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Developmental Effects and Selection Pressure of Genuity VT3 Pro Field Corn on Corn Earworm, Helicoverpa Zea (Lepidoptera: Noctuidae)

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Developmental effects and selection pressure of Genuity VT3 PRO field corn on corn earworm, *Helicoverpa zea* (Lepidoptera: Noctuidae)

By

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Transgenic corn and cotton expressing crystalline (Cry) insecticidal proteins from *Bacillus thuringiensis* (Bt) were commercially introduced in 1996. This technology has greatly improved the control of several key lepidopteran insect pests tobacco budworm, *Heliothis virescens* (Farbricius), pink bollworm, *Pectinophora gossypiella* (Saunders), southwestern corn borer, *Diatraea grandiosella* (Dyar), and European corn borer, *Ostrinia nubilalis* (Hübner). The corn earworm, *Helicoverpa zea*, has been more difficult to control using Bt crops and supplemental insecticide applications are often needed to prevent economic losses. A major threat to the longevity of transgenic technology is the evolution of resistance, especially when an insect pest infests both Bt corn and cotton in the same growing season. Similar Cry proteins are currently expressed in both corn and cotton commercial production systems. At least one generation per year develops on Bt corn before infesting cotton. Given that *H. zea* infests both crop hosts at some point every year, the objectives of this study were to evaluate the contribution and influence of Genuity VT3 PRO corn (expressing Cry1A.105 and Cry2Ab) on *H. zea* density, fitness, and selection on BollGard II cotton (expressing Cry1Ac and Cry2Ab). Non-Bt and VT3

PRO corn fields were sampled for larval density and any observed larvae were collected. Non-Bt field corn supported 61% more larvae compared to VT3 PRO fields. Larvae infesting non-Bt corn developed faster than those infesting VT3 PRO corn. Larvae collected from VT3 PRO corn had significantly higher pupal weight two out of the three years of this study. Pupae from VT3 PRO corn also had longer pupal duration two out of three years compared to pupae of larvae collected from non-Bt corn. Offspring from larvae reared on VT3 PRO corn had a higher LC_{50} compared to offspring from larvae reared on non-Bt corn. *H. zea* susceptibility to Bt is highly variable but the results presented here indicate that dual-gene corn hybrids such as VT3 PRO can select for *H. zea* populations with a higher propensity for causing damage in Bt cotton.

DEDICATION

I would like to dedicate this dissertation to my family for the love and support they have given me throughout my life. I owe them a debt I will never be able to fully repay. This dissertation and degree is the best means I have to show my appreciation for their impact on my life.

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CHAPTER I

REVIEW OF LITERATURE

Phylogeny

Hardwick established *Helicoverpa* (Lepidoptera: Noctuidae) in 1965 as a globally distributed genus comprising a total of 18 species. *Helicoverpa zea* (Boddie) is the only species of this genus present in North America (Hardwick 1965). *H. zea* was originally included in the genus *Heliothis* until Hardwick (1970) repositioned the species into *Helicoverpa* due to differences in morphology of male genitalia. A review of the older literature (i.e. work preceding that performed by Common in (1953) contains a great deal of ambiguity as *Noctua armigera* Hübner, *Bombyx obsoleta* Fabricius, *Chloridea obsoleta*, *Heliothis obsoleta*, *armigera*, and *umbrosus* are all used in reference to *H. zea* if the origin is referenced to the New World (Pogue 2004).

Common names of *H. zea* are as convoluted as the scientific names due to its geographic distribution, broad host range, and pest status. Quaintance and Brues (1905) referenced it as 'the worm', however, names such as shatterworm, vetchworm, false tobacco budworm, sorghum head worm, tomato fruitworm, bollworm, and corn earworm have all been used to describe *H. zea* (Sherman 1914; Burkett et al. 1983, and Dowd and Lagrimini 1997).

Life Cycle

H. zea generally completes 5 to 7 generations per year in the southern United States. The final generation enters diapause initiated by photoperiod and to some extent the nutritional quality of host plants (Stadelbacher 1981). Duration of pupal diapause ranges from 187 to 243 days for the Mid-South (Stadelbacher and Pfrimmer 1972). Eggs from female *H. zea* moths are oviposited singly on or near a fruiting structure or flower (Brazzel et al. 1953; Hardwick 1965). Female fecundity ranges from 500 to 300 eggs. Eggs hatch in 3 to 4 days (Ditman and Cory 1931; Hardwick 1996). Larvae will complete 5 to 6 instars in a 12 to 16 day period (Hardwick 1965). Adult eclosion occurs approximately 12 days after pupae have been fully sclerotized (Hogg and Calderon 1981). Female moths normally emerge a day before males and newly emerged males may have a green tone that is lost with age (Hardwick 1965; Hardwick 1996). The longevity of *H. zea* adults ranges from 5 to 17 days with a total lifespan of approximately 30 days (Quaintance and Brues 1905).

Geographic Distribution

The geographic range of *H. zea* includes the southern parts of Canada, most of North America, all of Central America, parts of Chile and Argentina in South America, all of the Caribbean Islands, and was (fairly) recently discovered in the Hawaiian Islands (Kogan et al. 1989; Hardwick 1996). Geographic regions between 40° N and 40° S experience annual populations (Fitt 1989); however, uncharacteristically warm temperatures in the extreme southern United States will allow populations to continue throughout the year without entering diapause (Hardwick 1965).

Host Plants

H. zea has been recovered from 240 plant species representing 36 plant families (Kogan et al. 1989). Seasonal abundance of *H. zea* is largely determined by the availability of suitable host plants throughout the year in North America. *H. zea* emerge from overwintering in the Mid-south region of the United States in April to May. The first 1 to 2 generations develop on wild host plants for feeding and development before moving into cultivated crops (Anonymous 1967; Sudbrink and Grant 1995). One third of the plant hosts are within the family Leguminosae; while the majority of remaining host plants can be divided among of Malvaceae, Solanaceae, and Asteraceae (Kogan et al. 1989). Specifically, the availability of spring legumes such as: crimson clover, *Trifolium incarnatum* (L.), white clover, *Trifolium repens* (L.), lupine, *Lupinus* spp., chickpea, *Cicer arietinum* (L.), alfalfa, *Medicago sativa* (L.), and vetch, *Vicia sativa* (L.) that are capable of sustaining 1 to 2 generations prior to the emergence of cultivated hosts (Isley 1935; Brazzel et al. 1953). Many of these species (especially clover species) have been seeded to road sides and ditch banks as a component of erosion prevention programs. This provides a vast amount of available host plants to perpetuate the initial population exiting diapause (Stadelbacher 1981). In fact, a spring-time drought can cause a decline in the availability of noncultivated host plants and lower *H. zea* densities throughout the entire growing season (Isley 1935; Stadelbacker and Pfrimmer 1972).

H. zea infest numerous agricultural commodities including: corn, (*Zea mays* L.); cotton, *Gossypium hirsutum* (L.); grain sorghum, *Sorghum bicolor* (L.); soybean, *Glycine max* (Merr.); wheat, *Triticum aestivum* (L.); tobacco, *Nicotiana tabacum* (L.); peppers, *Capsicum* spp; lettuce, *Lactuca sativa* (L.); and tomato, *Solanum lycopersicum* (L.) (Fitt

1989). Corn is the most preferred and suitable host for larval development. Females have higher fecundity and larvae complete development faster when reared on corn compared to other host plants or artificial diet (Isley 1942; Gore et al. 2003). Corn in the R1 growth stage (silking) is preferred for oviposition. As corn begins to mature during the latter part of the summer, moths migrate to more suitable agronomic hosts like cotton (Stadelbacher et al. 1986). Though *H. zea* is dependent on the availability of noncultivated spring hosts, the generations developing on corn are responsible for the high population densities that infest cultivated host plants like cotton, soybean, and grain sorghum (Isley 1942). Soybean and grain sorghum have generally harbored lower population densities compared to cotton, yet these crops are more suited for *H. zea* development (Anonymous 1967; Johnson et al. 1975). In recent years, cotton acreage in Mississippi has declined from 485,829 hectares in 2005 to 186,235 hectares in 2012 (Williams 2006; Williams 2013). Inversely, soybean acreage has increased from 647,773 hectares in 2006 to 769,230 hectares in 2012 (USDA 2007; USDA 2012).The increase in soybean acreage has resulted in soybean becoming a predominant host compared to cotton, and made soybeans a more favorable host for a greater length of time during the summer. As a result, the number of treated soybean acres to control *H. zea* has increased drastically within the past decade (Musser et al. 2012).

Economic Importance

The importance and volume of research surrounding *H. zea* is a result of it being a major arthropod pest of numerous agricultural and vegetable crops (Fitt 1989). This pest feeds almost exclusively on the reproductive parts of host plants; causing direct economic losses, and is the most common species within the *Heliothine* complex in the United

States (Williams 2013). Field corn remains the most significant producer of *H. zea* across much of the southern United States (Jackson et al. 2007). It has never been economically feasible to treat field corn for *H. zea* in the Mid-South. *H. zea* damage to cotton has been the most common complaint due to the traditionally high value and low insect tolerance of cotton (Isley 1935). Isley (1926) observed that *H. zea* outbreaks seldom occur in areas where corn is not grown. Quaintance and Brues (1905) noted *H. zea* as being the most destructive pest of cotton before the introduction of the boll weevil, *Anthonomus grandis grandis* (Boheman). Given the ability of *H. zea* to infest multiple crops of economic importance, great strides have been made to manage *H. zea* larvae and/or prevent damage.

Helicoverpa zea **Control**

Cultural practices were recommended prior to the reliance on insecticides for *H. zea* management. Adjustment of planting dates, field cultivation to destroy overwintering pupae, increasing canopy density, and elimination of non-crop hosts in early spring were all generally recommended in order to reduce *H. zea* densities within and among years (Fitt 1989). Planting a portion of the corn crop outside of the optimum planting period was recommended to use as a trap crop for cotton (Anonymous 1924). Alston et al. (1991) determined that egg and larval mortality increased due to predators and parasitoids as canopy density increased. These practices did not provide complete control, but used in combination with other control methods, theylessened the impact of *H. zea* after successive years of implementation.

Biological control agents or natural enemies (predators, parasitoids, and microbial organisms) may reduce *H. zea* population densities to manageable densities. The habit of

H. zea larvae to bore into plant tissue complicates control with insecticide applications because once larvae have entered the fruit (i.e. cotton boll or corn ear), maximizing exposure to the insecticide becomes difficult (Quaintance and Brues 1905). However, natural enemies are important because they are able to attack larvae concealed with in fruiting structures. King and Coleman (1989) performed a comprehensive review from more than 7,700 documents of all known arthropod predators and parasitoids of *H. zea.* In conclusion, they found 6 families of hymenopteran parasitoids comprising 60 different species, 4 families of dipteran parasitoids totaling 61 species, and 142 predatory species from 8 insect and 2 arachnid orders. Predators and parasitoids alike target specific life stages. The genus *Trichogramma* contains the most common egg parasitoids while the most frequently recorded larval parasitoids include *Cardiochiles nigriceps* (Viereck), *Microplitis croceipes* (Cresson), *Eucelatoria bryani* (Sabrosky), *Archytas marmoratus* (Townsend), and *Cotesia marginiventris* (Cresson) (King and Coleman 1989). Coleoptera, Neuroptera, and Hemiptera comprise the majority of predaceous insects.

Microbial agents of *Heliothines* in the United States include: the soil-borne bacterium *Bacillus thuringiensis* Berliner var. *kurstaki* (Bt), several species of fungi from *Entomophthora* and *Nomuraea rileyi* (Farlow), protozoans *Nosema heliothidis* and *Vairimorpha necatrix* (Kramer), and the baculovirus *H. zea* nuclear polyhedrosis virus (NPV) (Yearian et al. 1986). These pathogens seem to be ubiquitous across much of the United States and can be found in cotton, grain sorghum, field corn, and soybeans (Carner 1980; Gaugler and Brooks 1975; Schwehr and Gardner 1982). However, mainly NPV and Bt have been propagated and sold as commercial formulations to supplement synthetic insecticides when possible (King and Coleman 1989).

Management of beneficial arthropods and microbial organisms is an effective component of integrated pest management (IPM). The promotion of biological control agents aims to decrease the reliance on synthetic insecticides to control insect pests and is useful in insecticide resistance management (IRM) programs (Bottrell and Adkisson 1977). However, directions for facilitating the use of natural enemies are normally absent from the decision making process (King 1986). Natural enemies require an established *H. zea* infestation in order to be effective. Often times, the number of *H. zea* larvae that can be tolerated on a high-value, low-tolerance crop (e.g. cotton) is much lower than natural enemies can adequately achieve alone (King and Coleman 1989). Thus, much of *H. zea* control has traditionally been focused on insecticides.

Lincoln and Williams (1952) suggested that insecticide choice was not as important as the proper timing of application for controlling *H. zea*. It should be noted that during this time organophosphates and organochlorines (especially DDT) were being used for *H. zea* control. These insecticides provided good control of *H. zea* but were non-selective and disrupted beneficial-insect populations (Lincoln and Phillips 1970). These insecticidal chemistries provided excellent control when first introduced in the mid 20th century. DDT was used extensively in agriculture to control a number of pests; which culminated in complete field control failures of *H. zea* after just 10 years of use (Lincoln 1970). These insecticides were being applied at rates that not only decimated beneficial insect populations, but also resulted in phytotoxicity in the crops they were aiming to protect. The most common plant symptoms included leaf discoloration, reduction in plant height, and early maturation (Brazzel et al. 1953; Brown et al. 1962). Ewing and Ivy (1943) documented 12 beneficial insect species that were not able to survive in plots

treated with arsenicals and as a result, observed an increase in *H. zea* egg and larval densities. Newer, more effective insecticides continued to be developed after resistance to DDT was documented. The organophosphorus and carbamate insecticides were used to manage *H. zea* infestations into the early 1980s until being replaced by pyrethroids (Martinez-Carrillo and Reynolds 1983). Pyrethroid resistance has been documented in *H. zea* (Leonard et al. 1988). They are currently still recommended even though pyrethroids often fail to decrease larval density to sub-economic levels (Brown et al.1998). Flubendiamide and chlorantraniliprole represent the diamide class of chemistry and have provided enhanced control of *H. zea* compared to the previously recommended standards (Lorenz et al. 2011; Hardke et al. 2011).

***Bacillus thuringiensis* Berliner var. *kurstaki* (Bt)**

The insecticidal properties of Bt have been known for more than a century. Bt was first isolated by a Japanese scientist in 1901 from *Bombyx mori* (L.) larvae (Ishiwata 1901). Although Ishiwata (1901) never described the organism, credit was given to Ernst Berliner who formally documented Bt nearly a decade later (Beegle and Yamamoto 1992). Initial field trials were performed in the 1930s on European corn borer, *Ostrinia nubilalis*, in Hungary (Briggs 1986). However, development of Bt as an insecticide would come to a halt due primarily to World War I, II, and the great depression in the United States (Heimpel and Angus 1960). Once research on Bt resumed in the 1950s, there was considerable interest in culturing the bacterium for mass production to be used as foliar sprays and dusts. The first Bt products were β-exotoxins, considered nonspecific, and varied greatly with regard to efficacy. This was a result of inconsistencies in production methods that made standardizing a product nearly impossible (Heimpel 1967).

These efforts culminated in 1957 with the first Bt product, Thuricide (Beegle and Yamamoto 1992). Several commercialized Bt products were produced thereafter; although, these products failed to compete with synthetic insecticides because of low efficacy. In 1962, a subspecies of Bt was discovered by Edouard Kurstak (Kurstak 1962). Initially labeled as K-17, this subspecies is now known as *Bacillus thuringiensis* variety *kurstaki* and is specifically active against lepidopteran insect pests (Dulmage 1970). Dipel was later developed and contained the first Bt product composed of the *kurstaki* isolate available as a δ-endotoxin. Adoption and usage of *kurstaki*-based products as foliar treatments would experience several shortcomings. These products were not heat tolerant compared to the Bt precursors and were also subject to photodegradation (Beegle and Yamamoto 1992). Bt var. *kurstaki* products also could not compete with the inherently more toxic pyrethroids because of the specific pathway involved with affecting the target organism (must be ingested).

More than 200 parasporal crystalline proteins or protoxins (Cry proteins) have been identified from Bt as having biological activity against insects (Heckel et al. 2001). Cry proteins 1 and 2 of Bt var. *kurstaki* have activity against lepidopteran larvae. These protoxins are found in the reproductive sporangia during the sporulation phase and responsible for causing mortality in the target organism. Barton et al. (1987) revolutionized insect control by successfully transferring (unspecified) Cry proteins into tobacco. The greatest acceptance of this technology would be seen when commercial production of transgenic corn and cotton varieties began in 1996 (Perlak et al. 2001). Since that time, the number of acres planted to Bt crops has increased drastically. In the United States, the percentage of Bt corn and cotton varieties in 2013 was 76% and 75%,

respectively (USDA 2013). Although the introduction of these transgenic crops was to primarily control several lepidopteran pests, *H. zea* was not an initial target. Inability to control the pink bollworm, *Pectinophora gossypiella* (Saunders), tobacco budworm, *Heliothis virescens* (F.), and a corn borer complex composed of European corn borer, *Ostrinia nubilalis* (Hübner), and southwestern corn borer, *Diatraea grandiosella* (Dyar) with labeled insecticides led to the development of transgenic crops (Wilson et al. 1992; Stewart et al. 2001; Baldwin et al. 2005). Bt corn and cotton varieties have been successful with regard to controlling these pests since that time. However, controlling *H. zea* through transgenic Bt technology has proven to be more challenging.

Initial research illustrated that *H. zea* susceptibility to Bt was low and highly variable (Luttrell et al. 1999). BollGard® (Monsanto Company, St. Louis, MO) cotton was the first commercial Bt cultivar and expressed Cry1Ac. Cry1Ac was effective against *H. virescens*. However, *H. zea* was frequently observed infesting these first generation cultivars and BollGard® cotton frequently required supplemental insecticide applications to augment control (Mahaffey et al. 1995). Cry1Ac expression varies greatly in cotton squares (floral buds), flowers, and bolls but is overall lower compared to the leaf and terminal tissue. Furthermore, expression begins to drop below effective concentrations approximately 80 days after planting (Greenplate et al. 1998). Cotton becomes a more attractive and abundant host during this time period as corn begins to mature. Genuity™ Yieldgard® field corn (Monsanto Company, St. Louis, MO) was the first transgenic corn hybrid expressing a single-gene with insecticidal activity. Yieldgard® corn is offered containing either Cry3Bb1 (activity against corn rootworm, *Diabrotica* spp.) or Cry1Ab (activity on lepidopterous pests). Cry1Ab can be detected from leaves, tassels, stalk,

silks, and kernels. Commercial production of single-gene Bt cotton was eliminated from production beginning in 2010; however, single-gene corn varieties remain available but are not recommended for *H. zea* control.

Manipulation of plant genomes has allowed scientists to enhance and broaden the scope of target pests in Bt varieties (Halpin 2005). Utilization of Cry proteins has been developed in Bt crops through four primary strategies: rotation, simultaneous expression of different genes (gene stacking), sequentially, and expression of different genes into a single crop (gene pyramiding) (Tabashnik 1994; Roush 1997). Crops containing stacked genes have activity against different pest spectrums (e.g. having one herbicide tolerant gene and one Bt gene). Pyramided gene expression deploys two or more genes that focus on a specific group of pests (e.g. two Cry proteins targeting lepidopterous insect pests). BollGard II® cotton (Monsanto Company, St. Louis, MO) was the first commercialized transgenic event to express two (or dual-gene) Cry proteins in cotton varieties to improve resistance management (Chitkowski et al. 2003). BollGard II® expresses Cry1Ac combined with Cry2Ab. A second dual-gene cotton product became available in 2005 with the release of Widestrike™ (Widestrike Insect Protection, Dow Agrosciences, LLC, Indianapolis, IN) cotton varieties that express Cry1Ac and Cry1F. TwinLink™ (Bayer CropScience, Research Triangle Park, NC) cotton is the most recent Bt cotton variety on the market and contains Cry1Ab and Cry2Ae.

Bt corn varieties are available in many more trait combinations. There is a multitude of corn varieties that have either stacked, pyramided, or a combination of stacked and pyramided traits. The most efficacious trait-packages against *H. zea* in corn consist of Syngenta's Agrisure® Viptera™ (Syngenta Crop Protection, Inc., Greensboro,

NC) that express Cry1Ab and the novel Vip3A (vegetative insecticidal protein); Monsanto's Genuity™ Yieldgard® VT Pro™ and Triple Pro® are pyramids with Cry1A.105 and Cr2Ab; and SmartStax™ field corn that has a combination of multiple pyramided and stacked genes for herbicide and insect tolerance (including Cry1A.105, Cry2Ab2, and Cry1F) (Que et al. 2010).

There is a clear effort to enhance the efficacy of future transgenic varieties against *H. zea* through introducing new Cry and or Vip proteins (Lee et al. 2003). Vip proteins are secreted during the vegetative stages of Bt and have increased activity against a variety of lepidopteran and coleopteran pests (Estruch et al. 1996; Yu et al. 1997). Exploration of incorporating novel proteins such as Vip3A has developed as a result of resistance via laboratory selection and (seldom documented) field-evolved resistance to current commercial varieties (Ferre and Van Rie 2002). The possibility of resistance in *H. zea* to Bt crops has generated volumes of research attempting to determine resistance mechanisms, delay the onset of resistance, and properly manage current and future Bt traits.

***Bacillus thuringiensis* var. *kurstaki* Mode of Action**

Having a basic comprehension of the insect midgut is integral to understanding the mode of action of Bt proteins. Once plant material is ingested, it is broken down further by the foregut. The foregut also serves as a sieve to prevent larger particles from passing into the midgut where primary digestion occurs (Chapman 1998). The midgut is lined with a physical barrier known as the peritrophic membrane that protects the midgut epithelium cells. The peritrophic membrane differentiates the lumen into two compartments: the ectoperitrophic space that maintains the food bolus in the middle of

the gut, and the endoperitrophic space that lies between the peritrophic membrane and the cell membrane of the midgut (Lehane and Billingsley 1996). Enzymes begin breaking down food while in the ectoperitrophic space. Digested molecules are allowed to pass through the peritrophic matrix and be absorbed by microvilli on the epithelial cells of the midgut. Highly polyphagous insects such as *H. zea* require a midgut with extremely high pH values (a range normally 10 to 11) in order to digest plant material and detoxify volatile compounds produced through host-plant defenses (Dow 1992).

Plant tissue expressing Bt insecticidal proteins passes through to the midgut. The high pH of the insect midgut is responsible for proteolysis and activation of the protoxin (Whalon and Wingerd 2003). The toxin is activated when midgut proteases solubilize the protoxin in the insect midgut by cleaving both ends of the protein (Schuler 1998). The activated toxins are small enough to pass through the peritrophic matrix and bind to receptors on the membrane of the midgut epithelial cells. Toxins aggregate at the binding site and insert the activated protein into the membrane cells that causes a pore to form (Gringorten 2001). Pore formation causes two chemical imbalances in the larvae that lead to mortality of the insect. First, the pore allows for midgut contents to diffuse into the hemocoel causing the hemolymph pH to increase. Next, leakage of the midgut contents decreases the pH of the midgut (Whalon and Wingerd 2003). The primary purpose of the insecticidal pathway caused by the Cry proteins is not to cause insect mortality but rather to create an environment conducive for ensuring survivorship of the bacteria. Insect mortality is a result of Bt proteins circumventing insect host defenses to allow sporulation of the bacteria, even though spores are not expressed or present. Consequently, most insects die of starvation or septicemia rather than from a direct poisoning of the bacteria.

Resistance to *Bacillus thuringiensis* var. *kurstaki*

A major factor threatening the longevity of Bt crops is the development of resistance (Roush 1997). The Environmental Protection Agency (EPA) has taken a proactive stance on detection and prevention of resistance to Bt crops in order to preserve the benefits of this technology. The EPA requires all companies marketing Bt crops to conduct a resistance monitoring program. The most popular method thus far has been to subject progeny from field collections to a discriminatory dose with diet-incorporated bioassays (Bates et al. 2005).The primary insect pests infesting Bt crops have been monitored for evolving resistance evolution. This is notably so for *H. zea* due its inherent tolerance to Cry proteins that were first used in commercial Bt corn and cotton. Resistance is defined as "genetically heritable changes in a population resulting in a reduction in the susceptibility to a specific insecticide" (National Research Council 1986). This definition is well suited for resistance studies with conventional insecticides; however, it is not entirely accurate for studying Bt resistance in *H. zea*. Confirmation of resistance to conventional insecticides from field collections has traditionally been much easier to ascertain. Resistance to conventional insecticides involves repeated exposure to an insecticide as dosage increases with declining efficacy. Bt crops provide continuous exposure; however, expression of Bt proteins has a maximum boundary (Sumerford et al. 2013). Cry protein expression varies throughout the growing season but it is generally affected the most by environmental conditions and varietal background (Adamczyk et al. 2001). Resistance to Bt has been most often reported in two forms: 1) specimens are collected from a Bt crop and resistance is confirmed through bioassays on progeny and 2) laboratory documentation of differences in performance of field-collected individuals

(Sumerford et al. 2013). In either circumstance, it is unclear whether these measurements are adequately capturing the genetic influence of Bt traits on feral populations. For example, it is difficult to determine whether a field collection is resistant via bioassays or rather that the population is able to survive on a Bt host due to lower protein expression.

The mechanisms responsible for resistance to Bt have been grouped into three categories: 1) reduction in the number of binding sites or reduction in binding affinity 2) alteration of proteolytic processing and 3) regeneration of epithelial cells to prevent gut paralysis (Whalon and Wingerd 2003; Ferre and Van Rie 2002). Field evolved resistance has been confirmed in several insect species; the causes are still unclear but lack of compliance with regard to refuge requirements seems to be a common factor (Huang et al. 2011). Diamondback moth, *Plutella xylostella* (L.), developed resistance initially to Bt foliar sprays in the US (Shelton 1993; Gassmann et al. 2011). Fall armyworm, *Spodoptera frugiperda* (J.E. Smith), resistance to Cry1F in field corn has now been confirmed in Puerto Rico (Storer et al. 2010, 2012). Resistance to Cry1Ac has been established in the pink bollworm, *Pectinophora gossypiella* (Saunders), in isolated areas of India (Dhurua and Gujar 2011). The African stem borer, *Busseola fusca* (Fuller), has developed resistance to Cry1Ab in South Africa (Van Rensburg 2007). Laboratory selection constitutes the majority of resistance documentation in *H. zea*. Tabashnik et al. (2008) claimed that resistance had evolved to Cry1Ac based on conclusions drawn from work performed by Luttrell et al. (1999 and Ali et al. 2006) that described considerable variation in susceptibility. However, the findings of Tabashnik et al. (2008) have not been unanimously accepted in the scientific community and have been overall controversial. Therefore, it should be noted that elevated LC_{50} data obtained from field collections of *H.*

zea does not necessarily indicate a shift in the susceptibility of the population as a whole (Tabashnik et al. 2008; Sumerford et al. 2013). Initial estimates of baseline susceptibility of *H. zea* to Bt varied drastically (ca. 200 fold) (Luttrell et al. 1999); making detection of an actual shift in susceptibility difficult to observe.

Alleles responsible for resistance must be present in the initial population when the insecticide is first introduced; what is known as a pre-adaptive phenomenon (W.H.O. 1957). From that point on, selection pressure is placed on individuals with resistant genes and those genes must be inherited by successive generations. Alleles associated with resistance to Bt are believed to be inherited recessively and, therefore; extremely rare in natural populations (Bird and Akhurst 2006).

The EPA has mandated a high-dose refuge strategy to prevent or, at least, delay resistance in *H. zea* to Bt corn and cotton. This strategy is predicated on three assumptions: 1) resistance allele frequency is very low (<0.0001) 2) mating between resistant and susceptible individuals is random and 3) resistance alleles are recessively inherited (Carriére and Tabashnik 2001; Caprio and Sumerford 2007). A high dose is defined as a dose 25 times the concentration needed to kill 99% of susceptible larvae of the same age (US EPA-SAP 1998). In theory, a high-dose effectively kills 95% heterozygous individuals while the refuge provides homozygous susceptible individuals to mate with resistant individuals (Gould 1998); thus, maintaining low frequencies of resistant alleles in the natural population. A parameter not evaluated in the refuge strategy is the fitness cost associated with resistance alleles. Alleles conferring resistance that have a negative effect on the fitness of the insect can help thwart the evolution of resistance (Tabashnik and Carriére 2007). Resistant insects typically have higher

mortality, slower development, and/or produce fewer viable offspring compared to susceptible individuals in the absence of exposure to Bt (Gassmann et al. 2009; Huang et al. 2011).

Until 2008, Bt cotton had refuge requirements mandating any or one of the following: 5% allotment of unsprayed non-Bt acreage, 20% allotment of sprayed non-Bt acreage, or a 5% allotment of embedded non-Bt acreage. Structured cotton refuges have since been abolished because they do not provide sufficient numbers of *H. zea* to influence resistance and deployment of dual-gene corn and cotton reduces the number of susceptible moths needed for IRM (Tabashnik et al. 2003; Jackson et al. 2007). Structured refuges remain in place for Bt corn. Single-gene Bt corn varieties targeting lepidopteran insects in cotton growing regions have a non-Bt refuge requirement of 50% compared to pyramided Bt corn varieties that require a 20% non-Bt refuge (Que et al. 2010). Pyramided Bt corn varieties allow for a lower refuge percentage of 20% because resistance to multiple Cry proteins is extremely rare and, thus far, not been documented in the field (Sayyed et al. 2000). Also, expression of multiple insecticidal proteins in one plant has proven to delay resistance evolution more efficiently than single-gene Bt plants with larger refuge requirements (Zhao et al. 2003; Bates et al. 2005).

The Cry proteins that have been used in Bt crops display a great degree of structural resemblance and, therefore, may share similar functionality (de Maagd et al. 2001).Van Rie et al. (1989) developed the basic model for binding sites of Cry proteins. In effect, Cry1Aa binds only to receptor A, Cry1Ab can bind to both receptor A and B, and Cry1Ac can bind to receptors A, B, and C. Currently, the same Cry proteins are commercially available in both corn and cotton. Field corn is the largest contributor of *H.*

zea to the landscape in the southern United States during late spring to early summer before populations transition to cotton (Head et al. 2010). Season-long expression of the same or similar Cry proteins may be placing a tremendous amount of selection pressure on *H. zea*. For example, Cry1Ac provides a high dose for the first generation of *Heliothis armigera* in India; consequently, it qualifies as a low dose for the second generation (Bird and Akhurst 2004). This observation was made in isolated regions of India where Bt cotton was continuously grown with little to no refuge requirement, so direct correlations do not apply to Bt cropping systems in the United States. However, certain inferences can be made considering several Cry proteins do not constitute a high dose for *H. zea* (Jackson et al. 2003; Huang et al. 2011). The most commonly observed Bt resistance mechanism is an altered binding site on midgut epithelial cells when exposed to a high dose (Lee et al. 1995, Huang 2011; Sumerford et al. 2013). Exposure to moderate to low doses of Bt has a greater likelihood of conferring resistance via multiple resistance mechanisms (solubilization of protoxins, protease processing, passage of the toxin through the peritrophic membrane, site-specific binding, insertion of toxin into cells, formation of pores, and lysis of the midgut cells) (Heckel 1994; Sumerford et al. 2013). Such mechanisms would evolve gradually, lending to more subtle changes in susceptibility that may not be immediately apparent. The research presented herein was conducted to ascertain the impact of dual-gene Bt corn on *H. zea* at the landscape level. The results are not intended to directly illustrate resistance, but to help explain the temporal and spatial randomness of *H. zea* development on current Bt cotton cultivars.

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CHAPTER II

CONTRIBUTION OF *Helicoverpa zea* FROM GENUITY VT3 PRO CORN TO THE LANDSCAPE

Abstract

Genuity VT3 PRO field corn expresses Cry1A.105 and Cry2Ab and is considered to have good activity against corn earworm, *Helicoverpa zea* (Boddie). Twelve non-Bt and twenty VT3 PRO corn fields were surveyed across Mississippi during 2012 and 2013 to compare corn earworm populations among these hybrids across the landscape. *H. zea* larvae were collected from these fields to evaluate fitness costs associated with development on a transgenic host. Each year, populations of *H. zea* were observed in VT3 PRO fields, but non-Bt fields supported higher larval densities. Larvae also developed faster on non-Bt fields compared to VT3 PRO. Larval survivorship and development increased in VT3 PRO corn as ears matured. Pupal weight was higher and pupal duration was longer for larvae collected from VT3 PRO corn fields compared to larvae collected from non-Bt fields. These data will be important for estimating the impact of dual-gene Bt corn hybrids on *H. zea* resistance to these toxins.

Introduction

The corn earworm, *Helicoverpa zea* (Boddie), is a primary pest of cotton, *Gossypium hirsutum* (L.), grain sorghum, *Sorghum bicolor* (L.), and soybean, *Glycine*

max (L.), in the MidSouth region of the United States. A major factor contributing to the abundance of this insect is the influence of field corn, *Zea mays* (L.), on *H. zea* populations. A two year study performed by Jackson et al. (2007) found field corn to be the most significant producer of *H. zea* in the MidSouth and Southeastern United States. Head et al. (2010) ascertained the percentage of *H. zea* adults emerging from C_3 and C_4 plants in the cotton growing regions of the US by comparing C^{13}/C^{12} isotopic ratios. Additionally, they analyzed gossypol residues to separate individuals that had developed on cotton from other C_3 plants. The composition varied depending on state, however, at no period did the percentage of moths emerging from corn fall below 25%. Consequently, the composition of moths that developed on cotton peaked at 19% for the entire growing season. The study also found that the majority of adult moth traps surrounding cotton fields captured individuals that did not develop on cotton. Those data suggest that field corn is largely responsible for the generations infesting cotton in late summer. The implications of these studies led to the elimination of structured cotton refuges because the practice had a negligible influence on insect resistance management in *H. zea* (Head et al. 2010).

The first corn and cotton products expressing the crystalline (Cry) insecticidal proteins of *Bacillus thuringiensis* (Bt) were commercially introduced to help control the European corn borer, *Ostrinia nubilalis* (Hübner), and the southwestern corn borer, *Diatraea grandiosella* (Dyar), pink bollworm, *Pectinophora gossypiella* (Saunders), and tobacco budworm, *Heliothis virescens* (F.) (Stewart et al. 2001; Baldwin et al. 2005). First generation Bt corn hybrids expressed a single Cry protein and had limited activity against *H. zea* (Gould 1998; Siegfried et al. 2000). Since that time, commercial seed

companies have introduced hybrids expressing multiple insecticidal proteins targeting lepidopteran insects (gene pyramiding). These dual-gene products have served an integral function in delaying resistance evolution (Roush 1997; Zhao et al. 2003). Studies have demonstrated considerable variation in *H. zea* susceptibility to Cry proteins (Luttrell et al. 1999; Siegfried et al. 2000; Greenplate et al. 2003). Numerous studies have also documented fitness costs associated with exposure to a sublethal dose of Bt including: reduced fecundity, decreased larval and pupal weight, and delayed development (MacIntosh et al. 1990; Sims et al. 1996; Williams et al. 1998). Subsequent resistance management studies have focused on the influence of Bt corn on the susceptibility and ecology of *H. zea* (Sims et al. 1996). Fortunately, non-crop hosts and fitness costs have played an essential role in delaying *H. zea* resistance to Bt in the US (Tabashnik et al. 2003).

The Environmental Protection Agency (EPA) mandates that the cotton-growing regions of the US adhere to a 20% non-Bt refuge for pyramided corn varieties. Corn hybrids expressing a single Bt protein have a 50% structured non-Bt refuge requirement (Que et al. 2010). *H. zea* survival in Bt corn does not imply resistance because there are many factors that must be evaluated before confirmation. This is generally confirmed through dose-mortality bioassays in the laboratory. However, monitoring relative fitness of larvae collected from a Bt crop could detect changes in susceptibility that occur more subtly over time (Sumerford et al. 2013). Also, resistance monitoring studies that detect the impact of Bt proteins on various fitness parameters have the potential to serve as a diagnostic warning before resistance becomes widespread. The studies presented herein evaluate the contribution of *H. zea* developing on Genuity™ Yieldgard® VT Triple Pro®

(VT3 PRO) corn (Monsanto Company, St. Louis, MO) to the overall landscape population. VT3 PRO corn hybrids express Cry1A.105 and Cry2Ab2 and are considered one of the more efficacious Bt corn products against lepidopteran pests (Que et al. 2010). Cry1A.105 is a chimeric protein consisting of Cry1Ac and Cry1F (Hernández-Rodríguez et al. 2013). The impact on insect fitness from larval development on VT3 PRO was compared to that of larvae collected from non-Bt field corn.

Materials and Methods

Field Locations

Twenty VT3 PRO field corn fields were sampled in the 2012 and 2013 growing seasons to determine *H. zea* larval density across Mississippi. Paired non-Bt and VT3 PRO corn genotypes were planted on six planting dates at both the R.R. Foil Plant Science Research Farm in Starkville, MS and Delta Research and Extension Center in Stoneville, MS. The remaining non-Bt and VT3 PRO fields were located on commercial farms located in Humphreys and Sunflower County, Mississippi. Commercial VT3 PRO fields were chosen at random but were located near (within at least 0.8km) non-Bt fields and having similar planting dates. Standard agronomic practices for managing fertilization, weeds, and pathogens were implemented. All fields were furrow irrigated on 0.97m row width and a target plant population of 79,040 plants per hectare.

Ear Sampling

Fields were sampled at R1-R2 (silk emergence to blister), R3-R4 (milk to soft dough stage), and R6 (dent). Data for each sampled corn genotype were pooled each week to track development of *H. zea* populations across the landscape. During the first

two sampling dates, larval density was determined by destructively sampling one hundred ears for each corn genotype. Silks protruding from each ear were examined for damage and/or larvae. Ears were recorded as damaged if any visible injury to silk tissue or kernels consistent with Lepidoptera feeding was observed. Husks were then removed to expose kernels and the number of larvae present was recorded. Larvae were categorized as small (<0.635 cm), or large larvae (>0.635 cm). During the R6 sampling date, the total number of damaged kernels per 100 ears was recorded.

Insect Fitness

To evaluate fitness costs associated with survival on VT3 PRO corn, approximately 600 *H. zea* larvae were collected from fields of VT3 PRO and non-Bt corn with similar genetic backgrounds (300 from each corn genotype). Larvae were collected from the locations used to determine larval density in VT3 PRO and non-Bt field corn on research and commercial farms. Third instar larvae or larger were collected and placed in 36 mL Solo® cups containing a soy-protein, wheat-germ based artificial diet (FMC Biopolymer® Philadelphia, PA) with matching lids. Diet cups were kept in a rearing facility maintained at 25°C, 80% relative humidity, and a photoperiod of 16:8 (L:D). Larvae were monitored daily until pupation. Pupae were segregated by gender based on the presence of a ventral v-shaped suture on females or two rectal pads on males (Ditman and Cory 1931). Pupae were weighed and placed in empty Solo® cups with the sex and weight labeled on each lid. Pupae were monitored daily for adult eclosion to quantify pupal duration.

Data Analysis

Data collected on *H. zea* densities infesting VT3 PRO and non-Bt field corn were combined across all locations for analysis. Analysis of damaged kernel ratings and larval-size data excluded locations with larval densities <5% larvae infesting ears in the paired non-Bt corn fields. All data were analyzed with analysis of variance (PROC MIXED SAS Institute 2012). Year, location, larval size, corn genotype, and sample week were considered fixed effects to evaluate differences in larval development. Location by week nested in year was considered random. Pupal sex and corn genotype were considered fixed effects for analysis of fitness parameters. Analysis of variance (ANOVA) (SAS Institute 2012) was used to evaluate differences in field and laboratory measurements between non-Bt and VT3 PRO corn. Means were separated by using significant *F* test at the $P \leq 0.05$ level of significance.

Results and Discussion

Ear sampling

Before initiation of these experiments, *H. zea* infestations in VT3 PRO field corn were considered isolated events. The results presented here indicate these dual-gene varieties could possibly support high densities of *H. zea* across a broad area. *H. zea* infesting VT3 PRO corn was common throughout both years of this study. Essentially no larvae were observed infesting non-Bt or VT3 PRO in commercial fields because these sites were past the susceptible stage when *H. zea* populations began ovipositing in ear-stage corn. However, *H. zea* infested nearly all planting dates located on research farms. Initial analysis of total larval densities indicated there was no year by genotype by sample

week interaction (F=0.55; df=1, 104; P=0.76); therefore, sample data were pooled across years.

There was no corn genotype by sample week interaction (F=1.03; df=1, 104; P=0.42). The main effects of sample week (F=8.18; df=1, 104; P=<0.01) and genotype (F=11.56; df=1, 104; P<0.01) were significant for larval density (Fig. 2.1 and 2.2, respectively). Generally, *H. zea* density in field corn was low during the first two sample weeks for both years. Larval density gradually increased over the course of the sample period; suggesting migration of *H. zea* adults that contributed to overlapping generations. Larval density was lower in VT3 PRO corn fields when averaged across years (Fig. 2.2). Similarly to previous reports (Storer et al. 2001; Horner et al. 2003b), overall larval reduction by 61% across all VT3 PRO fields is considered to be a moderate dose. Because populations were observed over an eight week period, it appeared that multiple generations developed on Bt corn before all corn matured and populations may have dispersed to other plant hosts across the landscape. According to simulation models, selection for resistant individuals is heavily driven by the adoption rate of Bt crops across a region (Storer et al. 2003). Local populations occupying high Bt-adoption areas experience intense selection pressure compared to migrant populations developing on a mix of non-Bt and Bt host plants. Depending on the degree of isolation, localized populations developing on a Bt crop can drastically increase the frequency of resistance alleles within a population (Storer et al. 2003). Consequently, these resident populations may have higher survivorship on Bt cotton after completing several generations on Bt corn. *H. zea* surviving in Bt cotton is sporadic across the majority of the MidSouth with approximately 25% of the Bt cotton acreage treated with foliar insecticides for *H. zea*

annually (Williams 2013). Survivorship in Bt cotton is influenced by many factors, but the variation in *H. zea* infestation levels within and among years in Bt cotton can perhaps be explained by the intensity from which *H. zea* populations are selected in Bt corn.

Tang et al. (2001) recommended that non-Bt refuges should produce approximately 500 susceptible adults to mate with every resistant adult emerging from a Bt crop to maintain the frequency of resistance alleles in a population. Very large refuges would be needed when this ratio of susceptible to resistant adults is not achieved, especially when the target insect like *H. zea* is inherently tolerant to Bt (Gould et al. 2002). Also, because Bt corn does not provide a high dose for *H. zea*, it can be assumed that the proportion of heterozygous resistant individuals will be greater. Given the density of *H. zea* infesting Bt corn in 2012 and 2013, non-Bt corn refuges cannot feasibly produce enough susceptible individuals to prevent assortative mating. Natural refuges have helped meet this demand (Tabashnik et al. 2003; Jackson et al. 2007).

Although total larval density provides an indication of *H. zea* production in both non-Bt and Bt field corn, differences in larval size should provide a better indication of the relative production of each corn genotype over time. There was a significant corn genotype by corn growth stage by larval size interaction for numbers of larvae (F=7.91; df=1, 167; P<0.01) (Fig 2.3). Non-Bt corn fields had significantly more larvae compared to VT3 PRO at the R1-R2 growth stage and the majority of that population consisted of small larvae. The numbers of small larvae decreased by 62% in non-Bt fields at R3 while the number of large larvae increased by 61%. The numbers of small larvae infesting VT3 PRO increased by 3% from the R1 to R3 growth stage while the number of large larvae increased by 78%. Larval development is obviously being delayed due to feeding (or lack

thereof) on Bt corn. Also, larval survivorship on VT3 PRO corn remains nearly constant as ears develop; suggesting that a number of interactions may be at influencing *H. zea* survival and development on VT3 PRO corn. First, the increase of small larvae at R3-R4 ear stage suggests that VT3 PRO corn maintains a higher degree of suitability for oviposition. The quality of silks and ear tips greatly diminished due to larvae feeding on non-Bt ears after R1-R2 whereas the quality of VT3 PRO ears was noticeably less compromised. Second, protein expression (especially in silks) may decrease at later ear stages allowing larvae to survive and/or develop more rapidly. And last, there is a significant chance of cannibalism influencing *H. zea* larval density on either corn genotype; but, it may allow larvae infesting VT3 PRO corn to develop into more tolerant instars before ingesting any plant material expressing Bt proteins.

There was a significant year by genotype interaction for the number of damaged kernels in non-Bt and VT3 PRO corn ears (F=111.36; df-1, 3582; P<0.01). Therefore, the number of damaged kernels was analyzed by year between the two genotypes (2012 F=442.66; df=1, 1393; P<0.01, 2013 F=433.79; df=1, 2187; P<0.01). In 2012, the average number of damaged kernels was reduced from 15.82 kernels per ear in non-Bt corn to 5.77 kernels per ear in VT3 PRO corn (Fig. 2.4). In 2013, the number of damaged kernels averaged 3.42 kernels per ear in VT3 PRO corn compared to 7.58 per ear in non-Bt corn.

Insect Fitness

There was a significant year by genotype by sex interaction (F=3.39; df=2,1404; P=0.03) for pupal duration. Therefore, pupal duration was analyzed by year. There was no sex by corn genotype interaction (F=3.75; df=1,271; P=0.06) (F=3.0; df=1,172;

P=0.09) (*F*=0.02; df=1,961; *P*=0.88) for 2011, 2012, or 2013, respectively; indicating males and females responded similarly to corn genotype with regard to pupal duration. There were significant differences in pupal duration between males and females (*F*=29.4; df=1,272; *P*<0.01) in 2011 (Fig. 2.5A). There was also a significant difference in pupal duration between larvae collected from VT3 PRO and non-Bt corn (*F*=8.87; df=1,272; *P*<0.01) in 2011 (Fig. 2.5B). In 2012, there were significant differences in pupal duration between males and females (*F*=4.01; df=1,172; *P*=0.05) and corn genotype (*F*=13.31; df=1,172; *P*<0.01). Significant differences were observed in 2013 between pupal sex (*F*=270.84; df=1,961; *P*<0.01) and corn genotype (*F*=6.97; df=1, 961; *P*<0.01). Male pupae took longer to emerge all three years. Pupae of larvae collected from VT3 PRO took longer to pupate in 2011 and 2012; however, moths eclosed faster in 2013 compared to larvae collected from non-Bt corn.

There was no year by genotype by sex interaction for pupal weight (*F*=0.3; df=2, 4255; *P*=0.74). There was no sex by genotype interaction (*F*=0.73; df= 1, 4255; *P*=0.39) or sex by year interaction (*F*=2.88; df=2, 4255; *P*=0.06). However, there was a corn genotype by year interaction (*F*=24.18; df=2. 4255; *P*<0.01) (Fig. 2.6). There were no significant differences in pupal weights in 2011 and 2012. Pupae of larvae collected from VT3 PRO weighed significantly more than pupae collected from non-Bt corn in 2013; suggesting these larvae may have been more tolerant on VT3 PRO corn (or consequently other Bt crop hosts). Environmental conditions may have negatively influenced protein expression allowing more larvae to survive in VT3 PRO corn. However, this observation contradicts many studies (Siegfried et al. 2000; Storer et al. 2001; Horner et al. 2003a) on the side effects of sub-lethal doses of Bt. There was no difference in pupal weights of

larvae collected from non-Bt and VT3 PRO corn in 2012. Typically, some disadvantage on insect fitness is incurred by having the ability to develop on a transgenic crop. These fitness costs select against resistant individuals in the absence of Bt through several means, most often (but not always) being decreased survival (Gassmann et al. 2009). Larvae having larger body size were apparently selected for during the 2011 and 2013 growing season. There is a general association between larger body size and increased fecundity, but this correlation is weakly corroborated and difficult to estimate in insects (Honěk 1993).

As mentioned above, cannibalism can play a role in the survival and fitness of *H. zea* when one ear supports multiple larvae (Horner et al. 2003a). Horner and Dively (2003) determined *H. zea* larvae are less cannibalistic when reared on Bt corn leaf tissue incorporated into diet. Also, nutritional gain is negligible for cannibalistic larvae (Joyner and Gould 1985; Horner and Dively 2003). Chapman et al. (1999) reported cannibalistic fall armyworm, *Spodoptera frugiperda* (Smith), had lower pupal weight compared to non-cannibalistic larvae. Therefore, cannibalism appears to have no health benefit but serves a greater advantage with ensuring survival especially when numerous larvae inhabit a single ear as was observed for several locations in both 2012 and 2013.

Development was longer for larvae infesting Bt ears (Fig. 2.4). On average, larvae infesting non-Bt ears were entering the pre-pupal stage whereas larvae feeding on Bt ears were in the third instar or smaller during the R3-R4 sampling date. Upon fertilization of individual kernels, allele segregation results in a small percentage of kernels in each ear having no Bt expression (Horner et al. 2003b). Gore et al. (2005) determined *H. zea* larvae can detect artificial diet incorporated with Bt proteins. As a possible result, larval

duration may have been lengthened not only due to feeding on transgenic corn but also because of the time required to find kernels having little or no Bt expression. If larvae are able to develop on Bt corn varieties by feeding mostly on kernels expressing no toxin, selection may not be as high as previously theorized.

Bt expression had no effect on the pupal duration between males and females. Female pupae emerged approximately one day before males (Fig. 2.5); which is consistent with observations made by Hardwick (1965). Similar to observations made by Storer et al. (2001) and Horner and Dively (2003), pupal duration was longer for larvae collected from Bt corn. However, there is a drastic difference in the length of the duration between that study and the results presented here. Because *H. virescens* larvae have the ability to recover from intoxication (Dulmage et al. 1976), so it is likely that pupal duration was not as substantial here as in previous studies because our larvae completed development on clean diet as opposed to Bt corn. Larvae collected from non-Bt corn had significantly longer pupal duration than those collected from VT3 PRO corn in 2013. This again contradicts previous reports documenting delayed developmental time as a consequence of feeding on Bt plant tissue. There were no noticeable differences in adults reared on VT3 PRO and non-Bt corn that would indicate incomplete (or malformed) development of certain morphological features (e.g. wings, legs, primary body segments, etc.) that could be attributed to a hastened pupation period. Life history traits including adult life span and fecundity would certainly help explain the impact of a dual gene corn such as VT3 PRO on pupal duration. Unfortunately, this information is lacking in this dataset. Horner et al. (2003a) reported moths emerging from a single-gene Bt corn hybrid oviposited fewer eggs over a shorter time period compared to those emerging from non-

Bt corn. Attempts to obtain fecundity estimates, were inconclusive because of the low success rate of female *H. zea* to mate when paired individually with a male.

The impact of Bt corn on the susceptibility, ecology, and fitness on *H. zea* is a complex interaction of multiple factors. The ability of *H. zea* to complete multiple generations per growing season on dual-gene corn varieties like VT3 PRO without evolving resistance lends to this fact. A key component in the implementation of the refuge strategy is that mating is random among adults emerging from non-Bt and Bt plants. A delay in larval and pupal development can result in non-random mating with the possibility of increasing resistance allele frequency. The likelihood of assortative mating is higher in localized populations of *H. zea* without the genetic contribution of migratory adults. This could potentially lead resident populations of *H. zea* to have a higher propensity for causing damage in Bt cotton; but because the problem is not widespread, it is perhaps often overlooked. Future research should focus on the degree of selection occurring in individual Bt corn ears especially if larvae are feeding exclusively on kernels that have little or no expression.

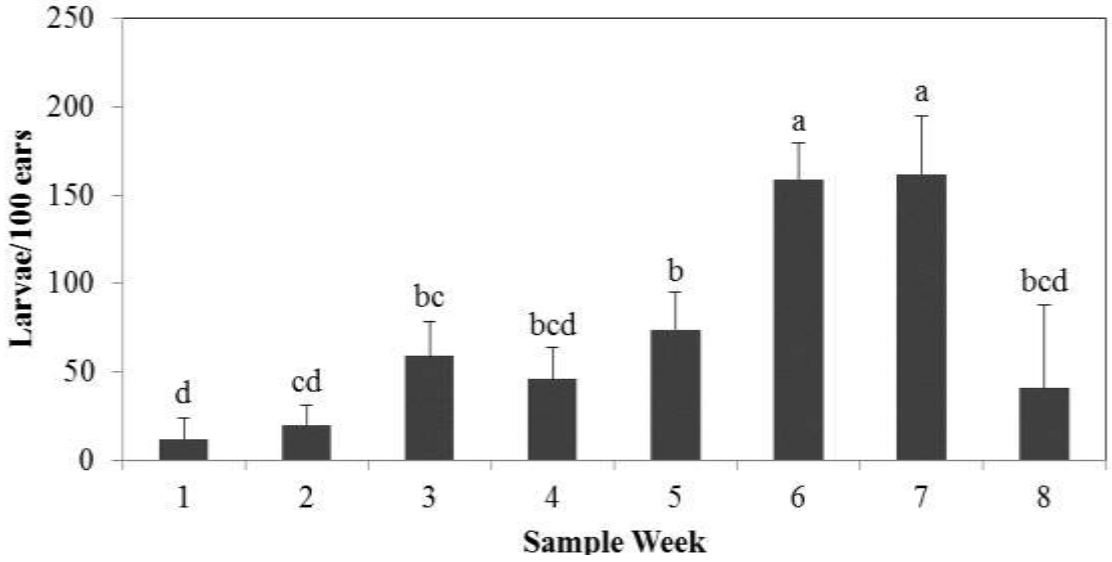

Figure 2.1 Mean (SEM) *H. zea* density infesting non-Bt and VT3 PRO corn ears across all locations.

Sampling initiated the first week of June and completed on the fourth week of July. Bars sharing the same letter groupings are not significantly different (P<0.05).

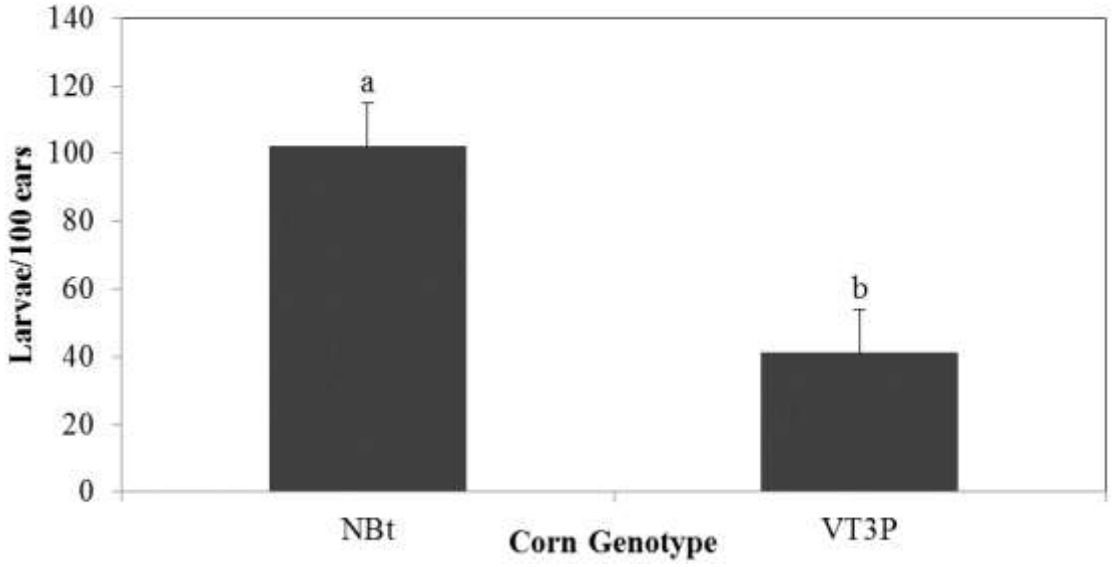

Figure 2.2 Mean (SEM) larval density of *H. zea* infesting non-Bt and VT3 PRO fields across growth stages and locations

Bars sharing the same letter grouping do not significantly differ (P<0.05).

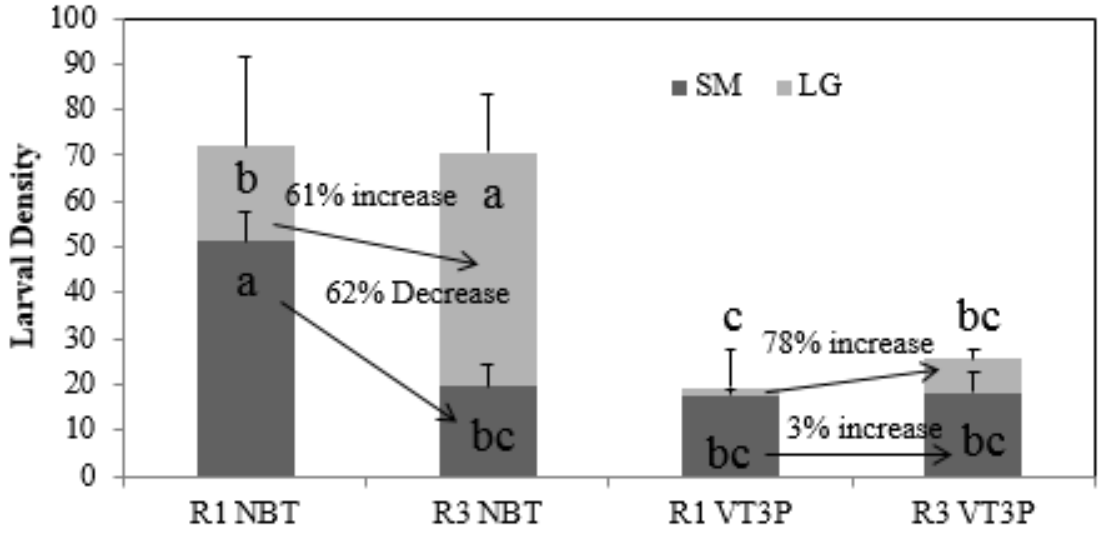

Figure 2.3 Differences in *H. zea* larval size infesting NBt and VT3 PRO corn fields at R1 and R3

Bars with the same letter grouping are not significantly different ($P<0.05$).

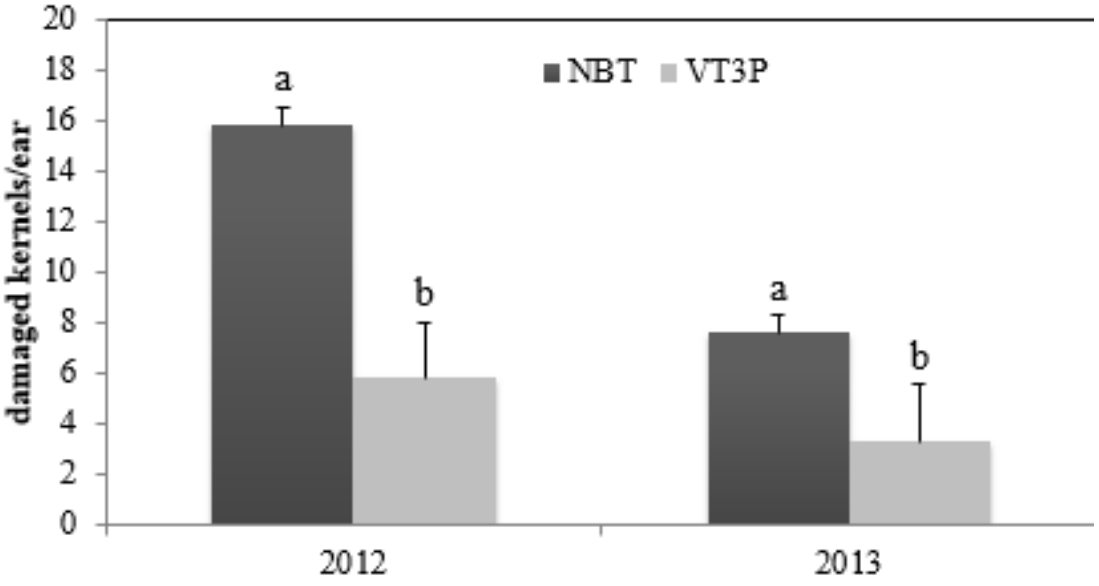

Figure 2.4 Mean (SEM) number of kernels damaged by *H. zea* larvae feeding on non-Bt and VT3 PRO corn

Damaged kernel counts were analyzed separately for each year. Bars within a year sharing the same letter grouping do not significantly differ ($P<0.05$).

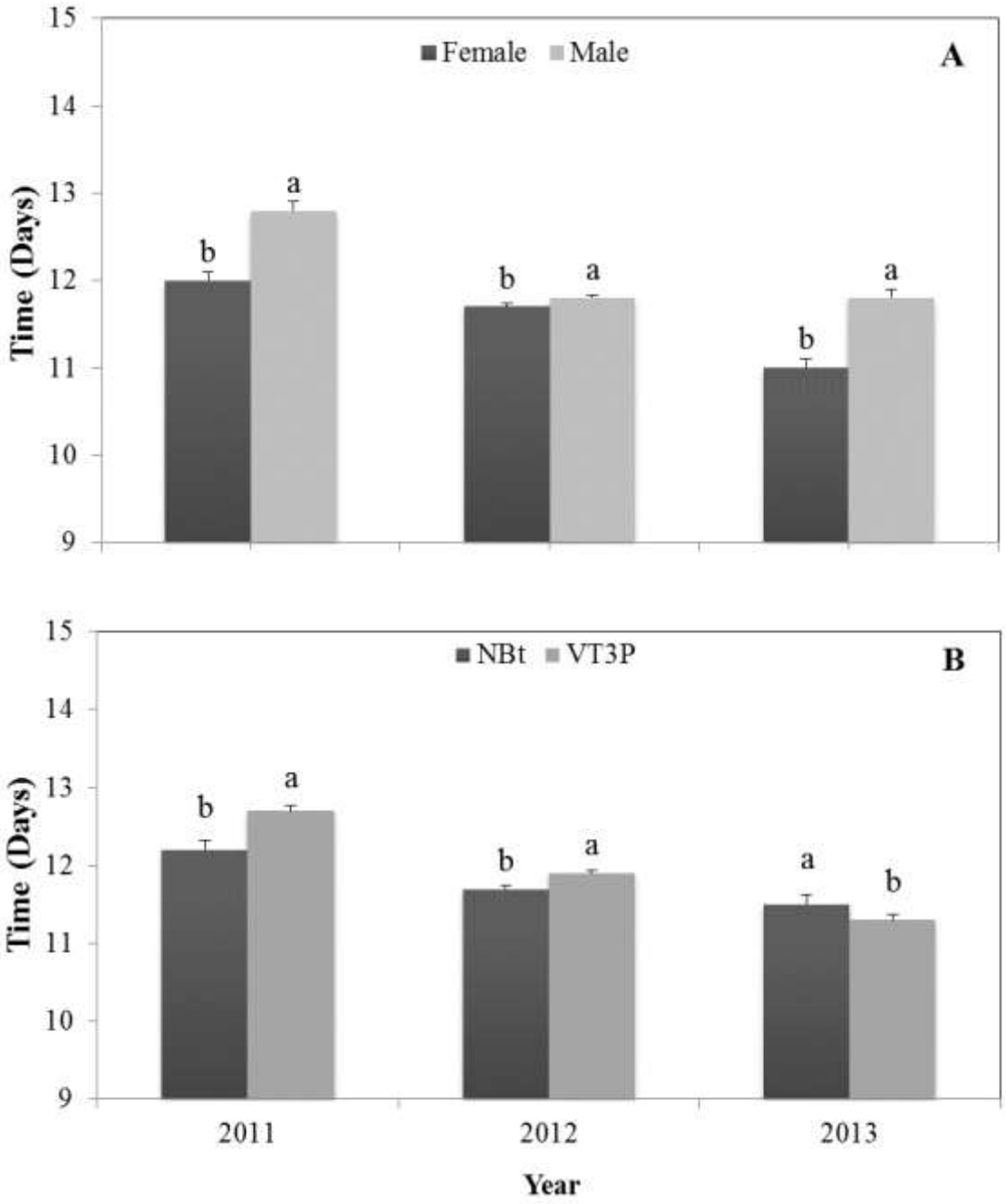

Figure 2.5 Mean (SEM) pupal duration (days) of male and female pupae and mean pupal duration (days) of pupae resulting from larvae collected from NBt and VT3 PRO corn

Pupal duration was analyzed separately by year. Bars within a year sharing the same letter grouping do not significantly differ ($P<0.05$).

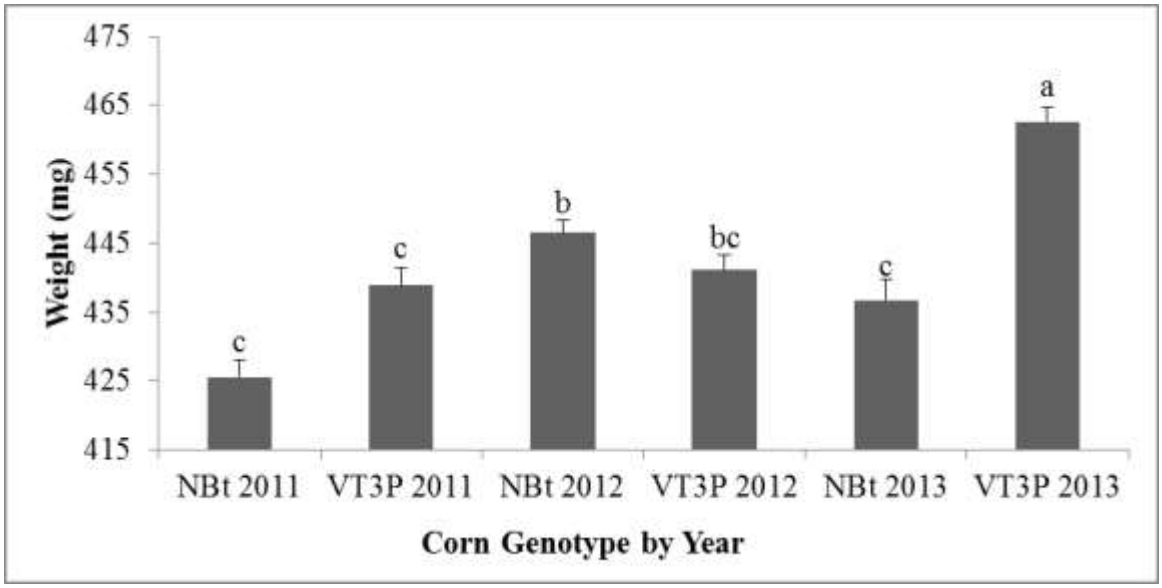

Figure 2.6 Mean (SEM) pupal weight (g) of larvae collected from NBt and VT3 PRO corn compared across years

Bars within a year sharing the same letter grouping do not significantly differ ($P<0.05$).

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CHAPTER III

INFLUENCE OF DUAL-GENE Bt CORN ON BOLLWORM, *Helicoverpa zea* (Boddie), SURVIVORSHIP ON BOLLGARD II COTTON

Abstract

Similar Cry proteins are expressed in both Bt corn, (*Zea mays* L.), and cotton, *Gossypium hirsutum* (L.), commercial production systems. At least one generation of corn earworm, *Helicoverpa zea* (Boddie), complete development on field corn in the MidSouth before dispersing across the landscape into other crop hosts like cotton. A concern is that Bt corn hybrids may select for *H. zea* populations with a higher probability of causing damage in Bt cotton. The objective of this study was to determine the susceptibility of *H. zea* offspring on lyophilized BollGard II cotton tissue that resulted from larvae reared on non-Bt and VT Triple Pro (VT3 PRO) field corn. Offspring from moths reared on VT3 PRO had a significantly higher LC_{50} two out of the three years this study was conducted. Excess larvae were placed on artificial diet and allowed to pupate to determine if there were any inheritable fitness costs associated with parental development on VT3 PRO corn. Offspring resulting from males collected from VT3 PRO had significantly lower pupal weight and longer pupal duration compared to offspring of individuals collected from non-Bt corn. However, offspring from females collected from VT3 PRO were not different from non-Bt offspring. Paternal influence on offspring in

insects is not commonly observed, but possibly illustrates the side effects of development on a transgenic plant expressing less than a high dose.

Introduction

The greatest threat to the longevity of transgenic crops expressing Bt crystalline (Cry) proteins is the widespread evolution of resistance (Roush 1997). Bt cotton has successfully managed a number of key lepidoptera pests including tobacco budworm, *Heliothis virescens* (F), and pink bollworm, *Pectinophora gossypiella* (Saunders). However, corn earworm, *Helicoverpa zea* (Boddie), is inherently more tolerant and larvae are often observed developing on Bt cotton (Mahaffey et al. 1995; Chitkowski et al. 2003). There have been no confirmed field-evolved cases of Bt resistance in *H. zea* to date (Huang et al. 2011). This is partly due to the implementation of the high-dose refuge strategy mandated by the U.S. Environmental Protection Agency (EPA) (Caprio and Sumerford 2007; Huang et al. 2011). Structured cotton refuges were eliminated in 2008 due to insufficient numbers of *H. zea* emerging from non-Bt cotton to have an impact on delaying resistance and because the need for susceptible moths is less when using dual-gene cotton varieties are perceived as a resistance management tool (Jackson et al. 2007). Structured refuges remain an integral part of resistance management in corn production especially in cotton producing regions of the U.S. In regions of the U.S. where Bt cotton is grown, corn varieties expressing a single gene have a refuge requirement of 50% of the total corn acreage. Pyramided corn varieties (expression of multiple insecticidal proteins targeting a specific group of pests (e.g. Lepidoptera) require a 20% non-Bt refuge (Que et al. 2010).

Currently, the same or similar Cry proteins are used in both Bt corn and cotton (Table 3.1). Van Rie et al. (1989) developed the basic model for binding sites of Cry proteins in the insect midgut. In theory, Cry1Aa binds to only receptor A. Cry1Ab binds to receptors A and B. Cry1Ac can bind to receptors A,B, and C. Cry proteins share structural similarities that may compromise the efficacy of one or more of these toxins. Fortunately, cross resistance in *H. zea* to Bt has only been observed in isolated studies and at low levels (Burd et al. 2003). However, deployment of similar Cry proteins in two crop hosts for *H. zea* increases this risk; especially when multiple generations develop on Bt corn before transitioning into cotton. Interestingly, basic physiology of a corn plant may be contributing to poorly understood events in resistance evolution. Allele segregation of individual corn kernels may result in an array of variability in Cry protein expression (Horner et al. 2003). Kernels may (and often do) express less than what is considered a high-dose. In pyramided varieties, allele segregation can also cause kernels to express one, both, or no toxin at all (Horner et al. 2003). Storer et al. (2001) determined that larvae have the ability to detect Bt expressing kernels and feed on kernels expressing little or no toxin until developing into less susceptible instars.

Table 3.1 Commercialized dual-gene Bt corn and cotton cultivars with activity against *H. zea*..

Crop/Cultivar	**Lepidoptera Active Traits**	**Refuge Requirement**
Cotton		
BollGard II	Cry1Ac + Cry2Ab	NA
Widestrike	Cry1Ac + Cry1F	NA
Twin Link	Cry1Ab + Cry2Ae	NA
Corn		
VT Double and VT Triple Pro	Cry1A.105* + Cry2Ab	20%
SmartStax	Cry1A.105 + Cry2Ab + Cry1F	20%
Agrisure Viptera	Cry1Ab + Vip3A	20%

Note: similar Cry proteins are expressed in both cropping systems
*Cry1A.105 is a chimeric protein structurally similar to both Cry1Ac and Cry1F.

Current insect resistance management (IRM) strategies for Bt crops involve the use of a high dose refuge strategy. For this strategy to be successful, three assumptions must be met: 1) alleles associated with resistance must be recessively inherited 2) resistance alleles must be rare and 3) mating among resistant and susceptible individuals must be random (Carriére and Tabashnik 2001). Unfortunately, criteria for one of these assumptions could possibly be in danger of being violated. Because survival on a sublethal dose delays developmental time (Horner and Dively 2003), assortative mating may be taking place at a higher frequency than expected (Liu et al. 2001) and thereby, increasing the number of homozygous individuals carrying resistance alleles. Additionally, this strategy also assumes mortality of heterozygous individuals is high on pyramided corn varieties that are most assuredly not true for insect pests like *H. zea* (Bates et al. 2005). Resistant allele frequency in *H. zea* is believed to be <0.001 (Carriére and Tabashnik 2001) and major genes conferring resistance have not been discovered in this species (Bates et al. 2005; Sumerford et al. 2013). This would suggest that multiple

alleles each having a minor effect will be involved in resistance evolution and as such, changes in susceptibility will occur gradually over time (Caprio and Sumerford 2007). Ironically, most resistance monitoring programs utilize methods aimed at capturing the increase of a single resistance allele. There are variations in technique but the most generally used method is to subject larvae to a diagnostic dose incorporated into artificial diet (Bates et al. 2005). A discriminatory dose allows for numerous individuals to be evaluated, but has limitations in the ability to detect resistance alleles that are minor, extremely rare, or recessive (Hawthorne et al. 2002). With that in mind, the objective of this study was to evaluate the influence feeding on Genuity™ Yieldgard® VT Triple Pro® (VT3 PRO) corn (Monsanto Company, St. Louis, MO) on *H. zea* susceptibility to lyophilized BollGard II (Monsanto Company, St. Louis, MO) cotton leaf tissue at a range of concentrations during the following generation. The goal of this research was to detect subtle changes in susceptibility that are associated with an inherent ability to develop on a transgenic host.

Materials and Methods

Leaf Tissue Collection

During the 2011 growing season, cotton leaf-tissue samples were collected from Delta & Pine Land® 0924B2RF (Monsanto Company, St. Louis, MO) cotton (expressing Cry1Ac and Cry2Ab) and Delta & Pine Land® 174RF (non-Bt) cotton. Cotton was grown according to standard agronomic practices and pest management recommendations with the exception that no insecticides were used with activity against Lepidoptera. Leaf tissue was collected during approximately the third week of flowering. One cotton leaf from each plant was selected from the third most upper node for a total of 500 leaves for each

cultivar. Leaves were placed in 3.785 L Ziploc® bags and then put in a -84°C cooler for 72h. After 72h, leaf tissue, now lyophilized, was finely ground into powder that would pass through a 40-mesh sieve, and then kept at -84°C until needed for bioassays.

Insect Rearing

H. zea larvae were collected from a VT3 PRO hybrid (variety 67-88, Monsanto Company, St. Louis, MO) and its non-Bt near isoline (variety 67-86, Monsanto Company, St. Louis, MO). Multiple collections were made each year in 2011, 2012, and 2013. Approximately 600 larvae were collected at a time (300 from each corn genotype). Only third instar larvae or larger were collected to maximize their selection to Bt and to minimize mortality from handling. Larvae were placed in 36 mL Solo® (Bio-Serv®, Frenchtown, NJ, USA) cups containing a soy-protein, wheat-germ based artificial diet with matching lids. Lids were labeled with the respective corn hybrids identifying where larvae were collected. Cups containing larvae were kept in a rearing facility maintaining 25°C, 80% relative humidity, and 16:8 (L:D) photoperiod. All other rearing was done under these environmental conditions. Larvae were monitored daily for emergence of pupae. Pupae were removed from individual cups to determine gender. Females were identified by the presence of a ventral, V-shaped suture near the tip of the abdomen. Males were identified by two rectal pads on the ventral tip of the abdomen (Ditman and Cory 1931). Pupae were then placed in empty 36 mL cups. Lids were labeled with the sex of the pupae and respective corn hybrid from which larvae were collected. Pupae were monitored daily for adult eclosion to arrange the following parental crosses: 1) NBt_F x NBt_M, 2) NBt_F x VT3 PRO_M, 3) VT3 PRO_F x NBt_M, 4) VT3 PRO_F x VT3 PRO_M. The capitalized abbreviation of each parental cross corresponds to the corn hybrid the larvae

were collected from with the subscript denoting the sex. Cohorts of moths were placed in identical 3.785 L cardboard containers with matching lids with the respective parental cross labeled on the outside of each bucket and fed a 10% sugar-water solution. The center of each lid was removed so that only the rim remained. Cotton cloth was placed over each bucket and fastened into place by a lid to serve as a oviposition substrate. Eggs were collected daily and new cloths were applied to every bucket. Collected egg sheets were kept individually in 1.83 L Ziploc® (S.C. Johnson & Johnson, Inc., Racine WI) bags until larvae hatched for use in bioassays.

***H. zea* Bioassays**

For bioassay, 0.5mL of warm soy protein-wheat germ based artificial diet (Bio-Serv®, Frenchtown, NJ, USA)was added to every well in a 128-well bioassay tray (Bio-Serv®, Frenchtown, NJ, USA). Diet was allowed to harden before application of slurry powder. A stock solution of powder slurry was made for each cotton variety by diluting 10 mg of leaf powder with 200 ml of a 0.02% agar (Bio-Serv®, Frenchtown, NJ, USA) (Greenplate et al. 2003). Eight concentrations of powder slurry were developed from each stock solution. Fifty µL of one concentration was applied to the diet surface of each well and a total of 16 wells were used for each concentration per tray (Greenplate et al. 2003). Assays were replicated based on the availability of larvae from parental crosses and offspring from each cross was assayed a minimum of two times each year. Overlay concentrations were allowed to dry under a laminar-flow hood (Agnew-Higgins, Inc, Garden Grove, CA) before one *H. zea* neonate (<24 h after hatching) from one the crosses was placed in each well. Cells were covered with perforated, clear 16-well lids (P.E. film, Bio-Serv®, Frenchtown, NJ, USA). Trays were labeled designating the

parental cross of the larvae and placed in a rearing chamber maintained at 25°C, 80% relative humidity, and a photoperiod of 16:8 (L:D). Mortality ratings were taken 7 d later. For the purpose of this study, mortality was defined as larvae or larvae that failed to molt to the second instar (weighing less than 10 mg) or larvae that failed to respond to a probe (Siegfried et al. 2000).

To observe parental influence on fitness costs in offspring, excess progeny from parental crosses were placed in 36 mL Solo® cups containing a soy-protein, wheat-germ based artificial diet (Bio-Serv®, Frenchtown, NJ, USA)with matching lids. Larvae were maintained as previously described. Larvae were monitored daily until pupation. Pupae were recovered from diet cups, weighed, and sexed. Sex of pupae was determined by the same procedure formerly described. Pupae were placed in empty Solo® cups with the sex labeled on each lid and monitored daily for adult eclosion to record pupal duration.

Analysis

Assay results were pooled across multiple collections within each year due to the difficulty in obtaining sufficient larvae to carry out replicated assays from one generation of a single collection. Data were analyzed separately for each year. Only first generation (F1) progeny were used for all assays. Concentration-mortality data were developed by evaluating F_1 progeny survivorship on eight Bt overlay concentrations eight non-Bt concentrations. Data were analyzed using Probit analysis (PROC PROBIT, SAS Institute 2012). Parental cross, cotton tissue type, concentration, and rep were all considered fixed effects. Mean LC_{50} values were calculated and separated by non-overlapping fiducial limits. Pupal weight and pupal duration were recorded from one collection of progeny in 2012. These data were analyzed using the PROC MIXED (SAS Institute 2012)

procedure. Sex and the origin of male and female adults were included as fixed effects. Analysis of variance (ANOVA) (SAS Institute 2012) was used to separate differences in pupal weight and pupal duration of progeny from parental crosses. Means were separated using a significant *F*-test at the $P \leq 0.05$ level of significance.

Results and Discussion

Based on results of these experiments, it appears that the source of males and females can influence *H. zea* susceptibility to Bt proteins. In 2011, the VT3 PRO_F x VT3 PRO_M homozygous cross (mating females and males collected from the same corn genotype) had an elevated LC_{50} value compared to the reciprocal crosses (mating females collected from VT3 PRO with males collected from non-Bt and vice versa) (Table 3.2). However, the NBt_F x NBt_M cross was not statistically different from any cross. In 2012, only offspring from the VT3 PRO_F x VT3 PRO_M cross had elevated LC_{50}s compared to other crosses that were not significantly different from each other. In 2013, no progeny resulting from any cross displayed statistically higher LC_{50}s. However the LC_{50} values generated for the VT3 PRO_F x VT3 PRO_M and NBt_F x NBt_M crosses are unreliable because there was no response to the range of concentrations of lyophilized tissue. The actual LC_{50} could not be generated because that value was above the maximum dose of the concentrations tested.

Larval mortality varied for each cross among years as expected. Numerous studies have documented the variation in the susceptibility of *H. zea* (Siegfried et al. 2000; Woodward et al. 2001; Ali et al. 2006; Ali and Luttrell 2009). Both homozygous crosses responded similarly in 2011 and 2012. This could suggest these populations originated from areas of similar Bt adoption such that selection resulted in these populations having

similar genetic background. Measuring actual shifts in susceptibility is difficult to determine with *H. zea* because of the wide range of responses observed when Luttrell et al. (1999) first documented baseline susceptibility to Bt. Additionally, if *H. zea* susceptibility is governed by multiple minor genes, then confirmation of whether a decrease in Bt susceptibility is due to a buildup of minor resistance genes or the natural variation of *H. zea* tolerance to Bt is difficult. These data illustrate that assortative mating of populations emerging from Bt corn can decrease insect susceptibility on Bt cotton. If it is assumed that resistant moths oviposited onto non-Bt corn at collection sites during 2011 and 2013, the susceptibility of those offspring should increase in the absence of Bt expression due to the instability of resistance alleles in a natural population. Genes associated with resistance in Lepidoptera are thought to be maternally inherited (Bird and Akhurst 2006). Progeny from reciprocal crosses could then be expected to display some evidence of inherited resistance if the female parent were truly resistant. Unfortunately, this was not observed in these experiments.

There was a significant interaction between the origin of female and male adults (F=.15.32; df=1, 421; P=0.01) for the pupal weight of progeny. Offspring from the VT3 PRO_F x NBt_M parental cross had the highest mean pupal weight compared to all other crosses (Fig. 3.1). Pupae from the NBt_F x VT3 PRO_M reciprocal cross had significantly lower pupal weight than the NBt_F x NBt_M cross, but higher than pupae from VT3 PRO_F x VT3 PRO_M. Progeny from VT3 PRO_F x VT3 PRO_M had the lowest pupal weight of all crosses. Similarly, an interaction between the origin of female and male adults (F=7.24; df=1, 345; P=0.01) had an impact on pupal duration of their respective offspring. Offspring from NBt_F x NBt_M cross had the shortest pupal duration compared to all other

crosses (Fig. 3.2). Pupae from VT3 PRO_F x VT3 PRO_M took longer to emerge than VT3 PRO_F x NBt_M pupae, but had a shorter duration compared to pupae from the NBt_F x VT3 PRO_M cross. Progeny from NBt_F x VT3 PRO_M cross had the longest pupal duration.

Offspring from parental crosses were affected by parental development on VT3 PRO corn. In both crosses consisting of males collected from VT3 PRO, the offspring had significantly lower mean pupal weight (Fig. 3.1). Similarly, offspring of males collected from VT3 PRO also had significantly longer mean pupal duration. These results do not conform to observations typically associated with inheritance mechanisms of fitness costs. As with resistance alleles, most fitness costs are recessively inherited (Gassman et al. 2009) and are often linked maternally (Wu et al. 2009). The male genetic contribution to progeny is minimal in comparison to maternal influence on offspring and lacks allelic components associated with insect fitness (Mike Caprio Personal Communication). Therefore, it is suggested that non-genetic paternal effects (seminal fluids or nuptial gift) are influencing differences in F_1 pupal weight and pupal duration.

Contrary to the amount of research performed on genetic inheritance, the concept of non-genetically inherited influence on offspring is a relatively new concept in insects; the depths of which are not quite fully understood. Seminal fluid is a nutrient-rich media that serves a variety of functions in insect mating to both the male and female (Poiani 2006). Seminal fluid aid in sperm competition and fertilization and can have negative effects on performance of offspring when the composition is altered or absent during copulation (Poiani 2006). Environmental factors like habitat and food quality can affect the quality of seminal fluid; thereby, influencing progeny (Rossiter 1996). For that reason, conclusions can be made as to the effect of Bt expression on paternal effects.

Survival of male *H. zea* on VT3 PRO corn may impose a cost on the quality of seminal fluid that negatively impacts the fitness of offspring. Anilkumar et al. (2008) noted *H. zea* males from a Cry1Ac resistant colony had more mating complications compared to females (although they were not attempting to evaluate paternal influence directly). Furthermore, this colony became heavily male biased after several generations of selection on Bt (Anilkumar et al. 2008). So in effect, male *H. zea* were more inept and more numerous compared to females when selected for resistance to Bt. In another study evaluating the dietary influence on male Lepidoptera, Delisle and Hardy (1997) discovered male and female reproductive capacity diminished greatly when males were reared on a low nutritional food source.

Assumptions can be made as to the origin and genetic background of the populations of *H. zea* collected over the course of this study, yet the hypotheses loosely support the results of these experiments. Resistance alleles are functionally recessive and perhaps, the concentrations of BollGard II tissue evaluated were such that the functionality of those alleles was not affected in reciprocal crosses. Lack of a significant concentration-mortality relationship with moths reared on non-Bt corn suggests reduced susceptibility in that cohort. This is a discouraging outcome, and frankly, complicates interpreting the implications of these data. Assortative mating of adults emerging from VT3 PRO corn has the potential to decrease susceptibility of offspring on BollGard II cotton. The degree of this susceptibility that makes sense in the context of actual square and boll damage remains in question.

Paternal influence on offspring is an interesting theory and one that requires further investigation. The majority of fitness costs are recessive, although dominant

alleles linked to disadvantages in fitness have been discovered (Gassmann et al. 2009). Non-genetic effects having deleterious effects on progeny represents another example of mechanisms preventing the increase of resistance alleles. Paternal effects may have a much larger impact on the evolution of resistance in *H. zea* than previously believed. Due to the small sample size of this observation, further experimentation should be performed in order to determine the consequences of such interactions on the ecology of *H. zea.*

Table 3.2 LC_{50} for progeny resulting from reciprocal and back crosses of *H. zea* adults reared on non-Bt and VT3 PRO field corn from 2011 to 2013.

Parental Cross	**χ^2**	***P*<0.05**	**Slope**	**LC_{50} (95% Fiducial Limits)**
2011				
NBt_F x NBt_M	5.82	0.56	0.88*	1.04(0.59-1.43)AB
NBt_F x VT3 PRO_M	4.88	0.77	0.28*	0.56(0.19-1.03)B
VT3 PRO_F x NBt_M	7.85	0.44	0.56*	0.5(0.22-0.86)B
VT3 PRO_F x VT3 PRO_M	4.90	0.77	0.28*	3.65(1.06-15.59)A
2012				
NBt_F x NBt_M	15.51	0.63	0.66*	0.75(0.53-1.02)B
NBt_F x VT3 PRO_M	6.11	0.30	0.98*	1.03(0.42-1.61)B
VT3 PRO_F x NBt_M	10.56	0.22	0.66*	0.76(0.48-1.12)B
VT3 PRO_F x VT3 PRO_M	7.43	0.76	0.55*	2.73(1.67-4.75)A
2013				
NBt_F x NBt_M	96.53	<0.01	0.66	>6.67
NBt_F x VT3 PRO_M	2.0	0.74	1.12*	0.63(0.34-0.92)A
VT3 PRO_F x NBt_M	11.89	0.68	0.55*	1.58(0.85-2.34)A
VT3 PRO_F x VT3 PRO_M	131.92	<0.01	0.64	>6.67

Highlighted rows represent analysis for all reps within respective parental crosses. LC_{50}s followed by the same letter grouping are not significantly different within a year.
*Slope is significant.

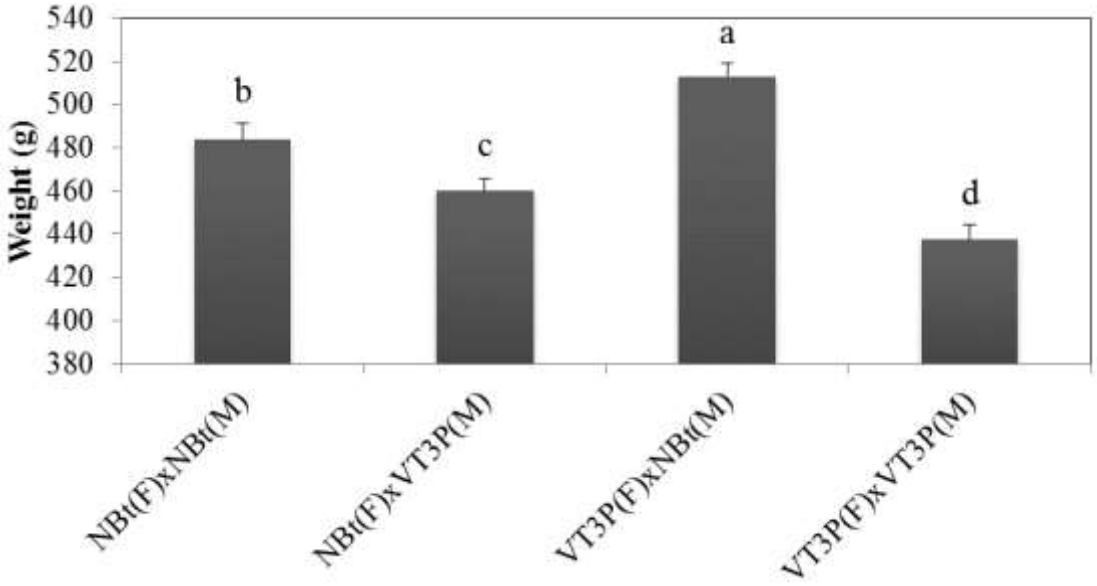

Figure 3.1 Mean (SEM) pupal weight (g) of F1 progeny resulting from parental crosses of larvae collected from VT3 PRO and non-Bt field corn

Bars sharing the same letter grouping are not significantly different (P<0.05).

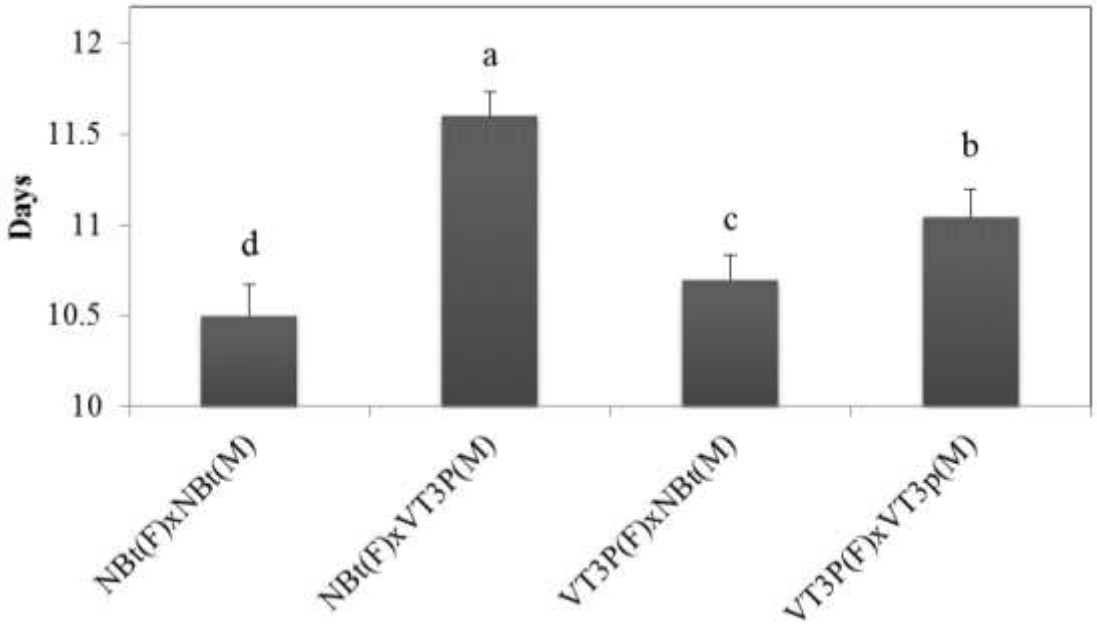

Figure 3.2 Mean (SEM) pupal duration (days) of F1 progeny resulting from parental crosses of larvae collected from VT3 PRO and non-Bt field corn

Bars sharing the same letter grouping are not significantly different (P<0.05).

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CHAPTER IV

CONCLUSIONS

VT3 PRO corn was considered to be one of the most efficacious Bt corn varieties in commercial production. Although there were significantly fewer larvae infesting VT3 PRO fields during 2012 and 2013, these hybrids did not provide a high dose with regard to controlling *H. zea* in the ear stage because larval density was reduced by only 61% across all locations. Larval development was delayed on VT3 PRO corn (Fig. 2.4) compared to non-Bt. The delay in larval development also translated into longer pupal duration for larvae collected from VT3 PRO corn two out of the three years of this study. In 2013, larvae collected from VT3 PRO fields had significantly shorter pupal duration and greater pupal weight (Fig. 2.5 and Fig. 2.6, respectively) compared to those collected from non-Bt fields. Even though larval development was delayed, an improvement in insect fitness suggests these individuals may be resistant (Gassmann et al. 2009). Larvae collected in 2011 and 2012 from VT3 PRO incurred fitness costs (pupal duration and weight) which are consistent with other reports (Storer et al. 2001; Storer et al. 2003).

Larval density increased in VT3 PRO fields from R1-R2 to R3-R4 growth stages; while larval densities on non-Bt corn remained the same. There are several possible explanations for this. First, larval development was significantly delayed on VT3 PRO corn compared to non-Bt corn. The proportion of small and large larvae changed significantly from R1-R2 to R3-R4 on non-Bt corn. Most of the larvae at R3-R4 on non-

Bt ears were classified as large. In contrast, a larger percentage of larvae were classified as small in VT3 PRO ears at R3-R4. Also, it appears that oviposition beyond the green silk stage continued beyond the green silk stage in these fields because larval densities increased from the R1-R2 stage to the R3-R4 stage on VT3 PRO. Protein expression in VT3 PRO corn kernels has not been quantified at different stages of kernel development. If protein expression decreases over time, as it does in cotton (Greenplate et al. 1999; Adamczyk et al. 2000), a greater proportion of larvae may be able to survive at the later stages of grain development. Future research should focus on *H. zea* survival at the later stages of grain development in Bt corn. Estimates on cannibalism are lacking but could also contribute to survivorship of larvae infesting VT3 PRO corn. If larvae are able to survive through cannibalism on Bt ears until protein expression decreases below effective levels, selection may not be as intense as previously theorized.

H. zea susceptibility on Bt cotton varied each year. Attempts to determine inheritance mechanisms of resistance through reciprocal crosses did not produce any results suggesting resistance alleles are passed maternally. Each year, the VT3 PRO homozygous cross had elevated LC_{50} values compared to the reciprocal crosses. The non-Bt and VT3 PRO homozygous crosses had similar responses in susceptibility in 2011 and 2013. Given the variation in susceptibility observed by Luttrell et al. (1999), it may be difficult to ever detect resistance before it has become widespread. *H. zea* susceptibility is not determined by a single gene conferring resistance (i.e. *P. xyllostella*) (Heckel et al. 2001), but is influenced by multiple minor genes. Under certain conditions, however, it may be possible to identify populations (or years) of *H. zea* that have higher survivorship on Bt cotton (or a Bt host plant). Correlating insect fitness with responses in bioassays

has the potential to provide further insight into actual changes in *H. zea* Bt susceptibility. For instance, bioassay results from 2011 and 2012 suggest these outcomes are measuring the natural variation in Bt susceptibility because insect fitness (pupal weight and pupal duration) of larvae collected from VT3 PRO corn was negatively affected. In 2013, larvae collected from VT3 PRO fields had higher pupal weight and shorter pupal duration than those collected from non-Bt fields, and their offspring had LC_{50} values on Bt cotton above the maximum concentration of lyophilized tissue. These results suggest there may have been a decrease in susceptibility in the populations evaluated in 2013. The difficulty lies in being able to reproduce these results with consistency.

Evaluating the impact of a transgenic host on *H. zea* ecology and susceptibility is a challenging task (Siegfried et al. 2000; Woodward et al. 2001; Ali et al. 2006; Ali and Luttrell 2009). Resistance to Bt in *H. zea* is influenced by many factors and there are physiological mechanisms at play to prevent the buildup of resistance alleles so that resistance does not become established. Current methods of assessing Bt susceptibility involve evaluating offspring of individuals collected from a Bt host. The research presented here indicates there may be an association between parental fitness and offspring susceptibility. In order to determine if susceptibility has truly been influenced, perhaps future research should continue to evaluate insect fitness along with conducting bioassays on Bt plant tissue.

The implications of these results also suggest that structured corn refuges are not serving their full purpose. First, the delay in larval and pupal development would lead to asynchronous emergence of moths causing non-random mating. Second, VT3 PRO homozygous cross had elevated LC_{50} values suggesting they are less susceptible to the Bt

proteins in BollGard II cotton. As a result, assortative mating of individuals emerging from VT3 PRO can produce offspring that have a higher tolerance on Bt cotton.

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