Research Article

Received: 30 November 2017 Revised: 13 March 2018 Accepted article published: 30 March 2018 Published online in Wiley Online Library: 10 May 2018

(wileyonlinelibrary.com) DOI 10.1002/ps.4927

Measuring rootworm refuge function: *Diabrotica virgifera virgifera* emergence and mating in seed blend and strip refuges for *Bacillus thuringiensis* (Bt) maize

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Abstract

BACKGROUND: Current insect resistance management plans rely on refuges of plants without *Bacillus thuringiensis* (Bt) toxins to provide a gene pool of unexposed insects. Insects from refuges must mate with insects from Bt maize to slow resistance evolution. We used stable isotope labeling to observe *Diabrotica virgifera virgifera* emergence, dispersal, physical characteristics, and mating in Bt and refuge maize planted in different refuge configurations. Our objective was to assess how refuge type facilitates mating between insects from Bt and refuge plants.

RESULTS: Mating between *D. v. virgifera* beetles from different plant types was more likely in seed blends compared with strip refuges. Adult *D. v. virgifera* from refuge plants emerged before those from Bt plants. In strip refuges, *D. v. virgifera* from refuge plants did not disperse far from refuge boundaries. Larval host plant type did not affect adult size. Larger males and females were more likely to mate. Low proportions of *D. v. virgifera* from refuge plants were found in 5% seed blend refuges.

CONCLUSION: Seed blend refuges can help to facilitate gene flow between *D. v. virgifera* beetles from Bt and refuge maize, but current approaches do not meaningfully contribute to delaying resistance because numbers of refuge beetles produced are insufficient.

Keywords: *Bacillus thuringiensis*; Cry3Bb1 toxin; Cry34/35Ab1 toxin

1 INTRODUCTION

Maize, *Zea mays* L. (Poaceae), genetically engineered to express *Bacillus thuringiensis* toxins (Bt maize) has simplified the logistics of production and provided farmers with unprecedented convenience in managing insect pests, along with environmental and yield protection benefits.[1] The primary belowground pest targeted by Bt maize is the corn rootworm complex, including western corn rootworm (WCR) (*Diabrotica virgifera virgifera* LeConte), the most economically damaging pest of corn in the USA. The Environmental Protection Agency (EPA) requires an insect resistance management (IRM) plan be included with the registration of all Bt maize. Currently, the only EPA-approved IRM plan is the refuge strategy, which relies on plants without the pest-specific Bt toxin (i.e. refuge plants) to produce abundant insects to mate with rare resistant insects from toxin-producing plants (i.e. Bt plants).[2,3]

Implicit in this approach is the assumption that insects from Bt and refuge maize mate at random, meaning that their likelihood of mating with one another is the same as their encounter rate – meeting equals mating. In field situations, there are indications that the situation is more complex. For example, mating opportunities may be limited by how far adults move prior to mating, delayed emergence of adults from toxic plants, and mate preferences resulting from lower fitness of insects exposed to toxins.[2,4,5] Multiple studies have observed dispersal,[6–9] emergence delays,[10–15] and reduced size[12,13,15,16] of WCR in Bt maize and used these data to predict how these factors will affect mating in refuge systems.

Several of the studies listed above have used gut content analyses of adult beetles to track movement. Our study is the first to label rootworms based on their larval host, in order to observe how field populations of WCR adults emerging from refuge and Bt maize disperse and mate in 20% strip, 20% seed blend, and 5% seed blend refuges. We tested strip and seed blend refuges of the same size (20%) for comparative purposes; 20% seed blend refuges are not currently an approved option for commercial maize producers. We used adult beetle head capsule size and dry weight to determine how physical size predicts mate choice in the field and if WCR fed from Bt maize differed in these parameters. Our previous field test of WCR refuge structures determined that non-random mating occurred when natural populations of WCR were confined

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to field cages.[17] The work described here explores refuge function further using unconfined natural populations of WCR, where the identity of mating insects (in terms of Bt or refuge natal host) was determined and refuge function, as measured by the key parameter of mating efficiency, was assessed. Based on our past observations and other published work, we hypothesized that non-random mating would occur in this system.

2 METHODS

2.1 Experimental design

Experiments were conducted in 2015 at Throckmorton Purdue Agricultural Center (TPAC) in Tippecanoe County, Indiana, and Pinney Purdue Agricultural Center (PPAC) in La Porte County, Indiana, USA. Three refuge configurations were tested: 20% seed blend, 20% strip, and 5% seed blend. Genuity VT Triple Pro® + Round-up Ready 2® (DKC 61–88) seeds (DeKalb, Monsanto, St. Louis, MO, USA), hereafter referred to as 'Bt', expressing Cry3Bb1 toxin for rootworm control were used in 20% refuge configurations. Refuge seed in 20% configurations were Genuity VT Double Pro® + Round-up Ready 2® (DKC 61–79) (DeKalb), hereafter 'refuge'. The 20% strip refuge had two strips (four rows each) of refuge plants per plot; strips were separated from each other and from the plot border by 10 or 11 rows of Bt plants. The 20% seed blend refuge was planted with seeds blended prior to planting in a 1:5 refuge to Bt ratio by seed weight. Bt and refuge seeds for 5% seed blend configurations were from a 'refuge in a bag' blend of Genuity SmartStax® (DKC 61–16) (DeKalb) Bt maize expressing Cry3Bb1 and Cry34/35Ab1 toxins for rootworm control. Genuity SmartStax® seeds were blended by the manufacturer. Seeds were separated visually prior to planting and refuge seeds removed to create pure stands of Bt plants. Seeds that contained rootworm-specific Bt traits were a different color (in this case, green) from refuge seeds (purple). Immediately following planting, 345 randomly chosen seeds (representing 5% of the 6900 seeds used per plot based on a planting rate of 68 419 seeds ha^{-1}) were removed and each Bt seed was replaced with two refuge seeds. Refuge seed locations were flagged and the smaller of the two refuge plants removed following germination. All seeds were treated by the manufacturer with the neonicotinoid clothianidin at the rate of 1.25 mg per kernel (Poncho® 1250; Bayer CropScience, Research Triangle Park, NC, USA).

Fields were planted on 8 May at TPAC and on 19 May at PPAC. Seeds were planted with a four-row planter (White 6100 series; AGCO, Duluth, GA, USA) at a rate of 68 419 seeds ha^{-1}. Plots measured 30.4 m by 30.4 m and consisted of 40 rows. Plots were spaced a minimum of 1000 m from each other and 200 m from any other maize to minimize movement of WCR. Fields were bordered by both wooded areas and soybeans. All plots were planted into fields where maize that did not express rootworm-specific Bt was planted the previous year to maximize the opportunities for WCR eggs and subsequent infestation. Standard agronomic practices for field maize production in Indiana were used, with the exception that plots were not given a second application of nitrogen fertilizer at the PPAC location because of excessive rainfall.

Plants were tested for Cry3Bb1 protein in the V2 stage using gene-check strips (Cry3B # AS 015 LS; EnviroLogix, Portland, ME, USA). All plants were tested in 20% seed blend refuges. Plants that tested negative for Cry3Bb1 (refuge) were flagged. Four randomly chosen plants per row were tested in 20% strip and 5% seed blend refuges to test for errors.

2.2 Stable isotope enrichment

An aqueous solution of ammonium nitrate ^{15}N (~98% ^{15}N) (Cambridge Isotope Laboratories, Inc., Andover, MA, USA) and distilled water was applied to a 10-cm-deep hole at the base of all refuge plants at the V2 growth stage. A rate of 0.6125 g of ammonium nitrate per liter of dH_20 was used; 10 mL of this solution was applied to each plant using a CO_2 pressurized backpack sprayer. We used ^{15}N, the stable isotope of nitrogen, because it is retained in insects that feed on enriched materials,[18–21] and ^{15}N feeding is not known to affect insect reproduction or behavior.[20,21] Maize plants use ^{15}N and ^{14}N in the same way. The rate we applied (~0.012 g per plant) represented a small fraction (0.441%) of the total nitrogen applied to each plant.

2.3 Insect sampling and measurements

Eight rows per plot were sampled three times per week beginning on the date that the first adult WCR was captured on a yellow sticky trap (Trece Pherocon® Unbaited AM Yellow Sticky Traps; Gempler's, Janesville, WI, USA) at each location. Plots were sampled between 8 am and 11 am to capture peak WCR mating hours.[7] Plots and rows were sampled in random order. Adult beetles were collected with aspirators (BugVac #2; Rose Entomology, Benson, AZ, USA) and placed into plastic bags (Ziploc; SC Johnson, Racine, WI, USA) labeled with the location, time, date, refuge configuration, and row. Mating pairs were stored together until processing. Samples were stored at −80 °C.

Using a Leica M125 stereo microscope with an attached digital camera (Leica model EC3; Leica Microsystems, Buffalo Grove, IL, USA) at 12.5× total magnification,[13] each head capsule was displayed as a live image using Leica Application Suite Imaging Software, v. 1.6.0 (Leica Microsystems). Head capsule width was measured for all mating pairs. Head capsule size of beetles in the non-mating population was estimated in random subsamples of 10% (with a minimum of ten beetles, or all beetles if fewer than ten) of beetles collected from each unique combination of location, refuge configuration, and collection date. Elytra and head capsules of female WCR were removed for isotope analysis. Female reproductive tracts were dissected for the presence of a spermatophore. If a spermatophore was not found, the spermatheca was removed and gently crushed on a microscope slide to observe for the presence of sperm at 10× magnification under a compound microscope (model SMZ445; Nikon Instruments, Inc., Melville, NY,USA). Dissections took place at room temperature in 0.1% saline solution.

Elytra and head capsules of female beetles and intact male beetles were placed in a laboratory oven (model LR270; Grieve-Hendry Co., Round Lake, IL, USA) at 90 ° C for 24 h to remove moisture. Dry weights of male beetles were measured to the nearest 0.1 mg (Mettler AE 100; Mettler Direct, Ventura, CA, USA). Male elytra and head capsules were removed after weighing.

2.4 ^{15}N testing and analysis

Elytra and head capsules were used for isotope analysis to avoid nitrogen from plant matter in the digestive tract and, in females, nitrogen from male spermatophores.[19] Dried elytra and head capsules were crushed between layers of wax paper, weighed to the nearest 0.0001 g, and placed into 4 × 6 mm mass spectrometry tin capsules (Costech Analytical Technologies, Inc., Valencia, CA, USA). Tins were placed into non-sterile 96-well plates (Sigma-Aldrich, St. Louis, MO, USA). New wax paper was used for every sample and all instruments and the workspace were cleaned with ≥70%

ethanol between samples. The Purdue Stable Isotope Laboratory, West Lafayette, IN, USA [using an isotope ratio mass spectrometer (IRMS)] performed the mass spectrometry. ^{15}N analysis was performed for all mating pairs. To determine the background populations in each field, proportions of non-mating Bt- and refuge-fed beetles were estimated using random subsamples of 10% (with a minimum of ten beetles, or all beetles if fewer than ten) of beetles collected from each unique combination of location, row, refuge configuration, and collection date.

Corrected ^{15}N values were used.[22] The percentage of ^{15}N in excess (excess %^{15}N) (i.e. amount over the average for known unenriched samples) was used to identify beetles that fed as larvae primarily on ^{15}N-treated refuge plants. A series of calculations was used to determine the amount of ^{15}N in excess for each sample.[17] The Purdue Stable Isotope Laboratory recommends that an excess %^{15}N > 0.5 be used as a threshold for identifying enriched samples. As larvae in our study had the potential to move between unenriched (toxic, Bt) and enriched (non-toxic, refuge) plants,[23] a conservative threshold of 1.5% (3 × 0.5%) was used.

2.5 Adult feeding trial

An experiment was conducted to determine if adult beetles could acquire ^{15}N in their elytra and head capsules from feeding upon aboveground tissues of enriched plants. This experiment was conducted on 21–23 July at TPAC and 29–31 July at PPAC in the same plots as described above and in rows not used for sampling field populations. Beetles used for this experiment were collected from natural populations at the Agronomy Center for Research and Education in Tippecanoe Co., Indiana, approximately 25 km from the nearest ^{15}N test plots. Beetles were confined on leaves, tassels, or silk of known ^{15}N-enriched and unenriched maize plants in custom-made bags of mesh cloth measuring 30.48 × 76.2 cm. Bags were placed over silk and leaves together when the ear had not elongated. Two beetles (one male and one female) were placed in each bag and bags were secured to plants with large binder clips. Beetles were collected after 48 h and processed for mass spectrometry. Uneven sample sizes occurred between locations as a result of fewer beetles being collected on 21 July; uneven sample sizes occurred between plants and refuge configuration as a result of individual beetles escaping from the bags or dying. Beetles that died in the bags were not analyzed.

2.6 Maize root testing

Root tissue from ^{15}N-enriched and unenriched maize plants was sampled 7 days following ^{15}N enrichment and analyzed for ^{15}N concentration. Eight plants (four enriched and four unenriched) were sampled from each plot. In seed blend refuges, two plants located side by side (one enriched and one unenriched) were removed from rows 5, 15, 25 and 35. In strip refuges, one enriched plant was removed from one interior and one exterior row per refuge strip; and one unenriched plant was taken from each row bordering a refuge strip. Plants in the approximate middle of the row (~16 m from row ends) were sampled. Plants were removed whole and washed three times in clean distilled water to remove soil particles. Plant tissue was placed in a laboratory oven at 90 °C for 24 h to remove moisture. Between 5 and 7 mg of primary root material was removed and processed for mass spectrometry.

2.7 Data analysis

All data were analyzed using SAS version 9.3 (SAS Institute, Cary, NC, USA). Data on beetle head capsule size and dry weight were analyzed using the PROC MIXED procedure. Fixed variables were refuge type, collection date, location (PPAC or TPAC), host plant (^{15}N-enriched refuge plant or unenriched Bt plant), mating status (mated or unmated), and the interaction of refuge type and host plant. The variable 'location' was random. Head capsule sizes for female and male beetles were analyzed separately. Females were considered mated when either a spermatophore or sperm was found during dissection. Males were considered mated if they were collected *in copulae*.

Fisher's exact test was used to test the relationship between the proportion of Bt/refuge beetles in the mating population and the proportion of Bt/refuge beetles in the non-mating, background population. The null hypothesis was that proportions of Bt and refuge beetles in these populations were the same. Rejection of the null hypothesis indicates that non-random mating occurred. Two-sided *P*-values were calculated using the method of summing small *P*-values.[24] Chi-square tests[25] were used to assess differences in expected and observed rates of mixed (Bt × refuge), Bt-only, and refuge-only mating pairs.

Data for the adult feeding experiment were analyzed using the PROC GLM procedure. Separate analyses were performed for each plot (i.e. every combination of location and refuge type). Explanatory variables were host plant (i.e. whether or not ^{15}N had been applied to the plant) and plant part (e.g. leaf, pollen, silk, or combination of leaf and silk). Only when significant differences ($\alpha < 0.05$) were detected in means between beetles from ^{15}N-enriched and unenriched plants was the %^{15}N excess in all beetles collected from that plot corrected using the equation:

$$\text{Adjusted atom excess}\%^{15}\text{N} = \text{atom excess}\% \; ^{15}\text{N} - \text{greatest mean atom excess}\%^{15}\text{N in beetles fed as adults on}^{15}\text{N} - \text{enriched plants}$$

Data from the maize root experiment were analyzed using the PROC GLM procedure. Explanatory variables were enrichment (i.e. whether or not ^{15}N had been applied to the plant), location, refuge configuration, and the interactions of these factors. Means for all tests were computed using LSMEANS and separated using the Tukey–Kramer method.

3 RESULTS

3.1 Mating

The proportions of refuge and Bt beetles in mating pairs did not differ from proportions in the non-mating population in 20% strip and seed blend refuges either by week or over the entire mating season (Fisher's exact test: seed blend: PPAC, $P = 0.9256$; TPAC, $P = 0.1008$; strip: PPAC, $P = 0.2155$; TPAC, $P = 1.0$) (Table 1). In both 20% strip refuge configurations, observed mating rates were different from expected (PPAC, df = 1, $\chi^2 = 8.36$, $P = 0.0038$; TPAC, df = 1, $\chi^2 = 13.29$, $P = 0.0003$) because there were more mating pairs from the same natal host (refuge × refuge and Bt × Bt) and fewer mixed pairs (refuge × Bt) (Table 2). In the 20% seed blend at TPAC, observed mating rates were different from expected (df = 1, $\chi^2 = 6.73$, $P = 0.0095$) because there were fewer mate pairs from the same natal host and more mixed pairs. In the 20% seed blend at PPAC, observed and expected mating rates were not different (df = 1, $\chi^2 = 1.62$, $P = 0.2809$). There were too few mating pairs collected from 5% seed blends for chi-square analysis.

Table 1. Fisher's exact test of the number of *D. virgifera virgifera* adults that fed as larvae on Bt and refuge maize in mating pairs and in the non-mating population. The null hypothesis was that proportions of Bt and refuge beetles in these populations were the same. Rejection of the null hypothesis indicates that non-random mating occurred. Two-sided *P*-values were used. Beetles were collected from ^{15}N-enriched (refuge) and unenriched (Bt) *Cry3Bb1*-expressing maize (*Z. mays* L.) in 20% strip refuges and 20% seed blend refuges at PPAC and TPAC, Indiana (July–August 2015)

		Mating		Non-mating		
Location – refuge	Date	Bt	Refuge	Bt	Refuge	*P*-value
PPAC – 20% seed blend	Week 1: 24 July	8	6	16	7	0.4948
	Week 2: 29 July to 31 July	34	58	43	70	0.8858
	Week 3: 3 August to 5 August	46	54	56	74	0.6893
	Overall: 24 July to 5 August	88	118	115	151	0.9256
PPAC – 20% strip	Week 1: 24 July	8	12	23	53	0.4295
	Week 2: 27 July to 31 July	98	102	201	212	1
	Week 3: 3–7 August	65	35	79	49	0.6786
	Overall: 24 July to 7 August	171	149	303	314	0.2155
TPAC – 20% seed blend	Week 1: 8–10 July	2	10	14	34	0.4858
	Week 2: 13–17 July	13	25	44	65	0.5651
	Week 3: 20–24 July	2	6	18	11	0.1090
	Week 4: 27–29 July	1	1	11	10	1
	Overall: 8–29 July	18	42	87	120	0.1008
TPAC – 20% strip	Week 1: 10 July	2	2	8	18	0.5840
	Week 2: 13–17 July	24	32	106	128	0.7669
	Week 3: 20–24 July	68	106	145	221	0.9252
	Week 4: 27–31 July	53	33	148	115	0.4510
	Overall: 10–31 July	147	173	407	482	1

Table 2. Chi-square analysis of mating rates in *D. virgifera virgifera* collected from ^{15}N-enriched (refuge) and unenriched (Bt) *Cry3Bb1*-expressing maize (*Z. mays* L.) in 20% strip refuges and 20% seed blend refuges at PPAC and TPAC, Indiana (July–August 2015)

Location – refuge	Observed	Expected	Observed – expected	(Observed – expected)^2	(Observed – expected)^2/ expected
PPAC – 20% strip					
Refuge × refuge	40	43.84	−3.84	14.75	0.34
Bt × Bt	51	35.82	15.18	230.43	6.43
Refuge × Bt	69	80.32	−11.32	128.14	1.60
				ΣChi^2	8.36
				df	1
				P-value	0.0038
PPAC – 20% seed blend					
Refuge × refuge	36	31.48	4.52	20.43	0.65
Bt × Bt	21	20.46	0.54	0.29	0.01
Refuge × Bt	46	51.05	−5.05	25.50	0.50
				ΣChi^2	1.62
				df	1
				P-value	0.2809
TPAC – 20% strip					
Refuge × refuge	58	48.82	9.18	84.27	1.73
Bt × Bt	45	31.95	13.05	170.30	5.33
Refuge × Bt	57	79.23	−22.23	494.17	6.24
				ΣChi^2	13.29
				df	1
				P-value	0.0003
TPAC – 20% seed blend					
Refuge × refuge	13	14.60	−1.60	2.56	0.18
Bt × Bt	1	5.14	−4.14	17.14	3.33
Refuge × Bt	16	10.25	5.75	33.06	3.23
				ΣChi^2	6.73
				df	1
				P-value	0.0095

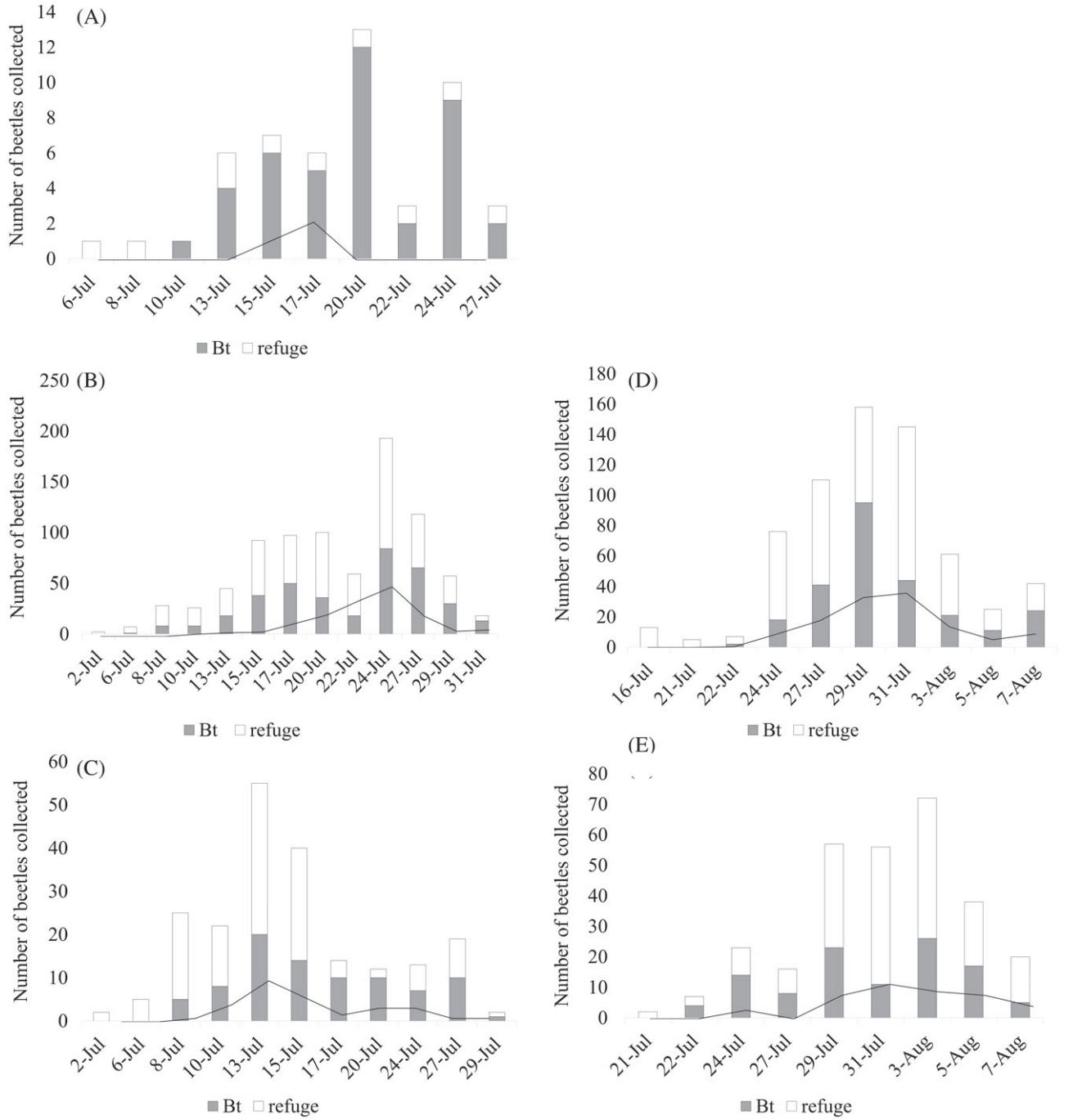

Figure 1. Number of adult *D. virgifera virgifera* from ^{15}N-enriched (refuge; white) and unenriched (Bt; gray) natal host plants collected by date (Indiana, 2015) in *Cry3Bb1*- and *Cry3Bb1* + *Cry34/35Ab1*-expressing Bt maize planted under different refuge configurations: (A) 5% seed blend refuge, TPAC; (B) 20% strip refuge, TPAC; (C) 20% seed blend refuge, TPAC; (D) 20% seed blend refuge, PPAC; and (E) 20% strip refuge, PPAC. The number of mating pairs collected is indicated with a black line.

3.2 Refuge and Bt populations

Refuge beetles represented 50–60% of the population in all 20% refuge configurations (seed blend: PPAC, 56.99%; TPAC, 60.68%; strip: PPAC, 49.42%; TPAC, 54.18%). Refuge beetles represented 11.11% of the population in the 5% seed blend at TPAC. Populations from the 5% seed blend at PPAC were not analyzed because insufficient numbers of beetles were collected.

All refuges had female-biased sex ratios (percent female: 20% seed blend: PPAC, 61.61%; TPAC, 57.14%; 20% strip: PPAC, 60.60%; TPAC, 56.11%; 5% seed blend: TPAC, 73.2%). Over 90% of female beetles were either mated or collected *in copulae* (20% seed blend: PPAC, 95.66%; TPAC, 96.06%; 20% strip: PPAC, 95.00%; TPAC, 92.26%; 5% seed blend: TPAC, 98.96%).

3.3 Adult emergence and dispersal

Refuge beetles were initially collected from plots 2 to 6 days before Bt beetles (5% seed blend: TPAC, 4 days; 20% seed blend: PPAC, 2 days; TPAC, 6 days; 20% strip: PPAC, 4 days; TPAC, 6 days) (Fig. 1).

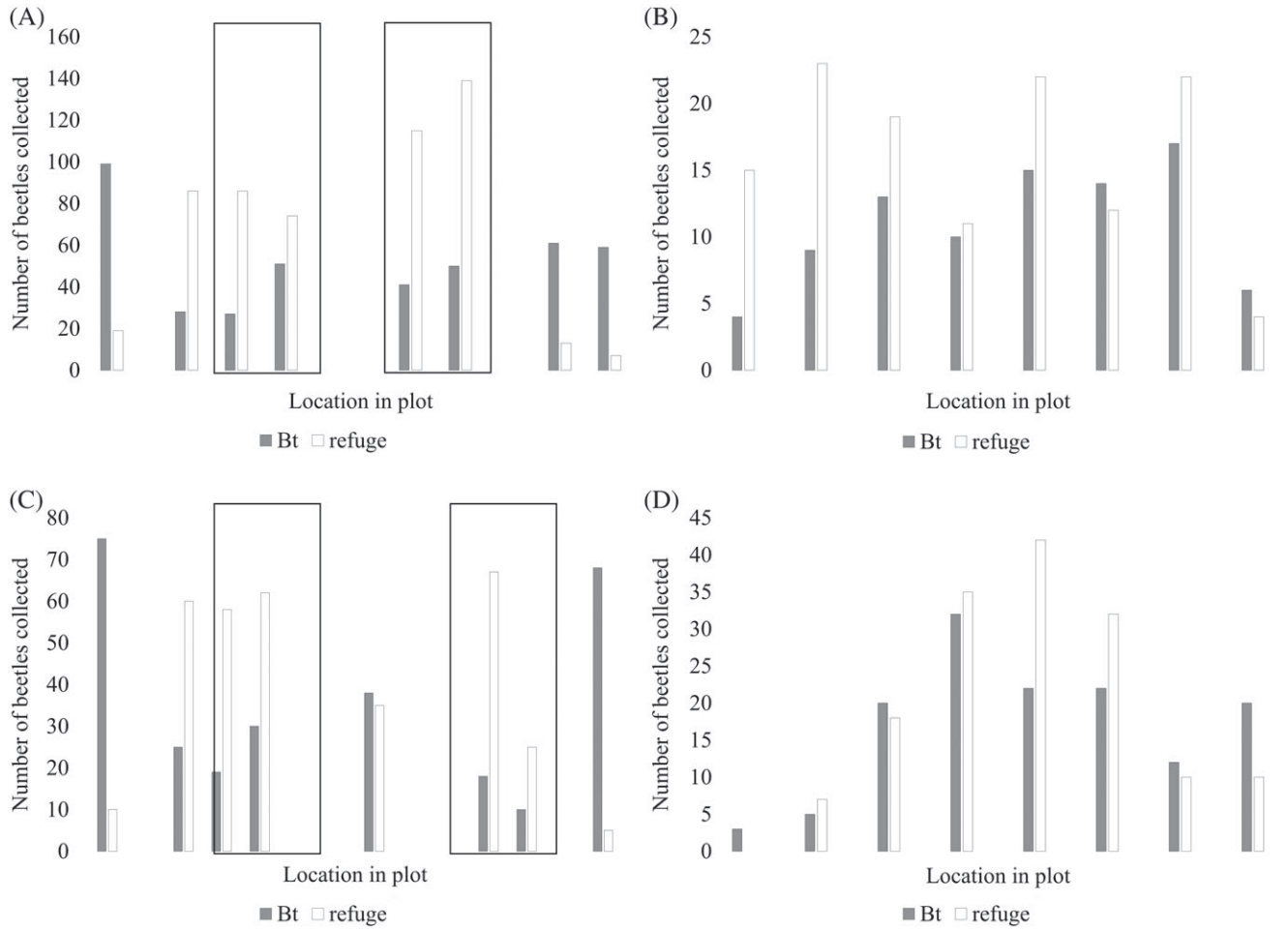

Figure 2. Number of adult *D. virgifera virgifera* from ^{15}N-enriched (refuge; white) and unenriched (Bt; gray) natal host plants collected by location in 40-row plots (Indiana, 2015) of *Cry3Bb1*- and *Cry3Bb1* + *Cry34/35Ab1*-expressing maize planted with different refuge configurations: (A) 20% seed blend refuge, TPAC; (B) 20% strip refuge, TPAC; (C) 20% strip refuge, PPAC; (D) 20% seed blend refuge, PPAC. The location of refuges in 20% strip refuges is indicated with a black box.

There were fewer days between the time at which the first beetle was collected and peak mating intensity (i.e. when the largest number of mating pairs were collected) in 20% seed blend refuges (PPAC, 9 days; TPAC, 11 days) compared with 20% strip refuges (PPAC, 14 days; TPAC, 22 days). With the exception of border rows, most Bt and refuge beetles were collected on rows containing their respective host plant in 20% strip refuges (Fig. 2).

3.4 Head capsule size and dry weight

Mated and unmated females had different head capsule sizes (df = 1, 1011, $F = 37.21$, $P < 0.0001$). Mated females had larger head capsules (mean ± standard deviation 1.5144 ± 0.0757 mm) than unmated females (1.4860 ± 0.0837 mm). Date also affected female head capsule size (df = 14, 1011, $F = 5.18$, $P < 0.0001$). There was no effect of refuge type (df = 2, 1011, $F = 0.98$, $P = 0.3743$), location (df = 1, 1011, $F = 1.48$, $P = 0.2234$), host plant (df = 1, 1011, $F = 0.31$, $P = 0.5787$), or the interaction between host plant and refuge type (df = 2, 1011, $F = 0.52$, $P = 0.5959$).

Mating and non-mating males had different head capsule sizes (df = 1, 1119, $F = 10.42$, $P = 0.0013$). Mating males had larger head capsules (1.4748 ± 0.0778 mm) than non-mating males (1.4662 ± 0.0859 mm). Date (df = 16, 1119, $F = 5.79$, $P < 0.0001$) and location (df = 1, 1119, $F = 9.76$, $P = 0.0018$) also affected male head capsule size. There was no effect of refuge type (df = 2, 1119, $F = 0.53$, $P = 0.5897$), host plant (df = 1, 1119, $F = 0.01$, $P = 0.9277$), or the interaction of host plant and refuge configuration (df = 2, 1119, $F = 1.45$, $P = 0.2359$).

Mating and non-mating males had different dry weights (df = 1, 1084, $F = 80.06$, $P < 0.0001$). Mating males were heavier (2.7721 ± 0.5258 mg) than non-mating males (2.3534 ± 0.6694 mg). Date (df = 16, 1084, $F = 5.20$, $P < 0.0001$) and the interaction of host plant and refuge type (df = 2, 1084, $F = 3.71$, P = 0.0247) also affected male dry weight. There was no effect of refuge type (df = 2, 1084, $F = 0.57$, $P = 0.2013$) or host plant (df = 1, 1084, $F = 0.57$, $P = 0.4522$).

3.5 Adult feeding trial

In TPAC, %^{15}N excess was not different in beetles caged on different host plants (5% seed blend: df = 1, 32, $F = 1.39$, $P = 0.2505$; 20% strip: df = 1, 45, $F = 1.80$, $P = 0.1340$; 20% seed blend: df = 1, 41, $F = 1.37$, $P = 0.2600$) (Table 3). Host plant affected %^{15}N excess in caged beetles from PPAC (5% seed blend: df = 1, 7, $F = 18.84$, $P = 0.0080$; 20% strip refuge: df = 1, 11, $F = 5.17$, $P = 0.0349$). No beetles survived on unenriched plants in the 20% seed blend refuge at PPAC. Thus, to be as conservative as possible in our designations of beetles as 'marked', %^{15}N excess was adjusted by the

Table 3. Mean %^{15}N excess in the elytra and head capsules of *D. virgifera virgifera* adults confined for 48 h on leaves, silk and tassels of ^{15}N-enriched and unenriched maize (*Z. mays* L.) plants at PPAC and TPAC (July 2015). Means used to correct %^{15}N excess in beetles collected and analyzed from research plots are in bold

				%^{15}N excess	
Location – refuge	^{15}N enrichment	Plant part	*n*	Mean	SD
PPAC – 5% seed blend	–	Leaf/silk	2	0.6220	0.5779
	–	Pollen	2	0.5012	0.6322
	+	Leaf/silk	2	0.9081	0.8394
	+	Pollen	2	**3.7047**	0.5378
PPAC – 20% seed blend	+	Leaf	4	0.5756	0.7276
	+	Pollen	4	**1.7122**	0.2047
	+	Silk	4	0.5803	0.6703
PPAC – 20% strip	–	Leaf	2	0.9204	0.1601
	–	Pollen	2	0.7200	0.5119
	–	Silk	2	0.1580	0.2378
	+	Leaf	2	**4.9222**	0.8150
	+	Pollen	2	1.7018	0.2470
	+	Silk	2	1.6898	0.4418
TPAC – 5% seed blend	–	Leaf	2	0.6464	0.2371
	–	Pollen	2	−0.0284	0.4258
	–	Silk	2	0.0055	0.3630
	+	Leaf	9	0.1797	0.3604
	+	Pollen	8	−0.0742	0.2294
	+	Silk	10	0.1162	0.2535
TPAC – 20% seed blend	–	Leaf	3	0.0234	0.3808
	–	Pollen	4	0.1501	0.5307
	–	Silk	3	0.0137	0.1184
	+	Leaf	12	0.3106	0.6147
	+	Pollen	12	0.8252	0.0890
	+	Silk	8	0.2205	0.3365
TPAC – 20% strip	–	Leaf	3	0.3479	0.1178
	–	Pollen	7	0.2476	0.2400
	–	Silk	8	0.0584	0.2566
	+	Leaf	5	0.6836	0.5180
	+	Pollen	12	0.6253	0.7076
	+	Silk	11	0.4364	0.4429

greatest mean %^{15}N excess from beetles caged on ^{15}N-enriched plants.

3.6 Maize root testing

The mean %^{15}N excesses in maize roots from ^{15}N-enriched and unenriched plants were different (df = 1, 47, $F = 25.56$, $P < 0.0001$). Location affected %^{15}N excess (df = 1, 47, $F = 8.95$, $P = 0.0050$). The mean %^{15}N excess in enriched plants from PPAC (304.29 ± 0.58) was over three times higher than that in enriched plants from TPAC (78.43 ± 0.55). There was no difference between the mean %^{15}N excesses in unenriched plants ($P = 1.00$). The interaction of enrichment and location also affected %^{15}N excess (df = 1,47, $F = 8.96$, $P = 0.0050$). There was no effect of refuge type (df = 2, 47, $F = 1.72$, $P = 0.1937$), the interaction of location and refuge type (df = 2, 47, $F = 0.56$, $P = 0.5789$), the interaction of location, refuge type and ^{15}N enrichment (df = 2, 47, $F = 0.56$, $P = 0.5774$), or the interaction of refuge type and ^{15}N enrichment (df = 2, 47, $F = 1.71$, $P = 0.1956$).

4 DISCUSSION

Nitrogen enrichment allowed us to observe how Bt and refuge beetles dispersed and mated in the field. Our results confirm that seed blend refuges facilitate mating between WCR beetles from different natal hosts. Seed blend refuges of 5% non-Bt seeds are only labeled for use with Bt maize that expresses two or more rootworm-specific Bt toxins (referred to as 'pyramided' or 'stacked'). Our 20% seed blend refuge treatment for single-gene Bt maize is not an approved IRM strategy and was used to gather information about the relative contributions of seed blends or block refuges to resistance management. While the 5% seed blend is, by a wide margin, the most common refuge implemented in Bt maize at the present time, our data indicate that mixed matings may be too infrequent to effectively delay resistance in these plantings because of the scarcity of adults from refuge plants (11% of the adults collected in those treatments).

Dispersal is a key determinant of mate selection in WCR. Typically, males will not travel unnecessarily to find mates,[5,7] and females will mate near emergence sites.[5,9] In our 20% strip refuges, few beetles from refuge plants moved four to ten rows from refuge borders; thus, Bt and refuge beetles mixed almost exclusively at refuge boundaries. Similar findings have been reported using gut content analysis to mark adult beetles after emergence.[9] Seed blend refuges lack defined boundaries and allow beetles from different hosts to emerge in close proximity throughout the field. The

probability of mixed mating increases greatly in the absence of other mate choice factors because adults do not have to travel to find mates from different natal hosts.[13]

Our results also indicate that natural WCR populations, like laboratory populations,[26] select mates based on size. Mated females and males found in mating pairs were larger and, in the case of males, heavier, than their unmated and non-mating counterparts. Mate preferences have resistance management implications if insects fed on toxic plants are smaller because this would encourage assortative mating in a species that prefers larger mates,[2] as is the case in WCR,[26] although there were no size differences between beetles from refuge and Bt plants in our study. Other studies have shown that larvae feeding only on *Cry3Bb1*-expressing plants develop into smaller adults.[12,13,27] It is possible that size similarities in our study resulted from larvae moving between refuge and Bt plants[28–30] or that there were no effects on size from Cry toxin feeding, as observed in some WCR populations.[11,29,31]

Emergence timing may also influence mate selection. WCR larvae fed on Cry3Bb1 develop more slowly than their unexposed counterparts, which may reduce the opportunity for partners from different host plants to mate.[10,13,32] Emergence delays are less pronounced in seed blends compared with strip or block refuges,[13] potentially because larvae can move more readily between Bt and refuge hosts.[28] Adults from Bt plants emerged later than adults from refuge plants in our study. There were more days between the time at which adults first emerged and the time at which the peak mating occurred in strip refuges compared with seed blends. It is possible that the longer period between emergence and peak mating disproportionately limited available mates both early and late in the emergence curve because: (i) males complete sexual development post-emergence,[5] (ii) males lose mating ability as they age,[33] and (iii) females typically mate within hours of emergence;[5] the latter is supported by the very high percentages of mated females in our field experiments.

In our study, WCR from Bt host plants represented 40–50% of the population in 20% refuge *Cry3Bb1*-expressing fields and nearly 90% of the population in 5% refuge *Cry3Bb1* + *Cry34/35Ab1*-expressing fields. Lower than expected (and desired, for IRM purposes) emergence from refuge plants in seed blends has been documented, though the mechanism remains unknown.[13,15,29] It is critical to the success of the refuge strategy that mating between insects from Bt natal hosts be relatively rare.[34] Given the high percentage of WCR that emerged after feeding on Bt plants in our 5% seed blend refuge, mating between Bt beetles is the dominant type of mating in this system. This conclusion is influenced by the large number of beetles emerging from Bt corn. As we are not aware of reports of resistance, or problem fields, within the state of Indiana at this writing, we assume that this is a typical rate of WCR survival in commercial Bt maize plantings. In any event, refuges for any transgenic crop have little value once resistance (which we define here as high rates of survival on the Bt crop) is common.

Our conclusions are based on field data from a single field season, and there are other considerations that may limit interpretation of our findings. Detectable concentrations of ^{15}N were found in adults confined on ^{15}N-enriched plants from one of our locations (PPAC). This probably occurred as a result of high levels in the plant: the mean ^{15}N concentration in maize roots from PPAC was over three times higher than that in maize roots from TPAC, possibly as a consequence of differences in the amount or timing of rainfall and soil type which affected nitrogen uptake. We corrected for this by adjusting baseline levels. Additional research is necessary to determine the extent to which a single larva feeds on both Bt and refuge plants; this is particularly important in seed blends where larval movement means that feeding on both plant types is likely to be common. This type of research may uncover the mechanism by which refuge beetle emergence is depressed in these plantings. We did not test WCR populations for resistance to Bt toxins, and are not aware of any confirmed field resistance in Indiana at the time of writing. Additionally, we have no information on egg densities in our study areas, so it is impossible to determine survivorship on Bt maize hybrids, although some level of survival to the adult stage is expected with all current Bt maize targeting WCR.[14,35–37] We located fields as far apart as practical given our available resources and the prevalence of maize in Indiana. It is likely that emigration and immigration occurred throughout the season,[8] but this should not be a factor in our results; there is no reason to assume that it is not equal across treatments.

In our 2014 study using natural populations and field cages, we found that non-random mating occurred in seed blend and strip refuges.[17] Our current results support these findings. In both studies, how adults disperse prior to mating is probably the key consideration, even though field cages changed dispersal and thus mating behavior.

Ensuring compatibility of IRM with insect biology and ecology is an important goal in this highly adaptable pest. Refuges that fail to delay resistance represent a fruitless effort and unnecessary expense to growers, in terms of lost yield. Our data, and those obtained in other work cited above, indicate that the vast majority of refuges currently implemented in Bt maize production are likely to be ineffective in terms of reducing the rate of resistance evolution, simply because too few refuge beetles are produced. We also report an instance of a 20% seed blend refuge (although we reiterate here that this seed blend is not currently an option for producers) that functions to facilitate mating between Bt and refuge populations. A 20% blended refuge may be an option for future maize hybrids expressing Bt toxins or other plant incorporated protectants. Importantly, we did not measure plant damage or yield as part of this study, key parameters in assessing the commercial viability of any IRM approach. Our work provides empirical evidence that the refuge strategy can be compatible for facilitating mating among WCR insects in mixed plantings, but that current seed blends may not contribute meaningfully to delaying resistance development. The key IRM challenge for current seed blends is the low production of refuge insects, which calls their utility into question. While our work demonstrates that the approach of increasing the proportion of refuge seeds should be investigated with these or future in-plant protectants, any gains in IRM functionality would have to be weighed against the likelihood of increased pest damage caused by feeding on refuge plants. Efficient protection of refuge seeds is a key consideration in managing these tradeoffs.

ACKNOWLEDGEMENTS

This work was supported in part by the Monsanto Corn Rootworm Knowledge Research Program, with special thanks to John Greenplate. We would like to express our gratitude to Larry Bledsoe, Steven Smith, Alejandro Gomez, Kirsten Brichler, Sarah Dietrich, and Chris Sieradzki. We thank the Purdue Agricultural Center staff for agronomic support and Tim Filley and Lindsey Crawley at the Purdue Stable Isotope Lab for mass spectrometry testing. We are grateful for training and knowledge generously provided

by Dr. Joseph Lee Spencer and his laboratory at the University of Illinois.

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