

# The impact of Bt maize as a natal host on adult head capsule width in field populations of western corn rootworm

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## Abstract

The success of the current resistance management plan for transgenic maize, *Zea mays* L. (Poaceae), targeting the rootworm complex hinges upon high rates of mating between resistant and susceptible beetles. However, differences in the fitness of adult beetles could result in assortative mating, which could, in turn, change the rate of resistance evolution. Adult head capsule widths of naturally occurring populations of western corn rootworm, *Diabrotica virgifera virgifera* LeConte (Coleoptera: Chrysomelidae), were examined in a variety of refuge configurations. Beetles were classified into treatments based on the hybrid – non-Bt refuge or Bt maize targeting larval rootworms (hereafter Bt-RW maize) – and location – proximity to other Bt-RW or refuge plants – of the natal host plant. Treatments included the following: a refuge plant surrounded by other refuge plants, a refuge plant located near a Bt-RW plant, a Bt-RW plant surrounded by Bt-RW plants, and a Bt-RW plant located near a refuge plant. The mean head capsule width of males emerging from Bt-RW plants was significantly smaller than the mean head capsule width of males emerging from refuge plants. These results indicate that males emerging from Bt-RW maize plants may be exposed to sublethal doses of the Bt toxin as larvae. No differences were detected between females emerging from refuge plants compared with Bt-RW plants. Overall mean head capsule width decreased as the season progressed, regardless of treatment. The diminished head capsule width of western corn rootworm males emerging from Bt-RW maize may act to enhance resistance management, particularly in a seed mix refuge system.

## Introduction

The use of transgenic Bt (*Bacillus thuringiensis* Berliner) maize, *Zea mays* L. (Poaceae), crops can reduce pesticide applications while improving yields and economic returns (Carpenter & Gianessi, 2001; Shelton et al., 2002; Brooks & Barfoot, 2005). Insect-resistant transgenic maize, including hybrids that control western corn rootworm, *Diabrotica virgifera virgifera* LeConte (Coleoptera: Chrysomelidae), has been rapidly adopted by North American growers (James, 2006), accounting for approximately 63% of the maize acreage in the USA in 2009 (NASS, 2009). However, as adoption rates (and therefore exposure of target insect populations) increase, the potential for long-term use of Bt maize may be limited without resistance

management strategies that take into account the impact of these host plants on significant life-history parameters of surviving beetles.

The resistance management plan used for all Bt maize crops is known as the refuge strategy, where a refuge of susceptible (i.e., non-Bt) plants is planted with the resistant crop plants (EPA, 1998) to produce a large population of susceptible insects that will mate with any resistant insects (Georghiou & Taylor, 1977; Roush & Daly, 1990; Gould, 1998; Bourguet et al., 2000; Tabashnik et al., 2004). In North American maize production, the refuge requirement for most Bt maize that controls rootworms (Bt-RW maize) is 20% of the acreage planted: the refuge areas are situated either in strips across the field or in a separate block in one section of the field (EPA, 2005; Mendelsohn, 2006). Planting a refuge in these configurations requires additional labor, and the refuge portion of the crop often suffers decreased yields because of insect damage; this

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provides growers with economic and logistical disincentives to comply (Mallet & Porter, 1992; Hurley et al., 2004; Alexander, 2007). Enforcement of refuge requirements is limited, and increasing rates of non-compliance have become a major concern (Goldberger et al., 2005; Jaffe, 2009).

Recently, the use of a seed mix refuge, in which the refuge seed is sold pre-mixed at the established percentage with the Bt-RW maize seed (Monsanto, 2010; Pioneer, 2010), was approved for sale in the USA by the Environmental Protection Agency (EPA, 2010). A seed mix refuge effectively eliminates the possibility of non-compliance, making this strategy appealing to seed companies and regulators. Additionally, yield increases (because of reduced refuge size), combined with the ease of planting compared to a block refuge, make seed mixes an attractive option for growers (Alexander, 2007; EPA, 2009). However, seed mix refuges present new biological considerations for resistance management. Most notably, they provide an increased opportunity for rootworm larvae to move between refuge and transgenic plants, possibly increasing exposure of the pest population to sublethal doses of Bt toxin, which can undermine resistance management (Mallet & Porter, 1992; Gould, 1998; Onstad & Gould, 1998; Davis & Onstad, 2000).

The refuge strategy requires that susceptible and resistant (or heterozygous resistant) western corn rootworm adults disperse and mate randomly in the field (Gould, 1986, 1994). Male rootworms normally emerge before females and are the primary dispersers prior to mating (Cates, 1968). The refuge strategy model (Gould, 1994; Onstad & Gould, 1998) assumptions of random mating between beetles from refuge and Bt-RW plants may be impacted by the size and fitness of emerging insects. Western corn rootworm males prefer larger females (Kang & Krupke, 2009a), and it follows that any environmental factors that lead to size differences may lead to non-random mating (Quiring & Timmins, 1990).

Head capsule width is commonly used as a biological fitness parameter to study corn rootworm larvae and adults (Branson & Ortman, 1970; Branson & Sutter, 1985; Weiss et al., 1985; Xie et al., 1992; Clark & Hibbard, 2004; Oyediran et al., 2004, 2008; Wilson & Hibbard, 2004; Chege et al., 2005; Ellsbury et al., 2005; Campbell, 2009; El Khishen et al., 2009; Li et al., 2009). Head capsule width has also been demonstrated to correlate moderately with lifetime fecundity for adult western corn rootworm females, and with longevity in young adult male western corn rootworms (Li et al., 2009). Unlike fresh or dry mass, head capsule width remains constant throughout each life stage (three instars and adult; Hammack et al., 2003; Li et al., 2009). Differences in the width of the adult head

capsule are often attributed to larval exposure to stressors in the western corn rootworm (Branson & Sutter, 1985), making it an ideal measure of fitness for adults exposed to a larval diet of varying quality. In this study, we measured and compared head capsules to determine whether exposure of western corn rootworm larvae to maize expressing a Bt toxin influenced adult fitness. We present data from a variety of possible refuge configurations and describe the potential implications for insect resistance management in seed mix refuges.

## Materials and methods

### Plot structure and planting

This is a companion study to Murphy et al. (2010), and plot orientation is described in further detail therein. There were seven refuge structure treatments that included 10 and 20% block, strip, and seed mix refuge structures, as well as a 100% refuge plot (hereafter referred to as 'control'). Each treatment was replicated in three 15.3 × 15.3 m (50 × 50 feet), 20-row plots at each location. Plots were planted in 2007 and 2008 at the Throckmorton Purdue Agricultural Center (TPAC) and the Agronomy Center for Research and Education (ACRE), both in Tippecanoe County, IN, USA. These two locations historically have high levels of western corn rootworm pressure; to enhance this pressure, the fields used were planted with maize the previous year. Planting occurred at TPAC and ACRE on 2 and 3 May in 2007, respectively, and on 5 and 6 May in 2008, respectively, within the conventional time frame for planting maize in northwestern Indiana in each year. The row spacing and plant density were 0.76 m and 68 448 kernels ha<sup>-1</sup>, respectively. Each treatment was bordered by one guard row of refuge maize on two sides.

All the hybrids used were glyphosate-tolerant and expressed the protein Cry1Ab (Event MON810, toxic to various lepidopteran larvae, including the European corn borer, *Ostrinia nubilalis* Hübner). The hybrids used for refuge and Bt-RW plantings were DKC61-73 and DKC61-69, respectively (Dekalb, St. Louis, MO, USA). The Bt-RW hybrids contained Event MON88017 and expressed the Cry3Bb1 Bt protein (toxic to *Diabrotica* spp.). The Bt-RW seed was treated with clothianidin insecticidal seed treatment at 0.25 mg per kernel (Poncho 250<sup>®</sup>; Bayer Crop Science, Research Triangle Park, NC, USA), a dose that is commonly applied to seed maize to control secondary insect pests in the USA. This rate offers no control of rootworm larvae. The refuge seed was treated with a higher rate of clothianidin (1.25 mg per kernel; Poncho 1250<sup>®</sup>) to avoid excessive rootworm damage in these high-pressure environments and better represent the situation in

commercial maize fields. Poncho 1250<sup>®</sup> is labeled for rootworm control and is sold commercially for this purpose (Bayer, 2005; Gray et al., 2006). Post-emergence glyphosate was applied for weed control at all locations, excluding ACRE in 2007.

#### **Beetle collection**

We collected emerging beetles from the field by randomly selecting and caging at least three refuge plants and three Bt-RW plants within a 15.3 × 7.6 m (maximum) sub-section of each plot. Plant selection was accomplished by randomly selecting a row number, where refuge structure permitted, and then a plant number within that row; though, modifications were made to accommodate trap size and spacing. The randomization was stratified across the inner 18 rows of a plot, placing only two cages in a section of six rows where refuge structure permitted. All of these plants were assayed for the presence or absence of the Cry3Bb1 protein using QuickStix<sup>™</sup> for Cry3B YieldGard<sup>®</sup> Rootworm Corn (Product No. AS 015; Envirologix, Portland, ME, USA) test strips. The selected plants, and the ground surrounding them, were covered with emergence cages in mid-June. The emergence cages were modified versions of those described by Musick & Fairchild (1970). They measured 0.8 × 0.5 m and were constructed of fine wire mesh (3 mm mesh size) covering wood frames. The upper portion of the cage was fitted with a single funnel trap, secured, and maintained upright using a wooden stake. Cage bases were sealed by embedding them into the ground and then securing them with two tent pegs. Strips of foam were wrapped around the stalk to seal the opening around the plant. The emergence cages were checked twice each week at both TPAC and ACRE locations beginning 25 June 2007 and 26 June 2008. To prevent escape, collection containers were placed on dry ice in 2007. Traps were monitored throughout the season, and beetles that had emerged were collected and stored individually in labeled vials at -10 °C.

#### **Head capsule measurements**

All beetles collected from emergence traps were separated into one of four treatments based on the type and locality of the plant they emerged from: (1) refuge plant surrounded by other refuge plants, (2) refuge plant near a Bt-RW plant, (3) Bt-RW plant surrounded by only other Bt-RW plants, or (4) Bt-RW plant near a refuge plant. According to Hibbard et al. (2003), western corn rootworms are capable of moving as much as 66 cm within maize rows and at least 46 cm across rows. With these movement capabilities in mind, we used the following designations in our study: a refuge plant was considered to be near a Bt-RW plant if there were at least two Bt-

RW plants within a radius of 19 cm within row and 76 cm across rows. Similarly, a Bt-RW plant was classified as near a refuge plant if there was at least one refuge plant within a 38-cm radius within row and a 76-cm radius across rows. Up to three male and three female beetles from each treatment, on each collection date (two collection dates per week), at each location, were randomly selected from the overall pool of beetles for measurement of the head capsule (see Tables 1 and 2). Sex was determined by examining the abdominal apex based on the methods described by White (1977). The head capsule of each beetle was viewed at 27× total magnification using a stereo microscope with an attached digital camera (models SZX12 and U-CMAD3, respectively; Olympus Optical, Tokyo, Japan). Each head capsule was displayed as a live digital image using AnalySIS MicroSuite imaging software (Soft Imaging System, Lakewood, CO, USA). A single measurement, accurate to within 0.01 mm, was taken at the widest point of the head capsule from eye to eye.

#### **Data analysis**

Statistical analysis was performed using Minitab statistical analysis software (version 16.1.0.0; Minitab, State College, PA, USA). Analysis of variance (ANOVA) and Tukey's multiple comparisons tests were performed separately on males and females for each year, following Box-Cox transformations for 2007 only. A one-way ANOVA followed by a Tukey's multiple comparisons test was also performed based on location (males and females combined) for 2007 and 2008, separately, following a Box-Cox transformation for the 2007 ACRE data. The results for the 2 years combined (males and females combined) were compared using a Kruskal-Wallis test, followed by multiple Mann-Whitney tests with a Bonferroni adjustment; these tests were used because the data failed to meet the normality assumptions necessary for ANOVA after transformation. The changes in mean head capsule width per week over time were obtained by pooling the measurements for all treatments (males and females) at both locations for the two sampling dates per week ( $n = 96$ , ideally). These data were then transformed using a Box-Cox transformation for 2007 data only and analyzed using ANOVA. In addition to the transformation of the 2007 data, weeks that included <30 total beetles were omitted for both years to reduce the chance of bias caused by incomplete samples or insufficient representation of a treatment. These included the weeks of 20 and 27 August for 2007, and weeks 23 and 30 June, 7 July, and 29 September for 2008. Degree day values (base 11 °C) for each calendar date were calculated using daily average soil temperatures from ACRE (Bergman & Turpin, 1986).

**Table 1** The number of beetles collected in 2007 that were used for head capsule measurements and analysis. Beetles are categorized by location, treatment, sex, and collection timing

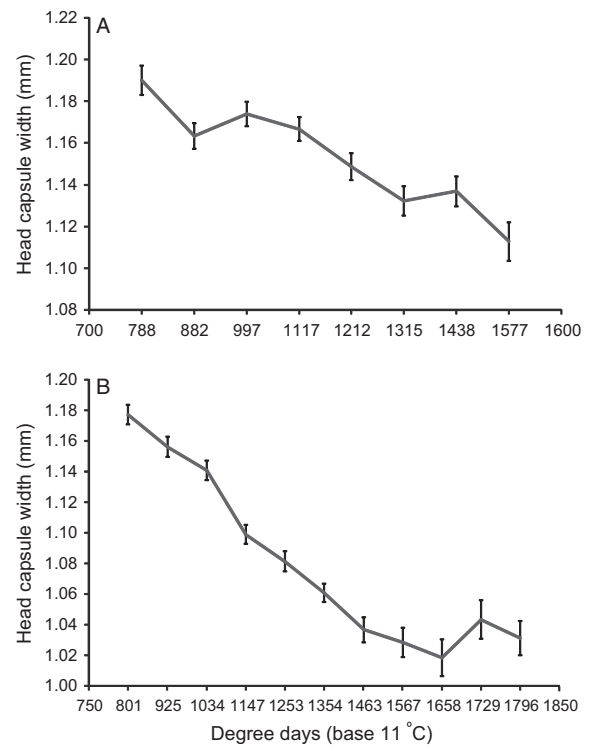
Week	Degree days	Refuge in refuge		Refuge near Bt-RW		Bt-RW in Bt-RW		Bt-RW near refuge	
		♀	♂	♀	♂	♀	♂	♀	♂
Agronomy Center for Research and Education									
25 June	788	4	5	5	6	0	0	0	1
2 July	882	6	6	6	6	1	2	5	5
9 July	997	6	6	6	6	6	6	6	6
16 July	1117	6	6	6	6	6	6	6	6
23 July	1212	6	6	6	6	6	6	6	6
30 July	1315	6	5	6	6	6	6	6	5
6 August	1438	6	6	6	6	6	6	6	5
13 August	1577	3	2	6	3	5	5	3	3
Throckmorton Purdue Agricultural Center									
25 June	788	6	6	6	6	0	1	3	0
2 July	882	6	6	6	6	4	6	5	6
9 July	997	6	6	6	6	6	6	6	6
16 July	1117	6	6	6	6	6	6	6	6
23 July	1212	6	3	6	6	6	6	6	5
30 July	1315	6	5	6	6	6	5	6	1
6 August	1438	6	4	6	5	6	5	6	2
13 August	1577	5	0	4	2	5	1	3	1

**Results**

In 2007, the mean head capsule width for males that emerged from Bt-RW plants surrounded by Bt-RW plants was significantly smaller when compared with males that emerged from refuge plants ( $F_{3,297} = 9.45, P < 0.001$ ). In 2008, males that emerged from refuge plants, or Bt-RW plants located near refuge plants, had significantly wider head capsules compared with males that emerged from Bt-RW plants surrounded by Bt-RW plants ( $F_{3,324} = 4.66, P = 0.003$ ). There was no difference in mean head capsule width for females in 2007 or 2008. As the season progressed, the overall mean head capsule width per week decreased in both 2007 ( $F_{7,624} = 10.39, P < 0.001$ ) and 2008 ( $F_{10,757} = 48.13, P < 0.001$ ; Figure 1).

When examined by location, the mean head capsule width for beetles that emerged from a Bt-RW plant surrounded by other Bt-RW plants was significantly smaller than the mean head capsule width of beetles that emerged from a refuge plant surrounded by other refuge plants for TPAC in 2007 ( $F_{3,316} = 8.81, P < 0.001$ ) and ACRE in 2008 ( $F_{3,400} = 9.25, P < 0.001$ ). The same trend was seen for ACRE in 2007.

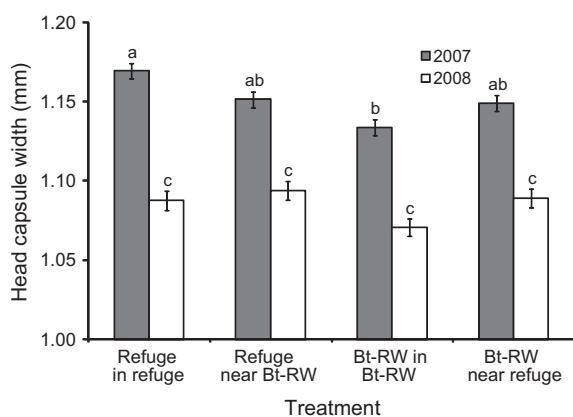
There was a significant difference between the mean head capsule widths for 2007 compared to 2008 in every treatment (Figure 2). In 2008, the mean head capsule widths were significantly smaller than each treatment in



**Figure 1** Mean ( $\pm$  SEM) head capsule width over time in (A) 2007 and (B) 2008. Head capsule widths of males and females were combined for each year.

**Table 2** The number of beetles collected in 2008 that were used for head capsule measurements and analysis. Beetles are categorized by location, treatment, sex, and collection timing

Week	Degree days	Refuge in refuge		Refuge near Bt-RW		Bt-RW in Bt-RW		Bt-RW near refuge	
		♀	♂	♀	♂	♀	♂	♀	♂
Agronomy Center for Research and Education									
14 July	801	6	6	6	6	1	0	4	6
21 July	925	6	6	6	6	3	6	6	6
28 July	1034	6	6	6	6	6	6	6	6
4 August	1147	6	6	6	6	6	6	6	6
11 August	1253	6	6	6	6	6	6	6	6
18 August	1354	6	6	6	6	6	6	6	4
25 August	1463	6	2	6	4	6	6	6	4
1 September	1567	6	1	6	0	6	4	6	2
8 September	1658	5	0	6	0	6	1	3	0
15 September	1729	2	0	4	0	2	0	4	0
22 September	1796	4	0	5	0	5	0	4	0
Throckmorton Purdue Agricultural Center									
14 July	801	4	3	1	3	0	0	0	1
21 July	925	6	6	6	6	5	6	4	6
28 July	1034	6	6	6	6	6	6	6	6
4 August	1147	6	6	6	6	6	6	6	6
11 August	1253	6	6	6	6	6	6	6	6
18 August	1354	6	6	6	6	6	6	6	5
25 August	1463	6	5	6	6	6	6	6	4
1 September	1567	6	3	6	2	6	3	6	3
8 September	1658	6	3	6	0	4	0	4	0
15 September	1729	6	1	5	0	3	0	4	0
22 September	1796	4	2	3	1	5	1	1	0

**Figure 2** Overall mean ( $\pm$  SEM) head capsule width for each treatment compared across and within years. Head capsule widths of males and females were combined for each year. The letters represent the grouping for significance at a level of  $\alpha = 0.05$ . Means capped by the same letter are not significantly different. These data were analyzed using a Kruskal–Wallis test followed by multiple Mann–Whitney tests with a Bonferroni adjustment.

2007 ( $H = 231.13$ , d.f. = 1,  $P < 0.001$ ). Additionally, the overall mean head capsule width for male and female beetles (combined) that emerged from a Bt-RW plant surrounded by other Bt-RW plants in 2007 was significantly smaller than the mean head capsule width of beetles that emerged from refuge plants surrounded by other refuge plants ( $W = 32\,966.5$ ,  $P < 0.001$ ). The sex ratio for all the beetles collected (all treatments combined) was female biased for both years, with females accounting for 73.4% of the population in 2007 and 57.9% of the population in 2008.

## Discussion

It can be difficult to discern the mechanism for differences in head capsule size in western corn rootworms (Meinke et al., 2009); though, density-dependent effects have been a commonly reported cause (Branson & Sutter, 1985; Weiss et al., 1985; Hibbard et al., 2010). Density-dependent effects have been associated with reduced head capsule width of adult females (Branson & Sutter, 1985) and



male-dominated sex ratios (Weiss et al., 1985). These differences were attributed to the early development of males, which is thought to give males an advantage over females when competing for scarce resources (Weiss et al., 1985). However, in our study, males were more severely affected than females, suggesting the involvement of additional, density-independent factors. There is some precedent to suggest that the Bt toxin may affect the sexes differently, as previous studies have reported female-biased emergence patterns from Bt hybrids expressing the Cry3Bb1 toxin (Al-Deeb & Wilde, 2005; Meinke et al., 2009; Murphy et al., 2010). Various western corn rootworm size parameters (i.e., head capsule width, elytra length, dry weight, longevity, fecundity) have been examined with several different Bt hybrids as hosts, using both laboratory and natural populations (Al-Deeb & Wilde, 2005; Storer et al., 2006; Oyediran et al., 2007; El Khishen et al., 2009). However, differences in head capsule widths of natural populations exposed to this particular event (MON88017, which was present in many of the most commonly planted maize hybrids in North America when this publication was written) have not been documented previously. We hypothesize that the differences in male head capsule size in our experiments may be because of sublethal effects of Bt-RW maize on male western corn rootworm larvae.

Several factors may explain the observed differences in head capsule size in males, but not in females. The mechanism responsible may be traced back to phenological differences between the sexes, specifically in the initiation and speed of larval development. Western corn rootworm males emerge from eggs first and develop more quickly than females (Hein et al., 1988; Jackson & Elliot, 1988; Meinke et al., 2009). These life-history parameters are important considerations in Bt maize systems if the relative toxicity of Bt-RW host plants changes based on maize phenology. Bt toxin expression in the root tissue of Event MON88017 may decline as the plant matures (Sidhu & Brown, 2004; Nguyen & Jehle, 2009), which means that earlier-emerging neonate larvae (primarily males) feeding on Bt-RW maize root tissue could be subjected to greater toxin concentrations than later-emerging females attempting to feed on the same plant. This provides females with a possible competitive advantage in Bt-RW maize systems. The female-biased sex ratios observed in both years of our study provide further support for the theory of a gender-biased advantage (Al-Deeb & Wilde, 2005; Meinke et al., 2009).

We noted no significant size differences in females (as predicted by head capsule size) in our study, and the head capsule width values described in our study are similar to those previously reported for this species (Oyediran et al., 2004; Li et al., 2009, 2010). This is encouraging from the standpoint of facilitating random mating: female weight

significantly influences male courtship behavior and mate choice, as males pursue large females more aggressively and remain in copula with them longer (Kang & Krupke, 2009a). However, we must emphasize that our study used refuge seed coated with a high rate of clothianidin insecticide (1.25 mg per kernel), labeled for control of rootworm larvae. Therefore, even in our 'refuge' treatments, we challenged the insects with significant stressors; thus, there was no true, toxin-free refuge for larvae, which may partially explain the lack of differences between Bt-RW and refuge females. In both block and strip refuges, the plants are consistently protected against rootworm larvae to preserve yield in North American commercial fields. The nature of these protective chemicals and their effects are largely uninvestigated in relation to beetle fitness from a resistance management standpoint. An experiment comparing seed treatments to unprotected isoline seed could offer useful information about the effects of various toxins on rootworm life-history parameters.

A review of climate data collected at our study locations reveals that the differences in mean head capsule width between 2007 and 2008 may be because of different environmental conditions. In 2007, both maize and western corn rootworm development progressed rapidly under warm, dry early-season conditions (May 2007 was 2.7 °C above the 30-year average with only 0.4× the 30-year average rainfall; Bowman, 2007a,b; Murphy et al., 2010). In contrast, crop and corn rootworm development in 2008 were delayed because of cool, wet weather conditions early in the season (1.4× the 30-year average precipitation and 2.3 °C below the 30-year average in May, 2.1× the 30-year normal precipitation in June; Mays, 2008; Murphy et al., 2010). The quality of maize as a host plant decreases as the season progresses (Hibbard et al., 2008), and it is likely that the mean head capsule widths for 2008 were significantly smaller than in 2007, regardless of treatment, because western corn rootworm larvae developed on older and/or less suitable maize plants. The decreases in mean overall head capsule width through each year were most likely caused by the same phenomenon.

Sublethal exposure to Bt toxin has been cited as a concern for resistance management in transgenic cropping systems, because of the possible fitness advantage provided to tolerant individuals (Gould, 1998; Onstad & Gould, 1998; Mallet & Porter, 1992). However, the results we present may actually mitigate these effects somewhat by furthering the goals of the refuge strategy in other ways. For example, if males emerging from refuge maize exhibit a fitness advantage over their counterparts emerging from Bt-RW plants, females emerging from Bt-RW plants may be more likely to mate with larger refuge males in a seed mix environment, where the distances between the refuge

and Bt-RW hosts are minimal. We also note that male mating ability declines with age (Kang and Krupke, 2009b), and proximity of plants to one another also favors resistance management by ensuring that refuge males are young enough to mate successfully once they locate females.

This research provides empirical evidence that male western corn rootworm larvae exposed to Bt-RW plants in a seed mix refuge exhibit diminished head capsule widths, which may be interpreted as evidence of reduced fitness. The implications of this result for resistance management as a whole are unclear; however, because the broad descriptor 'fitness' includes many parameters that can influence the mating success of beetles (dispersal, lifespan, mate finding, mate choice, etc.), our findings help inform the ongoing debate about how to best implement refuges in Bt maize systems.

These data highlight the necessity of revisiting elementary aspects of rootworm biology in resistance management research. Studies of the mating success of refuge beetles, which may or may not be exposed to non-Bt toxins (e.g., clothianidin), will help assess their compatibility (temporally, spatially, and with respect to size) with beetles that survive Bt toxin exposure. Furthermore, evaluating larval movement and feeding in the intertwined refuge and Bt-RW root systems is critical. This work, although logistically difficult, is likely essential to quantify the extent of sublethal exposure of larvae to Bt toxins. A better understanding of the nature and severity of these effects will be necessary to identify scientifically valid, commercially viable resistance management strategies in both current and future Bt maize systems.

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