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For the degree of Master of Science

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FACT OR FICTION:
RANDOM MATING IN FIELD POPULATIONS OF WESTERN CORN ROOTWORM (DIABROTICA VIRGIFERA VIRGIFERA LECONTE) EMERGING ON BT AND REFUGE CORN PLANTS

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ABSTRACT

Smith, Steven Joel. M.S., Purdue University August 2014. Fact or Fiction: Random Mating in Field Populations of Western Corn Rootworm (*Diabrotica virgifera virgifera* LeConte) Emerging from Bt and Refuge Corn Plants. Major Professor: Christian Krupke.

The western corn rootworm, or WCR, (*Diabrotica virgifera virgifera* LeConte) is the most significant pest of field corn (*Zea mays*) in the United States, and has recently expanded its range into Europe. Since 2004, hybrid corn containing Bt toxins targeting the corn rootworm complex have been heavily adopted and are now the primary control measure for this pest in North American corn production.

The evolution of resistance is an ongoing concern, and to ensure Bt products will retain their usefulness, insect resistance management (IRM) tactics using various refuge structures have been adopted. One of the key tenets of the refuge strategy is that males and females emerging from Bt and refuge plantings mate randomly. A violation of this largely untested assumption would lead to acceleration of resistance development.

To generate empirical field data on mating rates between beetles emerging from Bt and refuge plants, field cage studies using field populations of WCR in Indiana were utilized. Various refuge configurations were tested; all refuge plants were labeled using the stable isotope N^{15}. This mark persists in the adult beetles after eclosion, allowing for collection and analysis of isotopic ratios of beetles in mating pairs. This approach was

used to test the random mating assumption in Bt and refuge beetles collected from field cages. Other data collected include emergence rates, timing and sex ratios for each of the treatments.

Results indicate that mating based on natal host may not be as important of a factor as initially thought. Mixed mating occurs at a high rate when there are higher numbers of susceptible rootworms even though the measured fitness parameters between *Cry3Bb1* and refuge adults were significantly different ($p < 0.05$). The main indication from this study is that not enough susceptible individuals are produced from a 5% refuge-in-a-bag strategy which is the dominant form of refuge planting in the United States.

CHAPTER 1. INTRODUCTION AND BACKGROUND

The western corn rootworm (WCR) (*Diabrotica virgifera virgifera* LeConte (Coleoptera: Chrysomelidae)) was first identified as a pest of corn production in 1909 (Gillette, 1912) and today is the most economically important pest of corn (*Zea mays*) production in the US (Spencer et al., 2009). Historically, rootworms have easily developed resistance to insecticides, as well as the ability to overcome cultural practices (Spencer et al., 2009), causing an estimated annual economic loss to growers that exceeds $1 billion US (Metcalf, 1986). The eastward spread of rootworms in the US, both WCR and northern corn rootworm (NCR) (*Diabrotica barberi* Smith and Lawrence (Coleoptera: Chrysomelidae)), is thought to be largely caused by the practice of planting continuous corn that began in the late 1940's (Krysan and Branson, 1983). WCR is the dominant pest throughout most of the US Corn Belt. With the continuing high pest status of WCR, combined with historically high commodity prices, the new economic loss estimate far exceeds what Metcalf had proposed (Gray et al., 2009). This situation is also exacerbated by losses in rotated corn (Mitchell et al., 2004) and the introduction of WCR into Europe (Kiss et al., 2005). In 1992, the first reports of WCR adults were detected in a field of corn near the Belgrade Airport in Serbia (Baca, 1994). Because of the discovery being in the proximity of the airport, it is theorized that WCR made its initial establishment in Europe via commercial planes (Gray et al., 2009). By 2007, WCR had spread to 20

European countries (Gray et al., 2009) with at least three different points of introduction (Miller et al., 2005). Current WCR distributions in both North America and Europe can be found at http://extension.entm.purdue.edu/wcr/ (C. R. Edwards, 2012).

In 2013, growers in the US planted 95.4 million acres of field corn which produced 13.9 billion bushels and generated $62.7 billion in revenue (NASS, 2013), resulting in corn being the largest US crop in both volume and value. Corn production has significantly increased throughout US history and yield has been improved through production practices and technology, ultimately increasing US corn production to nearly 40% of the world supply (USDA-ERS, 2010). Corn primarily serves as the main feed grain for livestock and in human food products (Senti and Schaefer, 1972). Corn is also used for ethanol production (Pimentel and Patzek, 2005).

1.1 WCR Lifecycle and Behavioral Ecology

Larvae of WCR feed on the roots of corn and can cause reduced water and nutrient uptake, aid in pathogen entry and reduce the ability of the plant to resist lodging (Levine and Oloumi-Sadeghi, 1991). After three instars, larvae pupate for nearly two weeks and emerge as adults and begin to feed primarily on corn pollen and silks (Peairs and Pilcher, 2006). Male WCR are generally the first to emerge (approximately 5-7 d before females) (Branson, 1987), about 80% of which require post-emergence development to reach sexual maturity (Guss, 1976). Male response to the female pheromone primarily dictates male dispersal, although males will generally only travel as far as needed in order to find a mate (Marquardt and Krupke, 2009). Females are sexually mature upon emergence (Hammack, 1995). Most females mate within hours of emergence (Ball, 1957), and most often on the same plant where emergence occurs. Quiring and Timmins (1990) showed increased numbers of mating pairs coinciding with peaks of adult female emergence, indicating rapid mating of females.

Several important factors have been identified in recent years relating to the mating behaviors of WCR that could have implications for success of refuge strategies in Bt corn fields. Male WCR have greater mating ability when less than 10 d old, as discovered by Kang and Krupke (2009a). As males age, the ability to mate declines quite rapidly after sexual maturity is reached (Spencer et al, 2012), indicating that males may have less incentive to travel long distances to find a mate (Kang and Krupke, 2009a). Females remain close to where emergence occurred prior to mating, which means that males are the primary dispersers and promote gene mixing. After mating, females require a pre-ovipositional period that can last between 5-42 d (Bayar et al., 2002), during which females are more likely to disperse to locate optimal oviposition sites. Typically females will only mate once, while males will attempt to mate several times (Hill, 1975). Under optimal conditions, WCR females can produce an average of 440 viable eggs (Boetel and Fuller, 1997). Fisher et al. (1991) observed that over an 8 wk oviposition period, the percentage of viable eggs (eggs that hatch) declined from approximately 80% to 30%.

During copulation, males transfer a spermatophore to the female along with the sperm packet. The spermatophore in many species of insects is a “nuptial gift” and contains nutrients, mostly proteins and some carbohydrates, which benefit the female in egg development (Boggs and Gilbert, 1979; Bissoondath and Wiklund, 1995; Heller et al., 1998). Spermatophores can also serve as protection to the female and eggs. Male southern corn rootworms (*Diabrotica undecimpunctata howardi Barber)* actively ingest cucurbitacins found in cucurbits and transfer the toxin to the female via their spermatophore. The toxin then serves to protect the female from predation and is stored in the fat body, cuticle, haemolymph, and developing eggs (Ferguson and Metcalf, 1985; Andersen et al., 1988; Tallamy et al., 2000). In WCR, the spermatophore may constitute up to nearly 9% of the total body mass of the male (Quiring and Timmons, 1990) and may serve as paternal investment for the male's offspring (Murphy and Krupke, 2011).

Limited research has been reported on the mating behaviors of WCR. Lew and Ball (1979, 1980) discussed WCR courtship and mating, and developed an ethogram of exhibited behaviors. Quiring and Timmins (1990) gave evidence that ~70% of females mated within 24 hours after emergence. More recently, Kang and Krupke (2009a, 2009b) showed that females rarely mated more than one time and that males had a strong preference for, and mating occurred more readily with, larger females. The latter may be an adaptive trait because it has been shown that female WCR weight is positively correlated with fecundity (Branson and Sutter, 1985).

Coats et al. (1986) examined the dispersal characteristics of female WCR. Females were found to have sustained flights (>30 min) when aged 2-9 d and did not display prolonged flights after 9 d.. The general trend for ovarial development showed that sustained fliers had less developed ovaries than trivial fliers, but all were confirmed to have mated. In terms of periodicity, trivial flights occurred throughout the day and sustained flights were more likely to happen before sunset and after sunrise (Witkowski et al., 1975). Witkowski et al. (1975) noted that flight activity in both sexes is dependent on temperature with peaks in activity at 25^{o} C ± 0.55.

When WCR was first identified as a pest, corn fields were the sole habitat used by adults for oviposition and feeding (Shaw et al., 1978). In response to this behavior, crop rotation was aggressively promoted to limit WCR damage to roots (Levine and Oloumi-Sadeghi, 1991).

1.2 Integrated Pest Management Practices

Historical examples of integrated pest management (IPM) targeting the corn rootworm complex include: crop rotation, tillage, planting strategies, host-plant resistance (HPR), biological control, and soil/aerial applied insecticides (Levine and Oloumi-Sadeghi, 1991). Many of these practices are used in combination with one another to provide the highest degree of protection to the crop.

Crop rotation between corn and soybeans (*Glycine max*), as well as other crops, has long been implemented by growers to eliminate corn rootworm damage in corn fields (Levine and Oloumi-Sadeghi, 1991). This strategy is largely effective due to the corn rootworm's inability to feed and survive on soybean roots and other non-host crops (Crowder et al., 2005). However, WCR was able to adapt to this cropping system within two decades, demonstrating the rapid response of this pest to natural selection (Gray et al., 2009). Studies of the rotation-resistant rootworm variant have shown that gravid females are found in not only corn, but soybeans, oat stubble (*Avina sativa*), alfalfa (*Medicago sativa*) (Rondon and Gray, 2003) and also wheat (*Triticum spp.*) (Schroeder et al., 2005). WCR is also capable of prolonged embryonic diapause (Levine et al., 1992) in response to crop rotation, but the frequency of this trait is less than 1%. This indicates that a female's lack of distinct ovipositional preference is the primary cause of root damage to corn (Gray et al, 2009). Diapause is a delay in development due to unfavorable conditions and is typically found during the overwintering stage of insects in temperate zones. WCR overwinter as eggs, therefore diapause is found in the egg stage (Krysan, 1972). NCR have similar diapause habits as WCR, although the frequency of an extended, two year diapause is much greater in NCR.

Tillage practices have both direct and indirect benefits. Fall tillage can expose WCR eggs to more environmental conditions in the winter such as freezing and thawing, although this approach is only effective when winter conditions are harsh (Gray and Tollefson, 1988). Tillage can also allow for easier access to WCR eggs for natural enemies that are surface dwelling (Brust et al., 1986; Chiang, 1970; Stiner and House, 1990). The downside of tillage is that it has negative environmental effects. Top soil becomes easily removed through erosion (Van Oost et al., 2000), and tillage practices can reduce soil structure (Arshad et al,. 1999). As a result, there has been a strong trend towards minimum tillage across the upper Midwest.

Planting dates can influence the degree of larval damage to corn roots. Late planting can reduce root damage (Levine and Oloumi-Sadeghi, 1991) because larvae are only able to survive a few days after hatching in the absence of a suitable host (Branson, 1989). A cost of delayed planting is the potential for reduced yields. Another technique that has been used in corn production, but very rarely in the past and in recent history has not been used is a ‘trap crop’. A trap crop is late planted corn that attracts WCR adults (Hill, 1974) due to the higher availability of pollen. The following year a non-host crop (often soybeans) will be planted in the area to ensure no larval survival. Another option is to target the trap crop with insecticidal sprays to kill WCR adults. However studies so far have failed to demonstrate that this approach is an effective form of adult control (Witkowski and Owens, 1979).

Host plant resistance to WCR is rare in corn cultivars (Chiang and French, 1980) and is mostly attained through rigorous root growth and regeneration of roots (Branson et al., 1982). Another possibility is that some corn cultivars may be more nutritionally beneficial to WCR larvae and require less feeding than others (Levine and Oloumi-Sadeghi, 1991). More recently, antixenosis was discovered in a variety of corn. Antixenosis causes a behavioral non-preference in the pest species towards the host plant (Kogan and Ortman, 1978). This natural resistance to WCR is the first evidence of a non-preference mechanism to WCR larval feeding (Bernklau et al., 2010).

Since the introduction of corn to the Midwest, followed by the movement of WCR, few natural enemies of rootworms exist (Levine and Oloumi-Sadeghi, 1991). Ground beetles have been documented as an important predator in corn fields (Brust et al., 1986), although they are considered an opportunistic predator, rather than a specialist (Kirk, 1982) and only feed on eggs near the surface. Research by Lundgren and Fergen (2011) has studied the use of cover crops to enhance predator populations in fields to reduce WCR populations. Results indicate that cover crops can indeed reduce WCR larvae via predation by several generalist predators. Several studies in recent years have looked at generalist mite communities and the effects on young WCR larvae. Although mites are not a very good predator of WCR larvae, some predatory species of mites may help to reduce newly emergent WCR larvae in association with other generalist predators (Prischmann et al., 2011; Prischmann-Voldseth and Lundgren, 2011). Several entomopathogens of WCR have been identified, but little is known about the potential to minimize rootworm damage (Levine and Oloumi-Sadeghi, 1991). Various species of nematodes have been tested for efficacy against WCR larvae and have shown some potential (Gaugler, 1981; Jackson and Brooks, 1998; Munson and Helms, 1970; Poinar et al., 1983). There have been attempts recently to encapsulate entomopathogenic nematodes with promising results. The nematodes were able to break through the capsule and infect WCR, reducing damage to corn roots (Hiltpold et al., 2012). The only drawback of this control measure is that the capsules cannot be applied by existing equipment. Work is underway to improve the capsule so that it can be used with available equipment (Hiltpold et. al., 2012).

Insecticides have traditionally been used to control WCR populations and have been one of the most important tools. Insecticides targeting rootworms are typically granular (applied during planting), or seed treatments (coating the seed prior to planting with an insecticide) (Toepfer and Kuhlmann, 2006). But in recent years, more liquid formulations are becoming available and applied during planting, similar to granular insecticides. Ideally these insecticides should last throughout the most intensive larval feeding period (Levine and Oloumi-Sadeghi, 1991). Efficacy of soil-applied insecticides is dependent on an array of environmental and mechanical factors (Levine and Oloumi-Sadeghi, 1991).

There are several classes and formulations of insecticides targeting WCR. Organophosphates, pyrethroids and neonicotinoids are among the more commonly used insecticides. Organophosphates (OP's) target the insect nervous system by binding and inhibiting cholinesterases (O'Brien, 1963). Many OP insecticides have been banned in North America due to their high level of toxicity in mammals. Of the remaining OP's, the most common OP used today is chlorpyrifos (Lorsban 15G®, Dow AgroSciences). Pyrethroids are a neurotoxin and cause hyper-excitation and tremors, followed by paralysis in insects (Narahashi, 1971). Pyrethroids work by keeping the sodium channels open in the neuronal membranes. One of the most widely used pyrethroids is tefluthrin (Force 3.0G®, Syngenta). Neonicotinoids are a relatively new major class of insecticides and have a mode of action that blocks nicotinic acetylcholine receptors (Tomizawa and Casida, 2005). Neonicotinoids are commonly used as seed treatments, and are the most abundant class of insecticides used today, with many different formulations. Imidacloprid (Gaucho®, Admire®, etc., Bayer Crop Science), clothianidin (Poncho®, Bayer Crop Science), and thiamethoxam (Cruiser®, Syngenta) are the most common compounds applied to annual crop seeds. Although neonicotinoids are almost universally used to treat corn seeds, recent field research has shown that effectiveness of seed treatments against WCR is minimal and may not offer yield benefits (Cox et al., 2007, Petzold-Maxwell et al., 2013).

With the advent of genetically modified corn containing Bt crystalline proteins (discussed in section 1.3), the use of soil-applied insecticides has dramatically decreased. However, virtually all corn seed is still treated with neonicotinoid insecticides (Onstad et al., 2011). Hybrids with Bt provide potential for greater protection to corn roots than soil insecticides because the Bt is present in all root tissues and not localized in the soil profile.

1.3 Bt Corn Development and Adoption

Bacillus thuringiensis (Bt) is a spore forming bacterium that produces internal crystal (*Cry*) proteins, which in turn are protoxins active on several insect orders (Aronson and Shai, 2001). When an insect ingests these protoxins, proteases in the midgut activate the protoxin, allowing the activated toxins to bind to the midgut, causing a disruption in the membrane resulting in septicemia and death of the insect (Gill et al., 1992). The first effects of Bt toxins are evident within 12 h of feeding (Moellenbeck et al., 2001). As a means of utilizing *Cry* proteins as insecticides, seed companies have genetically modified several annual crops to express the Bt proteins targeting major pest species. The advent of these genetically modified crops provides effective control over many key insect pests and has additional benefits that include the reduction in the use of conventional insecticides and overall, better control of WCR (Carrière et al., 2003; Romeis et al., 2008).

Most corn producers throughout the country have adopted corn hybrids that express insecticidal *Cry* proteins targeting the corn rootworm (CRW) complex (primarily the WCR) as part of their pest management strategy. In 2009, Bt corn accounted for nearly 63% of the corn grown in the US (NASS, 2009). A downside to Bt corn is that currently registered Bt toxins for rootworm control are classified as low to moderate-dose toxins (Siegfried et al., 2005). Research has shown that numerous rootworm adults emerge from all currently available rootworm Bt products (Meihls et al., 2008). The *Cry3Bb1* toxin allows ~33% survival of WCR into adulthood (Binning et al., 2010). A high dose toxin is described as having 25 times the toxin concentration to kill susceptible larvae (EPA, 1998b). It cannot be assumed that Bt plants kill 100% of all susceptible individuals, so another definition for high dose specifies a plant that kills at least 99.99% of susceptible insects in the field (EPA, 1998a). Another possible downside to Bt hybrids targeting CRW is that the amount of Bt protein produced declines throughout the growing season (Nguyen and Jehle, 2009). In addition, WCR larvae are more tolerant to the effects of the toxins as the larvae age (Binning et al., 2010).

However, since the commercialization of Bt specifically targeting the CRW complex, in 2003 (NASS, 2006), there have been significant changes that affect how growers use and manage these products. One critical change that was introduced to the market in 2010 was the combination of several Cry toxins and herbicide tolerance traits, produced by formerly competing parent companies, into single hybrids (often called "stacked hybrids"). Stacked hybrids were developed with the goal of simultaneously simplifying weed management and increasing mortality rates in WCR, delaying resistance and allowing reduction in refuge size. These hybrids have been shown to potentially cause both weed control and resistance issues as "volunteer" plants when F1 seeds are allowed to germinate (Krupke et al., 2009). Not only are genetically modified volunteer plants resistant to glyphosate and/or glufosinate, which causes problems in rotated crops, but volunteer plants also express reduced rates of Bt toxins that could aid in the development of resistance in WCR populations (Krupke et al., 2009). Resistance to Bt hybrids among WCR populations in the US has been confirmed (Gassmann, 2011) and will be discussed in Chapter 2.

1.4 Refuge Planting for Bt Hybrids

Refuge corn is a critical component of the resistance management plan for Bt corn. A refuge consists of non-genetically modified crops that serve as a reservoir for susceptible individuals (Roush and Daly, 1980). The EPA has developed requirements for a refuge that growers must comply with when using all transgenic hybrids targeting insects, including corn. These refuge requirements were developed to delay WCR resistance to Bt. A refuge of 10-20% is required for hybrids containing a single Bt trait targeting WCR and must be planted in strips throughout the field or as a block in one section of the field (EPA, 2005). The refuge requirement for hybrids containing multiple Bt toxins targeting the same pests has been lowered to 5% (Ricketts and Heine, 2009), but usually utilizes a seed mix refuge. In any case, delaying resistance is essential for maintaining the usefulness of Bt as a management tactic against WCR as well as other Bt targeted pest species (Jaffe, 2009).

Using a refuge is a strategy designed to maximize the probability that resistant pests will find and mate with susceptible individuals that emerge from the refuge corn (Gould, 1998). The initial frequency of resistance alleles is presumed to be low, and resistant individuals surviving Bt events should be rare. These few rare individuals will find and mate with abundant susceptible pests that emerge from refuge plants, therefore keeping the frequency of resistance alleles at bay (Tabashnik and Gould, 2012). When the refuge requirements for rootworms were first established in 2003, there was a 90% compliance rate by growers (Jaffe, 2009). However, beginning in 2006 the compliance rate began declining sharply, and by 2008, 25% of growers were not using a refuge (Jaffe, 2009). The reduction of growers planting a refuge in association with a Bt crop may be a second factor that plays a role in the development of resistance of WCR to Bt products (Gassmann, 2011).

Shelton et al. (2000) measured the effects of refuge size, placement, and resistance of diamondback moth (*Plutella xylostella*) larvae exposed to Bt and non-Bt broccoli (*Brassica oleracea*). From this study it was determined that larger refuge sizes, planted separately produced the highest numbers of susceptible individuals as should be expected. Tabashnik et al. (2008) supports the case for larger refuges as well, and gives evidence that refuges can delay resistance.

Refuge-in-a-bag (RIB) seed mixes are now available and allow an alternative tactic for insect resistance management instead of relying on growers to plant a separate refuge (Onstad et al., 2011). The seed mix refuges forces growers to comply with EPA regulations while making planting more convenient. This approach allows for nearly synchronous emergence of WCR from both refuge and Bt corn plants, thereby increasing the probability of random mating (Murphy et al., 2010), a desirable goal for IRM. However, this synchrony may result from larval movement between plants (Hibbard et al, 2005), allowing for sublethal doses of Bt to be ingested (Mallet and Porter, 1992) and potentially enhancing the probability of resistance. Larval movement is restricted by several physical characteristics of the soil including bulk density (Strnad and Bergman, 1987a) and dampness or dryness (MacDonald and Ellis, 1990). As larvae develop, the larvae migrate to younger root tissues (Strnad and Bergman, 1987b) and may move up to three plants down a row (~60.96 cm) or across one row to another (~76.2 cm) (Hibbard et al., 2003). Host plant chemicals emitted by the roots allow larvae to find the roots with minimal searching effort (Gustin and Schumacker, 1989). As with other refuge approaches, mating rates between beetles arising from Bt and refuge plants has not been thoroughly quantified.

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CHAPTER 2. MATE SELECTION OF WESTERN CORN ROOTWORM UNDER VARYING REFUGE CONFIGURATIONS

Even though Bt hybrid corn technology targeting WCR is a relatively new technology, field-evolved resistance has been documented. Widespread planting of Bt crops creates intense selective pressure for a pest to evolve resistance (Gassmann et al., 2012). Meihls et al. (2008) reported that WCR could evolve resistance to genetically modified corn containing the *Cry3Bb1* protein (this includes Yieldgard® and VT Triple® hybrids, which are commonly planted in Indiana) within three generations under greenhouse conditions. This was further supported by Gassmann et al. (2011), where growers in Iowa reported severe rootworm damage to the *Cry3Bb1* expressing hybrids in the field beginning in 2008. Gassmann et al. (2011) collected eggs from mated females in fields showing signs of resistance and then under laboratory conditions, reared WCR larvae on corn plants expressing the *Cry3Bb1* trait and found significantly higher survival in these larvae as compared to control larvae with no Bt resistance. *Cry3Bb1* corn was grown in those Iowa fields for at least three consecutive years, but no information about refuge compliance levels were included in the paper. These discoveries lend support to the suggestion that resistance to Bt proteins targeting the corn rootworm complex are non-recessive (Meihls et al., 2008). Non-recessive inheritance occurs when the resistant phenotype in a population is higher than in recessively inherited resistance, speeding up resistance evolution.

To date, no resistance has been reported in hybrid corn expressing the *Cry34/35Ab1* proteins in the field (Gassmann, 2011), although resistance to *Cry34/35Ab1* has been documented in lab trials (Meihls et al., 2008). There is, however, a 5-7 d delay in adult emergence when WCR larvae are exposed to *Cry34/35Ab1* toxins (Storer et al., 2006). Male WCR emerging from Bt-RW expressing corn hybrids surrounded by Bt-RW plants tend to have smaller head capsules (Murphy et al., 2011) and lower dry weights than males emerging from refuge corn. These parameters are measured in the current study and will be discussed in more detail later. Size differences are important because they may potentially lead to non-random mating. It has been demonstrated that WCR males have a preference for larger females (Kang and Krupke, 2009a) and random mating is a crucial aspect of the refuge plan for resistance management.

The random mating hypothesis has been challenged by Spencer et al. (2012). In this paper, the authors describe how skewed male to female ratios, protandry, premating movement and delayed emergence from CRW-active corn affect reproductive behavior. All of these factors combined have the potential to allow for the evolution of CRW Bt resistance to develop at a much quicker rate.

2.1 Insect Marking Methods for Monitoring Movement

The ability to mass mark insects is key in developing and understanding insect dispersal and movement. Defining mating rates is partially dependent on knowing where the individuals originated from. Mark-recapture techniques to monitor WCR in fields have included fluorescent dusts on field-collected beetles, and feeding laboratory raised WCR beetles artificial diets with colored dyes (Naranjo, 1990). Although these methods are easy to implement and are somewhat effective, limiting factors exist. There are limitations on the number of WCR beetles that can be reared in a laboratory or collected from a field (Nowatzki et al., 2003). Additionally, these methods cannot be used to mark larvae, which is critical for any study that seeks to document Bt exposure during the period when selection occurs.

Elemental markers have been used in several cropping systems to label insects. These markers provide an environmentally safe method for marking insects through consumption of treated host plants (Berry et al., 1972). Rubidium (Rb) is a commonly chosen marker because it has similar chemical properties to potassium (K) and can readily be taken up by plants systemically (Berry et al., 1972). Because Rb is naturally found in the soil, it is necessary to determine the levels at which Rb is present and calculate a level of three standard deviations above the mean natural concentration to positively label insects (VanSteenwyk, 1991). Nowatzki et al. (2003) used Rb as a marker for WCR and showed that there were no significant effects on development. The problems with using Rb arise when beetles stop feeding on marked plants. The ability to distinguish between labeled and un-labeled WCR adults only lasts up to 3.2 d post-emergence (Nowatzki et al., 2003).

2.2 Pilot Study Materials and Methods

Pilot experiments were conducted in January through May 2012 to test the longevity of Rb in post-emergent beetles using differential doses and multiple applications of Rb to non-Bt corn plants infested with WCR eggs. This experiment took place under greenhouse conditions in the Environmental Entomology Laboratory (EEL) greenhouse at Purdue University. Non-Bt corn (DKC 62-55) was planted 2.54 cm deep into potting soil in 9.5 liter buckets. Screen mesh with a bungee cord strung around the edge was used to seal the edges and prevent the beetles from escaping. At the center of the mesh, a PVC pipe was installed to allow the corn plant to grow. The hole for the plant was sealed with strips of foam to prevent emergent adults from escaping.

WCR eggs were artificially infested into the soil using a pipette at a rate of 400 eggs per plant from colony beetles (USDA Northern Grain Insects Rearing Facility in Brookings, South Dakota). The eggs were deposited in two opposite holes at a depth of 10 cm near the corn plant at the V2 plant stage. Eggs prior to injection into the soil were suspended in a 0.15% agar solution to allow for even distribution of eggs. Corn plants were watered as needed.

Doses of Rb applications were as follows in grams: 0, 0.01, 0.025, 0.05, 0.075, and 0.1. Location of application was tested for each treatment dose; whorl, soil, and ½ whorl and ½ soil. Multiple applications were also assessed with each dose, but total dose to each plant added up to each respective dose. This was done in three weekly applications as suggested by Nowatzki et al. (2003) to potentially increase longevity of rubidium in WCR adults. This increased the number of treatments to 31. Rb was injected in solution to each corn plant at the V2 plant stage for the first application. Each treatment was replicated four times, giving a total of 124 treated plants. Due to space, resource and time demands, the replicates were separated into two planting dates. Plants were randomly assorted on the greenhouse bench.

WCR beetles that emerged were collected using an aspirator and placed into 2 oz plastic cups with lids (SOLO; Dart Container Corporation, Mason, MI) labeled with treatment and date. Beetles were then fed an artificial diet (Product #F9766B; Bio-serv, Frenchtown, NJ) until being freeze-killed to determine Rb concentration. Five male and five female beetles at 1-d-old, 2-d-old, and so on, up to 10-d-old, from each treatment, were tested for concentration of Rb. Concentration of Rb in adult beetles was measured by sending dried beetle specimens to the Purdue Rare Isotope Measurement (PRIMe) laboratory for ICP-OES analysis. Prior to sending the collected beetles to the PRIMe laboratory, samples were placed in a small laboratory oven (Grieve-Hendry Co., Round Lake, Illinois) and allowed to dry at 93º C for one hour. Results from this study were to be used to determine Rb application rates during the summer 2012 experiments.

2.3 Results and Discussion of Pilot Study

Results of this study were inconclusive. Due to a combination of experimenter error and greenhouse complications, the study was unable to be completed. A large number of the treated plants died potentially due to Rb application or failure of the greenhouse to regulate temperature and light. A majority of the remaining plants were stunted due to overexposure to light and heat. The timer for the lights did not work; therefore the corn plants were exposed to 24 h of light and heat for an unknown period of time (possibly weeks) before the fault was detected.

Of the WCR adult beetles that emerged, a good portion were deformed with wings hanging out from under the elytra and many died within 24 hrs. Remaining adults that were collected and survived until frozen were stored in an ultralow freezer (Model MDF-U52VA; Panasonic: Sanyo Scientific, San Diego CA). The next issue that arose was cooperation and communication with the PRIMe laboratory. Inability to maintain contact with PRIMe resulted in the samples not being processed to determine Rb levels.

Several lessons were learned from this experiment. First, there is a definite need to spend more time developing a plan and making it work. Many of the issues that occurred may have been avoided if more time and care were put into designing and maintaining the project (eg. making sure light timers worked, etc.). Secondly, the project felt daunting because of the large amount of work involved, which played a role in how much care was put into it. Because there were so many plants all growing at the same time, it was hard to invest a large amount of time with each individual plant. Lastly, there is a need to be more assertive when it comes to dealing with others to get done what needs to be done. Keeping communications flowing with another lab is essential to acquiring good, timely results when relying on them to process and relay findings.

With all of the complications encountered, efforts were turned away from using Rb as a marker and focused on a new approach. N^{15} has been proven extremely effective as a marker in many insect species and various ecosystems as well as remaining detectable indefinitely within plants and insects.

2.4 Stable Isotope Labeling

An isotope is defined as a material having the same atomic number of the parent element but a different number of neutrons giving it a different atomic weight. Stable isotopes occur naturally in the environment, however these isotopes are found at much lower levels than the elemental counterparts. For example, the natural abundance of N^{15} is approximately 0.3663% of all nitrogen atoms, and C^{13} makes up 1.108% of all carbon atoms (Hood-Nowotny and Knols, 2007). These isotopes are given the term stable because of being non-radioactive, in a non-decaying state (Hood-Nowotny et al., 2005), and no environmental impacts or biosafety issues are displayed. Stable isotopes are safe and non-invasive to target organisms, unlike other methods of labeling arthropods (eg. painting, radio-isotope labeling, etc.) (Le Maho, 2002). The most commonly used isotopes in ecological studies are hydrogen (H^2), carbon (C^{13}), nitrogen (N^{15}), and oxygen (O^{18}); all of which can easily be detected using isotope ratio mass spectrometry. A simplified explanation of how mass spectrometry works follows: the material is combusted at very high temperatures (~1800 °C) and converted to a gas before sending it through a chromatograph column. After passing through the column, the gasses are ionized, accelerated and separated by a magnetic field based on the mass to charge ratio (Hood-Nowotny and Knols, 2007). This allows for each isotope to be identified and a ratio of relative abundance in the sample can be determined.

There are a number of different techniques and applications that can be used when employing stable isotopes. Studies can be performed to look at movement and dispersal of arthropods, population dynamics, preferred hosts, multi-trophic studies and many other natural processes. The isotope of choice can be administered to the target organism by enrichment of the environment or a specific host, directly feeding it to the organism or using naturally occurring isotopes. The latter is useful when measuring dispersal due to specific geographical regions having distinctive isotopic profiles (Hood-Nowotny and Knols, 2007).

2.5 Methodology

A field study was conducted in the summers of 2012 and 2013 using Bt corn hybrids and non-Bt refuge corn plants to determine mating preference of beetles emerging from each type of corn. Some aspects of the methods vary from 2012 to 2013 and are discussed in section 2.6. Four treatments were replicated four times:

- strip refuge (20% refuge)
- seed mix or refuge-in-a-bag (RIB) (5% refuge)
- 100% Bt control
- 100% refuge control

Refuge size for the strip refuge treatment was approximately 20% with refuge (15 seeds) planted on one side, and the RIB treatment contained 5% refuge (4 seeds) plants randomly placed throughout the plot. Bt hybrid seeds used were Yieldgard VT Triple® + Round-up Ready 2® (DKC 62-54) expressing *Cry3Bb1* for WCR control. Refuge seeds were a near-isoline of this hybrid (DKC 62-55). In this study, isoline refers to refuge seeds that are nearly identical to the Bt seeds, with the exception that the refuge seeds do not contain the Bt toxin targeting WCR.

Sixteen plots (4 reps X 4 trt) measured 3.65 m by 3.65 m and included four rows of 20 corn plants spaced 76.2 cm apart and 15.24 cm spacing between plants in a row. Individual plots were set 2.44 m apart on all sides and a 3.05 m buffer was planted on all edges. This gave a total field area of 15.24 m by 52.43 m (0.20 acres). The strip refuge plots contained 15 refuge plants and 60 Yieldgard® plants (contains 75 plants in total to allow for 20% refuge) (Figure 2.1). RIB plots contained 4 refuge plants and 76 Yieldgard® plants to accommodate the 5% refuge requirement (Figure 2.1). Both controls contained 80 plants of their respective seed types. Each plot was enclosed by a screen house (3.65 m length X 3.65 m width X 2.13 m height). The edges of each screen house were covered with soil to seal the sides in order to keep beetles from moving in or out of the plots. Plots were planted with a four-row planter (White 6100 series) at a rate of 27,700 seeds per acre, minus the refuge in both the strip and RIB treatments. Refuge in the strip treatment was hand planted in row 1 of the plot. For the RIB treatment, 1 Bt seed per row was randomly chosen, dug up and replaced with 2 refuge seeds, which were then staked to identify location. After germination, the smaller of the two refuge plants was removed. When staked refuge plants reached the V2 plant stage, the plants were tested using gene-check strips (EnviroLogix Cry3B # AS 015 LS, Portland, ME) to confirm that plants were Bt-. After which, ammonium nitrate N^{15} (~98% N^{15}) (Cambridge Isotope Laboratories, Inc. Andover, MA) in the form of a dry powder was applied to the base of each plant by using a pencil to dig a 5 cm deep hole and applying the labeled fertilizer directly into the hole. Five percent of the total nitrogen needed per refuge plant was N^{15} to label individual refuge plants (~0.147 g per plant). Just prior to adult emergence (June, 18 2012; July, 3 2013) , all plants except the central 8 plants were cut to about 0.4 m and stripped of leaves to allow for easier spotting of mating pairs. This study took place at the Purdue Agronomy Center for Research and Education (ACRE) in a corn-after-corn field where WCR populations have historically been abundant.

Screen houses were monitored on a daily basis (Monday-Friday) to look for mating pairs. Monitoring took place in the morning during projected peak mating, 9-11am (actual time varied depending on temperature), without any time constraints on how long samplers had to be in each cage. Mating pairs were collected in individual

Ziploc baggies labeled with date, replicate and treatment. After collection, all samples were given an identification number and were frozen for later processing.

Individuals had head capsule measured and dry weight obtained to the nearest mg. Head capsules were measured using a stereo microscope with an attached digital camera (models SZX12 and U-CMAD3; Olympus Optical, Tokyo Japan). A picture was taken of the head capsule and measured within 0.01 mm using AnalySIS Microsuite imaging software (Soft Imaging System, Lakewood, CO, USA). A maximum of 12 mating pairs per treatment were assessed for head capsule width to provide a subsample for each treatment. Mating pairs were then placed into a small laboratory oven (Grieve-Hendry Co., Round Lake, Illinois) and allowed to dry at 93º C overnight to ensure that most of the moisture from each sample was removed. Following drying, individual beetles were weighed to the nearest 0.1 mg to obtain dry weight (Mettler AE 100; Mettler Direct, Ventura, California). Head capsule size and dry weight are used to estimate the fitness of each individual (Branson and Ortman, 1970). Fitness is defined as the numbers of viable offspring an individual is able to produce (Mitchell, 1981). As a general rule, larger individuals are able to produce more offspring (Mitchell, 1981), therefore size is often used as an indicator of fitness for insects and other organisms.

Samples were then prepared and sent to the Purdue Stable Isotope lab in which δN^{15} concentration was measured using Mass Spectrometry. The first step in preparation consists of removing the abdomen from each of the dried beetles. The purpose of removing the abdomen is to prevent the accidental inclusion of the spermatophore that is transferred from the male to the female during copulation (Lew and Ball, 1980). After removal of the abdomen, the elytra were crushed and placed into a mass spectrometry tin and weighed out between 0.300-0.400 g. Elytra were primarily used due to being heavily sclerotized and resistant to degradation (Klowden, 2002), and therefore have the greatest potential to retain the N^{15} label. If the elytra were small and did not weigh enough, additional ground-up WCR beetle heads were used. Sample tins used for elemental analysis and combustion were folded after weighing and placed into a well tray. After completion, the tray was delivered to the Purdue Stable Isotope lab (Purdue University, Hampton Hall of Civil Engineering). The samples were then combusted in an elemental analyzer (1050 °C), then analyzed by an isotope ratio mass spectrometer (Sercon 20-20 IRMS, continuous flow: PDZ Europa Elemental Analyzer; Crewe, Cheshire, England).

All of the beetles collected from the 20% strip refuge and 5% RIB were sampled for δN^{15} to give proportions of unexposed beetles to Bt-tolerant beetles. Resultant data from the Purdue Stable Isotope lab were reduced to corrected δN^{15} values and needed further reduction for more accurate readings (Dawson, 2002). The first calculation determines the ratio of N^{15}/N^{14} in each sample. This was done using the following equation:

$$(X_{\text{Sample } \delta N^{15}}/1000 + 1)*0.0036764$$

0.0036764 is the natural relative abundance of N^{15}. Next, the calculation for atom % N^{15} was conducted, which is essentially the percent of N^{15} relative to total N in the sample.

$$100*((X_{\text{Sample } N^{15}/N^{14}}/(X_{\text{Sample } N^{15}/N^{14}} + 1)))$$

This allows for the final calculation to determine Atom % Excess.

$$((X_{\text{Sample Atom } \%N^{15}} - 0.3679)/0.3679)*100$$

0.3679 is the average atom % N^{15} of known non-labeled samples from the control treatments to give a baseline constant. Atom % excess exposes the small differences between samples having slightly variable amounts of total N. It was then determined that a value of 1.5 or greater would be the threshold between labeled and non-labeled. This value allowed larvae that fed on a refuge plant for a small amount of time to be excluded from being labeled.

ANOVA tests followed by a Tukey HSD test were used to look for differences in the fitness parameters measured across treatments and sex. Chi-square (X^2) tests were conducted to look for differences in rates of mixed and non-mixed mating for each treatment that compare expected and observed rates of mating for each sampling event. Finally, simple models were developed to determine the rate at which resistance could conceivably develop if this system approximates whole field populations.

2.6 Problems Encountered and Amended Methodology

The first problem arose with the sampling technique. Due to relatively low WCR populations in the study area, sampling each tent for a short amount of time, even during peak mating, was not efficient enough to collect enough sample numbers. To correct this error, the research protocol for the summer of 2013 was amended to intensely sample three times a week (MWF) for a four hour time block (7am-11am), while rotating four individuals randomly from tent to tent every 15 min. This allowed each individual to sample each tent during all scouting events and thus minimizing sampling bias. This occurred from the time that the first female was found inside any tent and continued until no beetle captures occurred in each tent. Additionally, two repetitions of emergence cages for each treatment were added to identify peak emergence and male-to-female ratios throughout the season. These cages were sampled for all adult beetles following mating pair scouting.

Secondly, problems with the mass spectrometry results in 2012 were encountered. Due to over-labeling refuge plants, there was a large δN^{15} in the beetles that emerged from the labeled variety. This caused problems when running those individuals through the mass spec. Because samples contained so much of the label (2000-8000 ppm), N^{15} was detected in several subsequent samples, even though it was known that no N^{15} could possibly be in the sample. This was corrected in 2012 by running five blank mass spec tins though the machine to help clear out the system with the utilization of peach leaves to dilute the sample. Peach leaves are used because of neutral properties (commonly used as a standard and to dilute highly labeled samples) (Smodiš et al., 1992). To correct this problem in 2013, the amount of label was reduced and a different technique was used to apply the marker. Instead of using a dry powder, the ammonium nitrate N^{15} was dissolved into a water solution and injected near the base of the plant. The amount of label per plant was reduced to 1/6 of the original amount (0.0245g) as suggested by Dr. Greg Michalski (Purdue Stable Isotope Lab, Hampton Hall of Civil Engineering).

Finally, Tippecanoe County was under drought conditions for most of the 2012 summer months. Droughts can have the potential to reduce the numbers of WCR that survive to adulthood (Toepfer and Kuhlmann, 2006), influence mating patterns, and possibly negatively affect fitness of individuals. Effects of drought can also be noted in the plants by causing reduced nutrient uptake (Boyer, 1982).

2.7 Results

2.7.1 WCR Emergence

Delayed emergence in the treatments containing *Cry3Bb1* corn was observed as described in several previous studies. The first beetles were found in the 100% refuge treatment July 3, 2013, whereas the first beetles from the 100% Bt were noted on July 8, 2013; within the 5-7 d delay as noted by Storer et al., 2006. Even though delayed emergence was observed, peak emergence for all treatments occurred on the same day; July 22, 2013. After peak emergence, the numbers of emerging beetles sharply declined over the next few days for all treatments. Following the sharp decline in numbers, emergence somewhat stabilized and slowly decreased for several weeks until the end of August. The first treatment with no emergence was recorded on August 16, 2013 (100% Bt) (Figure 2.7). Subsequently following were the 5% RIB (August 19, 2013) (Figure 2.3-2.4), 20% strip refuge (August 23, 2013) (Figure 2.5-2.6), and finally the 100% refuge on August 28, 2013 (Figure 2.2).

Female biased sex ratios were found in all treatments. The 100% Bt treatment had the lowest ratio of males to females (M:F = 1:1.37). While the other three treatments were 1:1.75 (20% strip refuge), 1:1.73 (5% RIB) and 1:1.65 (100% refuge). In terms of total beetles emerging from each treatment, there were more adults emerging from the 100% refuge than any other treatment. As anticipated, the 100% Bt corn blocks produced the lowest numbers recorded. Total numbers are as follows for each treatment: 100% refuge = 639; 20% strip refuge = 278; 5% RIB = 276; 100% *Cry3Bb1* = 194.

63.73% of males and 61.93% of females emerging from the 20% strip refuge fed upon labeled, refuge plants as larvae. As for the 5% RIB treatment, 38.61% of males and

29.71% of females were detected as labeled. Tables 2.1 and 2.2 show daily collected adults from each treatment as well as separating N^{15} labeled adults from non-labeled adults. Atom % excess varied dramatically between samples throughout the experiment (Tables 2.3 and 2.4). This indicates that the label decays over time or is spread throughout the plant more evenly, and two or more labeled plants in close proximity create 'hot spots' in which labeled adults contain high amounts of N^{15}.

2.7.2 Natal Host Plant Effects on Fitness

Head capsule widths and dry weights of all individuals across treatments were examined from the emergence cages in 2013. Significant differences were found in both sexes and each of the variables measured. Male head capsule widths: 100% refuge = 1.15 (SE = 0.0058); 20% strip refuge = 1.15 (SE ± 0.0058); 5% RIB = 1.14 (SE ± 0.0064); 100% *Cry3Bb1* = 1.12 (SE ± 0.0070). For females: 100% refuge = 1.17 (SE ± 0.0070); 20% strip refuge = 1.15 (SE ± 0.0063); 5% RIB = 1.13 (SE ± 0.0069); 100% *Cry3Bb1* = 1.13 (SE ± 0.0083). Mean dry weights for males: 100% refuge = 3.17 (SE ± 0.0631); 20% strip refuge = 3.0 (SE ± 0.0685); 5% RIB = 2.94 (SE ± 0.0546); 100% *Cry3Bb1* = 2.69 (SE ± 0.0542). Female dry weights: 100% refuge = 2.97 (SE ± 0.0920); 20% strip refuge = 2.6 (SE ± 0.0832); 5% RIB = 2.64 (SE ± 0.1054); 100% *Cry3Bb1* = 2.43 (SE ± 0.0856). Beetles emerging from refuge plants have larger head capsules and greater dry weights than beetles emerging from Bt plants. As the amount of Bt plants increased, both head capsules and dry weights declined for both males and females. The mean head capsule width and dry weights of males and females from each treatment can be seen in Figures 2.8-2.11.

2.7.3 Mate Pairing

Peak mating generally occurred between 8:30 am and 10:30 am, although this varied depending on daily temperature. Based on personal observation, peak mating occurs when temperatures range from 20-24°C in microclimates within the field after most of the dew has evaporated.

Over the course of the summer, the curves for mating pairs collected follows trends of the emergence curves (Figure 2.2-2.7). Results from the 20% refuge show that there was a moderate amount of mixed mating between Bt and refuge adults. Along with moderate rates of Bt beetles mating with each other and low rates of refuges adults mating with one another (Figure 2.12). In the 5% RIB treatment, there were very high rates of Bt adults mating with each other and low rates of mixed mating and refuge adults mating with one another (Figure 2.13). Total mating pairs collected are as follows for each of the treatments: 100% refuge = 351; 20% strip refuge = 99; 5% RIB = 174; 100% *Cry3Bb1* = 107.

Because one of the objectives of the project was to compare how well refuges function in terms of facilitating mixed mating between refuge and Bt beetles, the most informative data for this project came from the 20% strip refuge and the 5% RIB treatments. Because females outnumber males, mating numbers are limited by numbers of males in each treatment. Expected results are calculated from the emergence cage totals (Table 2.1) and shown graphically in Figure 2.14.

20% strip refuge expected:

- mixed mating = 46.72%
- Bt/Bt pairings = 13.81%

- refuge/refuge pairing = 39.47%

5% RIB expected:

- mixed mating = 45%
- Bt/Bt pairings = 43.53%
- refuge/refuge pairing = 11.47%

Assuming that males and females collected from the field have only mated once, observed results taken from the field are shown below (Figure 2.15):

20% strip refuge observed:

- mixed mating = 16.7%
- Bt/Bt pairings = 49.0%
- refuge/refuge pairing = 34.4%

5% RIB observed:

- mixed mating = 27.5%
- Bt/Bt pairings = 65.6%
- refuge/refuge pairing = 6.9%

X^2 analyses for daily values show deviations from expected values.

2.7.4 Modeling Resistance

Simple models to predict WCR resistance were developed assuming that the collected data reflects what truly happens in a field. First, several assumptions must be made:

- This model assumes continuous corn within a field.

- Year one of the model uses mating rates and population densities collected from this research.
- All females mate once and males can mate multiple times to accommodate excess females.
- All females produce 440 viable eggs (Boetel and Fuller, 1997), a number commonly used in most models predicting WCR resistance (Onstad et al., 2001; Pan et al., 2011).
- In the 20% strip refuge, 36.7% adults are males and 63.3% are females as discovered in the emergence results. For the 5% refuge, 36.59% are male and 63.41% are female.
- Offspring of susceptible adults are also susceptible. Mixed mating also results in susceptible offspring.
- 80% of susceptible offspring are exposed to Bt in the 20% refuge and 95% of susceptible offspring are exposed to Bt in the 5% RIB each year, and die.
- Mating is calculated using ratios of Bt and refuge adults.
- The tipping point for observable damage occurs when ≥ 50% of mating adults are Bt/Bt pairs the previous year (Pan et al., 2011).

Year 1 - 20% Refuge:

34.48% Bt/Bt mating (33 pairs): produces 14520 offspring.

5329 are male (36.7%) and 9191 female (63.3%)

65.62% at least one refuge adult (63 pairs): produces 27720 offspring. 80% of larvae die leaving 5544 surviving to adulthood in year 2. 2035 are male and 3509 are female.

Year 2 - 20% Refuge:

Random crosses: proportion of Bt/ref males and females:

Resistant ♂ = 0.7237 Resistant ♀ = 0.7327

Susceptible ♂ = 0.2763 Susceptible ♀ = 0.2763

Mix = 40% Bt/Bt = **52.37**% Ref/Ref = 7.63%

Year 3 - 20% Refuge:

Observable damage in fields after ≥ 50% Bt/Bt mate pairing (Pan et al., 2011).

Year 1 - 5% RIB:

65.63% of mating is between resistant adults. Therefore observable damage will be found the following year.

2.8 Discussion

From this research, the main conclusion is that unexposed (i.e. susceptible) beetles are mating with Bt-exposed beetles, but there do not appear to be enough unexposed beetles produced from the refuge to reduce the rate of mating between Bt-exposed beetles. Ideally, mating between Bt-exposed beetles would be rare (< 5%) to insure a smaller chance of tolerant offspring being produced. This gives rise to concern about the viability of current refuge strategies to manage WCR resistance to Bt hybrids.

In the worst case scenario, the current study shows that a 5% RIB field allows for 65.63% Bt Bt pairings, which if planted in continuous corn, could show economic damage the following year. Indiana fields do not show this rapid resistance development because most are rotated from year to year.

Measured fitness parameters (head capsule widths and dry weights) do vary between treatments, which agree with results from Murphy et al., 2011 and Hoffmann, 2013. There was also a delay in emergence between refuge and Bt adults, also detected in Murphy et al., 2010. Though these parameters are statistically different between refuge and *Cry3Bb1* emergent adults, mixed mating between Bt and refuge adults still occurred. There are several potential explanations for this. The first may be that the fitness costs that are encountered are not sufficient to alter mate preferences. Another explanation may be that adults emerging from Bt corn may be able to produce higher quality, more resilient offspring. Therefore, selection may be towards Bt emergent adults, the opposite of what was originally thought. A third explanation could simply be due to the higher numbers of Bt adults compared to the refuge adults that were produced in the cages.

There has been much debate by researchers and regulators about the size requirements of a refuge, with the push for a 50% refuge rather than the current 5-20% parameters (Tabashnik and Gould, 2012). By decreasing the refuge from 20% to 5%, the number of susceptible beetles decreases to under half of what is produced in a 20% refuge. The thought behind this decrease in numbers is that beetles are moving away from the Bt plants towards the refuge plants (Hibbard et al., 2005). This decrease in susceptible adults is a problem when looking at the total number of susceptible vs. Bt-exposed WCR in each refuge design. The refuge plants in a 20% strip design produce approximately 63% of the total beetles collected in that treatment, which is not sufficient to effectively reduce mating between surviving Bt-exposed beetles, especially since multiple mating is typically rare (Kang and Krupke, 2009b; Hill, 1975; Branson and Johnson, 1973). From a predictive mathematical perspective, to reduce Bt pairings to 5%, ~77% of the population should consist of refuge adults. Reducing the refuge to 5% of the total plants in a field diminishes the percentage of susceptible beetles to 33% of the adult WCR population, further reducing the chances that susceptible beetles mate with Bt-exposed beetles.

According to data collected from this study, the 100% Bt treatment produces ~30% of the amount of beetles produces in the 100% refuge. This parallels numbers generated in Binning et al., 2011 who found 33% survival from *Cry3Bb1* plants in laboratory trials. The high rate of survival again demonstrates that the current Bt hybrids available commercially are low to moderate-dose toxins, when a high dose toxin would offer more sustainable control by causing higher rates of mortality to WCR larvae.

Looking at the susceptible beetles in the 20% refuge and the choice of mate, it was determined that 74.6% of refuge adults will mate with a Bt-exposed beetle, with that number being 80% in the 5% refuge. There did not seem to be a difference between refuge males versus females mating with Bt adults (18 refuge males and 26 refuge females mated with Bt adults in the 5% RIB; 19 refuge males and 28 refuge females mated with Bt adults in the 20% refuge). These high ratios of refuge adults mating with Bt adults are most likely due to the high (67% Bt-exposed in 5% RIB; 37% Bt exposed in 20% refuge) numbers of Bt-exposed beetles produced in each treatment. If the number of refuge plants increases, increasing the number of susceptible beetles, it may be expected that the number of Bt-exposed rootworms mating with susceptible rootworms would increase. Simple calculations using numbers generated from this study (comparison of the 100% refuge to the 100% Bt emergence totals), the recommended refuge should be a 50% refuge. A 50% refuge would reduce Bt pairings to 5.58% of the total mate pairs. The likelihood of an increase in refuge size is small though. The main reason is that refuges are inconvenient for the growers to plant when the refuge seeds are planted separately from the Bt seeds. A second reason is that the refuge is unprotected from WCR and growers must invest in insecticide treatments if they wish to protect their crop.

An interesting find that is worth mentioning is that there were more mating pairs collected in the 5% RIB than in the 20% refuge (174 in the 5% RIB; 99 in the 20% refuge). This result suggests that synchronous emergence of refuge and Bt emergent adults allows for greater mixing (Murphy et al., 2010). More mixing would support the case for seed mix refuges. But to determine the better planting strategy, larval movement between plants must be studied in more depth. If larvae frequently move between plants, which is likely, larvae can then acquire a sublethal dose of the Bt toxin (Mallet and Porter, 1992). Previous research has also shown that larvae tend to move away from a low-quality food source; for example a Bt host (Hibbard et al., 2005).

Observed rates of mating between refuge and Bt beetles differed from expected rates of mating calculated from the emergence data. The explanation for this may be that not all of the beetles in the mating cages were collected, whereas all beetles found in the emergence cages were collected. This means that not all of the mating pairs were collected over the course of the season. This is a product of sampling only three days per week, and sampling only in the mornings.

The question now is: why isn't resistance happening in Indiana now? To answer this we have to look at several different factors. For starters, most of the fields in Indiana are rotated, not corn after corn. Assuming any volunteer corn in the following year's soybeans are controlled, this prevents any eggs and subsequent larvae in that field from surviving the next year when the field is to be planted with soybeans or some other non-host crop. Another consideration is the relatively low abundance of WCR in Indiana fields. Lower abundance creates a reduced likelihood of resistant adults happening upon one another. Another question is: how long will current refuge practices maintain effectiveness and what recommendations can be made to prolong the durability of current Bt hybrids? This question is covered in Chapter 3.

There are some caveats associated with the methods in this experiment that must be pointed out. The cage nature of the study is the first limitation. First, field cages were utilized in this study, which can limit the dispersal of adults to distant areas of the field, although this may be of importance. Previous research shows that males tend to only travel as far as they need to in order to find a mate (Marquardt and Krupke, 2009). Anecdotally, many of the caged adults did not mate by the end of the sampling periods. The majority of non-mated beetles were females, raising the question of whether these females mated between sampling periods or the sex ratios were heavily female-biased and remained unmated throughout the season. To determine the mating status of remaining females, one could collect the females and conduct dissections to determine if these females were mated with or not. Presumably, because a male's ability to mate declines with age, many of the males that would have been flying around in the cages would have been the 'old males' and either previously mated or were less likely to mate due to their age (Kang and Krupke, 2009b). Another noteworthy point is that not every mating pair was collected because sampling only occurred 3 d per wk. These uncollected mating pairs could have influenced the results by shifting the numbers in favor of one mating strategy versus another (mixed pairing, Bt/Bt paring, or refuge/refuge pairing). To predict this potential shift, remaining adults in the cages could be sampled for N^{15} to give an idea of how many Bt versus refuge adults remained un-sampled. But the large numbers of samples collected over the summer give a strong indication of what happens in a field. This is attributed to mostly females remaining in the cages; therefore most of the males would have been collected throughout the study.

Second, some of the larvae that developed into N^{15} labeled adults could have fed on a Bt plant at one point. This may also play a part in the simultaneous emergence of both susceptible and tolerant beetles in the 5% RIB treatment (Murphy et al., 2010). It is also possible that adults could acquire the N^{15} label via feeding on the leaves/pollen of labeled plants. To reduce the influence of this variable, all of the labeled plants were cut down to 0.4 m and continuously stripped of new growth throughout the season. Next, because cages were not cleared of all beetles after each sampling event, it is impossible to know if mating pairs that were collected were previously mated (this is true for both sexes). Cages could not be cleared of all adult WCR, because the male's need for premating development. Although multiple mating is not the norm, females do mate multiple times in some cases, usually when her first mate's spermatophore is not of sufficient size (Lew and Ball, 1980). Males may also have reduced chances of mating more than one time due to the input needed to produce a second spermatophore (Murphy and Krupke, 2011) and the time restriction of mating within 10 days (Marquardt and Krupke, 2009).

With this in mind, the results obtained show that in a 5% RIB strategy, which is the current dominant form of refuge planting, higher numbers of Bt exposed adults compared to adults that were not exposed to the Bt event are surviving. These results also show that the method of using N^{15} to label larval WCR is an extremely useful tool and should be implemented in future research.

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Table 2.1 Emergence of adults from the 20% strip refuge and 5% RIB emergence cages for each sampling event in 2013.
* indicates peak emergence.

Table 2.1										
20% Strip Refuge Emergence						5% RIB Emergence				
Date	# M		# F			Date	# M		# F	
	Bt	Ref	Bt	Ref			Bt	Ref	Bt	Ref
3-Jul	0	0	0	0		3-Jul	0	0	0	0
5-Jul	0	1	0	0		5-Jul	0	0	0	0
8-Jul	2	2	0	1		8-Jul	3	1	0	0
10-Jul	0	1	3	1		10-Jul	2	0	0	0
12-Jul	2	3	0	2		12-Jul	2	0	1	2
15-Jul	4	3	0	4		15-Jul	7	*12	5	2
17-Jul	5	15	5	7		17-Jul	8	6	15	5
19-Jul	*14	11	11	17		19-Jul	5	1	17	6
22-Jul	5	*19	*30	*27		22-Jul	*18	*12	*43	*18
24-Jul	0	0	0	0		24-Jul	1	0	6	1
26-Jul	1	1	3	6		26-Jul	3	1	4	3
29-Jul	1	0	1	0		29-Jul	4	1	18	5
2-Aug	2	2	7	13		2-Aug	2	1	3	7
5-Aug	0	0	0	0		5-Aug	1	0	7	0
7-Aug	0	3	1	5		7-Aug	2	0	1	0
9-Aug	1	1	2	7		9-Aug	3	0	1	1
12-Aug	0	2	2	10		12-Aug	0	3	2	0
14-Aug	0	0	0	3		14-Aug	0	1	0	0
16-Aug	0	0	1	2		16-Aug	1	0	0	1
19-Aug	0	1	0	1		19-Aug	0	0	0	1
21-Aug	0	0	1	2		21-Aug	0	0	0	0
23-Aug	0	0	0	1		23-Aug	0	0	0	0
26-Aug	0	0	0	0		26-Aug	0	0	0	0
28-Aug	0	0	0	0		28-Aug	0	0	0	0
30-Aug	0	0	0	0		30-Aug	0	0	0	0
Total	37	65	67	109		Total	62	39	123	52
% of total	13.31	23.38	24.10	39.21		% of total	22.46	14.13	44.57	18.84
% of sex	36.69		63.3			% of sex	36.59		63.41	

Table 2.2 Emergence of adults from the100% refuge and 100% Bt emergence cages for each sampling event in 2013.
* indicates peak emergence.

Table 2.2						
	100% Refuge				100% Bt	
Date	# M	# F		Date	# M	# F
3-Jul	2	0		3-Jul	0	0
5-Jul	2	0		5-Jul	0	0
8-Jul	8	2		8-Jul	1	0
10-Jul	6	3		10-Jul	0	0
12-Jul	15	13		12-Jul	3	0
15-Jul	31	23		15-Jul	20	14
17-Jul	35	44		17-Jul	15	6
19-Jul	53	65		19-Jul	9	9
22-Jul	*54	*110		22-Jul	*22	*26
24-Jul	14	43		24-Jul	0	6
26-Jul	3	14		26-Jul	1	6
29-Jul	7	34		29-Jul	4	5
2-Aug	4	15		2-Aug	4	15
5-Aug	3	10		5-Aug	2	19
7-Aug	0	6		7-Aug	1	1
9-Aug	2	6		9-Aug	0	1
12-Aug	0	3		12-Aug	0	1
14-Aug	1	0		14-Aug	0	2
16-Aug	0	2		16-Aug	0	1
19-Aug	0	1		19-Aug	0	0
21-Aug	0	1		21-Aug	0	0
23-Aug	0	0		23-Aug	0	0
26-Aug	1	2		26-Aug	0	0
28-Aug	0	1		28-Aug	0	0
30-Aug	0	0		30-Aug	0	0
Total	241	398		Total	82	112
% of sex	37.72	62.28		% of sex	42.27	57.73

Table 2.3 Heat map of 2013 data showing atom % excess for 20% strip refuge mating pairs. Darker cells indicate sample contained high levels of the N^{15} label. As the cell gets lighter (more white) less N^{15} is found in the sample. The highest label amounts appear early in the study and fade throughout the season, and only in the 20% refuge. This indicates that plants close to one another serve as 'hot spots' and as the plants grow the N^{15} is spread throughout the rest of the plant structures or the N^{15} decays over time.

Date	20% Refuge Heat Map of Atom % 15N Excess							
12-Jul	4.9859	261.4135	2.5834	105.3643	38.8099	198.6772	105.2559	-0.6327
15-Jul	-0.2328	-0.1738	71.1313	1.2092	0.7054	-0.4043	172.2350	105.9173
17-Jul	0.2832	0.2375	55.0288	1.0711	116.9040	0.6346	-0.4079	267.5955
	103.3743	-0.1511	-0.8528	82.7340	0.4170	130.7791	0.9131	0.3558
19-Jul	0.2731	80.2829	49.4575	0.2394	201.3637	11.3693	1.6076	48.8050
24-Jul	0.1823	0.2265	0.2383	64.9740	0.8716	29.9118	0.4037	1.0921
	0.0403	-0.1052	0.5344	8.4096	0.9373	89.6599	12.5315	35.3966
	44.6133	1.4273	158.3436	2.7611	4.5768	-0.0337		
26-Jul	1.4037	1.4987	0.2392	20.0599	-0.0357	0.2600	0.0900	0.1577
	-0.1986	30.9344	0.3350	0.2456	55.9575	0.1476	41.8198	55.3665
	18.8368	24.5975	0.1284	-0.1299	5.3372	-0.2992	0.0207	0.0355
29-Jul	-0.0312	0.1323	35.5744	-0.0798	11.1147	0.0538	45.3852	0.5814
31-Jul	0.0035	0.5592	0.0231	9.7059	72.5214	-0.2762		
2-Aug	0.8748	0.1992	26.3378	7.9197	0.2660	2.2630	38.1335	0.2787
	70.6856	0.9319	37.3646	1.0431	57.7539	2.3134	19.4340	24.0826
	0.2061	26.1564	0.3940	0.4469				
5-Aug	0.4111	2.8491	2.8643	0.7509	40.2564	1.1464	1.3041	0.4967
	42.2391	2.4966	0.7489	15.2284	0.7880	0.7277	0.2636	0.4426
7-Aug	0.7165	0.7004	0.8745	0.7232	51.5981	28.0475	91.2851	2.9639
9-Aug	1.0317	1.1538	9.8769	0.1627	4.4170	0.8050	0.5975	0.3783
	67.9918	0.1790	0.9473	1.2787	1.4892	3.9335	1.3285	0.6582
	-0.0384	0.0446						
12-Aug	0.0563	0.6234	1.1307	0.7621	0.7694	0.9280	28.8170	0.9842
14-Aug	8.9678	0.1079	0.0876	-0.0029				
16-Aug	0.2516	-0.0506	16.7315	-0.2763	3.9710	0.4693	10.4456	6.1150
19-Aug	0.2675	-0.1701						
23-Aug	32.9163	-0.0151	60.9615	0.1071	14.6930	0.0696		
26-Aug	0.3309	0.4397						

Table 2.4 Heat map of 2013 data showing atom % excess levels of label found in the 5% RIB mating pairs using the same scale as the 20% refuge. In this treatment, there are less extreme values than seen in the 20% refuge. This is due to labeled plants being farther apart than in the strip refuge.

Date	5% RIB Heat Map of Atom % 15N Excess							
12-Jul	-0.2099	0.1703	1.6883	0.4456	-0.2962	-0.2091		
15-Jul	89.0908	63.8380						
17-Jul	0.8994	0.5488	1.1138	46.1001	0.6690	0.3237	5.1309	1.2968
	1.4643	49.6631	7.7461	44.5866	0.9676	0.4783		
19-Jul	1.2592	0.2833	-0.2549	-0.3413	-0.2423	-0.0516		
24-Jul	0.7991	1.0499	5.8448	0.5153	1.5924	1.1067	1.2074	0.9191
	-0.0263	-0.0221	6.3436	8.6272	1.5730	0.4927	0.7980	-0.4481
	1.0365	0.2075	0.0246	-0.5759	-0.0158	-1.0730	0.1440	-0.2229
	0.2475	-0.2758	2.4228	-1.2147	0.0064	-0.2428	-0.6637	-0.8134
	10.7919	-1.4492	-0.2249	-0.3641	2.1939	5.1501	3.8618	-1.5965
	-0.4613	-0.7612						
26-Jul	-0.0157	2.0902	0.4707	0.2434	1.0590	-0.8230	0.6182	0.3848
	5.1539	0.1047	-0.6307	-0.4072	-0.6617	-0.5644	0.4253	8.2396
	0.1541	0.1423	0.5134	-1.9761				
29-Jul	-0.3114	-0.7230	0.6467	0.4230	2.0365	0.9560	0.5242	-1.0006
31-Jul	0.8058	2.3226	31.2610	-0.2782	0.0242	-0.1552	-0.1106	-0.0340
	0.1645	5.5937						
2-Aug	10.3035	0.6815	4.7267	0.1725	0.4581	0.2231	1.1444	0.7479
	5.8924	-0.5735	1.6990	1.3289	2.6072	3.7377	0.9474	0.6243
	1.6352	4.0522	5.0686	5.4980	1.4821	12.0030	0.3703	-0.6526
	0.6755	7.6745	0.5229	0.5877	0.2059	0.1688	0.3557	1.7225
	-0.0920	12.0097	0.4311	-0.3094	0.0733	0.9194		
5-Aug	11.2745	-0.2510	0.8092	0.1782	0.3168	0.0621	0.5155	0.5669
	-0.1012	0.0491	-0.0794	-0.0987	0.4810	2.4880	-0.0829	-0.0796
	0.1893	0.0530	3.2470	3.6211	1.1368	1.7814	7.0585	1.3416
	10.1558	2.9456	1.4559	3.1591	0.0502	0.1592	0.3677	-0.2156
	0.2883	0.4712	-0.0178	0.4931	0.1654	0.1083	-0.4225	0.0729
7-Aug	0.3192	0.2435	0.3224	0.1253	1.6003	0.3197	2.4306	2.1533
	1.5164	3.6444	0.5795	0.6748	0.1816	-0.0716	-0.0618	0.1676
	1.9495	0.4791	-0.0666	0.1138	0.3088	0.1217	0.0138	0.2489
	0.0967	0.2267	-0.1262	-0.7083	0.4993	0.0905		
9-Aug	0.8452	0.8673	0.0394	0.0675	11.3026	0.2356	0.2406	-0.0701
	1.2252	0.5450	0.2022	-0.4155	0.0317	-0.0775	0.7370	1.1919
	7.1788	0.0437	0.1915	0.1984	0.2113	0.2860	0.1417	0.2544

	1.0517	0.0200	0.0689	-0.0838	1.3981	0.1191	3.4323	1.0110
	1.0503	-0.1035	0.7480	-0.0251				
12-Aug	0.4223	-0.1738	1.7720	0.9281	0.2738	0.2616	-0.0399	-0.3713
	0.9289	-0.0246	0.0502	0.0278	-0.0055	3.7228		
14-Aug	0.0538	0.5740	0.0488	0.1026	0.1470	0.1366	-0.0949	-0.1485
	-0.0854	0.4698	0.3853	0.0737	0.0120	0.2430		
16-Aug	-0.0807	0.1324	1.1517	9.9919	0.1482	-0.0637	0.0908	0.1255
	0.0358	0.2136						
19-Aug	1.6727	1.3463	0.1042	-0.1918				
21-Aug	0.0671	0.0796	0.8222	-0.0989	1.0817	0.3405	0.2766	-0.0529
23-Aug	0.3605	0.0059	0.0712	-0.0555	1.0497	0.8694	0.4282	0.0488
26-Aug	0.8375	1.7767	2.4622	0.3071	2.0128	0.8390	0.0037	16.0692
	0.0617	15.9462						

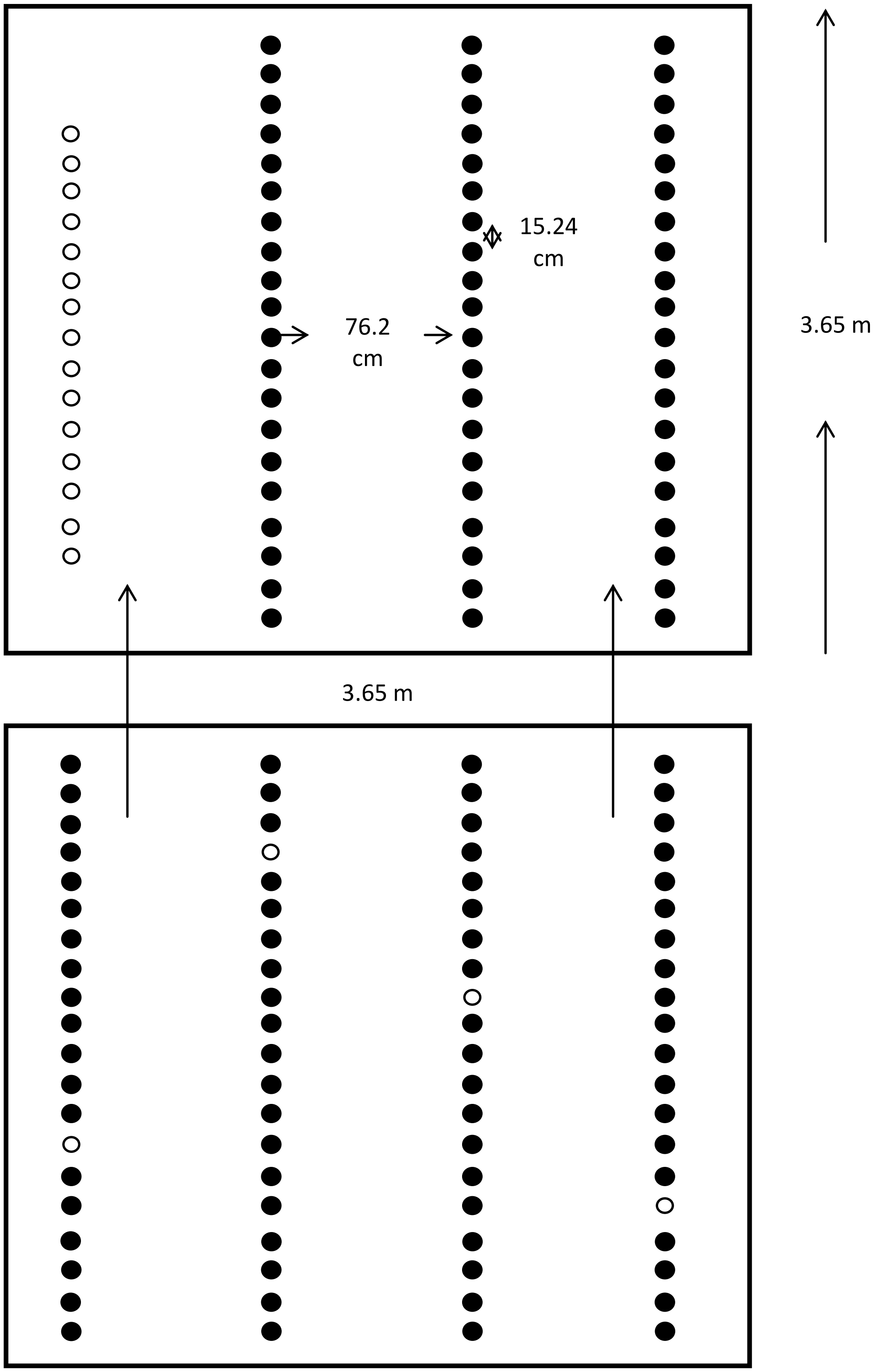

Figure 2.1 Plot design for the 20% strip refuge (top) and the 5% refuge-in-a-bag (bottom). White dots indicate refuge plants; black dots indicate Bt plants. (Not drawn to scale)

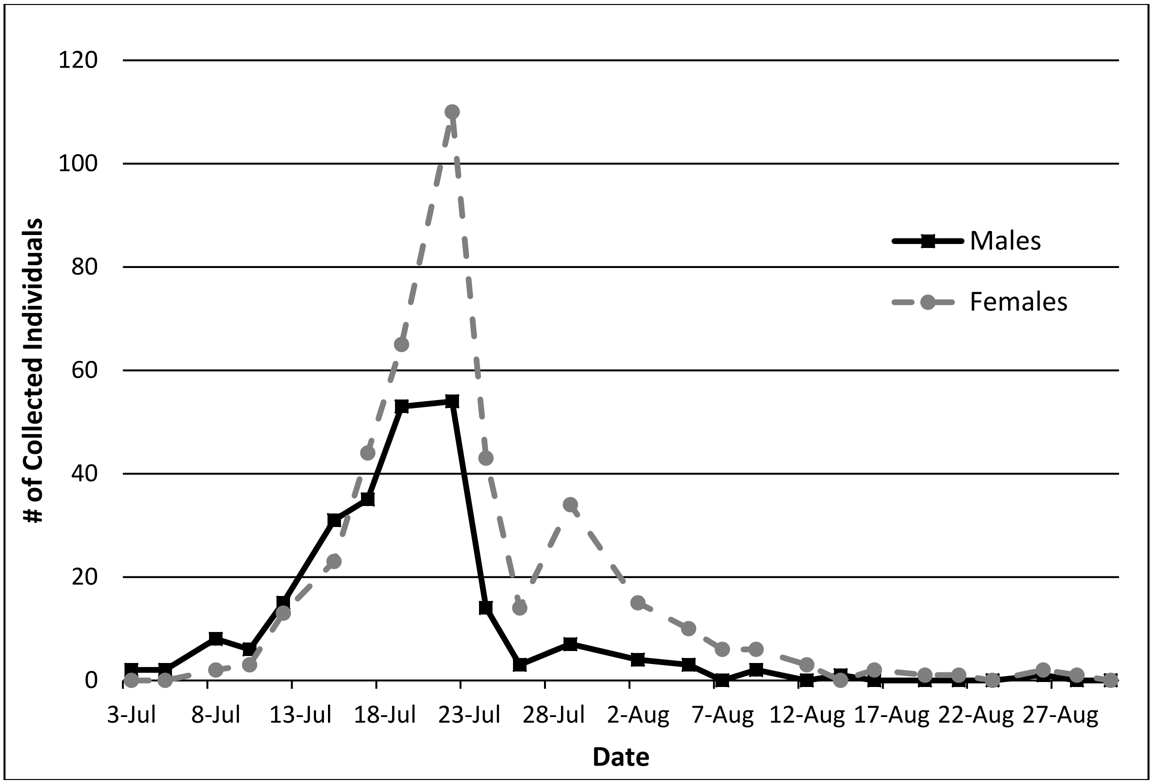

Figure 2.2 100% refuge WCR emergence in emergence cages from July 2 – August 28, 2013.

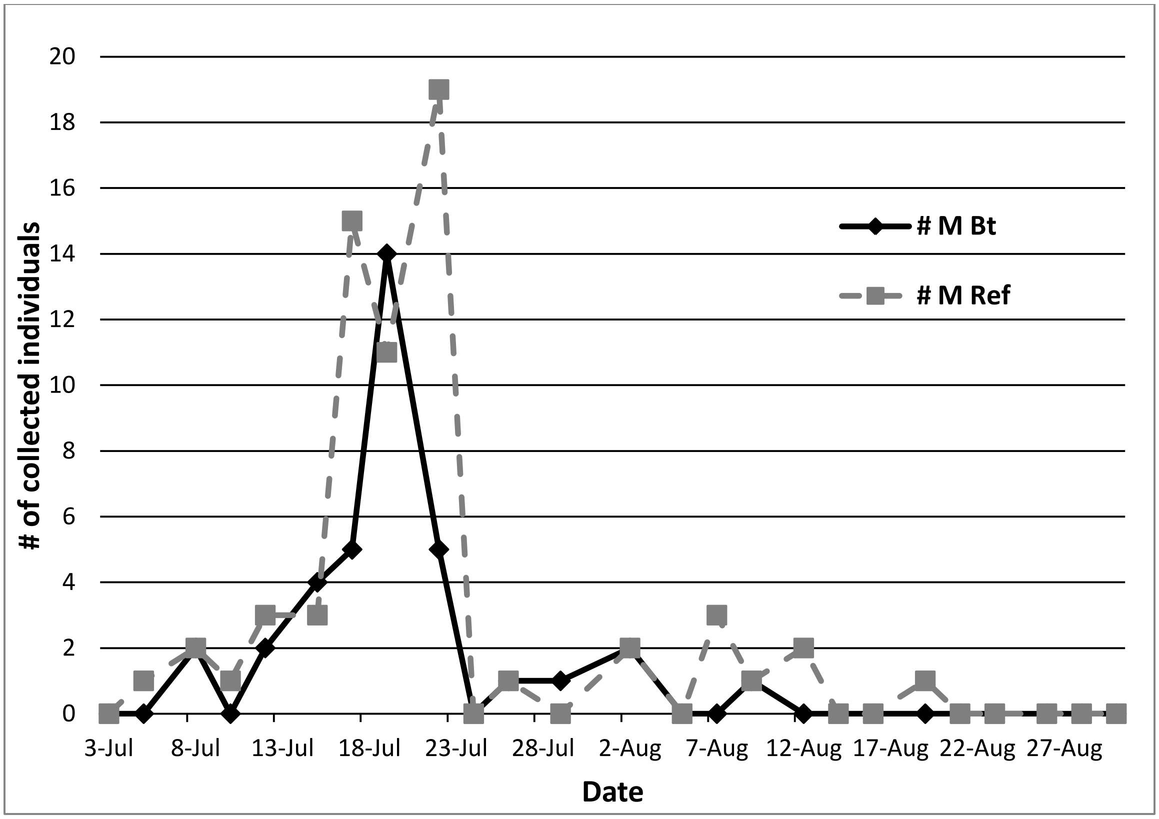

Figure 2.3 Male emergence from the 20% strip refuge emergence cages from July 5 – August 19, 2013.

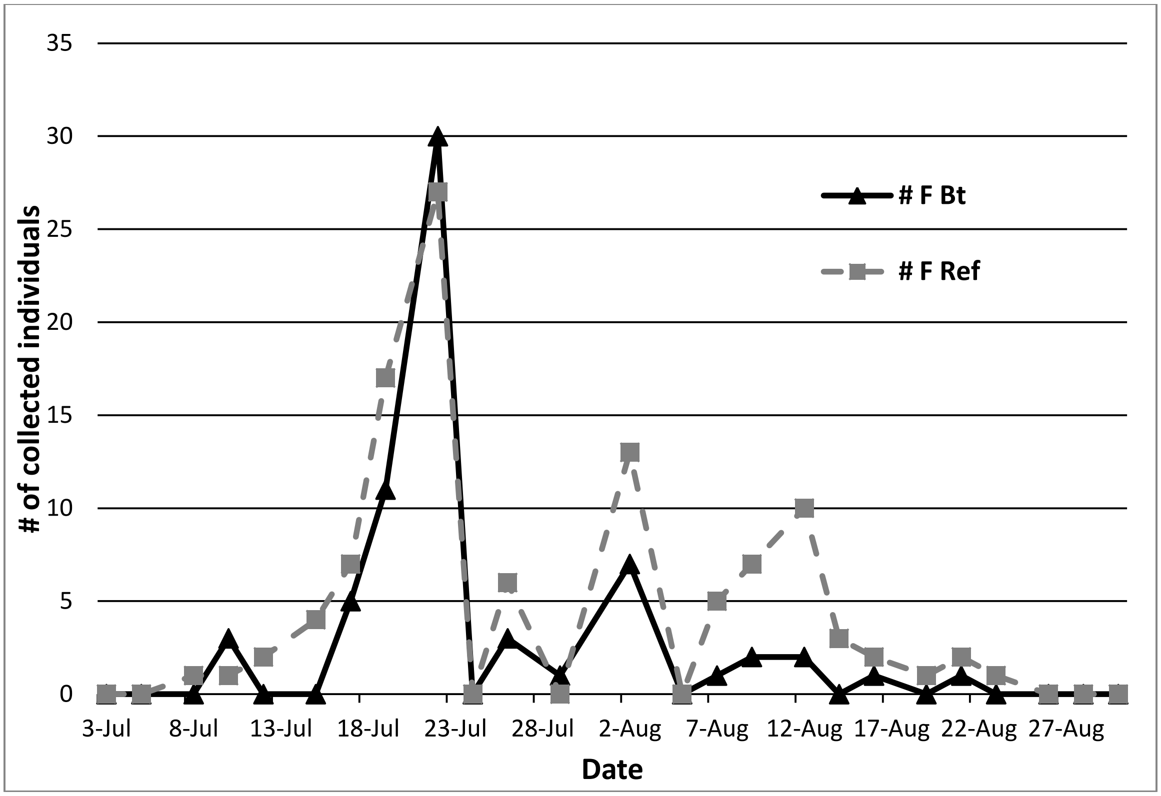

Figure 2.4 Female emergence from the 20% strip refuge emergence cages from July 8 – August 23, 2013.

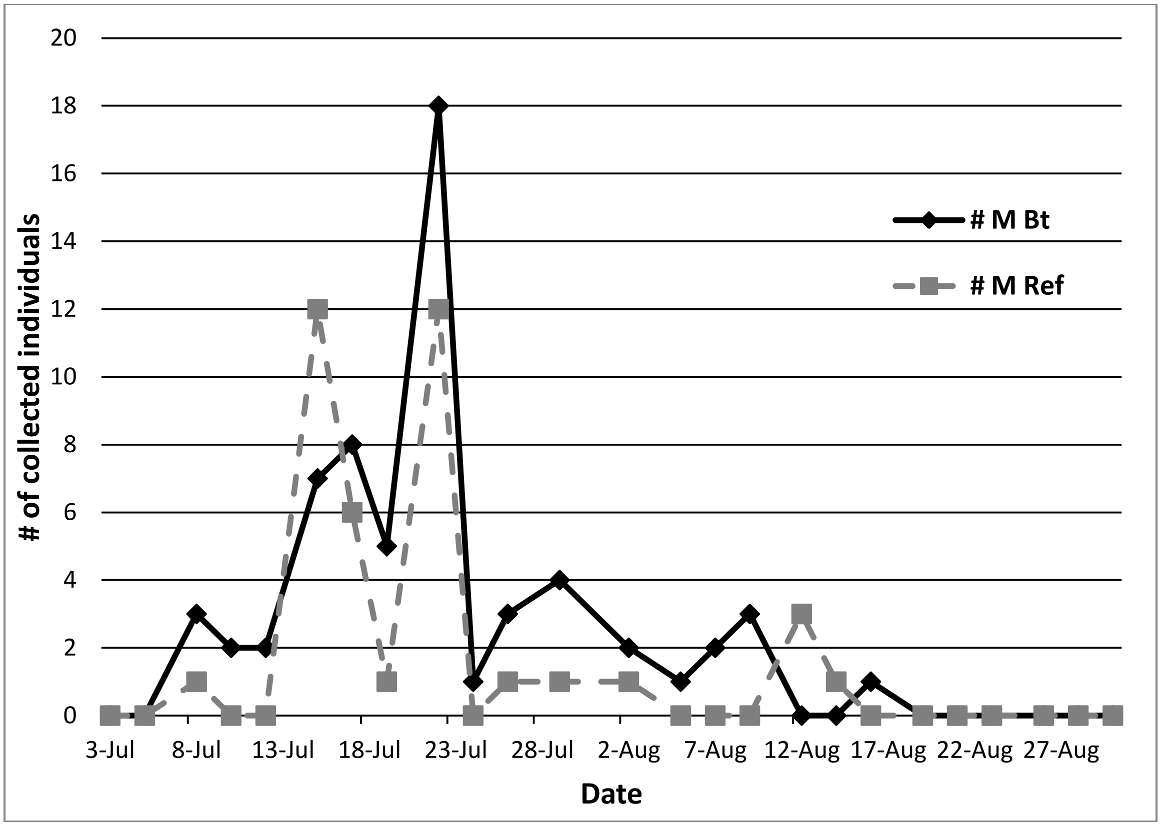

Figure 2.5 Male emergence from the 5% RIB emergence cages from July 8 – August 16 2013.

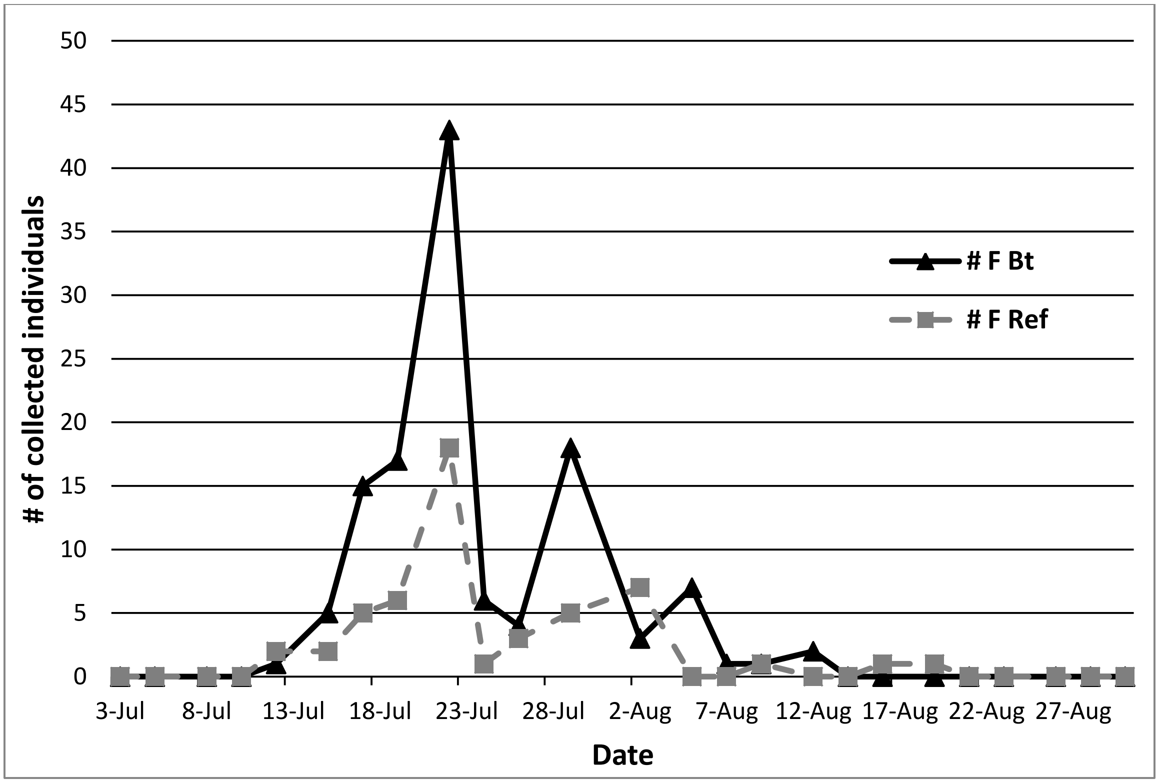

Figure 2.6 Female emergence from the 5% RIB emergence cages July 12 – August 19, 2013.

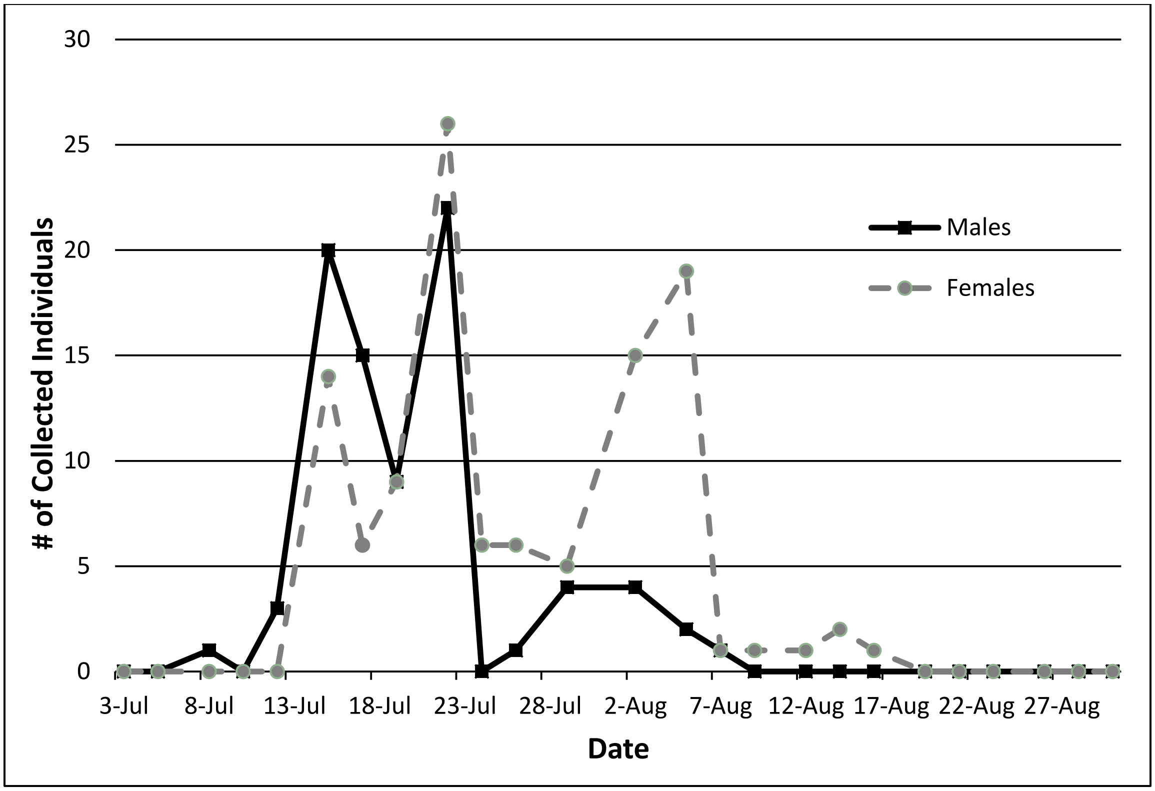

Figure 2.7 100% Bt WCR emergence July 8 – August 16, 2013 in emergence cages.

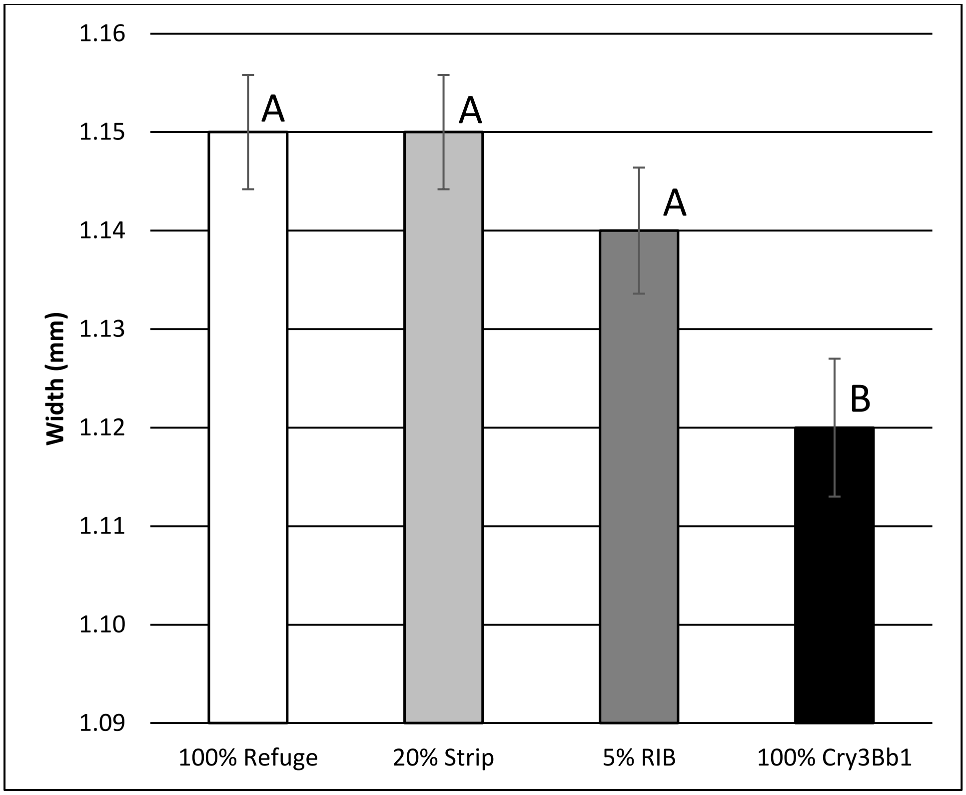

Figure 2.8 Head capsule width of male WCR for each treatment in 2013. Letters represent the grouping of significance at a level of $\alpha = 0.05$. Data were analyzed using a one-way ANOVA test followed by a Tukey test.

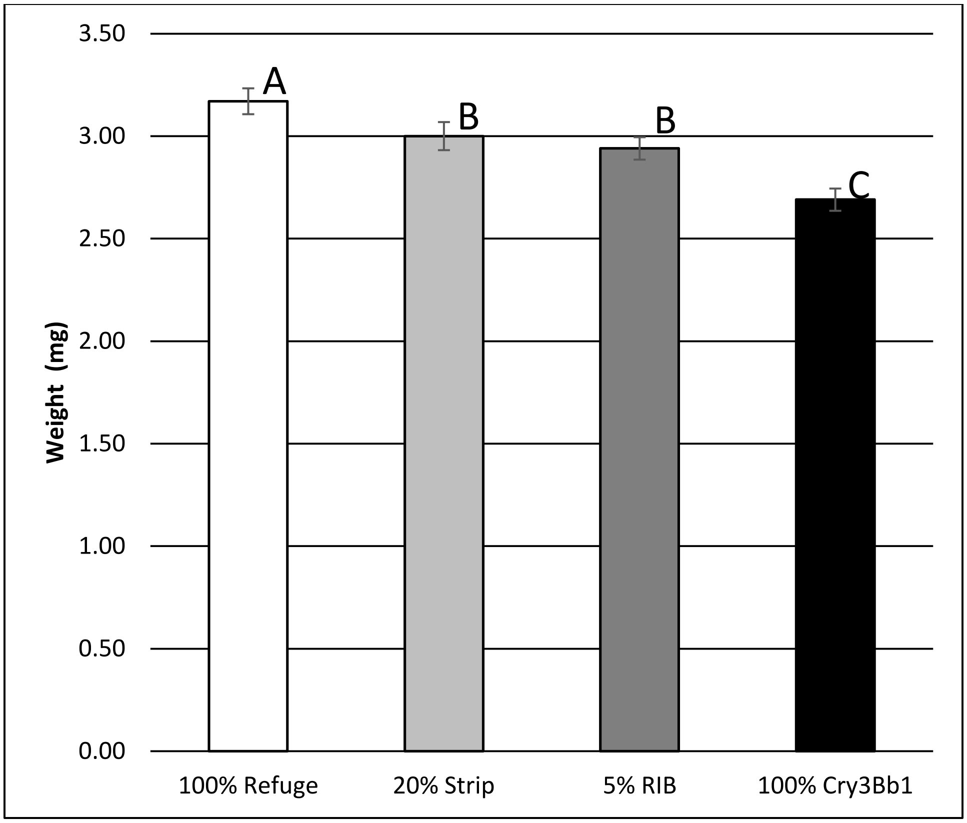

Figure 2.9 Dry weights of male WCR for each treatment in 2013. Letters represent the grouping of significance at a level of $\alpha = 0.05$. Data were analyzed using a one-way ANOVA test followed by a Tukey test.

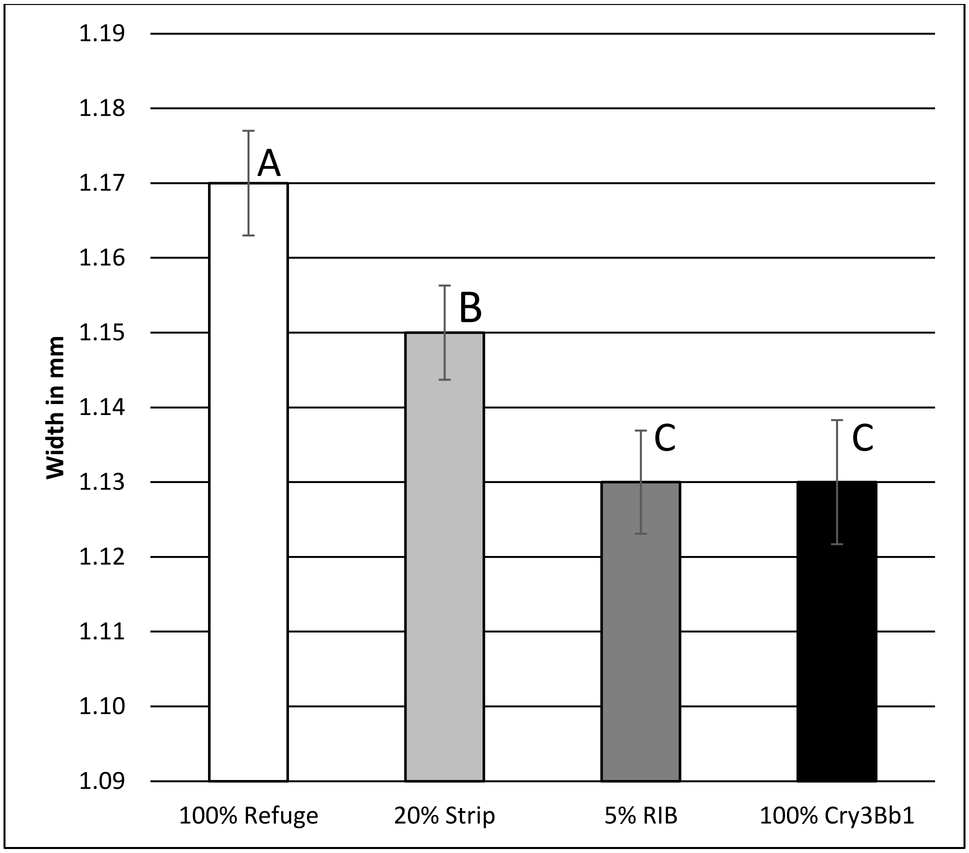

Figure 2.10 Head capsule widths of female WCR for each treatment in 2013. Letters represent the grouping of significance at a level of $\alpha = 0.05$. Data were analyzed using a one-way ANOVA test followed by a Tukey Honestly Significant Difference test.

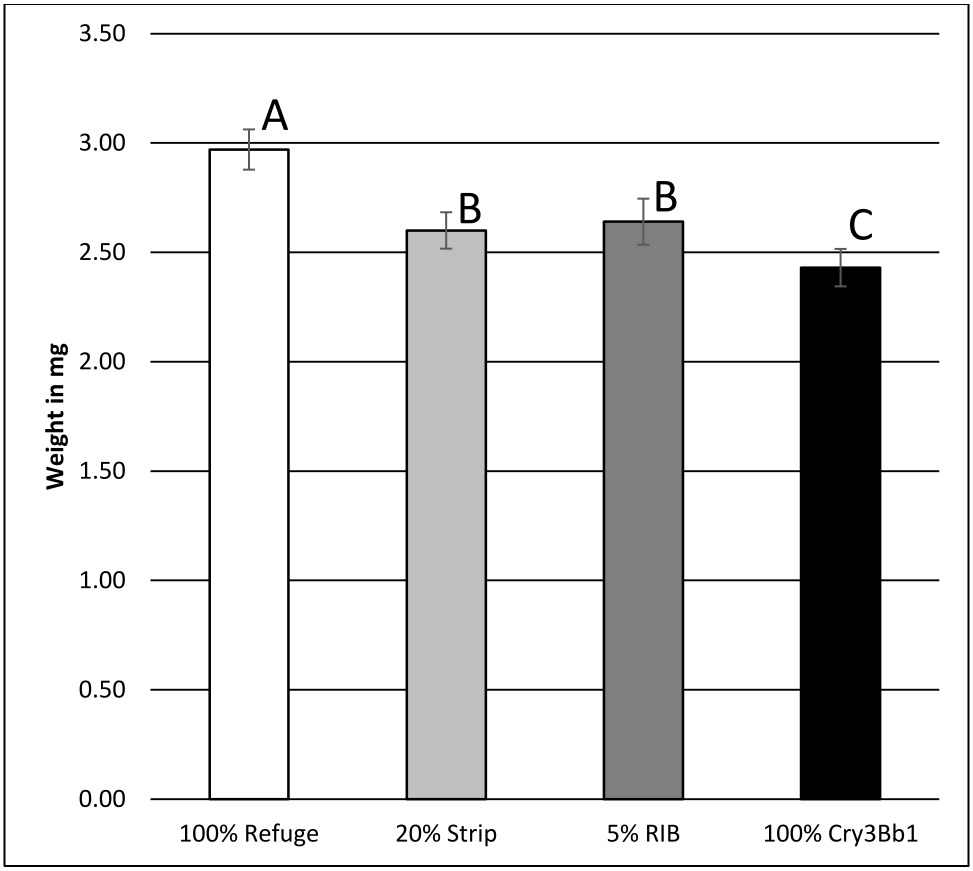

Figure 2.11 Dry weights of female WCR for each treatment in 2013. Letters represent the grouping of significance at a level of $\alpha = 0.05$. Data were analyzed using a one-way ANOVA test followed by a Tukey Honestly Significant Difference test.

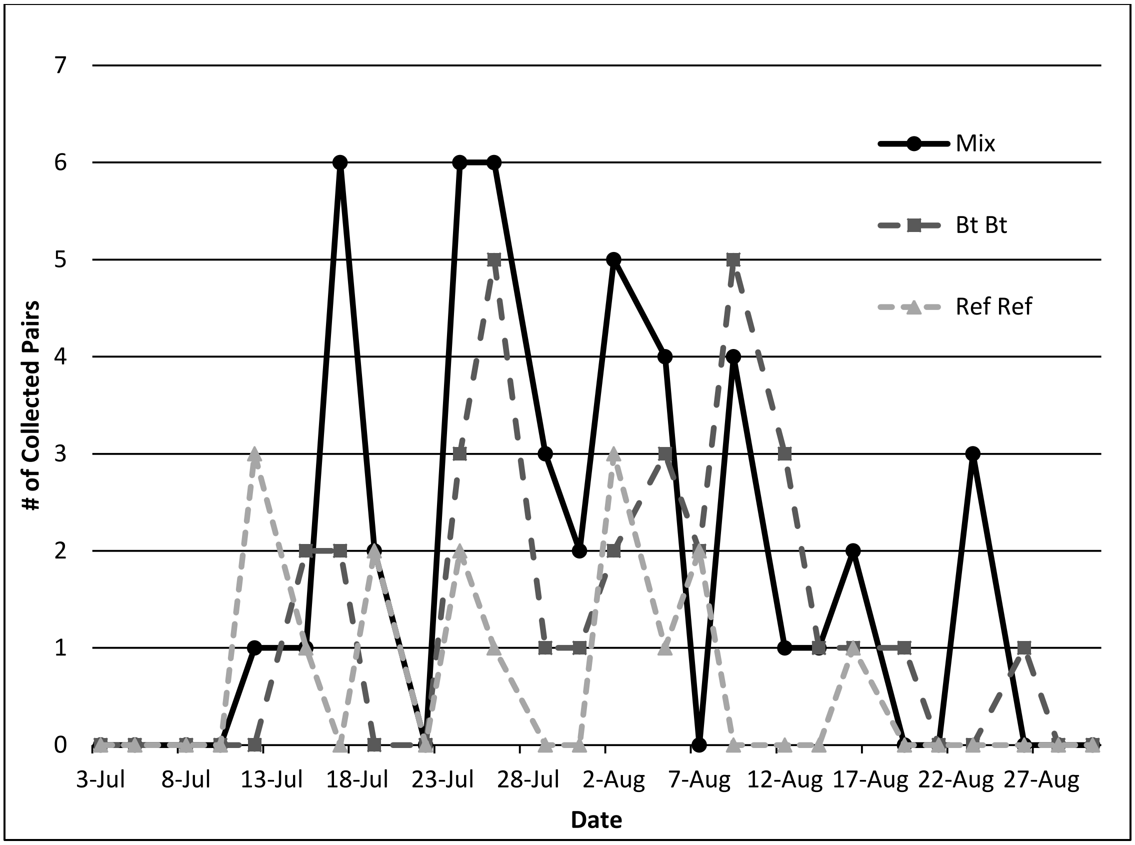

Figure 2.12 Total number of mating pairs collected in the 20% strip refuge from July – August 2013. Mix = one refuge beetle mating with a Bt beetle. Either sex can be Bt emergent.

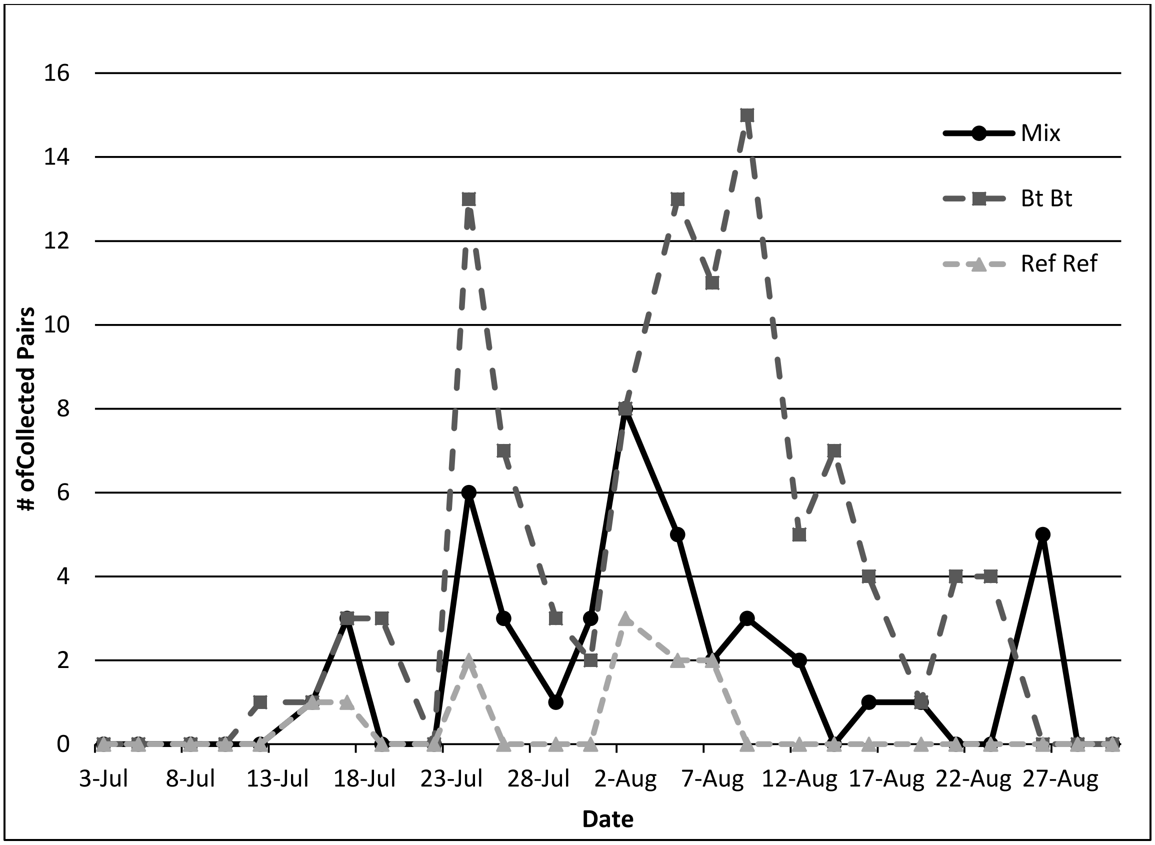

Figure 2.13 Total number of mating pairs collected in the 5% RIB treatment over time. Mix = one refuge beetle mating with a Bt beetle. Either sex can be Bt emergent.

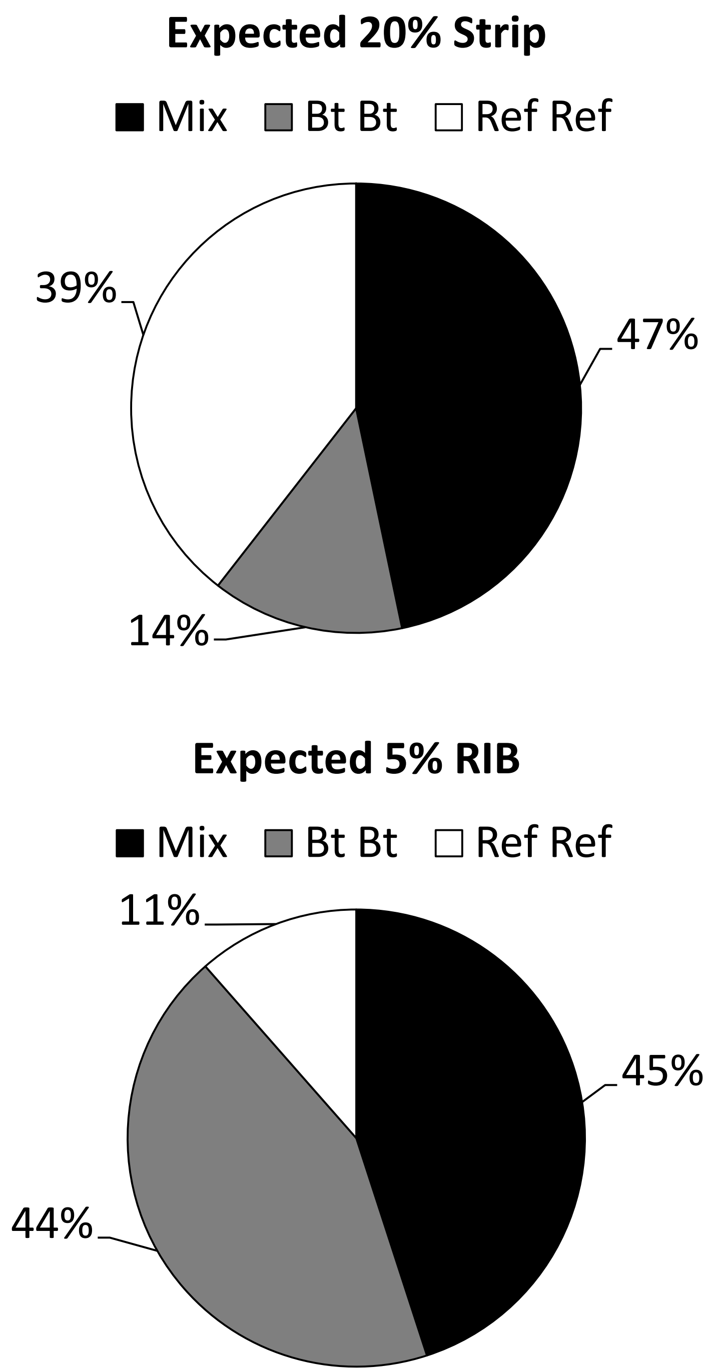

Figure 2.14 Expected percentage of mating pairs in each treatment in 2013 based on emergence of adults from the 20% and 5% refuge emergence cages. Expected values are calculated using daily ratios of Bt and refuge emergent males and females.

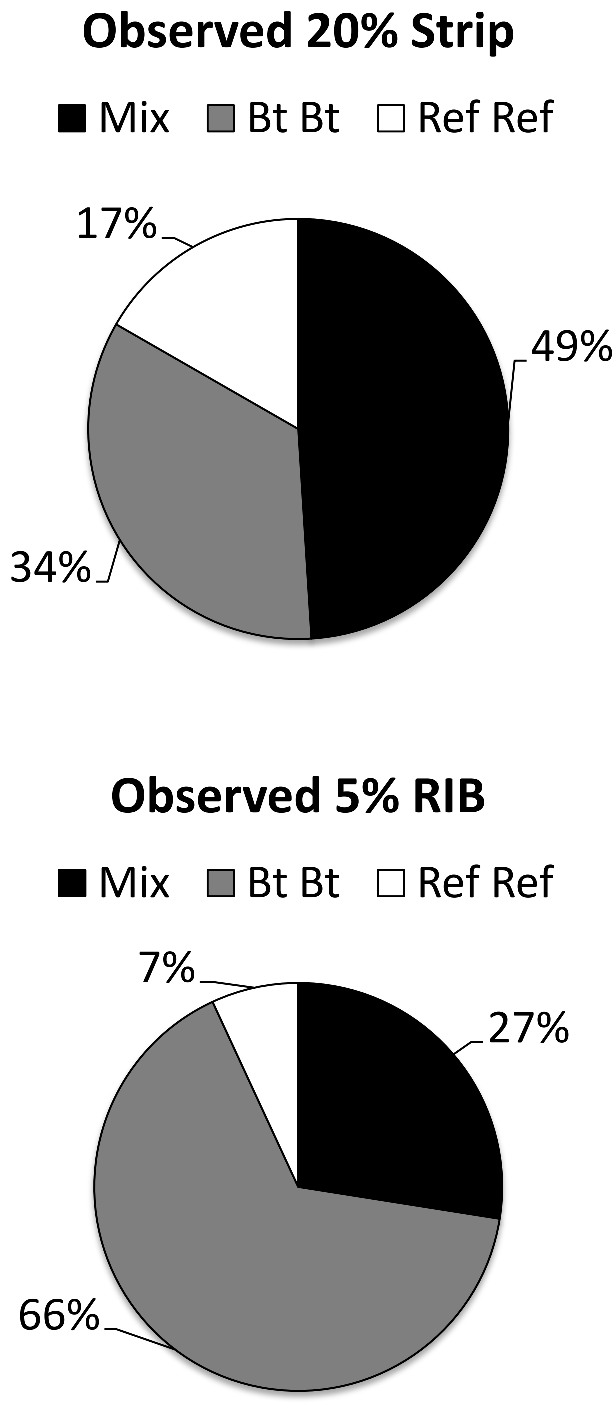

Figure 2.15 Observed percentage of mating pairs in 2013 for each treatment from the 20% and 5% refuge mating cages. Observed values calculated from daily ratios of Bt Bt pairs, refuge refuge pairs, and mixed mating pairs.

CHAPTER 3. SUMMARY

GM corn hybrids are an extremely successful mode of reducing pest populations and increasing yields with the advantage of significantly reducing pesticide usage. However, resistance is always a risk despite the effectiveness of any insecticide, including those expressed by GM crops. However, with careful practice and usage of these tools we can prolong their benefits. Therefore, pest management approaches to slow resistance development must be a top priority. With the recent resistance development of WCR to the *Cry3Bb1* event after less than a decade of commercial use, the next Bt events, or any other GM hybrid corn targeting WCR should be used and preserved in the most effective way.

The results in this study show that numerous adults are emerging from Bt hybrid corn, which is not how the technology in association with a refuge was originally intended to work. If the current Bt hybrids available are to be maintained, growers need to implement a multifaceted approach, or IPM. The following few paragraphs explain what growers can do to slow resistance development of the WCR to Bt events if used in association with one another.

First and foremost, growers should rotate crops in a field from year to year (Gassmann et al., 2011). This could be as simple as a corn/soybean rotation or more complex as to rotate in wheat or other crops giving a multi-year approach. Rotating to a crop other than corn guarantees that all of the WCR eggs laid in that field the previous season will die due to inability to find a suitable food source (Crowder et al., 2005), if adequate volunteer corn management practices are employed. Crop rotation is often not used because regional market pressures often make continuous corn the most economically attractive option.

Whether the field is to be planted corn after corn or rotated, a tactic that should be utilized is rotating Bt events in a field from year to year (Gassmann et al., 2011). The constraints here are that growers have loyalty towards seed vendors, and seed vendors usually only supply one variety of Bt hybrid targeting WCR. When a single event is used year after year the likelihood of resistance development is maximized. More and more beetles will become tolerant to the Bt event employed (Gassmann et al., 2011), eventually reaching numbers sufficient to cause economic damage. When Bt events are rotated, WCR are forced to feed on different toxins each year. The likelihood that a given beetle is tolerant to more than one Bt toxin with different modes of action, and that this tolerance is heritable, is rare (Bravo and Soberón, 2008). Cross resistance has been documented between Bt hybrid corn containing *Cry3Bb1* and *Cry3Aa* because the *Cry3Aa* event is simply a modified version of *Cry3Bb1* (Gassman et al., 2014). With this in mind, rotating Bt events reduces the potential for resistance development.

Since the Bt toxins available for WCR are not high-dose, the advent of pyramided Bt events (SmartStax®; collaboration between Monsanto, St. Louis, MO and Dow Agrosciences LLC, Indianapolis, IN) can be a vital tool, and is now the more common approach. SmartStax® exposes larval rootworm to both the Herculex® and Yieldgard® Bt events within the same plant. Although if a population is already resistant to one of the Bt toxins, the viability of the other trait is likely to weaken (Gassmann et al., 2011).

A way that growers can stay loyal to vendors and plant continuous corn would be to rotate a Bt event(s) with soil insecticides (Gassmann et al., 2011). Rotating between a Bt corn hybrid and a soil insecticide exposes larval WCR to two non-related insecticidal modes of action. Common insecticides used for WCR control are organophosphates (Fortress 5G®, Aztec 2.1G® and Lorsban 15G®) and pyrethroids (Force 3G® and Capture 2E®).

Finally, growers *must* plant refuges when using Bt hybrids, and if possible, increase the size of the refuge to allow more unexposed adults to disperse throughout the field (Tabashnik and Gould, 2012). In order to preserve Bt corn, there must always be abundant susceptible beetles in the WCR population. To have susceptible beetles, there must be plants in the field that do not contain the Bt toxin. Without the refuge, there is no means for resistance management because all surviving individuals will be tolerant to the Bt event and have an increased likelihood of passing that trait on to offspring. By adding sufficient susceptible beetles into the mix, tolerant beetles should, ideally, be outnumbered and the likelihood of mating with a susceptible beetle will be greater. The ideal planting configuration may be planting 4-6 row strips of refuge throughout the field with only 8-12 rows of Bt corn between them. This increases the likelihood that larval movement away from the original host has a greater chance of finding a plant of the same variety, while not forcing adult males to travel long distances to mate with a female emerging from the opposite variety of host plant.

All of the factors listed above are based on random mating (which appears to be occurring based on this study). But in the case that random mating does not occur, and some form of selection is occurring, different measures should be taken. If mating is not random due to size differences between Bt and refuge adults, the preferred refuge planting would be the seed mix approach along with a soil insecticide application. This planting method allows for synchronous emergence of Bt and refuge adults and size differences between refuge emergent and Bt emergent adults are not as variable. This is probably attributable to movement of larvae between plants. Increasing the refuge size may increase the level of sublethal exposure that larvae encounter. Having a higher chance for larvae to move from a Bt plant to a refuge plant or vice versa allows larvae a greater chance for surviving the Bt event. When in a 5% refuge, the likelihood of finding a refuge plant is very small compared to finding a Bt plant. Adding in the soil insecticide creates another hurdle that the larval rootworms must survive. These chemicals should only be used in fields where the threat of resistance is high and some damage has been observed on Bt corn roots. Soil insecticides reduce surviving adults by protecting the central root mass of the corn plant from larval feeding. In turn this reduces the number of Bt tolerant adults available to mate with. Consequently, this also reduces the number of susceptible adults, but low numbers of adults may promote more chance mating encounters. Chance mating refers to mating with the first individual one comes across without any selection due to the reduced chances of finding another mate (Cade and Cade, 1992).

Next steps in this research are to conduct field studies to determine how male dispersal and female mate selection are influenced on a larger scale versus caged studies.

Determining if susceptible males travel away from a host plant to distant Bt plants in a strip or block refuge system is key for these refuge planting strategies. Quantifying the mixed mating of susceptible and tolerant adults is important for all styles of refuge planting, and may have different results in large scale studies. Because this research determined that using N^{15} to label larval WCR is a useful and easily implemented tool, studies like this are feasible given careful planning and plot design.

Another study that needs to be completed is the further quantifying of larval movement in a Bt/refuge field. This will give insight in to how many larvae are getting a sub-lethal dose of the Bt toxin, or how many early instar larvae move from a Bt plant to a refuge plant and vice versa. Late instar larvae are highly tolerant to the Bt toxins (Binning et al., 2010) and therefore have a greater survival rate when exposed to the toxins. This study will likely need to be conducted on a small scale, at least initially. But again, N^{15} could serve as a label to determine larval host. With more testing, levels of N^{15} in larvae (and not just +/- data as in this study) could be used to determine the rate of movement between plants. This approach could also be used in association with other stable isotopes, namely C^{13}. Labeling the refuge plant(s) with one stable isotope and Bt plant(s) with the other would give better information about how much movement actually occurs.

Finally, identifying how much influence the size and content of the male spermatophore has on female selection could give some insight into the likelihood that males mate more than once. Determining how quickly a young or old male can produce a sizable spermatophore (the first or subsequent ones) may give insight to a male's ability to mate with multiple females. Work by Murphy and Krupke (2011) has shown that spermatophore volume has a positive linear relationship with male size. Another important note is that some studies, including this study, have shown that males emerging from a refuge plant are larger in size (head capsule width, dry weight) than males that emerge from a Bt host (Murphy et al., 2011; Gassmann et al., 2009). Previous work done by Quiring and Timmins (1990) has shown that larger males mate more quickly than smaller males. These points collectively suggest that spermatophore size may be indicative of a male's ability to mate with a female successfully, or mate a second time, potentially because it demonstrates male investment into offspring. Identifying the spermatophore effect may give better insight into mate selection in this species, ultimately leading to a better understanding of how WCR develops resistance to Bt corn hybrids.

3.1 Literature Cited

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