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Quantifying rates of random mating in western corn rootworm emerging from *Cry3Bb1*-expressing and refuge maize in field cages

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Abstract

The western corn rootworm (WCR), *Diabrotica virgifera virgifera* LeConte (Coleoptera: Chrysomelidae), is the most significant pest of field maize, *Zea mays* L. (Poaceae), in the USA. Maize plants expressing Bt toxins targeting the corn rootworm complex have been widely adopted and are the primary insecticidal control measure for this pest in North America. Insect resistance management tactics using various refuge structures have been adopted to ensure Bt products will retain durability. An assumption of the refuge strategy is that males and females emerging from Bt and refuge plantings mate randomly; this has not been tested in the field. We conducted cage studies using field populations of WCR in Indiana, USA, to generate empirical field data on mating rates between beetles emerging from *Cry3Bb1*-expressing Bt and refuge maize plants. Two refuge configurations were tested; all refuge plants were labeled using the stable isotope ^{15}N. This mark persists in adult beetles after eclosion, allowing for collection and analysis of isotopic ratios of all beetles. Additional data collected included adult emergence rates, timing and sex ratios for each of the treatments, and head capsule size and dry weights of beetles collected. Treatment had a significant effect on dry weight; mean dry weight decreased in Bt-only treatments. Fisher's exact test of proportions of mating pairs of refuge and Bt insects indicated that mating was not random in 20% strip refuges and 5% seed blend treatments. We found high percentages of beetles that fed on Bt-expressing plants as larvae, suggesting that mating between resistant beetles may not be rare even if random mating did occur.

Introduction

Widespread planting of maize that produces Bt toxins targeting western corn rootworm (WCR), *Diabrotica virgifera virgifera* LeConte (Coleoptera: Chrysomelidae), creates intense selective pressure for this pest to evolve resistance (Gassmann et al., 2012). Corn rootworm resistance was first documented in laboratory studies. WCR can evolve resistance to plants containing the Cry3Bb1 protein (referred to herein as 'Bt maize') within three generations under greenhouse conditions (Meihls et al., 2008). This work was later supported by field observations documenting severe rootworm damage to the Bt maize in commercial fields beginning in 2008 (Gassmann et al., 2011). Eggs collected from mated females in problem fields (i.e., showing high levels of root damage) were reared on Bt maize in the laboratory and demonstrated higher survival compared with those reared from females collected in non-problem fields (Gassmann et al., 2011).

Currently, the United States Environmental Protection Agency (US EPA) has adopted a refuge strategy, a plan that relies on abundant insects from plants without the toxic trait mating with relatively rare resistant insects from toxin-producing plants (US EPA, 1998). That resistant insects will mate primarily with susceptible insects is central to the success of refuges designed to promote resistance management (Tabashnik et al., 2008). Both, positive assortative mating (i.e., resistant insects mating with other resistant insects) and negative assortative mating (i.e., resistant insects mating with susceptible insects) would affect the speed of resistance evolution (Gould, 1998). Yet, individuals from Bt and refuge plants are assumed to mate at random in most model systems (Gould, 1986), and their likelihood of mating with one another is the same as their encounter rate with one

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another – in other words, meeting equals mating. The occurrence of random mating in the WCR/Bt maize system has been critically examined by a review of corn rootworm mating (Spencer et al., 2013). Skewed male-to-female ratios, protandry, premating movement, and delayed emergence from Bt maize-targeting WCR may all affect reproductive behavior and offer potential to hasten the evolution of resistance (Spencer et al., 2013).

There is a delay in adult emergence when WCR larvae are reared on Bt maize (Clark et al., 2012), which may provide a mechanism for increased levels of positive assortative mating. Some field studies have demonstrated that male WCR emerging from Bt maize tend to have smaller head capsules and lower dry weights than males emerging from non-Bt maize (Murphy et al., 2011; Petzold-Maxwell et al., 2013). Size differences, if they occur, could facilitate mate discrimination; it has previously been demonstrated that WCR males have a preference for larger females (Kang & Krupke, 2009). It follows that if larval exposure to Bt maize were correlated with adult size differences, this may predispose the adults in favor of non-random mating. The work described here examines this premise, and is the first to document mating rates between WCR adults from Bt and refuge maize where the identity of the natal host was known. We used field cage experiments to determine how refuges function in facilitating mating between field populations of beetles from Bt and refuge plants under field conditions.

Materials and methods

Plot arrangement

Field studies were conducted in 2013 using Bt hybrid and non-Bt maize plants to determine mating preference of beetles emerging from each maize variety. Four treatments were compared: a 20% strip refuge, a 5% seed blend refuge, a Bt-only control, and a refuge-only control. Bt seeds, Yieldgard VT Triple + Round-up Ready 2 (DKC 62-54) (DeKalb; Monsanto, St. Louis, MO, USA) were used, expressing *Cry3Bb1* for WCR control. Refuge seeds were in the same family as Bt seeds (DKC 62-55), but did not express *Cry3Bb1*. This study was conducted at the Purdue Agronomy Center for Research and Education in Tippecanoe County (IN, USA) in a continuous maize field where WCR populations have historically been abundant and rootworm-active Bt maize was not planted the prior growing season.

Plots measured 3.65 × 3.65 m with four rows of 20 maize plants spaced 76 cm apart and 15 cm spacing between plants in a row. Individual plots were spaced 2.4 m apart on all sides and a 3.1 m buffer was planted on all edges. Fields were left bare between plots. Strip refuge plots consisted of a single row of 15 refuge plants on one side of the plot and three rows of 20 Bt plants each (i.e., 75 plants in total to allow for 20% refuge). Seed blend plots contained four refuge plants placed randomly along with 76 Bt plants to accommodate the 5% refuge requirement. Bt only and refuge only plots contained 80 plants of their respective seed types. Seeds were planted with a four-row planter (White 6100 series, AGCO, Duluth, GA, USA) at a rate of 27 700 seeds per acre or 68 419 seeds per ha. All refuge seeds in 20% strip and 5% seed blend refuge treatments were planted by hand. Refuge seeds in the strip treatment were hand planted in the designated row. For the seed blend treatment, one Bt seed per row was randomly chosen, removed and replaced with two refuge seeds, and staked to identify location. The smaller of the two refuge plants was removed after emergence from the soil, a period of 7–10 days. Each treatment was replicated 6×: four replications were used to collect mating pairs only and two replications were used to measure WCR adult emergence timing and establish ratios of Bt and refuge beetles. Plots were planted on May 16 and arranged in a randomized block design.

Refuge plants in the V2 stage were tested for the presence of the Cry3Bb1 protein by crushing a small amount of a leaf with a buffer solution (QuickStix EB2 Extraction Buffer, Portland, ME, USA) in a 1-ml centrifuge tube. Using gene-check strips (EnviroLogix Cry3B # AS 015 LS, Portland, ME, USA), plants were identified as positive or negative for Cry3Bb1 protein expression. Ammonium nitrate ^{15}N (ca. 98% ^{15}N) (Cambridge Isotope Laboratories, Andover, MA, USA) was applied as a 1.225% ^{15}N solution to a 5-cm-deep hole at base of each refuge plant. A pipette (Eppendorf Research Plus, Hamburg, Germany) was used to deliver the labeled fertilizer directly into each of the holes at the rate of 1 ml of solution per hole. Natural abundances of stable isotopes have provided a field-tested method to track how generations of a polyphagous pest utilize different host plants (Gould et al., 2002; Hiltpold et al., 2014). The ^{15}N stable isotope of nitrogen provides a reliable, inexpensive, and efficient method of marking WCR (Murphy & Krupke, 2011). Maize plants uptake and utilize ^{15}N in the same manner as the more common ^{14}N. ^{15}N is likely to be retained in insects fed on enriched materials (Steffan et al., 2001) and is not known to affect insect behavior or fecundity (Haglar & Jackson, 2001; Hood-Nowotny & Knols, 2007). The rate we applied (ca. 0.024 g per plant) represented ca. 0.9% of the total initial nitrogen, as fertilizer, applied to the plant.

Approximately 2 weeks prior to predicted first adult emergence, plots were enclosed by a screen house (referred to herein as 'cages') custom built to the following specifications: 3.65 m long × 3.65 m wide × 2.13 m high

(Lumite Alto, GA, USA). The bottom edges of each cage were buried a minimum of 8 cm and covered with soil to keep adult beetles from moving in or out of the cages. All plants except the central eight plants were cut to ca. 0.4 m in height and stripped of leaves to facilitate spotting and collection of mating pairs. Allowing some plants to develop fully provided necessary nutrients, specifically pollen and silks, for the adult beetles to feed on (Hill, 1975). Determination of when to cut plants was estimated by digging up larvae from adjacent fields weekly and determining the predominant instar.

Beetle collection and ^{15}N determination

Mating and emergence cages were monitored 3× weekly beginning on 3 July 2013, the date the first female WCR was found. Sample collection continued until 28 August, the date that no beetle captures occurred in any treatment. Sampling was conducted during the peak period of mating initiation, between 07:00–11:00 hours (Marquardt & Krupke, 2009). Four workers were rotated to different field cages every 15 min. This allowed each individual to sample each treatment on each day to minimize sampling bias. Only mating pairs were collected from mating cages; mating pairs were stored together. All adult beetles were collected from emergence cages on each sampling date and stored together. Beetles from emergence and mating cages were collected by hand into clear plastic bags (Ziploc, SC Johnson, Racine, WI, USA). Samples were labeled with the date, replicate, and treatment, and stored in a freezer at −80 °C until processing.

Head capsules were measured and dry weights were obtained for all beetles collected in 20% strip and 5% seed blend refuge treatments. In Bt and refuge-only treatments, 10% of beetles collected (or at least five males, five females, and five mating pairs) per sample day were measured and weighed. Head capsules were measured using a stereo microscope with an attached digital camera (models SZX12 and U-CMAD3; Olympus Optical, Tokyo, Japan) at 27× total magnification. Each head capsule was displayed as a live image using Analysis Microsuite imaging software (Soft Imaging System, Lakewood, CO, USA) and a measurement was taken at the widest point of the head capsule from eye to eye, accurate within 0.01 mm (Murphy et al., 2011). Head capsule width is commonly used as a fitness parameter in WCR adults (Branson & Sutter, 1985; Li et al., 2009) and has been correlated with lifetime fecundity in females and longevity in males (Li et al., 2009). Head capsule width remains constant throughout each life stage and is a more reliable predictor of fitness than dry mass (Hammack et al., 2003; Li et al., 2009). Variation in head capsule width can be attributed to larval exposure to stressors (Branson & Sutter, 1985) making it a reliable indicator of larval nutrition and, in turn, adult fitness. Beetles were placed into a small laboratory oven (Grieve-Hendry, Round Lake, IL, USA) and allowed to dry at 93 °C for 18 h. Individual beetles were then weighed to the nearest 0.1 mg to obtain dry weight (Mettler AE 100; Mettler Direct, Ventura, CA, USA).

Beetles were analyzed at the Purdue Stable Isotope Laboratory to measure δ^{15}N concentration using mass spectrometry. Samples were prepared by first removing the abdomen from each dried beetle which prevented the accidental inclusion of the spermatophore, which is transferred from the male to the female during copulation, or any food material that may be in the gut. Previous work with this marking approach has demonstrated that ^{15}N is transferred from males, via the spermatophore, to females during mating (Murphy & Krupke, 2011). After removal of the abdomen, the elytra were removed and crushed. Between 0.3–0.4 mg of the crushed material was placed into a mass spectrometry tin. Elytra were used because they are heavily sclerotized and resistant to degradation (Klowden, 2002), and therefore offer the greatest potential to retain the ^{15}N label obtained during larval feeding. Ground head capsules were also used from beetles with elytra weighing less than 0.3 mg (<10% of total samples). Sample tins, used for elemental analysis and combustion, were folded after weighing and placed into a non-sterile 96-well plate (Sigma-Aldrich, St. Louis, MO, USA). Plates were delivered to the Purdue Stable Isotope Laboratory where samples were combusted in an elemental analyzer (1 050 °C) and analyzed by an isotope ratio mass spectrometer (Sercon 20-20 IRMS, continuous flow: PDZ Europa Elemental Analyzer, Crewe, UK).

All of the beetles collected from emergence cages and all of the mating pairs collected from mating cages in 20% strip refuges and 5% seed blends were sampled for δ^{15}N to give proportions of beetles that had not fed upon Bt plants (labeled) to beetles that had fed upon Bt plants (unlabeled). Corrected δ^{15}N values were determined for more accurate readings (Dawson et al., 2002). A series of calculations was used. The ratio of ^{15}N/^{14}N was calculated for each sample. This was done using the following equation:

$$0.0037 * \text{sample corrected } \delta^{15}\text{N}/(1000 + 1),$$

with 0.0037 being a correction factor that allows for the average natural abundance of ^{15}N (i.e., the ratio of atoms of ^{15}N/^{14}N is 0.0037 to 1 in an unlabeled standard). The calculation for atom % ^{15}N was conducted, which is the

percentage of ^{15}N relative to total N in the sample:

$$100*[\text{sample ratio } \frac{^{15}N}{^{14}N}/(\text{sample ratio } \frac{^{15}N}{^{14}N}+1)].$$

The final calculation to determine atom % excess is:

$$100*(\text{sample atom } \%^{15}N - 0.3679)/0.3679,$$

with 0.3679 as the average atom % ^{15}N of known, non-labeled samples. Atom % excess above the baseline constant of 0.3679 reveals the differences between samples having slightly variable amounts of total N.

Previous research has shown that larvae move more frequently from a low-quality food source to a higher-quality food source when available (Hibbard et al., 2003), creating the potential for larvae to move from unlabeled (toxic, Bt) plants to labeled (non-toxic, refuge) plants. With larval movement in mind, it was determined from the analyzed data that a value of 1.5 atom % excess ^{15}N was the threshold to identify a given sample as labeled. The Purdue Stable Isotope Laboratory recommends 0.5% as a threshold for identifying labeled samples. However, our system had to account for the potential movement of larvae between labeled and unlabeled plants. The conservative threshold of 1.5 (3 × 0.5%) decreases the number of false positives by reducing the likelihood that larvae that may have fed on a refuge plant for only a small amount of time being identified as 'labeled'.

One of the objectives of the project was to compare how the refuges currently in use in Bt maize systems function in terms of facilitating mixed mating between refuge and Bt beetles. Therefore, the most informative data came from the 20% strip refuge and the 5% seed blend treatments.

Data analysis

Analysis of variance (ANOVA) tests were used to assess differences in the fitness parameters (head capsule size and dry weight) measured across treatments and sex using the MIXED procedure in SAS v.9.4 (SAS Institute, Cary, NC, USA). Data on head capsule size and dry weight from adult beetles in mating and emergence cages were pooled for analysis. Data were pooled because trends within emergence and mating cages were in agreement with results of combined analysis; using a combined analysis did not change the significance of any variable. Explanatory variables used in these models were replicate, date collected, sex, treatment, and natal host type (Bt or refuge plant). The variable 'replicate' was treated as random. Tukey's honestly significant difference (HSD) test was used to separate means (Zar, 1999). Fisher's exact test was used to test the association between the proportion of Bt/refuge beetles in the mating population (collected from emergence cages) and the proportion of Bt/refuge beetles in mating pairs (collected from mating cages) for each refuge treatment by week. Each week consisted of three sequential collection days with the exception of the final week (26–30 August) where beetles were collected only on 1 day (26 August). A season-long analysis (i.e., combination of all weeks) was also performed. Because males take ca. 1 week to reach sexual maturity (Spencer et al., 2013), the number of Bt and refuge males in emergence cages from 1 week prior were paired with the number of Bt and refuge females in emergence cages from that week to estimate the operational sex ratio of Bt/refuge beetles. The null hypothesis was that Bt and refuge beetles were equally likely to mate. Rejection of the null hypothesis indicates that assortative mating occurred. Two-sided P-values were calculated using the method of summing small P-values (Agresti, 1992).

Results

Mate pairing

The percentage of refuge and Bt beetles collected in mating pairs was different from that in the potential mating population in both 20% strip (Fisher's exact test: $P = 0.0001$) and 5% seed blend refuges ($P = 0.0002$) over the mating season (20% strip: 8 Jul – 26 Aug; 5% seed blend: 15 Jul – 26 Aug) (Table 1). In both refuge treatments, the percentages of Bt beetles collected in mating pairs (20% strip: 55.7%; 5% seed blend: 80.7%) were higher than those in the background population (20% strip: 36.9%; 5% seed blend: 67.4%). Mean rates of mating combinations in the 20% strip refuge were: 43.3 ± 6.1% (mixed mating), 34.9 ± 8.9% (Bt × Bt), and 25.3 ± 11.1% (refuge × refuge). In the 5% seed blend refuge the following frequencies were observed: 35.1 ± 7.6% (mixed mating), 64.4 ± 11.0% (Bt × Bt), and 5.2 ± 3.2% (refuge × refuge).

WCR emergence

Delayed emergence in the treatments containing Bt maize was observed in our experimental cages. The first beetles were collected in the refuge-only treatment on 3 July 2013. The first beetles were collected from the Bt-only treatment on 8 July 2013. Despite this brief delay in initial emergence, peak emergence for all treatments occurred on the same sampling date, 22 July 2013. After peak emergence, the numbers of emerging beetles declined sharply over the next few collection days for all treatments and slowly decreased for several weeks until the end of August (Figure 1). The first treatment with no emergence was Bt only, on 16 August 2013. Following, in order, were the 5%

Table 1 Fisher's exact test of the number of *Diabrotica virgifera virgifera* adults that fed as larvae on Bt and refuge maize in mating pairs and in the potential mating population, under different refuge configurations (20% strip and 5% seed blend) of *Cry3Bb1*-expressing maize. Beetles were collected from caged plots in Tippecanoe County, Indiana, USA (2013)

		Mating (n = 4 plots)		Population (n = 2 plots)		
Refuge	Date	Bt	Refuge	Bt	Refuge	P
20% strip	8–12 Jul	1	7	3	5	0.57
	15–19 Jul	11	21	20	38	1
	22–26 Jul	28	18	56	62	0.16
	29 Jul – 2 Aug	18	16	14	33	0.041
	5–9 Aug	28	14	6	14	0.013
	12–16 Aug	14	6	4	19	0.0007
	19–23 Aug	5	3	1	6	0.12
	26 Aug	2	0	0	1	0.33
	Overall (8 Jul – 26 Aug)	107	85	104	178	0.0001
5% seed blend	15–19 Jul	18	8	44	14	0.59
	22–26 Jul	49	13	73	41	0.042
	29 Jul – 2 Aug	56	18	43	25	0.14
	5–9 Aug	89	17	15	3	1
	12–16 Aug	35	3	8	1	1
	19–23 Aug	19	1	1	5	0.0005
	26 Aug	5	5	0	0	1
	Overall (15 Jul – 26 Aug)	271	65	184	89	0.0002

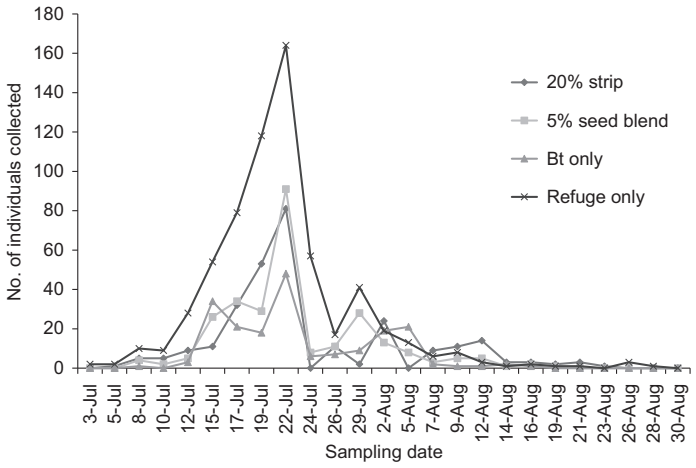

Figure 1 Total number of field-collected *Diabrotica virgifera virgifera* adults collected from caged plots (n = 2) in Tippecanoe County, IN, USA (2 July – 28 August 2013) under different refuge configurations of *Cry3Bb1*-expressing maize. Emergence begins earlier in plots containing only refuge plants. However, emergence peaks on the same date, 22 July, for all configurations.

seed blend and 20% strip refuge (26 August 2013), and the refuge-only treatment (28 August 2013).

Female-biased sex ratios were found in all emergence cage treatments. Ratios of males to females were as follows: 1:1.37 (Bt only), 1:1.75 (20% strip refuge), 1:1.73 (5% seed blend), and 1:1.65 (refuge only). Total numbers of adult captures by treatment were: 639 (refuge only), 278 (20% strip refuge), 276 (5% seed blend), and 194 (Bt only).

Of the adults collected from emergence cages in the 20% strip refuge, 63.7% of males and 61.9% of females were positively labeled with ^{15}N. In the 5% seed blend plots, 38.6% of males and 29.7% of females were labeled. Atom % excess decreased in labeled samples from the beginning to the end of the experiment ($F_{20,803} = 10.24$, $P<0.0001$) (Figure 2). This same trend occurred in beetles from mating cages (data not shown). This may be explained by the decay of the label over time or the dilution of the label concentration in root tissue as the label moves to other plant tissues in the growing plant. We did not investigate movement or spread of the label in plant tissue.

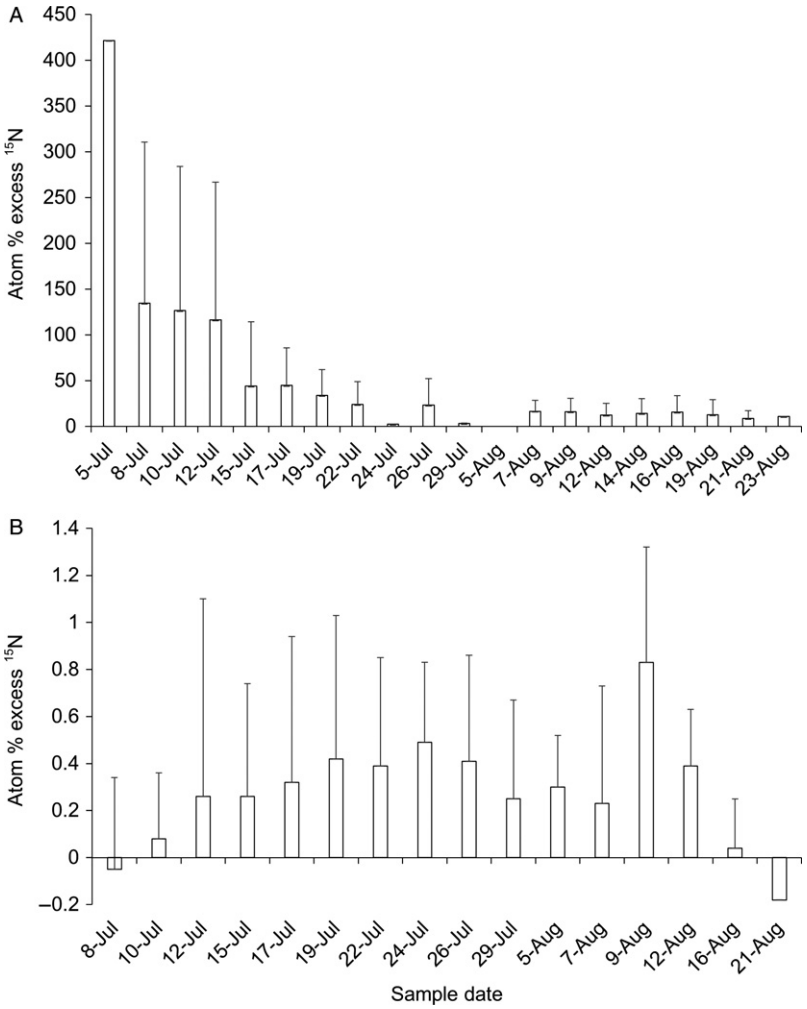

Figure 2 Mean (+ SD) atom % ^{15}N excess in elytra and head capsules of field-collected *Diabrotica virgifera virgifera* adults identified as (A) labeled and (B) unlabeled from caged plots (n = 2) in Tippecanoe County, IN, USA, under different refuge configurations of *Cry3Bb1*-expressing maize across sampling dates (2013). Positively labeled adults have high levels of ^{15}N early in the sampling period and low levels of ^{15}N late in the season. Unlabeled adults have atom % excess values <1.5.

Natal host plant effects

Significant differences were found in the head capsule widths of adult WCR by date collected only ($F_{20,803}$ = 3.02, P<0.0001). There were no differences in head capsule widths by natal host type ($F_{1,803}$ = 1.10, P = 0.30), treatment ($F_{3,803}$ = 2.22, P = 0.085; Figure 3), sex ($F_{1,803}$ = 0.00, P = 0.99), or replicate ($F_{5,803}$ = 5.65, P = 0.11).

Significant differences were found in the dry weight of adult WCR by date collected ($F_{20,803}$ = 2.07, P = 0.0041), treatment ($F_{3,803}$ = 3.36, P = 0.018), and sex ($F_{1,803}$ = 29.99, P<0.0001). Differences in dry weight were significant between refuge-only and Bt-only treatments (P = 0.035) (Figure 4). There were no differences between 20% strip vs. 5% seed blend treatments (P = 1.0), 20% strip vs. refuge only (P = 0.22), 20% strip vs. Bt only (P = 0.54), 5% seed blend vs. refuge only (P = 0.18), and 5% seed blend vs. Bt only (P = 0.23). Mean dry weight was highest in the refuge-only

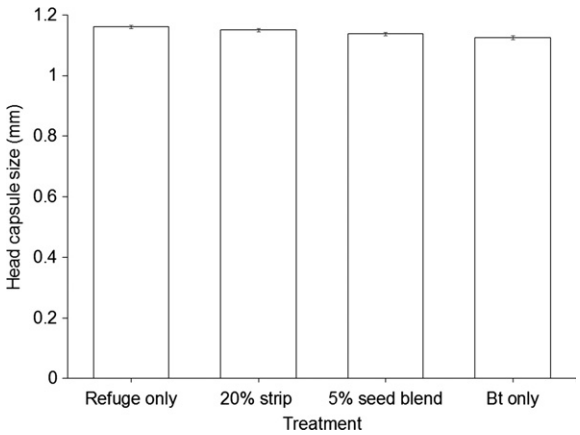

Figure 3 Mean (± SEM) head capsule width (mm) of field-collected *Diabrotica virgifera virgifera* adults from caged plots (n = 6) in Tippecanoe County, IN, USA (2013) under different refuge configurations of *Cry3Bb1*-expressing maize. Means did not differ significantly (Tukey's HSD test: P>0.05).

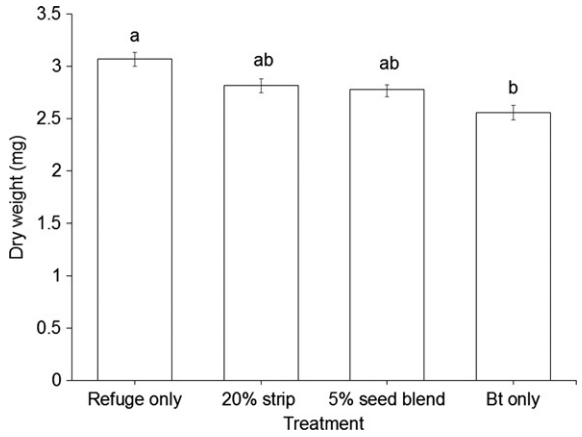

Figure 4 Mean (± SEM) dry weight (mg) of field-collected *Diabrotica virgifera virgifera* adults from caged plots (n = 6) in Tippecanoe County, IN, USA (2013) under different refuge configurations of *Cry3Bb1*-expressing maize. Means capped with the same letters are not significantly different (Tukey's HSD test: P>0.05).

treatment (3.100 ± 0.064 mg), followed by 20% strip (2.842 ± 0.059 mg), 5% seed blend (2.798 ± 0.047 mg), and Bt only (2.590 ± 0.071 mg). Males were heavier than females (2.985 ± 0.042 vs. 2.680 ± 0.042 mg dry weight). There were no differences in dry weight by replicate ($F_{5,803}$= 0.34, P = 0.80) or natal host type ($F_{1,803}$ = 2.19, P = 0.14).

Discussion

Results from our study indicated that 5% seed blend and 20% strip refuges do not facilitate random mating between WCR adults from different natal hosts when they are confined in field cages. Several factors have been hypothesized to influence mating between insects that emerge from refuge and Bt plants including: delayed adult emergence of insects exposed to toxins, decreased size of individuals fed from toxic plants, and limited in-field movement of adults prior to mating (Gould, 1998). This cage study presents the first empirical evidence that at least two of these factors, delayed emergence and decreased size, may influence mating rates between WCR from Bt and refuge host plants.

Besides the obvious limitation of caging plants and emerging beetles, there were other considerations that limited the scope of this study. Some ^{15}N-labeled beetles may have fed partially on Bt plants due to larval movement (Hibbard et al., 2005; Zukoff et al., 2012; Head et al., 2014). Our method was not sensitive enough to determine degree of refuge feeding. It is likely that refuge and Bt populations included beetles that fed, at least to some extent, on different hosts. Furthermore, although we limited opportunity for abdominal contents to affect ^{15}N marking, we cannot eliminate the possibility that beetles acquired some ^{15}N in their elytra and head capsules via feeding on labeled plants as adults. Enrichment from adult feeding could have only occurred in seed blend refuges because all refuge plants were cut prior to adult emergence in strip refuges. Results from trials conducted in 2015 indicated a small increase in ^{15}N in elytra and head capsules of beetles fed as adults from leaves, pollen, and silk of labeled plants (S Taylor & C Krupke, unpubl.).

Although not the central focus of this work, it is noteworthy that WCR from Bt plants represented a high percentage of the total population in our refuge treatments. Thus far, resistance to Bt maize has not been documented in Indiana, nor are we aware of any field-level performance problems at the time of this writing. We did not test populations in this study for levels of tolerance or resistance to Bt toxins. Susceptibility of a WCR field population to Cry3Bb1 toxin may vary based on genetic background, environmental conditions, and expression levels in the plant (Siegfried et al., 2005; Meihls et al., 2008; Clark et al., 2012; Devos et al., 2013). Cry3Bb1 is designated as a 'low to medium dose' toxin because WCR larvae have an inherent tolerance (i.e., low susceptibility to Cry3Bb1 prior to repeated exposure) and survival to the adult stage has been documented since the Cry3Bb1 toxin was first deployed in maize (Siegfried et al., 2005). One tenet of the refuge strategy is that mating between insects from Bt natal hosts should be rare because of the relatively large number of insects that emerge from refuge hosts (Onstad et al., 2011). Given the rates of survival of exposed WCR demonstrated by our work and others (Meihls et al., 2008; Gassmann et al., 2011, 2012; Clark et al., 2012), this central tenet is likely to be violated in the field with currently available Bt traits in maize. The relatively high survival rates of WCR exposed to current Bt maize hybrids has been known for some time, and there remains substantial debate in the literature regarding the refuge size needed to produce sufficient adults to delay resistance development. Some research suggests 50% refuge is advisable for single toxin varieties, yet transitioning to larger refuge sizes would be an arduous process, given the limited availability of refuge seed (Tabashnik & Gould, 2012) and likely resistance from the commercial market (i.e., farmers). Even if a 5% seed blend refuge supported truly random mating, our trials indicated that the predominant type of mating would be between pairs of beetles from Bt hosts (>65%).

WCR larvae fed on Cry3Bb1 toxin develop into adults more slowly compared with their unexposed counterparts (Murphy et al., 2010; Oswald et al., 2012). Asynchronous emergence of sexually mature adults from refuge and Bt

hosts may reduce opportunities for partners from different host plants to mate (Murphy et al., 2010, 2011). These emergence delays in beetles from Bt plants are less pronounced in seed blends compared to strip or block refuges (Murphy et al., 2010), potentially because larvae can move more readily between toxic and non-toxic hosts (Hibbard et al., 2005; Zukoff et al., 2012; Head et al., 2014). From the standpoint of resistance management, larval movement may hasten evolution of resistance if larvae can avoid lethal doses of toxin by changing hosts (Mallet & Porter, 1992; Hibbard et al., 2003, 2005; Zukoff et al., 2012; Head et al., 2014). Close proximity of non-toxic refuge plants to Bt plants in our seed blend refuge and small-scale strip refuge could have resulted in higher proportions of larvae moving between host plants. This, in turn, would result in higher levels of 'labeled' beetles that had fed on refuge plants as larvae, overlapping in emergence with beetles from Bt hosts. The inability to discriminate the extent to which larvae fed on refuge plants is a key limitation of our experimental design; this represents an area ripe for further exploration.

In addition to their longer development times, larvae feeding only on Bt maize develop into smaller adults (Murphy et al., 2011; Hoffmann et al., 2015), a finding that was replicated in our study. Differences in sizes between refuge and Bt-fed adults may lead to assortative mating because adult males have demonstrated preference for large mates in laboratory choice assays (Kang & Krupke, 2009). Our experiments revealed a trend where mean dry weight decreased for WCR adults from treatments containing only Bt plants. However, beetle head capsule sizes and dry weights were not different between treatments that contained both types of host plants. It is possible that larval movement between host plants has an equilibrating effect, and moderates the size-limiting effects of toxic plants in our study.

Small plot size (four rows) and use of field cages restricted our ability to test how adult dispersal affected mating rates. Male rootworms disperse on average 15 m per day based on previous field studies (Spencer et al., 2009); our field cages prevented males from dispersing more than ca. 4 m from their natal origin. Thus, cages may have retained potential mating males that would have otherwise dispersed. The degree to which this affected the observed rates of mating in our refuges cannot be determined given our experimental design. However, males do not travel farther than necessary to find mates (Marquardt & Krupke, 2009; Spencer et al., 2013), male capacity to mate declines with age (Kang & Krupke, 2009; Spencer et al., 2013), and most females mate within hours of emergence, often on their natal host plant (Marquardt & Krupke, 2009; Spencer et al., 2013). These characteristics of rootworm mating behavior make it likely that males mate early with the closest available female (Spencer et al., 2013), and may disperse prior to mating only when necessary. Proximity of refuge plants to Bt plants is likely to contribute significantly to rates of mixed mating depending on the distance adults move prior to mating. Adult WCR from refuge and Bt plants will likely mate more frequently in seed blends because widely dispersed refuge plantings increase opportunities for refuge WCR to encounter a mate from a different type host plant (Murphy et al., 2011). The reduced scale of our strip refuge may have inadvertently mimicked this effect by placing beetles from different hosts in closer proximity than they would otherwise be found in larger commercial fields. Mating rates in our 20% strip treatments do not likely characterize what occurs in six-row refuges, where Bt plantings are located further than three rows from refuge boundaries. However, mating rates in our strip treatment may adequately reflect how beetles mix at refuge boundaries, the location where beetles from different host plants are most likely to encounter and mate. Another potential factor affecting mating rates was the cutting of plants and removal of leaves to better facilitate the collection of adult beetles from field cages. Reducing or removing these physical barriers may have inadvertently increased mating rates between beetles from different natal hosts.

Our results add to a growing body of literature investigating viability of current refuge strategies to manage resistance by providing evidence on how emergence delays and size differences influenced WCR mating in the field. It is doubtful that refuges alone can slow the pace of resistance evolution given the high survival of beetles from Bt plants. Ideally, integrated pest management (IPM) and insect resistance management (IRM) are used in combination to address this goal. Using a greater diversity of management approaches between and within growing seasons, including crop rotation, avoiding use of Bt maize in areas where WCR pressure is low, rotation of Bt events, and application of soil insecticides as needed, all have potential to reduce selection pressure for insects to develop resistance (Gassmann et al., 2012). With documented WCR resistance to the Cry3Bb1 toxin after less than a decade of commercial use (Gassmann et al., 2011), the next Bt traits, or any other plant-expressed toxins targeting WCR (e.g., RNAi), should be safeguarded with research-based IRM strategies (i.e., research using empirical evidence) and not merely a blind reliance on refuges, which are in turn based upon assumptions about key aspects of pest biology. Our next steps for this research are to conduct field studies to determine whether refuges continue to facilitate random

mating between refuge and Bt-emergent WCR when beetles are able to move and disperse freely throughout commercial plantings.

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