., IA	May 11	June 30	July 6
Saunders Co., NE	May 17	June 30	July 6

Insects and Infestation. With the exception of the Missouri field sites, a trap crop of late planted maize (with pumpkins at some sites) was sown in each site in the years prior to the study to ensure a natural infestation of corn rootworm larvae the following year. In Missouri, a nonhost crop consisting of soybean, *Glycine max* (L.), was planted during both years prior to the study. Because central Missouri does not have rotation-resistant strains of corn rootworm that oviposit outside of maize in soybeans, all plots were artificially infested with western corn rootworm eggs obtained from the main diapausing colony of the USDA-ARS laboratory in Brookings, SD. For each year of the study, plots were infested at a rate of 1,000 eggs per 30.5 cm of row when maize had reached the V2-V3 stage (Ritchie et al. 2008). The total number of eggs needed for each plot was suspended into 400 ml of 0.15% agar solution. Trenches (5 cm deep) were dug on each side of each row using hoes, and 50 ml of egg solution was distributed evenly along the length of each side of each row. Percentage egg hatch was monitored in the laboratory from a subsample of eggs and averaged 83.8% in 2010 and 75.7% in 2011. Total viable eggs for each respective four row plot in the Missouri sites were ~30,840 for 2010 and ~30,280 for 2011.

Placement of Screen Tents and Beetle Collection. Emerging corn rootworm beetles from treatment plots were contained using screen tents (~3.4 by 4.0 m) that were placed over each individual plot. The bottom edges of each tent were buried 8–12 cm below the soil surface to prevent beetle escape and to securely anchor the tents against strong winds. Shortly after the first beetle emergence date (Table 1), all maize plants in tents at Missouri locations were stripped of leaves except for four central plants. The intact plants were left in tents to feed emerging corn rootworm beetles between collection dates and to concentrate beetles for easier collection. At other locations plants were cut just above the growing point of the plant. This height ranged from as short as 30 cm at the Illinois location to ~60 cm at the Nebraska location, where any new ear formation was also removed. Beetles were collected 2–3 times weekly from each tent using either mouth or battery operated aspirators (Catalog #s 1135A and 2820B, respectively, BioQuip, Rancho Dominguez, CA). Upon collection, beetles from each tent were placed in labeled containers and brought back to the laboratory where total beetle number, gender, and dry weight were recorded for each respective emergence tent.

Data Analysis. In order to meet the assumptions of normality and homogeneity of variances, data on beetle numbers and dry weight were log $(x + 0.1)$ transformed prior to analysis. Data for dry weight were considered as missing values in the analysis when no beetles were recovered from a particular tent. Data were analyzed using the generalized linear mixed model of the SAS statistical package (PROC GLIMMIX; SAS Institute 2008). The statistical model contained the main plot effect of environment, the subplot of maize treatment, the interaction of environment × treatment, the sub-subplot of gender, and the interactions of gender with all possible effects of the main plot and subplot. Replication within environment was the denominator of F for the main plot effects, replication within environment and treatment was the denominator of F for the subplot effects, and the residual mean square was the denominator of F for the sub-subplot effects. Least squares means (LSMEANS) of fixed effects were calculated separately for each environment and comparisons were performed using the t-test output of the SAS model. The western and northern corn rootworm data were analyzed separately. Results from all tests were considered statistically different at $P < 0.05$. Beetle emergence data were analyzed by plotting observed cumulative probabilities versus Julian dates. PROC PROBIT of the SAS statistical package (SAS Institute 2008) was used to model the occurrence of 50% beetle emergence and the 95% confidence intervals (CIs) among maize treatments.

Results

The number of western corn rootworm beetles emerging from tents varied significantly across environment, maize treatment, gender, and all possible two-factor interactions (Tables 2 and 3). The three-factor interaction of environment × treatment × gender was marginally significant at $P = 0.0507$ (Table 2). When averaged across years, environments, and genders, significantly more western corn rootworm beetles emerged from isoline maize than from all other treatments (Fig. 1A and B). Significantly fewer western corn rootworm beetles emerged from the 100:0 treatment (100% mCry3A + eCry3.1Ab) coupled with Force CS than from all other treatments (Fig. 1A and B). Overall, there was a slight, nonsignificant female bias in the number of beetles recovered from transgenic treatments (Fig. 1B). Gender and the interaction of gender and treatment were significant in the overall analysis (Table 2). The few significant differences between the number of female and male beetles that emerged from specific treatments were from the Bt plus insecticide treatments and the eCry3.1Ab treatment (Fig. 1B). The weight of western corn rootworm beetles that emerged from tents varied significantly among environment, maize treatment, gender, and all possible interactions except maize treatment × gender (Table 2). Overall, mean weights were greater for those beetles recovered from isoline treatments compared with transgenic treatments (Fig. 2A), and females generally weighed

Table 2. ANOVA table for factors impacting the number and dry weight of western corn rootworm (WCR) beetles recovered from tents during the study

Effect	WCR no.			WCR wt.		
	df	F	P	df	F	P
Environment (Env)	8, 19	54.99	<0.0001	8, 19	46.46	<0.0001
Treatment (Trt)	9, 171	85.69	<0.0001	9, 141	10.64	<0.0001
Env × Trt	72, 171	2.10	<0.0001	68, 141	3.49	<0.0001
Gender	1, 190	19.18	<0.0001	1, 132	44.55	<0.0001
Env × gender	8, 190	27.58	<0.0001	8, 132	3.71	0.0006
Trt × gender	9, 190	2.95	0.0027	9, 132	1.46	0.1710
Env × Trt × gender	72, 190	1.36	0.0507	62, 132	1.83	0.0020

Table 3. Mean (±SE) number of female, male and total WCR beetles recovered from tents in each environment in 2010 and 2011

Treatment	2010			2011		
	Female no.	Male no.	Total no.	Female no.	Male no.	Total no.
Tippecanoe Co., IN						
80:20	36.7 ± 11.8 bcd	16.3 ± 6.8 bcd	53.0 ± 18.3 bcd	60.3 ± 20.1bc	88.0 ± 25.0 bc	148.3 ± 45.0 bc
90:10	21.7 ± 5.4 bcde	12.3 ± 2.7 cde	34.0 ± 8.0 cd	43.7 ± 40.2 cd	69.3 ± 58.9 cd	113.0 ± 99.0 cd
95:5	14.3 ± 4.9 cde	4.7 ± 1.5 def	19.0 ± 6.4 de	37.0 ± 17.0 cd	44.3 ± 20.2 cd	81.3 ± 37.0 cd
95:5 + Force	3.7 ± 1.2 ef	2.0 ± 0.6 ef	5.7 ± 0.7 e	6.7 ± 3.5 de	16.0 ± 12.1 de	22.7 ± 15.5 de
100:0	4.7 ± 1.7 ef	1.0 ± 0.0 fg	5.7 ± 1.7 ef	11.3 ± 3.2 cd	19.7 ± 8.8 cde	31.0 ± 11.9 cd
100:0 + Force	2.0 ± 1.5 f	0.0 ± 0.0 g	2.0 ± 1.5 f	1.3 ± 0.9 e	3.0 ± 1.2 e	4.3 ± 0.9 e
eCry3.1Ab	7.0 ± 2.5 de	4.7 ± 0.9 def	11.7 ± 2.6 de	17.7 ± 4.3 cd	15.7 ± 5.9 cde	33.3 ± 10.2 cd
mCry3A	69.3 ± 22.5 abc	40.7 ± 9.9 bc	110.0 ± 31.2 c	99.7 ± 67.7 bc	136.7 ± 103.9 bc	236.3 ± 171.4 bc
Isoline	376.7 ± 74.7 a	264.7 ± 54.9 a	641.3 ± 129.5 a	723.7 ± 444.7 a	803.7 ± 501.8 a	1527.3 ± 946.5 a
Isoline + Force	129.0 ± 22.3 ab	88.3 ± 11.3 ab	217.3 ± 30.7 ab	305.7 ± 120.0 ab	360.7 ± 157.5 ab	666.3 ± 277.2 ab
Champaign Co., IL						
80:20	52.0 ± 19.2 ab	6.5 ± 1.8 ab	58.5 ± 20.7 ab	213.3 ± 30.7 abc	152.3 ± 15.7 bcd	365.7 ± 46.3 bc
90:10	13.8 ± 5.6 bc	2.5 ± 1.2 bc	16.3 ± 6.3 bc	165.3 ± 37.2 bcd	88.0 ± 18.1 bcde	253.3 ± 51.6 bcd
95:5	19.5 ± 11.9 bc	3.5 ± 1.9 bc	23.0 ± 13.8 bc	81.7 ± 25.3 bcde	50.7 ± 11.6 cdef	132.3 ± 36.1 cde
95:5 + Force	13.8 ± 6.5 bc	2.3 ± 0.9 bc	16.0 ± 7.2 bc	17.7 ± 2.3 ef	10.3 ± 3.4 fg	28.0 ± 5.7 ef
100:0	4.0 ± 3.3 d	1.5 ± 1.2 cd	5.5 ± 4.5 d	31.3 ± 10.2 def	18.7 ± 5.5 efg	50.0 ± 14.4 def
100:0 + Force	3.0 ± 0.4 cd	0.0 ± 0.0 d	3.0 ± 0.4 d	18.0 ± 12.1 f	3.7 ± 1.2 g	21.7 ± 13.2 f
eCry3.1Ab	4.3 ± 1.4 cd	1.3 ± 0.6 c	5.5 ± 2.0 cd	60.3 ± 24.4 cdef	34.7 ± 8.7 def	95.0 ± 33.0 cde
mCry3A	2.0 ± 0.7 d	1.3 ± 0.8 cd	3.3 ± 1.3 cd	402.0 ± 107.2 ab	307.7 ± 67.1 abc	709.7 ± 169.8 ab
Isoline	169.0 ± 14.5 a	26.0 ± 3.8 a	195.0 ± 17.2 a	1152.0 ± 179.8 a	1198.0 ± 315.9 a	2350.0 ± 494.5 a
Isoline + Force	115.8 ± 66.8 a	16.0 ± 10.7 a	131.8 ± 77.5 a	393.3 ± 5.0 ab	397.0 ± 54.6 ab	790.3 ± 59.0 ab
Boone Co., MO						
80:20	34.3 ± 6.1 bc	44.7 ± 8.2 bc	79.0 ± 11.7 bc	8.3 ± 1.2 bc	21.7 ± 4.1 bc	30.0 ± 4.4 b
90:10	11.0 ± 7.6 cd	15.7 ± 7.2 cd	26.7 ± 14.6 cd	1.7 ± 1.2 d	7.3 ± 2.4 cd	9.0 ± 3.6 cd
95:5	4.0 ± 1.7 d	5.7 ± 3.2 de	9.7 ± 3.8 de	0.0 ± 0.0 e	3.0 ± 0.6 de	3.0 ± 0.6 de
95:5 + Force	0.3 ± 0.3 e	1.3 ± 0.9 ef	1.7 ± 1.2 fg	1.3 ± 0.9 d	1.0 ± 0.6 ef	2.3 ± 1.5 de
100:0	0.7 ± 0.7 e	0.3 ± 0.3 fg	1.0 ± 1.0 fg	0.0 ± 0.0 e	0.0 ± 0.0 f	0.0 ± 0.0 f
100:0 + Force	0.0 ± 0.0 e	0.0 ± 0.0 g	0.0 ± 0.0 g	0.0 ± 0.0 e	0.0 ± 0.0 f	0.0 ± 0.0 f
eCry3.1Ab	2.0 ± 2.0 e	2.7 ± 1.2 de	4.7 ± 3.2 ef	0.0 ± 0.0 e	0.3 ± 0.3 ef	0.3 ± 0.3 ef
mCry3A	53.0 ± 18.7 b	45.3 ± 10.7 bc	98.3 ± 29.5 b	4.7 ± 1.9 cd	22.0 ± 6.7 c	26.7 ± 8.2 bc
Isoline	398.0 ± 163.3 a	626.3 ± 265.9 a	1024.3 ± 427.2 a	161.0 ± 63.3 a	737.0 ± 274.8 a	898.0 ± 338.2 a
Isoline + Force	112.7 ± 20.9 ab	161.0 ± 12.3 ab	273.7 ± 28.7 ab	44.0 ± 20.0 ab	109.3 ± 59.4 ab	153.3 ± 79.3 a
Story Co., IA						
80:20	21.0 ± 17.5 abc	11.7 ± 10.7 bcd	32.7 ± 28.2 ab	5.0 ± 1.5 b	3.7 ± 2.7 b	8.7 ± 3.8 b
90:10	13.0 ± 11.0 abc	5.7 ± 3.7 bcd	18.7 ± 14.7 ab	0.7 ± 0.3 cd	1.0 ± 0.6 bc	1.7 ± 0.7 c
95:5	13.3 ± 10.9 bcd	13.0 ± 12.5 cde	26.3 ± 23.4 b	1.7 ± 1.7 cd	2.0 ± 2.0 bc	3.7 ± 3.7 cd
95:5 + Force	0.7 ± 0.7 e	0.0 ± 0.0 f	0.7 ± 0.7 c	1.7 ± 1.7 cd	0.7 ± 0.7 c	2.3 ± 2.3 cd
100:0	5.0 ± 3.2 cde	6.3 ± 3.3 de	11.3 ± 5.8 b	0.3 ± 0.3 cd	0.7 ± 0.7 c	1.0 ± 0.6 cd
100:0 + Force	7.3 ± 4.4 abc	1.7 ± 1.7 ef	9.0 ± 6.0 b	0.0 ± 0.0 d	0.0 ± 0.0 c	0.0 ± 0.0 d
eCry3.1Ab	0.7 ± 0.3 de	0.0 ± 0.0 f	0.7 ± 0.3 c	2.7 ± 1.5 bc	0.3 ± 0.3 c	3.0 ± 1.7 cd
mCry3A	19.7 ± 6.7 a	11.3 ± 0.9 ab	31.0 ± 7.5 a	3.0 ± 3.0 cd	3.0 ± 1.5 b	6.0 ± 4.5 bc
Isoline	76.3 ± 61.4 a	76.7 ± 66.7 a	153.0 ± 127.9 a	48.7 ± 15.3 a	26.7 ± 11.8 a	75.3 ± 26.9 a
Isoline + Force	17.7 ± 8.2 ab	11.3 ± 4.8 abc	29.0 ± 12.8 a	49.0 ± 11.7 a	31.7 ± 4.3 a	80.7 ± 16.0 a
Saunders Co., NE						
80:20	-	-	-	33.7 ± 5.5 bcd	34.7 ± 6.0 bcd	68.3 ± 3.4 bcd
90:10	-	-	-	28.3 ± 6.2 bcd	30.0 ± 11.8 bcd	58.3 ± 18.0 cd
95:5	-	-	-	20.3 ± 3.5 cd	17.3 ± 2.4 cd	37.7 ± 5.8 cde
95:5 + Force	-	-	-	15.7 ± 0.3 cd	11.7 ± 2.4 cde	27.3 ± 2.7 cde
100:0	-	-	-	11.3 ± 2.8 d	10.3 ± 4.3 de	21.7 ± 6.9 de
100:0 + Force	-	-	-	7.0 ± 2.5 d	7.7 ± 4.6 e	14.7 ± 7.1 e
eCry3.1Ab	-	-	-	29.3 ± 10.2 bcd	16.0 ± 2.0 cd	45.3 ± 12.1 cde
mCry3A	-	-	-	75.0 ± 17.5 abc	68.0 ± 19.3 abc	143.0 ± 34.4 abc
Isoline	-	-	-	344.7 ± 12.5 a	281.0 ± 91.0 a	625.7 ± 214.2 a
Isoline + Force	-	-	-	158.0 ± 40.1 ab	149.0 ± 19.3 ab	307.0 ± 52.4 ab

Different lowercase letters indicate significant differences ($P < 0.05$) between treatments within a column and location.

more than males (Fig. 2B), though an estimate of LSMEANS was not possible because of the large number of missing values (beetles were not always recovered, especially from transgenic treatments).

When averaged across years, environments, and gender, there was nearly a 14-d delay in time to 50% emergence from the 100:0 treatment coupled with Force CS compared with isoline maize as calculated by PROC PROBIT (Table 4). CIs (95%) overlapped for just three combinations: the 95:5 seed blend and 90:10 seed blend; 100:0 seed blend and eCry3.1Ab; and 100:0 seed blend and the 95:5 seed blend coupled with Force CS. The 50% emergence date was significantly different for all other treatments in the combined analysis.

The number of northern corn rootworm beetles emerging from tents varied significantly among environment, maize treatment, environment × maize treatment, and environment × gender (Tables 5 and 6). When averaged across years, environments, and gender, significantly more northern corn rootworm beetles

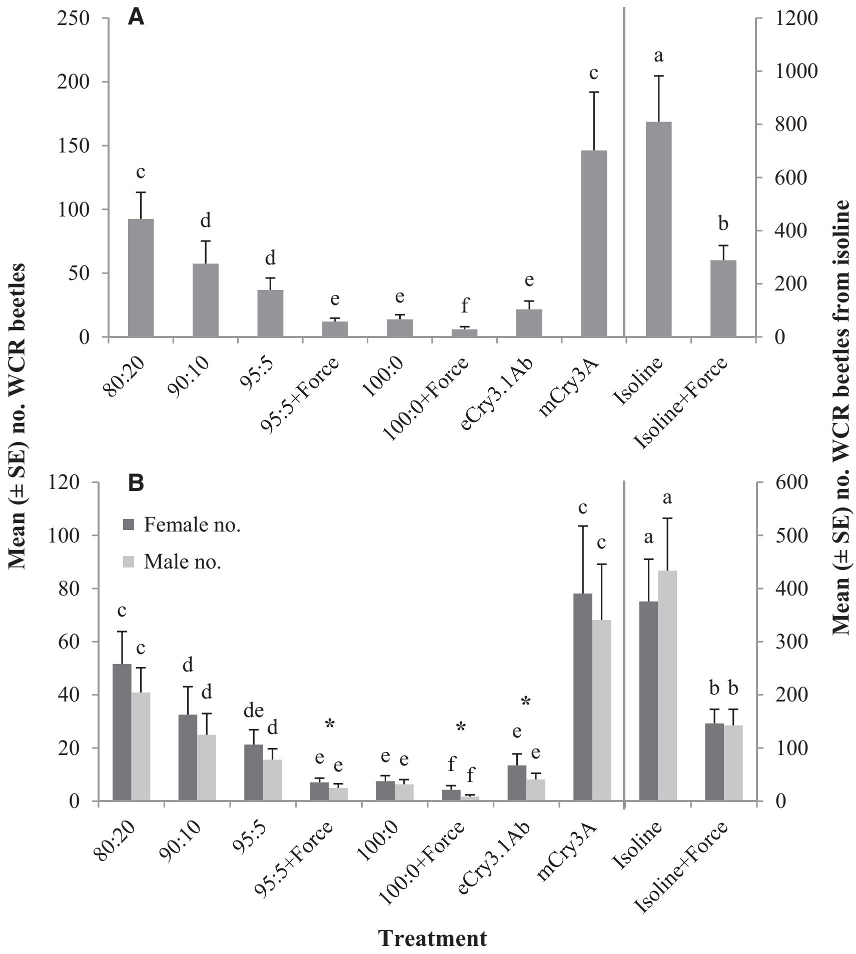

Fig. 1. Mean (±SE) number of western corn rootworm beetles recovered from maize treatments (**A**) data averaged across years, environments, and gender (**B**) data averaged across years and environment. Lowercase letters indicate comparisons among maize treatments within each gender. The vertical line indicates a switch from the left axis to the right axis. The * indicate significant differences between gender within treatment.

emerged from isoline treatments compared with transgenic treatments (Fig. 3). In addition, significantly fewer northern corn rootworm beetles emerged from the 100:0 treatment and 95:5 seed blend coupled with Force CS than from all other maize treatments except for the 100:0 treatment coupled with Force CS and eCry3.1Ab treatments (Fig. 3). The weight of northern corn rootworm beetles that emerged from tents varied significantly among environment and gender (Table 5). Treatment was marginally significant at $P = 0.0553$ (Table 5). Female beetles weighed significantly more than male beetles overall but this difference was not significant within the 100:0 treatment, 100:0 treatment coupled with Force CS, 90:10 seed blend, mCry3A, and isoline treatments (Fig. 4).

Discussion

Relatively few studies involving comparisons of beetle emergence from seed blend refuges to block refuges have been published in the refereed literature. Petzold-Maxwell et al. (2013b) compared blended refuges of near-isoline and Cry34/35Ab1 to pure stands of non-Bt and Cry34/35Ab1. In their study, rootworm survival in the blended refuge treatments was not significantly greater than survival in the pure stand of Bt maize. Head et al. (2014a) conducted a series of laboratory and field experiments comparing mixtures of non-Bt seed and seed containing Cry3Bb1 + Cry34/35Ab1. Again, there was no significant difference in western corn rootworm beetle emergence between the blended refuge and the pure Bt stand. Both studies also evaluated insecticidal seed treatments, but in general, seed treatments did not significantly impact beetle emergence. Both Cry34/35Ab1 and Cry3Bb1 + Cry34/35Ab1 significantly delayed 50% beetle emergence when compared with the pure non-Bt refuge (Petzold-Maxwell et al. 2013b, Head et al. 2014a). In this study, when averaged across gender and all environments, significantly more beetles of both western and northern

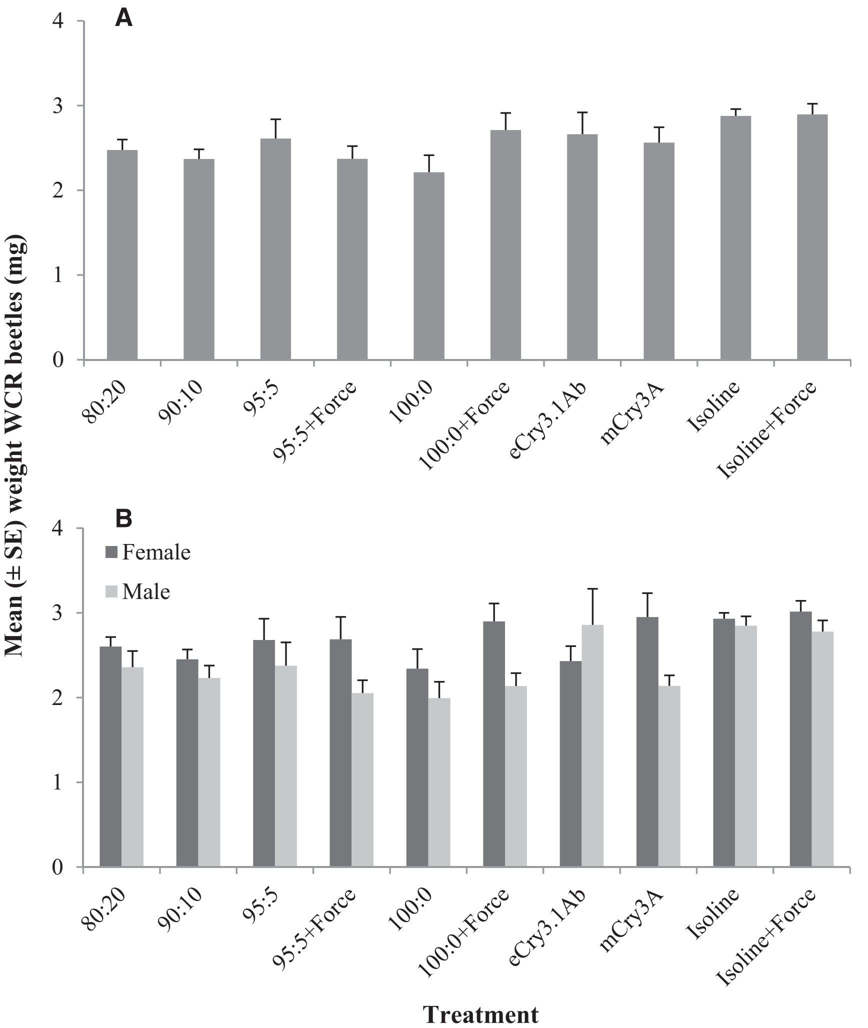

Fig. 2. Mean (±SE) weight of western corn rootworm beetles recovered from maize treatments (**A**) data averaged across years, environments, and gender (**B**) data averaged across years and environment.

Table 4. Mean Julian date for 50% emergence of WCR beetle emergence when averaged across 2 yr, 5 environments, and 28 total replications

Treatment	50% emergence	95% confidence interval
Isoline	196.23 h	196.196–196.26
Isoline + Force	197.00 g	196.938–197.056
80:20	201.64 f	201.531–201.757
mCry3A	202.88 e	202.795–202.964
95:5	203.39 d	203.213–203.577
90:10	203.58 d	203.425–203.726
95:5 + Force	205.67 c	205.317–206.030
100:0	205.94 bc	205.659–206.213
eCry3.1Ab	206.29 b	206.051–206.528
100:0 + Force	209.99 a	209.401–210.369

Different lowercase letters indicate significant differences ($P < 0.05$).

corn rootworm emerged from the 5% seed blend refuges when compared with the pure pyramid treatment (Figs. 1A and 4). For western corn rootworm, an average of 14.1 beetles per plot emerged from 100% eCry3.1Ab + mCry3A. Significantly more western corn rootworm beetles emerged from the 5% seed blend when male and female data were combined (Fig. 1A).

Table 5. ANOVA table for factors impacting the number and dry weight of northern corn rootworm (NCR) beetles recovered from tents during the study

Effect	NCR no.			NCR wt.		
	df	*F*	*P*	df	*F*	*P*
Environment (Env)	3, 8	16.93	0.0008	3, 8	13.91	0.0015
Treatment (Trt)	9, 72	14.46	<0.0001	9, 63	1.99	0.0553
Env × Trt	27, 72	1.69	0.0410	27, 63	1.26	0.2230
Gender	1, 80	0.69	0.4080	1, 59	41.63	<0.0001
Env × gender	3, 80	7.62	0.0002	3, 59	0.59	0.6220
Trt × gender	9, 80	1.80	0.0818	9, 59	1.05	0.4140
Env × Trt × gender	27, 80	0.87	0.6516	27, 59	1.17	0.3021

Table 6. Mean (±SE) number of female, male, and total NCR beetles recovered from tents in each environment in 2010 and 2011

Treatment	2010			2011		
	Female no.	Male no.	Total no.	Female no.	Male no.	Total no.
Story Co., IA						
80:20	6.0 ± 4.0 ab	11.7 ± 9.2 abc	17.7 ± 13.2 ab	1.3 ± 0.9 b	2.0 ± 0.6 b	3.3 ± 1.2 b
90:10	3.7 ± 1.9 bc	4.7 ± 2.6 bcd	8.3 ± 4.4 bc	4.0 ± 2.1 b	1.0 ± 0.6 bc	5.0 ± 2.5 b
95:5	9.7 ± 7.3 ab	14.0 ± 7.9 ab	23.7 ± 15.2 ab	1.0 ± 0.0 b	1.0 ± 0.6 bc	2.0 ± 0.6 b
95:5 + Force	2.3 ± 1.2 bcd	0.3 ± 0.3 e	2.7 ± 1.3 cd	1.3 ± 0.7 b	0.3 ± 0.3 c	1.7 ± 0.9 b
100:0	0.3 ± 0.3 d	0.3 ± 0.3 e	0.7 ± 0.3 d	2.3 ± 1.5 b	0.3 ± 0.3 c	2.7 ± 1.2 b
100:0 + Force	7.0 ± 3.6 ab	4.7 ± 4.2 cde	11.7 ± 6.4 bc	3.3 ± 1.2 ab	0.7 ± 0.3 bc	4.0 ± 1.5 b
eCry3.1Ab	0.7 ± 0.7 cd	1.7 ± 1.2 de	2.3 ± 1.9 cd	2.3 ± 1.3 b	0.7 ± 0.7 c	3.0 ± 1.2 b
mCry3A	7.7 ± 3.2 ab	5.0 ± 2.1 abcd	12.7 ± 5.2 ab	4.0 ± 2.1 ab	2.0 ± 1.5 bc	6.0 ± 3.6 b
Isoline	13.0 ± 4.7 a	31.7 ± 12.6 a	44.7 ± 15.3 a	14.7 ± 5.8 a	19.3 ± 6.0 a	34.0 ± 11.8 a
Isoline + Force	6.7 ± 3.5 ab	9.7 ± 1.2 abc	16.3 ± 4.7 ab	12.0 ± 0.6 a	17.0 ± 1.0 a	29.0 ± 1.2 a
Environment 2[a]						
80:20	27.3 ± 5.7 bc	22.3 ± 5.0 bc	49.7 ± 10.6 bc	10.0 ± 4.0 a	13.0 ± 1.0 a	23.0 ± 4.6 a
90:10	19.0 ± 5.0 bcd	12.0 ± 3.1 bcd	31.0 ± 8.0 cd	7.0 ± 2.0 a	16.3 ± 3.7 a	23.3 ± 1.7 a
95:5	12.0 ± 3.5 cde	8.3 ± 1.2 cde	20.3 ± 4.5 cde	5.7 ± 1.5 a	6.7 ± 1.7 a	12.3 ± 3.0 a
95:5 + Force	4.7 ± 1.2 cdef	3.7 ± 1.8 def	8.3 ± 3.0 def	8.3 ± 1.9 a	7.7 ± 1.2 a	16.0 ± 1.7 a
100:0	1.7 ± 0.7 f	1.7 ± 0.9 f	3.3 ± 1.2 f	5.0 ± 1.0 a	9.3 ± 1.8 a	14.3 ± 2.6 a
100:0 + Force	3.0 ± 1.0 ef	1.7 ± 0.3 ef	4.7 ± 1.3 ef	4.3 ± 1.5 a	7.0 ± 0.6 a	11.3 ± 0.9 a
eCry3.1Ab	3.3 ± 0.3 def	2.7 ± 0.3 def	6.0 ± 0.6 def	9.0 ± 2.6 a	17.0 ± 4.4 a	26.0 ± 3.0 a
mCry3A	18.0 ± 9.3 cde	12.7 ± 2.3 bcd	30.7 ± 10.2 cd	10.0 ± 3.8 a	15.3 ± 1.9 a	25.3 ± 5.6 a
Isoline	251.3 ± 66.7 a	189.0 ± 50.8 a	440.3 ± 117.5 a	12.7 ± 4.3 a	29.7 ± 5.5 a	42.0 ± 7.6 a
Isoline + Force	112.0 ± 50.5 ab	92.0 ± 56.3 ab	204.0 ± 106.7 ab	7.0 ± 1.7 a	18.0 ± 4.5 a	25.0 ± 6.1 a

[a]Environment 2; Kossuth Co., IA in 2010 and Saunders Co., NE in 2011.
Different lowercase letters indicate significant differences ($P < 0.05$) between treatments within a location and column.

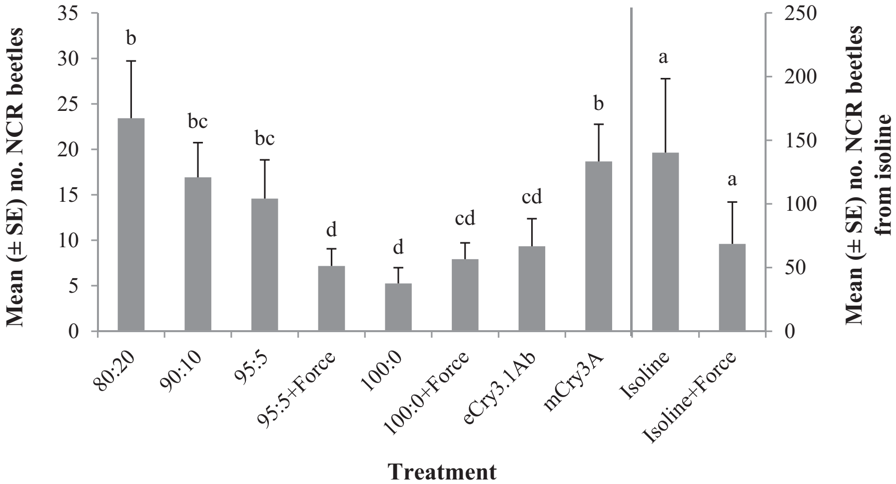

Fig. 3. Mean (±SE) number of northern corn rootworm beetles recovered from maize treatments when averaged across years, environments, and gender. The vertical line indicates a switch from the left axis to the right axis.

The 5% blended refuge produced 2.6-fold more western corn rootworm beetles than the pure pyramid, while the 10–20% seed blends had 4.2- and 6.7-fold more beetles emerged, respectively (Fig. 1A). To put this in perspective, an average of 832.2 western corn rootworm beetles per tent were produced from 100% near isoline (Fig. 1A). A pure stand of near-isoline refuge corn that was 5% of this area would produce 5% of 832.2 or 41.6 beetles, but these beetles would emerge a considerable distance away from any beetles produced from a pure stand of Bt if block refuges were utilized. In this study, an average of 37.3 western corn rootworm beetles per tent were produced from the 95:5 seed blend (Fig. 1A). This is nearly 90% of the beetles from a similar number of near-isoline plants in a pure stand, but emerging in the same vicinity as any beetles from Bt. Although only 2.6-fold more beetles emerged from 5% near-isoline seed blend than from the 100% eCry3.1Ab + mCry3A, unlike the 5% seed blend for SmartStax (Head et al. 2014a) and Cry34/35Ab alone (Petzold-Maxwell et al. 2013b), this number was significantly more than the number of beetles from the pure pyramid (Fig. 1A). However, some of these actually emerged from the 95% Bt rather than

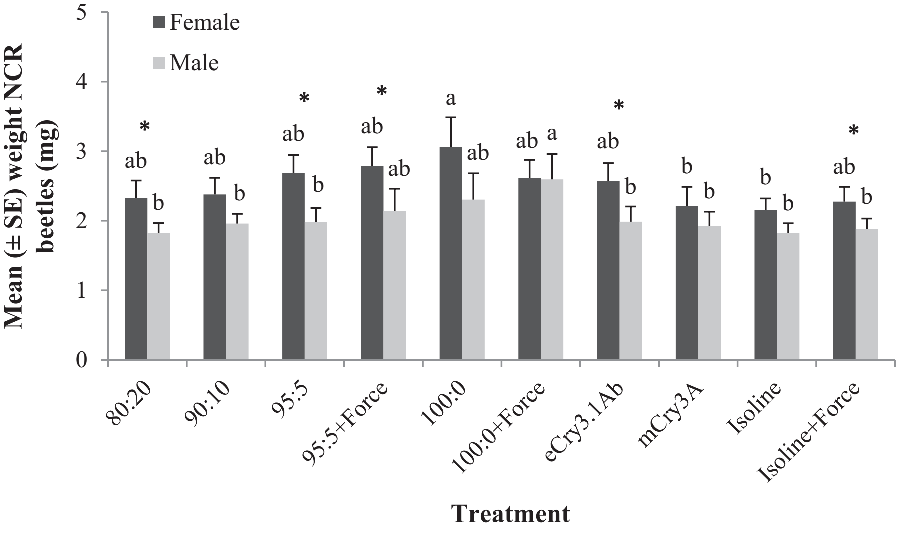

Fig. 4. Mean (±SE) weight of northern corn rootworm beetles recovered from maize treatments when averaged across years and environment. Letters indicate comparisons among maize treatments within each gender. * indicate significant differences between gender within treatment.

the 5% refuge, so the number that likely emerged from just isoline plants might be closer to 24 [37.3 from the 5% seed blend—number expected from Bt plants (0.95 × 14.1)].

The high dose refuge strategy for IRM in single trait events involves planting Bt crops that produce a high concentration of toxin (a minimum of 99.99% mortality in the field) to ensure that individuals that are heterozygous for resistance do not survive on the Bt crop, thus making resistance functionally recessive (EPA, Scientific Advisory Panel 1998). None of the previously registered individual rootworm-active Bt products express a concentration of Cry proteins considered high-dose (Storer et al. 2006, Hibbard et al. 2010a, Clark et al. 2012, Head et al. 2014b), effectively making the strategy for single trait rootworm products simply a refuge strategy. According to Roush (1998) "much can be gained from pyramiding if the mortality of susceptible insects [to individual components] is consistently >95%." Previously, Hibbard et al. (2011) showed that eCry3.1Ab and mCry3A caused a reduction in western corn rootworm emergence of 99.79% and 97.83% when compared with emergence from isoline maize in the specific western corn rootworm populations and the environments evaluated, suggesting that pyramiding would be useful. Efficacy of rootworm-active Bt maize can vary with environment and insect population (Storer et al. 2006; Hibbard et al. 2010a, 2011; Clark et al. 2012). In this study, the 100:0 treatment (100% eCry3.1Ab + mCry3A) caused a greater reduction in beetle emergence than each protein used separately (98.3% compared with 97.4% for eCry3.1Ab and 81.9% for mCry3A), but none of the treatments caused as much of a reduction in beetle emergence as from similar treatments in Hibbard et al. (2011). Because egg densities in most locations were unknown in this study, it is possible that density-dependent mortality reduced emergence on isoline corn in some locations resulting in an underestimate of mortality due to the Bt toxins (Hibbard et al. 2010b). It is also possible that the strain used by Hibbard et al. (2010b) was more susceptible than most of the field strains evaluated here. Pyramiding mCry3A and eCry3.1Ab will be effective in delaying resistance as long as cross resistance is not present (Roush 1998).

Petzold-Maxwell et al. (2013a) and Tinsley et al. (2015) concluded that combining insecticide with Bt maize did not increase yield or reduce root injury to Bt maize. Furthermore, Petzold-Maxwell et al. (2013a) concluded that delays in emergence from Bt maize combined with insecticides could promote assortative mating among Bt-selected individuals, which may hasten resistance evolution. We did not collect yield or plant injury information, but in our study, insecticides significantly increased delays in emergence compared with Bt maize alone. The greatest delay in time to 50% emergence was from the 100:0 treatment coupled with Force CS (13.8 d relative to 50% emergence from near-isoline). The delayed emergence of western corn rootworm beetles from Bt fields relative to refuge fields is a commonly observed phenomenon. Hibbard et al. (2011) showed that there was an 8.0 d delay in time to 50% emergence for western corn rootworm beetles from eCry3.1Ab compared with isoline maize, but for eCry3.1Ab + mCry3A the delay was 4.6 d. Although this study similarly showed a greater delay in the time to 50% emergence for eCry3.1Ab (10.1 d) compared with the 100:0 treatment (9.7 d), delays in beetle emergence were greater than those reported previously by Hibbard et al. (2011). The addition of Force CS exacerbated this delay and would make the risk of assortative mating greater if used on pure stand pyramid maize and to a lesser extent, for seed blends as well. The addition of insecticides also increased western corn rootworm mortality compared with Bt maize alone. The 95:5 seed blend and 100:0 treatment coupled with Force CS caused a reduction in western corn rootworm beetle emergence of 98.6–99.3%, respectively, versus 95.5–98.3%, respectively, for these same treatments without insecticide. When averaged across the locations of the present study, the addition of Force CS significantly affected western corn rootworm beetle emergence for each of treatments to which it was applied (Fig. 1), but this was not the case for northern corn rootworm (Fig. 3). Gray et al. (1992) found that soil insecticide may actually result in greater beetle emergence in some instances when damage to nontreated control plots is so high that density-dependent mortality may be occurring. Apparently, density-dependent mortality (Hibbard et al. 2010b) was less for western corn rootworm under the current conditions than for Gray et al. (1992). Northern corn rootworm is highly sensitive to interspecific competition from western corn rootworm (Woodson 1994), so density effects may have disproportionately affected the insecticide data for this species.

In total, 1,683 northern corn rootworm beetles emerged from isoline corn in 2010 and 2011. In comparison, a total of 63 northern corn rootworm beetles emerged from the 100:0 treatment and 112 beetles emerged from the eCry3.1Ab treatment, which was significantly fewer beetles than all other treatments except both the 100:0 and 95:5 seed blend treatments coupled with Force CS (95 and 86 beetles, respectively). From the information available herein and Hibbard et al. (2011), the eCry3.1Ab protein and especially eCry3.1Ab + mCry3A proteins appear to be similarly effective in controlling northern corn rootworm as western corn rootworm, which is not the case for all rootworm Bt products (Head et al. 2014a).

Overall, we found a significantly greater number of both western and northern corn rootworm beetles emerging from a 5% seed blend of near-isoline with mCry3A + eCry3.1Ab refuge than from a pure stand of mCry3A + eCry3.1Ab. The timing of 50% beetle emergence was significantly delayed and the total number of beetles emerged was significantly reduced when the recommended rate of Force CS was added, suggesting this insecticide should not be used with pure stand pyramid corn or 5% seed blends from a resistance management perspective due to increased risk of assortative mating. Given the history of western corn rootworm adaptation to management tactics, it is difficult to predict with confidence the success of any resistance management tactic. As pointed out by Porter et al. (2012), it will be important to implement a long term integrated approach to corn rootworm management that includes multiple tactics such as rotation of Bt hybrids that express different Cry proteins for corn rootworm, use of soil insecticides at-planting with whole farm or field use of non-Bt hybrid, rotation to a nonhost crop, adult suppression programs where appropriate, and field scouting information and knowledge of corn rootworm densities.

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