THE MOVEMENT BEHAVIOR AND REPRODUCTIVE ECOLOGY OF WESTERN CORN ROOTWORM BEETLES (COLEOPTERA: CHRYSOMELIAE) IN BT CORNFIELDS WITH STRUCTURED AND SEED BLEND REFUGES

BY

SARAH A. HUGHSON

DISSERTATION

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Doctoral Committee:

Principal Research Scientist Joseph L. Spencer, Chair, Director of Research
Professor May R. Berenbaum
Professor Lawrence M. Hanks
Research Scientist Marianne Alleyne
Professor Christian H. Krupke, Purdue University

ABSTRACT

In the United States, western corn rootworm (Coleoptera: Chrysomelidae; *Diabrotica virgifera virgifera* LeConte) larvae have been managed using transgenic Bt (*Bacillus thuringiensis* Berliner) corn hybrids expressing insecticidal Cry proteins since 2003. To slow the evolution of resistance to Cry proteins, the Environmental Protection Agency mandated an insect resistance management (IRM) plan requiring the planting of non-Bt refuges as structured blocks or randomly distributed (seed blends) within Bt cornfields. Refuges allow susceptible larvae to develop without exposure to Cry proteins expressed elsewhere in the field. The modest populations of susceptible beetles from the refuge are expected to disperse across cornfields and mate with most of the rare, potentially resistant, beetles that survived Bt exposure. These "mixed-matings" are expected to produce heterozygous Bt susceptible offspring, delaying resistance because few resistant individuals will have opportunities to mate and produce homozygous Bt resistant offspring. Because the efficacy of the rootworm refuge strategy relies heavily on western corn rootworm movement and mating behavior, it is important to understand these behaviors in Bt cornfields. This dissertation presents data on daily movement within Bt cornfields and details of western corn rootworm mating relevant to resistance management.

The spatial and temporal distribution of beetle mating activity and movement were measured in four refuge treatments 1) 20% structured refuge, 2) 5% structured refuge, 3) 5% seed blend refuge and 4) 0% refuge from 2010 to 2012. By testing beetle gut contents for the presence of specific Cry proteins and comparing them to the Cry proteins expressed by plants at their collection site, recent movement could be detected. These findings are presented in Chapters 2 and 3 of this dissertation.

In Chapter 2, gut content analysis revealed that 8.5% of mating pairs were mixed-matings: they included a beetle that moved between refuge and Bt corn prior to mating. Most mixed-matings occurred in refuge corn, meaning that most resulted from an individual traveling from Bt to refuge corn before it mated. Dissection of mating females confirmed that most mated while they were newly emerged and that at least 4.5% of females mated more than once in short succession or more than a week after their initial mating.

Chapter 3 of this dissertation revealed that the daily proportion of beetles moving between refuge and Bt corn was 17 to 25% collected during the vegetative period but dropped off dramatically to 3 to 10% during the pollination and post-pollination periods. When beetles moved within cornfields, they traveled at a rate of 26 to 31 m/day.

In Chapter 4, the effect of high and low beetle density with three different M:F sex ratios (1:3, 1:1 and 3:1) on mating frequency was evaluated. Sex ratio significantly influenced mating regardless of density. More matings per female were recorded in male skewed treatments and more matings by males were recorded in female skewed treatments. Female dissections revealed that matings occurred throughout the five day experiment.

Chapter 5 describes a sperm precedence study, in which females were given the opportunity to mate with a new male each day and paternity was assessed for family groups of egg batches as well as the female beetle and her two mates using microsatellite genotyping. Of the females in the study, 8.5% of females accepted a second mate and the mean interval between the first and second mating was 7.5 days. Genotyping results were inconclusive.

The assumptions of the high-dose refuge strategy are poorly matched to the reality of western corn rootworm biology. Structured refuges do not facilitate movement and mating patterns that favor production of heterozygous susceptible offspring. A seed blend generated a

more even distribution of beetles and mating activity; yet, detailed mating analyses suggest seed blends may not promote more desirable mixed-matings than structured refuges.

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INTRODUCTION

Western corn rootworm (*Diabrotica virgifera virgifera* LeConte) is the most economically important pest of corn in the United States (US; Gray et al. 2009) and has been called the world's most expensive pest to control (Cock 2011). In the mid-1980s it was estimated that, together with the northern corn rootworm (*Diabrotica barberi* Smith and Lawrence), western corn rootworms may cost US$1 billion annually in yield losses and control costs (Metcalf 1986). Today, this cost is thought to be greater (Dun et al. 2010, Tinsley et al. 2013).

Western corn rootworm was first identified as a pest of corn in 1909 (Gillette 1912). The larvae feed on corn roots and are responsible for most of the yield loss and costs associated with this species while the adults feed on the foliage, tassels, silks and kernels of corn plants (Branson and Krysan 1981, Moser and Vidal 2005) and usually do not cause economic losses (Levine and Oloumi-Sadeghi 1991). The univoltine rootworm life cycle is closely tied to corn and they have few alternate hosts (Branson and Ortman 1967, 1970, 1971; Clark and Hibbard 2004). Their strong ovipositional fidelity to corn and the larval inability to survive on other crops led to the suggestion that rootworms could be easily controlled using an annual crop rotation to a non-host crop (Forbes 1883, Gillette 1912), which is still one of the primary methods used to control rootworms in the Corn Belt (Shaw et al. 1978, Levine and Oloumi-Sadeghi 1991, Wilson et al. 2005). However, the great efficacy of crop rotation was its undoing, it selected for females with reduced ovipositional fidelity to cornfields creating a pest whose eggs could be found in almost

any crop (including corn) (Onstad et al. 1999, 2001; Levine et al. 2002). The evolution of behavioral resistance to crop rotation resulted in reports of severe injury in first-year corn (Levine and Oloumi-Sadeghi 1991, Levine et al. 2002). As rotation resistance spread to the surrounding areas, corn growers began to rely heavily on soil applied insecticides and foliar sprays (Levine and Oloumi-Sadeghi 1991, Rice 2004). Rotation resistance has expanded from its IL-IN epicenter into MI, OH, WI, IA and Ontario, Canada (Gray et al. 2009). However, recent data indicate that rotation-resistant WCR densities are currently below economic thresholds in Iowa (Dunbar and Gassmann 2013).

The first Bt (*Bacillus thuringiensis* Berliner) corn hybrids, targeting root-feeding larvae were introduced in 2003 (US EPA OPP 2001) and offered efficacy similar to that of soil applied insecticides (Rice 2004). Bt technology proved to be an effective management tool and was rapidly adopted by farmers. By 2015, 92% of corn in the US expressed one or more Bt toxins to manage rootworm and lepidopteran pests (USDA ERS 2016). Because if the inherent risk of resistance evolution, the Environmental Protection Agency (EPA) required a refuge of non-Bt corn to be planted in each Bt cornfield (US EPA OPP 2001, US EPA BPPD 2012). This Insect Resistance Management (IRM) plan was adapted from the high-dose refuge strategy already employed to delay resistance in lepidopteran pests, including European corn borer (*Ostrinia nubilalis* Hübner; US EPA 1998) which have very different biology and behavior than corn rootworms. While the body of literature regarding the western corn rootworm is broad, there are still gaps that require better understanding. This review, outlines western corn rootworm movement and mating behavior and explore the role this pest's biology plays in insect resistance management.

LIFE CYCLE AND MATING BEHAVIOR

Life cycle

The western corn rootworm is a univoltine species, with individuals overwintering in the soil of agricultural fields as eggs (Ball 1957) in an obligate diapause (Krysan and Branson 1977). Larvae begin to eclose from their eggs between late May and early June (Levine et al. 2002), then quickly begin to feed on the roots of young corn plants (Krysan and Branson 1983). Larval feeding injury can cause reductions in yield by rendering the plants susceptible to disease (Palmer and Kommedahl 1969) and disrupts water and nutrient flow (Kahler et al. 1985). It can also reduce the structural integrity of stabilizing roots, causing the plants to lodge, which makes them difficult to harvest, further reducing yield. Adult feeding does not usually cause economic damage, but when populations are high, excessive silk feeding may disrupt pollination which prevents kernel formation and reduces yield (Levine and Oloumi-Sadeghi 1991).

After completing three instars (George and Hintz 1966, Hammack et al. 2003), larvae form an earthen cell in which they pupate (Chiang 1973). Adult emergence begins in late June and early July (Murphy et al. 2010, Hughson and Spencer 2015) and continues until mid to late August (Hughson and Spencer 2015). Western corn rootworms are protandrous. Males typically emerge before females, reaching peak cumulative emergence before females (Darnell et al. 2000, Nowatzki et al. 2002), although 97.8% of male and female emergence periods overlaps (Quiring and Timmins 1990). Hughson and Spencer (2015) reported that males emerged 5.2 to 6.0 days before females based on mean emergence date, which is consistent with the five to seven day difference in emergence previously reported (Branson 1987, Quiring and Timmins 1990). Delays in emergence between the sexes are thought to be caused by differences in the rates of post-diapause embryonic development and post-hatch larval development between males and

females (Branson 1987). Time differences in emergence are also thought to allow males time to reach sexual maturity before most females emerge and become available to mate (Guss 1976). Emergence from Bt corn is also delayed compared to refuge corn (Storer et al. 2006, Murphy et al. 2010, Hughson and Spencer 2015). This delay can be 4.5 to 7.8 days, creating a 9.6 to 12.9 day difference between the emergence of refuge males and Bt females (Hughson and Spencer 2015).

Because of the delay in emergence between males and females, populations tend to have a male biased sex ratio early in the season, which shifts to a female bias as the season progresses (Short and Hill 1972). Bt cornfields often have a female biased sex ratio (Al-Deeb and Wilde 2005, Meinke et al. 2009, Murphy et al. 2010). Female biased sex ratios in emergence from Bt corn are thought to be caused by slower development on Bt plants (Beckler 2006).

Mating behavior

Western corn rootworms females usually mate shortly after emergence, often within 12 to 24 hours, while they are still teneral (Cates 1968, Quiring and Timmins 1990). While males may require time to reach sexual maturity (Guss 1976), females are mature upon emergence and begin to release sex pheromone (calling) to attract males shortly thereafter (Hammack 1995). Female sex pheromone is primarily composed of 8R-methyl-2R-decenyl-proponoate (Guss et al. 1982) and is recognizable by males five to seven days after emergence (Guss 1976). On their first day after emergence, 54% of females began calling, on the second day 96.4% began calling and by the third all of the females had begun to call (Hammack 1995). When males detect sex pheromone, they engage in mate-seeking flight and move toward the source of the pheromone (Ball and Chaudhury 1973, Guss 1976, Lew and Ball 1979, Marquardt and Krupke 2009). When

males and females encounter one another, they have a brief period of courtship which may include up to one hour of mounting and mate-guarding prior to mating (Lew and Ball 1979). Mating occurs for three to four hours (Ball 1957, Lew and Ball 1979, Sherwood and Levine 1993). During the first hour of mating, the male produces a spermatophore in the female's bursa copulatrix (Lew and Ball 1980). Spermatozoa (hereafter referred to as sperm) from the spermatophore migrate to the female's spermatheca within two to four hours (Lew and Ball 1980), where they can be stored for 40 to greater than 76 days after mating (Branson and Johnson 1973, Hill 1975, Lew and Ball 1980). The spermatophore is a nutrient rich nuptial gift, consisting of an ampulla containing enough sperm that a female may not need to remate (Hill 1975) and a spermatophylax containing proteins and carbohydrates (Murphy and Krupke 2011) that can be absorbed by the female (Lew and Ball 1980). The spermatophore can be completely absorbed by a female after five to 7 days (Lew and Ball 1980) and some compounds from the spermatophore are incorporated into the eggs (Murphy and Krupke 2011). The size of the spermatophore is substantial, on average, equivalent to up to 9% of the male's mass, making each mating a significant investment for males (Quiring and Timmins 1990, Tallamy et al. 2000). Without a quality food source, males mated less frequently and produced smaller spermatophores (Quiring and Timmins 1990), reinforcing the idea that spermatophore production is a significant investment for males.

Classic literature on western corn rootworms suggests that females mate only once (Ball 1957) and males may mate as many as 8 or 14 times throughout their life (Branson et al. 1977). While recent research has shown that males have a reproductive life span of ten days after their initial mating, during which they mate, on average, an additional 2.24 times (Kang and Krupke 2009). Determining the number of times a female mates can be quite difficult (Chapter 2 and

chapter 5 of this dissertation). Branson et al. (1977) reported 23% of females mated more than once in the laboratory based on an egg viability study using females that were mated to sterile males followed by unaltered males. Hughson (Chapter 2 of this dissertation) reported 4.5% of females mating more than once in the field, based on dissection of females collected while mating and analysis of the number of spermatophores or combination of spermatophore and sperm presence in each individual. In a mating study, Hughson (Chapter 5 of this dissertation) also reported that 8% of females were observed mating a second time when presented with a new male each day after emergence. When females mate more than once, it is unclear which male will fertilize all or a proportion of the resulting eggs (Chapter 5 of this dissertation) although research on southern corn rootworm (*Diabrotica undecimpunctata* Howardii) suggests they may have last male sperm precedence (Oyediran et al. 2007).

Oviposition

Female western corn rootworms may begin ovipositing 12 to 21 days after emergence (Short and Hill 1972, Branson and Johnson 1973, Hill 1975). On average females produce 266 to 441 viable eggs in their lifetime (Ball 1957, Elliott et al. 1990, Fischer et al. 1991, Boetel and Fuller 1997). However, they are capable of producing over 1000 total eggs in their lifetime under ideal laboratory conditions (Branson and Johnson 1973, Hill 1975).

Most oviposition occurs in the morning between 8.00 h and 12.00 h (Ball 1971). Females preferentially oviposit on moist soil (Gustin 1979), depositing most of their eggs within the first 10 cm of the soil (Ball 1957, Hein et al. 1988, Pierce and Gray 2006). Females have also been observed entering drought cracks to oviposit (Kirk 1979). Within the drought cracks, females were recorded ovipositing at a variety of depths depending on the moisture content of the soil

(Kirk 1979). Females may prefer locations with moist soil because the young within the eggs require moisture to complete development (Krysan 1978).

Females were reported to deposit eggs almost exclusively in cornfields from July to August (Ball 1957). However, a proportion of the population leaves their natal cornfield (Spencer et al. 2005) and can be found in weedy field margins, volunteer corn and other crops where they may feed on available pollen (Shaw et al. 1978). Since rootworms oviposit wherever they feed (Branson and Krysan 1981), this portion of the population likely played an important role in the evolution of behavioral resistance to crop rotation. While the offspring of females that oviposited in cornfields could not survive when soybeans were planted in the field during the following year, rotation from soybeans to corn provided a fitness advantage to females with reduced ovipositional fidelity to corn. High adoption of rotation in portions of the Corn Belt and the resulting increase in the proportion of beetles entering soybeans prior to oviposition led to behavioral resistance to crop rotation in western corn rootworm (Levine and Oloumi-Sadeghi 1996, Levine et al. 2002).

ADULT MOVEMENT BEHAVIOR

Intrafield distributions and movement

Intrafield movement in western corn rootworms is a topic of particular importance to the success of the high-dose refuge strategy and sustainability of Bt technology. Movement within cornfields is usually discussed in terms of mate-seeking movement. Factors influencing beetle distributions within fields are not well understood (Meinke et al. 2009). Measures of population distributions and density have shown that beetle populations are often aggregated within cornfields (Midgarden et al. 1993, Park and Tollefson 2006a). Adult distributions are closely

related to the distributions of larvae in cornfields, suggesting that adults may remain near their emergence sites (Toepfer et al. 2007). Similarly, within Bt cornfields, measures of beetle abundance and mating activity revealed that beetle populations were concentrated in refuge corn throughout the season (Hughson and Spencer 2015, Chapter 2 of this dissertation). These distributions suggest that few beetles move out of the refuge after emergence or prior to mating and may remain near their emergence sites.

In addition to direct observation in the field and laboratory, a variety of methods have been used to mark beetles and measure their movement (Nowatzki et al. 2003, Spencer et al. 2003, Taylor et al. 2016). Mark-release-recapture studies have proven difficult to use because high population densities prevent adequate recapture (Spencer et al. 2003). By placing ^{15}N (Taylor et al. 2016) or rubidium (Nowatzki et al. 2003) in soil where refuge corn is planted, the materials are taken up by the plants and can be detected in the herbivorous insects that feed on them. Insects feeding on untreated plants do not have detectable concentrations of the applied materials in their bodies. When insects that fed on treated plants emerge from the soil as adults, they can be collected and tested for the isotope or rare earth element allowing the distribution of or distance traveled by refuge origin beetles to be determined (Nowatzki et al. 2003, Taylor et al. 2016). While placing isotopes or rare earth elements in the field can be expensive and difficult to detect in small subjects like insects (Hagler and Jackson 2001), these methods allow researchers to be sure of each beetle's larval origin.

The crystalline (Cry) proteins expressed by the Bt plants can also be used to detect beetle movement (Spencer et al. 2003). Using this method, adult beetles mark themselves by feeding on Bt corn. When they are collected their gut contents can be tested for the presence of ingested Cry proteins using ELISA test strips sensitive to specific proteins. This allows a variety of Cry

proteins expressed in both refuge and corn rootworm Bt corn plants to be detected. Because the proteins are only detectable in a beetle's gut for 12 to 48 hours (Chapter 2 of this dissertation), this cannot confirm an individual's larval host (refuge or Bt corn) but it does reveal a snapshot of approximately daily movement activity based on where an individual was feeding. If a beetle's collection location and the Cry proteins expressed by corn plants in that location are known, the presence of Cry proteins not expressed in that location can allow the researcher to determine distributions of moving beetles, proportion of beetles collected that have moved and calculate daily movement rates of beetles that have recently fed on refuge and Bt corn within a Bt cornfield (Spencer et al. 2003, Chapter 2 of this dissertation).

Field observations have revealed that females usually do not disperse from their emergence location prior to mating (Ball 1957, Cates 1968, Lew and Ball 1979) although they may walk a short distance from their emergence site prior to mating (Marquadt and Krupke 2009). Males are thought to move readily through a cornfield in mate-seeking flight (Ball 1957, Spencer et al. 2009), although few publications have measured this movement. Mate-seeking movement occurs when males move toward sex pheromone produced by females. Studies using sticky traps, have shown that more males are collected from traps baited with virgin females than unbaited traps (Marquardt and Krupke 2009). However, because female baited traps are biased toward the collection of mate-seeking males (Meinke et al. 2009), these counts cannot determine the proportion of the population represented by mate-seeking males.

By analyzing Cry proteins in the gut contents of beetles collected on sticky traps in a cornfield with a 20% structured refuge, Marquardt and Krupke (2009) determined that there was no difference in the proportion of refuge beetles collected on sticky traps near the refuge-Bt interface or in the center of a Bt cornfield, ~200 m from the refuge. Spencer et al. (2003)

analyzed the gut contents of beetles collected from refuge and Bt corn in cornfields planted with refuge strips and determined that beetles traveled a maximum of 13.9 to 27.4 m/day. Similarly, Hughson (Chapter 2 of this dissertation) calculated rates of travel using Cry protein detection in Bt cornfields with 20% and 5% structured refuges and found that beetles traveled 26 to 31 m/day. Both of these values were similar to the < 30 m/day recorded by Coats et al. (1987) using tethered flight mills. In both the Spencer et al. (2003) and Hughson (Chapter 2 of this dissertation) studies, there were no difference in the movement rates of males and females. And in the Hughson (Chapter 2 of this dissertation) study, there was no difference in the proportions of moving males and females. Similarly, there were no differences in the number or duration of flights between males and females in tethered flight mills (Naranjo 1990, Coats et al. 1986).

Based on these observations, it seems that there is little detectable difference in intrafield movement behavior between males and females, suggesting males may not readily distribute themselves across fields. Given that males, on average, mate 3.24 times within their ten day reproductive lifespan (Kang and Krupke 2009), a period of mate-seeking movement may represent a brief portion of their lives. Males emerging in an area of high beetle abundance, such as a refuge, may only need to move a short distance to encounter calling females, making the scale of mate-seeking movement smaller than anticipated. If these hypotheses are true, mate-seeking movement would be difficult to detect without using methods specifically targeting mate-seeking males. However, without additional study, the movement capabilities of mate-seeking males within cornfields remains unclear.

Interfield movement and long-distance dispersal

During the mornings and evenings, western corn rootworm adults can be observed ascending and dispersing from cornfields (Isard et al. 2004). Among dispersing beetles, collected from 10 m tall scaffolding towers, 85% were female and, among those females, 84% contained a spermatophore, indicating that a majority of interfield dispersers were recently mated females (Isard et al. 2004). In flight mill studies, females did not engage in sustained flight more than six to nine days after emergence (Naranjo 1990, Coats et al. 1986) and physiological research revealed that post-mating dispersal is influenced by juvenile hormone (Coats et al. 1987).

Studies of interfield movement often focus on movement between corn and soybean fields (Isard et al. 2000, Levine et al. 2002; O'Neal et al. 1999, 2004, Spencer et al. 2005, Pierce and Gray 2006). Beetles that enter soybeans are unable to survive on soybean foliage and may return to corn to feed (Mabry and Spencer 2003). Beetles may initially be enticed to leave maturing corn by pollen from other plants, including weeds (Moeser and Vidal 2005), or volatiles from pollen and silks in late-planted (less mature) cornfields (Naranjo 1994, O'Neal et al. 2004). Beetles are capable of moving up to 300 m into soybean fields (Toepfer et al. 2006) and travel 4.6 to 9.1 m/day, up to a maximum estimate of 36.5 to 73.0 m/day within soybean fields (Spencer et al. 2003).

In studies of long-distance dispersal, females under direct observation in tethered flight mills were recorded to fly up to 24 km in a single flight or 39 km in a series of flights (Coats et al. 1986). There is documentation of western corn rootworms traveling tens of kilometers in thunderstorms (Grant and Seevers 1989). This capacity for long-distance flight may explain their ability to spread to new locations (Onstad et al. 1999).

BT CORN HYBRIDS

Introduction and adoption

After the discovery of rotation resistance in the Corn Belt, corn growers in affected areas began to rely more heavily on soil applied and foliar insecticides (Spencer et al. 2011, Rice 2004), highlighting the need for a new means of managing western corn rootworm. Bt hybrids were an attractive option because they offered an efficacy similar to insecticides, reduced occupational exposure to chemicals, specific targeting of pest species and promised a reduction in broad-spectrum insecticide application (Rice 2004).

Bt corn hybrids targeting root-feeding western and northern corn rootworm larvae were first commercialized in 2003 (US EPA OPP 2001). Bt hybrids are genetically engineered to express Cry proteins normally produced in the inclusion bodies of the soil microbe *Bacillus thuringiensis* during sporulation (Schnepf et al. 1998). The Cry proteins expressed by Bt crops are active toxins rather than the protoxins found in whole bacteria (Clark et al. 2005) which require processing in the alkaline larval gut to be converted to their active form (Schnepf et al. 1998). When rootworm larvae feed on corn roots expressing Cry proteins, the Cry proteins bind with receptors on the insect's midgut epithelial cells and insert themselves into the microvillus membrane (de Maagd et al. 2001). When multiple Cry proteins penetrate the microvillus membrane, they form a pore in the tissue (Xu et al. 2014) and, eventually kill the larvae. Cry proteins are most effective in killing neonate larvae and are less effective on later instars (Binning et al. 2010).

Bt hybrids were first commercialized in1996 to control European corn borer and, with high rates of adoption, have achieved area-wide suppression of their populations in portions of the Mid-west (Hutchison et al. 2010). In MN, IL and WI, European corn borer populations were

suppressed to the point that both Bt and non-Bt corn growers gained economic benefits (Hutchison et al. 2010). With hopes of similar success, Bt hybrids targeting corn rootworms were commercialized in 2003. Since the introduction of rootworm active Bt traits, low beetle populations have been reported in corn and soybeans in IL (Gray 2013, Hughson and Spencer 2015) which may also be a result of an area-wide effect of Bt hybrids adoption.

Initially, only Bt hybrids expressing single corn rootworm targeting Bt traits were introduced, but over time, combinations of traits became commercially available (see Tabashnik et al. 2009, Table 1). In 2005, stacked hybrids were commercialized, expressing a corn rootworm targeting Bt trait and a lepidopteran targeting Bt trait. By 2008, pyramided hybrids were introduced, expressing two or more rootworm traits and/or two or more lepidopteran traits. Following their introduction, Bt hybrids were rapidly adopted and now represent 89% of all corn grown in Illinois (USDA ERS 2016). Long term area-wide adoption of Bt technology (USDA ERS 2016) and incomplete compliance with refuge requirements (Jaffe 2009) increased the selection pressure on western corn rootworm populations and played an important role in the evolution of Bt resistance.

The first case of field-evolved rootworm resistance to a Bt trait was identified in 2009 (Gassmann et al. 2011). The fields in this study had a history of planting continuous Bt corn and had experienced unexpected injury (Gassmann et al. 2011). Beetles were collected from the fields and their offspring were tested in plant-based bioassays, comparing the survival or larvae on Bt and non-Bt plants. Assays revealed that the population had reduced susceptibility to the Cry3Bb1 trait (Gassmann et al. 2011), which was the first commercialized and the most commonly used rootworm Bt trait. Since then, Cry3Bb1resistance in western corn rootworm has been detected in fields throughout the Corn Belt (Gassmann et al. 2014, Wangila et al. 2015,

Schrader et al. 2016, Zukoff et al. 2016, Ludwick et al. 2017). Cross-resistance, a reduced susceptibility to one Bt trait caused by exposure to a different Bt trait, is a problem caused by the structural similarities among several rootworm Bt toxins (Jakka et al. 2016). Populations that have been reported as resistant to Cry3Bb1 have also had a reduced susceptibility to mCry3A and eCry3.1Ab (all are Cry3 toxins; Gassmann et al. 2014, Wangila et al. 2015, Jakka et al. 2016, Zukoff et al. 2016, Ludwick et al. 2017). While some populations maintained susceptibility to Cry34/35Ab1 (Gassmann et al. 2014, Wangila et al. 2015, Jakka et al. 2016), significantly reduced susceptibility to Cry34/35Ab1 was documented for other populations (Zukoff et al. 2016, Ludwick et al. 2017).

High-dose refuge strategy

The inherent risk of insect resistance from control methods with high efficacy and adoption led the EPA to adapt the high-dose refuge strategy (Tabashnik 1994, Gould 1998) to prolong the lifetime of Bt technology targeting corn rootworms (US EPA OPP 2001). Using this strategy, which was previously employed to delay resistance in European corn borer, growers of Bt corn were required to plant a specific percentage of their Bt cornfield with non-Bt corn. Refuges are intended to preserve insect susceptibility within Bt cornfields, by allowing a portion of the insect population to develop without exposure to Cry proteins and survive to adulthood (Tabashnik 1994, Gould 1998). The abundant susceptible adults of refuge origin, are expected to distribute themselves across Bt cornfields and mate with any potentially resistant adults that survived Bt exposure as larvae (Tabashnik 1994, Gould 1998). Because susceptible insects are expected to be homozygous dominant and resistant insects are expected to be homozygous

recessive for resistance alleles, matings between these individuals should result in heterozygous susceptible offspring, slowing the evolution of resistance (Tabashnik 1994, Gould 1998).

The high-dose refuge strategy for European corn borer required a 20% structured refuge to be planted up to 0.5 mi away from its corresponding Bt cornfield (US EPA 1998, US EPA OPP 2001). To compensate for the reduced dispersal capability of coleopterans compared to lepidopterans, in the rootworm version of the strategy planting a structured refuge was required within or immediately adjacent to a Bt cornfield expressing traits that targeted corn rootworms (US EPA OPP 2001). Initially, a 20% structured refuge was required for Bt hybrids expressing a single trait (US EPA OPP 2001), but as stacked and pyramided hybrids were introduced, refuge requirements were reduced to 10% and 5%. In addition, refuges could be planted in a variety of new configurations, depending on the number or combination of Bt traits expressed by the hybrids, including structured row-strips and seed blends (also called "refuge-in-a-bag"). Seed blends were introduced (US EPA BPPD 2010), in part, to address concerns about falling grower compliance with refuge regulations (Jaffe 2009). They are sold with the appropriate amount of refuge seed pre-mixed in each bag of Bt corn, offering growers the convenience of deploying the appropriate amount of refuge without handling separate bags of Bt and refuge seed.

The success of high-dose refuge strategy for rootworms relies on satisfying four main assumptions: 1) Bt traits targeting corn rootworms are high-dose, 2) resistance alleles are initially rare in a population, 3) resistance alleles are monogenic and recessively inherited, and 4) beetles from refuge and Bt corn move readily across the field and intermate (Gould 1998, Tabashnik 2008). While each of these assumptions are met for European corn borer (Bourguet et al. 2003, Huang et al. 2011, Tabashnik et al. 2013, Siegfried et al. 2014), they are not met for western corn rootworm.

The EPA defines a high-dose trait as allowing the survival of < 0.01% of feeding individuals (US EPA 1998). While high-dose Bt traits exist for lepidopteran pests, none of the currently available corn rootworm Bt traits express Cry proteins at a high-dose (Meihls et al. 2008, Binning et al. 2010, Clark et al. 2012, Frank et al. 2015). In studies using susceptible populations, 1.51% and 3.79% of the insects survived exposure (Meihls et al. 2008, Clark et al. 2012). These moderate doses ensure the survival of some Bt origin beetles each generation, which can accelerate the rate of resistance evolution by increasing the frequency of resistance alleles in subsequent generations (Gould 1998, Tabashnik et al. 2013). Alleles for rootworm resistance to Bt traits should be rare in a population prior to Bt exposure and those alleles should be recessively inherited. However, resistance models by Onstad and Meinke (2010) suggest that resistance allele frequencies are 20 to 200 times greater than have been observed in pests that have been successful in delaying resistance and 2000 times greater than is standard for use in IRM modelling. Studies examining the inheritance of resistance suggest that in some populations resistance is non-recessive while in other populations resistance is polygenic (Petzold-Maxwell et al. 2012, Thompson 2014).

Recent studies have illustrated a lack of synchrony in the emergence of male and female beetles (Hughson and Spencer 2015) and beetles from refuge and Bt corn (Murphy et al. 2010, Hughson and Spencer 2015), which created a delay of 9.6 to 12.9 days between refuge male and Bt female emergence (Hughson and Spencer 2015). The ten day reproductive lifespan of males (Kang and Krupke 2009) may widen the temporal barrier, prevent them from intermating.

After emergence, beetles appear to reside near their location of emergence (Toepfer et al. 2007, Hughson and Spencer 2015) and do not move far prior to mating (Chapter 2 of this dissertation). Modest daily intrafield movement rates (Spencer et al. 2003, Chapter 2 of this

dissertation) and lack variation between male and female movement capabilities (Naranjo 1990, Coats et al. 1986, Spencer et al. 2003, Chapter 2 of this dissertation) suggest that male intrafield movement is less extensive than anticipated and may be insufficient to ensure the adequate mixing of beetles from refuge and Bt corn to delay Bt resistance. Seed blends were thought to provide better mixing of refuge and Bt beetles (Pan et al. 2011) because they would integrate refuge plants within the Bt crop. However, the proportion of beetles collected in mixed-matings (including a beetle from refuge and a beetle from Bt corn), was no different in seed blends compared to structured refuges of the same percentage (Chapter 2 of this dissertation). Mate-seeking movement may be inadequate to delay resistance.

Among the assumptions of the high-dose refuge strategy, corn rootworm behavior features most prominently in the assumption that males will move extensively across Bt cornfields before they mate. At the time this strategy was developed for use against corn rootworms in Bt corn, the available literature supported this assumption. Rootworm males moved significant distances in search of mates and that males were capable of mating many times (Ball 1957, Branson et al. 1977). However, these studies were carried out long before Bt hybrids became available and dominated the Midwest's agricultural landscape. These changes have made it essential to revisit and expand upon our assumptions about basic western corn rootworm biology and ecology associated with Bt corn exposure and cultivation.

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CHAPTER 2: CHARACTERISTICS AND ABUNDANCE OF MATING WESTERN CORN ROOTWORMS (COLEOPTERA: CHRYSOMELIDAE) IN BT CORNFIELDS WITH STRUCTURED AND SEED BLEND REFUGES

INTRODUCTION

Since 2003, western corn rootworm (*Diabrotica virgifera virgifera* LeConte) has been controlled throughout the United States and Canada using transgenic Bt (*Bacillus thuringiensis* Berliner) corn hybrids (US EPA OPP 2001). Bt hybrids were engineered to express toxic Cry proteins that target young root-feeding larvae (Binning et al. 2010), offering a specific and effective method of controlling rootworms that was intended to reduce reliance on, and exposure to, chemical insecticides (Rice 2004). Bt hybrids were rapidly adopted by corn growers and represented 92% of corn grown in the United States in 2015 (USDA ERS 2016).

To delay the evolution of rootworm resistance to Bt hybrids, the US Environmental Protection Agency required growers to plant a non-Bt refuge within each Bt cornfield as part of an insect resistance management (IRM) plan (US EPA OPP 2001). A refuge is an area of non-Bt corn where Bt susceptible rootworms can develop without exposure to Bt toxins expressed elsewhere in the field. Beetles emerging from refuges are expected to disperse broadly across Bt cornfields and mate with rare potentially resistant beetles that survived Bt exposure yielding susceptible heterozygous offspring (Tabashnik 1994, Gould 1998); the proportion of the population needed to engage in this behavior is undefined. Unfortunately, grower compliance with the size of the required refuge (initially 20% of field area) was never high which may have left many Bt cornfields with fewer refuge beetles than were needed (Jaffe 2009). Based on the high-dose refuge strategy (Gould 1998), it was assumed that > 99.9% of susceptible larvae would die after feeding on the Bt crop (US EPA 1998). This dosage assumption proved to be

optimistic; none of the Bt hybrids targeting rootworms meet this criterion (Meihls et al. 2008, Clark et al. 2012). The lack of high-dose efficacy, wide-spread use of Bt technology and poor refuge compliance all increased the risk that rootworms would rapidly evolve resistance to this technology.

In addition to concerns about product efficacy and refuge compliance, the adequacy of the refuge-based IRM plan for corn rootworm was limited by inadequate knowledge of pest biology. Expectations about beetle movement and mating in the field were integral to the success of refuge strategy, leaving it vulnerable to erroneous assumptions about western corn rootworm behavior and reproductive ecology. Despite a long pest history going back for more than a century (Gillette 1912, Spencer et al. 2011), western corn rootworm field biology remains poorly understood, particularly in the context of the changing agricultural landscape.

Western corn rootworms overwinter as eggs in the soil of cornfields (Ball 1957) and larvae begin to hatch in central Illinois during late May or early June (Levine and Oloumi-Sadeghi 1991, Levine et al. 2002). The larvae feed on corn roots (Krysan and Branson 1983) and have the potential to cause extensive economic damage (Metcalf 1986, Dun et al. 2010, Tinsley et al. 2012). After pupation, adults begin to emerge from the soil in late June or early July (Levine and Oloumi-Sadeghi 1991, Murphy et al. 2010, Hughson and Spencer 2015) and feed on corn foliage, silks, tassels and kernels (Ball 1957, Moser and Vidal 2005). Rootworms are protandrous, with males emerging 5.2 to 6.0 days before females (Branson 1987, Hughson and Spencer 2015). Males may require this interval to reach sexual maturity (Guss 1976), but females are sexually mature upon emergence (Hammack 1995). After emergence, females begin to release sex pheromone (Hammack 1995) and males orient their movements toward the pheromone in mate-seeking flights (Ball and Chaudhury 1973, Guss 1976, Marquardt and

Krupke 2009). Females usually do not disperse before mating (Ball 1957, Cates 1968, Lew and Ball 1979), although short distance movement near the emergence site has been recorded (Marquardt and Krupke 2009). The females are usually mated within 12 to 24 hours after emergence, many while they are still teneral (Cates 1968, Quiring and Timmins 1990). Females are thought to mate only once in the field (Ball 1957, Cates 1968), although they have been recorded mating a second time in the laboratory (Branson et al. 1977).

Mating is completed in three to four hours (Ball 1957, Lew and Ball 1979, Sherwood and Levine 1993). During the first hour of mating, the male produces a spermatophore in the female's bursa copulatrix (Lew and Ball 1980). Within two to four hours, spermatozoa (hereafter sperm) acquired from the spermatophore migrate to the female's spermatheca, where they are stored (Lew and Ball 1980). The spermatophore is a significant contribution from the male. It can be equivalent in size to up to 9% of the male's mass (Quiring and Timmins 1990, Tallamy et al. 2000) and consists of proteins, carbohydrates and water (Murphy and Krupke 2011), which may be absorbed by the female during the following five to seven days (Lew and Ball 1980).

Hughson and Spencer (2015) found that western corn rootworm adults were concentrated in the refuge portions of Bt cornfields with structured refuges for much of the growing season. Apparently, many beetles remain near the location where they emerge, rather than dispersing across the fields (Hughson and Spencer 2015). Persistent aggregations of beetles in structured refuges may result in a high likelihood of mating between beetles of refuge origin rather than mixing between refuge and Bt beetles. The extent to which areas with concentrated female abundance (and many calling females) may attract mate-seeking beetles from nearby areas of low abundance needs investigation. Uniform distributions of mating across Bt cornfields would

suggest that beetles are readily moving and mating within Bt cornfields as expected in high-dose refuge strategy. Studying the reproductive characteristics of mating beetles and spatial and temporal patterns of mating activity in Bt cornfields may reveal whether adequate mixing of susceptible and potentially resistant beetles, required for the success of refuge strategy, occurs in the field.

This study examines physiological characteristics of mating beetles to confirm that measures recorded in laboratory studies (i.e. age of mating, number of matings, size preferences of males and females) are consistent with field populations and whether they may provide insights about mating distributions that are relevant to IRM. This study also examines the spatial and temporal distributions of mating activity in Bt cornfields with structured and seed blend refuges and reveals the proportion and distribution of matings that include one individual from refuge and one of Bt origin, a condition necessary for the success of refuge strategy. Based on the distributions of adults reported in Hughson and Spencer (2015), I predict that most mating activity in Bt cornfields with structured refuges will occur in refuge rows and that mating activity will be uniformly distributed in fields with seed blend refuges. In addition, because beetles were evenly distributed in seed blend refuges (Hughson and Spencer 2015), I expect to find more mixed-matings will be identified in seed blend refuge treatments.

MATERIALS AND METHODS

Location and treatments

The research site was located at the University of Illinois' "Shaw Farm" in Urbana, IL. Four 1.66 ha cornfields were each subdivided into three 0.53 ha plots separated by 8 m alleyways. Each of the twelve plots was randomly assigned one of four unique refuge treatments

in a completely randomized design with three replicates; treatment locations were randomized each year (Hughson and Spencer 2015). Each plot was 110 to 116 rows wide planted on a north-south axis and each row was 0.76 m wide. Planting dates were similar to the planting dates in the surrounding commercial fields (see Hughson and Spencer 2015 for planting dates).

The refuge treatments were 1) 20% structured refuges (22 rows of refuge corn and 88 rows of Bt corn), 2) 5.2% structured refuges (6 rows of refuge corn and 109 rows of Bt corn), 3) 5.2% seed blend refuges and 4) 0% refuge treatments as positive controls. The 5.2% refuge treatments will be referred to as 5% refuge treatments for simplicity. In structured refuge treatments, the refuge rows were always located on the west side of their respective plots (Hughson and Spencer 2015). Refuge percentage and configurations were selected based on the refuge standards when the experiment was developed. Cornfields planted with a single rootworm active Bt trait required a 20% structured refuge within or adjacent to the field (US EPA OPP 2001), while pyramided hybrids could be planted in a 5% structured refuge adjacent to or 5% seed blend refuge with the refuge seeds randomly distributed across the Bt cornfield (US EPA OPP BPPD 2010).

The rootworm active Bt portion of each plot was planted with Herculex® XTRA (Pioneer Hi-Bred International, Inc., Johnston, IA; 2010: Hybrid 34B41, RM 109 days; 2011 and 2012: Hybrid 33W84, RM 111 days) which expressed Cry1F protein, targeting lepidopteran larvae, and Cry34/35Ab1 protein, targeting corn rootworm larvae. The refuge portion of each treatment was planted with YieldGard® Corn Borer (Monsanto Company, St. Louis, MO; 2010: Hybrid 34B94, RM 110 days; 2011 and 2012: Hybrid 33B54, RM 113 days) which expressed Bt Cry1Ab targeting lepidopteran larvae (Hughson and Spencer 2015). A non-Bt refuge for lepidopteran pests was planted adjacent to the research fields. Corn phenology was recorded based on

observations of plant development in the Bt hybrids which comprised 80 to 100% of the plants in each plot. Phenology stages (Ritchie et al. 1993, Abendroth et al. 2011) were defined and recorded as vegetative, pollination and post-pollination each year (Hughson and Spencer 2015).

Single adult and mating pair collections

Western corn rootworm mating pairs and single adults were collected in 11 designated sampling rows in each of the 12 plots. Collections began at the center of each row to avoid collecting beetles from the edge of the field, where beetle populations may have been inflated by movement between fields (see Hughson and Spencer 2015 for the details of plot labeling).

Mating pair and individual adult collections were carried out three to five days per week in each plot and sampling row. To ensure that the earliest emerging insects were not overlooked, collections began when third instar larvae were observed on corn roots excavated from the field. Collections occurred from 8:30 to 11:00 a.m., when mating activity was at its peak (Bartelt and Chiang 1977, Dobson and Teal 1986) and newly emerged beetles were still teneral (Cates 1968). Collections were carried out in each sampling row for four minutes but shortened to three or two minutes when beetles were abundant to avoid collecting excessively large samples (Hughson and Spencer 2015). Collection duration was recorded so mating abundance could be reported as mating pairs collected per minute (mating pairs/min). During collections, single adults and mating pairs were hand collected by knocking them from corn foliage into collection jars. Collection jars consisted of a funnel fixed to the top of a 0.95 L glass jar (Ball Corporation, Bloomfield, CO) which contained a small piece of dry ice. The beetles were killed on contact by freezing or asphyxiated by the CO_2 gas emitted by the dry ice (Hughson and Spencer 2015). When an apparent mating pair was killed, those *in copula* remained attached to one another,

making it possible to differentiate mating from single or mate-guarding beetles and identify the individual members of each mating pair. If an apparent mating pair was only engaged in mate-guarding, the beetles separated in the jar. At the end of each collection, the samples were transferred to plastic bags where data including the number of observed mating pairs collected and their corresponding refuge treatment, sampling row identification, date, collection duration and collection time, were recorded. The bags were placed in a cooler of dry ice for transportation to the laboratory, where they were sorted and stored in a freezer to await processing (Hughson and Spencer 2015).

Reproductive characteristics

To assess relative size, single and mating beetles (males and females) were weighed and the length of the left elytron was measured (the right was measured if the left was damaged or missing). Female maturity (teneral or mature) was recorded to determine whether partner size played a significant role in mate selection. To understand the mating status and reproductive development of female beetles, single and paired females were dissected. During dissections, mating status (individual or paired), number of apparent matings and an ovary development rating were recorded. A mating was confirmed if a female 1) was *in copula*, 2) had a spermatophore in her bursa copulatrix, 3) had sperm in her spermatheca or 4) had any combination of those characteristics. Presence of a spermatophore was confirmed visually using a stereo microscope (StereoZoom 4, Leica Microsystems Inc., Buffalo Grove, IL). Relative size was recorded for each spermatophore. A large spermatophore (Figure 2.1) included both an ampulla and a larger spermatophylax with little apparent degradation, indicating that mating had occurred recently. A small spermatophore had a spermatophylax that was partially absorbed

(similar in size to the ampulla) or completely absorbed and absent with only the ampulla remaining. Since spermatophores are completely absorbed in five to seven days (Lew and Ball 1980), a large spermatophore indicated recent mating, a small spermatophore indicated that mating occurred at least a few days prior to collection and the absence of a spermatophore when sperm was present indicated that mating occurred more than five to seven days prior to collection. Sperm presence was determined by removing the spermatheca, placing it on a slide in a droplet of saline solution (0.75% NaCl) and crushing it beneath a cover slip. The slide was inspected through a compound microscope (Microstar 10, American Optical Company, Buffalo, NY) under 200 x magnification, to confirm the presence of sperm, which resemble a mass of hair-like structures. Ovary development was rated using a modification of the ovary maturity scale developed by Short and Hill (1972). According to this scale, stage 1 ovaries have no egg development, stage 2 ovaries have only small ovules, stage 3 ovaries have some full-size eggs that had not completed choriogenesis, and stage 4 ovaries contain some mature eggs with chorions. If present, the mature eggs were removed and the total number of eggs was recorded.

In some circumstances it was possible to determine that a paired or individual female had mated more than once. A female was assumed to have mated at least twice if she was collected with more than one spermatophore in her bursa copulatrix or if her spermatheca contained sperm but she was *in copula* with a male that had not yet produced a spermatophore. All paired females collected from 2010 to 2012 were dissected and their mating characteristics were recorded.

Many individually collected females were also dissected. Because 132 row samples containing 0 to > 50 beetles were collected each day, subsets of individual beetles were taken from the row samples for processing in 2010 and 2011. In 2010, no more than ten male and ten

female beetles were randomly selected, weighed and their elytra were measured for each row collection. In 2011 each of these females were also dissected. However, in 2010 the females were subsampled further; only females from one replicate of each treatment (randomly selected) per collection day were dissected.

Detection of ingested Bt proteins in rootworm gut contents

The specific Bt proteins found in the insect gut were used to infer patterns of beetle movement when compared to those expressed in corn plants at the beetle's collection location (Spencer et al. 2003). Spencer et al. 2003 found that in two to four hour long laboratory observations, western corn rootworm beetles fed 35 to 60% of the time they were on corn tissues. This suggested that beetles in Bt cornfields likely spend a significant portion of their time feeding on tissues expressing Cry proteins which can be detected in their gut contents using EnviroLogix™ QuickStix™ (EnviroLogix Inc., Portland, ME) ELISA test strips and regularly renewing that detectable material. The high proportion of individuals that tested positive for the tissue in the field and the ability to easily detect the Cry proteins expressed by those tissues confirmed the efficiency of using Cry proteins as markers.

A feeding calibration experiment was conducted to determine how long the Cry1Ab and Cry1F proteins expressed in corn tissue could be detected after beetle feeding. Groups of beetles were fed on Bt corn plants expressing either Cry1Ab or Cry1F in a greenhouse. Each plant was enclosed with a mesh bag and the beetles inside were allowed to feed for 24 hours. At the end of the Bt feeding period, the beetles were gently shaken off the foliage, then moved within the mesh bags and slipped over fresh non-Bt plants. Groups of eight beetles were removed from each bagged non-Bt plant at 0, 2, 4, 8, 12, 16, 24, 36 and 48 hours and tested for the presence of

Cry1Ab or Cry1F proteins using EnviroLogix™ QuickStix™ test strips. This procedure generated a binary dataset that was analyzed to identify how long each Cry protein could be detected after feeding.

Before dissection and gut content analysis, beetles were rinsed to remove Bt plant debris or frass, preventing surface contamination. Care was taken not to disturb the gut during dissection so the contents could be analyzed afterward. The Cry proteins detected in a beetle's gut contents allowed us to determine whether a beetle was feeding on refuge or Bt corn within the last day, but cannot confirm the corn type of the larval host. To determine if beetles had previously consumed refuge or Bt corn, EnviroLogix™ QuickStix™ test strips (EnviroLogix Inc., Portland, ME) were used to detect the presence of the Cry1Ab protein, expressed exclusively by refuge plants, and Cry1F, expressed exclusively by Bt plants. Beetles were individually placed in a microcentrifuge tube (Fisherbrand, Hampton, NH) with 0.5 mL of QuickStix™ Extraction Buffer and pulverized using the Sonifier® Cell Disruptor (Branson Ultrasonics Corporation, Danbury, CT). Male beetles were tested for three Cry proteins: Cry1Ab, indicating the beetle had recently fed on refuge corn, Cry1F, indicating feeding on Bt corn and Cry3Bb1. Cry3Bb1 was expressed by the Bt hybrids planted in all of the commercial cornfields surrounding the research site but was not expressed by the hybrids planted for this experiment. The presence of this Cry3Bb1 in beetle gut contents indicated that the male entered the research site after feeding in a different cornfield. Female beetles were tested for two Cry proteins: Cry1Ab and Cry1F. Based on the original assumptions of refuge strategy for corn rootworms that males from refuge corn would move broadly across Bt cornfields and mate with potentially resistant females from Bt corn, females were not tested for the Cry3Bb1 Bt protein from the surrounding landscape.

By testing the gut contents of individual beetles collected in mating pairs for the presence of Cry proteins, it was possible to identify "mixed-matings." Mixed-matings were defined as a mating pair including one individual from refuge corn (testing positive for Cry1Ab) and the other from Bt corn (testing positive for Cry1F); this type of mating is essential for the success of western corn rootworm IRM in Bt cornfields. Using this method, the proportion of mixed-matings among all of the mating pairs collected from 2010 to 2012 was identified.

In addition to identifying whether mating pairs were mixed-matings, this method revealed which member(s) of a mixed-mating had moved and the direction of travel (from refuge to Bt corn or vice versa). If the Cry protein(s) in a beetle's gut differed from the proteins expressed by the corn plants where it was collected, the beetle was designated a "mover." When the mover in a mixed-mating was identified, the sex and direction of travel were recorded. Mixed-matings were identified that included nearly every possible combination of movers (e.g. male, female, both as movers) and direction of travel (e.g. refuge to Bt corn, Bt to refuge corn, entry from a nearby field). These descriptive measures are referred to as the "composition" of mixed-matings. The composition breakdown of mixed-matings collected in each treatment was presented as the proportion of mixed-matings represented by each combination.

Statistical analyses

Mating pair abundance was calculated as mating pairs collected per minute (mating pairs/min) in each sampling row. Abundance data for each sampling row were non-normal and log (x + 0.5) transformed. The transformed abundance data were analyzed among treatments, corn phenology and sampling rows using three-way analysis of variance (ANOVA) and pairwise

multiple comparisons using the Tukey-Kramer method in JMP Pro 10 (SAS Institute Inc. 2012). All three years differed and were analyzed separately.

The measures of reproductive characteristics (maturity, number of spermatophores, presence of sperm in spermatheca and ovary rating) were non-normal and log (x + 0.5) transformed. The characteristics of paired females were compared to individual females to determine whether mating females differed in these measures, using t-tests (JMP Pro 10, SAS Institute Inc. 2012). The mean weights and elytron lengths of paired and individual males and females were tested for normality and log (x + 0.5) transformed. The mean weights and elytron lengths of paired males and females were compared to individually collected males and females to determine whether the size of actively mating or individual beetles differed, using t-tests (JMP Pro 10, SAS Institute Inc. 2012).

The binary data produced by the feeding calibration experiment were converted to proportions of Bt positive beetles per time interval for each Bt protein and replicate tested. Each proportion per replicate represented eight beetles (a total of 432 beetles were used) with each Bt protein treatment and time interval. The proportions of Bt positive beetles were analyzed using ANOVA to compare the time intervals (JMP Pro 10, SAS Institute Inc. 2012). The longest time intervals within which positive tests were read for each protein were reported.

Very few mixed-matings were detected during this experiment so mixed-matings from all three years were pooled for analysis. The Kruskal-Wallis test and Steel-Dwass test of multiple comparisons (JMP Pro 10, SAS Institute Inc. 2012) were used to determine whether refuge treatment or corn phenology had an impact on the total number of mixed-matings collected. There were too few mixed-matings to analyze their abundance by sampling row. However, the total numbers of mixed-matings and mating pairs were presented in a figure depicting the year,

refuge treatment and sampling row where they were collected. The composition (Bt origin and direction of male and female movement) of individuals in mixed-matings were summarized for each treatment to reveal the direction that moving individuals in each pair traveled.

RESULTS

Mating pair abundance

In 2010, 2011 and 2012, 3,990, 3,036 and 4,354 row samples were collected, yielding 290, 319 and 218 mating pairs, respectively. During sampling, 11,469, 17,901 and 30,219 single adults were collected in 2010, 2011 and 2012, respectively, for a total of 59,589 single adults.

The distribution of mating pair abundance (mating pairs/min) among sampling rows differed by refuge treatment and phenology (2010: df = 8, $F = 3.49$, $P = 0.0005$; 2011: df = 8, $F = 8.87$, $P < 0.0001$; 2012: df = 8, $F = 20.20$, $P < 0.0001$). In structured refuge treatments, the mating abundance was greater in refuge rows than in Bt rows during the vegetative and pollination phenology periods all three years (Figures 2.3 through 2.5). However, during post-pollination, refuge rows did not differ from Bt rows except in the 5% structured refuge in 2010 (Figures 2.3 through 2.5). In seed blend and 0% refuge treatments, mating abundance did not differ and was evenly distributed across the fields in all three phenology periods from 2010 through 2012 (Figures 2.3 through 2.5). These data are presented in numerical detail in Tables (A.1 through A.3).

Reproductive characteristics

Paired females were more likely to be young compared to individual females. In 2010 and 2011, many more paired females were teneral than individually collected females (2010: $t =$

15.53, $P < 0.0001$; 2011: $t = 18.96$, $P < 0.0001$; Table 2.1). The mean stage of ovary development for paired females was lower than that of individual females (2010: $t = 6.36$, $P < 0.0001$; 2011: $t = 11.06$, $P < 0.0001$; Table 2.2). Paired females were more likely to contain a spermatophore than individual females (2010: $t = -12.44$, $P < 0.0001$; 2011: $t = -13.74$, $P < 0.0001$; Table 2.1). Individual females were more likely to have sperm in their spermathecae (2010: $t = 6.71$, $P < 0.0001$; 2011: $t = 9.33$, $P < 0.0001$; Table 2.1). The mean proportions and means for paired females in 2012 were reported in Tables A.4 and A.5.

Evidence of multiple mating was found during dissections; some females possessed more than one spermatophore. On average, mating females had more spermatophores than single females (2010: $t = -12.60$, $P < 0.0001$; 2011: $t = -13.38$, $P < 0.0001$; Table 2.2). Among paired females, 1.5% had more than one spermatophore. In addition, some paired females were discovered with stored sperm; however, the fact that their current mate had not yet transferred a spermatophore indicated that the sperm was the result of a previous mating. These females, which apparently mated more than once over a longer period of time, represented 3.0% of all paired females. Combining these two types of multiple matings, at least 4.5% of females mated more than once. The corresponding measures for paired females collected in 2012 are presented in Tables (A.4 and A.5).

Elytron length and weight between individual and paired males or females were variable among the years, groups and measurements (Table 2.3) preventing a consistent pattern from being identified.

Detection of ingested Bt proteins in rootworm gut contents

The calibration experiment revealed that the Cry1Ab protein could be detected using EnviroLogix™ QuickStix™ in the western corn rootworm gut for up to 12 hours and the Cry1F protein could be detected for up to 48 hours (Figure 2.2). This allowed beetle movement to be detected based on recent herbivory.

Although 827 mating pairs were observed, 52 had to be excluded from gut content analysis because they were damaged or the individuals had been separated in storage making them indistinguishable from the individually collected beetles stored in the same vial. Of the 775 intact mating pairs, 66 were categorized as mixed-matings (a mating pair with two beetles of different field location origins based on the Cry proteins detected in their guts), representing 8.5% of all mating pairs collected.

The refuge treatment significantly influenced the proportion of mixed-matings that were recovered ($\chi^2 = 21.15$, df = 3, $P < 0.0001$). There was no difference in the proportion of mixed-matings collected in the 20% structured, 5% structured or 5% seed blend treatments, although fewer were collected in the 0% refuge treatment. Of all mating pairs collected, 9.2% were mixed-matings in the 20% structured refuge treatments, 16.5% were mixed-matings in 5% structured refuge treatments, 12.8% were mixed-matings in 5% seed blend treatments and 0.0% were mixed-matings in the 0% refuge treatments. Phenology also influenced the proportion of mixed-matings recovered ($\chi^2 = 58.66$, df = 2, $P < 0.0001$). A greater proportion of mixed-matings were recovered during the vegetative period (0.284) than the pollination (0.050) and post-pollination periods (0.052), which did not differ.

In the 20% structured refuge treatment, 88.9% of mixed-matings in refuge corn rows (Figure 2.6) included a male that moved from Bt to refuge corn, while just 11.1% included a

female that moved from Bt to refuge corn. No mixed-matings included either a male or female that moved from refuge to Bt corn.

In the 5% structured refuge treatment, 61.1% of mixed-matings were collected in refuge corn and included a male that moved from Bt to refuge corn and 5.6% included a female that moved from Bt to refuge corn (Figure 2.6). In 8.3% of the mixed-matings, the male and female tested positive for both Cry proteins and were collected in refuge corn, indicating they each moved from Bt to refuge corn and fed there before mating. The remaining mixed-matings in 5% structured refuge treatments were collected in Bt corn rows, one included a male that moved to Bt corn from refuge corn and the other pair included a female that moved from refuge to Bt corn (Figure 2.6).

In the 5% seed blend treatments, mixed-matings were more evenly distributed across the cornfields than in structured refuge treatments (Figure 2.6). Because Bt and refuge plants are randomly distributed across the field, gut content analysis cannot indicate the direction of movement in seed blend fields.

Some mating pairs included a male that had entered the research site from another location (n = 12), representing 1.5% of all matings. Of these matings, 66.7% included a male positive for Cry3Bb1 and a female positive for Cry1F, indicating that both individuals had Bt corn origins. Because these matings resulted from interfield movement rather than intrafield movement, the did not meet the criterion of a mixed-mating because neither partner contained Cry1Ab, refuge corn. The remaining 33.3% were mixed-matings, including a male from another field (Cry3Bb1 positive) and a female from refuge corn. One of these mixed-matings was collected in the 20% structured refuge, two were in the 5% structured refuge and one was in the 5% seed blend.

DISCUSSION

Mating characteristics

These analyses of female reproductive characteristics suggest that mating female western corn rootworms are younger compared to the general field population of females. Fewer mating females were teneral (~ 50%) than reported in laboratory studies (96%; Quiring and Timmins 1990) which may be a result of ecological variability in the field rather than ideal laboratory conditions or emergence in an area where rootworm populations have been suppressed by Bt hybrids. Accordingly, females may require additional time to attract a mate at low beetle densities (Spencer et al. 2012). Most of the individually collected females in the field were mature and had characteristics indicating that they were mated. They were also more likely to have mature eggs than mating females. No gravid females were collected in mating pairs.

The first record of field-collected females mating multiple times was discovered during dissections. While females have been recorded mating more than once in a laboratory setting (Branson et al. 1977), there was no previous evidence that they may mate multiple times in a dynamic field environment. Based on dissections, it was estimated that at least 4.5% of females mated more than once in the field, either in short succession or more than five to seven days after the first mating. This is only a minimum estimate because multiple mating is difficult to detect. Based on dissections, multiple matings could be confirmed only by direct observation of a combination of mating activity (a pair *in copula*), spermatophore presence and sperm presence in the spermatheca. However, beetles remain *in copula* only for three to four hours (Ball 1957, Lew and Ball 1979, Sherwood and Levine 1993) and spermatophores are absorbed after five to seven days (Lew and Ball 1980) making it likely that some multiply mated females were not identified because they no longer had a spermatophore in their body or were not actively mating

when collected. The proportion of females mating more than once in the field may be even greater than observed in this study; a laboratory study suggested that 23% of females may take a second mate (Branson et al. 1977). Confirmation of multiple mating in a field population may be more precisely explored using genomic methods to analyze the allelic admixture of sperm in a female's spermatheca.

A concern arising from the identification of polyandrous females, is that they may have additional opportunities to mate with putative-resistant males from Bt corn. While this risk may seem small, it has not been explored in resistance management modeling. Because Bt emergence is delayed compared to refuge corn (Storer et al. 2006, Murphy et al. 2010, Hughson and Spencer 2015) and males have a limited reproductive lifespan (Kang and Krupke 2009b), a female may be more likely to encounter a Bt emerging male than an early emerging refuge male if she takes a second mate over a week after her initial mating. If western corn rootworms have second male sperm precedence, as has been recorded in southern corn rootworm (*Diabrotica undecimpunctata* Howardii; Oyediran et al. 2007), the possibility that alleles from a potentially resistant second male are being passed to all or a majority of a female's offspring should be considered.

Some researchers have shown that rootworm adults prefer larger beetles as mates (Kang and Krupke 2009a, French and Hammack 2010). However, in the present study, body size did not appear to be a significant factor in mate choice. In previous studies, individual beetles were given the option between partners designated large and small (Kang and Krupke 2009a, French and Hammack 2010). In the field, perhaps individuals are less likely to encounter two potential mates of noticeably different sizes. It may be that partner size is not the deciding factor in mate

selection in the field, but one of a variety of trait criteria an individual may assess when selecting a mate.

Mating pair abundance

In structured refuge treatments, mating pair abundance was greater in refuge rows compared to Bt corn rows during the vegetative and pollination periods, while in seed blend and 0% refuge treatments, mating abundance was evenly distributed across fields all season. These patterns closely reflect the patterns of single adult abundance reported in Hughson and Spencer (2015), suggesting that adult beetles did not distribute themselves broadly across fields while mate-seeking. The differences in distributions between structured and seed blend treatments for both mating activity and adult abundance suggest that the distribution of refuge and Bt plants, may play an important role in establishing beetle distributions (Hughson and Spencer 2015).

In the 5% seed blend and 0% structured refuge treatments during 2011 and 2012, some rows along the west side of the plots had apparently increased, although not significantly greater, mating abundance. This likely occurred because one of those treatments was planted where a structured refuge treatment had been located during the previous year. The west sides of the fields corresponding to former refuge must have had more oviposition leading to elevated emergence, abundance (Hughson and Spencer 2015) and mating activity. In 2010 weather may also have affected mating abundance. Heavy rains early in the season submerged portions of refuge corn for nearly two weeks, killing some of the larvae in the soil and causing lower beetle and mating abundance in 20% structured refuges compared to the 5% refuges. Low abundance in these areas later in the season suggests that beetles did not move back into these locations

when the standing water was gone. Thus, not only the refuge treatment, but other factors that affect beetle abundance, such as flooding, may influence mating patterns.

Examination of the gut contents of individuals in mating pairs revealed that 8.5% of all mating pairs were mixed-matings. This number may be lower than expected given that the success of refuge strategy was predicated on the idea that males emerging from refuge corn move readily throughout Bt cornfields and mate with potentially resistant females that emerged from Bt corn. This number is much lower than the 43.3 ± 6.1% of mixed-matings collected in 20% structured refuges by Taylor et al. (2016) in a study using ^{15}N labeling of experimental plots inside 3.65 x 3.65 x 2.13 m screen tents. In the present study, a smaller proportion of mixed-matings were collected in larger areas (0.53 ha), which may indicate that mate-seeking movement occurred on a smaller scale than previously anticipated. This suggests that beetles may be more likely to move one to four rows to cross the refuge-Bt interface prior to mating, rather than dozens of rows. In commercial cornfields many times the size of these plots, the proportion of mixed-matings may be even smaller.

Taylor et al. (2016) also reported 35.1 ± 7.6% mixed-matings collected in 5% seed blend refuges. Regardless of plot size, seed blends with the same percent refuge should, hypothetically, yield a similar proportion of mixed-matings. The discrepancy between these studies may have occurred because the Taylor et al. (2016) beetles were unable to disperse from the experimental plots, inflating the local frequency of interactions between refuge and Bt corn beetles.

In structured refuge plots, most mixed-matings occurred in the refuge rows, early in the season, constituting 28% of all matings, compared to 5% during the pollination and post-pollination periods. This pattern suggests that mixed-matings occurred where and when beetle

emergence, abundance (Hughson and Spencer 2015) and mating activity were greatest, reinforcing the conclusion that beyond the vegetative period, little movement occurred among the mate-seeking populations.

Analyzing the composition of mixed-matings revealed that most mixed-matings in structured refuge treatments included males that moved from Bt corn to refuge corn. While this finding is consistent with the assumption that most mate-seeking movement is carried out by males (Ball and Chaudhury 1973, Guss 1976, Marquardt and Krupke 2009), the absence of many refuge males paired with Bt females in Bt corn rows contradicts the related expectation that abundant refuge males would be moving out of refuge and mating with Bt females. The combination of delayed female emergence compared to males (Ball 1957, Hughson and Spencer 2015) and delayed rootworm emergence from Bt corn compared to refuge emergence (Storer et al. 2006, Murphy et al. 2010, Hughson and Spencer 2015) may have increased the synchrony of emergence between Bt males and refuge females that made those pairings more likely. Furthermore, the greater female density in refuges may create a higher concentration of sex pheromone than the more sparsely distributed Bt females emerging from Bt corn. The higher concentration could attract mate-seeking Bt males from nearby Bt rows into refuge. If mate-seeking refuge males are encountering abundant calling females in the refuge, they may be unlikely to disperse and encounter sparse Bt females. Attraction of some mate-seeking Bt males into nearby refuge rows, could leave potentially resistant Bt emerging females isolated, prolonging their pre-mating interval and delaying oviposition.

Analysis of mixed-mating composition also revealed that some unmated females moved across the refuge-Bt interface prior to mating. A female that emerged within a structured refuge has entered a habitat with high abundance and mating activity making it possible for her to

encounter multiple mate-seeking males before accepting one. Conversely, a female emerging from Bt corn, an area of low beetle abundance and low mating activity, may not encounter a male for some time. While unmated Bt emerging females may continue to call for multiple days (Hammack 1995) and wait for a male, they may also begin to move within the field until they encounter a mate-seeking male (Spencer et al. 2012).

Homogeneity of the Bt and refuge plant landscape in seed blends appears to contribute to homogeneous beetle distributions (Hughson and Spencer 2015) and mating activity. The random distribution of refuge plants among Bt plants in seed blends were expected to yield more mixed-matings because they increased the effective area of refuge-Bt interface in these fields, allowing beetles to encounter mates of different corn origins without broad dispersal (Hughson and Spencer 2015). However, this research revealed that, while matings are more evenly distributed across the fields, the patterns of recent feeding revealed that seed blends did not produce a greater proportion of mixed-matings than a structured refuge of the same size.

Although seed blends did not result in a greater proportion of mixed-matings, they provided lower overall beetle abundance (Hughson and Spencer 2015) and fewer total mating beetles than structured refuges. Seed blends also provided a uniform distribution of mating beetles and more diverse combinations of individuals in mixed-matings which may make seed blends a better long-term option compared to structured refuges. While planting structured refuges in the same location year after year was viewed as beneficial to IRM since it would promote establishment of a persistent susceptible population (Pan et al. 2011), this research suggests that the heterogeneous distribution of refuge and Bt plants across structured refuge treatments may increasingly concentrate beetle abundance and mating in the refuge portion of the field, producing a preponderance of matings between refuge beetles.

Interestingly, mixed-matings were not collected from any 0% refuge treatments and, overall, only four mixed-matings involved a male that entered from outside the research site (i.e. a male positive for Cry3Bb1 protein). The experimental plots used in this research were separated from the other experimental plots and neighboring cornfields by 8 m alleyways; the absence of mixed-matings on 0% refuge and the presence of few offsite males suggests that interfield movement contributed little to mating activity. The lack of males from other cornfields means that male mate-seeking movement may contribute little to the spread of Bt resistance. Post-mating long-distance dispersal by females has the potential to spread Bt resistance over long-distances. Coates et al. (1986) found that 15% of females in tethered flight mills engaged in sustained flight with an average rate of 10 km/hour, representing a greater percentage of the population than was recorded for interfield movement by males in this research. Given the rate at which long-distance dispersing beetles travel and the proportion of the population they represent, it will be important to determine whether beetles emerging from refuge and Bt corn share similar capabilities for long-distance dispersal to those recorded in the laboratory.

Recent reports of Bt resistance in multiple locations (Gassmann et al. 2011, 2014; Gray 2012, 2013; Wangila et al. 2015, Schrader et al. 2016, Zukoff et al. 2016, Ludwick et al. 2017) highlight concerns that rootworm behavior may not meet the expectations of refuge strategy. This research revealed that only 8.5% of mating pairs included individual beetles with different Bt origins and most mating was concentrated around refuge plants suggesting that many beetles did not move significant distances prior to mating. When mate-seeking beetles moved prior to mating, most moved early in the season and they traveled from Bt corn to refuge corn.

While some simulation models suggest that seed blends may provide longer durability of Bt traits than structured refuges when rootworm reproductive biology is incorporated (Pan et al.

2011, Spencer et al. 2012), this research does not support the expectation that seed blends would produce a greater proportion of mixed-matings. Without the detailed analyses of where the partners in mating pairs originated, the more even distribution of mating pairs in the seed treatment versus block refuges presents an overly optimistic picture of mating in seed blends.

Regardless of whether a particular refuge configuration facilitates desirable or undesirable beetle behavior, none of the currently available Bt traits expressed in Bt corn hybrids satisfy high-dose expectations (Meihls et al. 2008, Clark et al. 2012) that are fundamental to the use of high-dose refuge strategy. The problem of field evolved Bt resistance in western corn rootworm populations is growing (Gassmann et al. 2011, 2014; Gray 2012, 2013; Wangila et al. 2015, Schrader et al. 2016, Zukoff et al. 2016, Ludwick et al. 2017), in part, because the expectations of technology developers, scientists and corn growers for beetle behavior were unrealistic. Although the reality of beetle behavior in Bt corn was not known when the rootworm IRM plans were established, this research has revealed that rootworm biology does not meet any of the assumptions required by high-dose refuge strategy to delay resistance to Bt technology. Expanding the body of knowledge about rootworm behavior and ecology in the field may ultimately enhance the remaining efficacy of current technology and help develop new IRM and management plans that are better suited to this pest's biology.

TABLES

Table 2.1. Proportions of paired and individual female western corn rootworms that were mature, had at least one spermatophore present in their bursa copulatrix or had sperm in their spermatheca upon collection in 2010 and 2011.

	2010				2011			
	Paired		Individual		Paired		Individual	
Reproductive measure	n [a]	Proportion [b]	n	Proportion	n	Proportion	n	Proportion
Maturity [c]	278	0.37 b	5066	0.78 a	298	0.51 b	5317	0.89 a
Spermatophore Presence	278	0.87 a	2293	0.49 b	298	0.85 a	3223	0.45 b
Sperm in Spermatheca	278	0.46 b	2286	0.66 a	298	0.38 b	3214	0.65 a

[a] Number of paired and individual females dissected and observed in 2010 and 2011.
[b] Proportion of females testing positive for a given measure within the same mating status and year combination. Proportions in the same row, within the same year, followed by the same letter are not significantly different ($\alpha = 0.05$). T-tests were performed on log (x + 0.5) transformed data. Untransformed proportions are shown. Paired females from 2012 are presented in the Table A.4.
[c] Proportion of females that were non-teneral.

Table 2.2. Mean (± SE) ovary development, number of spermatophores and number of mature eggs observed in paired and individual female western corn rootworms collected in 2010 and 2011.

	2010				2011			
	Paired		Individual		Paired		Individual	
Reproductive measure	n [a]	Mean ± SE [b]	n	Mean ± SE	n	Mean ± SE	n	Mean ± SE
Ovary Development [c]	278	1.10 ± 0.02 b	2294	1.38 ± 0.02 a	298	1.03 ± 0.01 b	3223	1.58 ± 0.02 a
No. Spermatophore	278	0.89 ± 0.02 a	2293	0.49 ± 0.01 b	298	0.86 ± 0.02 a	3224	0.46 ± 0.01 b
Mature Eggs	0	--	41	99.4 ± 3.67	0	--	128	73.7 ± 3.04

[a] Number of paired and individual females dissected and observed in 2010 and 2011.
[b] Means (mean ± SE) in the same row, within the same year, followed by the same letter are not significantly different ($\alpha = 0.05$). T-tests were performed on log (x + 0.5) transformed data. Untransformed means are shown. Paired females from 2012 are presented in the Table A.5.
[c] Scored on a scale of 1 to 4 based on Short and Hill 1972. During stage 1 no ovary development was observed and during stage 4 mature eggs were observed.

Table 2.3. Mean (± SE) weight and elytron length of paired and single western corn rootworm males and females collected in 2010 and 2011.

		2010				2011			
		Paired		Individual		Paired		Individual	
Sex	Size Measure	n [a]	Mean ± SE [b]	n	Mean ± SE	n	Mean ± SE	n	Mean ± SE
Male	Weight	283	0.0082 ± 0.0001 b	4943	0.0083 ± 0.0000 a	307	0.0093 ± 0.0001 a	4657	0.0071 ± 0.0000 b
	Elytron Length	282	3.78 ± 0.02 a	4741	3.70 ± 0.00 b	307	3.86 ± 0.01 a	4657	3.85 ± 0.00 a
Female	Weight	278	0.0084 ± 0.0001 b	5068	0.0092 ± 0.0001 a	297	0.0093 ± 0.0001 a	5333	0.0089 ± 0.0001 a
	Elytron Length	278	3.93 ± 0.02 a	5050	3.84 ± 0.00 b	298	3.93 ± 0.02 b	5331	4.05 ± 0.00 a

[a] n represents the number of paired and individual male or female beetles measured in 2010 and 2011.
[b] Means (mean ± SE) in the same row, within the same year, followed by the same letter are not significantly different ($\alpha = 0.05$). T-tests were performed on log (x + 0.5) transformed data. Untransformed means are shown.

FIGURES

Figure 2.1. A spermatophore, removed from a female's bursa copulatrix. The spermatophore consists of two major portions, the spermatophylax, on the left and the ampulla, which contains sperm, on the right.

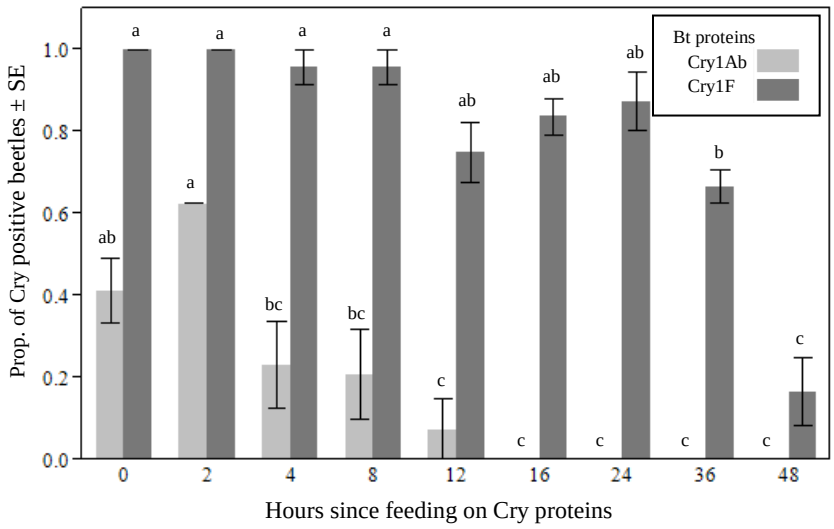

Figure 2.2. Proportions (± SE) of beetles testing positive for Cry1Ab and Cry1F proteins expressed specifically by the refuge or Bt corn hybrids, respectively, by time interval after feeding. Beetles were fed the Cry1Ab or Cry1F proteins for 24 hours and then moved to non-Bt corn plants and tested for the presence of Cry1Ab or Cry1F in their gut contents at regular intervals for 48 hours. Gray bars and dark bars represent the proportion of beetles testing positive for Cry1Ab or Cry1F, respectively, at each post-feeding interval. ANOVA and Tukey-Kramer test of multiple comparisons were performed ($\alpha = 0.05$). Bars of the same color with the same letter are not significantly different.

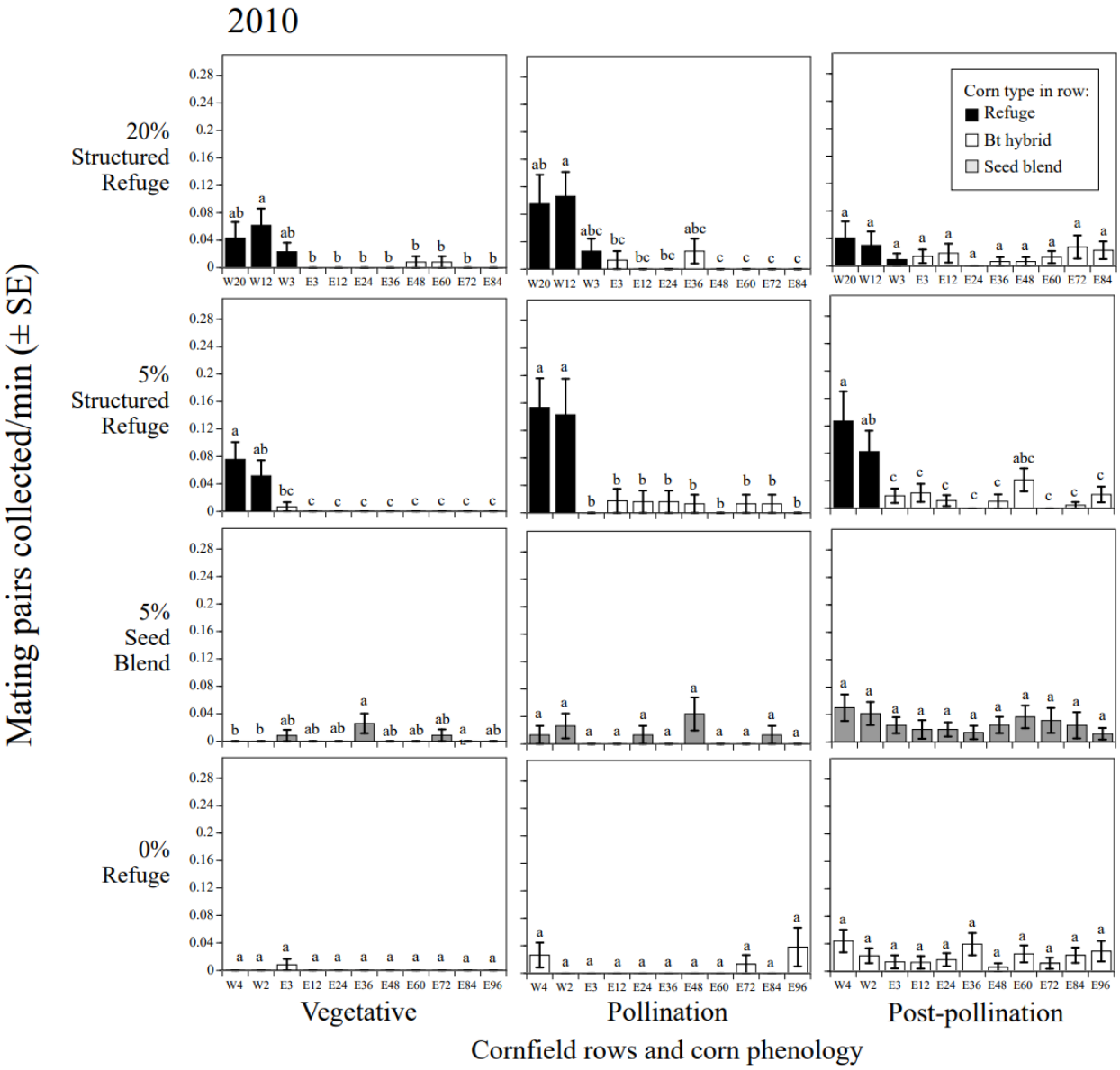

Figure 2.3. The mean number of mating pairs collected per minute (mating pairs / min ± SE) in each sampling row of each refuge treatment and period of corn phenology in 2010. ANOVA was performed on log (x + 0.5) transformed means and the Tukey-Kramer of multiple comparisons were performed to determine differences in mating pair abundance among sampling rows. Bars bearing the same letter are not significantly different ($\alpha = 0.05$). Untransformed means are shown. Corn phenology was determined based on Ritchie et al. 1993 and Abendroth et al. 2011. See Hughson and Spencer (2015) for row labeling details.

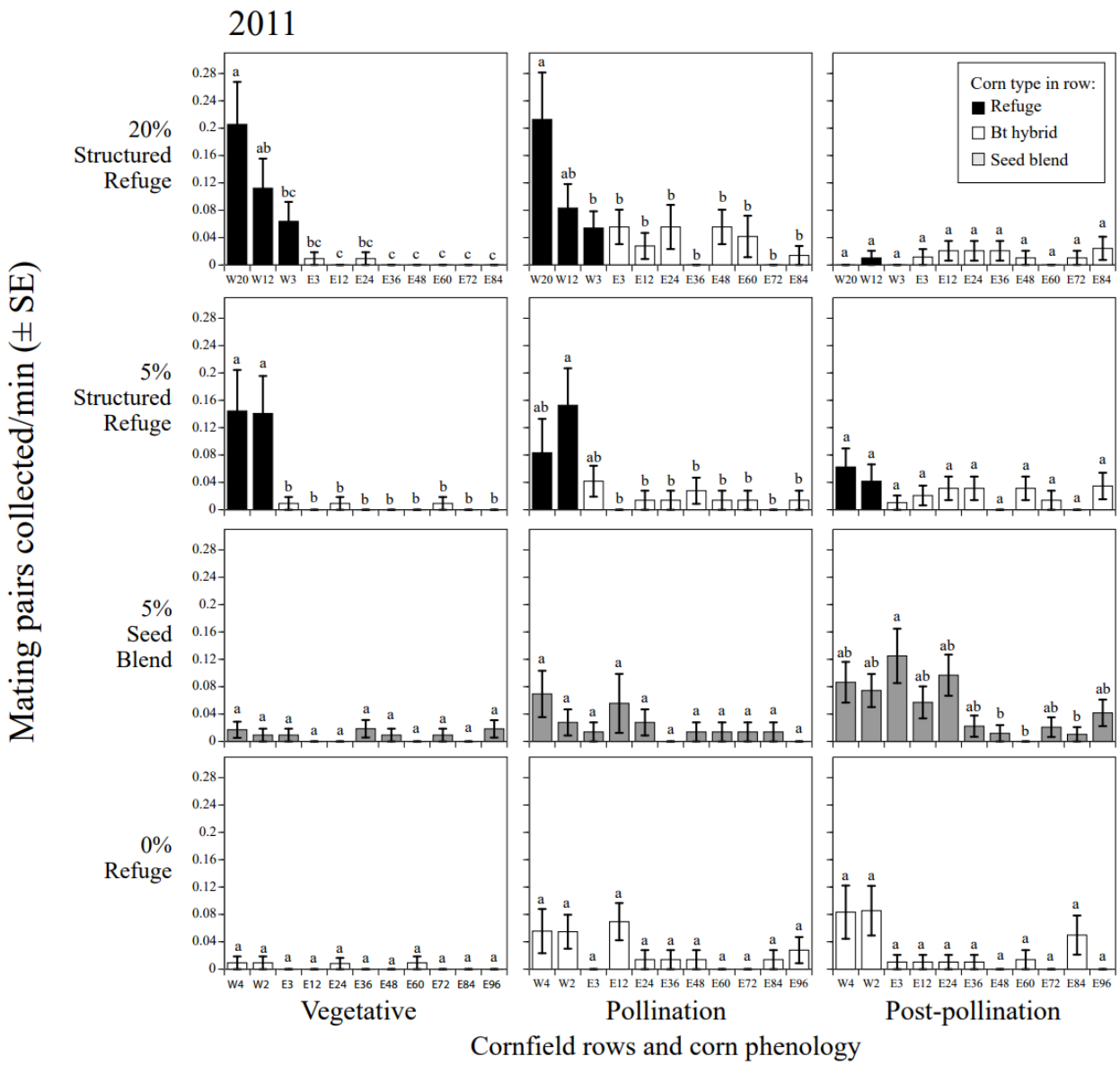

Figure 2.4. The mean number of mating pairs collected per minute (mating pairs / min ± SE) in each sampling row of each refuge treatment and period of corn phenology in 2011. ANOVA was performed on log (x + 0.5) transformed means and the Tukey-Kramer of multiple comparisons were performed to determine differences in mating pair abundance among sampling rows. Bars bearing the same letter are not significantly different ($\alpha = 0.05$). Untransformed means are shown. Corn phenology was determined based on Ritchie et al. 1993 and Abendroth et al. 2011. See Hughson and Spencer (2015) for row labeling details.

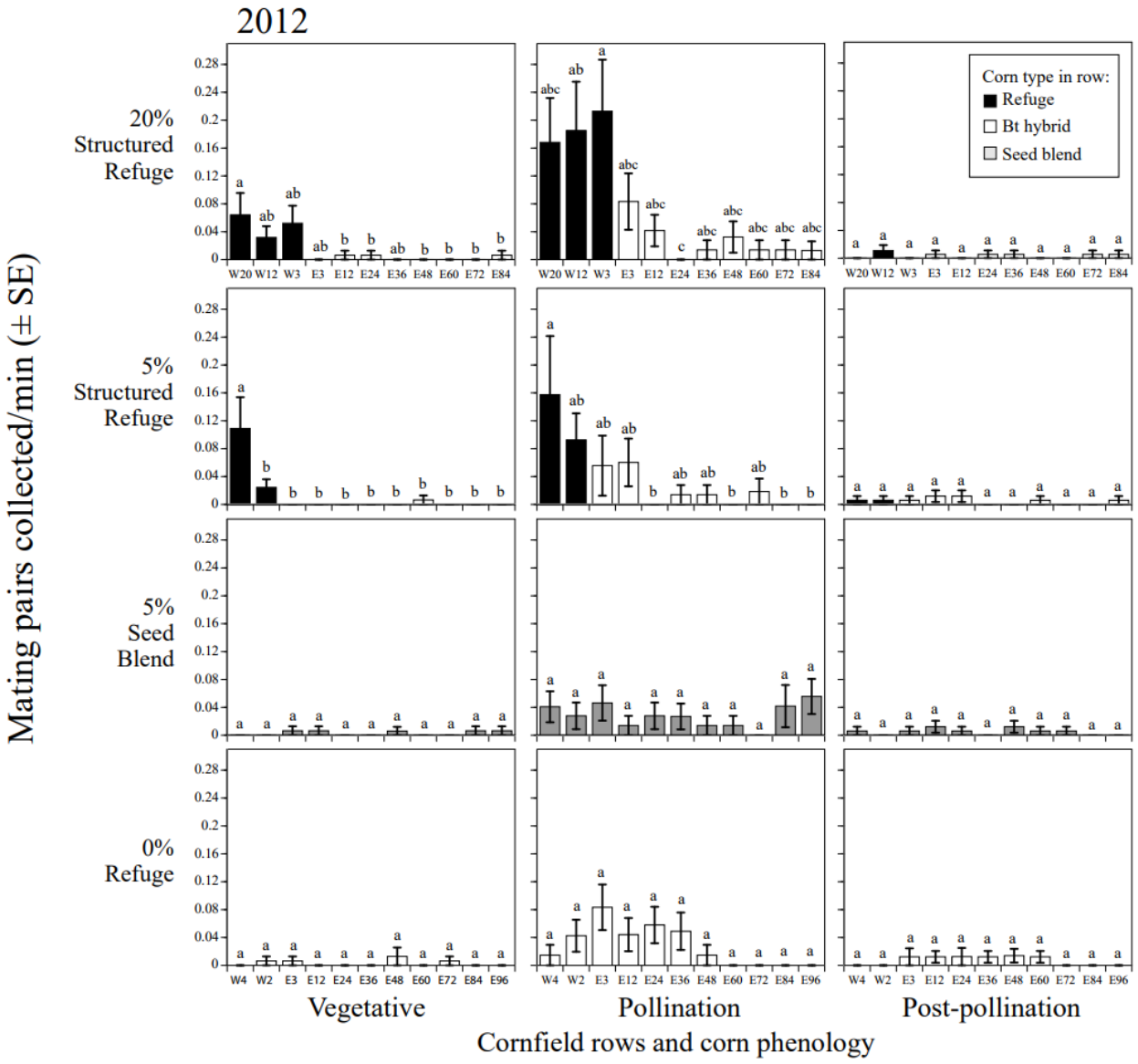

Figure 2.5. The mean number of mating pairs collected per minute (mating pairs / min ± SE) in each sampling row of each refuge treatment and period of corn phenology in 2012. ANOVA was performed on log (x + 0.5) transformed means and the Tukey-Kramer of multiple comparisons were performed to determine differences in mating pair abundance among sampling rows. Bars bearing the same letter are not significantly different ($\alpha = 0.05$). Untransformed means are shown. Corn phenology was determined based on Ritchie et al. 1993 and Abendroth et al. 2011. See Hughson and Spencer (2015) for row labeling details.

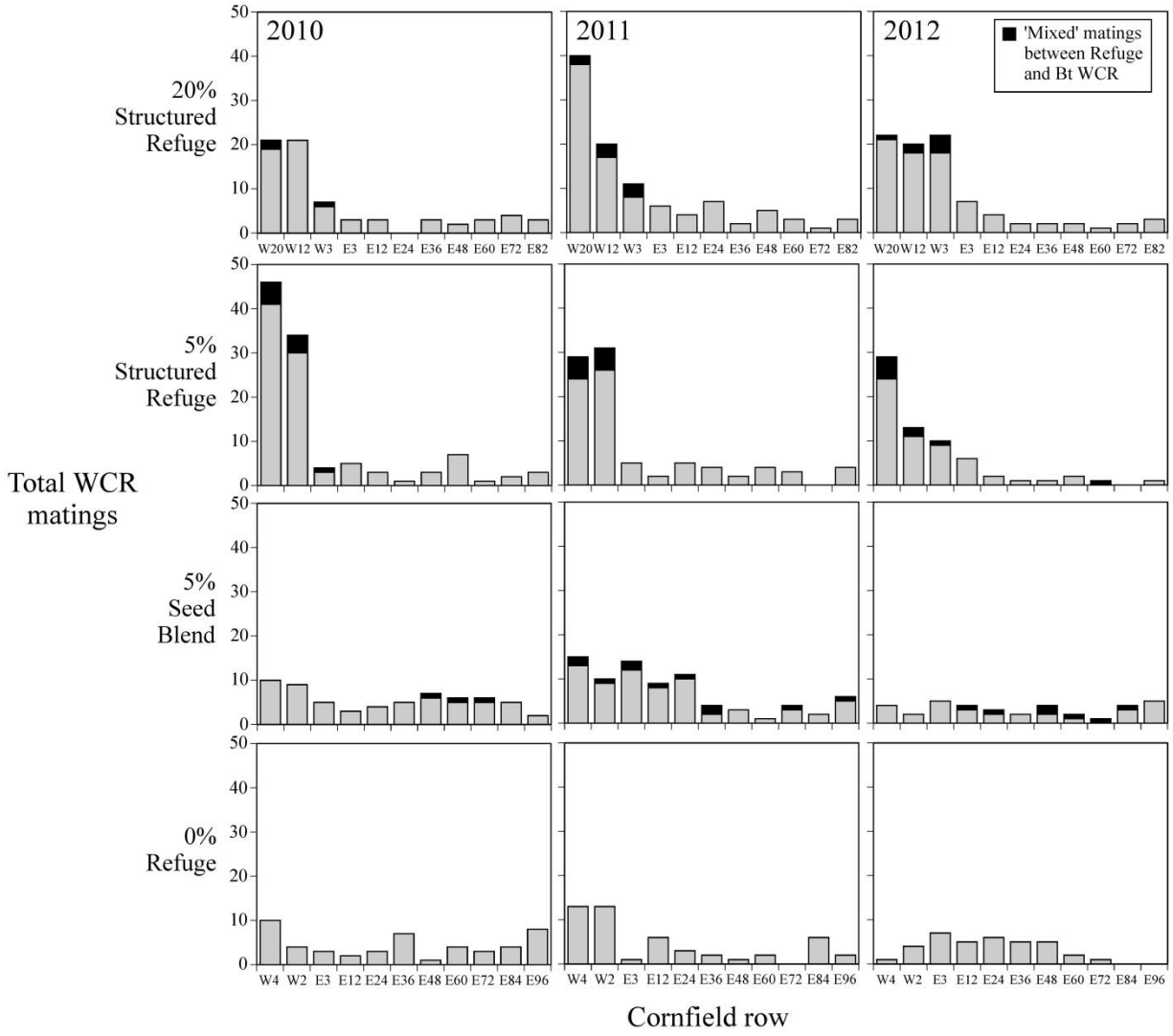

Figure 2.6. Total number of mating pairs and mixed-matings in each sampling row across each refuge treatment from 2010 through 2012. Height of bars represents the total number of mating pairs collected in each sampling row, refuge treatment and year combination. In each bar, the black portion of the bar represents the total number of mixed-matings, while the gray portion represents the total number of non-mixed-matings. These were not analyzed because too few mixed-matings were collected to compare among the sampling rows. See Hughson and Spencer (2015) for row labeling details.

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CHAPTER 3: INTRAFIELD MOVEMENT OF WESTERN CORN ROOTWORMS (COLEOPTERA: CHRYSOMELIDAE) IN BT CORNFIELDS WITH STRUCTURED AND SEED BLEND REFUGES

INTRODUCTION

Western corn rootworm (*Diabrotica virgifera virgifera* LeConte) is one of the most economically important pests of corn in North America (Gray et al. 2009) and Europe (Baca 1994, Kiss et al. 2005). In the United States and Canada, western corn rootworms are controlled using Bt (*Bacillus thuringiensis* Berliner) corn hybrids, engineered to express Cry proteins that are toxic to young root-feeding larvae (Binning et al. 2010). This pest has a long history of evolving resistance to previous chemical (Bigger 1963, Metcalf 1983, Spencer et al. 2011) and cultural control methods (Levine et al. 2002). Just as it had for the 1996 commercialization of Bt corn hybrids targeting European corn borer (*Ostrinia nubilalis* Hubner, Lepidoptera: Crambidae), the Environmental Protection Agency (US EPA) also mandated an insect resistance management (IRM) plan for rootworm resistant Bt corn hybrids. All growers of rootworm resistant Bt corn were required to plant a non-Bt refuge within or adjacent to all Bt cornfields (US EPA OPP 2001). The refuge provides a location where Bt susceptible rootworm larvae can develop without exposure to Bt proteins. The susceptible beetles emerging from refuge will mate and pass susceptible alleles to the following generation. Refuges have been deployed in a variety of sizes and configurations including structured refuges consisting of 20% or 5% (depending on whether there was expression of one or more rootworm active traits in the Bt hybrid) blocks of refuge planted adjacent to, or strips planted at regular intervals within, Bt cornfields. As adoption of Bt hybrids increased and the deployment of refuges became more complicated, refuge compliance began to decline (Jaffe 2009) because growers may view the

refuge as an area of the field that does not receive the yield benefits of Bt hybrids (Hurley and Mitchell 2011). Concerns about farmer compliance were addressed by approval of seed blend refuges where refuge and Bt seeds are pre-mixed in bags of seed and refuge plants become randomly distributed across Bt cornfields at planting (US EPA OPP BPPD 2010).

The theoretical basis for the successful application of the refuge strategy to delay resistance is predicated on the fulfillment of four major assumptions: 1) resistance alleles are initially rare in the pest population, 2) resistance alleles are inherited recessively, 3) the Bt toxin are expressed in plant tissues at a high-dose and 4) the movement of mate-seeking adults leads to a broad distribution of beetles and facilitates mating between refuge and Bt corn individuals, that will produce heterozygous susceptible offspring (Tabashnik 1994, Gould 1998).

While these assumptions were well-suited for Bt hybrids targeting a highly mobile lepidopteran species such as the European corn borer, not all of the assumptions are met for Bt traits targeting corn rootworms. Resistance allele frequencies in rootworm populations are estimated at 0.2, which is 20 to 200 times greater than that estimated for other pests and 2000 times greater than is standard in successful IRM modelling (Onstad and Meinke 2010). When western corn rootworm resistance was selected for in the laboratory, its inheritance was found to be non-recessive, dominant or polygenic rather than recessive (Meihls et al. 2008, Petzold-Maxwell 2012, Thompson 2014). Ingber and Gassmann (2015) reported that Cry3Bb1 resistance in a field western corn rootworm population (Hopkinton) was non-recessive. A high-dose Bt trait is defined as having a susceptible beetle survival rate of < 0.01% of the population (US EPA 1998). However, mortality studies carried out by Clark et al. (2012) and Meihls et al. (2008) determined that 1.51 to 3.79% of western corn rootworms survived exposure to Bt proteins, indicating that Bt traits targeting corn rootworms are not high-dose. In addition, plant-based

resistance bioassays using beetles from field populations revealed that resistance was present in multiple populations (Gassmann et al. 2011, 2014; Gray 2012, 2013; Wangila et al. 2015, Schrader et al. 2016, Zukoff et al. 2016, Ludwick et al. 2017). This is not surprising; laboratory studies have demonstrated that Bt resistance can be selected in the laboratory for all commercialized Bt traits in as few as three generations (Meihls et al. 2011, 2012; Oswald et al. 2011). While some studies have shown that mating is non-random (Taylor et al. 2016, Chapter 2 of this dissertation). the assumption of broad movement across Bt cornfields has not been evaluated on a field-scale. This study is focused on evaluating the accuracy of western corn rootworm movement assumptions by measuring patterns of beetle movement within Bt cornfields with different refuge configurations.

Western corn rootworms engage in two types of movement, long-distance migratory flight and short-distance flight within fields or between nearby fields. During long-distance flight, western corn rootworms may be carried tens of kilometers by storms (Grant and Seevers 1989) and females have been recorded in tethered flight for an equivalent of 24 km in a single flight or 39 km in a series of flights during a single night (Coats et al. 1986). Substantive long-distance dispersal capabilities have been invoked to explain the rootworm's ability to spread to new locations (Onstad et al. 1999). Rootworms are also capable of movement among neighboring corn and soybean fields (Isard et al. 2000, Levine et al. 2002; O'Neal et al. 1999, 2004). Certainly, these insects have the capacity to disperse readily across a single cornfield but different types of movement may have unique motivations or occur at different times in the insect's life. The ability to disperse long distances may not reflect the actual likelihood of flight or the rate of travel achieved by beetles moving within Bt cornfields.

Intrafield movement is associated with mate-seeking and reproductive behavior in western corn rootworm adults and is largely unexplored on a field scale with some notable exceptions (Coats et al. 1986; Naranjo 1990a, b; Stebbing et al. 2005, Marquardt and Krupke 2009). Because male intrafield movement is mate-seeking, it is important to evaluate the ecology and behavior that occur prior to mating, such as emergence and mate attraction. Males emerge from the soil on average 5.2 to 6.0 days before females (Branson 1987, Hughson and Spencer 2015) and may require five to seven days to reach sexual maturity (Guss 1976). Females are sexually mature upon emergence (Hammack 1995) and begin to release sex pheromone shortly thereafter (Ball 1957, Hill 1975). Males are attracted to the pheromone (Ball and Chaudhury 1973, Guss 1976, Marquardt and Krupke 2009) and engage in mate-seeking flight, orienting themselves toward calling females, which usually are mated within 12 to 24 hours of emergence (Quiring and Timmins 1990). Females usually do not disperse prior to mating (Ball 1957, Cates 1968, Lew and Ball 1979) although their movement prior to mating has been recorded (Marquardt and Krupke 2009). Males were thought to move readily in the field while mate-seeking, but they were less likely to engage in sustained flight in the field (Isard et al. 2004) and there was no difference in the number or duration of flights in tethered flight mills between the sexes (Naranjo 1990b, Coats et al. 1986). No research has explored the proportions of rootworm populations that move within cornfields, the timing of movement or the rate of intrafield movement.

The field research presented here, examines the distributions of moving beetles in Bt cornfields, proportions of beetles that move between refuge and Bt corn within cornfields and estimates the daily movement rates of beetles collected in Bt cornfields with different refuge percentages and configurations. Based on the heterogeneous distributions of beetle abundance

(Hughson and Spencer 2015) and mating activity (Chapter 2 of this dissertation) recorded in structured refuge cornfields and the uniform distributions recorded in seed blends, I expect to find greater proportions of moving beetles and uniform distributions of moving beetles across fields with seed blend refuges. Developing a better understanding of western corn rootworm activity in Bt cornfields is necessary to assess whether their ecology and intrafield movement matched the widely-held literature-based assumptions about their behavior.

MATERIALS AND METHODS

Location and treatments

The field study was carried out on the University of Illinois' "Shaw Farm" in rural northeast Urbana, IL (Champaign County; 40° 09' N, 88° 08' W) from 2010 to 2012. This location was planted with twelve 0.53 ha plots separated by 8 m alleyways. Each plot was randomly assigned one of four refuge treatments as a completely randomized design with three replicates, treatment locations were randomized each year (see Hughson and Spencer 2015 for additional planting details).

The refuge treatments consisted of a 20% structured refuge (22 rows of refuge corn and 88 rows of Bt corn), a 5.2% structured refuge (6 rows of refuge corn and 109 rows of Bt corn), 5.2% seed blend refuge, and 0% refuge treatment. The 5.2% refuges will, hereafter, be referred to as 5% refuge treatments for simplicity. In structured refuge treatments, the refuge rows were always located on the west side of the plots. The 0% refuge treatment served as a Bt positive control. The 5% seed blend treatment was prepared by weighing out and thoroughly mixing the appropriate amounts of refuge and Bt seed (Hughson and Spencer 2015). These refuge

percentages and configurations were chosen based on those available to commercial growers during 2010 (US EPA OPP BPPD 2010), when this experiment began.

The Bt transgenic portion of each treatment was planted with a Herculex® XTRA hybrid (Pioneer Hi-Bred International, Inc., Johnston, IA; 2010: Hybrid 34B41, RM 109 days; 2011 and 2012: Hybrid 33W84, RM 111 days) which expressed the Cry1F and Cry34/35Ab1 Bt proteins that targeted lepidopteran larvae and corn rootworm larvae, respectively. The rootworm refuge portion of each field was planted with YieldGard® Corn Borer (Monsanto Company, St. Louis, MO; 2010: Hybrid 34B94, RM 110 days; 2011 and 2012: Hybrid 33B54, RM 113 days) that expressed Bt Cry1Ab targeting lepidopteran larvae. The commercial cornfields adjacent to the study location were planted with a variety of YieldGard® Rootworm hybrids (Monsanto Company, St. Louis, MO) expressing the Cry1Ab and Cry3Bb1 proteins that targeted lepidopteran larvae and corn rootworm larvae, respectively (Hughson and Spencer 2015). The stages of corn phenology (Ritchie et al. 1993, Abendroth et al. 2011) were recorded throughout the study as vegetative, pollination and post-pollination each year (Hughson and Spencer 2015). Plant maturity and phenology were estimated based on the Bt corn plants alone because they represented the vast majority (80 to 100%) of the plants per plot (Hughson and Spencer 2015).

Adult field collections

Western corn rootworms were collected from 11 sampling rows in each plot (see Hughson and Spencer 2015 for details about row labeling). Adult field collections were carried out in each sampling row and plot three to five times per week, beginning when third instar larvae and pupae were first detected in cornfield soil at the field site. Collections occurred between 8:30 and 11:00 a.m., when adult activity and mating were high (Bartelt and Chiang

1977, Dobson and Teal 1986) and newly emerged beetles were still teneral (Cates 1968). Each collection was timed for two to four minutes during which single beetles and mating pairs were hand collected from corn foliage into collection jars with dry ice, where they were killed by the CO_2 liberated from the ice or frozen in contact with the ice (as described by Hughson and Spencer 2015, Chapter 2 of this dissertation). The samples from each row were stored in a cooler with dry ice and returned to the laboratory, where they were stored in a freezer (-20º C) until they could be processed. At the time of collection, date, collection time, collection duration, refuge treatment and sampling row identification code were recorded for each sample. Sampling row location and treatment were later used to identify the corn type (refuge or Bt corn) available in the sampling row where each beetle was recovered and to determine the distance between each beetle's collection location and the nearest interface between refuge and Bt corn.

Adult gut protein identification of beetle distributions

Beetle movement between the refuge and Bt portions of a plot was confirmed by identifying the Cry proteins in beetle gut contents and determining whether they differed from the refuge or Bt corn specific Cry proteins expressed by the plants in the sampling row where the beetle was collected (Spencer et al. 2003, Chapter 2 of this dissertation). After corn tissue had been ingested, the Cry proteins expressed by the plants could be detected in a beetle's gut contents using EnviroLogix™ QuickStix™ ELISA test strips (EnviroLogix Inc., Portland, ME). Spencer et al. (2003) determined that western corn rootworm beetles actively fed on corn tissue 30 to 65% of the time they were on corn plants. Thus beetles continually marked themselves and replenished the Cry protein markers in their guts through normal feeding activity (Spencer et al. 2003, Chapter 2 of this dissertation). The presence of Cry1Ab proteins, expressed exclusively by

refuge plants, or Cry1F proteins, expressed exclusively by Bt plants targeting corn rootworms, in beetle guts were used to identify where individual beetles had been feeding in each plot prior to capture. By testing beetles for the presence of Cry proteins at regular intervals after feeding, Hughson (Chapter 2 of this dissertation) identified that Cry1Ab was detectable in the insects' guts up to 12 hours after feeding and Cry1F could be detected up to 48 hours after feeding. Beetles collected in Bt corn that were later found to have refuge-specific Cry proteins in their guts must have recently moved from refuge to the Bt portion of a field or vice versa.

Identification of the specific Cry protein(s) in beetle gut contents was accomplished using EnviroLogix™ QuickStix™ test strips as described in (Spencer et al. 2003, Chapter 2 of this dissertation) at a cost of US $0.90 for each QuickStix™ test strip. Female beetles were tested for Cry1Ab and Cry1F while males were tested for Cry1Ab, Cry1F and a third Cry protein, Cry3Bb1. A positive test for Cry1Ab indicated that a beetle had recently fed on refuge corn, whereas a positive test for Cry1F indicated a beetle had recently fed on Bt corn. Because corn in all of the nearby commercial cornfields expressed the Cry3Bb1 protein, which was not present at the study site, the detection of Cry3Bb1 in the gut contents of males provided a way to detect interfield movement from an offsite cornfield. Males were tested for Cry3Bb1 in 2011 and 2012 (Chapter 2 of this dissertation).

The determination of beetle movement depended on knowing where an individual had been collected. The protein(s) detected in each beetle's gut contents was compared to the protein expressed by the plants in the row where it was collected to determine whether it had moved between corn types. If the Cry protein from corn tissue in the beetle's gut differed from that expressed by the corn at its collection location, that beetle was designated a "mover". The mean proportions of movers were estimated relative to the abundance in each row. Because an

assumption of the refuge strategy for corn rootworm was that males moved widely while mate-seeking (Ball 1957, Spencer et al. 2009) and females mated shortly after emergence before any significant movement takes place (Marquardt and Krupke 2009), only males were tested for the presence of Cry3Bb1 proteins that could reveal interfield movement.

Each day of collections generated 132 row samples containing 0 to > 50 beetles per sample. Because time and resource constraints prevented every beetle from being analyzed, a subset of those in a collection were processed to detect ingested Cry proteins each year. In 2010 and 2011, up to ten males and ten females from each row sample were processed. As a result of higher beetle populations and time limitations at the time of processing for Cry protein detection, in 2012, up to three male and three female beetles were processed from each row sample. Even with subsampling, a total of 23,099 beetles were processed and analyzed in this study, producing detailed datasets.

Distribution of Cry positive adults

When male and female beetles from 2010 through 2012 were tested for the presence of Cry1Ab and Cry1F, they were given a Cry positive or Cry negative score producing binary datasets. These datasets were used to calculate the mean proportion of Cry1Ab and Cry1F positive beetles in each row sample. The mean proportions were tested for homogeneity using the Shapiro-Wilk test and found to be non-normal. The mean proportions were then analyzed using the Kruskal-Wallis test to determine whether treatment and phenology were significant main effects (JMP Pro 10, SAS Institute Inc. 2012). The Steel-Dwass test of multiple comparisons was used to identify which phenologies and treatments had greater mean proportions of Cry positive beetles (JMP Pro 10, SAS Institute Inc. 2012). Pairwise multiple comparisons of the mean proportions of Cry1Ab and Cry1F positive beetles were also carried out

using the Marascuilo procedure (R version 3.2.1, R Core Team 2015) among sampling rows in each treatment and phenology.

Interfield movement

Male beetles from 2011 through 2012 were also tested for the presence of Cry3Bb1 and given a Cry positive or Cry negative score producing a binary dataset. The mean proportion of Cry3Bb1 positive males was calculated. The Shapiro-Wilk, Kruskal-Wallis and Steel-Dwass analyses were also carried out for the mean proportion of Cry3Bb1 positive males and used to evaluate interfield movement by phenology and refuge treatment.

The proportions of intrafield movers

Beetles in structured refuge treatments that tested positive for a Cry protein that was not expressed by the corn plants in the row where they were collected, were designated as "movers." The proportions of movers were calculated for structured refuge treatments, representing the proportion of all beetles collected in a treatment that moved across the refuge-to-Bt interface within the 12 (Cry1Ab) to 48 hours (Cry1F) each Cry protein could be detected in their bodies (Chapter 2 of this dissertation). The proportions of movers were tested for homogeneity using the Shapiro-Wilk test (JMP Pro 10, SAS Institute Inc. 2012). The proportions were non-normal each year and analyzed using the Kruskal-Wallis test (JMP Pro 10, SAS Institute Inc. 2012) for main effects of phenology and treatment. The Steel-Dwass test of multiple comparisons was used to determine which levels of phenology and treatment had the greatest proportions of movers (JMP Pro 10, SAS Institute Inc. 2012).

When beetles tested positive for both Cry1Ab and Cry1F, they were designated as "double positive". In structured refuge treatments, these beetles must have moved between and recently fed in both refuge and Bt corn areas. They could have moved across a wide distance or

across the 0.76 m wide interface between the refuge and Bt areas of each plot. In the 5% seed blend, where there was no identifiable refuge-Bt interface, beetles could become double positives by moving very little and feeding on adjacent refuge and Bt plants. These double positive beetles were used to investigate short distance plant-to-plant movement. The proportions of double positive beetles per sampling row were also analyzed using the Kruskal-Wallis test and Steel-Dwass test of multiple comparisons (JMP Pro 10, SAS Institute Inc. 2012) to determine whether the proportion of double positive beetles differed among refuge treatments.

Estimations of daily movement rates

Intrafield movement rates in the structured refuge treatments were reckoned relative to the interface between refuge and Bt corn. Because the seed blend refuge treatment and the 0% refuge treatment lacked a defined refuge-to-Bt corn interface, movement rates could not be calculated for those treatments. To estimate the minimum daily movement rate among movers collected at various distances from the nearest sources of Cry protein, both their distance traveled and the longest post-ingestion time interval during which beetles could test Cry positive were identified (Chapter 2 of this dissertation). The distance each beetle had traveled (in meters) was calculated by multiplying the number of rows between the location where the individual was collected and the nearest source of that Cry protein detected in that beetle's gut by the 0.76 m row width. Hughson (Chapter 2 of this dissertation) identified that Cry1Ab (refuge corn) could be detected for up to 12 hours after feeding and Cry1F (Bt corn) could be detected for up to 48 hours after feeding. Minimum daily movement rates for each mover were calculated by dividing the distance the individual traveled (m) by the post-ingestion detection interval (in hours). Movement rate estimations were calculated for beetles that traveled from refuge to Bt corn but

were not calculated for beetles traveling from Bt corn into refuge corn because 5% and 20% structured refuge treatments only had 6 and 24 rows of refuge corn, respectively. Calculating movement rates over these short distances that many beetles could easily traverse would significantly underestimate their movement rates.

The daily movement rates were tested for normality using the Shapiro-Wilk test and log (x + 0.5) transformed. The transformed movement rates were analyzed to determine whether phenology, treatment and beetle sex influence the rate of travel, using ANOVA and the Tukey-Kramer test of multiple comparisons (JMP Pro 10, SAS Institute Inc. 2012).

RESULTS

Distribution of Cry positive adults

The proportions of beetles positive for Cry1Ab (refuge corn) were greatest in 20% structured refuge treatments and lowest in 0% structured refuge treatments each year; in 2011 and 2012, there was no difference between the 5% structured refuge and 5% seed blend treatments, while in 2010 the proportion of Cry1Ab positive beetles was greater in 5% structured refuge than 5% seed blend treatments (2010: $\chi^2 = 374.03$, df = 3, $P < 0.0001$; 2011: $\chi^2 = 330.82$, df = 3, $P < 0.0001$; 2012: $\chi^2 = 326.55$, df = 3, $P < 0.0001$). The proportions of Cry1Ab positive beetles were greater during the vegetative period than the pollination period and lowest during the post-pollination period each year (2010: $\chi^2 = 328.19$, df = 2, $P < 0.0001$; 2011: $\chi^2 = 203.83$, df = 2, $P < 0.0001$; 2012: $\chi^2 = 222.77$, df = 2, $P < 0.0001$). Row-by-row multiple comparisons revealed that, in structured refuge treatments, the proportions of beetles testing positive for Cry1Ab were greatest in refuge rows throughout the season (Figures 3.1 through 3.3). Cry1Ab positive beetles were collected in some Bt rows during the vegetative period of corn phenology,

but the proportion of beetles testing positive for refuge corn diminished as the distance between each sampling row and the refuge increased (Figures 3.1 through 3.3). As corn phenology progressed, the proportions of Cry1Ab positive beetles collected in Bt rows diminished (Figures 3.1 through 3.3).

The proportions of beetles positive for Cry1F (Bt corn) were greatest in 0% structured refuge treatments and lowest in 20% structured refuge treatments each year (2010: $\chi^2 = 275.44$, df = 3, $P < 0.0001$; 2011: $\chi^2 = 322.54$, df = 3, $P < 0.0001$; 2012: $\chi^2 = 334.31$, df = 3, $P < 0.0001$). In 2010 and 2011, the proportions of Cry1F positive beetles were greater during the pollination period than the post-pollination period and lowest during the vegetative period each year (2010: $\chi^2 = 111.88$, df = 2, $P < 0.0001$; 2011: $\chi^2 = 171.91$, df = 2, $P < 0.0001$). In 2012, there were no differences in the proportions of Cry1F positive beetles during the pollination and post-pollination periods, but they were significantly lower during the vegetative period (2012: $\chi^2 = 222.77$, df = 2, $P < 0.0001$). Row-by-row multiple comparisons revealed that the proportions of beetles positive for Cry1F were greatest in Bt rows and did not differ among the rows throughout the pollination and post-pollination periods. During the vegetative period, the proportions of beetles positive for Cry1F were also greater in Bt rows although there was some variation among Bt rows with low beetle abundance (Figures 3.1 through 3.3).

In 5% seed blend treatments, low proportions of Cry1Ab positive beetles were collected in nearly every corn row throughout the season; those proportions did not differ among the rows (Figures 3.1 through 3.3). Few Cry1Ab positive beetles were collected in any 0% refuge treatment sampling rows; most were collected during the vegetative period (Figures 3.1 through 3.3). The proportions of Cry1Ab positive beetles in 0% refuge treatments diminished during the pollination and post-pollination periods (Figures 3.1 through 3.3). The proportions of Cry1F

positive beetles collected in the 5% seed blend and 0% refuge treatments did not differ among sampling rows and remained high across the phenology periods (Figures 3.1 through 3.3). As with structured refuge treatments, the row-to-row variability in the proportion of Cry1F positive beetles in 5% seed blend and 0% refuge was greatest during the vegetative period.

The proportions of intrafield movers

Each year, there was no difference in the mean proportion of beetles that were "movers" between refuge and Bt corn in 20% and 5% structured refuge treatments (2010: $\chi^2 = 0.72$, df = 1, $P = 0.3950$; 2011: $\chi^2 = 0.25$, df = 1, $P = 0.6136$; 2012: $\chi^2 = 0.10$, df = 1, $P = 0.7477$; Table 3.1). However, significantly more movers were identified during the vegetative period than during pollination or post-pollination (2010: $\chi^2 = 92.38$, df = 2, $P < 0.0001$; 2011: $\chi^2 = 171.23$, df = 2, $P < 0.0001$; 2012: $\chi^2 = 52.83$, df = 2, $P < 0.0001$; Table 3.2).

The direction of travel was also considered. With respect to movement from refuge to Bt corn in structured refuges, the mean proportion of movers was greater in 20% structured refuges than 5% structured refuges in 2010 and 2011 (2010: $\chi^2 = 7.10$, df = 1, $P = 0.0077$; 2011: $\chi^2 = 4.34$, df = 1, $P = 0.0372$; Table 3.1) but there was no difference in the proportion of movers between those treatments in 2012. A greater proportion of beetles also moved from refuge to Bt corn during the vegetative period when compared to the pollination and post-pollination periods (2010: $\chi^2 = 118.50$, df = 2, $P < 0.0001$; 2011: $\chi^2 = 108.89$, df = 2, $P < 0.0001$; 2012: $\chi^2 = 56.63$, df = 2, $P < 0.0001$; Table 3.2).

Regarding beetles moving from Bt corn into the refuge, the mean proportion traveling from Bt corn to refuge corn was greater in the 5% structured refuge than the 20% structured refuge each year (2010: $\chi^2 = 4.19$, df = 1, $P = 0.0406$; 2011: $\chi^2 = 20.24$, df = 1, $P < 0.0001$; 2012:

χ^2 = 17.44, df = 1, P < 0.0001; Table 3.1) and there was no impact of corn phenology on these proportions (Table 3.2).

The mean proportion of double positive beetles (those testing positive for both refuge and Bt corn Cry proteins) was influenced by refuge treatment each year (2010: χ^2 = 45.62, df = 3, P < 0.0001; 2011: χ^2 = 166.44, df = 3, P < 0.0001; 2012: χ^2 = 201.30, df = 3, P < 0.0001). In 2010, there was no difference in the mean proportion of double positives among the 20% structured, 5% structured or 5% seed blend refuge treatments and each was greater than the proportion in the 0% refuge treatments (Table 3.3). In 2011 and 2012, the 5% seed blend treatments had the greatest mean proportion of double positive beetles (Table 3.3). The 20% structured and 5% structured refuge treatments did not differ and the 0% refuges had the lowest proportion of double positive beetles (Table 3.3).

Estimations of daily movement rates

When beetles moved from refuge to Bt corn, they traveled at 26.23 ± 3.09 m/d in 2010, 30.31 ± 2.34 m/d in 2011 and 31.22 ± 2.43 m/d in 2012. Rate of movement did not differ between 20% and 5% structured refuge treatments, except in 2010, when the movement rate for the 20% structured refuge treatments, 32.28 ± 4.46 m/d, was greater than that for 5% structured refuge treatments, 19.43 ± 4.10 m/d (F = 4.43, df = 1, 135, P = 0.04). During 2011, movement rates in 20% and 5% structured refuge treatments were 29.60 ± 3.00 m/d and 31.30 ± 3.74 m/d, respectively. During 2012, movement rates in 20% and 5% structured refuge treatments were 30.38 ± 2.81 m/d and 32.62 ± 4.50 m/d, respectively. Corn phenology did not affect the rate of travel in 2010 but was a significant factor in 2011 (F = 5.90, df = 2, P = 0.0032) and 2012 (F = 4.16, df = 2, P = 0.0170). In 2010, beetles traveled 28.35 ± 4.28 m/d during the vegetative

period, 25.07 ± 6.52 m/d during the pollination period and 21.91 ± 6.07 m/d during post-pollination. In 2011, the rate of travel was greatest during the pollination period when they moved 50.33 ± 7.17 m/d compared to the vegetative and post-pollination periods when beetles moved 24.58 ± 2.49 m/d and 28.93 ± 4.78 m/d, respectively. In 2012, beetles traveled at the greatest rate during the post-pollination period at 42.24 ± 5.77 m/d and at the lowest rate during the vegetative period at 24.76 ± 3.15 m/d. During the pollination period, beetles traveled 32.32 ± 4.36 m/d, which did not differ from either the vegetative or post-pollination periods.

Interfield movement

The mean proportion of males collected in sampling rows that originated from an offsite location (Cry3Bb1 positive males) was annually influenced by the phenology (2011: $\chi^2 = 32.41$, df = 2, $P < 0.0001$; 2012: $\chi^2 = 63.72$, df = 2, $P < 0.0001$). The greatest proportion of Cry3Bb1 positive males was collected during the vegetative period while the Cry3Bb1 positive proportions collected in the pollination and post-pollination periods did not differ (Table 3.2).

DISCUSSION

Cry detection and beetle movement

Based on detection of ingested Cry proteins specific to refuge and Bt portions of refuge treatments, the proportions of beetles that moved between refuge and Bt corn changed dramatically during the growing season. The greatest proportions of beetles moved between refuge and Bt corn during the vegetative period of corn phenology; significantly lower proportions moved during the pollination and post-pollination periods. During the vegetative period in structured refuge treatments, refuge (Cry1Ab) positive beetles were concentrated in

sampling rows within and immediately adjacent to refuge rows with few detected in Bt rows; fewer refuge positive beetles were detected in Bt rows during the pollination and post-pollination periods. Likewise, Bt (Cry1F) positive beetles were concentrated in Bt rows and proportions of Bt positive beetles collected in refuge corn decreased as the season progressed (Figures 3.1 through 3.3). This pattern was observed again in the mean proportions of beetles that crossed the refuge-Bt interface in structured refuge treatments (movers). Movers represented a greater proportion of the population during the vegetative period, 17 to 25% of the beetles collected, and dropped to 3 to 10% during pollination and post-pollination periods. These spatial and temporal distributions of Cry positive beetles were very similar to the distributions of free-moving beetles (Hughson and Spencer 2015) and mating activity (Chapter 2 of this dissertation) collected in the same fields in which most mating activity occurred during the vegetative period. These pattern suggest that a majority of the beetle movement occurs early in the season with much less movement occurring later in the season. Even when beetle movement is at its peak, only up to a quarter of the beetles moved between refuge and Bt corn on a daily basis.

Movement rates

Moving western corn rootworm adults traveled at an average rate of 26 to 31 m/day. This rate is close to the range of maximum intrafield movement rates (18.3 to 36.6 m/day) estimated by Spencer et al. (2003), but larger than a later average intrafield movement rate estimate of 6 to 17 m/day (Spencer et al. 2009). While this means that a beetle could travel nearly one third the length of the 0.53 ha fields used in this experiment within a day, in the context of Illinois farms, which averaged 145 ha in 2014 (Illinois Dept. of Agriculture 2014), beetles travel a relatively short distance each day. Considering that only 17 to 25% of the beetles

moved at this rate daily during the vegetative period, and many fewer did so during the pollination and post-pollination periods, only small proportions of the population are moving at even modest rates. This finding makes it apparent that many western corn rootworms do not readily distribute themselves across these research fields because movement behavior has strong seasonality. The lack of significant movement is also evident in the adult abundance distributions for these same fields (Chapter 2 of this dissertation, Figures 2.2, 2.3, and 2.4). Based on beetle abundance patterns over the season (Hughson and Spencer 2015) and these movement patterns, it is clear that many beetles did not cross the refuge-to-Bt interface in structured refuge treatments. It is difficult to imagine that beetles would travel the distance required to cross the refuge-Bt interface to encounter a mate in a commercial cornfield more than 250 times larger. With low intrafield movement rates, the distance between refuge and Bt portions of commercial cornfields with structured refuges would prevent many refuge and Bt origin beetles from encountering one another. Given the limited intrafield movement and mixing of refuge and Bt beetles on a small scale, an important assumption of the high dose refuge strategy, it is not surprising that field-evolved resistance has been documented across a broad geographic range (Gassmann et al. 2011, 2014; Gray 2012, 2013; Wangila et al. 2015, Schrader et al. 2016, Zukoff et al. 2016, Ludwick et al. 2017).

Despite evidence that the highest proportions of beetles moved early in the season, the greatest movement rates among movers were observed among the low proportions of beetles later in the season. This movement may occur when late emerging beetles from Bt corn (Murphy et al. 2010, Hughson and Spencer 2015) are seeking mates in areas with a low abundance of potential mates and may travel much farther than an early season beetles before encountering a mate (Spencer et al. 2012). Some beetles may also disperse later in the season in

search of better quality food as local plants mature (O'Neal et al. 2002). After pollination, corn plants redirect energy and nutrients from vegetative growth of foliage and production of pollen to maturing ears (Ritchie et al. 1993, Abendroth et al. 2011); nitrogen content in foliage begins to decline as corn enters reproduction and nitrogen is moved into reproductive tissues such as kernels (Abendroth et al. 2011). At this time, the moisture content in foliage and preferred foods, such as pollen and silks (Ball 1957) also declines (Abendroth et al. 2011). Western corn rootworm females that fed on silks and pollen were more fecund than females that fed on other corn tissues like foliage, suggesting that synchrony between female emergence and pollination improves their fitness (Elliott et al.1990).

Given the significant investment males make while producing a spermatophore (Tallamy et al. 2000), they likely also require high-quality food to produce nutrient rich spermatophores (Quiring and Timmins 1990). Later emerging beetles may seek pollinating plants to benefit from these high-quality foods. Studies of western corn rootworm interfield dispersal into trap crops showed that flying beetles oriented themselves toward pollinating corn (Darnell et al. 2000, O'Neal et al. 2004). Similarly, in studies of interfield movement from corn to soybean fields, more beetles were recorded dispersing from cornfields during post-pollination (O'Neal et al. 2004, Pierce and Gray 2006). These studies support the idea that movement later in the season may be related to finding better quality food (Darnell et al. 2000, O'Neal et al. 2004). Although the above studies were intended to quantify aspects of interfield movement, intrafield movement may be similarly influenced by the changing nutritional quality of maturing corn since western corn rootworm adults are generally attracted to plant volatiles associated with corn pollination (Naranjo 1994).

Short-distance movement and seed blends

While structured refuges created marked heterogeneity of beetle distributions and dramatic changes in proportions of movers as the season progressed, seed blend refuges showed little variation in the proportions of Cry positive beetles across the fields and throughout the season (Figures 3.1 through 3.3) which may indicate that these populations are staying near their site of emergence or that there could be ongoing movement throughout the seed blend treatments. Identifying the proportions of beetles positive for both refuge and Bt Cry proteins (double positives) allowed for a comparison of short-distance plant-to-plant movement among structured and seed blend refuge treatments, revealing that more plant-to-plant movement was detected in the seed blend treatments than in the structured refuge treatments in 2011 and 2012. Assuming that refuge plants are randomly distributed in the 5% seed blends, the higher proportions of double positive beetles may indicate that the refuge plants are encountered with some greater than expected probability, or that a portion of beetles emerging from refuge plants may remain near their emergence site and encountered the same refuge plant more than once as they visited and fed upon adjacent Bt plants. The homogeneous distribution of refuge plants in seed blends, rather than the quantity of refuge plants, may have facilitated more detectable plant-to-plant movement because it maximized the amount of refuge-to-Bt interface in the field providing beetles with more opportunities to move between individual refuge and Bt expressing plants. Increased plant-to-plant movement in seed blends may increase the likelihood that beetles move between refuge and Bt plants over a short time scale, potentially resulting in more mixed-matings. However, Hughson (Chapter 2 of this dissertation) reported no difference in the proportion of mixed-matings collected in seed blends compared to structured refuge treatments of the same size. This result supports the idea that adults tend to remain near the plant from

which they emerged. Since adults do not incapable of distinguishing between refuge and Bt expressing plants (Spencer et al. 2003), they may be encouraged to linger near refuge plants by chemical cues from newly emerged calling females or because they are females attempting to attract a mate. This seeming tendency for beetles to remain near refuge plants may also explain why most mixed-matings identified by Hughson (Chapter 2 of this dissertation) were found in the refuge and included an individual that entered refuge corn from Bt corn prior to mating (Chapter 2 of this dissertation). These behaviors suggest that the scale at which mate-seeking movement is occurring is much smaller than many anticipated. These refuge strategies, even when seed blends are deployed, seem to be a poor fit with western corn rootworm ecology.

Interfield movement

Very small proportions of males were detected as interfield movers (Table 3.2). The proportions of males testing positive for Cry3Bb1 were greatest during the vegetative period suggesting that male interfield movement, like intrafield movement, occurred more frequently early in the season and dropped off during the pollination and post-pollination periods. Even during the vegetative periods when interfield movement was greatest, the proportions of interfield movers were lower than the proportions of intrafield movers (Table 3.2). However, the fact that both inter- and intrafield movement were greater during the vegetative period, may suggest that both types of movement are similarly motivated by either mate-seeking or food-seeking early in the season.

In 2012, the proportions of interfield movers were noticeably higher than in 2011, while proportions of intrafield movers were consistent between the years. This difference between interfield movers in 2011 and 2012, likely occurred because the adjacent commercial fields were

planted with corn in 2012 and soybeans in 2011. In 2012, the minimum distance these beetles would have to travel to enter the research site was an 8 m (the width of the alleyway between the fields), while interfield movers in 2011 would have had to cross the surrounding soybean fields to enter the research site, suggesting that interfield movers are more likely to be observed crossing short distances during a single day.

Conclusions

This research shows that western corn rootworm intrafield movement had strong seasonality, the proportions of movers detected in the field were limited and individuals that did move, had only modest daily movement rates. This suggests that the assumption of extensive intrafield movement across Bt cornfields, which is essential to the success of high-dose refuge strategy in delaying the evolution of resistance to Bt hybrids, is not met.

In structured refuges, the distribution of refuge beetles across Bt cornfields suggested that most beetles emerged, resided (Hughson and Spencer 2015) and mated (Chapter 2 of this dissertation) in the same locations, indicating that little movement occurred. Seed blends generated uniform distributions of beetles and the potential for more short-distance plant-to-plant (refuge-to-Bt plant) movement suggesting that seed blends might produce substantially more mixed-matings than structured refuge treatments. However, seed blends produced no more mixed-matings than a structured refuge treatment of the same size (Chapter 2 of this dissertation), suggesting that seed blends may not be any more effective in delaying resistance than structured refuges.

Although integrating refuge eliminates the spatial heterogeneity and concentrated areas of high beetle abundance, at the scale of beetle-to-beetle mating interactions, the desirable

interactions do not occur between beetles of refuge and Bt corn origins even when 5% refuge is maximally integrated into Bt corn as part of a seed blend. If such intimate integration did not increase the proportion of mixed-matings, perhaps the percentage of the refuge corn is too small. However, achieving a level of interaction between mate-seeking refuge and Bt beetles that generates a significantly higher level of intermating may not be achievable, even with a larger refuge, as long as rootworm development on refuge and Bt plants exacerbates the lack of synchrony in emergence of an already protandrous species. Ultimately, the evolution of Bt resistance may not be a result of lack of grower compliance with refuge regulations or inadequate refuge size, but the lack of a high-dose Bt trait and the fact that western corn rootworm ecology is ill-matched with this IRM strategy.

Based on extensive field sampling, the expectations of rapid western corn rootworm dispersal and extensive intermating between refuge and Bt beetles were not met in any of the refuge treatments in this research. Failure of a key IRM plan assumption about rootworm behavior along with the other failed IRM assumptions have done nothing to protect the long term durability of Bt traits for corn rootworm management. A limited future for the current Bt traits is supported by increasing numbers of reports of field evolved resistance to various Bt traits (Gassmann et al. 2011, 2014; Gray 2012, 2013; Wangila et al. 2015, Schrader et al. 2016, Zukoff et al. 2016, Ludwick et al. 2017). While it may be attractive to improve Bt performance, geographically widespread reports of Bt resistance suggest that we are arguably approaching the end of the Bt era of pest management (Porter 2016). It may be wise to invest in expanding studies of western corn rootworm behavior and ecology and use those results to design a biologically inspired IRM plan reflecting the most effective deployment of a highly effective

mode of action, rather than an operationally convenient deployment that imposes unrealistic expectations on pest biology.

TABLES

Table 3.1. Mean proportions of moving western corn rootworm adults detected in 20% versus 5% structured refuge treatments during 2010, 2011 and 2012. A mover was defined as a beetle testing positive for a Cry protein that was not expressed by the corn growing in the sampling row where it was collected.

	2010				2011				2012			
	20% Struc Ref		5% Struc Ref		20% Struc Ref		5% Struc Ref		20% Struc Ref		5% Struc Ref	
Direction moved	n [a]	Prop. movers [b]	n	Prop. movers	n	Prop. movers	n	Prop. movers	n	Prop. movers	n	Prop. moved
Overall	569	0.07 a	578	0.05 a	702	0.08 a	715	0.10 a	447	0.11 a	444	0.14 a
Refuge to Bt [c]	357	0.08 a	426	0.04 b	363	0.09 a	441	0.06 b	312	0.11 a	354	0.11 a
Bt to refuge [d]	212	0.14 b	152	0.16 a	182	0.10 b	125	0.21 a	135	0.12 b	90	0.24 a

[a] Number of beetles tested for Cry proteins in each corn type, structured refuge treatment and year.
[b] Proportions within the same row and year with the same letter designation were not different ($\alpha = 0.05$). Comparison of structured refuge treatment effects on proportions of movers were performed with a Kruskal-Wallis test.
[c] Proportion of beetles collected in Bt sampling rows that tested positive for Cry1Ab, the protein expressed by refuge corn.
[d] Proportion of beetles collected in refuge sampling rows that tested positive for Cry1F, the protein expressed by Bt corn.

Table 3.2. Mean proportions of moving western corn rootworm adults detected in structured refuge treatments by corn phenology periods during 2010, 2011 and 2012. A mover was defined as a beetle testing positive for a Cry protein that was not expressed by the corn growing in the sampling row where it was collected.

	2010						2011					
	Vegetative [a]		Pollination		Post-pollination		Vegetative		Pollination		Post-pollination	
Direction of movement	n [b]	Prop. movers [c]	n	Prop. movers	n	Prop. movers	n	Prop. movers	n	Prop. movers	n	Prop. movers
Overall	217	0.17 a	336	0.05 b	594	0.03 c	237	0.23 a	431	0.05 b	749	0.07 b
Refuge to Bt [d]	117	0.27 a	234	0.04 b	432	0.01 b	136	0.27 a	284	0.01 b	381	0.06 b
Bt to refuge [e]	100	0.16 a	102	0.14 a	162	0.15 a	98	0.17 a	89	0.14 a	120	0.14 a
Interfield movement [f]		---		---		---	230	0.03 a	373	0.01 b	501	0.01 b

[a] Phenology was determined based on Ritchie et al. (1993) and Abendroth et al. (2011).
[b] Number of beetles tested for Cry proteins in structured refuge treatments during each phenology period.
[c] Proportions within the same row and year with the same letter designation were not different ($\alpha = 0.05$). The Kruskal-Wallis test and Steel-Dwass test of multiple comparisons were performed to evaluate the effect of corn phenology on the proportions of movers.
[d] Proportion of beetles collected in Bt sampling rows that tested positive for Cry1Ab, the protein expressed by refuge corn.
[e] Proportion of beetles collected in refuge sampling rows that tested positive for Cry1F, the protein expressed by Bt corn.
[f] Proportion of male beetles in any sampling row that tested positive for Cry3Bb1, a Bt trait that was not expressed by corn plants at the study site. These beetles engaged in interfield movement. This test was not performed in 2010.

Table 3.2 (Cont.). Mean proportions of moving western corn rootworm adults detected in structured refuge treatments by corn phenology periods during 2010, 2011 and 2012. A mover was defined as a beetle testing positive for a Cry protein that was not expressed by the corn growing in the sampling row where it was collected.

	2012					
	Vegetative		Pollination		Post-pollination	
Direction of movement	n	Prop. movers	n	Prop. movers	n	Prop. moved
Overall	201	0.25 a	296	0.08 b	394	0.10 b
Refuge to Bt [d]	141	0.27 a	221	0.07 b	304	0.07 b
Bt to refuge [e]	60	0.18 a	75	0.12 a	90	0.20 a
Interfield movement [f]	187	0.14 a	279	0.03 b	378	0.04 b

Table 3.3. Mean proportions of western corn rootworms testing positive for both refuge (Cry1Ab) and Bt (Cry1F) proteins in each refuge treatment during 2010, 2011 and 2012.

Refuge treatment	2010		2011		2012	
	n [a]	Prop. [b]	n	Prop.	n	Prop.
20% Structured Refuge	568	0.06 a	568	0.06 b	447	0.07 b
5% Structured Refuge	578	0.04 a	590	0.07 b	444	0.07 b
5% Seed Blend	510	0.06 a	594	0.14 a	468	0.20 a
0% Structured Refuge	475	0.01 b	546	0.03 c	448	0.02 c

[a] Number of beetles tested for Cry proteins in each corn type in structured refuge treatments.
[b] Proportions of beetles testing positive for both refuge and Bt Cry proteins in each refuge treatment, each year. Proportions within the same column with the same letter designation were not different ($\alpha = 0.05$). The Kruskal-Wallis test and Steel-Dwass test of multiple comparisons were performed to analyze the effect of refuge treatment on the proportions of double positive beetles.

FIGURES

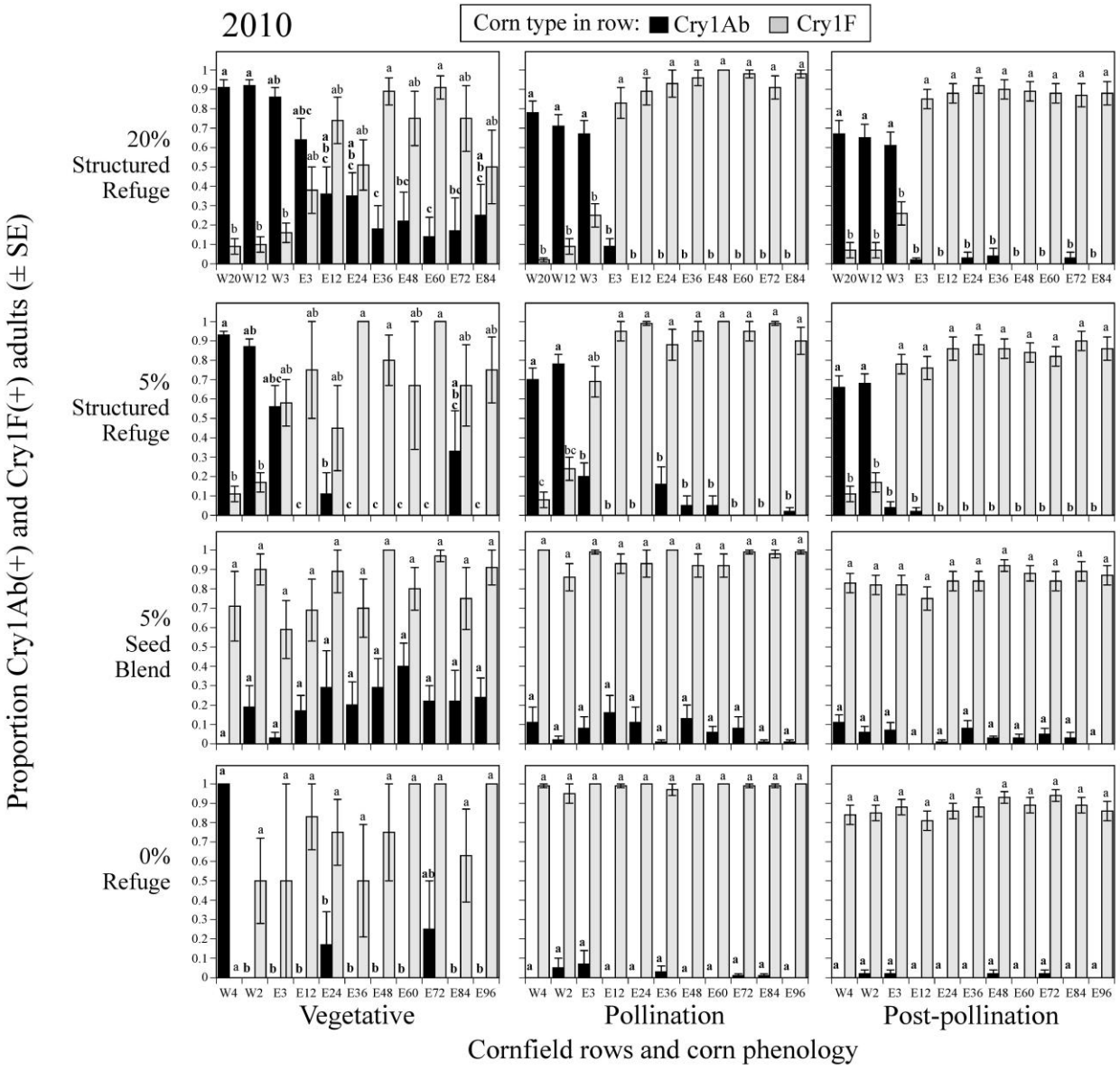

Figure 3.1. Proportion of adult western corn rootworms that tested positive for the indicated Cry proteins (± SE) in each sampling row by corn phenology and treatment. Black bars indicate the proportion of beetles testing positive for refuge corn (Cry1Ab) in the sampling row where they were collected. Gray bars indicate the proportion of beetles testing positive for Bt corn (Cry1F) in the sampling row where they were collected. Bars bearing the same letter are not significantly different based on multiple comparisons using the Marascuilo test, untransformed means are shown. See methods for row labeling explanation. Corn phenology was determined based on Ritchie et al. 1993 and Abendroth et al. 2011.

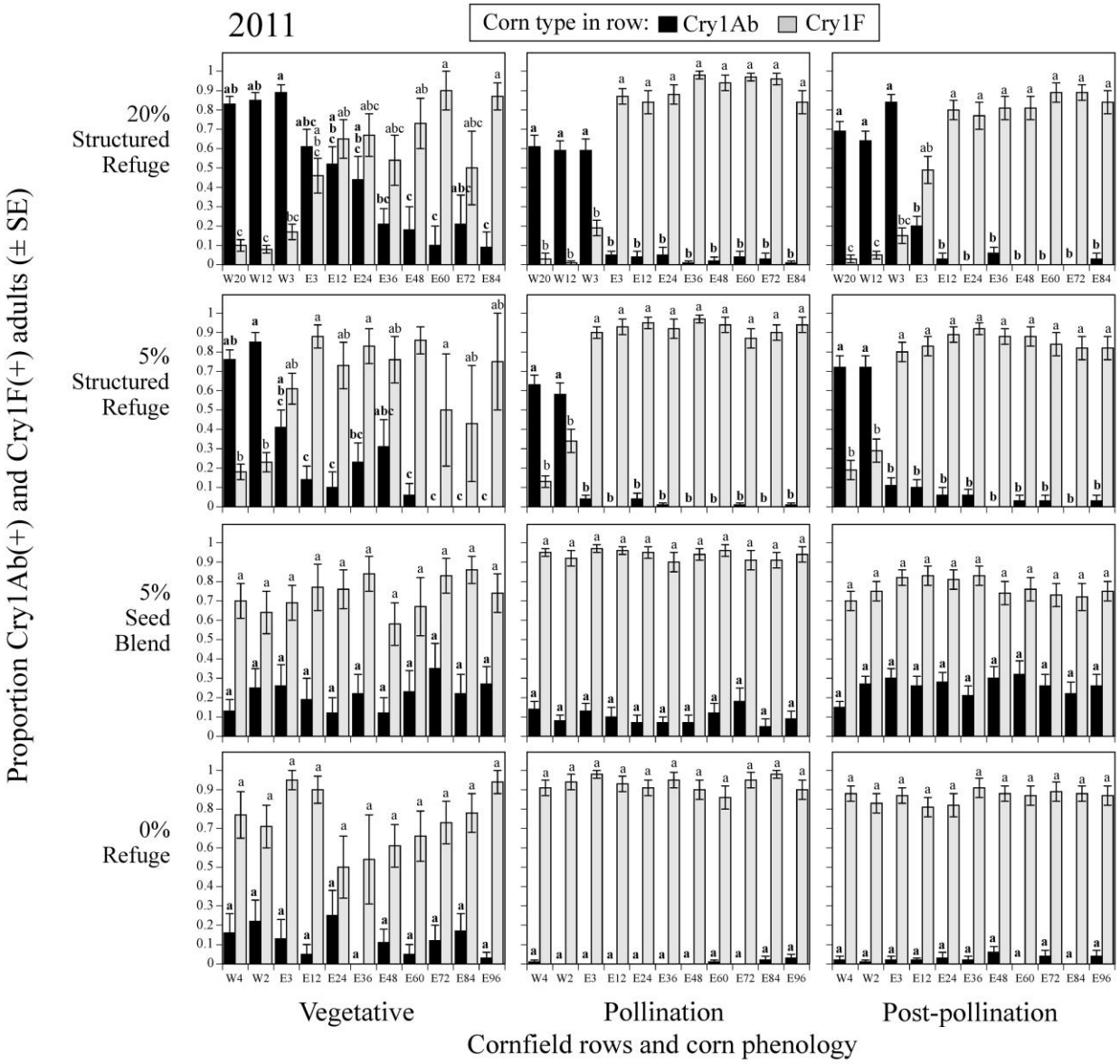

Figure 3.2. Proportion of adult western corn rootworms that tested positive for the indicated Cry proteins (± SE) in each sampling row by corn phenology and treatment. Black bars indicate the proportion of beetles testing positive for refuge corn (Cry1Ab) in the sampling row where they were collected. Gray bars indicate the proportion of beetles testing positive for Bt corn (Cry1F) in the sampling row where they were collected. Bars bearing the same letter are not significantly different based on multiple comparisons using the Marascuilo test, untransformed means are shown. See methods for row labeling explanation. Corn phenology was determined based on Ritchie et al. 1993 and Abendroth et al. 2011.

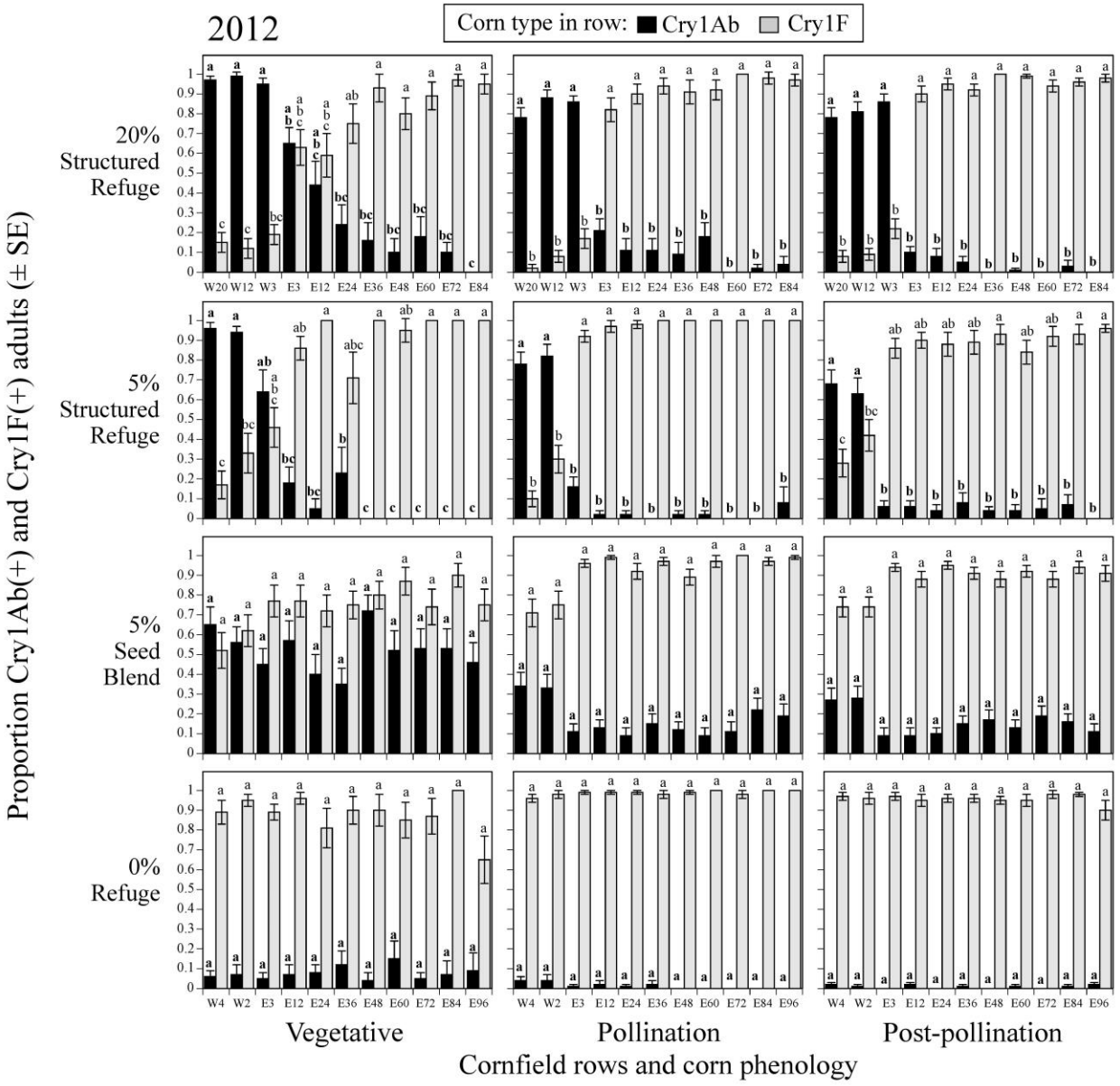

Figure 3.3. Proportion of adult western corn rootworms that tested positive for the indicated Cry proteins (± SE) in each sampling row by corn phenology and treatment. Black bars indicate the proportion of beetles testing positive for refuge corn (Cry1Ab) in the sampling row where they were collected. Gray bars indicate the proportion of beetles testing positive for Bt corn (Cry1F) in the sampling row where they were collected. Bars bearing the same letter are not significantly different based on multiple comparisons using the Marascuilo test, untransformed means are shown. See methods for row labeling explanation. Corn phenology was determined based on Ritchie et al. 1993 and Abendroth et al. 2011.

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CHAPTER 4: EFFECTS OF SEX RATIO AND BEETLE DENSITY ON WESTERN CORN ROOTWORM (COLEOPTERA: CHRYSOMELIDAE) MATING BEHAVIOR

INTRODUCTION

Western corn rootworm (*Diabrotica virgifera virgifera* LeConte; Coleoptera: Chrysomelidae) is a serious pest of corn throughout North America and is controlled predominantly, using Bt (*Bacillus thuringiensis* Berliner) corn hybrids that express Cry proteins toxic to rootworm larvae. To delay the evolution of rootworm resistance to Bt corn, the United States Environmental Protection Agency has mandated an insect resistance management (IRM) plan, based on the the high-dose refuge strategy, that requires growers of Bt corn to plant a non-Bt refuge as a structured block, multi-row strips or as a seed blend, within each Bt cornfield (US EPA OPP 2001, US EPA OPP BPPD 2010). Among the assumptions of the high-dose refuge strategy, mate-seeking rootworm beetles emerging from refuge and Bt plants are expected to readily move across these fields and intermate (Tabashnik 1994, Gould 1998). Many aspects of rootworm reproduction have been well investigated in the laboratory and the field (see reviews Spencer et al. 2009, Chapter 1 of this dissertation). However, portions of the literature may be due for re-examination because the studies were carried out long before the Bt hybrids were commercialized, and thus do not reflect the effects of Bt corn on the spatial and temporal landscape of rootworm abundance. For example, delays of 4.5 - 7.8 days between emergence from Bt corn compared to refuge corn (Storer et al. 2006, Murphy et al. 2010, Hughson and Spencer 2015) create seasonally variable areas of high and low beetle density within Bt cornfields with structured refuges, which may affect the ability of beetles to encounter mates from either refuge or Bt corn (Spencer et al. 2012, Hughson and Spencer 2015, Chapters 2 and 3 of this dissertation). The behavioral and physiological

consequences of mating under such heterogeneous conditions in Bt cornfields with structured refuges were not studied prior to commercialization of rootworm Bt corn. Understanding how field population characteristics, like sex ratio and density, affect western corn rootworm reproductive ecology, will help refine efforts to model the durability of control methods in IRM strategies which rely on rootworm mating behavior for success.

Western corn rootworms are univoltine pests that overwinter as eggs in the soil of corn or soybean fields (Levine and Oloumi-Sadeghi 1991, Levine et al. 2002). Egg hatch begins in late May and early June (Levine and Oloumi-Sadeghi 1991) and larvae begin to feed on young corn roots, which can result in significant economic damage (Metcalf 1986, Dun et al. 2010, Tinsley et al. 2012). After pupation, rootworm adults emerge from the soil beginning in late June (Ball 1957, Murphy et al. 2010, Hughson and Spencer 2015) and feed on the above-ground portions of the corn plants (Ball 1957).

Males emerge from the soil, on average, 5.2 to 6.0 days before females (Branson 1987, Hughson and Spencer 2015) and may require this post-emergence period to reach sexual maturity (Guss 1976). Males were previously thought to mate many times (Branson et al. 1977, Quiring and Timmins 1990). However, recent research has suggested that males mate on average only 2.24 times during the ten day period after their initial mating and few males mated thereafter (Kang and Krupke 2009).

Females are sexually mature upon emergence and begin releasing sex pheromone ("calling") to attract males shortly thereafter. Hammack (1995) reported that 54% of females begin calling on the first day after emergence, 96% begin calling by the second day and by the third day, 100% had begun calling. Within the first 24 hours of emergence, 70% of females mated (Quiring and Timmins 1990).

The duration of mating is three to four hours (Ball 1957; Lew and Ball 1979, 1980; Sherwood and Levine 1993), during which the male requires at least 1.5 hours to pass a spermatophore to the female (Lew and Ball 1980). In addition to providing the female with spermatozoa (hereafter referred to as sperm), the spermatophore is a nutrient rich nuptial gift containing protein, carbohydrates and water (Murphy and Krupke 2011) that is absorbed by the female after five to seven days (Lew and Ball 1980). The spermatophore is metabolically costly for a male to produce, representing up to 9% of the male's mass (Quiring and Timmins 1990, Tallamy et al. 2000). After deposition, sperm from the spermatophore migrate to the female's spermatheca after two to four hours (Lew and Ball 1980). A single mating likely provides enough sperm that a female does not need to mate again (Branson and Johnson 1973, Hill 1975). Although females were previously thought to mate only once in the field (Ball 1957, Cates 1968), they have been reported mating more than once in the laboratory (Branson et al. 1977) and in the field (Chapter 2 of this dissertation).

Mating studies have the potential to generate new insights that have significant implications for IRM because the success of the current strategy relies on several assumptions about western corn rootworm behavior related to mating. This research investigates the role of population characteristics such as insect density and sex ratio in rootworm mating success. While male skewed sex ratios are often associated with greater female mating success (Arnqvist and Nilsson 2000), Bt cornfields often produce female skewed sex ratios compared to non-Bt cornfields (Al-Deeb and Wilde 2005, Meinke et al. 2009, Murphy et al. 2010). It is unclear how variation in sex ratio might affect rootworm mating behavior. Population densities vary greatly between the refuge and Bt portions of the same cornfield (Hughson and Spencer 2015). It has been suggested that low population density in Bt portions of a field

might influence mate selection; sparsely distributed beetles emerging in Bt corn may wait longer to encounter a potential mate (Spencer et al. 2012). If they wait long, the aging females may begin to feed and move, activities that may bring a female closer to a refuge where mate-seeking males may be encountered more frequently (Spencer et al. 2012, Hughson and Spencer 2015). The experiment described here investigated the role of high and low population densities, three sex ratio treatments and other factors (including some female reproductive characteristics) in the mean number of matings by female western corn rootworm beetles. The goal was to identify whether females in areas of low density and female skewed sex ratios mate less frequently than females held at higher density with male skewed sex ratios. Cultivation of Bt cornfields with vast acreage of refuge and Bt corn creates areas of high and low beetle density with female skewed sex ratios. I hypothesize that newly emerged females in areas with low beetle densities and female skewed sex ratios will be less likely to mate than females in areas with higher density or male skewed sex ratios.

MATERIALS AND METHODS

Field location and planting

The field location for this study was at University of Illinois at Urbana-Champaign (UIUC) Ag Engineering Farm in southeast Urbana, IL (40° 07' N, 88° 21' W). In 2015, the location was planted with a Roundup Ready® Pioneer hybrid (P0987R, RM 109 days, Pioneer Hi-Bred International, Inc. Johnston, IA) that expressed no lepidopteran or coleopteran management traits was planted. On May 22, 2016, a Pioneer AcreMax® hybrid (P1197AM, RM 111 days) expressing both Cry1Ab and Cry1F traits for lepidopteran management was planted in

the study area. Corn rows were planted on a north-south axis at a rate of 84,014 seeds/ha (34,000 seeds per acre) with 0.76 m between each row.

Screen emergence tents (3.7 x 3.7 x 2.1 m; Redwood Empire Awning and Furniture Co. Inc., Santa Rosa, CA) spanning four corn rows were erected in the cornfields on June 13, 2016. All but eight or nine plants inside the emergence tents were cut off 0.5 m above the ground. This allowed the plants and beetles feeding on their roots to survive while removing the majority of the foliage in the tents so emerging beetles could be easily observed and collected. The tops of the remaining plants were trimmed to ca. 2 m to prevent them from pushing on the tops of the tents and to reduce the locations where beetles could hide.

Beetle collection and treatments

Newly emerged, field beetles were collected from screen tents daily using an insect vacuum (BioQuip Products Inc., Rancho Dominguez, CA) from June 20 through August 4, when beetle emergence from the tents ceased. After collection, adults were separated into single-sex reservoir cages (22 x 22 x 22 cm, BioQuip Products Inc., Rancho Dominguez, CA) and labeled with the date, so insect age was known. The experimental cages were 22 x 22 x 22 cm screen cages (MegaView Science, Taichung, Taiwan and BioQuip Products Inc., Rancho Dominguez, CA). Insects in cages were furnished with a water source and corn foliage, tassels and corn silks or ears to match the field phenology of the surrounding corn plants. Water was refreshed once or twice daily and food was replaced every other day.

To test the influence of population density and sex ratio on the likelihood of mating, a factorial experiment with treatments that combined two population densities, low density (n = 4 beetles / cage) and high density (n = 8 beetles / cage), with three sex ratios, 3:1, 1:1 and 1:3

(M:F), was developed. The six treatments were: 1) low density 3:1 (3M:1F), 2) low density 1:1 (2M:2F), 3) low density 1:3 (1M:3F), 4) high density 3:1 (6M:2F), 5) high density 1:1 (4M:4F), and 6) high density 1:3 (2M:6F). Each time enough beetles had emerged to complete one replicate (n = 36), males and females were distributed among cages housing the six treatments. Female beetles were placed in the experiment on their first or second day after emergence because most females begin to release sex pheromone during this interval (Hammack 1995). Males were placed in the experiment five to 15 days after emergence because males are thought to require five to seven days after emergence to reach sexual maturity (Guss 1976) and have, on average, a ten day reproductive lifespan (Kang and Krupke 2009). The treatment cages were then placed inside the screen tents in the field (Figure 4.1). The experiment was carried out in the field to provide natural temperature and humidity conditions as well as a background of pheromone and plant volatile cues that would be experienced by a field population. Enclosure in screen tents also prevented disturbance by birds and small mammals. The cages were positioned so a screen panel, rather than the impermeable cage floor panel, was on the bottom of the cage, thereby allowing rain to pass through the cages, rather than pooling to prevent the beetles from drowning.

Beetles remained in the treatment cages and were allowed to mate over a five day period. At the end of the fifth day, the beetles were removed from each treatment cage, placed in vials and stored in a freezer at -20° C until they could be processed. A total of 12 replicates were completed in 2016 (n = 432 beetles). The beetles from each treatment cage were weighed and the length of their left elytron was measured. The females were dissected under a stereo microscope (StereoZoom 4, Leica Microsystems Inc., Buffalo Grove, IL) in a drop of saline solution (NaCl 0.75%) and their mating characteristics were recorded as described by Hughson

(Chapter 2 of this dissertation), including the presence of a spermatophore in the bursa copulatrix, number of spermatophores, presence of sperm in the spermatheca and stage of ovarian development. Spermatophores break down completely after five to seven days (Lew and Ball 1980) and sperm may be stored in the spermatheca until the end of a female's natural life (Branson and Johnson 1973, Hill 1975). This sequence of events allowed the observation of new whole spermatophores (recorded as large), older degraded spermatophores (recorded as small) and the detection of sperm in the spermathecae of females in which a spermatophore had degraded completely.

When beetles were retrieved from the freezer for dissection, it was discovered that a power outage left some of the specimens desiccated or with mold growth. This prevented reliable weight measurements, so elytron length was used as a measure of size.

Many of the methods described above were developed and tested in an initial feasibility study conducted in 2015 using beetles from the USDA non-diapause population (USDA-ARS North Central Agricultural Research Laboratory (NCARL), Brookings, SD). Concerns and limitations associated with the use and field relevance of laboratory colony subjects led to an improved design based on a field population in which beetles could be collected as they emerged in screen tents adjacent to those used to house the treatment replicates.

Statistical analyses

During dissection of female beetles, the presence of a spermatophore and stored sperm confirmed that mating had occurred. Reproductive characteristics of mated and unmated females, including mean elytron length, weight and ovary rating (Short and Hill 1972), were compared using t-tests in JMP Pro 10 (SAS Institute Inc. 2012). The mean proportions of

females with spermatophores, with large spermatophores, with sperm in the spermatheca and the mean ovary development ratings were compared among density and sex ratio treatments to assess the role of these population characteristics in female behavior and physiology. Among the females with spermatophores, the proportions of females with large and small spermatophores were compared to determine if most mating occurred at the beginning of or throughout the experiment. Comparisons were performed using the Kruskal-Wallis test and the Steel-Dwass test of multiple comparisons (JMP Pro 10, SAS Institute Inc. 2012).

The mean numbers of matings per male and female among treatments were analyzed to determine whether the likelihood of mating was influenced by population density and sex ratio using the Kruskal-Wallis test (JMP Pro 10, SAS Institute Inc. 2012). The Steel-Dwass test of multiple comparisons (JMP Pro 10, SAS Institute Inc. 2012) was also used to analyze whether the likelihood of mating was significantly affected by treatment levels.

RESULTS

Body size, measured as mean female elytron length did not differ between mated and unmated females averaging 3.96 ± 0.04 mm and 3.94 ± 0.06 mm, respectively. Females had ovary development ratings that ranged from 1 to 3, 81% had stage 1 ovaries with no visible oocyte development, 17% had stage 2 ovaries with some small but visible oocytes in the vitellarium and 2% had stage 3 ovaries with some large oocytes characterized by advanced yolk deposition in the vitellarium (Short and Hill 1972). Ovary development was greater in mated females than unmated females averaging 1.28 ± 0.05 and 1.12 ± 0.05, respectively ($t = 2.38$, $P = 0.0195$).

Three females were found with two spermatophores, representing 1.4% of all females. One female had two large spermatophores and was in a high density 3:1 treatment. A second female had two small spermatophores and was in a high density 1:1 treatment. A third female had one large and one small spermatophore and was in a low density 1:1 treatment. Both large and small spermatophores were found in females after the experiment. Among females with a spermatophore present, 52% had small and 57% had large spermatophores, indicating that mating occurred throughout the experiment; there was no difference in the number of females with large or small spermatophores. The mean proportions of females with a spermatophore present were not affected by the population density but were affected by the male to female sex ratio in the population ($\chi^2 = 8.42$, df = 2, $P = 0.0148$). The mean proportion of females with a spermatophore was greatest in 3:1 sex ratio treatments and lowest in the 1:3 sex ratio treatments, while the 1:1 treatments did not differ from either the 3:1 or 1:3 treatments (Table 4.1). Mean proportions of females with large spermatophores did not vary based on population density or sex ratio (Table 4.1). More than half of the females with a spermatophore had a large spermatophore, indicating that the females had mated recently and thus had little time for absorption of the spermatophore. The mean proportion of females with sperm present in their spermathecae varied with both population density ($\chi^2 = 4.29$, df = 1, $P = 0.0384$) and sex ratio ($\chi^2 = 6.70$, df = 2, $P = 0.0351$). Mean proportions of females with sperm present were greater in treatments with low population densities (Table 4.1). The proportions with sperm present were greater in populations in the 3:1 sex ratio, lowest in the 1:3 sex ratio treatments and the 1:1 sex ratio treatments did not differ between the 3:1 and 1:3 sex ratio treatments (Table 4.1). The mean ovary development did not differ between density or sex ratio treatments (Table 4.1).

The mean number of matings per female in each treatment were not influenced by population density but they were influenced by sex ratio ($\chi^2 = 11.77$, df = 2, $P = 0.0028$; Figure 4.2). The mean numbers of matings per female were greater in treatments with 3:1 sex ratios, while the 1:1 and 1:3 did not differ (Figure 4.2). Similarly, mean numbers of matings among males in each treatment were influenced by sex ratio ($\chi^2 = 35.08$, df = 2, $P < 0.0001$; Figure 4.2) but not population density. The mean numbers of matings per male differed among all three sex ratios with the greatest mean numbers of matings per male occurring in 1:3 treatments and the fewest numbers of mean matings in the 3:1 treatments (Figure 4.2).

DISCUSSION

A male biased sex ratio led to a greater number of matings per female. Females in treatments with a female biased sex ratio had the lowest matings per female. Thus, it is surprising that female biased sex ratios in beetle emergence and abundance were consistently observed in Bt cornfields (Tables B.1 and B.2; Al-Deeb and Wilde 2005, Meinke et al. 2009). In the Bt portions of structured refuge treatments, emergence sex ratios averaged less than 0.5 (1:2), while refuge areas were still female biased at 0.768 (3:4; Tables B.1 and B.2). Sex ratios in field collections of free-moving adults in the same treatments were also female biased (Tables B.1 and B.2). Western corn rootworm emergence patterns were female biased throughout the 2010 to 2012 field experiment. Sex ratios of free moving adults were less skewed (some were male biased in structured refuge), but still generally female biased. Thus western corn rootworm females must overcome a relative scarcity of males to mate. Releasing sex pheromones is an effective way for females to attract mate-seeking males and increase the operational sex ratio in their immediate vicinity.

The fact that a male skewed sex ratio increased the females' probability of encountering potential mates may be especially important for females in areas where males are locally scarce. Pheromone release may allow females to increase their opportunities to encounter mate-seeking males, overcoming mate finding problems for isolated females. However, in female skewed treatments, some females went unmated during the five days after emergence, when the literature indicates they are most likely to mate (Ball 1957, Quiring and Timmins 1990). These females may be typical of females that have been isolated in the field; if they are unable to increase the operational sex ratio in their vicinity by releasing sex pheromone, they may remain unmated.

Males mated more often in treatments with female skewed sex ratios, than males in treatments with an equal or male skewed sex ratios. In the 1:3 sex ratio treatments, about half of the males mated a second time during the five day experiment, at an age when they were most likely to mate (Guss et al. 1972, Kang and Krupke 2009). The presence of both small and large spermatophores in females indicates that some were mated at the beginning of the experiment allowing time for the spermatophores to be partially absorbed (Chapter 2 of this dissertation) and others were mated near the end of the experiment. These observations suggest that, even within their reproductive lifespan, males may be limited in their ability to mate multiple times in short succession. Males may require time to feed and replenish nutrients before they are capable of mating and producing a second spermatophore (Quiring and Timmins 1990). As host plants begin to mature and their quality declines (Abendroth et al. 2011) males may be unable to consume enough nutrients to produce an adequate spermatophore. In addition, if males are concentrated in refuge corn where female abundance is high, males may encounter nearby calling females and expend their reproductive capabilities before leaving the refuge to encounter calling females from Bt corn.

In areas with greater beetle abundance like refuges, males may have multiple opportunities to mate with calling females. Similarly, a calling female in a high density area may be approached by multiple males. The phenomenon of multiple mating, as evidenced by the presence of two spermatophores in a female's reproductive tract, may also be related to male skewed sex ratios.

Unlike the conditions faced by a calling female who just emerged on an isolated host plant, females in a cornfield are surrounded by an abundance of potential mates who are competing for numerous calling females. Alternatively, under high competition circumstances, a mating male may be dislodged from his mate by another male before completing a mating. Males require up to1.5 hours to transfer a complete spermatophore to a female (Lew and Ball 1980) and when matings are terminated after less than one hour sperm usually is not transferred (Sherwood and Levine 1993). When mating was interrupted at one hour, the subsequent ovarian development of these females was reduced compared to females whose matings went to natural completion (Sherwood and Levine 1993). Accordingly, a female separated from her mate before she receives an adequate spermatophore may retain receptivity and accept another mate (Leopold et al. 1971) to gain the developmental benefits of the spermatophore.

Western corn rootworm spermatophores also provide nutritional benefits such as carbohydrates, water and a high percent protein content compared to some other species that have been analyzed (Murphy and Krupke 2011). Nutrients from the spermatophore have been detected in the eggs (Murphy and Krupke 2011) and fat body of mated females (Tallamy et al. 2000) suggesting that females benefit both indirectly, in increased fitness from egg production, and directly, in nutrient absorption, from receiving a spermatophore (Arnqvist and Nilsson 2000). Although food-limitation was not evaluated in this study, it is conceivable that some

twice-mated females may accept a second mate because they lack nutritional resources that could be supplemented by acquiring a second spermatophore.

Lower survival of male larvae on Bt plants is likely responsible for the shift in sex ratios reported for insects emerging from Bt cornfields (Al-Deeb and Wilde 2005, Meinke et al. 2009). Coupled with delayed emergence of beetles from Bt plants (Storer et al. 2006, Murphy et al. 2010, Hughson and Spencer 2015), this shift in male abundance and availability could reduce opportunities for females to mate shortly after emergence, despite their calling behavior. If the probability of calling decreases over time among unmated females or they release pheromone at lower rates or less consistently, aging early-emerging females may become less attractive than newly emerged females and may not have as many opportunities to mate. Some may mate later or not at all (Spencer et al. 2012, Chapter 2 of this dissertation).

Females that had mated during the experiment had greater mean ovarian development than same-aged females that had not, which was consistent with Hill (1975) and Sherwood and Levine (1993) in which western corn rootworm females had differences in ovarian development based on the duration of mating. Females mating for two hours had greater ovarian development compared to females interrupted after one hour (Sherwood and Levine 1993), thus it is not surprising to find that mated females had greater ovarian development, even a short time after mating. In many insects, physical or chemical cues that stimulate female reproductive development are passed from male to female during mating (Spencer et al. 1992, Miller et al. 1994, Sherwood and Levine 1993, Klowden 2007). These cues include compounds produced by male accessory glands, physical cues from stretch receptors in the female reproductive tract, or a combination of cues (Spencer et al. 1992, Miller et al. 1994, Sherwood and Levine 1993, Klowden 2007) providing females with an additional benefit of mating.

Refuge males are expected to move across cornfields to mate with newly emerged Bt females (Tabashnik 1994, Gould 1998), an assumption derived from research conducted well before Bt corn hybrids were commercialized. While classic literature has paved the way for current research, the local agroecosystem has changed and the western corn rootworms and management strategies within it have evolved. Because the landscape of corn production has changed dramatically, it was critical to revisit some of the field biology behind the expectations that are fundamental to IRM. Exploring fine points of male-female mating interactions, in this case population density and sex ratios, expands upon mere basic biology to provide a more mechanistic understanding of western corn rootworm behavioral dynamics.

This research has demonstrated that changes in population dynamics created by pest control methods may alter western corn rootworm mating behavior to the detriment of our IRM goals. The combination of a general delay in emergence of females relative to males (Ball 1975, Hughson and Spencer 2015), delays in emergence from Bt corn (Crowder et al. 2005, Storer et al. 2006, Murphy et al. 2010, Petzold-Maxwell et al. 2013, Hughson and Spencer 2015) and sparse abundance in Bt corn may result in isolated Bt females that are unable to mate rapidly. Even if isolated females are in the presence of males, delays in emergence from Bt corn (Crowder et al. 2005, Storer et al. 2006, Murphy et al. 2010, Petzold-Maxwell et al. 2013, Hughson and Spencer 2015) and the short male reproductive lifespan (Kang and Krupke 2009) may limit the operational sex ratio in Bt corn (Spencer et al. 2012) and ultimately result in undesirable matings between males and females that emerged from Bt corn. While the current IRM plan is not well suited to western corn rootworm biology, the knowledge gained from applying this strategy and exploring basic aspects of pest biology can shape future investigations and improvement of management strategies.

TABLES

Table 4.1. Mean (± SE) reproductive characteristics of mating female western corn rootworms held at different densities and sex ratios.

	Population Density				Population Sex Ratio					
	Low		High		3:1		1:1		1:3	
Reproductive Measure	n	Mean ± SE [a]	n	Mean ± SE	n	Mean ± SE	n	Mean ± SE	n	Mean ± SE
Spermatophore Present	34	0.69 ± 0.06 a	36	0.61 ± 0.05 a	23	0.78 ± 0.08 a	24	0.62 ± 0.07 ab	23	0.55 ± 0.05 b
Large Spermatophore Present [b]	31	0.54 ± 0.07 a	34	0.63 ± 0.07 a	20	0.60 ± 0.11 a	24	0.62 ± 0.07 a	21	0.54 ± 0.09 a
Sperm Present	35	0.64 ± 0.07 a	36	0.48 ± 0.05 b	24	0.70 ± 0.08 a	24	0.51 ± 0.08 ab	23	0.47 ± 0.05 b
Ovary Development [c]	30	1.19 ± 0.07 a	30	1.21 ± 0.06 a	19	1.18 ± 0.08 a	23	1.23 ± 0.09 a	18	1.11 ± 0.05 a

[a] Means (± SE) in the same row and population measure (density or sex ratio) with the same letter designation are not significantly different (Steel-Dwass test of multiple comparisons , $\alpha = 0.05$).
[b] Among females with a spermatophore, the mean proportion of females with a large spermatophore in in their bursa copulatrix.
[c] Mean ovary development was scored on a scale of 1 to 4 based on Short and Hill (1972). During stage 1 no ovary development was observed and during stage 4 mature eggs were observed.

FIGURES

Figure 4.1. A screen emergence tent (3.7 x 3.7 x 2.1 m) spanning four corn rows.

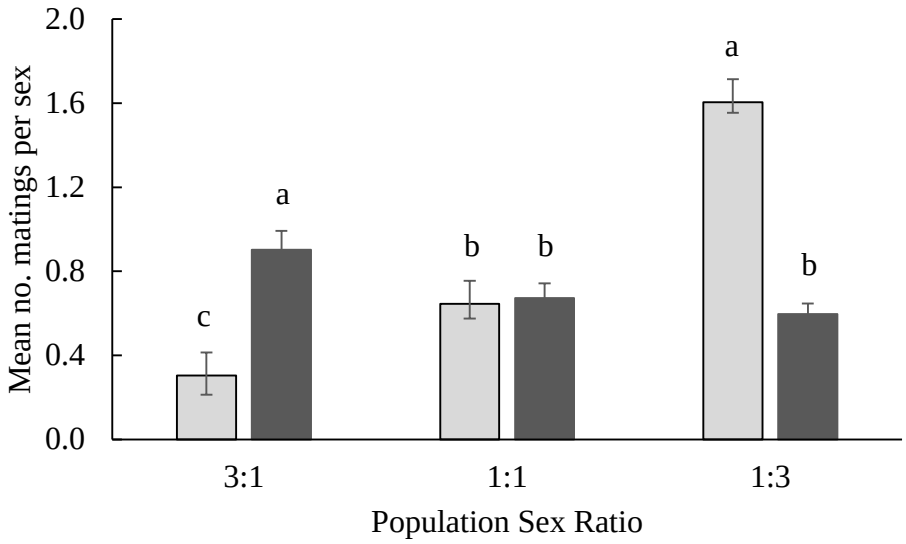

Figure 4.2. Mean number of matings (± SE) per male (gray bars) and female (dark bars) held in cages with 3:1, 1:1 or 1:3 M:F sex ratios. The mean number of matings per treatment was calculated based on the total number of matings based on spermatophores and/or presence of sperm observed in dissected females. The Kruskal-Wallis test was performed to determine whether population density influence the mean number of matings. Bars of the same shade bearing the same letter are not significantly different ($\alpha = 0.05$).

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CHAPTER 5: ASSESSING SPERM PRECEDENCE IN WESTERN CORN ROOTWORM (*DIABROTICA VIRGIFERA VIRGIFERA* LECONTE) USING MICROSATELLITE GENOTYPING

INTRODUCTION

Western corn rootworm (*Diabrotica virgifera virgifera* LeConte) is a serious pest of corn in the United States (US), costing growers more than $1 billion in management costs and yield losses annually (Metcalf 1986, Dun et al. 2010). Corn hybrids expressing proteins from Bt (*Bacillus thuringiensis* Berliner) that are toxic to root-feeding larvae have been used to control rootworm populations since 2003 (US EPA OPP 2001). To slow the evolution of resistance to Bt toxins expressed in Bt corn hybrids, the US Environmental Protection Agency mandated use of an insect resistance management (IRM) plan (US EPA 1998, US EPA OPP 2001) based on the high-dose refuge strategy where Bt corn was grown. According to this strategy, a 'refuge' of non-Bt corn must be planted in a portion of every Bt cornfield, providing a location where Bt susceptible beetles can develop without exposure to Bt toxins. These susceptible beetles are expected to move across cornfields and mate with rare potentially resistant beetles that survived larval exposure to Bt corn, producing heterozygous susceptible offspring (Tabashnik 1994, Gould 1998).

Because this IRM strategy relies on rootworm movement and mating behavior to create the conditions that delay resistance, understanding these behaviors is important to ensure the strategy's efficacy. Using simulation models, researchers evaluate the durability of Bt hybrids based on parameters including the dose of the Bt trait, refuge size, refuge configuration and pest biology (e.g. Pan et al. 2011, Spencer et al. 2012, Kang et al. 2013). Field research leading to insights regarding movement, mate choice, mating frequency, sperm precedence and oviposition in Bt cornfields can broaden the information base used by modelers to refine their predictions.

Adult male western corn rootworms emerge from the soil, on average, 5.2 to 6.0 days before females (Branson 1987, Hughson and Spencer 2015) and may require five to seven days to reach sexual maturity (Guss 1976). Adult females are sexually mature upon emergence (Hammack 1995) and usually mate during the first 24 hours while they are still teneral (Cates 1968, Quiring and Timmins 1990). Mating lasts for three to four hours on average (Ball 1957, Lew and Ball 1979, Sherwood and Levine 1993), during which the male deposits a spermatophore in the female's bursa copulatrix (Lew and Ball 1980). Males make a significant contribution to mating when they provide a spermatophore, which amount to up to 9% of the male's mass (Tallamy et al. 2000). The spermatophore is a nuptial gift that has two major components (pictured in Chapter 2 of this dissertation, Figure 2.1). The first is the ampulla, which contains spermatozoa (hereafter referred to as sperm) that migrate to the female's spermatheca (Lew and Ball 1980), where they can be stored for 40 to more than 76 days (Branson and Johnson 1973, Hill 1975, Lew and Ball 1980). The second component is the spermatophylax, which is rich in proteins (Gillott 2003, Murphy and Krupke 2011), carbohydrates and water (Murphy and Krupke 2011). A spermatophore can be absorbed by the female within five to seven days (Lew and Ball 1980). Egg-laying begins when females are between 12 and 21 days old (Short and Hill 1972, Branson and Johnson 1973, Hill 1975). During their lifetime, females that mated once produced an average of 266 to 441 viable eggs (Ball 1957, Elliott et al. 1990, Fischer et al. 1991, Boetel and Fuller 1997) but some individuals are capable of producing more than 1000 total eggs (Branson and Johnson 1973, Hill 1975).

Western corn rootworm females were thought to mate only once in the field (Ball 1957, Cates 1968), but studies have shown that a portion of the population mates more than once in the field (Chapter 2 of this dissertation) and in the laboratory (Branson et al. 1977, Chapter 4 of this

dissertation). The proportion of females that mate more than once may range from 4.5% in field populations (Chapter 2 of this dissertation) to 23% of the population in the laboratory (Branson et al. 1977). Females do not accept mates while gravid (Chapter 2 of this dissertation) or actively ovipositing (Branson et al. 1977), although they may accept a second mate before oviposition or during intervals between dates of oviposition (Branson et al. 1977).

Multiple mating by females is common among insects and usually occurs with different partners (polyandry), rather than repeated matings with the same male because insects typically lack pair bonds (Thornhill and Alcock 1983). Polyandrous behavior may not occur in all western corn rootworm females, but because matings between Bt resistant and Bt susceptible individuals are fundamental to the success of rootworm IRM, variation in the timing and frequency of female mating could change the abundance of Bt susceptible offspring. If multiple matings are occurring, the type of sperm precedence could further affect the likelihood of Bt susceptible offspring.

No research specifically examining sperm precedence in western corn rootworm has been published, but three studies (Branson et al. 1977; Oyediran 2002, 2007) suggest that the second or last male to mate may have precedence in fertilizing the offspring. Branson et al. (1977) carried out an experiment with three treatment groups in which females were first mated to a sterile irradiated male and were then housed continuously with an unaltered male beginning 1) immediately after the first mating, 2) before the first oviposition or 3) after the first oviposition. Females introduced to the unaltered male immediately after mating produced viable eggs as did females introduced to unaltered males after first oviposition, but none of the females introduced to unaltered males before first oviposition appeared to mate a second time (Branson et al. 1977). While this finding suggests last male sperm precedence, or mixed paternity, it cannot rule out

first male sperm precedence because none of the females were mated first to an unaltered male followed by a sterile male (Branson et al. 1977). The use of sterile males also raises questions about the possibilities that females may respond differently to the presence or absence of viable sperm (Gromko et al. 1984) or that the sperm themselves are unable to migrate to the female's spermatheca.

Genotyping may allow sperm precedence to be evaluated without raising questions about the capabilities of irradiated males or how females respond to them. Oyediran (2002 Chapter 2, Chapter 4; 2007) evaluated sperm precedence in two studies using southern corn rootworm (*Diabrotica undecimpunctata* Howardi). In the first study, females were mated to two males with different abdominal color phenotypes. Their offspring's abdominal phenotypes were evaluated to determine sperm precedence (Oyediran 2002 Chapter 2). In the second study, RAPD-PCR and electrophoresis were used to compare DNA fragments from the offspring and parents (Oyediran 2002 Chapter 4). In both studies, Oyediran (2002 Chapter 2, Chapter 4; 2007) concluded that southern corn rootworm had second male sperm precedence.

Last male sperm precedence in western corn rootworm may further complicate management of insect resistance especially if multiple mating is fairly common among females. Delays in female emergence compared to males (Ball 1957, Hughson and Spencer 2015), delays in adult emergence from Bt corn compared to non-Bt corn (Storer et al. 2006, Murphy et al. 2010, Hughson and Spencer 2015), female-biased sex ratios in Bt corn versus non-Bt corn (Al-Deeb and Wilde 2005, Meinke et al. 2009) and the 10 day reproductive lifespan of males (Kang and Krupke 2009) already create a situation where late-emerging females are more likely to encounter and mate with Bt emerging (potentially resistant) males (Spencer et al. 2012, Hughson and Spencer 2015, Chapter 2 and chapter 3 of this dissertation). In addition, simulation models

suggest that these conditions may reduce the durability of Bt traits (Spencer et al. 2012). The possibility that some proportion of females may mate more than once with the last male fertilizing the offspring may create two different scenarios. If the female mates twice early in the season while mate-seeking refuge-emerging males are present, the offspring are likely to be susceptible to Bt. If the female mates again later in the season, however, there is a risk that she will mate with a male that may carry Bt resistance alleles, passing them to her offspring. In contrast, if the first male's sperm have precedence, polyandry may have little additional impact on resistance management.

This study was designed as a field and laboratory experiment with an associated genomic experiment. The field and laboratory portions were intended to identify the proportion of females that accept a second mate, identify the interval between acceptance of a first and a second mate, as well as providing measures of fecundity. The genomic portion of the study was intended to evaluate sperm precedence among the offspring of twice-mated females, by comparing the genotypes among the offspring, parent beetles and sperm stored in the female's spermatheca using known western corn rootworm microsatellite loci. The operative hypothesis was that there would be last male sperm precedence in western corn rootworm and thus sperm from the most recent mate would fertilize all or a majority of the resulting eggs.

MATERIALS AND METHODS

Field location and beetle collection

The University of Illinois at Urbana-Champaign Ag Engineering Farm (AEF; 40° 07' N, 88° 21' W) was selected as the site of this experiment. In 2015, the field was late-planted with corn and pumpkins, creating a cornfield that pollinated after the surrounding fields, to attract

egg-laying western corn rootworm beetles and increase the rootworm population the following year. In 2016, a Pioneer AcreMax® hybrid (P1197AM, RM 111 day, Pioneer Hi-Bred International, Inc., Johnston, IA, USA) expressing both Cry1Ab and Cry1F traits for lepidopteran management was planted at the AEF field site on May 22. Four screen emergence tents (3.7 x 3.7 x 2.1 m; Redwood Empire Awning and Furniture Co. Inc., Santa Rosa, CA, USA) were installed over vegetative corn on June 13, 2016. The emergence tents spanned four 0.762 m wide rows of corn; all but nine plants per emergence tent were cut off at 0.5 m above the ground to reduce the complexity of vegetation within the cages to facilitate beetle collection. Trimmed plants continued to grow and support developing larvae; tents eventually produced several thousand beetles.

Beetles emerged from the emergence tents from Jun. 20 through Aug. 4. Newly emerged beetles were collected from the emergence tents each day during this period. Because the emergence tents were emptied of beetles each day, all beetles encountered in the emergence tents had emerged fewer than 24 hours prior to collection (day one). Single adults were collected from each emergence tent using an insect vacuum (BioQuip Products Inc., Rancho Dominguez, CA, USA); males and females were separated into single-sex reservoir cages (22 x 22 x 22 cm; BioQuip Products Inc., Rancho Dominguez, CA, USA; MegaView Science, Taichung, Taiwan) and labeled with the date of emergence so their age was known. All reservoir cages and experimental cages (described below) were maintained in the field within the large emergence tents (pictured in Chapter 4 of this dissertation) to provide protection from local wildlife. Food and water were continuously available to the beetles. Foliage, tassels, silks and corn ears were provided as food, matching the phenology of the surrounding cornfield. Water containers were refreshed once or twice daily and food was replaced every other day. The screen cages were

turned so a screen, rather than solid, side was on the bottom of the cage, allowing rain to pass through the cage and preventing the beetles from drowning in pooled water.

Mating arrangements

Each female in the mating study was housed in a 17.5 x 17.5 x 17.5 cm screen experimental cage (MegaView Science, Taichung, Taiwan). The experimental cages were maintained inside an emergence tent in the cornfield where the female emerged for the duration of the mating portion of the experiment. Insects thus could be maintained under field temperature and humidity conditions while experiencing normal field stimuli, such as host plant volatiles and western corn rootworm pheromones that may influence the insects' behavior.

Each newly emerged female was originally collected while mating in an emergence tent and assigned an individual identification number, thereby allowing the first mating to be initiated and confirmed with minimal handling and disruption. When a mating pair was encountered, they were gently coaxed to walk into a vial and allowed to rest for a short time to minimize stress, after which the vial was opened and placed inside an experimental cage. When the initial mating ended naturally, the male was removed from the cage with a vial, labeled to correspond to the female identification number and stored in the laboratory freezer at -20º C. The female was left in the cage and a new male was introduced each day thereafter.

The subsequent new males were between five and 15 days old, to ensure they had completed post-emergence maturation (Guss 1976) but had not exceeded their 10 day reproductive life span (Kang and Krupke 2009). Each morning a new male was introduced into a female's cage between 7:00 and 9:00 am, when mating activity was greatest (Bartelt and Chiang 1977, Dobson and Teal 1986). Mating lasts on average between three and four hours

(Ball 1957; Lew and Ball 1979, 1980; Sherwood and Levine 1993), therefore the cages were observed to confirm mating at two hour intervals until late afternoon or early evening. Observations were made without disturbing the cage whenever possible, to avoid interrupting courtship behavior or mating. Because western corn rootworm males may mount females and remain in place for up to an hour prior to mating (Lew and Ball 1979), mounted pairs required close inspection to confirm mating (visual confirmation of aedeagus insertion). If mating was not observed, the male was removed from the cage and released at the end of the day. When mating was observed, the beetles were allowed to continue until mating ended naturally. After mating ended, the male was removed, labeled as the second male for that individual female and stored in the freezer. After the second mating, the female was transferred to an oviposition container in a climate-controlled laboratory chamber (Percival Scientific Inc., Perry, IA, USA) maintained at 24° C and 13L:11D schedule. Females may begin to oviposit six to ten days after mating (Sherwood and Levine 1993) and do not mate while gravid (Chapter 2 of this dissertation). Therefore, females that did not mate a second time within ten days or became visibly gravid, were removed from the experiment. The ages of females at the first mating, second mating and death were recorded. The age of males accepted in the first and second matings were recorded.

Egg collection

The oviposition containers consisted of a stacked pair of 6 cm diameter Petri dishes (ThermoFisher Scientific, Waltham, MA, USA). The top petri dish contained a small amount of corn tissue as a food source and a piece of moist cotton as a water source; each had a 1 cm hole cut in the bottom, allowing the female to move into the bottom Petri dish. The bottom surface

and the sides of the lower 6 cm petri dish were colored with permanent marker to create a dark area for oviposition. This dish contained a smaller 3.5 cm diameter Petri dish (Falcon, Corning, NY, USA) of 80 mesh field soil for oviposition (Figure 5.1). The soil was moistened, spread evenly in each oviposition dish and scored three or four times to simulate natural fractures in the soil where females prefer to oviposit.

Females were observed twice weekly to determine whether oviposition had occurred and to provide fresh food, water and clean containers. The entire container, including the small oviposition dish, was observed using a stereomicroscope at 6 - 12 x magnification (StereoZoom 4, Leica Microsystems Inc., Buffalo Grove, IL, USA) to detect oviposition and to assure that even eggs deposited in small crevices or on food items were not overlooked. When eggs were present, the oviposition dish was replaced with a fresh dish of soil. The dish containing eggs was lidded, labeled with the female identification number, oviposition date and held at room temperature (ca. 21º to 22º C) for 14 days to allow completion of early embryonic development. After 14 days, the oviposition dish lids were secured with rubber bands, placed in a plastic bag and moved to a chamber maintained at 6º C in 24 hours darkness. Eggs could then naturally enter diapause so they could be stored for at least five months.

Egg data were recorded as weekly egg batches. Females were maintained and allowed to oviposit until they died naturally. After death, females were placed in vials with their identification number and stored in the laboratory freezer at -20º C. The age at which twice-mated females began oviposition, duration of oviposition, weekly egg data and total eggs produced were recorded for each female.

Sample preservation

Before DNA extraction, the eggs were separated from the soil in each oviposition dish. The soil was washed through an 80 mesh sieve with tap water; the soil passed through and the eggs were collected in the sieve. If two oviposition dishes with eggs were collected from a female during the same week, they were pooled during the washing step to create a single weekly egg batch. After washing, the eggs were rinsed from the sieve into a container and observed under a stereomicroscope at 6 - 12 x magnification (StereoZoom 4, Leica Microsystems Inc., Buffalo Grove, IL, USA). The eggs were counted, any remaining soil or debris was removed using a fine probe and the eggs were transferred into a microcentrifuge tube in a small amount of water with a disposable 7 mL pipette. The egg batches were stored in a refrigerator overnight before they were transported to Illinois Institute of Technology in Chicago, IL in coolers.

Dissection of spermathecae and isolation of sperm

After allowing the frozen females to thaw at room temperature, their spermathecae were isolated under a stereomicroscope at 6 - 12 x magnification by pulling away the pygidium of each female's abdomen with a pair of fine forceps (Dumont #5/45, Fine Science Tools Inc., Forest City, CA, USA) and iridectomy scissors (BioQuip Products, Inc., Rancho Dominguez, CA, USA) and picking up the dark, question mark shaped spermatheca with the forceps. Each spermatheca was placed on a microscope slide in a 5 μL droplet of 0.065% NaCl aqueous solution. Under a stereomicroscope at 50 x magnification (Wild M5-53540, Heerburgg, Switzerland), the spermatheca was cut into two pieces using two fine steel probes, a coverslip was placed on top of the pieces and gently tapped to expel sperm (Bermond et al. 2014). Under

the microscope, sperm resembled a cloud of small hairs emanating from the spermatheca fragments. The coverslip was carefully removed and 5 μL of 0.065% NaCl was added to facilitate the transfer of the sperm into a micro-centrifuge tube for DNA extraction using a P20 micropipette as described in Bermond et al. (2014).

DNA extraction

DNA was extracted from sperm using the commercial kit prepGeM (ZyGeM Ltd, Hamilton, New Zealand) according to the manufacturer's instructions with an elution volume of 20 μL. The DNA from adults was extracted from the head and the thorax of each beetle with the DNAzol method (Molecular Research Center, Inc., Cincinnati, OH, USA) using an elution volume of 100 μL (TE buffer, 10 mM Tris-HCl, 0.1 mM EDTA, pH 8). This method relies on the use of a single extraction buffer to solubilize the different cellular components and on the precipitation of DNA in the presence of ethanol (Chomczynski et al. 1997). The abdomen of an adult was removed and the head and thorax of each adult was crushed with a pestle in 100 μL of DNAzol. The abdomens of females were saved for subsequent sperm extraction. Each pestle was rinsed with 880 μL of DNAzol and 10 μL of PolyAcryl Carrier (Molecular Research Center, Inc., Cincinnati, OH, USA), 10 μL of RNase A added and the sample allowed to incubate at 37º C for one hour. Then 10 μL of proteinase K (Qiagen, Germany) was added to the homogenate, the mixture was stored at room temperature on the bench for three hours, then centrifuged at 13,200 rpm for ten minutes. DNA was precipitated from the kept supernatant at room temperature with 0.5 mL of 100% ethanol and centrifuged at 13,200 rpm for ten minutes. The pellets of DNA were subsequently washed twice in 0.8 mL of 70% ethanol, with two steps of centrifugation at 5,000 rpm for five minutes after each wash. Ethanol was then removed from

the DNA pellets using a P20 micropipette. Finally, the pellets were resuspended in 60 μL of TE buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 8).

Genotyping

Thirteen microsatellite loci of WCR (DVV-D2, DVV-D4, DVV-D8, DVV-D9, DVV-D 11, DVV-D12bis, DVV-T1, DVV-T2, DVV-T3, Dba-01, Dviz11, Dba-05 and Dba-07; Kim et al. 2008) were used to genotype individual beetles, egg batches and spermathecae contents. These loci were selected because they could be reliably amplified and many had previously been used in western corn rootworm population genetics studies (Ciosi et al. 2008).

These loci were amplified using three multiplex PCR performed in a T100 Thermal Cycler (Bio-rad Laboratories, CA, USA) and were subsequently analyzed by replicating some of the conditions described in Miller et al. (2007): the multiplex PCR was carried out in a total volume of 25 μL containing 2 μL of template DNA solution, 2X Type-It Qiagen Multiplex Master mix (Qiagen, Germany) and each primer at 0.2 μM. Forward PCR primers were 5' labelled with a fluorescent dye, allowing the PCR products to be detected on an automated DNA capillary sequencer. The thermal cycling program for all three multiplex PCRs was 95° C for 15 minutes followed by 25 cycles of 94° C for 30 seconds, 55° C for 90 seconds and 72° C for 60 seconds followed by a final incubation at 60° C for 30 minutes. DNA amplification consisted of 25, 35 and 43 PCR cycles for beetles, eggs and sperm samples, respectively. Amplified microsatellites were analyzed by capillary electrophoresis in combination with GeneScan-600 LIZ size standards (Applied Biosystems, Foster City, CA, USA) on an ABI 3730 XL automated DNA sequencer (Applied Biosystems, Foster City, CA, USA) at University of Illinois at Chicago. The calculation of the sizes of amplified microsatellites and their assignment to allele

classes was partially automated using the Geneious version 10.05 (http://www.geneious.com, Kearse et al. 2012). The primers used for DVV-D12 amplified were modified from those described by Kim et al. (2008; primers used here were F: 5'-GATTCTCAGTAATGGGGAAACG-3'; R: 5'- CACACGCTTTCTCGTAATCTATC-3'), to decreased the frequency of null allele detection at this locus. All individual beetles were assigned to a diploid multilocus genotype showing two peaks per individual per locus, maximum. When spermathecae contained the sperm of two males, four peaks at maximum were expected to be detected (tetraploid profile). When egg batches were assigned, up to six peaks were expected because the egg batches contained multiple offspring produced by three potential parents.

RESULTS AND DISCUSSION

Female mating, longevity and oviposition measures

During the experiment, 71 females were collected in a mating pair within 24 hours of emergence (designated day 1). Of these females, 8.5% (n = 6) eventually mated a second time (designated Fam02 - F to Fam07 - F), with a different male. The mean age at which these females accepted a second mate was 7.5 ± 1.6 days (mean ± SE; range 3 to 12 days; Table 5.1). This interval is similar to the five day interval used in the laboratory by Hughson (Chapter 4 of this dissertation) showing that females mated twice within their first week as adults. The Branson et al. (1977) laboratory study also showed that females mated twice, those females accepted a second male either during the interval between the first mating and beginning of oviposition or during intervals between dates of oviposition. These studies have shown that

western corn rootworm females will mate a second time, although this can be difficult to observe and quantify.

The mean age of the second male mated by the females was 9.0 ± 0.9 days (range 6 to 11 days; Table 5.1). Detailed information about male age at mating is lacking in the available western corn rootworm literature, stating only that males require five to seven days to reach sexual maturity (Guss 1976) and that males mate 2.24 times during the ten days after their first mating (Kang and Krupke 2009) although old publications suggest may mate many times (Ball 1957, Branson et al. 1977). While five to 15 days old unmated males were placed in the experiment to improve the likelihood of a second mating, only day one males were collected in first matings, contradicting the literature-based expectation that all males require a post-emergence maturation period to reach sexual maturity (Guss 1976). The proportion of the male population represented by these males could not be quantified here because the total number of males that had emerged were not recorded but it was estimated that several thousand beetles were collected from the emergence tents. It is notable that Guss's (1976) experiment did not measure mating activity, but focused on the timing of male response to sex pheromone. Guss (1976) found that within the first 24 hours, 0% of males responded to sex pheromone, at one to two days 10% responded and the response percentages increased progressively until five to seven days when 80% of males responded. The large population of emerging insects may have increased the likelihood of observing an uncommon behavior such as mating by western corn rootworm males within one day of adult emergence. In addition, Hammack (1995) reported that some males attempted courtship with females they encountered before the females began to release sex pheromone (based on visual observation of female calling posture), which may indicate that females have other cues (e.g. contact pheromones in epicuticular waxes; Chung and

Carroll 2015) that are attractive to males or that males may simply attempt courtship when they encounter females. Further research to document male mating propensity by age would clarify the common assumptions based on the work of Guss (1976).

Twice-mated females began oviposition at 22.6 ± 4.2 days (range 15 to 39 days; Table 5.2), a finding consistent with other studies that reported oviposition beginning 20 to 23 days post-emergence (Short and Hill 1972). It is important to note that the date of first oviposition may have been prolonged in the present study because females were not provided with an oviposition substrate until the second mating was complete.

The mean duration of oviposition was 41.2 ± 11.3 days (range 6 to 74 days; Table 5.2). Females oviposited for a mean of 5.4 ± 1.3 weeks (Table 5.2). The oviposition duration was variable and some females ceased oviposition for one or more weeks within the oviposition range, an observation mentioned elsewhere in the literature (Short and Hill 1972, Branson et al. 1977, Sherwood and Levine 1993). Most of the females laid eggs until their death, although some ceased oviposition a week or two prior to death (Branson and Johnson 1973). The females produced an average of 31.2 ± 9.5 eggs / week and a lifetime mean total of 145.4 ± 49.5 eggs / female (Table 5.2). This mean total eggs laid per female was within the mean range of 66 to 173 eggs laid by field females collected and maintained in the laboratory by Short and Hill (1972), albeit far less than the 1023 eggs presented by Branson and Johnson (1973). This wide variation may be a product of the small sample size.

The mean female lifespan was 57.0 ± 15.8 days (range 11 to 101 days; Table 5.1) was similar to the 64 to 67 days recorded by Short and Hill (1972) and later emerging females appeared to have shorter longevity (Boetel and Fuller 1997). In some insects, females may gain fitness in terms of longevity and fecundity from an additional mating while in others multiple

mating may lead to a loss in fitness due to risks associated with mating such as predation (Arnqvist and Nilsson 2000). In western corn rootworm, an initial mating is beneficial and can stimulate egg production (Sherwood and Levine 1993) and may reduce longevity (Murphy and Krupke 2011) but it is unclear whether multiple mating may be beneficial or detrimental in terms of longevity and egg production. For late emerging females, this may not matter, if they cannot oviposit until they are 20 days old; they may not have the opportunity to find adequate food resources to provision eggs and oviposit before cornfields mature and become poor foraging locations. If late emerging females oviposit and produce a mean of only 31.2 eggs per week, they may be contributing very little to the next generation.

Female dissection

During dissection, a small amount of sperm was collected from the spermatheca of each female except Female 2, whose spermatheca lacked any visible sperm. Female 6 was the only female with eggs remaining in her body at death; her ovaries contained 67 mature eggs. Only small quantities of sperm were collected from each female's spermatheca, possibly because it had been depleted during oviposition or because the sperm had been stored within the females for a long period of time. Some studies suggest that viable sperm can be stored for 40 to greater than 76 days (Branson and Johnson 1973, Hill 1975, Lew and Ball 1980); however, some of these females stored sperm within or beyond that range. Collecting sperm from within these tiny sclerotized organs (much smaller than the period at the end of this sentence) is also a technically difficult task and sperm could be lost during the collection process. It is recommended that researchers allow adequate time to practice the dissection prior to working with the test samples. This will ensure that the spermathecae and their contents to be handled deftly, to avoid the

samples drying onto the slide or being lost on the surface of the slide.

DNA extraction and genotyping

DNA was extracted successfully from egg batches containing more than ten eggs. DNA extractions for all of the beetles were successful, although three of the females had DNA quantities that may have been too low to be successfully amplified and genotyped. Only one of the sperm samples was successfully extracted. Despite this problem, two family groups had DNA quantities great enough to evaluate the parent beetles and the egg batches. If this experiment were to be repeated, the male and female beetles would be killed in liquid nitrogen and stored at -80° C until processing to ensure the highest level of DNA preservation.

Genotyping was completed but the results were unclear. Some of the samples did not include any readable allele peaks. Others samples that had readable allele peaks but did not have the expected number of alleles for that sample type (i.e. beetles, egg batches or sperm). In some cases samples that had previously been identified as containing genomic DNA did not produce peaks after genotyping, in other cases the opposite occurred. Females and their eggs often contained the same diploid genotypes, despite the egg batch samples containing multiple individual offspring. When male samples had readable peaks, they often had the same genotypes as the females, making it difficult to differentiate the male and female contributions to the eggs. One or two males showed four clear allele peaks indicating tetraploid profiles. A tetraploid profile would be a reasonable observation in a sperm sample, when the females mated twice and contributions from two males would be present, or in egg batches, because they contained multiple offspring. The various discrepancies suggest that there may have been inconsistencies between the sample location in the electrophoresis capillary system and the original sample

identification during genotyping. The genotyping facility indicated that there were damaged electrophoresis capillaries and that the sample locations had been shifted. However, that was accounted for and did not resolve all of the discrepancies in the results.

Successful amplification and genotyping should be possible for the beetle and egg batch samples from the two family groups from which adequate quantities of DNA were extracted. While genotyping and determining sperm precedence in two families would not produce strong conclusions, they could provide a proof of method. Previous research has shown that both beetles and sperm collected from the spermathecae can be genotyped using this method and the same microsatellite loci (Bermond et al. 2014). Results from egg batches in this experiment demonstrated that they can also be genotyped using this method. If a greater sample size of twice-mated females could be collected (possibly by leaving females housed continuously with males of known genotypes), this genotyping method and these microsatellite loci could be used to identify sperm precedence. In addition, this method could be used to genotype field females and their offspring to produce, not only an estimation of the proportion of the population that mates more than once, but also a minimum estimation of the number of mates a female accepts based on the number of alleles present at a given locus in the offspring.

Conclusions

Multiple mating by females is difficult to identify because visible evidence of a mating (the spermatophore) can be completely absorbed by the female within five to seven days (Lew and Ball 1980). Unless a female was directly observed mating twice or was collected shortly after a second mating, it could be nearly impossible to determine the number of times a female had mated without using genomic methods. Since the mean interval between first and second

matings was similar to the time required for the spermatophore to be absorbed, the absorption of the spermatophore may play a role in determining when or if a female mates again. In some species, the presence of the spermatophore is a physical barrier to an additional mating (Thornhill and Alcock 1983). Observation of some western corn rootworm females with two spermatophores, including two large spermatophores in their bursa copulatrix, suggests the presence of a rootworm spermatophore may not physically prevent all subsequent matings. If females may mate again when a spermatophore is completely or partially absorbed (Chapter 2 and chapter 4 of this dissertation) and the females live on average 57 days, they may have the capacity to mate several times before and between oviposition.

This research has shown that 8.5% of females mated more than once. While multiple mating was observed in a small proportion of females, it highlights the fact that there is still much to be learned about western corn rootworm reproduction. One of the major benefits of polyandry is that females may gain advantages from nuptial gifts, like spermatophores, which provide sperm as well as nutrients that can be absorbed by females (Arnqvist and Nilsson 2000). The large size of the male's spermatophore (equivalent to up to 9% of their body mass; Tallamy et al. 2000) and the need to replenish resources contributed to the female during mating may explain why males mate fewer times than was previously thought. Western corn rootworm spermatophores have a fairly high protein content when compared to other insects (Murphy and Krupke 2011).. Western corn rootworm spermatophores have a fairly high protein content when compared to other insects (Murphy and Krupke 2011). Nutrients and other compounds provided to females in spermatophores have been identified in their developing eggs (Murphy and Krupke 2011) and in fat body surrounding the ovaries (Tallamy et al. 2000).

Detecting females that remate later in life may be particularly difficult by direct observation of dissection, yet these females could still benefit greatly from remating. Females require a continuous source of energy and nutrients to continue producing eggs (Branson and Krysan 1981). As the growing season progresses and host plant quality begins to decline (Darnell et al. 2000), the nutrients gained from mating and absorbing a spermatophore may provide nutritional benefits to the female. Evidence for this type of late mating may come from mating activity observed late in the season when adult emergence is low (Chapter 2 of this dissertation, Figures 2.3 through 2.5).

The refuge strategy for Bt corn targeting corn rootworm beetles relies heavily on our understanding of western corn rootworm mating behavior. By investigating rootworm mating behavior in the field, practical knowledge about the efficacy of our current IRM strategies can be obtained. A deeper understanding of beetle reproductive capabilities (i.e. mating frequency) should also inform the modeling and design of future IRM plans.

Use of microsatellites to genotype beetles and their offspring to identify sperm precedence eliminates the subjectivity of using sterile males (Branson et al. 1977) and time intensive nature of breeding a population of beetles to express marker phenotypes (Oyediran 2002 Chapter 2). While polyandrous females may represent only a small proportion of the population, twice-mating females may be more likely to encounter Bt emerging males during their second mating. If last male sperm precedence is the rule in western corn rootworm, even Bt susceptible females that initially mated a refuge male may eventually produce offspring carrying resistance alleles. Understanding western corn rootworm sperm precedence and the proportion of females likely to mate more than once will enhance the prospects for effective insect resistance management.

TABLES

Table 5.1. Date of emergence, age (d) at mating and death for each female western corn rootworm that mated twice and the age of the second male in each family group.

Female ID	Female emergence date	Age of female at 1st mating (d) [a]	Age of female at 2nd mating (d)	Age of 2nd male (d)	Age of female at death (d)
Fam02 - F	6/24/2016	1	12	7	101
Fam03 - F	7/07/2016	1	5	10	58
Fam04 - F	7/07/2016	1	5	11	93
Fam05 - F	7/14/2016	1	9	10	51
Fam06 - F	7/22/2016	1	3	6	28
Fam07 - F	7/12/2016	1	11	10	11

[a] Age dates were recorded based of the date of adult emergence, "1 d" represents day one which included the first 24 hours after emergence.

Table 5.2. Age of first oviposition (d), duration of ovipositional period (d), weeks within ovipositional period when eggs were laid, mean eggs laid per week and total fecundity of female western corn rootworms that mated twice.

Female ID	Age of first oviposition (d)	Ovipositional period (d)	Weeks with oviposition [a]	Mean eggs per week	Total eggs laid
Fam02 - F	39	54	5 / 8	8.0	40
Fam03 - F	20	39	5 / 6	31.0	155
Fam04 - F	20	74	10 / 11	30.5	305
Fam05 - F	19	33	5 / 5	65.4	185
Fam06 - F	15	6 [b]	2 / 2	21.0	42
Fam07 - F [c]	-	-	-	-	-

[a] The number of weeks a female oviposited with the oviposition range, excluding weeks when oviposition did not occur.
[b] Female 6 oviposited over a 6 day period but the dates of oviposition spanned on two different designated collection weeks.
[c] This female died before oviposition.

FIGURES

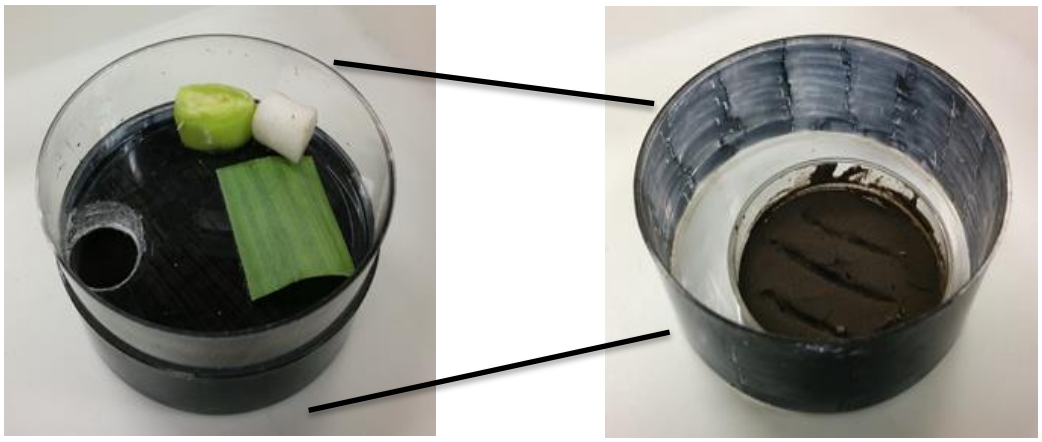

Figure 5.1. Western corn rootworm oviposition container. Consisting of two stacked 6 cm petri dishes, the lower dish contained a 3.5 cm diameter petri dish of moistened 80 mesh field moist soil for oviposition. A 6 cm petri dish containing food and a water in a cotton wick with a 1.5 cm hole was stacked atop the lower dish. Individual beetle housed in each dish accessed the darkened lower petri dish through the 1.5 cm hole. When in use, the top dish was covered with a clear lid.

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APPENDIX A: ADDITIONAL MEASUREMENTS OF MATING CHARACTERISTICS AND ABUNDANCE

1) Statistical analyses

The distribution of western corn rootworm (*Diabrotica virgifera virgifera* LeConte) mating abundance in four refuge treatments is presented in Tables A.1 through A.3. The mating pairs collected per minute (mating pairs / min ± SE) each year, during the vegetative, pollination and post-pollination periods within each refuge treatment were analyzed by sampling row location. The data were log (x + 0.5) transformed and analyzed using ANOVA and the Tukey-Kramer test of multiple comparisons using SAS 9.3® PROC MIXED (SAS Institute Inc., Cary, NC, 2011).

In 2012, only paired females were dissected to record reproductive characteristics because of time constraints. As a result, the reproductive characteristics could not be compared to those of individually collected females and are presented here. The proportion of paired females that were mature, had one or more spermatophores in their bursa copulatrix and had sperm in their spermatheca are presented in Table A.4. The mean ovarian development, number of spermatophores and number of mature eggs collected in paired females in 2012 are presented in Table A.5.

2) Patterns of mating abundance among rows

Mating abundance in 20% and 5% structured refuge treatments was greatest in refuge rows during the vegetative and pollination periods form 2010 through 2012 (Tables A.1 through A.3). During post-pollination in 20% and 5% structured refuge treatments, there was no difference in mating abundance among refuge and Bt sampling rows (Tables A.1 through A.3),

except in the 5% structured refuge treatments in 2010, then mating activity remained higher in refuge rows compared to Bt sampling rows (Table A.1). Mating activity in 5% seed blend and 0% refuge treatments was low each year and evenly distributed across the fields all three years (Tables A.1 through A.3).

3) Reproductive measures for paired females in 2012

Among paired females collected in 2012, 74% were mature (non-teneral) at the time of mating suggesting that they were over 24 hours old, 87% had a received a spermatophore, indicating that they had been mating at least 1.5 hours (Lew and ball 1980), and 42% had sperm in their spermatheca, indicating that they had been mating at least two hours (Lew and Ball 1980) at the time of collection (Table A.4). Paired females collected in 2012 had a mean ovary development rating of 1.08 ± 0.02 indicating that these females were young. They contained an average of 0.89 ± 0.02 spermatophores, indicating that most females had only one spermatophore in their bodies, although some were collected with two spermatophores (Table A.5). None of the paired females had stage 4 ovaries or mature eggs (Table A.5).

TABLES

Table A.1. The spatial and temporal abundance of mating western corn rootworms (mating pairs / min; mean ± SE) analyzed by sampling row in each refuge treatment and corn phenology in 2010.

Treatment	Row [b]	n [c]	Vegetative [a] Mating pairs /min (± SE)	n	Pollination Mating pairs /min (± SE)	n	Post-Pollination Mating pairs /min (± SE)
20% Structured Refuge	W20-Rfg	30	0.04 ± 0.02ab	19	0.10 ± 0.04ab	41	0.04 ± 0.02a
	W12-Rfg	30	0.06 ± 0.02a	19	0.11 ± 0.04a	41	0.03 ± 0.02a
	W3-Rfg	30	0.02 ± 0.01ab	19	0.03 ± 0.02abc	41	0.01 ± 0.01a
	E3-Bt	30	0.00 ± 0.00b	19	0.01 ± 0.01bc	41	0.01 ± 0.01a
	E12-Bt	30	0.00 ± 0.00b	19	0.00 ± 0.00bc	41	0.02 ± 0.01a
	E24-Bt	30	0.00 ± 0.00b	19	0.00 ± 0.00bc	40	0.00 ± 0.00a
	E36-Bt	30	0.00 ± 0.00b	19	0.03 ± 0.02abc	40	0.01 ± 0.01a
	E48-Bt	30	0.01 ± 0.01b	19	0.00 ± 0.00c	40	0.01 ± 0.01a
	E60-Bt	30	0.01 ± 0.01b	19	0.00 ± 0.00c	40	0.01 ± 0.01a
	E72-Bt	30	0.00 ± 0.00b	19	0.00 ± 0.00c	40	0.03 ± 0.02a
	E84-Bt	29	0.00 ± 0.00b	19	0.00 ± 0.00c	40	0.02 ± 0.01a
5% Structured Refuge	W4-Rfg	30	0.08 ± 0.03a	19	0.15 ± 0.04a	43	0.13 ± 0.04a
	W2-Rfg	29	0.05 ± 0.02ab	19	0.14 ± 0.05a	43	0.08 ± 0.03ab
	E3-Bt	30	0.01 ± 0.01bc	19	0.00 ± 0.00b	43	0.02 ± 0.01c
	E12-Bt	30	0.00 ± 0.00c	19	0.02 ± 0.02b	43	0.02 ± 0.01c
	E24-Bt	30	0.00 ± 0.00c	19	0.02 ± 0.02b	42	0.01 ± 0.01c
	E36-Bt	30	0.00 ± 0.00c	19	0.02 ± 0.02b	43	0.00 ± 0.00c
	E48-Bt	30	0.00 ± 0.00c	19	0.01 ± 0.01b	44	0.01 ± 0.01c
	E60-Bt	30	0.00 ± 0.00c	19	0.00 ± 0.00b	44	0.04 ± 0.02abc
	E72-Bt	30	0.00 ± 0.00c	19	0.01 ± 0.01b	44	0.00 ± 0.00c
	E84-Bt	30	0.00 ± 0.00c	19	0.01 ± 0.01b	44	0.00 ± 0.00c
	E96-Bt	30	0.00 ± 0.00c	19	0.00 ± 0.00b	44	0.02 ± 0.01c
5% Seed Blend	W4-Sb	30	0.00 ± 0.00b	19	0.01 ± 0.01a	41	0.05 ± 0.02a
	W2-Sb	30	0.00 ± 0.00b	19	0.03 ± 0.02a	41	0.04 ± 0.02a
	E3-Sb	30	0.01 ± 0.01ab	19	0.00 ± 0.00a	41	0.02 ± 0.01a
	E12-Sb	29	0.00 ± 0.00ab	19	0.00 ± 0.00a	41	0.02 ± 0.01a
	E24-Sb	29	0.00 ± 0.00ab	19	0.01 ± 0.01a	41	0.02 ± 0.01a
	E36-Sb	29	0.03 ± 0.01a	19	0.00 ± 0.00a	41	0.01 ± 0.01a
	E48-Sb	29	0.00 ± 0.00ab	19	0.04 ± 0.02a	41	0.02 ± 0.01a
	E60-Sb	29	0.00 ± 0.00ab	19	0.00 ± 0.00a	41	0.04 ± 0.02a
	E72-Sb	29	0.01 ± 0.01ab	19	0.00 ± 0.00a	41	0.03 ± 0.02a
	E84-Sb	29	0.00 ± 0.00ab	19	0.01 ± 0.01a	41	0.02 ± 0.02a
	E96-Sb	29	0.00 ± 0.00ab	19	0.00 ± 0.00a	41	0.01 ± 0.01a
0% Refuge	W4-Bt	29	0.00 ± 0.00a	19	0.03 ± 0.02a	43	0.04 ± 0.02a
	W2-Bt	30	0.00 ± 0.00a	19	0.00 ± 0.00a	43	0.02 ± 0.02a
	E3-Bt	30	0.01 ± 0.01a	19	0.00 ± 0.00a	43	0.01 ± 0.01a
	E12-Bt	30	0.00 ± 0.00a	19	0.00 ± 0.00a	43	0.01 ± 0.01a
	E24-Bt	30	0.00 ± 0.00a	19	0.00 ± 0.00a	43	0.02 ± 0.01a
	E36-Bt	30	0.00 ± 0.00a	19	0.00 ± 0.00a	43	0.04 ± 0.02a
	E48-Bt	30	0.00 ± 0.00a	19	0.00 ± 0.00a	43	0.01 ± 0.01a
	E60-Bt	30	0.00 ± 0.00a	19	0.00 ± 0.00a	43	0.03 ± 0.01a
	E72-Bt	30	0.00 ± 0.00a	19	0.01 ± 0.01a	43	0.01 ± 0.01a
	E84-Bt	30	0.00 ± 0.00a	19	0.00 ± 0.00a	43	0.02 ± 0.01a
	E96-Bt	30	0.00 ± 0.00a	19	0.04 ± 0.03a	43	0.03 ± 0.01a

The mean number of mating pairs collected per minute (mating pairs / min ± SE) in each sampling row for each refuge treatment and period of corn phenology in 2010. ANOVA and the Tukey-Kramer of multiple comparisons were performed on log (x + 0.5) transformed means to determine differences in mating pair abundance among sampling rows, illustrating the spatial distribution of mating activity within each refuge treatment and phenology. Means followed by the same letter are not significantly different ($\alpha = 0.05$). Untransformed means are shown.

[a] Phenology was determined based on Ritchie et al. 1993 and Abendroth et al. 2011.

[b] Letters and numbers indicate the location of each sampling row relative to the interface between refuge and Bt corn types. The suffixes Rfg, Sb and Bt indicate that the sampling row contained refuge, seed blend or Bt corn, respectively. See text for further explanation.

[c] n indicates the number of mating pair collections in each sampling row within each refuge treatment and phenology period.

Table A.2. The spatial and temporal abundance of mating western corn rootworms (mating pairs / min; mean ± SE) analyzed by sampling row in each refuge treatment and corn phenology in 2011.

Treatment	Row [b]	n [c]	Vegetative [a] Mating pairs /min (± SE)	n	Pollination Mating pairs /min (± SE)	n	Post-Pollination Mating pairs /min (± SE)
20% Structured Refuge	W20-Rfg	27	0.21 ± 0.06a	18	0.21 ± 0.07a	24	0.00 ± 0.00a
	W12-Rfg	27	0.11 ± 0.04ab	18	0.08 ± 0.04ab	24	0.01 ± 0.01a
	W3-Rfg	27	0.06 ± 0.03bc	18	0.05 ± 0.02b	24	0.00 ± 0.00a
	E3-Bt	27	0.01 ± 0.01bc	18	0.06 ± 0.03b	24	0.01 ± 0.01a
	E12-Bt	27	0.00 ± 0.00c	18	0.03 ± 0.02b	24	0.02 ± 0.01a
	E24-Bt	27	0.01 ± 0.01bc	18	0.06 ± 0.03b	24	0.02 ± 0.01a
	E36-Bt	27	0.00 ± 0.00c	18	0.00 ± 0.00b	24	0.02 ± 0.01a
	E48-Bt	27	0.00 ± 0.00c	18	0.06 ± 0.03b	24	0.01 ± 0.01a
	E60-Bt	27	0.00 ± 0.00c	18	0.04 ± 0.03b	24	0.00 ± 0.00a
	E72-Bt	27	0.00 ± 0.00c	18	0.00 ± 0.00b	24	0.01 ± 0.01a
	E84-Bt	27	0.00 ± 0.00c	18	0.01 ± 0.01b	24	0.02 ± 0.02a
5% Structured Refuge	W4-Rfg	30	0.14 ± 0.06a	19	0.08 ± 0.05ab	43	0.06 ± 0.03a
	W2-Rfg	29	0.14 ± 0.05a	19	0.15 ± 0.05a	43	0.04 ± 0.02a
	E3-Bt	30	0.01 ± 0.01b	19	0.04 ± 0.02ab	43	0.01 ± 0.01a
	E12-Bt	30	0.00 ± 0.00b	19	0.00 ± 0.00b	43	0.02 ± 0.01a
	E24-Bt	30	0.01 ± 0.01b	19	0.01 ± 0.01b	42	0.03 ± 0.02a
	E36-Bt	30	0.00 ± 0.00b	19	0.01 ± 0.01b	43	0.03 ± 0.02a
	E48-Bt	30	0.00 ± 0.00b	19	0.03 ± 0.02b	44	0.00 ± 0.00a
	E60-Bt	30	0.00 ± 0.00b	19	0.01 ± 0.01b	44	0.03 ± 0.02a
	E72-Bt	30	0.01 ± 0.01b	19	0.01 ± 0.01b	44	0.01 ± 0.01a
	E84-Bt	30	0.00 ± 0.00b	19	0.00 ± 0.00b	44	0.00 ± 0.00a
	E96-Bt	30	0.00 ± 0.00b	19	0.01 ± 0.01b	44	0.03 ± 0.02a
5% Seed Blend	W4-Sb	27	0.02 ± 0.01a	18	0.07 ± 0.03a	24	0.09 ± 0.03ab
	W2-Sb	27	0.01 ± 0.01a	18	0.03 ± 0.02a	24	0.07 ± 0.02ab
	E3-Sb	27	0.01 ± 0.01a	18	0.01 ± 0.01a	24	0.13 ± 0.04a
	E12-Sb	27	0.00 ± 0.00a	18	0.06 ± 0.04a	24	0.06 ± 0.02ab
	E24-Sb	27	0.00 ± 0.00a	18	0.03 ± 0.02a	24	0.10 ± 0.03ab
	E36-Sb	27	0.02 ± 0.01a	18	0.00 ± 0.00a	24	0.02 ± 0.02ab
	E48-Sb	27	0.01 ± 0.01a	18	0.01 ± 0.01a	24	0.01 ± 0.01b
	E60-Sb	27	0.00 ± 0.00a	18	0.01 ± 0.01a	24	0.00 ± 0.00b
	E72-Sb	27	0.01 ± 0.01a	18	0.01 ± 0.01a	24	0.02 ± 0.01ab
	E84-Sb	27	0.00 ± 0.00a	18	0.01 ± 0.01a	24	0.01 ± 0.01b
	E96-Sb	27	0.02 ± 0.01a	18	0.00 ± 0.00a	24	0.04 ± 0.02ab
0% Refuge	W4-Bt	27	0.01 ± 0.01a	18	0.06 ± 0.03a	24	0.08 ± 0.04a
	W2-Bt	27	0.01 ± 0.01a	18	0.05 ± 0.02a	24	0.09 ± 0.04a
	E3-Bt	27	0.00 ± 0.00a	18	0.00 ± 0.00a	24	0.01 ± 0.01a
	E12-Bt	27	0.00 ± 0.00a	18	0.07 ± 0.03a	24	0.01 ± 0.01a
	E24-Bt	27	0.01 ± 0.01a	18	0.01 ± 0.01a	24	0.01 ± 0.01a
	E36-Bt	27	0.00 ± 0.00a	18	0.01 ± 0.01a	24	0.01 ± 0.01a
	E48-Bt	27	0.00 ± 0.00a	18	0.01 ± 0.01a	24	0.00 ± 0.00a
	E60-Bt	27	0.01 ± 0.01a	18	0.00 ± 0.00a	24	0.01 ± 0.01a
	E72-Bt	27	0.00 ± 0.00a	18	0.00 ± 0.00a	24	0.00 ± 0.00a
	E84-Bt	27	0.00 ± 0.00a	18	0.01 ± 0.01a	24	0.05 ± 0.03a
	E96-Bt	27	0.00 ± 0.00a	18	0.03 ± 0.02a	24	0.00 ± 0.00a

The mean number of mating pairs collected per minute (mating pairs / min ± SE) in each sampling row are presented for each refuge treatment and period of corn phenology in 2011. ANOVA and the Tukey-Kramer of multiple comparisons were performed on log (x + 0.5) transformed means to determine differences in mating pair abundance among sampling rows, illustrating the spatial distribution of mating activity within each refuge treatment and phenology. Means followed by the same letter are not significantly different ($\alpha = 0.05$). Untransformed means are shown.

[a] Phenology was determined based on Ritchie et al. 1993 and Abendroth et al. 2011.

[b] Letters and numbers indicate the location of each sampling row relative to the interface between refuge and Bt corn types. The suffixes Rfg, Sb and Bt indicate that the sampling row contained refuge, seed blend or Bt corn, respectively. See text for further explanation.

[c] n indicates the number of mating pair collections in each sampling row within each refuge treatment and phenology period.

Table A.3. The spatial and temporal abundance of mating western corn rootworms (mating pairs / min; mean ± SE) analyzed by sampling row in each refuge treatment and corn phenology in 2012.

Treatment	Row [b]	n [c]	Vegetative [a] Mating pairs /min (± SE)	n	Pollination Mating pairs /min (± SE)	n	Post-Pollination Mating pairs /min (± SE)
20% Structured Refuge	W20-Rfg	39	0.06 ± 0.03a	18	0.17 ± 0.06abc	45	0.00 ± 0.00a
	W12-Rfg	39	0.03 ± 0.02ab	18	0.19 ± 0.07ab	45	0.01 ± 0.01a
	W3-Rfg	38	0.05 ± 0.03ab	18	0.21 ± 0.07a	45	0.00 ± 0.00a
	E3-Bt	39	0.00 ± 0.00ab	18	0.08 ± 0.04abc	45	0.01 ± 0.01a
	E12-Bt	39	0.01 ± 0.01b	18	0.04 ± 0.02abc	45	0.00 ± 0.00a
	E24-Bt	39	0.01 ± 0.01b	18	0.00 ± 0.00c	45	0.01 ± 0.01a
	E36-Bt	39	0.00 ± 0.00ab	18	0.01 ± 0.01abc	45	0.01 ± 0.01a
	E48-Bt	39	0.00 ± 0.00b	18	0.03 ± 0.02abc	45	0.00 ± 0.00a
	E60-Bt	39	0.00 ± 0.00b	18	0.01 ± 0.01abc	45	0.00 ± 0.00a
	E72-Bt	39	0.00 ± 0.00b	18	0.01 ± 0.01abc	45	0.01 ± 0.01a
	E84-Bt	39	0.01 ± 0.01b	18	0.01 ± 0.01abc	45	0.01 ± 0.01a
5% Structured Refuge	W4-Rfg	39	0.11 ± 0.04a	18	0.16 ± 0.08a	42	0.01 ± 0.01a
	W2-Rfg	39	0.02 ± 0.01b	18	0.09 ± 0.04ab	42	0.01 ± 0.01a
	E3-Bt	39	0.00 ± 0.00b	18	0.06 ± 0.04ab	42	0.01 ± 0.01a
	E12-Bt	39	0.00 ± 0.00b	18	0.06 ± 0.03ab	42	0.01 ± 0.01a
	E24-Bt	39	0.00 ± 0.00b	18	0.00 ± 0.00b	42	0.01 ± 0.01a
	E36-Bt	39	0.00 ± 0.00b	18	0.01 ± 0.01ab	42	0.00 ± 0.00a
	E48-Bt	39	0.00 ± 0.00b	18	0.01 ± 0.01ab	42	0.00 ± 0.00a
	E60-Bt	39	0.01 ± 0.01b	18	0.00 ± 0.00b	42	0.01 ± 0.01a
	E72-Bt	39	0.00 ± 0.00b	18	0.02 ± 0.02ab	42	0.00 ± 0.00a
	E84-Bt	39	0.00 ± 0.00b	18	0.00 ± 0.00b	42	0.00 ± 0.00a
	E96-Bt	39	0.00 ± 0.00b	18	0.00 ± 0.00b	42	0.01 ± 0.01a
5% Seed Blend	W4-Sb	39	0.00 ± 0.00a	18	0.04 ± 0.02a	41	0.01 ± 0.01a
	W2-Sb	39	0.00 ± 0.00a	18	0.03 ± 0.02a	41	0.00 ± 0.00a
	E3-Sb	39	0.01 ± 0.01a	18	0.05 ± 0.03a	41	0.01 ± 0.01a
	E12-Sb	39	0.01 ± 0.01a	18	0.01 ± 0.01a	41	0.01 ± 0.01a
	E24-Sb	39	0.00 ± 0.00a	18	0.03 ± 0.02a	41	0.01 ± 0.01a
	E36-Sb	39	0.00 ± 0.00a	18	0.03 ± 0.02a	41	0.00 ± 0.00a
	E48-Sb	39	0.01 ± 0.01a	18	0.01 ± 0.01a	41	0.01 ± 0.01a
	E60-Sb	39	0.00 ± 0.00a	18	0.01 ± 0.01a	41	0.01 ± 0.01a
	E72-Sb	39	0.00 ± 0.00a	18	0.00 ± 0.00a	41	0.01 ± 0.01a
	E84-Sb	39	0.01 ± 0.01a	18	0.04 ± 0.03a	41	0.00 ± 0.00a
	E96-Sb	39	0.01 ± 0.01a	18	0.06 ± 0.03a	41	0.00 ± 0.00a
0% Refuge	W4-Bt	39	0.00 ± 0.00a	17	0.01 ± 0.01a	41	0.00 ± 0.00a
	W2-Bt	39	0.01 ± 0.01a	17	0.04 ± 0.02a	41	0.00 ± 0.00a
	E3-Bt	39	0.01 ± 0.01a	17	0.08 ± 0.03a	41	0.01 ± 0.01a
	E12-Bt	39	0.00 ± 0.00a	17	0.04 ± 0.02a	41	0.01 ± 0.01a
	E24-Bt	39	0.00 ± 0.00a	17	0.06 ± 0.03a	41	0.01 ± 0.01a
	E36-Bt	39	0.00 ± 0.00a	17	0.05 ± 0.03a	41	0.01 ± 0.01a
	E48-Bt	39	0.01 ± 0.01a	17	0.01 ± 0.01a	41	0.01 ± 0.01a
	E60-Bt	39	0.00 ± 0.00a	17	0.00 ± 0.00a	41	0.01 ± 0.01a
	E72-Bt	39	0.01 ± 0.01a	17	0.00 ± 0.00a	41	0.00 ± 0.00a
	E84-Bt	39	0.00 ± 0.00a	17	0.00 ± 0.00a	41	0.00 ± 0.00a
	E96-Bt	39	0.00 ± 0.00a	17	0.00 ± 0.00a	41	0.00 ± 0.00a

The mean number of mating pairs collected per minute (mating pairs / min ± SE) in each sampling row are presented for each refuge treatment and period of corn phenology in 2012. ANOVA and the Tukey-Kramer of multiple comparisons were performed on log (x + 0.5) transformed means to determine differences in mating pair abundance among sampling rows, illustrating the spatial distribution of mating activity within each refuge treatment and phenology. Means followed by the same letter are not significantly different ($\alpha = 0.05$). Untransformed means are shown.

[a] Phenology was determined based on Ritchie et al. 1993 and Abendroth et al. 2011.

[b] Letters and numbers indicate the location of each sampling row relative to the interface between refuge and Bt corn types. The suffixes Rfg, Sb and Bt indicate that the sampling row contained refuge, seed blend or Bt corn, respectively. See text for further explanation.

[c] n indicates the number of mating pair collections in each sampling row within each refuge treatment and phenology period.

Table A.4. The proportions of paired female western corn rootworms that were mature, had at least one spermatophore present in their bodies or had sperm in their spermathecae upon collection in 2012.

	Paired	
Reproductive measure	n [a]	Proportion [c]
Maturity [b]	238	0.74
Spermatophore Presence	238	0.87
Sperm in Spermatheca	238	0.42

[a] Number of paired females dissected and observed in 2012.
[b] Proportion of females testing positive for a given measure within the same mating status and year combination.
[c] Proportion of females that were non-teneral.

Table A.5. The mean (± SE) ovary development, number of spermatophores and mature eggs observed in paired female western corn rootworms upon collection in 2012.

	Paired	
Reproductive measure	n [a]	Mean ± SE
Ovary Development [b]	238	1.08 ± 0.02
No. Spermatophores	238	0.89 ± 0.02
Mature Eggs	0	--

[a] Number of paired females dissected and observed in 2012.
[b] Ovary development was scored on a scale of 1 to 4 based on Short and Hill 1972. During stage 1, no ovary development was observed and during stage 4, mature eggs were observed.

APPENDIX B: SEX RATIO OF BEETLE EMERGENCE AND ABUNDANCE

Table B.1. The total western corn rootworm adults collected from single-plant emergence traps by plant type and refuge treatment and male:female sex ratios from 2010 through 2012.

		Refuge plants			Bt plants		
Year	Refuge treatment	Total males	Total females	M:F sex ratio [a]	Total males	Total females	M:F sex ratio
2010	20% Structured	30	86	0.349	6	8	0.750
	5% Structured	176	126	1.397	12	20	0.600
	5% Seed Blend	40	92	0.435	16	28	0.571
	0% Refuge [b]	N.A.	N.A.	N.A.	18	36	0.500
2011	20% Structured	140	290	0.483	26	66	0.394
	5% Structured	92	160	0.575	4	48	0.083
	5% Seed Blend	68	206	0.330	32	68	0.471
	0% Refuge	N.A.	N.A.	N.A.	98	200	0.490
2012	20% Structured	310	434	0.714	66	146	0.452
	5% Structured	508	570	0.891	74	124	0.597
	5% Seed Blend	70	188	0.372	60	130	0.462
	0% Refuge	N.A.	N.A.	N.A.	148	254	0.583

[a] Sex ratios > 1.0 are male biased and those < 1.0 are female biased. Refer to Hughson and Spencer 2015 for details about emergence and field abundance collections.
[b] There are no refuge plants in 0% refuge.

Table B.2. The total free-moving western corn rootworm adults collected from refuge, Bt and seed blend refuge treatments and male:female sex ratios from 2010 through 2012.

		Refuge rows			Bt or seed blend rows		
Year	Refuge Treatment	Total males	Total females	M:F sex ratio [a]	Total males	Total females	M:F sex ratio
2010	20% Structured	955	666	1.434	622	680	0.915
	5% Structured	966	784	1.232	895	971	0.922
	5% Seed Blend [b]	N.A.	N.A.	N.A.	1188	1387	0.857
	0% Refuge [c]	N.A.	N.A.	N.A.	1085	1266	0.857
2011	20% Structured	1143	2361	0.484	741	1099	0.674
	5% Structured	737	1734	0.425	807	1514	0.533
	5% Seed Blend	N.A.	N.A.	N.A.	1622	2667	0.608
	0% Refuge	N.A.	N.A.	N.A.	1149	2327	0.494
2012	20% Structured	2141	2323	0.922	3008	2601	1.156
	5% Structured	1388	1274	1.089	2353	2334	1.008
	5% Seed Blend	N.A.	N.A.	N.A.	3264	3166	1.031
	0% Refuge	N.A.	N.A.	N.A.	3034	3333	0.910

[a] Sex ratios > 1.0 are male biased and those < 1.0 are female biased.
[b] Seed Blend rows contain both Refuge and Bt plants; beetle collections cannot be assigned to one plant type in particular. Refer to Hughson and Spencer 2015 for details about emergence and field abundance collections.
[c] There are no refuge plants in 0% refuge.