Quantifying western corn rootworm emergence and fitness parameters from different Bt corn hybrids

Steven J. Smith and Christian H. Krupke

Department of Entomology, Purdue University, 901 W. State St., West Lafayette, IN 47907

Introduction

Western corn rootworm (WCR) (*Diabrotica virgifera virgifera* LeConte) is a major pest of corn production across the corn belt in the United States and recently has been introduced into Central and South-Eastern Europe (Ciosi et al., 2011). Indiana corn producers, like many throughout the country, have adopted hybrid corn containing genes coding for insecticidal crystals from the bacterium *Bacillus thuringiensis* (Bt) targeting the corn rootworm complex (primarily the western corn rootworm) as part of their pest management arsenal. Bt corn accounted for approximately 63% of the corn grown in the United States in 2009 (NASS, 2009). However, since its introduction in 2004, there have been significant changes that affect how growers will use and manage these products. One critical change that was introduced to the market in 2010 is the combination of Bt and herbicide-tolerance traits, produced by formerly competing seed companies, into single hybrids (often called "stacked hybrids"). Stacked hybrids were developed in hopes of simplifying weed management and increasing mortality rates in WCR, delaying resistance and allowing reduction in refuge size.

Bt is a bacterium occurring in the soil. It expresses a toxin that was introduced into corn in hopes of controlling western corn rootworm populations. This approach has previously been used to control lepidopteran pests in corn as well (Meihls, et. al., 2009). The protein is produced by the plant and when ingested, will bind to the gut of the larvae to cause the insect to stop feeding. Shortly after, the gut wall breaks down and bacteria from the gut invade the body cavity and cause death (Bessin, 2004). These proteins are produced in decreasing amounts as the plant ages (Nguyen & Jehle, 2009). In contrast, as the beetle develops it becomes more tolerant to the Bt toxins, therefore giving it a greater chance of survival (Meihls, et. al., 2009).

Because the doses of Bt expressed by corn are relatively low-dose (ca. 1-4% of insects survive to adulthood) (Meihls, 2009), there may be opportunity for WCR to develop resistance. To determine how quickly resistance can be acquired, a series of experiments were conducted under both greenhouse and field conditions. Under greenhouse conditions, using Yieldgard (Monsanto Corp.) hybrids expressing the Cry3Bb1 protein, WCR was able to develop resistance within three generations. In field experiments with the same hybrid, WCR developed resistance within seven generations (Meihls, 2009).

Refuge corn is a critical component of the resistance management plan for Bt corn. The EPA has set requirements for a refuge that farmers must comply with when using genetically engineered corn hybrids. These refuge requirements were developed to help delay WCR resistance to Bt. A refuge of 20% is required for hybrids containing a single Bt trait targeting WCR and must be planted in rows throughout the field or as a block in one section of the field (EPA, 2005). The refuge requirement for hybrids containing stacked traits has been lowered to 5%, but still utilizing the same planting patterns (Ricketts & Heine, 2009). Using a refuge attempts to maximize the probability that resistant pests will find and mate with susceptible individuals that emerged from the refuge corn. When the compliance requirements were first established in 2003 there was a 90% compliance rate (Jaffe, 2009). However, beginning in 2006 the compliance rate began decreasing, and by 2008 25% of farmers were not using a refuge. "Delaying resistance is important because it protects the benefits of Bt for future use by biotech farmers as well as farmers who use microbial insecticides with Bt bacterium" (Jaffe, 2009).

Methods

Structure and planting

Four treatments replicated nine times, were randomly arranged on a greenhouse bench and re-randomized once per week to prevent uneven growth between plants. The four treatments were composed of a Yieldgard hybrid (YG) (Mycogen: 2T789) expressing Cry3Bb1 for WCR, Herculex hybrid (HX) (Pioneer: 33F88) expressing Cry34/35 for WCR, SmartStax hybrid (SS) (Mycogen: 2T784) expressing the Bt toxins Cry34/35 and Cry3Bb1 for WCR, and a non-Bt

refuge control (Mycogen: 2T777). Seeds were planted August 31, 2010 into 2 ½ gallon buckets with screw on lids. A wire mesh center and two PVC pipes were attached to the lids, one allowing for the corn plant to grow through and the other for a collection container. The collection container was a plastic cup with lid and fit snugly into the PVC pipe. The bottom of the cup was cut out and a small funnel was inserted allowing adult beetles to get into the cup, but not out. The hole for the plant was sealed with strips of foam to prevent emergent adults from escaping the bucket. The soil used for the experiment was Sunshine Redi-earth Plug and Seedling (Sun Gro, Bellevue, Washington) and was placed into the buckets leaving two inches from the soil surface to the rim of the bucket. Seeds were planted at a depth of 2.54 cm for optimal germination and root development. Watering was limited to two times per week and progressively increased as the corn developed. The final watering schedule was watering two days and off one day. The plants were fertilized once a week with a Miracle-Gro Garden Feeder hose attachment (The Scott's Miracle-Gro Company, Marysville, Ohio). An additional two plants per treatment were planted and infested at the same time, these were excavated October 15, 2010 and confirmed that third instar WCR larvae were present.

Infestation and collection

WCR eggs were acquired in mid August from the USDA Northern Grain Insects Rearing Facility in Brookings, South Dakota. The eggs were maintained in an environmental chamber (Percival Scientific, Perry, Iowa) at 8º Celsius and 50% relative humidity. The target was to infest each plant with approximately 400 WCR eggs. Upon receipt, eggs were stored in a petri dish in a soil medium and had to be separated from this medium. To do this, the eggs and soil medium were placed in a size 60 sieve (Fisher Scientific, Suwanee, Georgia) and washed with cold water (approximately 8^{o} C). The eggs were then transferred into a previously prepared, refrigerated 0.15% agar solution in which the eggs were suspended. The agar solution was made by boiling 880 ml of de-ionized water and mixing in 1.32 ml of agar (Bacto™ Agar; Becton, Dickenson and Company, Difco Laboratories, Sparks, MD), until all the granules were dissolved. After addition of the WCR eggs, the solution contained approximately 200 eggs per 10 ml of solution. Two 7.62 cm holes on either side of the corn plant were made using a pencil in which the WCR eggs were injected on September 17, 2010. 10 ml of the egg solution was injected into the holes using a 10 ml pipette (Eppendorf North America, Hauppauge, New York). The plants were then

watered and fertilized as needed and examined for adult emergence. First adult emergence occurred November 2, 2010 and continued until December 15, 2010. Emerging adults were collected daily and plants were checked for adults until one week without any emergence was recorded. Collection vials were labeled for each plant, each day. Vials contained a small amount of 70% ethyl alcohol to prevent escape and kill beetles rapidly. After collection, the vials were placed into a freezer for storage. After a week of no emergence activity within a single plant, the plant was excavated and roots were washed to examine root density and damage.

Post emergence

All beetles collected were separated by sex and counted. Five males and five females per treatment per week were selected at random and their head capsules were measured to provide an estimate of beetle fitness. Head capsules were measured using a stereo microscope with an attached digital camera (models SZX12 and U-CMAD3; Olympus Optical, Tokyo Japan). A picture was taken of the head capsule and measured within 0.01 mm using AnalySIS Microsuite imaging software (Soft Imaging System, Lakewood, CO, USA). The head capsule was measured at the widest portion of the head capsule from eye to eye. The final step was to obtain the dry weight of the beetles. Beetles were separated by gender for each treatment/week and placed into a small laboratory oven (Grieve-Hendry Co., Round Lake, Illinois) and allowed to dry at 93º Celsius for one hour. The beetles were then weighed in milligrams to obtain their dry weight (Mettler AE 100; Mettler Direct, Ventura, California). The last two steps give us an overall idea of the fitness of the beetles coming from each treatment.

Results

Delayed emergence of beetles associated with Bt hybrid corn has been observed in this experiment (Figure 1). The first male and female beetles emerged from the refuge hybrid November 2, 2010. The first SmartStax female emerged November 11, 2010, and the first SmartStax male emerged November 12, 2010. For Herculex and Yieldgard the first emergence of both sexes was November 9, 2010.

There were also significantly more beetles that emerged from the non-Bt refuge than the Bt hybrids (Figure 2). The mean males emerging per plant in the refuge corn was 67.88 ± 5.47, and mean females was 91.25 ± 7.82. For SmartStax plants the mean number of males per plant was 9.22 ± 2.59, and females: 8.78 ± 2.49. Herculex males averaged 12.00 ± 3.65, and females averaged 11.38 ± 4.40. Yieldgard males averaged 19.56 ± 3.17, and females 36.56 ± 5.88.

Head capsule measurements in millimeters are as follows. Refuge males had an average width of 1.11mm ± 0.03, females 1.10mm ± 0.02. SmartStax males averaged 1.04mm ± 0.01, females 1.06mm ± 0.03. Herculex males averaged 1.05mm ± 0.02, and females 1.06mm ± 0.02. Yieldgard males averaged 1.10mm ± 0.02, females 1.13mm ± 0.03. The head capsules of males in both the non-Bt refuge and Yieldgard were significantly larger than those from the SmartStax and Herculex (Figure 3).

Average weight/beetle/week/sex was also calculated in milligrams. Refuge males weighed 1.41mg ± 0.15, females weighed 1.55mg ± 0.15. SmartStax males weighed 1.13mg ± 0.08, females 1.12mg ± 0.06. Herculex males weighed 0.98mg ± 0.09, females 1.19mg ± 0.05. Yieldgard males weighed 1.31mg ± 0.15, females 1.59mg ± 0.19. Females emerging from the non-Bt hybrid and Yieldgard were significantly heavier than the SmartStax and Herculex as well (Figure 4).

Discussion

The Bt traits for Yieldgard and Herculex are touted as having different modes of action. This means that they attack the beetle in different ways and act independently of one another. Therefore I expected to find that SmartStax would be statistically superior (i.e. fewer beetles emerging) when compared to Herculex, but instead I find that the two are very similar in the number of beetles that emerged. This suggests that the combination of traits from Herculex plus Yieldgard for rootworm control is not significantly different from Herculex alone. In other words, the Herculex trait is causing most of the mortality in the SmartStax hybrid and the

Yieldgard is seemingly inactive or having little input. If the traits were truly independent I would expect to find that individually Herculex and Yieldgard would be similar in their results and the combination of the two (SmartStax) would have significantly less beetles emerge. Further investigation is needed to confirm whether or not the Yieldgard trait in the SmartStax hybrid is having any additive effect on WCR mortality.

Emergence timing is important because females have shown a preference to mate with younger individuals (Kang & Krupke, 2009). This study has shown that refuge individuals are larger than individuals from Bt hybrids, especially SmartStax and Herculex, and it has been previously demonstrated that male WCR beetles prefer larger females (Kang & Krupke, 2009), which means that mating may not be random (Quiring & Timmins, 1990) potentially reducing the efficacy of a refuge.

The next step that I intend to investigate is determining if these factors influence mating behavior. To do this, I will rear individuals from both SmartStax and the non-Bt refuge plants. The beetles will be separated out by sex and corn hybrid and allowed to feed and mature for a few days. Each chamber will be connected to a central container but have valves so that the beetles cannot go from one chamber to another. After the maturation period, males and females from the refuge corn will be marked with a fluorescent dye, and then all of the beetles will be released into the central chamber. Mating pairs will be recorded and then from this I can determine the lingering question about random mating.

Acknowledgements

I would like to thank Paul Marquardt for the use of greenhouse space and the bucket traps, Alex Murphy for help with using the computer software associated with head capsule measurements and Dr. Douglas Richmond for assistance with the Statistica program. This project was funded through the Agricultural Research Fund Scholarships at Purdue University.

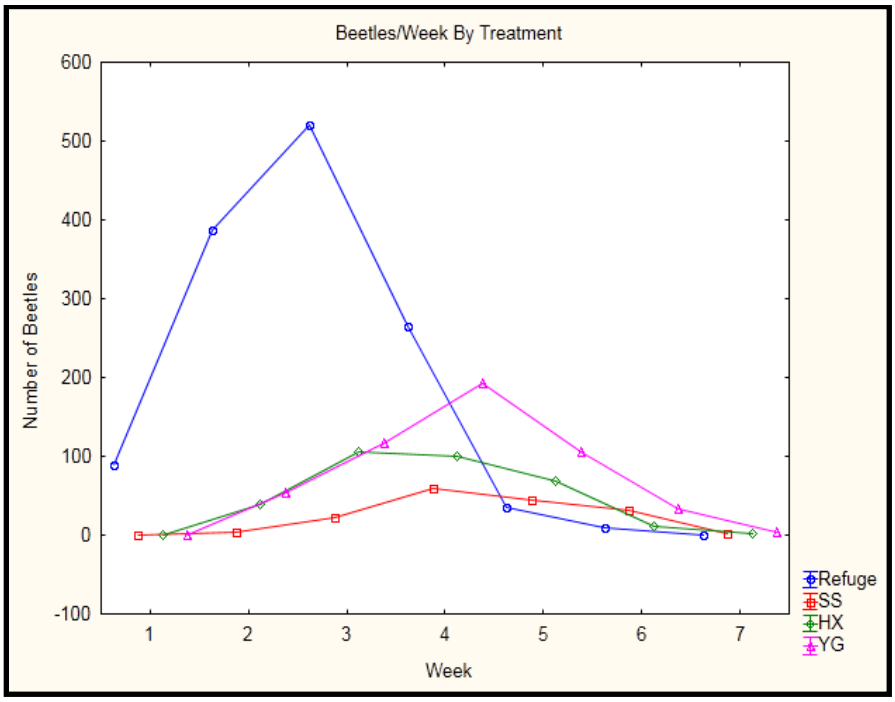

Figure 1: Emergence timeline

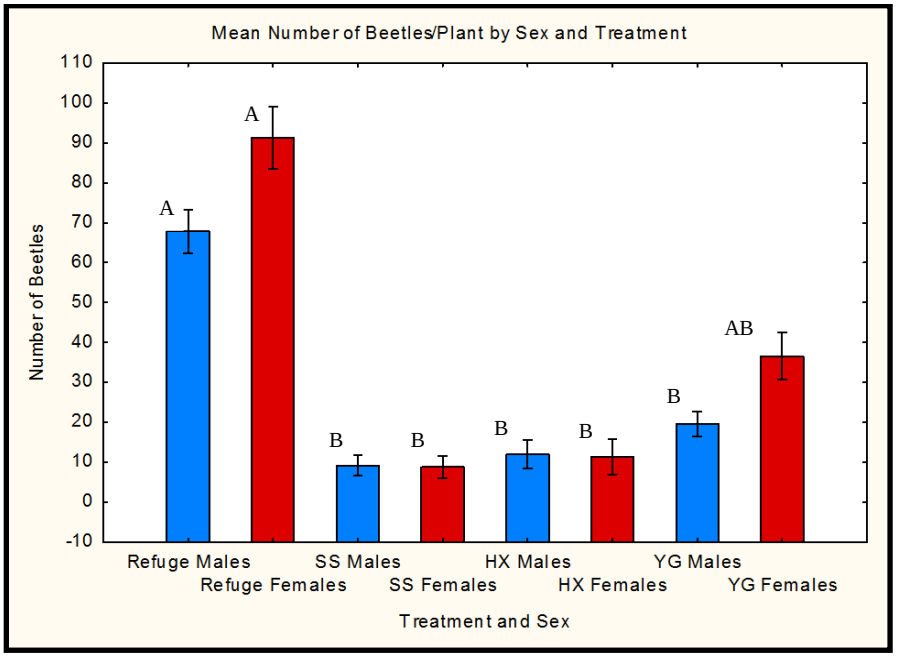

Figure 2: Mean beetles/plant/treatment
Analyzed using ANOVA followed by a Tukey Test

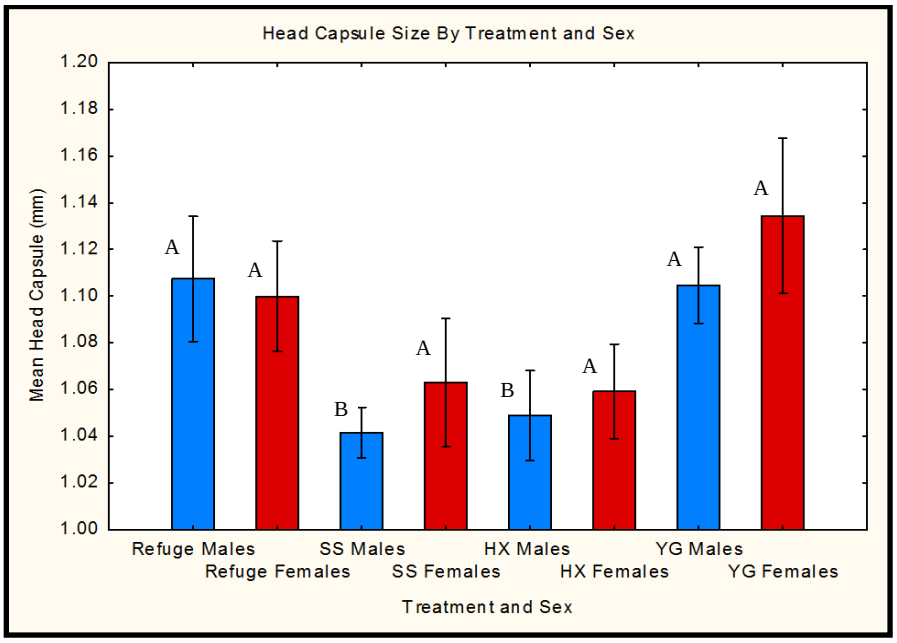

Figure 3: Mean head capsule/beetle/treatment
Analyzed using ANOVA followed by a Tukey Test

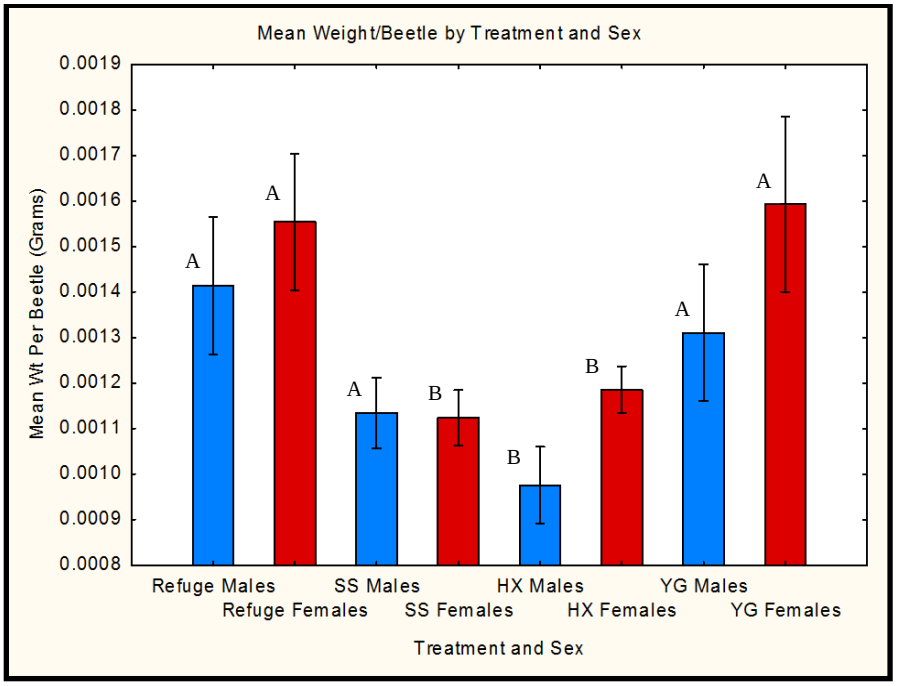

Figure 4: Mean weight/beetle/treatment
Analyzed using ANOVA followed by a Tukey Test

References

Bessin, R. (2004, January). *Bt-corn: what is it and how it works*. http://www.ca.uky.edu/entomology/entfacts/ef130.asp.

Ciosi, M., Miller, N. J., Toepfer, S., Estoup, A., & Guillemaud, T. (2011). Stratified dispersal and increasing genetic variation during the invasion of central Europe by the western corn rootworm, Diabrotica virgifera virgifera. *Evolutionary Applications,* 4(1): 54-70.

EPA (2005) *Bascillus thurigiensis* Cry3Bb1 protein and genetic material necessary for its production (Vector ZMIR13L) in event MON863 corn (006484) fact sheet. United States Environmental Protection Agency, Washington, DC, USA. http://www.epa.gov/oppbppd1/biopesticides/ingredients/factsheets/factsheet_006484.htm#reports.

Jaffe, G. (2009, November). Complacency on the farm: Significant noncompliance with the EPA's refuge requirements threatens the future effectiveness of genetically engineered pest-protected corn. *Center for Science in the Public Interest.* Washington, DC, USA. http://cspinet.org/new/pdf/complacencyonthefarm.pdf.

Kang, J., & Krupke, C. H. (2009) Influence of weight of male and female western corn rootworm (Coleoptera: Chrysomelidae) on mating behaviors. Annuals of the Entomological Society of America 102: 326-332.

Meihls, L. N., *et al*. (2009). Increased survival of western corn rootworm on transgenic corn within three generations of on-plant greenhouse selection. *PNAS,* 105 (49).

NASS (2009). Acreage. National Agriculture Statistics Service, United States Department of Agriculture, Washington, DC, USA. http://usda.mannlib.cornell.edu/usda/current/Acre/Acre-06-30-2009.pdf.

Nguyen, H. T., & Jehle, J. A. (2009). Expression of Cry3Bb1 in transgenic corn MON88017. *Journal of Agricultural and Food Chemistry*, 57: 9990-9996.

Ricketts, M. & Heine, R. (2009, July 20). *Monsanto, Dow AgroSciences complete U.S. and Canadian regulatory authorizations for SmartStax corn; Plans set to launch seed platform on 3 million- to 4 million-plus acres* (Press Release). http://monsanto.mediaroom.com/index.php?s=43&item=729.

Quiring, D. T., & Timmons, P. R. (1990). Influence of reproductive ecology on feasibility of mass trapping *Diabrotica virgifera virgifera* (Coleoptera: Chrysomelidae). *Journal of Applied Ecology*, 27: 965