MOVEMENT BEHAVIOR OF THIRD INSTAR EUROPEAN CORN BORERS, *OSTRINIA NUBILALIS,* ON BT CORN

by

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ABSTRACT

The European corn borer (ECB), *Ostrinia nubilalis* (Hübner), is a major lepidopteran pest of maize in the United States and Canada. One of the most effective control methods for ECB is the use of transgenic crops that encode for insecticidal crystalline (Cry) proteins that are derived from the soil bacterium *Bacillus thuringiensis* (Bt). The continued effective use of Bt corn relies on compliance with insect resistance management practices; where planting a small percentage of non-Bt refuge corn adjacent to or as seed blends within Bt corn fields provides a population of susceptible insects that can mate with rare resistant individuals. Little is known about how these non-Bt plant blends affect movement behaviors of ECB in the third instar, an intermediary stage where the larva begins to bore into corn stalks. The objective of my study was to assess and characterize the amount of movement off of infested plants in the field using a variety of five plant arrays containing lepidopteran Bt corn plants and non-lepidopteran protein marked refuge corn plants. Additional studies sought to determine the presence and persistence of three different Bt Cry proteins in third instars using monoclonal ELISA test strips. Based on two years of field movement studies I found that the range of third instars moving off of an infested plant when it was a Cry1Ab corn plant is approximately 30% to 65%. The range for third instars moving off of an infested plant when it was a Cry1F corn plant was approximately 20% to 60%, and that the range for third instars moving off of an infested plant when it was a non-lepidopteran protein marked refuge corn plant was about 6% to 18%. The range of movement off of the infested plant for the protein marked refuge plants was consistent with that for near isoline control plants. The lepidopteran targeted Bt

Cry proteins were found to be consistently detected for seven sampling points over a 24 hour feeding period. However, the non-lepidopteran Bt Cry proteins could not be consistently detected over the same 24 hour period. Additionally, the persistence of Bt plant material in third instars varied depending on the Cry protein. Future research using ELISA plate methods could yield more consistent results for the non-lepidopteran Bt Cry proteins.

Chapter 1

INTRODUCTION

Life History and Ecology

European corn borer (ECB), *Ostrinia nubilalis* (Hübner), is a major economic pest of maize, *Zea mays L*., and other crops in the United States and Canada. ECBs costs corn growers an estimated $1 billion in annual losses (Mason et al. 1996). ECB is a moth species in the family Crambidae, whose larvae are typically stem borers (Kristensen, 1999). This pest, which typically has two generations per year, is capable of infesting a wide range of alternate host plants of agricultural significance when preferred hosts are not available (Hodgson, 1928), such as: soybeans, sorghum, cotton, tomatoes, potatoes, and some varieties of weeds (Bohnenblust and Tooker, 2010). ECBs have economically impacted growers considerably in management costs and yield losses. Larvae feed primarily on whorls, tassels and leaf ribs in early developmental stages and burrow into plant stems and corn ears in later development stages (Velasco et al., 2002). ECB can significantly reduce corn yield. Major concerns for corn growers comes when damage caused by ECBs leads to yield loss due to physiological damage and in some cases lodging, in which the corn plant breaks below the ear and is no longer able to support itself upright and lies flat on the ground (Sibale et al., 1992). In cases where no plant protection is used, ECB can lead to yield losses of around 6 bushels per acre per larvae on average per plant (Mason et al., 1996) in the U.S. where corn is the number one agricultural product (EPA, 2000).

As its name suggests, the European corn borer was introduced to the United States from Europe. The exact origins are not known though it is speculated that they arrived from Hungary or Italy in the early 1900s. ECBs were thought to have been brought over in imported broomcorn, a variety of sorghum used to make brooms until the mid-1900s. The first recorded presence of ECB in the United States occurred in 1917, near Boston, Massachusetts and again in 1920 near Lake Erie (Mason et al., 1996). It is probable that there were multiple introductions at several locations during that time, with only a few actually leading to a successful reproductive establishment (Caffrey and Worthley, 1927).

Since its discovery in the United States, ECB has spread across the eastern two-thirds of the United States. ECBs can be found as far north as Canada, as far west as the Rocky Mountains and as far south as northern regions of Texas and Florida (Mason et al. 1996). In the Corn Belt states such as, Illinois, Iowa, Minnesota and Nebraska, which produce approximately 50 percent of the total U.S. corn; ECB control has been a major concern.

In North America ECBs have two distinct pheromone strains, known as the E and Z races. Both races can be found in the eastern U.S. with some states such as Delaware having both populations, as well as, hybrids of the two races. Individuals from the Z race populations are dominantly found west of Pennsylvania and it is rare to encounter populations of the E race in these locations (Iowa State University, 2011). The pheromone races refer to the dominant portion of E and Z isomers of 11-tetradecenyl acetate found in the pheromone mixture produced by the females.

Individuals with the dominantly E isomer are mostly found along the eastern seaboard of the United States (Klun et al., 1975, Kochansky et al., 1975). They emerge early in the spring and can be found on a wider variety of plants species other than corn as corn has protective plant defenses present in its early growth stages. Z pheromone strain populations tend to be found west of the Appalachian Trail and westward throughout the Corn Belt; they emerge later in the growing season and feed primarily on corn. Delaware has a sympatric distribution of E and Z pheromone strain populations throughout the state which results in the presence of hybrids in the population (Mason et al., 1996).

European corn borer has four life stages; egg, larvae, pupae and adult. ECBs lay eggs in clusters of 15-30 on the undersides of leaves. Larvae have five stages of development, the first referred to as neonates. Young larvae primarily feed in the corn whorls, along leaf mid-ribs, and even on corn ears. More developed larvae tend to bore directly into the stems and corn ears, and continue to feed by tunneling inside the plant. Because ECBs feed internally, this disrupts the physiological processes of the plant leading to yield loss (Mason et al., 1996). During the first generation, fifth instars stop feeding and pupate in the soil. However second generation ECB do not pupate until the following year. They overwinter by diapausing in old corn material. Larvae break diapause when ambient temperatures average around 50º F. The larvae pupate and emerge as adults usually a week later, depending on daily temperatures. An estimated ECB lifecycle for areas in which they are bivoltine along with corn growth stages can be seen in Figure 1 (Mason et al., 1996).

Management Strategies

With such a high potential for yield loss and costs to the growers, a variety of control practices have been implemented over the years. Some of the earliest control measures used were cultural and mechanical controls such as shredding and plowing under corn stalks to eliminate ECB larval overwintering sites and good weed control (Bohnenblust and Tooker 2010). Cultural pest control methods included the selection of corn varieties that were more resistant to ECB larvae feeding. Some of these hybrids produced plant chemical defenses, such as 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA) (Brindley and Dicke, 1963). In addition, some of these early varieties contained other physiological herbivore tolerance traits such as, plant rind toughness and the ability to keep producing full ears even under high infestation levels. In addition, growers could avoid high damage by implementing additional cultural control methods such as: planting later in the season or harvesting fields earlier.

In addition to cultural and mechanical controls, natural mortality from environmental and biological factors such as, weather and natural enemies can have influence on ECB populations. Although natural control is not capable of consistently reducing pest populations below economic thresholds it can significantly impact population dynamics of ECBs. Some of the predators that are known to prey on ECBs are birds who feed on overwintering larvae, and ladybird beetles, and lacewing larvae which feed on the eggs and early instars. Other natural enemies include parasitic Hymenoptera. One species of ladybird beetles, *Coleomegilla maculate*, is known to be

a significant predator of ECB (Bhalgat et al., 2004). These control methods are hard to regulate for agricultural pest control as they are dependent on many different naturally occurring environmental and biological parameters.

One of the most commonly practiced methods for ECB control is the use of synthetic organic insecticides. However, these have to be well timed for stalk boring insects and thus require additional time and costs in labor to scout and spray (Armstrong et al., 1995). For insecticide use, ECB larvae are most vulnerable after they first emerge and before they can bore into the plant stems. It becomes exceedingly difficult to treat ECBs once they leave the whorls and bore into the plant (Iowa State University, 2010). The types of insecticides that have been used to control ECBs vary and include granular and spray formulations (Hyde et al., 1999). Most conventional insecticides have to be applied under strict guidelines and their persistence in the environment can have harmful effects on beneficial organisms in the surrounding habitats. Insecticides can become expensive and using one type of insecticide continuously against the same pest will lead to increased selection for resistance to the insecticide. This often means that growers have to rotate with other insecticides that have different modes of action (McCaffrey and Nauen, 2006). Another side effect of some insecticide control measures is that they can cause a resurgence of other pest insect populations due to mortality of their natural enemies.

One of the most frequently used microbial insecticides are granular or liquid sprays that contains the bacterium *Bacillus thuringiensis* (Bt) (Ostlie et al., 2008). Bt is considered a safer method of ECB control compared with traditional chemical

control methods as its mode of action is specific for Lepidoptera and has reduced toxicity levels to non-target organisms. Bt proteins must be consumed before they become toxic and only work on species that have the right binding sites in the gut to activate the toxin, making them very specific to certain insect groups or species (Huang et al., 2002). Once the toxin is ingested and binds to receptors in the gut it disrupts the cells and allows for gut bacteria to enter the haemolymph (Ostlie et al., 2008) (Figure 2). However, like other insecticides Bt sprays must be applied when larvae are actively feeding on the outside of the plant or it will not be effective.

As stated before the biggest problem with most conventional pesticides is application timing to reach larvae before they bore into the stalk. Thus, in the early 1990's genetically modified crops were introduced that incorporated the crystalline (Cry) proteins of Bt into the plant tissues. Multiple Cry proteins were discovered, some that targeted lepidopteran pests such as ECBs and others that targeted coleopteran pests such as corn rootworm (Mason et al., 1996, Bravo et al., 2007). Similar to the granular spray the proteins enter the insect midgut, where enzymes in the gut cleave the protein turning it into its toxic form. The now activated toxin binds to receptors in the cell lining of the insect midgut causing ruptures (i.e. pores) in the insect gut lining that quickly reduces or stops the insects from feeding, and eventually leads to mortality if they have consumed a lethal dose (Ostlie et al., 2008 and Tabashnik, 1994).

Bt corn has been very successful in its ability to control ECB populations and has led to the wide spread adoption of Bt corn varieties across North America (Hurley

et al., 2002). However, similar to use of traditional insecticides, evolution of ECB resistance to these proteins has been a major concern and a Resistance Management Plan is currently a condition of registration by the U.S. Environmental Protection Agency (EPA) for all genetically modified plants containing Bt proteins (EPA, 2009). These plans are most effective when based on a high dose refuge strategy that require that the plants express a dose high enough to kill all heterozygous resistant individuals and that a refuge of non-Bt corn be planted to produce susceptible offspring that could then mate with any homozygous resistant individuals that survive Bt exposure (Figure 3 A-B). The purpose is for susceptible ECB adults to mate with any potentially resistant individuals that may have survived in the Bt corn, maximizing the chance that their offspring carry genetic traits to remain susceptible to the toxin.

Ideally, this process would allow Bt technology to successfully control ECB populations for many years, possibly indefinitely, but it relies on the compliance of growers to actually plant the refuge when planting Bt corn fields. Recent studies suggest that an increasing number of corn growers are not complying with IRM refuge standards, leading to increased risk of resistance evolving sooner than expected (EPA, 2009). One way that companies have sought to deal with concerns of increased rate of resistance evolving is by pyramiding multiple Cry proteins with different unique modes of action targeting the same pest species in the same corn variety (Zhao et al., 2003).

Another way the corn seed production industry is attempting to address concerns about low compliance rates is by marketing a refuge-in-the-bag (RIB)

product where the required amount of non-Bt seed that serves as the refuge is blended with Bt corn seed (Comis, 2003) and distributed to growers in single bags. This makes sure that there is the minimum required percentage of refuge plants in the field (Figure 3C); and the grower does not have to calculate what proportion of the field needs to be planted with non-Bt. It saves the growers in time and costs as they no longer have to switch out seed for standard refuge while planting large fields. However, some concerns with these RIB products are that resistance may increase due to the presence of non-Bt corn adjacent to Bt corn plants. The concern is that larvae who may begin on non-Bt plants but, at some point during their development, switch over to Bt corn may survive to reproduce due to increased tolerance to the toxins as older larvae. ECB adults that survive may produce offspring with partial resistance (Gould, 2000). It has been shown that later instars are more capable of surviving on Bt corn than neonates (Huang et al., 1999).

Dispersal and Feeding Behavior on Bt Corn

ECBs have very strong dispersal behavior early in their development. Particularly, the movement and dispersal behaviors in the highly mobile neonate stage have been the focus of prior studies. These studies show that the largest proportion of larval movement occurs in the first 48 hours (Ross and Ostlie, 1990) and that upon hatching 50% of the larvae will move from their natal plant whether it is Bt or non-Bt (Razze et al., 2011, Razze and Mason, 2012). However, if larvae are exposed to natal plants that contain Bt toxins and are able to assess that the plant is toxic or inadequate at

providing essential nutrients a higher rate of natal plant abandonment is observed (Goldstein et al., 2010). The movement of larvae from plant to plant becomes important in determining the appropriate amount of refuge that growers should use, as a higher frequency of movement behavior, particularly of older larvae, can lead to individuals surviving to adulthood, thus increasing likelihood of resistance developing (Mallet and Porter, 1992).

As stated before most movement and feeding studies have focused on neonate and early instars of ECB which are most susceptible to Bt toxin; however, less is known about the proportion of larval movement in mid- to late development ECB instars. Previous speculation was that mid- to late instar movement was predicted to be very low as larvae have begun to bore into corn stalks and are actively feeding. However, prior field observations from individuals who have worked with ECBs in the field for many years led them to questions whether these larvae were as sedentary as previously thought (personal correspondence, Rich Hellmich). In addition, very little is known about how the movement behavior of these mid- to late instars is changed when given the option to feed on Bt versus non-Bt corn plants. After the second instar, ECBs typically begin to bore into the stalk of corn plants (Mason et al., 1996), leading to a shift in movement behavior from the initial dispersal phase of early ECB instars. Because of this change in behavior there is a need for a better understanding of the movement behavior of third instar ECBs and how movement is influenced by host plant factors, such as the presence of Bt toxins.

The objectives of my research project are 1) evaluate movement behavior of third instar larvae from the infested plant, 2) determine what proportion of ECB larvae move off infested plants during a 72 hour feeding period when the infested plant is a Bt versus marked refuge Bt corn plant, 3) determine if there is a difference in movement behavior between simulated first and second generation in the field, and 4) learn more about the persistence and decay rate of Bt Cry proteins in the ECB larvae gut.

I hypothesized that in ECB, third instars would be subjected to density dependent movement and that there would be a significantly higher proportion of larval movement from the infested plant in cases where the infested plant was Bt corn compared to where the infested plant is non-Bt corn. In addition I hypothesized that a higher proportion of larvae would remain on the infested plant when the infested plant was non-Bt. I also expected that if larvae did move onto adjacent Bt corn plants that they would not remain on these plants and continue to seek more suitable corn host plants nearby. This may lead larvae to continue to move out further from their infested host plant or could lead to movement back and forth between the infested plant and the adjacent plant. Having a better knowledge of third instar movement could provide important information for modelers in determining the proportion of refuge required and for implementation of blended seed refuge in Bt corn fields. Finally I hypothesized that there could be observed differences in movement behavior between simulated first and second generation third instar ECBs due to factors such as the development stage of the corn plants.

Chapter 2

MATERIALS AND METHODS

Insect Specimens

The insects used for these experiments were obtained from the USDA Corn Institute Research Laboratory located in Ames, Iowa. Each year a new colony was created by collecting egg masses from Iowa fields at the end of the growing season and then brought into the laboratory where the larvae were reared. These field populations were then allowed to mature, pupate, and mate for several generations prior to shipping. For my studies, depending upon availability or the type of experiment planned, either egg masses or larvae were shipped from the Iowa laboratory via overnight transport. Egg masses were received from Iowa, where they had been laid on wax paper strips which could be easily cut or peeled off. Larvae arrived in insect containers which housed ECB diet at the base and a cardboard ring for pupation. When egg masses arrived they were placed inside of insect containers that contained ECB diet, which was also obtained ready made from the Iowa laboratory. Insect containers were then sealed at the edges with tape to prevent neonates from escaping. Developing insects were housed in regulated growth chambers (Percival Scientific, Perry, IA) in the entomological laboratory at the University of Delaware (UD), in Newark. Two growth chambers were set up with temperatures that were maintained at 20º C in one growth chamber and 25º C in the other in order to vary the development rate. The light cycle for the growth chambers

were maintained on L:D 16:8h schedules. Larvae were reared in growth chambers to the third instar before use in all experiments and bioassays. In cases where larvae were used in the field, ECB larvae were fed blue diet a minimum of one week prior to being placed in the field. The blue diet was obtained from the Iowa laboratory and it served to visibly dye the alimentary canal of larvae blue so that they could be distinguished from naturally occurring ECB individuals in the field. The dye that was used to make the blue diet was Sudan Blue II, dye content 98% (Sigma-Aldrich, St. Louis, MO).

Plants

Corn varieties used in the experiments included a series of Bt and non-Bt traited corn plants. The Bt corn plants included two single trait lepidopteran Bt corn varieties containing either Cry1Ab and Cry1F, and a coleopteran targeted Bt corn variety, Cry34/35Ab1, that served as a marked control for later gut analysis. In addition a non-Bt near isoline was used that had the same genetic background as the Bt corn varieties. The non-Bt near isoline was used in the field treatments in 2011 to determine if it does not differ from Cry34/35Ab1 that would serve as a suitable marked refuge control to compare with the Bt plant varieties. All corn plant varieties contained the Round-up Ready® gene (NK603) and contained a fungicidal seed treatment. For trials run in the laboratory, corn plants were grown in the greenhouse. Corn plants were potted in 3gallon plastic pots, large enough for adequate root growth. Soil was filled to within an inch of the rim to allow for appropriate watering and two seeds were planted per pot. Plants were labeled by corn variety and date planted. One

week after seedlings emerged they were assessed and thinned. Plants were watered daily and monitored for pest and/or nutrient problems. Iron was added periodically to minimize leaf chlorosis. No insecticides were used on any of the corn plants. Greenhouse plants were raised to at least the V7 (vegetative stage) for use in all feeding and movement trials to minimize the effects of plant chemical defenses such as DIMBOA. DIMBOA is a powerful antibiotic found in maize and other plants that serves as a natural deterrent of European corn borer. Concentrations of DIMBOA are highest in seedlings and young corn plants and decrease significantly as the plant ages. For movement trials in the greenhouse, plants were utilized at corn development stages V7 or R3 to simulate when third instar ECB larvae would encounter specific corn development stages as they would occur in the field for each of the respective generations.

Presence and Persistence of Larval Dye

ECB egg masses and blue diet were acquired from the Iowa laboratory. Egg masses were transferred from wax paper strips into insect containers with the blue ECB diet. Larvae were allowed to feed and mature on the diet until they reached third instar. Larvae were examined to determine the extent of visible coloring and whether coloration was sufficient to distinguish from the natural coloration of ECB larvae. Larvae were then removed from the blue diet and fed on corn plant material for three days and observed for retention of blue coloration. Larvae that were dyed using the blue ECB diet were monitored and compared with other laboratory reared ECBs from

the same group who were fed on the regular ECB diet to see if the dye had any effects on larval development. Overall larval health and size was compared by visual observation. ECB pupae and adults that fed continuously on the blue artificial diet as larvae were also observed for retention of blue coloration.

Density Dependence and Recapture Trial on Non-Bt Corn

Non-Bt near isoline-corn plants were grown in the greenhouse to the V7 stage and arranged in arrays of five plants placed in a line with the base of the pots and leaves touching. Initial trials were run inside the greenhouse. Three treatments were designed with the middle plant in each array as the infested plant receiving either 1, 5, or 9 third instar larvae that were dispersed amongst the whorl leaves of the center plant. Larvae were starved for roughly two hours prior to being placed on plants to encourage feeding behavior during the trial and to discourage movement behavior associated with being disturbed. Larvae were placed on plants using soft bristle paintbrushes to minimize effects from handling. Larvae were then observed, including interactions between larvae for one hour. Each treatment was replicated six times.

Observations were distinguished on the amount of movement that took place during the one hour observations. Once infested the normal observed behavior was determined to include larvae that silked down into the leaf whorl or axil; had little change in behavior; and remain at their leaf whorl or axil but would move slightly away from other larvae present. More significant movement included movement of

larvae off onto edges of the leaf, silking down onto another leaf axil or whorl and possibly moving onto another plant completely.

For the recapture portion of the trial, the same five non-Bt plant were used. Treatments of one or five third instar larvae were infested on the middle plant, dispersed amongst whorl leaves. Each treatment was repeated six times, with one set of three conducted inside the greenhouse and the other set of three conducted in potted plants outside the greenhouse to simulate environmental factors that would normally occur in the field. Larvae were placed on the plants for 72 hours and then recaptured and counted by destructive plant dissections in which the corn plants were cut at the base and systematically dissected.

Third Instar Movement Field Trials

In the summer of 2011 and 2012 field experiments were conducted to assess the movement behaviors of third instar ECB larvae on the University of Delaware Newark Farm. The overall field measured 48 rows by 100 feet. Plants that were used in the field trials were planted near the middle to end of May as is appropriate for the Mid-Atlantic region when daily temperatures averaged around 50º to 55 º F (Farnham 2001). A stacked corn variety that contained Cry1Ab + Cry34/35Ab1 and herbicide resistance served as background plants for the overall field. Background corn seeds were planted using a tractor with a six row planter. Rows were spaced 30 inches apart and planting rate was 29,000 seeds per acre. The overall field received standard fertilizer application at planting and pre-emergence herbicide spray to control for

weeds. The overall field was broken down into eight blocks that were labeled A through H. Half of the blocks were selected at random and designated for use in either the simulated first generation or second generation ECB field trials. Simulated first generation took place when plants were in corn growth stage V7 and simulated second generation took place during corn reproductive stage R3. These corn growth stages were picked to correspond to when naturally occurring third instar ECB larvae are normally found in the field (Mason et al. 1996). Each block was further broken down into five replicate plots. Each replicate plot consisted of six treatments in 2011 (Figure 4) and five treatments in 2012 (Figure 5). A complete randomized block designed was used on all treatments for each replicate. There were a total of twenty replicates for each generation in each year.

The first treatment designated as NAN consisted of a Cry1Ab Bt corn plant as the middle plant, the two immediately adjacent plants were marked refuge plants that contained the non-lepidopteron Bt protein Cry34/35Ab1, and the plants two away from the middle plant were non-Bt near isoline corn plants. The second treatment, NFN, consisted of a Cry1F Bt corn plant as the middle plant, the two immediately adjacent plants were marked refuge, and the plants two away from the middle plant were non-Bt near isoline corn plants. The third treatment, ANA, consisted of a marked refuge plant for the middle plant location, the two immediately adjacent plants were Cry1Ab Bt corn plants, and the plants two away from the middle plant were non-Bt near isoline corn plants. The fourth treatment, FNF, consisted of a marked refuge for the middle plant, the immediately adjacent plants were Cry1F Bt corn plant, and the

plant two away from the middle plant were non-Bt near isoline Bt plants. There were two control treatments in 2011. Control 1contained a non-lepidopteran Bt (Cry34/35Ab1) as the middle infested plant, which in principle should not have any effect on ECB larvae (Figure 4). Control 2 contained all non-Bt near isoline corn plants, which serve as a comparator for the Control 1 treatment and was used to determine if there was any effect of the marked refuge Cry34/35Ab1 on ECB larvae. The two control treatments were compared to each other to determine if the non-lepidopteran Bt plant variety (Cry34/35Ab1) was an effective marked refuge to use in the mixed Bt and non-Bt treatments. Because there were no significant difference between the two controls during the 2011 field season, Control 2 was removed from the field during the 2012 field season, resulting in only five treatments (Figure 5).

The specific Bt corn seed and the near isoline seed appropriate for each five-plant treatment were planted by hand. Two seeds were placed at each plant location at an estimated two inches adjacent to the tractor planted row and marked with colored stakes that corresponded to the specific corn variety (Figure 6). One week after emergence hand planted treatments were thinned and checked for damage or missing plants and any excess background plants were removed. All field corn seed was provided by DuPont- Pioneer Hi-bred, International, Johnston, IA.

In preparation for infesting plants in the field, larvae reared on blue ECB diet that showed considerable blue coloration were collected and placed into Solo® condiment cups (Solo Cup Company, Lake Forest, IL) in groups of five the morning of larval infestation. Larvae were placed in Solo® cups without food for

approximately two hours before being released on plants to encourage feeding on the host plant. Larvae were transported to the field in coolers. Larvae were placed on the middle plant of each treatment using soft bristle paintbrushes to gently brush larvae into the leaf whorl or axil depending on plant age or location. ECB Larvae were dispersed at a rate of one to two larvae per leaf whorl or axil on middle to upper regions of the appropriately staged corn plant. All larvae that had died before being infested were replaced. Any larvae that fell off the infested plant and could not be found or retrieved were replaced. Once all treatments had been infested with ECB larvae they were left in the field with minimum human disturbance for 72 hours. After 72 hours, all five plants in each treatment were destructively dissected. Any larvae found during the dissections had their plant location recorded and were collected into 1.5 microliter centrifuge tubes. Each tube was labeled with the simulated generation number, plot letter, block number, treatment number and plant location. Collected centrifuge tubes were immediately placed in coolers full of ice packs until they could be transported back to the laboratory. Once back in the laboratory larvae were examined for blue coloration to ensure no naturally occurring ECB larvae were collected and recorded. Finally, larvae were stored in a -80ºC freezer.

Presence and Retention of Bt in Third Instars

To determine if Bt material that had been fed upon could be detected in third instar ECB larvae, ELISA based Bt test strips (Envirologix, Portland, ME) were used to detect the specific protein. This part of the study was broken up into two parts: 1. to

determine if Bt proteins showed up consistently over a 24 hour period of feeding on Bt corn and 2. to determine how long Bt plant material Cry proteins could be detected in the larvae after cessation of feeding. Three different Cry proteins in corn plants were used for this study; Cry1Ab and Cry1F (both lepidopteran targeted Bt proteins) and Cry34/35Ab1 (non-lepidopteran Bt protein that served in the field trials as a marker for the refuge plants).

For the first part of my study, I obtained third instar larvae from the Iowa laboratory that had been fed on regular ECB diet prior to the study. Three feeding containers were set up with only one of the three types of Bt plant material inside each one. Each container was infested with ninety larvae. Ten larvae from each of the feeding containers were removed and sacrificed during time intervals that consisted of 0, 2, 4, 6, 8, 12, and 24 hours past initial infestation in the feeding cages. Larvae were chosen on the basis of whether they showed signs of feeding. Signs of feeding included visible plant material in gut when turned over and fresh frass adjacent to larvae. Once larvae were removed from their feeding containers larvae were placed in 2 µl microcentrifuge tubes and 0.5 µl of buffer solution was added to each tube. Using a clean plastic pestle for each sample the larvae were crushed and ground up until the entire contents were thoroughly homogenous in the buffer solution. After grinding, an Envirologix® Bt test strip corresponding with the Bt protein material the larva had fed upon was inserted applicator end down in the buffer solution. Test strips were incubated at room temperature for ten minutes in solution. Once tests were completed

results were recorded as negative or positive for presence of Bt plant material based on the color that could be visually discerned on the test strip.

For the second part of my study, ninety larvae were placed in feeding cages with only one type of Bt plant material and allowed to feed for 24 hours. After feeding, the larvae were removed and placed in new feeding cages that contained only the non-Bt near isoline corn plant material. At time intervals of 0, 2, 4, 6, 8, 12, and 24 hours after being transferred to the near isoline feeding cages, ten larvae that showed signs of feeding were removed and insect content was analyzed using the same method as before from each feeding cage.

The assay sensitivity for Bt was measured by first placing ninety larvae on Cry34/35Ab1 (marked refuge) plant material. Larvae were allowed to feed on the plant material for 24 hours before being moved to a new feeding cage containing either Cry1Ab or Cry1F plant material, each cage containing thirty larvae each. Ten larvae from each cage were removed and sacrificed after being moved to cages containing lepidopteran targeted Bt plant material at intervals of 0, 2, 4 6, 8, 12, and 24 hours past when they were first placed in the new feeding cages. Larvae body contents were analyzed using the same grinding and ELISA Bt test strip method as described earlier.

To characterize the reciprocal ninety larvae were each placed in feeding cages containing either Cry1F or Cry1Ab plant material. After 24 hours larvae were moved into feeding cages that contained only Cry34/35Ab1 (marked refuge) plant material. Ten larvae were removed and their contents analyzed at intervals of 0, 2, 4 6, 8, 12,

and 24 hours after they were transferred into the marked refuge containers. Insect contents were analyzed using the same ELISA Bt test strips method from before.

A follow up study was conducted to determine if the amount of Cry34/35Ab1 Bt plant material that could be expected to be found in ECB larvae guts was too small to detect using the Bt test strips. The estimated amount of plant material a larvae consumes daily was determined to be at least 17 milligrams. This was calculated by estimating that a larva eats about its weight in plant material daily (personal observation). Three third instar larvae were weighed and the mean weight used to measure the plant material that would be used. From that mean weight I calculated what the wet plant material weight would be for $1/10^{th}$ of the total daily amount of plant material consumed; which was determined to be 1-2 milligrams of Cry34/35Ab1 plant material (Figure 7).This part of the study was to determine if Cry34/35AB1 could be consistently detected as pure plant material (not consumed) at both the estimated daily amount of plant material and the $1/10^{th}$ amount of plant material that could be found in an ECB larva. This test was repeated on ten larvae.

Data Analysis

Larval movement behavior in the overall field plot was analyzed by comparing the results from the field data collected in 2011 and 2012 between treatments and by corn growth stage. A 3x2 Fisher's exact test was conducted on all treatments in a pairing method; with the categories of proportions that were compared being divided into the (1) percentage of larvae that remained on the infested plant, (2) the percentage

of larvae that moved onto an immediately adjacent plant and (3) the percentage of larvae that moved two plants away from the infested plant. All treatments that were conducted in the same year and the same corn growth stage (either V7 or R3) were compared against one another, two at a time, to determine if there was a significant change in movement behavior given different arrangements of Bt and marked refuge corn plants. Significance was determined using a p-value of 0.05, given that the null hypothesis is that there will be no significant change in movement behavior whether the larvae begin on Bt or non-Bt corn.

Chapter 3

RESULTS

Presence and Persistence of Larval Dye

Larvae that had fed on blue diet showed various levels of blue coloration of the overall body depending on how long they had fed on the diet. In all cases the larvae that had fed on the diet for more than two weeks showed considerably more coloration (enough to distinguish from natural coloration of ECB larvae). Larvae that had been reared from egg hatch on the blue diet to the fifth instar showed the highest accumulation of blue coloration in the body. Blue third instar ECBs that had been fed the blue diet continuously after explosion and then transferred and fed on corn plant material for three days showed some loss of coloration at the end of this period. In extreme cases, most of the body coloration was lost; however, blue coloration could usually still be observed on the underside of the larvae by peering through the exoskeleton with the use of a microscope. Observational comparisons between larvae fed on blue ECB diet versus those fed on the regular ECB diet showed observable signs of difference in size and overall heath. Pupae and adults reared continuously on the blue artificial diet from egg hatch did not appear to have any residual blue coloration from the artificial diet after pupation.

Density Dependence and Recapture Trial on non-Bt Corn

For the density dependence portion of the trial, in treatments where only one larva was infested on the host plant no signs of change in their movement behavior were observed. For treatments where there were five larvae infested on the middle plant, larvae showed little to no change in movement behavior based on visual observations compared to treatments where only one larva was infested. In treatments where nine larvae were infested on the middle plant there was no observed movement on to an adjacent plant. However, because more larvae had to share the same whorl or leaf axil on the infested plant, there was more observed dispersal on the infested plant. Where more than two larvae were placed in the same whorl or leaf axil had a higher tendency for one of the larvae to move out to the leaf edge or silk down to the next leaf axil. Eventually all larvae settled into feeding on the infested plant, and none of them moved off the plant during the hour of observation.

For the recapture portion of the trial conducted inside the greenhouse, where only 1 larvae was infested per plant, the mean recapture rate was 0.8 larvae per infested plant of six (Table 1), with one plant that had no larvae found. For treatments where five larvae were infested on the middle plant the mean of recaptured larvae was 4.4 larvae, with the majority of treatments having all five larvae recaptured. There was one case where only three larvae were recaptured; however, a spider was found nesting on the infested corn plant. In the set of treatments that were conducted outside, the treatments with only one larva infested on the middle plant had a mean recapture rate of 0.5 larvae per infest plant of six, with half of the treatments resulting in no

larvae recaptured . For treatments where five larvae were infested on the middle plant the average recapture rate was 3.8 larvae per treatment (Table 1). The lowest number of larvae recaptured was three. No outside factors were observed that could explain what happened to the missing larvae.

Third Instar Movement Field Trials

2011

In the 2011 field studies, the number of larvae recovered during the V7 growth stage was 329 of total 600 larvae infested. The percentages of larvae for the V7 corn growth stage that were recaptured in the NAN treatments that remained on the Cry1Ab infested plant were as follows: 73, 27, and 0 percent; representing those that stayed on the center plant moved to the adjacent plants, and moved to two plants away from the infested plant, respectively (Figure 8). The percentages of larvae that were recaptured on the NFN treatments that remained on the Cry1F infested plant, moved to one of the immediately adjacent plants, and moved to two plants away from the infested plant were 83, 15, and 2 percent, respectively. The percentages of larvae that were recaptured on the ANA treatments that remained on the infested plant, moved to one of the immediately adjacent plants, and moved to two plants away from the infested plant were 94, 6, and 0 percent compared to the percentages of larvae recaptured on the FNF treatments that were 88, 12, and 0 percent, respectively. The percentages of larvae recaptured on the Control 1 marked refuge treatment that remained on the infested plant, moved to one of the immediately adjacent plants, and moved to two

plants away from the infested plant were 93, 5 and 2 percent, respectively. The percentages of larvae recaptured from the Control 2 non-Bt treatments that remained on the infested plant, moved to one of the immediately adjacent plants, and moved two plants away from the infested plant were 92, 5, and 3 percent, respectively (Figure 8).

The 2011 treatment NAN was significantly different from treatments NFN, ANA, FNF, Control 1 and Control 2 (having the highest percentage of larvae that moved off the infested plant) (Table 2). The treatment NFN was significantly different from treatment ANA. NFN was not significantly different from treatment FNF, but was significantly different from the Control 1 and Control 2 treatments (Table 2). The treatment ANA was not significantly different from treatments FNF, Control 1 and Control 2. The treatment FNF was significantly different from treatments Control 1 and Control 2. The treatment Control 1 is not significantly different from the Control 2 treatment.

The number of larvae recaptured during the 2011 R3 growth stage was only 148 of the total 600 larvae put out (ca. 25%). The percentages of larvae that were recaptured for the R3 growth stage on the NAN treatments that remained on the infested plant, moved to one of the immediately adjacent plants, and moved two plants away from the infested plant were 65, 35 and 0 percent, respectively (Figure 9). The percentages of larvae that were recaptured on the NFN treatments that remained on the infested plant, moved to one of the immediately adjacent plants, and moved two plants away from the infested plant were 74, 26, and 0 percent. The percentages of larvae that were recaptured on the ANA treatments that remained on the infested plant,

moved to one of the immediately adjacent plants, and moved two plants away from the infested plant were 86, 11, and 3 percent, respectively. The percentages of larvae that were recaptured on the FNF treatments that remained on the infested plant, moved to one of the immediately adjacent plants, and moved two plants away from the infested plant were 87, 13 and 0 percent. The percentages of larvae recaptured on the Control 1 marked refuge treatment that remained on the infested plant, moved to one of the immediately adjacent plants, and moved two plants away from the infested plant were 88, 12 and 0 percent. The percentages of larvae recaptured from the Control 2 non-Bt treatments that remained on the infested plant, moved to one of the immediately adjacent plants, and moved two plants away from the infested plant were 79, 21, and 0 percent, respectively (Figure 9).

The 2011 R3 treatment NAN was not significantly different from treatment NFN though it was significantly different from treatments ANA, FNF, Control 1 and Control 2 (Table 2). Treatment NFN was significantly different from treatments ANA, FNF, and Control 1; however, it was not significantly different from Control 2 (Table 2). Treatment ANA was not significantly different from treatments FNF and Control 1; however, it was significantly different from Control 2. Treatment FNF was not significantly different from the Control 1 and the Control 2 treatments. Treatment Control 1 was not significantly different from treatment Control 2.

2012

In the 2012 field studies, the number of larvae recaptured for the V7 corn growth was 376 of the total 600 larvae put out (62%). The percentages of larvae for the V7 corn growth stage that were recaptured on the NAN treatments that remained on the infested plant, moved to one of the immediately adjacent plants, and moved two plants away from the infested plant were 39,45, and 16 percent, respectively (Figure 10). The percentages of larvae that were recaptured on the NFN treatments that remained on the infested plant, moved to one of the immediately adjacent plants, and moved two plants away from the infested plant were 50, 31 and 19 percent, respectively. The percentages of larvae that were recaptured on the ANA treatments that remained on the infested plant, moved to one of the immediately adjacent plants, and moved two plants away from the infested plant were 92, 6, and 2 percent compared to the percentages of larvae recaptured on the FNF treatments that were 91, 6, and 3 percent, respectively. The percentages of larvae recaptured on the Control treatment that remained on the infested plant, moved to one of the immediately adjacent plants, and moved two plants away from the infested plant were 86, 13 and 1 percent, respectively (Figure 10).

The 2012 V7 treatment NAN was not significantly different than treatment NFN; however, it was significantly different than treatments ANA, FNF, and the Control (Table 3). Treatment NFN was also significantly different from treatments ANA, FNF, and the Control (Table 3). Treatment ANA was not significantly different

from treatment FNF and the Control. Treatment FNF was not significantly different from the Control as well.

The number of larvae recaptured during the 2012 R3 corn growth stage was 314 of the total 600 larvae infested (52%). The percentages of larvae that were recaptured during the R3 corn growth stage on the NAN treatments that that remained on the infested plant, moved to one of the immediately adjacent plants, and moved two plants away from the infested plant were 31, 40, and 29 percent, respectively (Figure 11). The percentages of larvae that were recaptured on the NFN treatments that remained on the infested plant, moved to one of the immediately adjacent plants, and moved two plants away from the infested plant were 33, 58, and 9 percent. The percentages of larvae that were recaptured on the ANA treatments that remained on the infested plant, moved to one of the immediately adjacent plants, and moved two plants away from the infested plant were 91, 2, and 7 percent. The percentages of larvae that were recaptured on the FNF treatments that remained on the infested plant, moved to one of the immediately adjacent plants, and moved two plants away from the infested plant were 83, 4, and 13 percent. The percentages of larvae that were recaptured on the Control treatments that remained on the infested plant, moved to one of the immediately adjacent plants, and moved two plants away from the infested plant were 78, 14, and 8 percent, respectively (Figure 11).

The treatment NAN was significantly different from treatments NFN, ANA, FNF, and the Control with the highest percentage of larvae that moved off the infested plant (Table 3). Treatment NFN was significantly different from treatments ANA,

FNF, and the Control (Table 3). The treatment ANA was not significantly different than the FNF treatment but was significantly different from the Control with a higher percentage of larvae remaining on the infested plant. Treatment FNF was significantly different from the Control with a higher percentage of larvae moving two away from the infested in the FNF treatment.

Presence and Retention of Bt in Third Instars

For the first part of the Bt test strip trial in which larvae were analyzed over the course of 24 hours, those larvae that had been sacrificed prior to feeding on Bt corn material showed no positive results for Bt in the insect. For treatments in which larvae had fed on Cry1Ab plant material over the 24 hour period, all larvae showed positive for Bt material after 0,2,4,6,8,12 and 24 hours (Figure12). For treatments in which larvae had fed on Cry1F plant material over the 24 hour period, all larvae showed positive for Bt material after 0,2,4,6,8.12 and 24 hours (Figure 12). However, the coleopteran Cry protein, Cry34/35Ab1, was quite variable in its detection of plant material in the insect body content and no trend was apparent (Figure 12). At intervals of 0,2,4,6,8,12 and 24 hours after feeding on the Cry34/35Ab1 material the percent of larvae that had positive detections were 0, 10, 40, 40, 0, 40 and 30, respectively (Figure 12).

For the second part of the trial in which larvae had been fed for 24 hours on specified Bt plant material and then moved to non-Bt plant material, those larvae that had fed on lepidopteran Bt plant material showed a high level of retention compared to

those larvae that had fed on Cry34/35Ab1. Larvae that had fed on Cry1Ab prior to being transferred to non-Bt plant material had positive detections at intervals of 0,2,4,6,8,12 and 24 hours post transfer of 100, 100, 90, 90, 100, 60, and 20 percent, respectively, of the total larvae (Figure 13). For larvae that had fed on Cry1F prior to being transferred to non-Bt plant material had positive detection at intervals of 0,2,4,6,8,12 and 24 post transfer of 100, 80, 80, 70, 30, 20, 0 percent, respectively, of the total larvae (Figure 13). The larvae that had fed for 24 hours on the Cry34/35Ab1 before being transferred to non-Bt plant material resulted in positive detections at intervals 0, 2, 4, 6, 8, 12, and 24 post transfer of 30, 10, 0, 20, 10, 0, and 0 percent, respectively, of the total larvae (Figure 13).

For the first test for Bt retention when larvae were fed for 24 hours on Cry34/35Ab1 and then transferred to Cry1Ab, there was a consistent trend over time that the number of positive detections for Cry34/35Ab1 decreased while the detection of Cry1Ab remained consistent. At intervals of 0, 2, 4, 6, 8, 12 and 24 hours post transfer larvae had positive detections for Cry34/35Ab1 of 50, 20, 10, 10, 20, 0, 0 percent, respectively, of the total larvae and for Cry1Ab they had positive detections of 0, 80, 100, 100, 100, 100 and 100 percent, respectively, of the total larvae (Figure 14). When larvae were fed for 24 hours on Cry34/35Ab1 and then transferred to Cry1F, similar to the corresponding treatment, there was a steady decline in the number of positive detections for Cry34/35Ab1 Bt plant material in the insect with a consistent detection of Cry1F Bt material. At intervals of 0, 2, 4, 6, 8, 12, and 24 hours post transfer larvae had positive detections for Cry34/35Ab1 of 50, 20, 10, 10, 0, 0, 0

percent, respectively, of the total larvae and for Cry1F they had positive detections of 0, 90, 90, 90, 100, 100, and 100 percent, respectively, of the total larvae (Figure 15).

For the second test for Bt retention in which larvae were fed for 24 hours on Cry1Ab and then were transferred to Cry34/35Ab1, larvae showed that Cry1Ab material could be detected even 24 hours after they had last fed on that particular type of Bt material and there was a consistent trend for decrease in detection over time. However, the detection rate for Cry34/35Ab1 remained inconsistent (Figure 16). Larvae that had fed at intervals of 0, 2, 4, 6, 8, 12, and 24 hours had positive detections for Cry1Ab of 100, 100, 100, 90, 100, 80, 30 percent, respectively, of the total larvae and positive detections for Cry34/35Ab1 of 0, 50, 60, 60, 50, 20, and 70 percent, respectively, of the total larvae (Figure 17). For larvae that had been fed on Cry1F for 24 hours and then transferred to Cry34/35Ab1 plant material, there was again a trend for a decrease in positive detection of Cry1F plant material in the insect but at least one larva resulted in positive detection 24 hours after being transferred and similar to prior results the Cry34/35Ab1 remained inconsistent for positive detection of that specific type of Bt plant material in the insect (Figure 17). Larvae that had fed at intervals of 0, 2, 4, 6, 8, 12, and 24 hours had positive detections for Cry1F of 100, 100, 100, 90, 40, 30, 10 percent, respectively, of the total larvae and positive detections for Cry34/35Ab1 of 0, 20, 50, 60, 50, 80, and 50 percent, respectively, of the total larvae (Figure 17). For the follow up study, in which pure Cry34/35Ab1 plant material at $1/10^{th}$ the estimated daily amount consumed showed positive detection using the Envirologix Bt test strips for all replicates.

Chapter 4

DISCUSSION

Presence and Persistence of Larval Dye

The use of blue dye allowed for a more accurate determination of artificially infested larvae in the field trials when naturally occurring populations were also in the field. The use of dyes in artificial insect diets to mark larvae has been used for a variety of insect studies (Hagler and Jackson, 2001) and species that include *Helicoverpa armigera*, *Diatraea grandiosella*, *Spodoptera frugiperda*, and *Pectinophora gossypiella* (Graham and Mangum, 1971, Zhao et al., 2008). My results showed for larvae reared on blue diet that were transferred to a non-blue dye food source after the third instar that overall body coloration faded. Despite this, specific regions of the larvae's body would still show remnants of blue coloration after all other coloration had faded.

In the study, some larvae were collected that did not have visually obvious blue coloring and these were checked more thoroughly in the laboratory. Even when most of the blue coloring was gone, larvae could be placed upside down and observed under a microscope where at least some residual blue coloration was observed through the exoskeleton. This is consistent with studies by Zhao et al. (2008) on cotton bollworm, *H. armigera*, in which they dissected larvae that had fed on artificial diet that contained a similar dye to what I used and they were able to detect the dye in the fat bodies and the digestive track.

Other indicators that helped to determine whether field collected larvae were from the group that was artificially infested or if these were from naturally occurring populations was to identify any unexpected developmental stages, because even though the treatments were designed to coincide with the naturally occurring populations of ECB, exact overlap was not possible. Naturally occurring larvae tended to be at least one to two instars older than the infested larvae. The blue dye worked satisfactorily through the three days of my experiment but beyond that the method of marking insects using the blue dye would likely be less reliable, and not very practical for long term use in the field for ECB larvae. No additional side effects or changes in behavior were observed from using the dye, though prior studies using other similar dyes did report that factors such as larval development, pupation, eclosion, and survival can be and have been affected depending on the dye used (Ostlie et al., 1984, Zhao et al., 2008).

Another potential issue that could have occurred in the field is that larvae containing the blue dye may have been more susceptible to visual predators, for example by birds, due to their bright coloration. Vulnerability would be strongest during the time it took from artificial infestation to the point in which the larvae were able to tunnel into the corn stalk. However, individual larvae that remained on leaves or stalk would continue to be more vulnerable.

Density Dependence and Recapture Trial on non-Bt corn

To determine how many larvae to infest on without stimulating a density dependent migratory response for the field study; it was important to determine a suitable number of larvae that was high enough that we could successfully recapture at least half of the larvae infested but also to minimize the chance that third instar movement would be affected by density dependence. The density dependence portion of my results indicated that when one or five larvae were infested on the middle plant that there was either no change or little change in movement behavior. In those treatments in which nine larvae were infested on the middle plant no larvae moved completely off the infested plant, but some movement behavior was observed in which larvae moved around on the same leaf blade, moved out to the leaf edge or silked down to another leaf. It was noted that larvae would have to come in contact with other larvae to exhibit this behavior.

In a study that observed the number of ECBs per 100 plants from 1935 to 1985 in various location in Delaware; the highest proportion of larvae per plant was found in 1977 with about 7 per plant (Burbutis et al., 1984). This is prior to the use of Bt corn, but the average capture rate from the 1950's to 1985 was between 200 and 400 larvae per 100 plants with an increasingly higher number of larvae per 100 plants as time went on. The study by Burbutis et al. (1984) also predicted that the population could peak at 900 borers per 100 plants in 1986. This supports the idea that plants infested with up to nine ECB larvae per plant would be within the expected range of possible naturally occurring ECBs recaptured. However, that would be the highest

expected level of infestation per plant. Despite the fact that larvae can survive with that many other ECBs present in the same plant, there is probable interspecific competition and possible density dependent movement of larvae on those plants.

It is speculated that if enough larvae infested the same plant that it could lead to direct competition for the same resource and this could influence larval movement. Some of ECB neonates' initial dispersal behavior in which more than 50% of neonates will disperse off of the natal plant, this rate increases when the natal plant contains Bt endotoxins, as documented by Goldstein et al. (2010) and Razze et al. (2012), could be in response to an adaptation to try to limit sibling competition for the same resource. Therefore in my study I wanted to select a reasonable range in which to test possible effects of density dependence when multiple larvae were all infested on the same plant. Unlike previous neonate studies in which egg masses were applied to infested plants, larvae were placed in specific areas of the corn plants and distributed more evenly to minimize effects of density dependence. Treatments with only one larva served as a basis for comparison when looking for changes in behavior for the rest of the experiment. It is important to note that despite efforts to stabilize larvae and to reduce stress factors when infesting plants, most larvae did move around a bit upon being placed on the plants with paintbrushes. In most cases the larvae dropped down further into the whorl or leaf axil in which they were infested, though a few would crawl out onto the leaf's edge; however, they would eventually return to the leaf axil or whorl.

The most distinguishable changes in behavior due to the presence of other larvae usually occurred when more than two larvae were placed in the same axil or whorl. Larvae that came in direct contact with other larvae often would move out to the edge. The more frequent this event became, particularly in those treatments where nine larvae were infested on the same plant, the more often it happened. Usually in treatments where two larvae were placed in the same axil or whorl there was enough room for both larvae to exist without much contact with each other. Additionally, after three days of being able to move freely, larvae that were recovered had managed to disperse enough to eliminate overlapping of tunnels. It is quite possible that larvae are able to detect the presence of other ECB larvae in the plant to avoid direct contact with each other. My personal observations were that it was rare to see two ECB larvae in the same tunnel during dissections. Again, no movement off the infested plant occurred immediately following infestation. Following this portion of the trial, it was determined that nine larvae was too high to simulate what could occur in the field even under high population numbers without influence of density dependence.

Originally the recapture rate experiment was run inside the greenhouse to gauge the maximum number of larvae that I could expect to retrieve after three days from field treatments. The greenhouse trials showed a higher number of larvae being recaptured than originally expected due to exclusion or limiting of outside factors such as heat, desiccation, and predation, though as noted, not all predation was excluded in the greenhouse. It was also important to note that plants that were used for the outside portion of this trial were placed outside almost a week prior to running the trial so that

plants could be acclimated to outside conditions. As expected less larvae were recovered in the outdoor trial compared to the indoor trial. The outdoor trial was a closer example of what was expected in the field. However, it helped to determine that plants should be infested with five larvae as it ensured that at least one larva would be recovered after three days and that on average we could expect to recover half of the larvae even with the additional outside factors.

Third Instar Movement Field Trials

The movement study in the field supported my hypothesis that third instar movement would differ depending on whether larvae started out on Bt corn versus starting out on the marked refuge. In 2011, the fields went through near drought conditions and by the R3 stage many of the plants in the treatment were desiccating and showing evidence of drought stress. This may have had effects on why we recovered a lower proportion of larvae, particularly in the late growth stage treatments for that year compared to 2012. Despite recovering less larvae during the R3 growth stage the trends of movement behavior of the third instar larvae continued to be similar between both growth stages and both years **(**Figures 8, 9, 10, and 11**)**.

When analyzing how larval movement compared in regard to corn growth stage, the results for the majority of the treatments showed that growth stage had no significant effect on movement of third instars. This is logical despite the fact that the plant is changing, particularly in its chemical and physical characteristics. The lack of change in movement behavior may have more to do with the fact that all the larvae are

of the same instar and therefore their nutritional needs and plant host choices are going to be influenced by similar factors. However, the only treatment that did not follow this pattern was the NFN treatment in 2012. In this treatment the infested plant was a Cry1F single trait Bt corn plant. When comparing the early vegetative growth stage (V7) with the later reproductive growth stage (R3) the percentage of larvae recaptured on the immediately adjacent plant was much higher during the R3 growth stage compared to V7 stage (Figures 10 and 11). Because no other treatments had the same issues, particularly because this occurred during the 2012 field season and not the 2011 drought season, it is likely that it was not influenced by an environmental factor. Other potential explanations for why these results were observed for this specific treatment could include bias in either infesting or recapturing larvae, random chance for the data resulting this way, or outside factors such as location specific predation. However, this particular outcome does not follow the trend for the rest of the treatments and should be viewed with some skepticism.

When looking at the actual percentages of larval movement between similar types of treatments, some strong trends are noted. The control treatments showed that under normal conditions the majority of third instars should be expected to remain on the infested plant. On average approximately 90 percent of the larvae remained on the infested plant, with about 10 percent moving off to immediately adjacent plants over the three day period. Only 1-2 percent of the total larvae moved more than one plant away from the infested corn plant. There were concerns that the marked refuge, Cry34/35Ab1 Bt, would cause some larvae to move off at a higher frequency than the

near isoline that contained no Bt proteins; however, in most cases the marked refuge was nearly identical to the near isoline control or more larvae remained on the infested marked control plant than on the near isoline control plant. Based on the findings Cry34/35Ab1 had no influence on ECB third instars and it can serve as a good marker in future studies of this insect.

Treatments in which larvae started out on the marked refuge with a single trait Bt corn plant immediately adjacent to them did not show any significant difference when compared with the controls. So in cases of a field planted with blended refuge seed where third instars happen to move onto or begin on a non-Bt refuge plant, there is the possibility that some of the larvae are unlikely to move off onto an adjacent corn plant. However, third instars in treatments where the infested center plant was Bt showed significantly higher movement off of the infested plant onto the adjacent marked refuge plant. This could mean that larvae naturally occurring in a seed blend field who either move onto or started out on a Bt plant in a seed blend field would be more likely to move off of the infested plant. However, the percentage of larvae moving off the Bt plant could have a high range of variation due to outside factors.

Based on the field studies the range of third instars moving off of Cry1Ab corn is approximately 30% to 65% and the range for third instars leaving Cry1F corn is about 20% to 60%. This is the first study to show these percentages of third instar movement off of Bt corn. It was not possible to determine if larvae had fed on the infested plant before moving onto adjacent plants; however, larvae were withheld from food about two hours prior to being put on the plants with the idea that this

would stabilize the larvae and encourage feeding on the infested plant. Under ideal conditions the larvae that were infested in the treatments would feed on the infested plant before moving off. It is possible that larvae who did feed on the infested plant when it was Bt became ill or, through lack of ability to gain actual nutrition from the plant material, would be more likely to leave the plant seeking a more suitable host.

It is important to note that this experiment only took into account the movement behaviors of third instar larvae for three days and did not track the mortality rate of larvae. Nor did we test if larvae were more susceptible to the Bt toxins after moving off of Bt corn plants. Because all of the larvae collected from the research field were frozen, future research could be done on the insect body contents to determine what specific Bt proteins the larvae consumed and, potentially, the quantities of the different types of plant material. The Bt presence and retention results will provide a baseline of information for this kind of future research.

Presence and Retention of Bt in Third Instars

The results from the Bt presence trials in which larvae were fed for hours 0 to 24 on their specific Cry proteins showed that the lepidopteran Cry proteins were able to be detected the longest and most consistently during feeding. This supports the idea that these proteins are binding to receptors in the gut and; therefore, are detectable for a much longer range of time compared to Cry34/35Ab1. Because Cry34/35Ab1 is not targeted for receptors in ECB midguts it is possible that very little of the protein remains in the gut and is expelled with other waste products. Another idea is that the

Cry34/35Ab1 proteins are being altered in the gut so that they are no longer detectable by the ELISA Bt test strip's monoclonal antibodies. This is supported by similar research methods to detect Bt material in corn rootworm, *Diabrotica virgifera,* in which similar Cry protein test strips were used (Spencer et al., 2003). In this case the lepidopteran Cry proteins were less detectable. The results in the follow-up portion of the current study support the idea that some transformation of the coleopteran Cry protein is occurring after the insect consumes the corn tissue because it was easily detected in the corn tissue by itself. It is clear that the ELISA test strips are capable of detecting even minute levels of Cry34/35Ab1 protein as shown by the small sized of plant tissue tested (Figure 7).

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APPENDIX

TABLES AND FIGURES

Table 1. The mean percentage of larvae recaptured for the preliminary density dependence and recapture trial conducted at locations inside and outside the greenhouse on non-Bt corn.

	Greenhouse		Outside	
	1 Infested	5 Infested	1 Infested	5 Infested
Recaptured Larvae Mean	0.8	4.4	0.5	3.8

Table 2. Fisher's Exact Test for 2011, in which treatments were compared for significant change in larval movement for both growth stages V7 and R3. Significance: p=0.05.

Growth Stage V7		Growth Stage R3	
Comparisons:	**Fisher's Exact Test (p-value):**	**Comparisons:**	**Fisher's Exact Test (p-value):**
NAN vs. NFN	0.0301	NAN vs. NFN	0.219
NAN vs. ANA	< 0.0001	NAN vs. ANA	< 0.0001
NAN vs. FNF	0.0118	NAN vs. FNF	0.0004
NAN vs. Control 1	< 0.0001	NAN vs. Control 1	0.0002
NAN vs Control 2	< 0.0001	NAN vs Control 2	0.0401
NFN vs. ANA	0.0192	NFN vs. ANA	0.0044
NFN vs. FNF	0.3106	NFN vs. FNF	0.0313
NFN vs. Control 1	0.0471	NFN vs. Control 1	0.0183
NFN vs. Control 2	0.0505	NFN vs. Control 2	0.505
ANA vs. FNF	0.2159	ANA vs. FNF	0.2991
ANA vs. Control 1	0.6047	ANA vs. Control 1	0.3837
ANA vs. Control 2	0.347	ANA vs. Control 2	0.0287
FNF vs. Control 1	0.0828	FNF vs. Control 1	> 0.9999
FNF vs. Control 2	0.0491	FNF vs. Control 2	0.1871
Control 1 vs. Control 2	0.8405	Control 1 vs. Control 2	0.0556

Table 3. Fisher's Exact Test for 2012, in which treatments were compared for significant change in larval movement for both growth stages V7 and R3. Significance: p=0.05.

Growth Stage V7		Growth Stage R3	
Comparisons:	**Fisher's Exact Test (p-value):**	**Comparisons:**	**Fisher's Exact Test (p-value):**
NAN vs. NFN	0.1414	NAN vs. NFN	0.0008
NAN vs. ANA	< 0.0001	NAN vs. ANA	< 0.0001
NAN vs. FNF	< 0.0001	NAN vs. FNF	< 0.0001
NAN vs. Control	< 0.0001	NAN vs. Control	< 0.0001
NFN vs. ANA	< 0.0001	NFN vs. ANA	< 0.0001
NFN vs. FNF	< 0.0001	NFN vs. FNF	< 0.0001
NFN vs. Control	< 0.0001	NFN vs. Control	< 0.0001
ANA vs. FNF	> 0.9999	ANA vs. FNF	0.2531
ANA vs. Control	0.2274	ANA vs. Control	0.0049
FNF vs. Control	0.1881	FNF vs. Control	0.0341

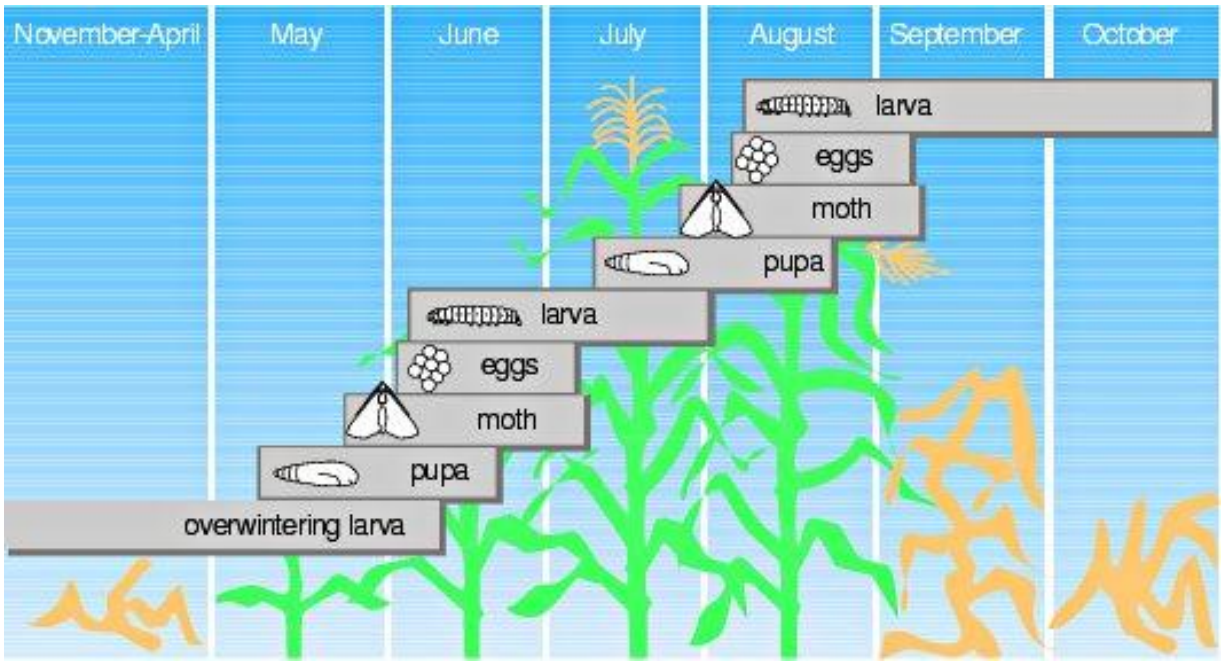

Figure 1. Typical life history of European corn borer in relationship to corn phenology in the United States.

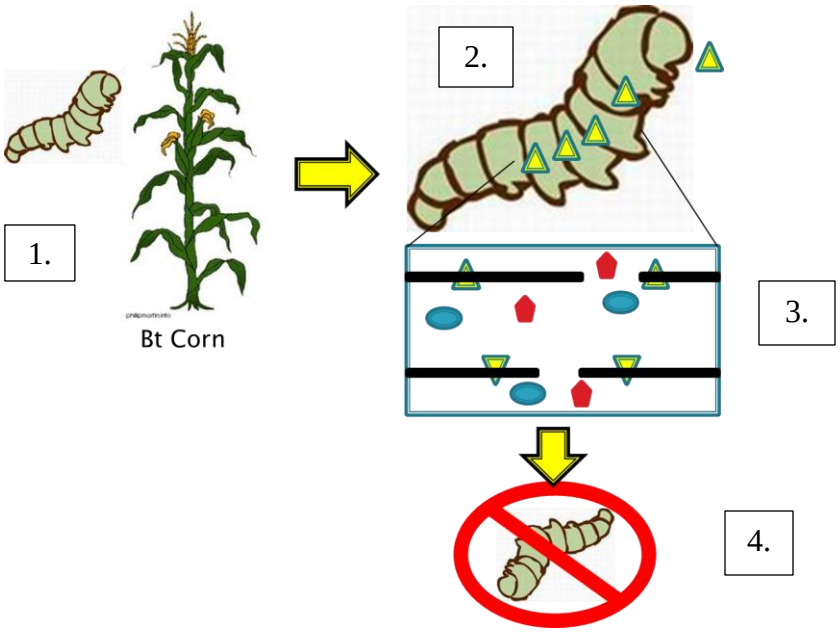

Figure 2. Mode of Action of *Bacillus thuringiensis* var. *kurstaki* on caterpillars' midguts. 1) Larvae consume plant material containing Bt, 2) Toxin binds to specific receptors in the gut, larvae stops feeding, 3) The gut wall begins to break down, allowing spores and normal gut bacteria to enter the body cavity, and 4) In 1-2 days, the larvae dies from septicemia as spores and gut bacteria proliferate in the blood.

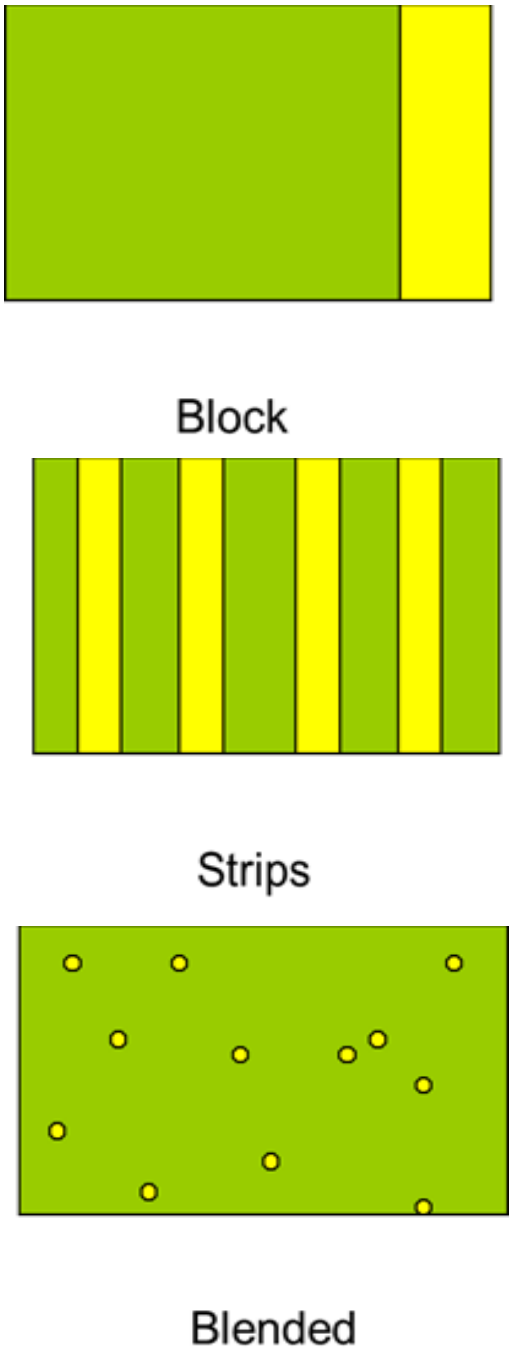

Figure 3. (A)(B)(C). Possible Bt refuge field configurations for insect resistance management of European corn borer. Light shaded areas represent refuge plants that do not contain Bt proteins and dark shaded areas represent Bt corn plants.

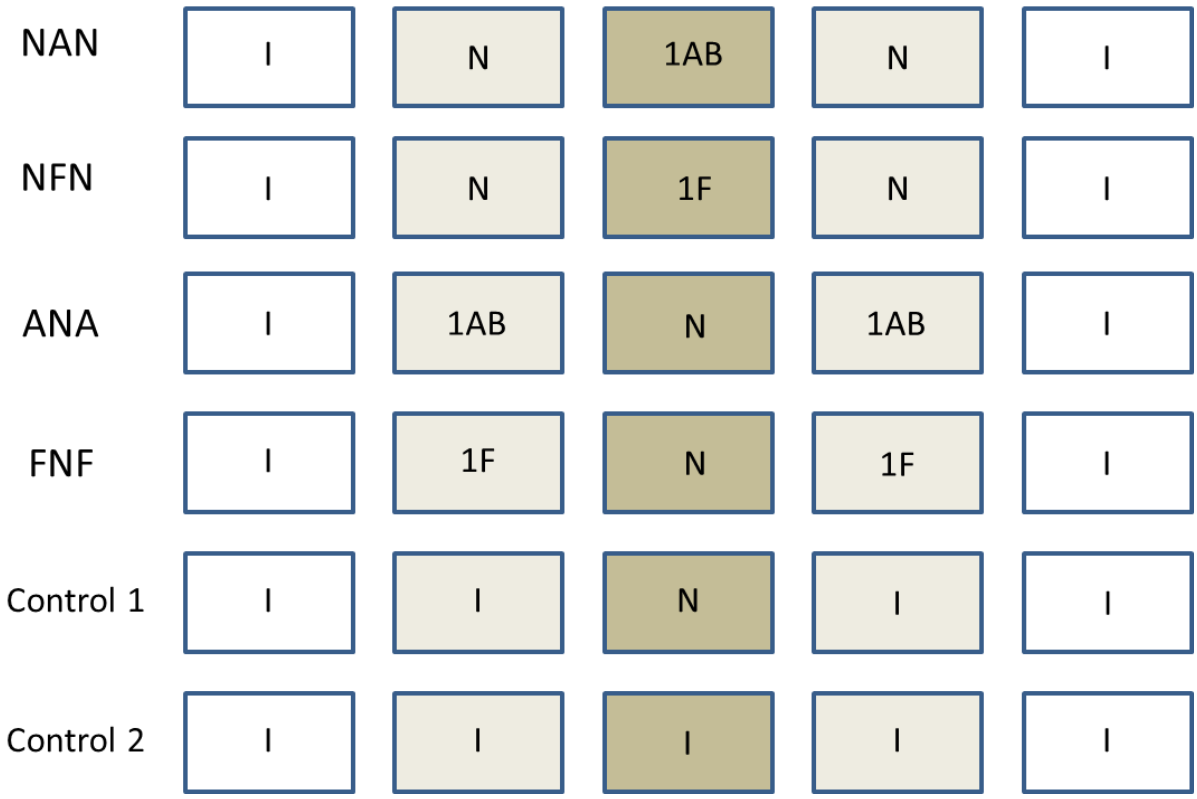

Figure 4. 2011 larval movement field treatments of Bt and non-Bt plant arrays (I= non-Bt near isoline, N= marked refuge with Cry34/35Ab, 1AB=Cry1Ab, and 1F=Cry1F).

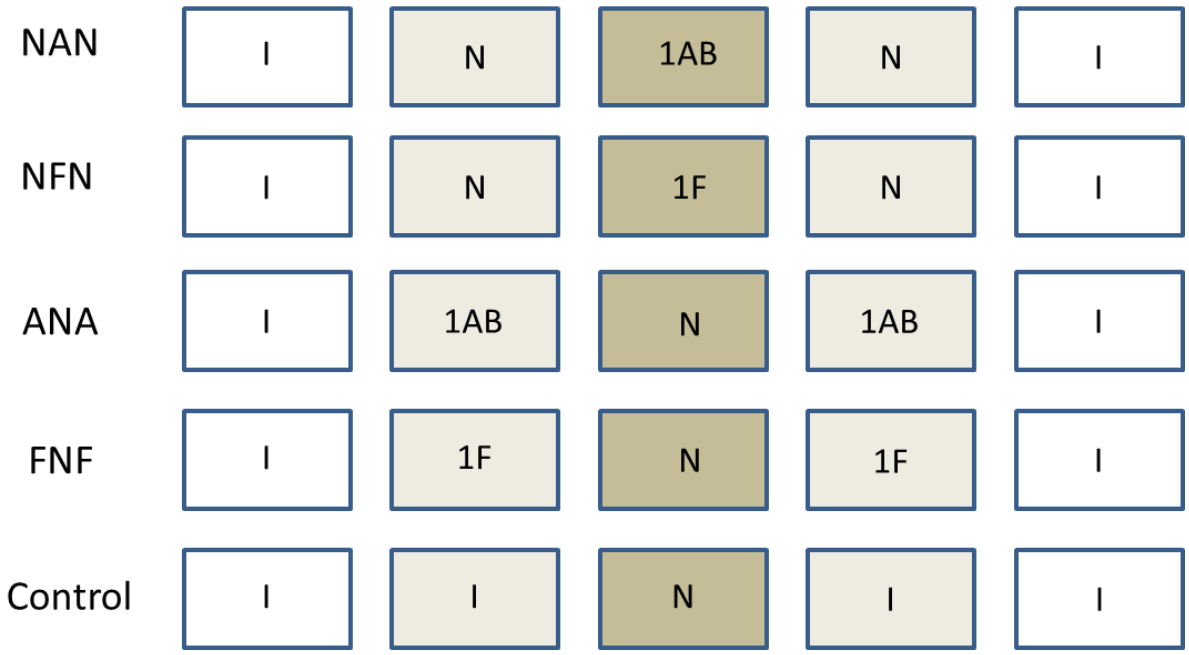

Figure 5. 2012 larval movement field treatments of Bt and non-Bt plant arrays (I= non-Bt near isoline, N= marked refuge with Cry34/35Ab, 1AB=Cry1Ab, and 1F=Cry1F).

Figure 6. Color plant stake markers used in third instar movement field trials to associated with Bt plant variety.

Figure 7. Corn leaf tissue respresenting an estimated 1/10th daily comsumption for third instar ECB larvae of Cry34/35Ab1 used in Bt ELISA test analysis.

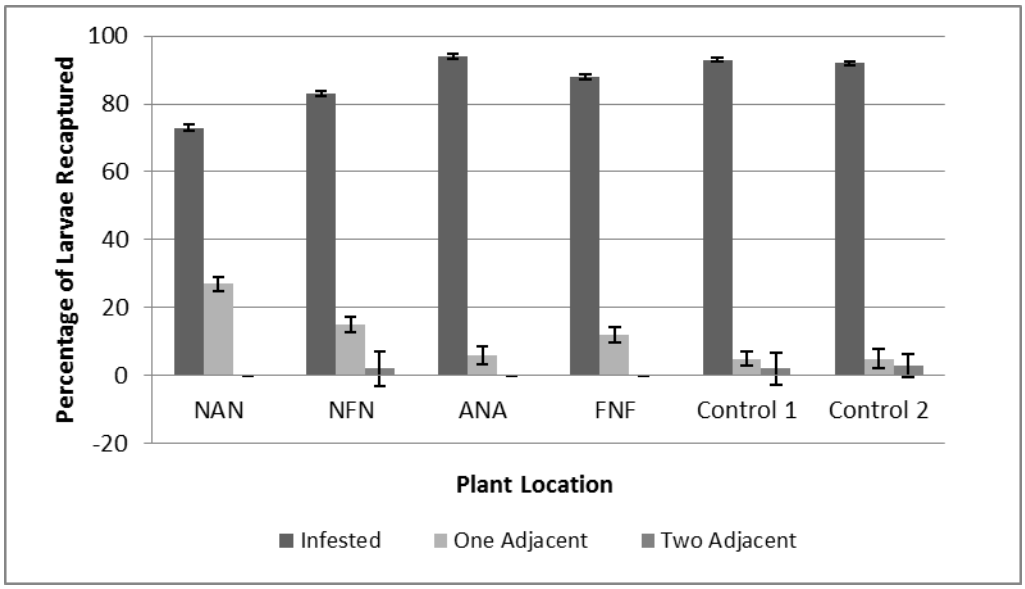

Figure 8. Percentage of larvae found per plant location during the 2011 V7 corn growth stage.

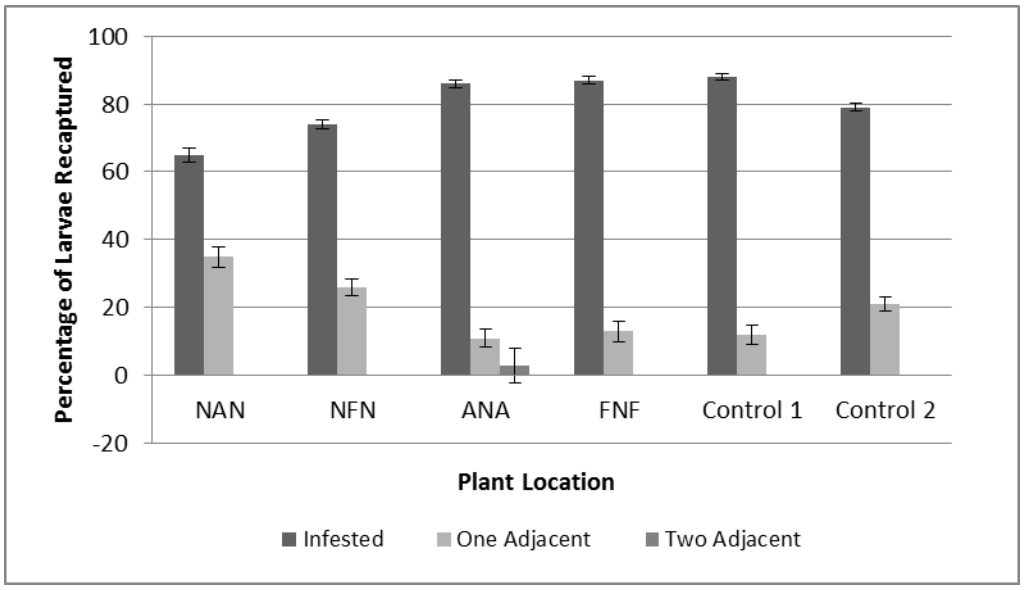

Figure 9. Percentage of larvae found per plant location during the 2011 R3 corn growth stage.

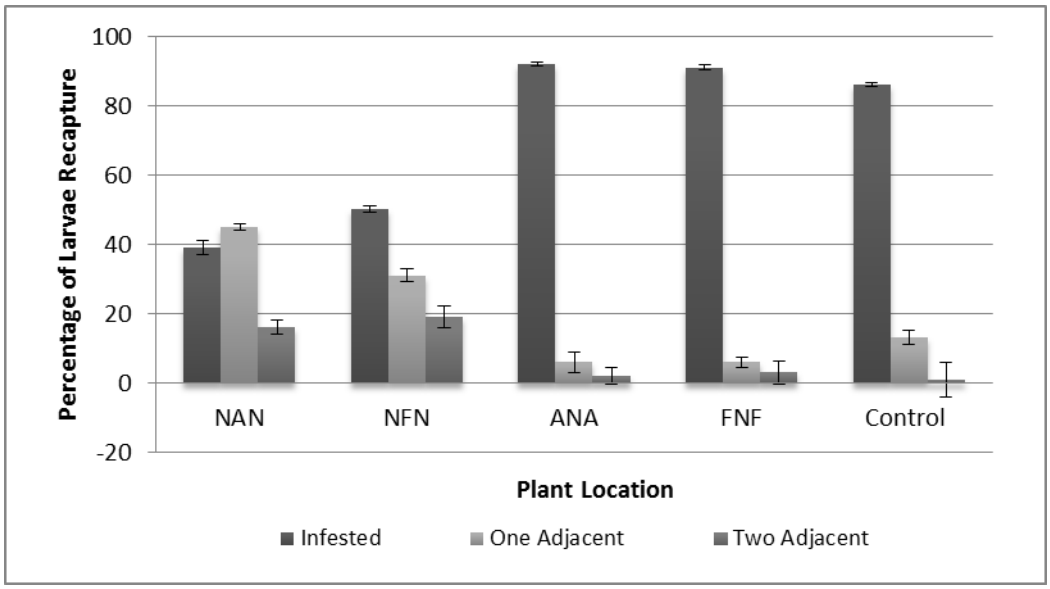

Figure 10. Percentage of larvae found per plant location during the 2012 V7 corn growth stage.

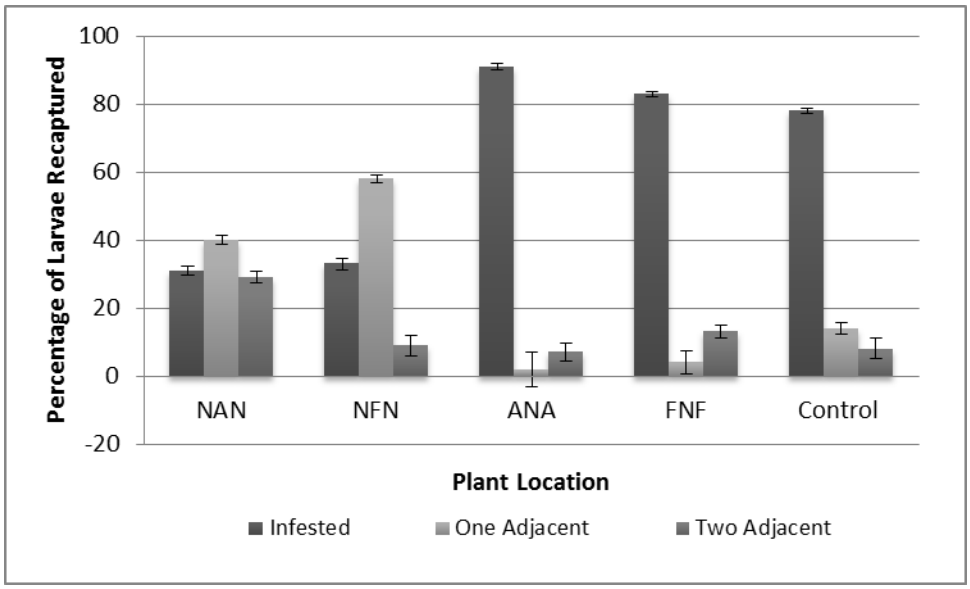

Figure 11. Percentage of larvae found per plant location during the 2012 R3corn growth stage.

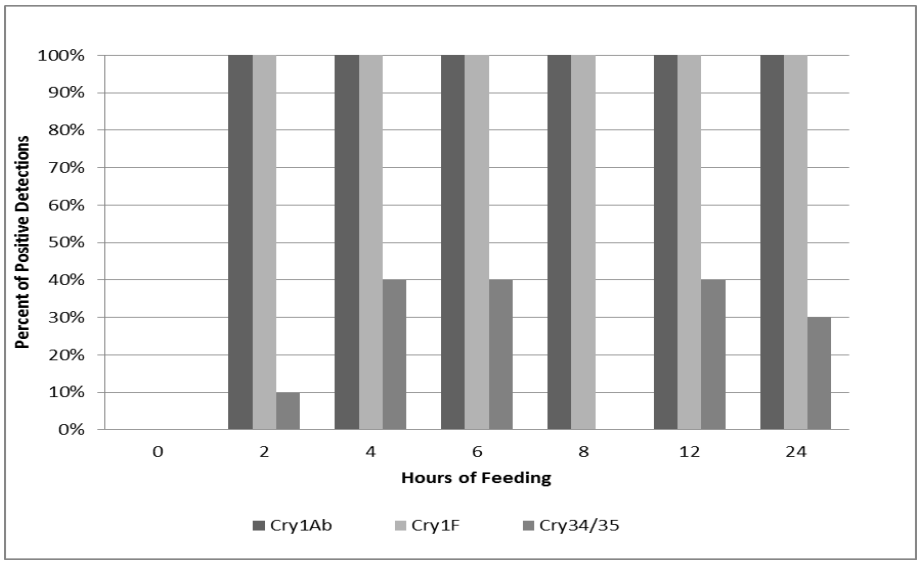

Figure 12. Percent of third instar ECB that were positive for presence of various Cry proteins using ELISA Bt test strips over a 24 hour feeding period on the designated Bt plant material.

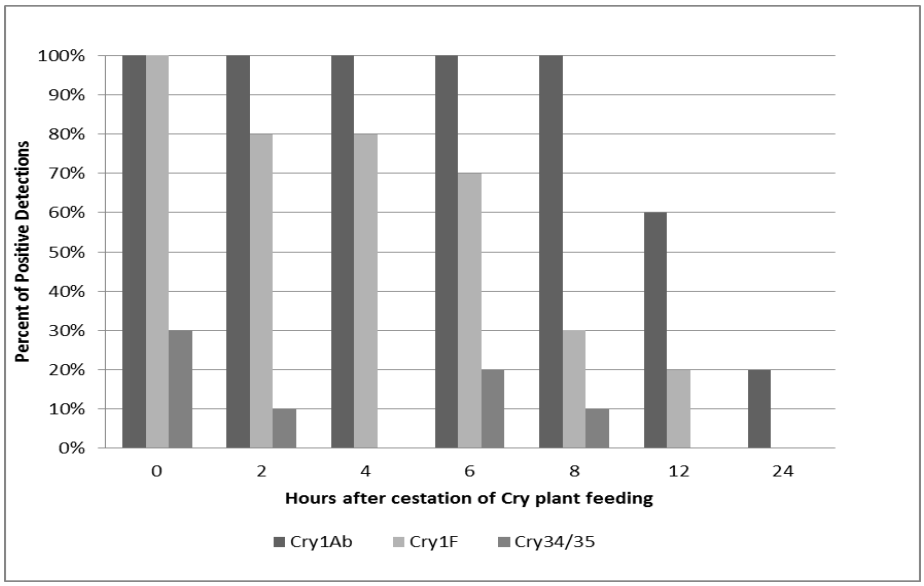

Figure 13. Percent of third instar ECB that were positive for presence of various Cry proteins using Bt ELISA test strips after feeding for 24 hours on Bt plant material and then being transferred to non-Bt plant material.

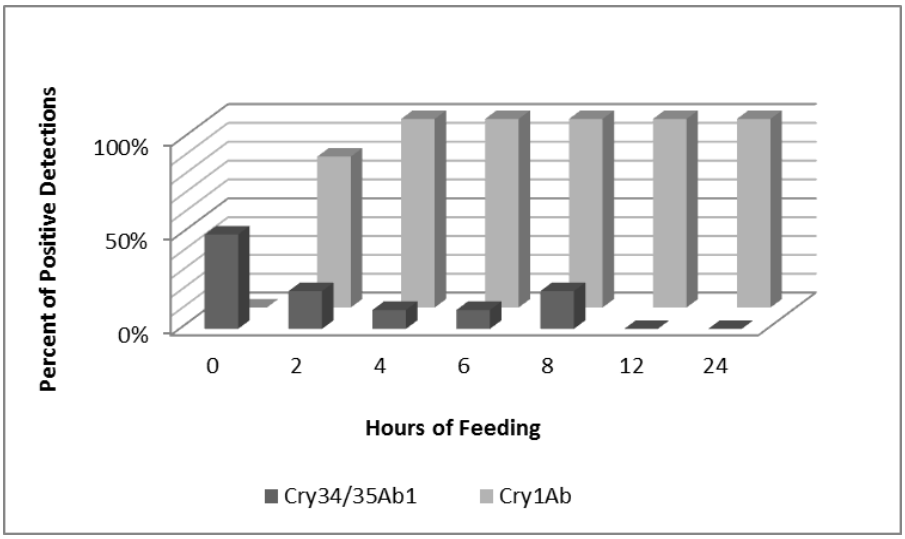

Figure 14. Percent of third instar ECB that were positive for presence of Bt using ELISA test strips in which larvae had fed on Cry34/35Ab1 plant material for 24 hours and then were transferred to Cry1Ab for the following 24 hours.

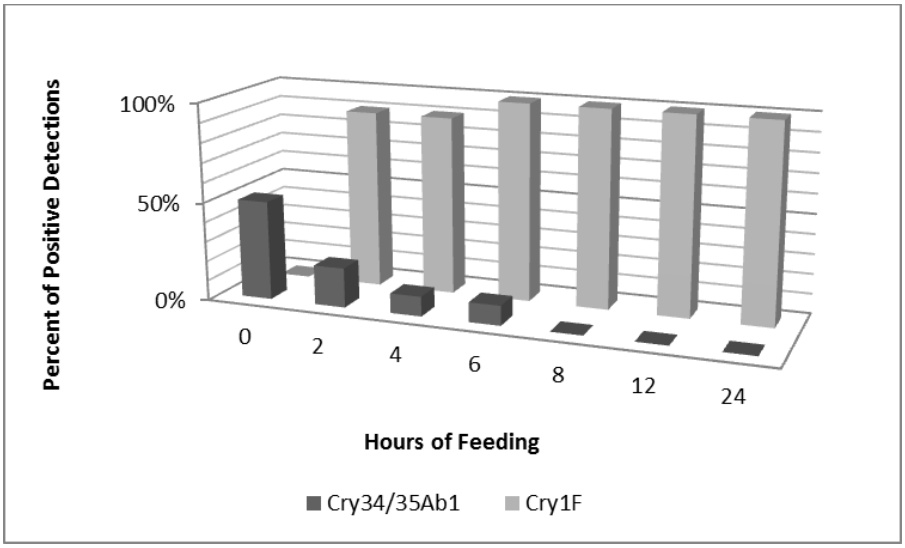

Figure 15. Percent of third instar ECB that were positive for presence of Bt using ELISA test strips in which larvae had fed on Cry34/35Ab1 plant material for 24 hours and then were transferred to Cry1F for the following 24 hours.

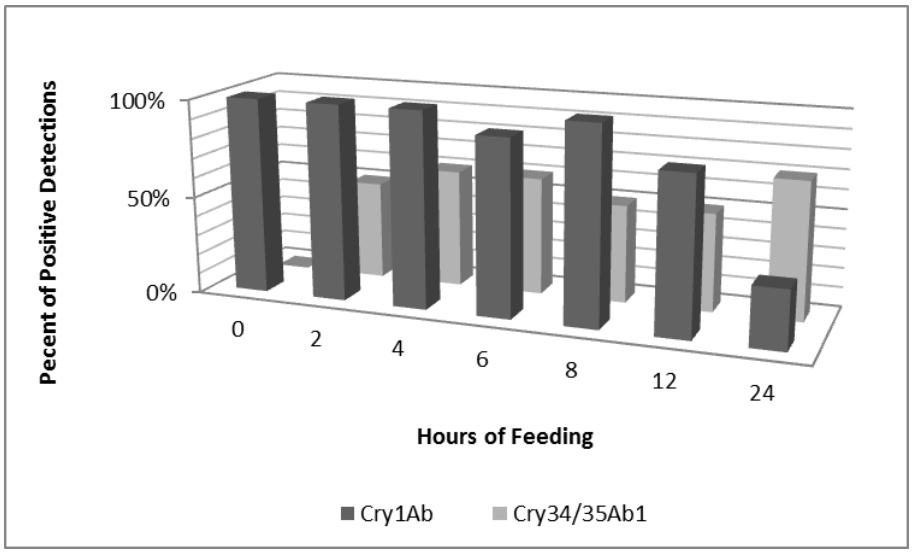

Figure 16. Percent of third instar ECB that were positive for presence of Bt using ELISA test strips in which larvae had fed on Cry1Ab plant material for 24 hours and then were transferred to Cry34/35Ab1 for the following 24 hours.

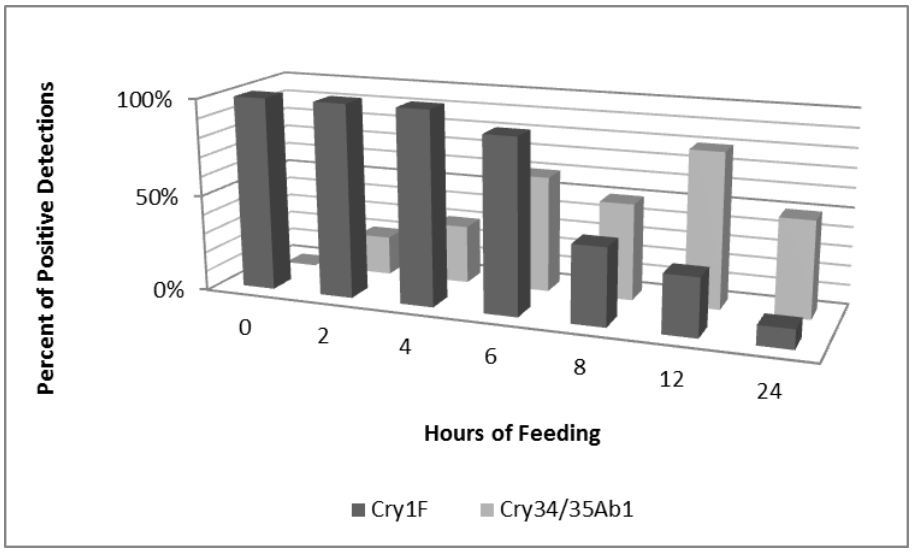

Figure 17. Percent of third instar ECB that were positive for presence of Bt using ELISA test strips in which larvae had fed on Cry1F plant material for 24 hours and then were transferred to Cry34/35Ab1 for the following 24 hours.