Fig. 1.

Fig. 2.

Fig. 5.

Fig. 6.

Fig. 7.

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2 **Evaluation of European corn borer *Ostrinia nubilalis* (Lepidoptera: Crambidae) larval**
3 **movement and survival in structured and seed blend refuge plantings**

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ABSTRACT

Blending *Bacillus thuringiensis* (*Bt*) corn and non-*Bt* corn seeds in the same bag is a convenient and easier compliance solution to satisfy refuge regulations. However, there has been considerable debate and concern about larval movement of the target insects in a seed blend because of its potential effect on the development of resistance. Reported here are studies to determine the larval movement of European corn borers, *Ostrinia nubilalis* (Hübner), in a seed blend compared to a structured refuge and their effects on survival, feeding injury and larval fitness. Also evaluated is the relative performance of a purple-seeded corn hybrid as a surrogate host plant for tracking gene flow. Seed blend and structured refuge arrangements of Agrisure GT/CB/LL *Bt* corn expressing Cry1Ab and isoline plants were evaluated at two locations in 2013 and 2014 by manually infesting refuge plants with first and second generation corn borer larvae. The majority of leaf and stalk injury was recorded on the refuge plant, which amounted to 66-83% of the total tunnel length. In both refuge arrangements, plant injury and the number of larvae recovered significantly decreased on neighboring plants with increasing distance away from the refuge plant in the same row. The relative decline in injury was much more pronounced in the seed blend, with neighbor *Bt* plants experiencing very minor tunneling or no injury at all due to the high dose trait. Eighty-nine percent fewer live larvae were also found on neighbor *Bt* plants in the seed blend compared to those found in the structured refuge plots. Larvae that moved to *Bt* plants as well as older instars that were manually placed on *Bt* plants failed to complete development. Comparison of the number of tunnels and larvae recovered per refuge plant indicated that the seed blend may not produce as many susceptible individuals as those produced in a structured refuge arrangement. Although the purple-seeded hybrid showed lower levels of ear and stalk injury, it could serve as an appropriate surrogate refuge plant to track gene flow.

Key words: *Bacillus thuringiensis*, larval movement, structured refuge, seed blend refuge,

1. Introduction

The high-dose-refuge strategy is the current foundation for managing pest resistance in genetically modified corn expressing insecticidal proteins derived from *Baccillus thuringiensis* Berliner (*Bt*) (Gould 1998, Matten et al. 2012). This approach requires that the high dose expression in *Bt* plants kills homozygous susceptible and heterozygous individuals in the population, leaving only rare homozygous resistant insects. A refuge of non-*Bt* plants in close proximity to the *Bt* crop is intended to provide large numbers of susceptible insects to mate with any rare resistant insects that may survive on the transgenic crop (Ostlie et al. 1997, Gould, 1998, Huang et al. 2011).

The refuge requirement for corn products with most single *Bt* genes for lepidopteran control is a structured block or strip of non-*Bt* corn planted in or near to the *Bt* field. Since 2010, the refuge-in-bag approach has been approved for many *Bt* corn products with pyramided genes and adopted by growers in the U.S. corn belt. This concept of blending 95% *Bt* seed with 5% non-*Bt* seed in the same bag provides farmers with a convenient and easier compliance solution to satisfy federal regulations. A seed blend simplifies refuge management by eliminating the structured refuge and allows farmers to plant *Bt* corn in every field, while still complying with refuge requirements. The non-*Bt* refuge plants serve as the refuge which allows target insects such as European corn borer (ECB), *Ostrinia nubilalis* (Hübner), and corn earworm (CEW), *Helicoverpa zea*, to survive as susceptible adults and randomly mate with any rare resistant adults. Refuge plants interspersed within *Bt* corn fields also facilitates random mating of resistant and susceptible moths (Davis and Onstad 2000). Irrespective of the refuge type, models predict that resistance will be delayed substantially if the assumptions of the high dose-refuge strategy hold true (Gould, 1998, Alstad and Andow, 1995, Onstad and Gould 1998, Onstad et al. 2012, Zhao et al. 2003).

However, there has been considerable debate and concern about larval movement of the target insects in a seed blend because of its potential effect on the development of resistance (Mallet and Porter 1992, Davis and Onstad 2000, Alyokhin 2011, Onstad et al. 2011, Wangila et al. 2013, Yang et al 2014). ECB is the major stalk borer species in corn in the United States and known to disperse among plants as larvae (Razze and Mason 2012). Larval movement among *Bt* and non-*Bt* plants in a seed blend may create a more favorable environment for resistance development in target pest populations (Razze et al. 2011). For example, movement of susceptible larvae from refuge plants to *Bt* plants could lead to greater mortality of susceptible insects than in a structured refuge planting, thus resulting in a lower refuge population (Onstad and Gould 1998; Davis and Onstad 2000). On the other hand, heterozygous resistant individuals or those insects containing minor resistance alleles could feed on non-*Bt* plants first and later move to *Bt* plants and survive because older instars are much less susceptible to *Bt* toxins (Huang et al. 1999; Walker et al. 2000; Huang et al. 2006, Carrol et al 2012). Therefore, differential susceptibility among instars and larval movement among *Bt* and non-*Bt* plants could create a sub-lethal dose exposure of the target pests and promote build-up of resistance by increasing survival of heterozygotes. To address this issue, reported here are studies to determine the larval movement of ECB infestations in a seed blend compared to a structured refuge and their effects on survival, feeding injury and fitness of the target insect. The study also evaluated the relative performance of a purple-seeded hybrid as a surrogate host plant for tracking gene flow.

2. Materials and methods

2.1. Study sites and treatments

The study was repeated over two years at the Louisiana State University Agricultural Center research stations in St. Joseph (2013) and Winnsboro (2014), and at the Central Maryland Research and Education Center, Beltsville (2013 and 2014). A *Bt* corn hybrid

Agrisure GT/CB/LL containing the gene expressing the Cry1Ab protein for control of lepidopteran pests and its closely related non-*Bt* isoline were used at all sites. In certain treatments, a Syngenta's purple non-*Bt* hybrid corn which is similar in phenology and maturity period to the yellow corn hybrids was also used as the refuge plant. In each study, ECB larval movement, survival and plant injury were evaluated in four different refuge arrangements as follows: 1) Pure stands of non-*Bt* plants with infested yellow seeded non-*Bt* plants as refuges (Y-Structured); 2) Pure stands of non-*Bt* plants with infested purple seeded non-*Bt* plants as refuges (P- Structured); 3) Seed blend of *Bt* plants with infested yellow seeded non-*Bt* plants as refuges (Y-Seed blend); and 4) Seed blend of *Bt* plants with infested purple seeded non-*Bt* plants as refuges (P-Seed blend).

2.2 Experimental design and refuge placement

At each study site, the experimental design was a Latin-square consisting of four treatment plots replicated four times. Each plot measured 12 rows wide and 7.5 m long. Plots were planted side by side within a row block, whereas a buffer strip of 12 rows of male-sterile non-*Bt* corn was planted the opposite way between blocks. All seeding was done with a no-till corn planter to establish a 64,200 plant population per hectare. Each row contained about 38 plants, 20 cm apart. Plots were planted in early May at the Louisiana sites and in late May at the Maryland site.

Non-*Bt* refuge plants were selected and marked with small stakes after plant emergence in each plot of treatment 1. Refuge plants in treatments 2, 3, and 4 were hand planted immediately after each plot was machine-seeded. To ensure successful seedling emergence of refuge plants, two seeds were inserted into the soil at the appropriate depth. Each seed was planted slightly off center from the planter seed slot and marked to identify the refuge plant from other seedlings. The purple plants were easily identified by the color of their stem and leaves. At the two-leaf stage, the less vigorous non-*Bt* seedling was removed. Three refuge

plants were established in each row 3, 5, 7, and 9 and staggered about 1.8 m apart within each row, so that they were not directly across from refuge plants in the nearest adjacent rows.

2.3 Insect infestations

European corn borer egg masses were obtained from the USDA-ARS, Corn Insects and Crop Genetics Research Unit, Ames, IA. Upon hatching, corn borers were reared in the laboratory on artificial diet to second instars and then manually transferred to refuge plants in each plot. The refuge plants in all four treatments were infested at two growth stages. At the V8-V10 growth stage, 25 second ECB instars were transferred from diet cups to the inner whorl leaves of each refuge plant in rows 3 and 5 to simulate first generation infestation. A second introduction of 25 second instars was made two days later to the same plants for a total of 50 larvae per plant. Refuge plants in rows 7 and 9 were infested at anthesis to simulate second generation by transferring 50 second instars from diet cups to the ear leaf axil and axils above and below the primary ear.

Additional infestations were conducted during the 2014 study in Maryland to determine whether late instars could survive if they moved to *Bt* plants. Five fourth instars were manually placed on each of eight *Bt* plants away from the cluster sets in seed blend plots in early July and again in mid-August to simulate potential movement of first and second generation larvae.

2.4 Data collection

Movement and survival of ECB larvae during each generation were measured on cluster sets of individual refuge plants and the neighbor plants within and across rows. At the Louisiana sites, sets of the central refuge plant with four neighbor plants on each side within the row were labeled according to replicate, treatment and plant position (coded 0=refuge, N1, N2, N3 and N4 relative to the number of plants within the row away from the refuge plant). In addition, three plants on each adjacent row directly across from the refuge plant were

sampled as part of the cluster set (coded N5, N6 and N7). At the Maryland sites, sets of only five plants including the central refuge and two neighbor plants on each side within the row were labeled to replicate, treatment and plant position (coded 0=refuge, N1 and N2). For each generation infestation, data were recorded on six cluster sets of plants per treatment plot.

For the first generation, visual ratings of leaf damage were recorded 1 to 3 weeks after infestation (varied by study site) on each cluster sets of plants using the 0-9 modified leaf rating scale (Guthrie et al., 1960). At 3 to 4 weeks after infestation, each set of plants were then removed at ground level from rows 3 and 5 and brought back to the laboratory where they were carefully dissected and examined for leaf and stalk feeding injury, number of larvae and pupae, and body weights. For the second generation, cluster sets of refuge and neighbor plants were removed at the hard dough stage from rows 7 and 9. Each plant was examined carefully to record stalk, ear, and shank feeding injury. Each stalk and ear shank was dissected to record number of individual tunnels and total tunnel length. Dead, parasitized, and surviving larvae and pupae in the ear and stalk were recorded and live stages were removed to record body weight. All *Bt* plants infested with late larvae were removed from the seed blend plots and examined to record the same endpoints.

At all study sites, five consecutive plants in each structured refuge plot were removed prior to crop maturity from sections of rows farthest away from refuge plants. These non-*Bt* plants were dissected and examined to measure the natural occurring infestation level of ECB, recording the same stalk injury measurements described above. In Maryland, visual inspections were also made during early July to assess first generation leaf injury in the same plots.

2.5. Data analysis

All endpoints were analyzed using SAS software programs to compute means and standard errors, and various statistical indices and residual plots were generated to check the

assumptions of ANOVA. The mixed model procedure was used to test for treatment effects, within row plant position differences, and interactions, after data transformations were made for lack of normality and unequal variances. Data for each generation infestation were pooled over both years for each study site and analyzed separately. Response variables were leaf damage rating, number of tunnels, total tunnel length, and number and weight of larvae and pupae, with treatment and plant position relative to the refuge plant as fixed factors, and row and year as random blocking factors. Plots were treated as replicates, whereas cluster sets of plants within plots were subsamples and averaged before analysis. Tukey's option was used to test for significance ($P < 0.05$) among multiple mean comparisons. Contrast tests were also performed for specific comparisons between treatments with yellow and purple-seeded refuge plants.

3. Results

3.1. Naturally occurring ECB infestation

The background activity of the naturally occurring ECB population at all study sites was very low, particularly at the two Louisiana sites where there was no discernible evidence of ECB inflicted leaf injury, stalk tunneling, or any other related plant damages during both years. In the Maryland studies, ECB injury resulting from the natural population was present during the first generation on less than 2% of the non-*Bt* plants that were not in any way affected by the manual infestations. This low level of background activity was insignificant and had negligible effect on the data interpretation. Stalk samples examined during the second generation revealed low injury levels, averaging less than 0.5 tunnels per non-*Bt* plant. This background level may have had a slight effect on the overall level of second generation damage on the manually-infested refuge plants and neighboring non-*Bt* plants in the structured refuge plots, but relative differences would still be consistent among treatments.

3.2. First generation damage and movement

At the Louisiana sites, feeding injury to the whorl leaves was relatively light during both years, with most refuge plants showing less than a leaf damage rating of 2.5 (**Fig. 1**). The effects of treatment ($P = 0.48$) and its interaction ($P = 0.84$) with plant position within row were not significant but the main effect for plant position relative to the refuge plant was significant ($F = 21.65$; $df = 4,96$; $P < 0.001$). Leaf injury on neighboring plants decreased abruptly as the distance from the refuge plant increased. Pooled over treatments, leaf damage ratings averaged 2.25 ± 0.18 a, 1.19 ± 0.05 b, 1.07 ± 0.03 c, 1.06 ± 0.02 c, and 1.02 ± 0.01 c for the refuge plant and neighboring N1, N2, N3 and N4 plants, respectively. Leaf injury on the adjacent rows was absent, indicating no movement between rows during the whorl stage infestations. A contrast test of only refuge plant data revealed significant differences ($F = 4.31$; $df = 3,24$; $P = 0.014$), indicating that the purple refuge plants had slightly lower levels of leaf injury than the yellow-seeded refuge plants. Stalk injury from the first generation infestation in 2014 was nearly absent in all plots and no larvae were recovered. Thus, results in **Fig. 2** show the number of tunnels, tunnel length, and number of larvae per plant recorded from 432 plants dissected in 2013. Similar to leaf injury, the number of tunnels ($P = 0.085$) and tunnel length per plant ($P = 0.128$) showed no treatment or interaction effects (**Figs. 2A and 2B**) but the main effect for plant position within the row was significant for both measurements (tunnels: $F = 15.34$; $df = 4,40.7$; $P < 0.001$; tunnel length: $F = 24.47$; $df = 4,40.9$; $P < 0.001$). Furthermore, contrast tests comparing the purple and yellow-seeded refuge plants showed no significant differences (tunnels: $P = 0.170$; tunnel length: $P = 0.319$). The interaction effect for larvae recovered was significant ($F = 2.83$; $df = 12,59$; $P = 0.004$) but none were found on the four neighboring plants on both sides of the refuge. However, a range of 0.54 to 0.19 larvae per plant was recovered from refuge plants (**Fig. 2C**) and treatment differences were near significant ($P = 0.079$), indicating numerically more larvae recovered from purple refuge plants.

Leaf and stalk damage caused by the first generation was higher and less variable at the Maryland location. For leaf damage rating, the interaction effect was significant ($F = 4.6$; $df = 6,90.6$; $P < 0.001$), indicating that leaf injury was highest on the refuge plant and decreased disproportionately on the neighboring N1 and N2 plants depending on the treatment (**Fig. 3A**). Leaf damage on neighbor *Bt* plants in the seed blend treatments was significantly lower compared to the refuge plant and always below a rating of 2 (only a few shot holes present). Interaction effects were also significant for number of tunnels ($F = 3.62$; $df = 6,90.5$; $P < 0.001$) and tunnel length ($F = 7.6$; $df = 6,89.3$; $P < 0.001$), showing similar patterns across plant positions relative to the refuge plant (**Figs. 3B and 3C**). In the structured refuge treatments, stalk injury was recorded on 88, 74 and 43% of the refuge and neighbor N1 and N2 non-*Bt* plants, respectively. The number of tunnels on the refuge plants averaged 4.2 per plant with 11.4 cm of total length, while the neighboring N1 and N2 non-*Bt* plants had 48 and 79% fewer tunnels and 45 and 79% less tunnel length, respectively. In the seed blend treatments, stalk injury was recorded on 77, 8 and 5% of the refuge and neighbor N1 and N2 *Bt* plants, respectively, and the number and length of tunnels were consistently lower on the refuge plants, averaging 3.0 per plant with 8.4 cm of total length. The presence of stalk damage on neighbor *Bt* plants indicated some movement of ECB off the refuge plant but the number of tunnels and extent of tunneling were very low. The neighbor N1 and N2 *Bt* plants next to the refuge had significantly 88 and 97% fewer tunnels and 94 and 98% less tunnel length, respectively.

3.3. Second generation damage and movement

At the Louisiana location, moderate levels of stalk injury resulted from the manual infestation of larvae during anthesis in both years, with the highest injury recorded on the refuge plants (**Fig. 4**). Refuge plants in the seed blend plots had numerically less stalk injury compared to the Maryland location but there was no consistent evidence of differences

between purple and yellow seeded plants. The number of tunnels and total tunnel length were significantly affected by the treatment (tunnels: $F = 17.59$; $df = 3,94.2$; $P < 0.001$; tunnel length: $F = 22.97$; $df = 3,180$; $P < 0.001$) and plant position (tunnels: $F = 31.53$; $df = 7, 172$; $P < 0.001$; tunnel length: $F = 86.1$; $df = 7, 184$; $P < 0.001$) relative to the refuge but not by their interactions (tunnels: $P = 0.188$; tunnel length: $P = 0.122$). The extent of stalk injury in the seed blend plots was 51 to 55% less than that in the structured refuge plots. Pooled over treatments, the number and length of tunnels declined rapidly but at a lower rate from the nearest to most distant neighboring plants from the refuge within the row. The number of tunnels recorded on neighboring N1, N2, N3 and N4 plants was 86, 95, 96, and 93% less, respectively, compared to the refuge plant (**Fig. 4A**). Similar trend within the row was reflected in the total tunnel length (**Fig. 4B**). N5, N6 and N7 plants, located on adjacent rows across from refuge, also showed evidence of injury but only on a very small percentage of the plants had tunnels. Statistically, there were no significant differences among the neighbor plants within and across rows, except for the N1 plants which had higher stalk injury than the other neighboring plants.

Infestations of second generation ECB caused 2 to 5 times more stalk damage in the Maryland studies compared to the Louisiana sites, although trends were generally similar. In both years, data were recorded separately for ear and stalk injury. Ear damage consisted primarily of tunneling in the tip and shank portion of the ear. This injury was significantly different among treatments ($F = 6.62$; $df = 3,44$; $P < 0.001$) but the tunnel length was dependent on the within row position of the plants, as indicated by a significant interaction effect ($F = 5.14$; $df = 6,44$; $P < 0.001$) (**Fig. 5A**). Very little ear damage occurred in ears of the neighboring N1 and N2 plants. The purple refuge plants had virtually no ear damage, comparable to neighboring plants, while the yellow-seeded refuge plants had consistently >4 cm of tunneling in most ears. Differences in stalk injury by the second generation showed a

similar pattern to that of the first generation. Stalk injury was recorded on 94, 61 and 47% of the refuge and neighbor N1 and N2 non-*Bt* plants in structured refuge plots. The incidence of stalk injury in the seed blend plots was lower, with 65, 11 and 3% on the non-*Bt* refuge and the neighbors N1 and N2 *Bt* plants. The interaction effect for number of tunnels was not significant indicating that differences among treatments were relatively the same at each plant position. However, there were significantly more tunnels per plant in structured refuge plots than in seed blend plots ($F = 17.0$; $df = 3,44$; $P < 0.001$) (**Fig. 5B**). Tunnel length was also significantly lower in refuge and neighbor plants in seed blend plots but differences varied across the plant position ($F = 2.84$; $df = 6,44$; $P = 0.020$) (**Fig. 5C**). The number and length of tunnels per plant were considered indirect indicators of ECB fitness in terms of the number of larvae that successfully invaded stalks and caused tunneling. Levels of stalk injury in the neighbor N1 and N2 non-*Bt* plants in the structured refuge plots were 56% and 74% less than that of refuge plants, respectively. Neighbor *Bt* plants in seed blend plots also had some stalk injury but significantly less. Comparatively, overall stalk injury was 87% and 97% less on the *Bt* plants at the N1 and N2 positions, respectively. Although stalk injury was lower in these *Bt* plants, the presence of ECB tunnels indicated that some larvae moved off the refuge and survived long enough to cause feeding injury.

3.4. Number and weight of ECB larvae and pupae

For the Louisiana studies, larvae were only recovered from refuge plants after the first generation infestation in 2013, and none were found on the neighboring plants. Based on a contrast test, the number of larvae recovered from refuge plants in the structured and seed blend plots averaged 0.49 ± 0.08 and 0.25 ± 0.05 larvae per plant, respectively, and was significantly different ($F = 7.22$; $df = 1,14$; $P = 0.018$). No live larvae were recovered from cluster sets of plants during the second generation in 2013 or after both generations in 2014.

Manual infestations during both generations in Maryland resulted in more consistent and

higher numbers of ECB recovered. After the first generation infestation, destruct sampling of plants was scheduled to record larvae before they were lost due to parasitism or pupation outside the plant. Consequently, a total of 445 larvae and 65 pupae were extracted, of which the majority of larvae were late instars. Of the structured refuge plots, 32.0, 29.2 and 10.0% were recovered from the refuge plant and neighboring N1 and N2 plants, compared to 23.9, 2.4 and 2.5% found on the refuge plant and neighboring N1 and N2 plants in the seed blend plots, respectively. **Fig. 6** shows the average number per plant and body weight of ECB larvae and pupae recovered from first generation infestations by treatment and within row plant position. The number of larvae and pupae recovered was significantly less in the seed blend treatments ($F = 12.23$; $df = 3,91.3$; $P < 0.001$) and neighboring plants next to the refuge ($F = 72.67$; $df = 2,91.3$; $P < 0.001$). Few larvae and only one pupa were recovered from the N1 and N2 *Bt* plants in the seed blend treatments, and differences compared to the refuge plant were greater (near significant interaction effect; $P = 0.07$) (**Fig. 6A**). The weight of recovered larvae was recorded as a measure of fitness, with the expectation that growth inhibition may result if larvae moved off refuge plants to neighboring *Bt* plants and survived bouts of intoxication. Weights of larvae were significantly affected by plant position but the differences depended on the refuge arrangement ($F = 5.16$; $df = 6,59.5$; $P < 0.001$) (**Fig. 6B**). Statistically, there was no difference between weights of larvae extracted from purple and yellow-seeded refuge plants. Overall larvae recovered from the refuge, N1 and N2 plants in the combined structured and seed blend treatments averaged 82.0 ± 7.44 , 84.6 ± 5.34 and 74.4 ± 9.63 mg, and 88.8 ± 5.95 , 46.7 ± 8.40 and 35.3 ± 1.07 mg, respectively. However, sample size was low for the seed blend treatments because only 25 larvae were found at the N1 and N2 neighbor *Bt* plant positions. Nevertheless, results suggest that the growth of the larvae that moved off the refuge may have been inhibited in the seed mix treatments.

A total of 125 live fifth instars and 5 dead or parasitized ones were recorded from the

refuge and four neighbor plants after the second generation in Maryland studies. No pupae were found because second generation ECB normally develop to the last instar which overwinters as a late instar. Across the four treatments, the movement of larvae indicated by the number recovered from the neighboring plants relative to the refuge was similar to the movement indicated by the first generation infestation (**Fig. 7A**). Of the structured refuge plots, 39.2, 22.4 and 14.4% were recovered from the refuge plant and neighboring N1 and N2 plants, compared to 21.6, 2.4 and 0.0% found on the refuge plant and neighboring N1 and N2 plants in the seed blend plots, respectively. The average number of larvae was significantly affected by the interaction of treatment and row position of the plants ($F = 4.47$; $df = 6,48$; $P = 0.001$). Comparing differences among refuge plants only, the number of larvae recovered from structured refuge plots was significantly higher than the number recovered from the seed blend plots. Furthermore, the purple refuge plants produced fewer larvae than the yellow-seeded refuge plants. Of the seed blend treatments, no larvae were recovered from the neighbor *Bt* plants at the N2 position and only three larvae were found at the N1 position. These results provide evidence that most larvae moving off the refuge in a seed blend did not survive. Larval weights were not significantly different among treatments or across neighboring plants relative to the refuge plant (**Fig. 7B**), but there were very few surviving larvae recovered from the neighboring *Bt* plants to test for differences.

3.5. Injury and survival of late instars on Bt plants.

Of the 320 fourth instars manually introduced on *Bt* plants, none were recovered and thus assumed to have not survived. Injury measurements recorded as damaged leaves, attempted entry holes, and minor tunneling showed similar levels comparable to those recorded on neighbor *Bt* plants in the seed blend treatments. Of the older larvae introduced on leaf axils during mid-August, they immediately tried to enter stalks but were restrained by intoxication from the expressed toxins and caused only attempted entry holes.

4. Discussion

Most lepidopteran pests of corn exhibit larval movements in cornfields (Davis and Onstad, 2000). Ross and Ostlie (1990) showed that about 50-56% of ECB neonates abandon the natal plant after hatching and most of those larvae disperse within 0.5 m to other corn plants in the same row. Additionally, it is known that considerable larval movement occurs on a corn plant after the third instar which may trigger movement to other plants (White and Andow, 2007). Many scenarios have been proposed by which ECB larval movement may cause faster resistance development in a seed blend refuge arrangement (US EPA, 2010; Glaum et al. 2011). In this study, the primary focus was the movement of ECB larvae from the non-*Bt* refuge plant to neighboring *Bt* or non-*Bt* plants in the same row. The number of larvae recovered provided direct evidence of movement within cluster sets of plants. Even though larvae were not found, feeding injury differences between refuge and neighboring plants reflected the relative movement of larvae off a non-*Bt* refuge plant infested with 50 early instars during each generation. One might argue that this relatively high infestation rate could cause in an unrealistic high level of stalk injury, resulting in a density dependent effect on movement. However, average tunnel length per plant (<20 cm) was well below what has been reported for this pest (>40 cm of injury) before the adoption of *Bt* corn. We choose the conservative approach to compensate for natural mortality and ensure enough larval survival to rigorously test whether larvae moved and survived off the refuge plant. The background activity of the naturally occurring ECB population was relatively low in all studies and had a negligible effect on the interpretation of these injury differences. Although all refuge plants were infested at the same rate, ECB caused 2 to 5 times more plant injury during both generations in Maryland compared to injury levels at the Louisiana sites. Survival of larvae was also higher in Maryland as evident by the number of larvae recovered after each generation infestation. Differences in mortality factors, environmental conditions and

density-dependent effects between the two locations may have resulted in the disproportionate survival of larvae and subsequent level of injury.

The majority of leaf and stalk injury was recorded on the refuge plant, which amounted to an overall 83% of the total tunnel length in the Louisiana studies and 66% in Maryland studies. In both structured refuge and seed blend treatments, plant injury decreased on neighboring plants with increasing distance away from the refuge plant. The relative decline in injury across neighbor plants from the refuge was significantly more pronounced in the seed blend treatments due to the high dose trait of the neighboring *Bt* plants. Studies in Louisiana reported no injury inflicted or larvae recovered on all neighbor *Bt* plants in both years, yet damage patterns indicated that ECB larvae moved from the infested refuge plants to at least four plants away within the same row of the structured refuge plots. Maryland results did show evidence of stalk injury on neighbor *Bt* plants in the seed blend but the number of tunnels and extent of tunneling were overall 94% less than the injury on neighbor non-*Bt* plants in the structured refuge plots. Moreover, only 28 live larvae were found on the neighbor *Bt* plants during both years, compared to 235 larvae extracted from the neighbor non-*Bt* plants. Of those larvae that survived on *Bt* plants at least until stalks were sampled, body weights were more variable and generally lower, suggesting that larval development was inhibited in the seed blend treatments. The fate of the fourth instars that were manually placed on *Bt* plants in the 2014 study provides additional evidence that larvae moving off the refuge in a seed blend will likely not survive. These older larvae caused minor feeding injury in an attempt to bore into stalks but did not survive. Taken altogether, these findings clearly show ECB larval movement among plants in a seed blend; however, additional studies are needed to determine if this dispersal behavior could occur in ways that favor survival of heterozygote individuals and thus increase the rate of resistance development. Similar movement patterns have been shown for larvae of the sugarcane borer, *Diatraea saccharalis*

(F.), which can disperse from infested plants and move at least four plants away within the same row (Wangila et al., 2013). Although larval movement of ECB off refuge plants in a seed blend is very likely, heterozygous resistant larvae or those containing minor resistance alleles must survive in order to increase resistance development. In this study, ECB larvae that moved to *Bt* plants as well as the older instars that were manually placed on *Bt* plants failed to complete development. This observation is very important in resistance management with regard to sub-lethal exposure. The *Bt* hybrid used in this study contained a single gene that is high dose for ECB, whereas hybrids in current seed blend corn products contain pyramided traits of multiple genes for ECB and other lepidopteran pests; thus, redundant killing by their expressed proteins should further reduce the probability of heterozygote survival, even in situations where young larvae feed on non-*Bt* plants first and later move as older larvae to *Bt* plants.

Comparison of stalk injury and larval recovery data from refuge plants between the two refuge arrangements gave an understanding of the relative refuge size in terms of the number of potential susceptible individuals produced for resistance management. The number of individual tunnels was direct evidence that ECB larvae entered stalks and potentially survived to produce susceptible adults, even though not all larvae or pupae were recovered from each tunnel. In most studies, refuge plants in the seed blend plots had significantly fewer tunnels than did the refuge plants in structured refuge plots. Similarly, significantly lower numbers of larvae were recovered from the seed blend plots as a result of movement of larvae to neighbor *Bt* plants, where they were killed by the high dose trait. Collectively, results indicated that the seed blend may not produce as many susceptible ECB as those produced in a structured refuge arrangement. For example, based on the number of recovered larvae pooled over all studies, a 5% seed blend is likely to produce 30 to 51% fewer susceptible individuals than a 5% structured refuge.

Finally, this study provided information to assess the relative performance of the purple-seeded hybrid as a surrogate host plant for tracking gene flow. Compared to the yellow-seeded hybrid, the purple trait did not significantly affect measurements of leaf and stalk injury or the number of ECB larvae recovered during the first generation. Larval and pupal weights did not show any treatment effects comparing yellow- and purple-seeded corn. However, lower incidence of ear and stalk injury and fewer ECB larvae recovered in the second generation were significantly affected by the purple trait. Thus, the purple-seeded hybrid may not serve as an appropriate surrogate refuge for producing susceptible individuals but could be a convenient means to track gene flow between refuge and neighbor plants.

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Fig. 3

Fig. 4

Ms. Ref. No.: CROPRO-D-15-00417

Figure Captions.

Figure 1. Mean damage rating ($\pm$ SE) caused by first generation European corn borer on the refuge corn plant and neighbor N1-N4 plants within the same row. Means are averaged over two years of data from structured and seed blend planting arrangements with yellow (Y) or purple (P) seeded non-*Bt* plants as refuges. Louisiana, 2013 and 2014.

Figure 2. Mean ($\pm$ SE) number of galleries, total gallery length, and number of first generation European corn borer larvae on the refuge corn plant and neighbor N1-N4 plants within the same row. Means are averages per plant of data from structured and seed blend planting arrangements with yellow (Y) or purple (P) seeded non-*Bt* plants as refuges. Louisiana, 2013.

Figure 3. Mean ($\pm$ SE) leaf injury rating, number of galleries, and total gallery length caused by first generation European corn borer on the refuge corn plant and neighbor N1-N2 plants within the same row. Means are averages per plant of data over two years from structured and seed blend planting arrangements with yellow (Y) or purple (P) seeded non-*Bt* plants as refuges. Mean with the same letter above bars are not significantly different ($P < 0.05$). Maryland, 2013 and 2014.

Figure 4. Mean ($\pm$ SE) number of galleries and total gallery length caused by second generation European corn borer on the refuge corn plant and neighbor N1-N4 plants within the same row and neighbor N5-N7 plants on adjacent rows. Means are averages per plant of data over two years from structured and seed blend planting arrangements with yellow (Y) or purple (P) seeded non-*Bt* plants as refuges. Louisiana, 2013 and 2014.

Figure 5. Mean ($\pm$ SE) ear damage, number of galleries, and total gallery length caused by second generation European corn borer on the refuge corn plant and neighbor N1-N2 plants within the same row. Means are averages per plant of data over two years from structured and seed blend planting arrangements with yellow (Y) or purple (P) seeded non-*Bt* plants as refuges. Maryland, 2013 and 2014.

Figure 6. Mean ($\pm$ SE) number and weight of European corn borer larvae and pupae recovered after the first generation infestation from the refuge corn plant and neighbor N1-N2 plants within the same row. Means are averages per plant of data over two years from structured and seed blend planting arrangements with yellow (Y) or purple (P) seeded non-*Bt* plants as refuges. Maryland, 2013 and 2014.

Figure 7. Mean ($\pm$ SE) number and weight of European corn borer larvae recovered after the second generation infestation from the refuge corn plant and neighbor N1-N2 plants within the same row. Means are averages per plant of data over two years from structured and seed blend planting arrangements with yellow (Y) or purple (P) seeded non-*Bt* plants as refuges. Maryland, 2013 and 2014.