ABSTRACT

ARENDS, BENJAMIN ROBERT. *Helicoverpa zea* (Lepidoptera: Noctuidae) Incidence and Susceptibility to Bt Across North and South Carolina in Relation to Bt Crop Composition. (Under the direction of Drs. Dominic Reisig and George Kennedy).

Helicoverpa zea (Boddie) is a polyphagous pest that can feed on important crops in the Southeast such as corn (*Zea mays* L), cotton (*Gossypium hirsutum* L), and soybean (*Glycine max* L). Different generations of *H. zea* feed on these hosts in a predictable pattern through the growing season. First generation *H. zea* feed on whorl-stage corn and non-crop hosts, followed by ear-stage corn, and finally cotton and soybean. Both corn and cotton express *Bacillus thuringiensis* to control a suite of lepidopteran pests; however, Bt expression in corn and cotton provides only a moderate dose to *H. zea* and complete control is not achieved. In the Southeast, *H. zea* is resistant to some of the Bt toxins expressed in these crops. In instances of resistance, the temporal and spatial composition of Bt crops (corn and cotton) may provide insight where resistance may be evolving. To investigate this, we related the temporal and spatial composition of Bt crops (corn and cotton) surrounding sample sites across North and South Carolina to the level of Cry1Ac resistance in *H. zea*, to the magnitude of whorl-stage corn damage from first generation *H. zea*, and to the the amount of ear damage and larval exit holes in Bt and non-Bt corn caused by 2nd generation *H. zea*.

Because 2nd generation *H. zea* that complete development on corn potentially oviposit in cotton, we collected *H. zea* larvae from non-Bt corn across North and South Carolina and subjected them to a Cry1Ac diagnostic-dose bioassay. Results from our bioassay were then related to the Bt crop (corn and cotton) composition during the preceding year in a 1 km buffer radius. While the abundance of cotton during the previous year in a 1 km buffer radius was associated with higher levels of Cry1Ac resistance, the presence of corn had a strong negative

effect on the level of Cry1Ac resistance, indicating cotton potentially being a source of Cry1Ac resistant selection.

To investigate *H. zea* dynamics in ear-stage corn, paired Bt and non-Bt corn plots were sampled for ear damage and larval exit holes across North and South Carolina and related to the proportional area of corn and cotton during the preceding year and the proportional area of corn during the current year within a 1 km buffer radius. Across all locations, we found Bt corn had significantly less damage and fewer larval exit holes compared to non-Bt corn. Furthermore, there was a negative relationship between the amount of ear damage and larval exit holes in Bt and non-Bt corn and the proportional area of corn and cotton in a 1 km buffer radius from the previous year and the proportional area of corn from the current year, indicating damage and larval exit holes in Bt corn is related to Bt resistance and the area of corn and cotton.

Lastly, damage in whorl-stage corn was measured in Bt and non-Bt corn in plots across North Carolina to understand dynamics in the first generation of *H. zea* and implications they may have throughout the season in North Carolina. Here, we found that whorl-stage corn is damaged by *H. zea* and *Spodoptera frugiperda* (J.E.Smith). Overall, non-Bt corn damaged whorl-stage plants is rare and even rarer in Bt corn. We found no relationship between the amount of damaged whorl-stage plants and the amount of subsequent ear damage and the abundance of late-season hosts (cotton and soybean) that can serve as an overwintering location.

Overall, these results provide a new understanding of how Bt toxins expressed in common host crops affects *H. zea* development and resistance selection. This landscape-level assessment of selective filters will provide useful context to improve resistance management for *H. zea*.

Helicoverpa zea (Lepidoptera: Noctuidae) Incidence and Susceptibility to Bt Across North and South Carolina in Relation to Bt Crop Composition

by
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DEDICATION

To my wife, Kayla: You have encouraged and supported me throughout my entire undergraduate and graduate degree and had to deal with my stress associated with it. Also, you were the best at rearing earworms!

To my parents: You both encouraged my creativity as a kid and asking a million questions about how things work.

To my uncle, Jim: You exposed me to the world of entomological research and have been a mentor throughout my life.

BIOGRAPHY

Benjamin Arends grew up on a farm near Ackley, Iowa. Growing up a farm, an interest in agriculture was a given but that interest further developed when he worked at Innovative Ag Services. Wanting to learn more and advance his career, he pursued his undergraduate degree at Iowa State University, majoring in Biology and minoring in Entomology. Benjamin thoroughly enjoyed his entomology classes and wanted to join two of his interests, agriculture and entomology. His interests brought him to North Carolina State University where he could blend the two together to pursue his Master’s degree in Entomology.

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Susceptibility of *Helicoverpa zea* (Lepidoptera: Noctuidae) to Cry1Ac in Relation to Bt Crop Composition and the Potential Importance of Cotton to Select for Bt-resistant *H. zea* Individuals

Abstract

Since its commercialization, the planting of Bt corn and cotton has markedly increased, resulting in significant selection pressure for resistance in insects that are not completely managed by Bt. One indication of resistance development are increasing foliar sprays in cotton for *Helicoverpa zea* (Boddie) due to Bt resistance. In this study, we related the level of Cry1Ac resistance (a toxin expressed in the majority of Bt cotton) in the generation of *H. zea* that typically develops on cotton to the abundance of Bt crops during the preceding year in a 1 km buffer radius. To accomplish this, we collected *H. zea* larvae from non-Bt corn during 2017 and 2018 at 59 locations across North and South Carolina and bioassayed their progeny for Cry1Ac resistance. Our results classified 57 out of the 59 assayed populations as potentially heterozygous for a resistant allele to Cry1Ac at a diagnostic-dose of 29µg/cm^2. When coupled with our landscape analysis, we demonstrated that the effects of the proportional area of corn and cotton during the preceding year are interdependent on odds of survival in our Cry1Ac bioassays. Larger area of cotton was related to more *H. zea* survival, but the presence of corn was related to less Cry1Ac survival. While cotton is not thought to an important *H. zea* host relative to other crops, our results indicate that it could be related to a higher frequency of Bt resistant individuals.

1 Introduction

Helicoverpa zea (Boddie) is a multivoltine polyphagous pest that feeds on economically important crops to the Southeast such as corn (*Zea mays* L.), cotton (*Gossypium hirsumtum* L.), and soybean (*Glycine max* L.) (Quaintance & Brues 1905, Neunzig 1969, Fitt 1989). In the Southeast, *H. zea* complete at least four generations per year, with each generation feeding on a predictable sequence of crops at various phenological stages (Isely 1935, Kennedy and Storer 2000). Silking-stage corn is the preferred ovipositional site for second-generation adult *H. zea* and is an excellent host for developing larvae (Barber 1943, Johnson et al. 1975). Moths emerging from corn fields commonly oviposit in later flowering crops, such as cotton and soybean (Kennedy and Storer 2000). Of the main crop hosts of *H. zea* in the Southeast, both corn and cotton express insecticidal endotoxins derived from *Bacillus thuringiensis* (Bt) Berliner to control a suite of lepidopteran pests, but these toxins only have moderate activity on *H. zea* (FIFRA SAP 1998); therefore, complete control is not achieved (Storer et al. 2001, Reay-Jones and Reisig 2014). As a result, resistance has been documented to both Cry1 and Cry2 proteins expressed in both corn and cotton due to low- to moderate-dose of toxins against *H. zea* (Bowen et al. 2014, Bibb et al. 2018, Reisig et al. 2018, Bilbo et al. 2019). Over time, this has led to an increase in foliar insecticide sprays to manage this pest in Bt crops where economic damage can occur (Dively et al. 2016, Reisig et al. 2018).

Bt corn and cotton were first commercialized in 1996 and expressed a single-toxin, Cry1Ab and Cry1Ac, respectively (US EPA 2018). In corn, the primary target pests were *Ostrinia nubilalis* (Hübner) and *Diatraea graniosella* (Dyar) (Storer et al. 2001). In cotton, the primary target pests were *Chloridea virescens* (Fabricius), *Pectinophora gossypiella* (Saunders), and *H. zea* (FIFRA SAP 1998). However, in corn, *H. zea* was not a target pest since the concentration of the toxin expressed is not considered a high dose (FIFRA SAP 1998, Storer et al. 2001). The situation has changed over time, because *P. gossypiella* has been eradicated from the U.S. (Anderson et al. 2019) and because, similar to corn, the expression profile from commercialized cotton traits are not considered a high dose for *H. zea* (FIFRA SAP 1998, Reisig and Kurtz 2018, US EPA 2018). This is important, since expression at less than a high dose can speed resistance development (Gould 1998).

Over the past several decades, the abundance of Bt crops in the landscape has rapidly increased since their commercialization (ISAAA 2017). In 2018, the United States Department of Agriculture estimated that 83% of the corn and 92% of the cotton planted in the U.S. expressed one or more Bt toxins (USDA 2019). Because of widespread Bt crop deployment, selection pressure for resistant insects has also increased (Tabashnik 1994, Gould 1998). Insecticide Resistance Management (IRM) strategies to delay resistance rely on multiple tactics (FIFRA SAP 1998, Gould 1998). One tactic, the high-dose refuge strategy, allows rare Bt resistant (assuming the resistance that develops is a single allele and recessive) insects that develop on Bt plants to mate with susceptible insects that develop on non-Bt plants (Tabashnik 1994, Gould 1998). A functionally high-dose Bt toxin occurs when a plant produces enough toxin to kill at least 95% of the heterozygotes that carry a single allele for resistance (FIFRA SAP 1998, Gould 1998). This limits the number of heterozygotes that develop on Bt plants and passing on resistant alleles to the next generation. The spatially structured refuge consists of non-toxic plants planted near the toxic plants or as a seed-blend where a known percentage of non-toxic plants are randomly distributed within toxic plants throughout the field (Bates et al. 2005). In the Southeast, corn growers that plant hybrids expressing two or more toxins are required to plant a 20% refuge (US EPA 2018). However, in North Carolina, refuge compliance is low among growers (Reisig 2017) which explain the emergence of incidence of resistant *H. zea* in the region (Reisig and Kurtz 2018). In contrast, cotton growers are not required to plant a refuge as research has shown that non-Bt cotton is not an important crop host to for *H. zea* (Head et al. 2010). As a result, IRM strategies for Bt cotton depend on a natural refuge consisting of uncultivated hosts and non-Bt crops, as well as a spatially structured non-Bt corn refuge (US EPA 2018).

In addition to the high-dose refuge strategy, pyramiding multiple toxins in the same plant can delay the evolution of resistance (Roush 1998, Carrière et al. 2015). Pyramided plants are most effective at delaying resistance if each individual toxin has a unique mechanism to kill the target pest (Carrière et al. 2015). When each toxin has a unique pathway, insects with minor resistance alleles to a single toxin or those that carry a heterozygous resistance allele to a single toxin have a greater mortality probability for the other toxin, thereby delaying the evolution of resistance (Gould 1998, Huang et al. 2011, Brevault et al. 2013, Carrière et al. 2015). However, modeling efforts by Brevault et al. (2013) illustrate that the rate of resistance evolution to two-toxin pyramid varieties increases when individual toxin expressed in the pyramid are not high doses. Early hybrids of corn and varieties of cotton expressed a single toxin (US EPA 2018). Over the past decade, corn hybrids expressing multiple toxins for lepidopteran pests have become very common in the southern row crop production system (i.e., Cry1Ab + Cry1F, Cry1A.105 + Cry2Ab2, Cry1Ab + Vip3Aa20, Cry1Ab + Cry1F + Vip3Aa20, and Cry1A.105 + Cry2Ab2 + Cry1F) (Reisig et al. 2015, Reay-Jones et al. 2016). Moreover, cotton varieties expressing similar toxins are also common in these production systems (i.e., Cry1Ac + Cry2Ab2, Cry1Ac + Cry1F, Cry1Ab + Cry2Ae, Cry1Ab + Cry2Ab2 + Vip3Aa19, Cry1Ac + Cry1F + Vip3Aa19, and Cry1Ab + Cry2Ae + Vip3Aa19)(US EPA 2018). Unfortunately, the effectiveness of pyramiding toxins to delay resistance can be diminished by cross-resistance between different toxins that share similar pathways to kill the insect (Welch et al. 2015). For example, strong cross-resistance has been documented between Cry1Ac, Cry1Ab, and Cry1A.105 (Anilkumar et al. 2008, Welch et al. 2015). In systems where multiple generations of a single pest feed on multiple crops that express similar toxins, cross-resistance can hasten resistance. In corn-cotton agroecosystems in the Southeast, second generation *H. zea* feed on ear-stage corn followed by the third generation feeding on cotton (Jackson et al. 2008), allowing for multiple generations to be exposed to multiple toxins with potential for sequential resistance selection events.

Helicoverpa zea feed on different hosts as they become suitable throughout the growing season. Head et al. (2010) characterized ^{13}C and ^{12}C isotope ratios in adult male *H. zea* to estimate if larvae developed on C_3 or C_4 photosynthetic pathway plants. Their study found that the majority of *H. zea* captured during the months of June and July developed on C_4 plants, and male moths captured during August and September developed on C_3 plants. Because corn utilizes a C_4 photosynthetic pathway, and is the most abundant and attractive host during late June and July, it is likely a significant source of *H. zea* moths that disperse in late July and early August, and oviposit in other flowering crops, including cotton and soybean (Head et al. 2010). This has also been indirectly corroborated through high densities of *H. zea* found in corn fields from June to August (Jackson et al. 2008) accompanied by high densities of moth captures in traps adjacent to corn fields (Allen and Luttrell 2011). Similarly, during late July and August, flowering cotton and soybean (both C_3 plants) are highly attractive hosts to *H. zea* and high larval densities are often found in these crops combined with increased trap captures adjacent to these crop fields (Jackson et al. 2008, Allen and Luttrell 2011). In North Carolina, it is these hosts (cotton and soybean) that produce the majority of the overwintering population. In the mid-south region, there are positive correlations among the abundance of corn surrounding cotton fields and abundance of *H. zea* and *C. virescens* eggs present in cotton fields (Allen and Luttrell 2009). Because *H. zea* and *C. virecens* eggs were not differentiated in their study, and because corn is not a host of *C. virescens*, the majority of their egg detections are likely *H. zea*. Finally, this host shift from corn to cotton can have implications for resistance because the Bt toxins expressed in corn are similar to the toxins expressed in cotton and, as a result, tend to favor selection for cross-resistance via sequential Bt host crop use within the season (Welch et al. 2015).

To understand Bt resistance selection, spatial and temporal abundance of crop hosts and toxins need to be characterized at a local landscape level (Onstad and Carrière 2013). Previous modeling studies have examined how resistance evolution is affected by the abundance of Bt and non-Bt crops in a region (Storer et al. 2003a & b, Sisterson et al. 2004). Storer et al. (2003a & b) used a stochastic, spatially explicit simulation model to investigate how the deployment of Bt crops in North Carolina would affect resistance evolution in *H. zea*. Similarly, Sisterson et al. (2004) used a stochastic, spatially explicit model to examine *P. gossypiella* susceptibility to Bt cotton in Arizona. Although different pests were studied, both models drew similar conclusions; resistance evolves faster in regions where Bt crops are abundant in relation to non-Bt refuge because of the increased selection intensity (Storer et al. 2003a & b, Sisterson et al. 2004). Furthermore, pest behavior, such as dispersal, mating, and oviposition may also influence the rate of resistance evolution (Onstad and Carrière 2013).

Multiple studies have documented resistance to Bt toxins expressed in corn and cotton (Bowen et al. 2014, Bibb et al. 2018, Reisig et al. 2018, Bilbo et al. 2019). Because early generations of *H.* zea develop on corn and later generations on cotton (Kennedy and Storer 2000, Jackson et al. 2008), the objective of our study was to understand the level of Cry1Ac resistance in the generation of *H. zea* that emerge from corn to infest cotton, nearly all of which expresses Cry1Ac in North Carolina. Moreover, by sampling from multiple locations in North and South Carolina, this study examines how the spatial abundance of Bt crops (corn and cotton) at fine scales is related to Cry1Ac susceptibility in *H. zea*. Following the results from Storer et al. (2003a & b) and Sisterson et al. (2004), we hypothesize that landscapes of abundant Bt-expressing crops (corn and cotton, which express similar Cry1 proteins) during the preceding year will be related to increased *H. zea* survival to Cry1Ac.

2 Materials and Methods

2.1 Corn Plots

In 2017 and 2018, non-Bt corn plots were planted in corn-growing counties across North Carolina and South Carolina. The non-Bt hybrid used was Dekalb 67-70RR (Bayer CropScience, St. Louis, MO, USA). All corn plots were four to twelve rows wide and > 40 m long. Larger plots were preferred and planted when possible to minimize cross-pollination among Bt and non-Bt hybrids. Plots were planted using commercial corn planters at various populations depending on soil type and fertility. Planting dates ranged from late March to early June depending on planting conditions at each site. In 2017, 42 plots were planted in North Carolina. In 2018, 29 plots were planted in North Carolina and 22 plots were planted in South Carolina. To document that a corn plot was non-Bt, an ELISA strip test was performed following manufacturer's instructions using a one composite leaf sample from three random plants within each plot (QuickStix Kit for Cry1Ab Corn Leaf & Seed, Envirologix Inc., Portland, ME). Corn plots at each location were maintained using agronomic recommendations from their respective

Cooperative Extension Services. GPS coordinates were recorded at each location using a cellular phone.

2.2 Determination of diagnostic dose and survival threshold

To classify *H. zea* populations as susceptible or potentially heterozygous for a resistant allele to Cry1Ac, a diagnostic dose was determined by performing a repeated multiple dose diet overlay bioassay on four known Cry1Ac susceptible colonies during 2017. Susceptible *H. zea* colonies were obtained from Mississippi State University (field collected during 2017 and maintained continuously in the laboratory), Louisiana (field collected during 2016 and maintained continuously in the laboratory in North Carolina), North Carolina (field collected during 2016 and maintained continuously in the laboratory in North Carolina), and from Benzon Research Inc. (Carlisle, PA). To prepare diet trays, a 0.75 ml aliquot of a modified commercial diet (Southland Products, Lake Village, AR) was pipetted into each well of a 128 well tray. Commercial diet was modified with the addition of casein to produce a 1.6:1 protein to carbohydrate ratio and anti-microbials (Deans et al. 2017). Once cooled to room temperature, a 40 µL aliquot of a liquid Cry1Ac protein (94-96% pure, trypsin activated, ion exchange HPLC purified, desalted, and freeze dried, obtained from Case Western Reserve University), dissolved in Triton X-100 (0.1%) buffer, was pipetted on the surface of the diet in each well at eight different concentrations. Those concentrations ranged from 0, 0.1, 1, 5, 10, 50, 100, and 200 µg/cm^2 and an untreated control. Once diet dried, one neonate was applied to each well using a fine-tipped paintbrush and covered with a self-adhesive plate to prevent insect escape. Bioassay trays were incubated in a growth chamber (27°C, 60%RH, 14:10 photoperiod) for 7 days, after which, larval mortality was recorded. Larvae were considered dead if they did not move after being prodded with a paintbrush. Eighty larvae were tested at each concentration to estimate a dose response for each population. Using probit analysis (PROC PROBIT, SAS Institute, 2012), 95% lethal concentrations (LC_{95}) to Cry1Ac were estimated for each population. To determine the diagnostic dose, the average LC_{95} across the four susceptible populations was calculated to be 29µg Cry1Ac/cm^2 (Table 1.1). The diagnostic dose bioassay was then tested four independent times on the Louisiana susceptible laboratory colony to determine threshold of survivorship in order to classify assayed populations as susceptible or potentially heterozygous for a resistant allele. The average survivorship among the four assays was 7 ± 4.76% (mean percentage survivorship ± SD; min. 0.9; max 14%). Therefore, bioassays with a survivorship of greater than 7% were classified as potentially heterozygous for a resistant allele. By establishing a survival threshold for classifying populations as potentially heterozygous for a resistant allele or susceptible allowed us to ensure that 95% of individuals that were heterozygous resistant were killed by the toxin. This aligns with the assumptions of the high-dose approach of Bt crops where toxin expression kills at least 95% of heterozygous and 99.99% of susceptible individuals (Gould 1998).

2.3 *H. zea* larval sampling and bioassay

During 2017 and 2018, all non-Bt corn plots were sampled for *H. zea* to test for Cry1Ac susceptibility. Sampling was initiated when corn plants were at the R2-R3 growth stage and *H. zea* larvae were at least at the third instar stage. Larvae were collected from randomly selected ears in the middle rows of each plot and at least 5 m in from end rows to reduce the probability of Bt exposure due to cross-pollination. Fifty to 120 larvae were collected from each plot and were immediately placed on a casein-wheat germ-based artificial diet in 30 mL plastic cups sealed with a breathable lid. Diet consisted of wheat-germ and casein to achieve a protein: carbohydrate ratio of 1.6:1 and was supplemented with sucrose, vitamins, anti-microbials, and preservatives (Deans et al. 2017). Collected larvae were placed in coolers and immediately transported to the Vernon James Research Center in Plymouth, NC. Larvae were reared to adulthood replacing diet as needed. Once pupation occurred, pupae were surfaced sterilized in a 5% bleach solution with water. The sex of each pupa was determined for each collected population and randomly placed in a 1.8 L container at a 3:1 (female: male) ratio, with a maximum of 28 pupae per container. Containers were covered with cheesecloth to allow air movement and to provide an ovipositional substrate. Adults were reared in rooms at a temperature of 25°C, 50% RH, and natural photoperiods. After pupation, adult moths were maintained on a 10% sucrose solution *ad libitum*. When eggs were present on the cheesecloth, the cloth was removed and placed into 0.5-liter container to allow neonates to hatch. Neonates less than 24 h old from each collection were used in a diet-based diagnostic dose bioassay. Bioassays were prepared as described previously, except Cry1Ac protein was dissolved in Triton X-100 (0.1%) to achieve the Cry1Ac diagnostic dose (LC_{95}) of 29μg/cm^2 determined earlier. 100 μL of Cry1Ac liquid solution was individually pipetted into 112 wells and 100 μL of aqueous Triton X-100 buffer was individually pipetted into 16 wells to serve as a control. Once applied, trays were allowed to dry.

One neonate was then placed in each well using a fine-tipped paintbrush and then covered with a self-adhesive cover plate. Because colony size varied, not all assays had 112 larvae in the treated wells, but all assays had 16 larvae in the control wells. To prevent static build-up on the cover plate, which could cause the neonate to adhere to the cover plate, a static gun (Milty Zerostat 2 Anti-Static Gun, Darmstadt, Germany) was applied to each cover plate to eliminate static. In 2017, treated trays were then incubated in a growth chamber (27°C, 60% RH, 14:10 photoperiod). In 2018, trays were incubated on the lab bench (25°C, 50% RH, natural photoperiod) due to a change in manufacturing of cover plates allowing condensation buildup inside each well if placed in the growth chamber. Larvae from each population were exposed to the treatment for 7 days. Following the 7-day exposure period, trays were assessed for larval mortality. Larvae were considered dead if they did not move after being prodded with the paintbrush. Proportion survival was corrected for control mortality using Abbott's method (Abbott 1925).

2.4 Landscape analysis

Landscape composition surrounding each sample site was determined using remotely sensed data from the USDA National Agricultural Statistics Service- Cropland Data Layer (CDL) (USDA-NASS 2019). Because selection for Bt resistant *H. zea* occurs only in crops expressing Bt (corn and cotton), total area of corn and cotton production in a 1 km buffer radius surrounding each sample site was quantified using ArcGIS (Version 10.1 ESRI 2018, Redlands, CA). A 1 km buffer was selected based on findings by Graham et al. (1978) that the majority of rubidium-marked *H. zea* emerging from corn fields were captured in traps 0.8 km from the source field. In our study, we hypothesized that landscapes with relatively high proportional areas of corn and cotton surrounding our sample sites during the previous year exerted a greater influence on Cry1Ac resistance in *H. zea* than sample sites with lower proportional areas of corn and cotton. Therefore, annual production during the previous year was used to estimate total corn and cotton production area within each 1 km buffer radius surrounding sample sites.

2.5 Statistical analysis

To test for a difference in bioassay survival by year, results were analyzed using a general linear model (PROC GLM, SAS Institute, 2012) where proportion of survival per assayed population was coded as the dependent variable and year as the independent variable.

To relate bioassay survival to proportional area of Bt-expressing crops (corn and cotton) in the landscape, a multiple logistic regression model, modeled as a binomial distribution using the logit link function, (PROC GLIMMIX, SAS Institute, 2012) was constructed with bioassay survival per location coded as the dependent variable and the proportional area of corn during the preceding year (up to a 1 km buffer radius), the proportional area of cotton during the preceding year (up to a 1 km buffer radius), and their interaction as the independent variables. Intercept for each year was included as a random effect. Prior to analysis, a Moran's I test was conducted to test for spatial autocorrelation among sample sites (Overmars et al. 2003). Using survival in bioassays as the predictor, there was no evidence of autocorrelation among sample sites (P = 0.29), indicating that bioassay survival among sample sites are independent. Moran's I test was conducted in R version 3.6.0 using the *ape* package (R Core Team 2019).

3 Results

During 2017 and 2018, 7,058 larvae from 59 total collected *H. zea* populations were subjected to a Cry1Ac diagnostic dose diet-based bioassay. In 2017, 28 collected populations were assayed and in 2018, 31 collected populations were assayed. Survival to the Cry1Ac bioassay varied by each location ranging from 1% to 96% (Fig 1.1). Using the 7% threshold survivorship to classify populations as potentially heterozygous for a resistant allele, our bioassays revealed only two populations classified as susceptible to Cry1Ac.

Comparing percent survivorship across all bioassays by year sampled, 2018 had significantly higher bioassay survivorship than 2017 (F = 25.38; df = 1, 58; P < 0.0001)(Fig. 1.2). The average percent survivorship was 27.4 ± 3.29% SEM in 2017 and 53.5 ± 3.81% in 2018.

There was a significant interaction between the proportional area of corn during the previous year and the proportional area of cotton during the previous year (up to a 1 km buffer radius) on the log odds of bioassay survivorship ($F = 74.45$; df = 1, 54; $P < 0.0001$)(Fig. 1.3; Table 1.2). This analysis suggests that bioassay survival varied with proportional area of corn and cotton in a 1 km buffer radius during the previous year, with the effect of each depending on the proportional area of the other.

4 Discussion

Our study assayed *H. zea* populations from 59 locations across North and South Carolina in 2017 and 2018. We hypothesized that landscapes of relatively high proportional areas of Bt-expressing crops (corn and cotton) during the previous year (up to a 1 km buffer radius) surrounding our sample sites would have greater selection pressure for Bt resistance, resulting in collected populations having greater survivorship in our diagnostic dose bioassays. Our results indicated the proportional area of corn and cotton in a 1 km buffer radius during the previous year effects are related to the odds of bioassay survival. Higher abundance of cotton in the system during the previous year resulted in increased log odds survivorship in our bioassays; the presence of corn exerted a strong negative effect on log odds bioassay survivorship. These results indicate resistance selection complexity in the corn-cotton agroecosystem.

Out of those 59 populations, 57 were considered potentially heterozygous for a resistant allele to Cry1Ac at the diagnostic dose of 29µg/cm^2, indicating Cry1Ac resistant populations of *H. zea* are widespread across the Carolinas. Our survival threshold for classify populations as resistant was low, ensuring that 95% of individuals heterozygous for the resistant allele were being killed by the toxin. Furthermore, Burd et al. (2003) collected a single *H. zea* female adult in North Carolina that carried a dominate gene for resistance, which further supported the need for a low survival threshold to classify resistance as this violates the high-dose assumption. Additionally, this minimized chances for miss-classifying populations as susceptible when, in fact, they may have been heterozygous for the resistant allele. Finally, our results showed that 2018 bioassay survival was greater, on average than in 2017, indicating that the incidence of field-evolved resistance to Cry1Ac may be changing (Tabashnik et al. 2014). Reisig et al. (2018) collected *H. zea* populations from both Bt and non-Bt hosts across the Southeast and subjected them to a diet-based bioassay overlaid with Cry1Ac. Their study found high variability in Cry1Ac susceptibility among collected populations at a dose of 31.6 $\mu g/cm^2$, a similar dose used in our study. Bilbo et al. (2019) subjected *H. zea* populations collected from North and South Carolina to Cry1A.105 and Cry2Ab2, toxins expressed in some Bt corn hybrids, and found that all populations were highly resistant to Cry1A.105, but had some susceptibility to Cry2Ab2. Since the amino acid sequence in Cry1A.105 is 93% similar to Cry1Ac, it is possible that resistance to one toxin confers resistance to the other (US EPA 2010, Welch et al. 2015). Results from our study align with results from previous studies, as we demonstrated varying resistance to Cry1Ac across the Carolinas; however, in addition to measuring Cry1Ac resistance across the Carolinas, we related the level of resistance (through Cry1Ac diagnostic dose bioassay survivorship) to Bt crop (corn and cotton) composition during the previous year surrounding the sample sites. Since the majority of corn and cotton planted in the Southeast is Bt, it is crucial to understand how the local composition of these primary hosts can affect Bt resistance.

Multiple studies have documented insecticide resistance in landscapes where intensive use of a specific insecticide selected for resistance over time (Huseth et al. 2015, Huseth et al. 2018, Reinders et al. 2018). These studies found that landscapes of intense use of a specific insecticide resulted in pests that were more likely to be resistant to that particular insecticide. Our results showed a similar trend; here the presence of cotton during the previous year was related to Cry1Ac resistant *H. zea* populations. However, an increased abundance of corn was negatively related to the incidence of resistant populations. Because cotton is a late-season host and a site for overwintering pupae (Fitt 1989), increased abundance of Bt cotton in the system may exert a greater effect on the source of resistant alleles, increasing resistance the following year. In contrast, as corn is increased in the system, the potential of late-season host selection of overwintering resistant alleles may be decreasing. In the landscapes surrounding our sample sites, we observed yearly shifts in abundance of corn and cotton, indicating potential changes in yearly contributions of resistant alleles. Moreover, studies have demonstrated seasonal declines in Bt cotton toxin concentration, allowing for increased survival on older Bt cotton (Carrière et al. 2017, Carrière et al. 2019). In *H. zea* populations that are highly resistant to Cry1Ac, the effectiveness of pyramided Bt cotton varieties could be reduced as the season progresses, allowing for increased survival and contribution of resistant alleles through sub-lethal selection events (Brevault et al. 2013). Although Cry1 toxins expressed in corn and cotton exhibit cross-resistance (Welch et al. 2015), the toxins expressed in corn are different than those expressed in cotton. Bt expression profiles between corn and cotton are likely different in specific tissues where larvae feed, and, independent of Bt expression, host quality is different between corn and cotton (Deans et al. 2017); therefore, selection taking place in corn could result in different resistant phenotypes than selection that takes place in cotton.

Reisig et al. (2018) observed that increased foliar sprays in Bt cotton was associated with survival of *H. zea* populations on Cry1Ac dose response assays in North Carolina suggesting field-evolved resistance for *H. zea*. To further expand our study, it would be beneficial to understand the Bt crop composition in those landscapes where foliar sprays on Bt cotton took place. In combination with our study, this could provide a roadmap for growers to focus scouting efforts or to identify locations to plant Bt cotton fields that would be at a reduced risk for economic damage. Additionally, resistance monitoring should go beyond single protein bioassays since the majority of corn and cotton planted in the U.S. expresses multiple toxins. Although the effect of each toxin acts independently (Greenplate et al. 2003), multiple toxins acting in tandem could result in unique phenotypes of resistant insects that single toxin bioassays would not capture.

Modeling by Storer et al. (2003a & b) and Sisterson et al. (2004) indicated that resistance should evolve faster in areas where there are relatively high proportions of Bt crops compared to refuge. Moreover, the Storer et al. (2003a & b) model indicated that selection for resistance is more intense in Bt cotton than Bt corn (assuming cross-resistance between Cry1Ac and Cry1Ab); however, as both crops increased in the landscape, resistance increased. Our results illustrate similar findings in that they indicate selection in cotton is more important in influencing resistance levels present in the following year. However, they also demonstrate that as the proportional area of corn in the landscape increases the impact of cotton on resistance levels in the population the following year is reduced. Results from our study will benefit IRM modeling efforts for *H. zea* by documenting the potential effect Bt cotton has on resistance. They also suggest that cotton fields in landscapes characterized by a high proportional area of corn in the previous year may be at a reduced risk for damage by *H. zea*.

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TABLES

Table 1.1. LC_{95} values from multiple dose bioassays on susceptible colonies.

Colony	LC_{95} (μg/cm^2)	Fiducial limits
Mississippi State	25.49	14.61, 158.01
Louisiana	27.58	13.50, 214.35
North Carolina	28.23	11.94, 403.65
Benzon	34.58	14.61, 158.01

Table 1.2. Summary of multiple logistic regression model parameters of log odds of bioassay survival in relation to the proportional area of corn and cotton during the previous year for 2017 and 2018.

Response variable	Model Parameters	Log odds parameter estimate	Standard error	Confidence interval		t value	*P* value
				Lower	Upper		
Bioassay survivorship	Intercept	-0.2877	0.556	-7.355	6.78	-0.52	0.6961
	Corn	-0.148	0.878	3.69	7.21	-0.64	0.5266
	Cotton	5.451	0.233	-0.61	0.32	6.21	<.0001
	Corn* cotton	-73.564	8.53	-90.65	-56.47	-8.63	<.0001

FIGURES

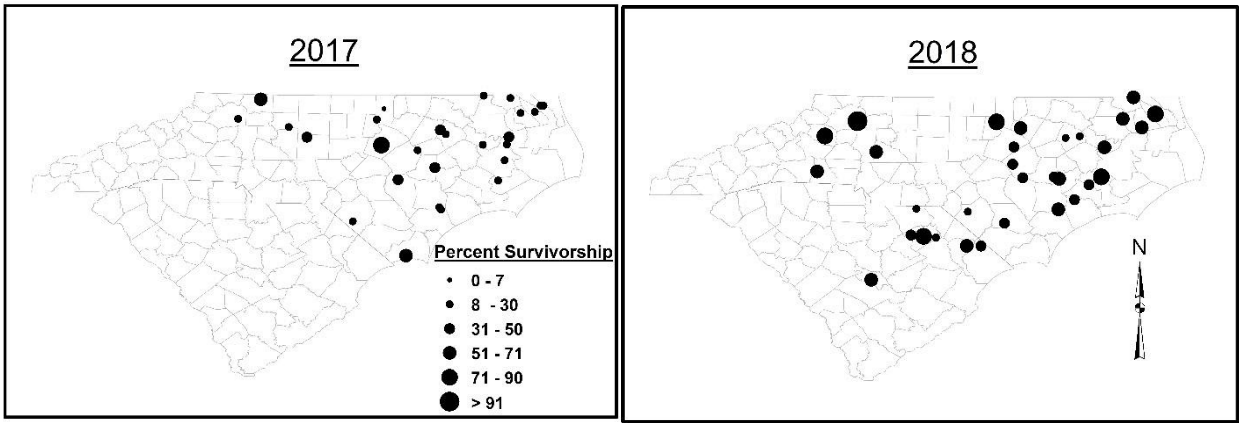

Figure 1.1. Survivorship of *H. zea* to diagnostic dose bioassays in 2017 (n=28) and 2018 (n=31). Each dot represents the sample site where *H. zea* were collected. Dot size in each figure represents percent survivorship in each diagnostic dose bioassay.

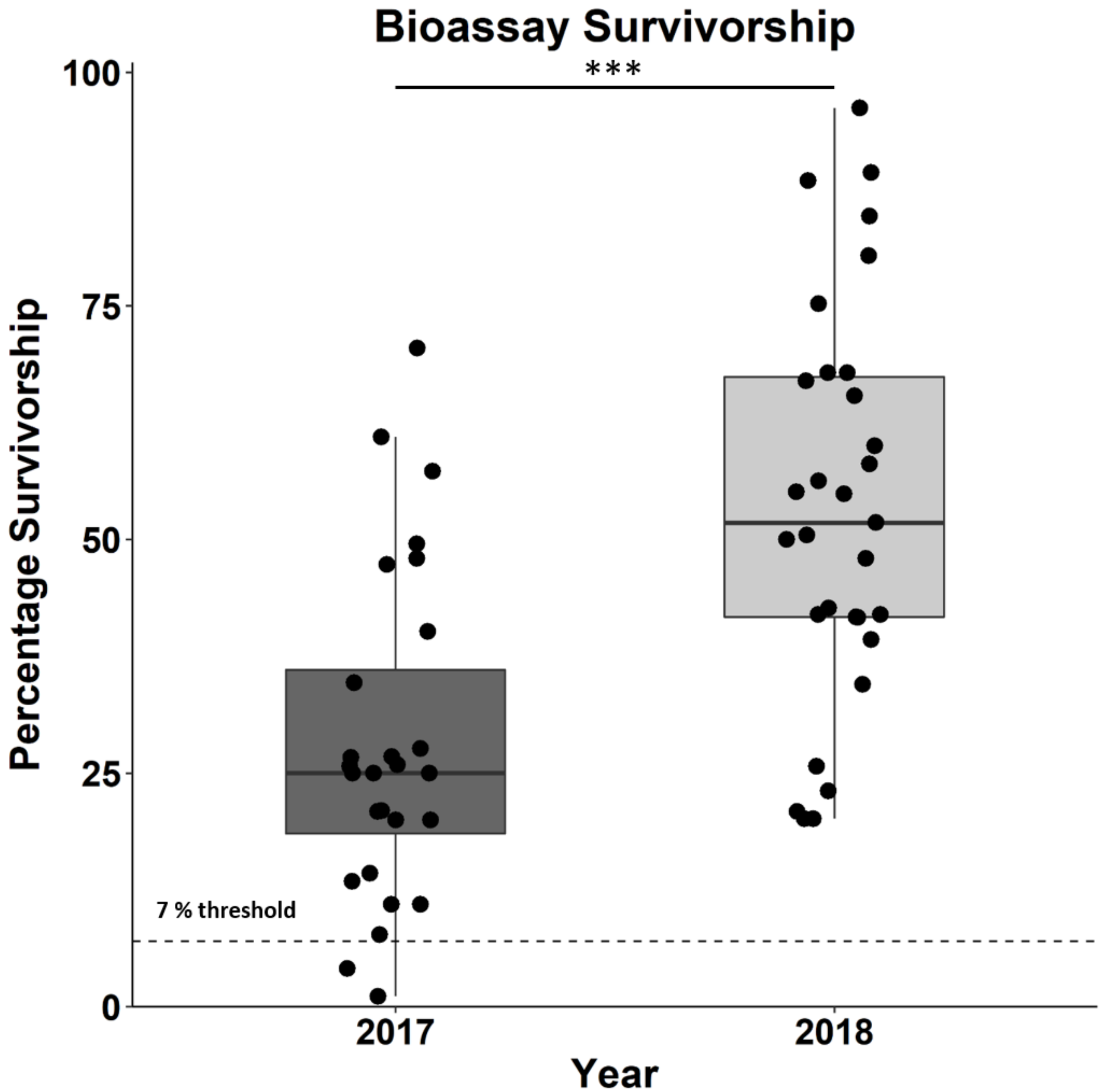

Figure 1.2. Survival distribution of field collected *H. zea* to a diagnostic dose by year. Points below the 7% threshold line were considered susceptible and points above the 7% threshold line were considered potentially heterozygous for a resistant allele to Cry1Ac at the diagnostic dose of 29µg/cm^2.

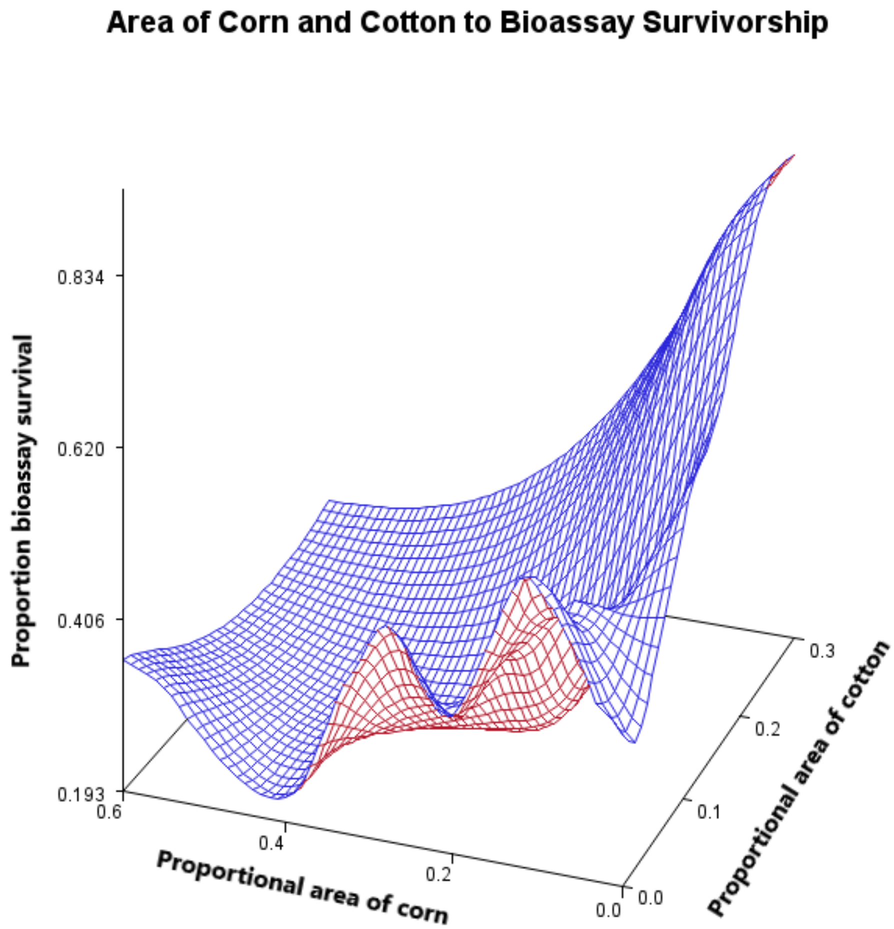

Figure 1.3. Relationship of the proportion of Cry1Ac bioassay survivorship to the proportional area of corn during the previous year and the proportional area of cotton during the previous year in a 1 km buffer radius.

Chapter 2

Helicoverpa zea (Lepidoptera: Noctuidae) Incidence and Susceptibility to Bt Corn Across North Carolina and South Carolina in Relation to the Bt Crop Composition in the Landscape

Abstract

Characterizing *Helicoverpa zea* (Boddie) damage to corn in relation to the spatiotemporal composition of Bt crops is essential to understand how landscape composition affects *H. zea* abundance and selection for Bt resistance. To examine this relationship, paired Bt and non-Bt corn plots were sampled across locations in North and South Carolina during 2017-2019. Kernel damage and larval exit holes were measured following larval development. To understand how corn and cotton composition surrounding sample sites related to feeding damage, landscape-level data were used to quantify corn and cotton composition surrounding the sample site since the vast majority of acreage of these crops is planted to Bt varieties. We then examined the relationship between corn and cotton abundance during the preceding year in a 1 km buffer surrounding sample sites relative to *H. zea* damage and larval exit holes. Across the years and locations, there was significantly less damage in Bt corn compared to non-Bt corn. However, damage in Bt corn was still widespread, indicating the presence of resistance to Bt manifest as a reduction in efficacy, but not complete loss of control. There were negative relationships between both ear damage and larval exit holes in both Bt and non-Bt corn and the proportion of corn in a 1 km buffer during the current year and the proportion of corn and cotton in a 1 km buffer during the previous year. Our study suggests that damage observed in Bt corn is dependent on both the presence of Bt resistance in the *H. zea* population and the abundance of two hosts, corn and cotton.

1 Introduction

Helicoverpa zea (Boddie) is a polyphagous pest that can feed on multiple crop and non-crop hosts (Quaintance and Brues 1905, Fitt 1989). In the Southeast, *H. zea* is multivoltine, completing four or more generations per year (Neunzig 1969, Kennedy and Storer 2000). *Helicoverpa zea* can feed on economically important crops such as corn (*Zea mays* L.), cotton (*Gossypium hirsutum* L.), and soybean (*Glycine max* L.) (Neunzig 1969). Overwintering *H. zea* typically emerge in May and feed on whorl-stage corn and other non-crop hosts (Fitt 1989, Kennedy and Storer 2000). Generally, the majority of first-generation adult oviposition coincides with silking-stage corn (Barber 1943, Hardwick 1965). In North Carolina, corn is also an excellent developmental host for the larvae (Johnson et al. 1975) and can act as a sink for second-generation *H. zea* and a source for third-generation *H. zea* larvae that infest later-season hosts when they are more suitable for oviposition, such as cotton and soybean (Kennedy and Storer 2000). Because soybean is a late-season non-Bt host, it can influence overwintering populations and abundance of *H. zea* that could potentially infest corn and other suitable hosts the following year.

Corn hybrids that express certain insecticidal endotoxins derived from *Bacillus thuringiensis* (Bt) Berliner, have activity against *H. zea*; however, since these toxins are only expressed at a moderate dose (US EPA 2018), complete control is not achieved (Storer et al. 2001, Reay-Jones and Reisig 2014). Furthermore, *H. zea* is resistant to both Cry1 and Cry2 toxins expressed in corn pyramids (Bilbo et al. 2019, Kaur et al. 2019); however, in timely planted corn, yields are not significantly impacted by *H. zea* damage (Bowen et al. 2014, Reay-Jones and Reisig 2014, Bibb et al. 2018).

Helicoverpa zea adult males captured during the months of June and July develop as larvae from hosts that utilize a C_4 (likely corn) photosynthetic pathway and adult males captured during late August and September develop from hosts that utilize a C_3 (likely cotton and soybean) photosynthetic pathway (Head et al. 2010). Additionally, high densities of *H. zea* moths have been captured in traps adjacent to corn suggesting that corn is a major source of *H. zea* (Allen and Luttrell 2011) and many larvae can be found in corn fields from June to August (Jackson et al. 2008). Subsequent *H. zea* moth captures later in the season increased in traps adjacent to cotton and soybean fields (Allen and Luttrell 2011), accompanied by high larval densities in those crops (Jackson et al. 2008). Furthermore, Allen and Luttrell (2009) found significant positive correlations between areas of corn surrounding a cotton field and number of heliothine (referring to both *H. zea* and *Chloridea virescens* Fabricius) eggs present in cotton fields during the month of August, after *H. zea* has completed development in corn. Combined with results from Head et al. (2010), these studies show a *H. zea* host shift among crops during the cropping season. Additionally, this host shift aligns with the reproductive stage of these crops (silking corn and flowering cotton and soybean), which is the stage most suitable for oviposition (Isely 1935).

Bt corn and Bt cotton were first commercialized in 1996 and expressed Cry1Ab and Cry1Ac, respectively, to control a suite of lepidopteran pests (US EPA 2018). The primary target pests for Bt corn were *Ostrinia nubilalis* (Hübner) and *Diatraea graniosella* (Dyar) and the target pests for Bt cotton were *C. virescens*, *Pectinophora gossypiella* (Saunder), and *H. zea* (FIFRA SAP 1998, Storer et al. 2001). In corn, *H. zea* was considered a non-target pest; however, Cry1Ab corn expressed a moderate dose of toxin killing 65-95% of the larvae (FIFRA SAP 1998). The Federal Insecticide, Fungicide and Rodenticide Act Scientific Advisory Panel

(1998) defined a non-target pest as: (1) a pest that is directly or potentially affected by a low to high dose expressed in a Bt crop but is of less economic importance than the targeted pest, or (2) a pest that is indirectly affected by a Bt crop through changes in patterns of insecticide use. Since its commercialization, adoption rates for Bt crops have dramatically increased. As of 2018, 83% of the corn and 92% of the cotton planted in the U.S. expressed one or more Bt toxins (USDA 2019). Moreover, widespread planting of Bt crops has been associated with a regional decline in target pest populations (Hutchison et al. 2010) as well as non-target pests, such as *H. zea*, benefiting both growers of non-Bt row and vegetable crops (Dively et al. 2018).

Concomitant with the widespread planting of Bt crops, selection pressure for Bt resistant pests has markedly increased (Tabashnik 1994, Gould 1998). One tactic to delay resistance is the high-dose refuge strategy, in which Bt plants and non-Bt plants are grown alongside each other (Gould 1998). The high-dose refuge strategy allows susceptible insects to develop on non-Bt plants and to mate with rare resistant insects that complete development on Bt plants (Gould 1998). Because Bt crops express a high dose for their primary target pests, this has been an effective strategy to manage these pests; however, since the Cry toxins expressed in Bt crops are only moderately toxic to *H. zea* (Storer et al. 2001, Horner et al. 2003, Sivasupramaniam et al. 2008).

In addition to the high dose-refuge strategy, transgenic Bt pyramids, which express two or more toxins in the same plant, are also used to delay resistance (Carrière et al. 2015). Pyramided transgenic crops are most effective at delaying resistance if each toxin has a unique pathway to kill the insect (Carrière et al. 2015). However, the longevity of pyramided crops can be reduced by cross-resistance between toxins that share similar binding sites in the insect's midgut (Welch et al. 2015). In geographical regions where a single pest feeds on multiple crops that express similar toxins, the probability of cross-resistance can increase. Because *H. zea* develops on corn before dispersing to cotton and other suitable hosts (Jackson et al. 2008) and because both corn and cotton express similar Bt toxins to which pests can have cross-resistance (Welch et al. 2015, US EPA 2018), there is considerable potential for corn to act as a selective filter to produce Bt resistant *H. zea* that may infest Bt expressing cotton (Kennedy and Storer 2000, Reisig and Kurtz 2018). Moreover, in North Carolina, Bt corn is widely planted across the landscape; a minority of growers comply with refuge requirements (Reisig 2017), which could increase the rate of Bt resistance development (Reisig and Kurtz 2018).

Storer et al. (2003a & b) used a stochastic, spatially explicit simulation model to examine how the abundance of Bt crops in the landscape can affect resistance evolution in *H. zea*. They found that landscapes of abundant Bt expressing crops (corn and cotton) would accelerate the evolution of Bt resistance in *H. zea*. Additionally, Storer (2003b) found that regions modeled with increased proportions of Bt corn increased the rate of resistance evolution. In empirical studies of a different pest, Huseth et al. (2018) discovered that landscapes with intense use of neonicotinoid seed treatments on cotton and soybean were related to increased resistance in populations of *Frankliniella fusca* (Hinds), a highly polyphagous economic pest of cotton. Similarly, Reinders et al. (2018) found varying degrees of *Diabrotica virgifera virgifera* (LeConte) susceptibility to Cry3Bb1 and mCry3A within two Nebraska counties. Fields with a history of intense corn production expressing Cry3Bb1 and mCry3A were associated with *D. virgifera virgifera* populations that exhibited decreased susceptibility to the Bt toxins, suggesting resistance management at the local level may be needed to preserve Bt durability (Reinders et al. 2018). Additionally, Carrière et al. (2012) found a positive association with *Bemisia tabaci* (Gennadius) resistance to pyriproxyfen and area of cotton treated with pyriproxyfen. In combination, these studies illustrate how intense use of insecticides at local levels drive resistance development.

Dively et al. (2016) observed *H. zea* infestation levels and damage in Bt sweet corn (*Zea mays L.* convar. *saccharata* Koern) plots that expressed Cry1A.105 + Cry2Ab2 from 2010 to 2016. In the Bt-expressing hybrids, they found that, over time, *H. zea* larvae were infesting more ears, consuming more kernels, and reaching later instars, illustrating decreased Bt susceptibility. Similar to Dively et al. (2016), we designed a study to observe field performance of Bt corn hybrids expressing Cry1A.105 + Cry2Ab2 paired with non-Bt hybrids across various agricultural production locations in North Carolina and South Carolina. Because the presence of a larval exit hole in the corn husk indicates that a larva has completed development in the ear and potentially burrowed in the ground to pupate and later emerge as an adult (Olmstead et al. 2016). Therefore, we used the presence of a larval exit hole as a proxy for completion of larval development and Bt toxin survival. We assumed that Bt-resistant populations of *H.* zea are dynamic in time and space and that the amount of feeding and larval development on Bt corn will vary across North Carolina and South Carolina. We hypothesized that there are definable spatial patterns in the extent of feeding and completion of larval development on Bt corn. To further describe this variation, we examined the landscape composition surrounding each sample site. Following results from Storer et al. (2003a & b), we assumed the landscapes of abundant corn and cotton production were associated with higher levels of Bt resistance. Accordingly, we hypothesized that landscapes of intense corn and cotton production were associated with higher levels of ear damage and larval exit holes in Bt corn due to high levels of resistance. Our objective was to describe the variation in levels of ear damage and larval exit holes in paired plantings of Bt and non-Bt corn in relation to the abundance of corn and cotton in the landscape.

2 Materials and Methods

2.1 Corn Plots

From 2017 to 2019, paired Bt and non-Bt corn plots were annually planted in corn-growing counties across North Carolina and South Carolina. The Bt hybrids included Dekalb 67-72 VT2P and 67-44 VT2P, Genuity VT Double Pro (VT2P) (Bayer CropScience, St. Louis, MO, USA). VT2P expresses the Bt toxins Cry1A.105 + Cry2Ab2. The non-Bt hybrid was Dekalb 67-70RR (Bayer CropScience). All corn plots were four to twelve rows wide and > 40 m long. Larger plots were preferred and planted when possible to minimize cross-pollination by other hybrids. Plots were planted using commercial corn planters at various populations depending on soil type and fertility. Planting dates ranged from late March to early June depending on planting conditions at each site. In 2017, 42-paired (Bt/non-Bt) plots were planted in North Carolina. In 2018, 29 plots were planted in North Carolina and 22 plots were planted in South Carolina. In 2019, 31 plots were planted in North Carolina and 16 plots were planted in South Carolina. Not all plots had Bt and non-Bt hybrids planted directly adjacent to one another, but across all plots they were planted within 50 m of each other. Plots planted during 2017 and 2019 included pairs planted to 67-70 RR and 67-72 VT2P, exclusively. In 2018, we were unable to plant 67-72 VT2P in some plots. However, we planted 27 plots to both Bt hybrids (67-44 VT2P, 67-72 VT2P) and to 67-70 RR to compare performance of both Bt hybrids to each other, as well as 67-70 RR. Seventeen plots were planted exclusively to pairs of 67-70 RR and 67-44 VT2P and 7 plots were planted exclusively to pairs of 67-70 RR and 67-72 VT2P. To confirm presence or absence of a Bt toxin in each plot, an ELISA strip test was performed using a one composite leaf sample from three random plants within each plot and following the manufacturer's instructions (QuickStix Kit for Cry1Ab Corn Leaf & Seed, Envirologix Inc., Portland, ME). Corn plots at each location were maintained using agronomic recommendations from their respective Cooperative Extension Services. GPS coordinates were recorded at each location using a cellular phone.

2.2 Ear-stage damage sampling

To ensure ear-damage to corn was caused by *H.* zea, all plots were sampled for *H. zea* larvae when plants were at the R2-R3 growth stage and when larvae were at least third instars. After *H. zea* had exited the ears but before secondary pests and pathogens had obscured the effects of *H. zea* injury, 25-35 mature ears from both Bt and non-Bt plots were sampled at random from each location and assessed for ear damage and the presence of larval exit holes. To reduce the probability of cross-pollinated ears, ears were randomly sampled from the middle rows of each plot and at least 5 m inside the plot, avoiding previously sampled ears with husks pulled to base of ear. The area damaged (cm^2) was visually measured on each ear using a gridded 6 cm by 3 cm ruler and the total number of larval exit holes per ear was recorded.

2.3 Landscape analysis

Landscape composition surrounding each corn plot was determined using remotely sensed data from the USDA National Agricultural Statistics Service-Cropland Data Layer (CDL) (USDA-NASS 2019). Because selection for Bt resistant *H. zea* occurs only in crops expressing Bt (corn and cotton), total area of corn and cotton production, in a 1 km buffer radius surrounding each sample site was quantified using ArcGIS (Version 10.1 ESRI 2018, Redlands, CA). The area within the buffer planted to soybean, which does not express Bt Cry toxins but is also an important late-season host supporting substantial populations of *H. zea*, was also quantified. A 1 km buffer radius was selected based on findings by Graham et al. (1978) that the majority of rubidium-marked *H. zea* emerging in cornfields were captured in traps within 0.8 km radius of the source field. In our study, we hypothesized that higher proportions of corn, cotton, and soybean production surrounding each sample site during the year prior to the sample collection exerted a greater influence on *H. zea* overwintering population size and Bt resistance in corn, affecting the abundance and incidence of resistance of *H. zea* in corn fields the following year. Additionally, we hypothesized that higher proportions of corn surrounding the sample sites during the sampling year will have an effect on the level of damage and larval exit holes. Therefore, annual production from the previous year was used to quantify total corn, cotton, and soybean production area within each 1 km buffer radius surrounding sample sites as well as the area of corn during the sampling year.

2.4 Statistical analysis

A Wilcoxon signed-rank test was conducted to determine if there were differences in the total area of damage (cm^2) per ear between the two VT2P hybrids, 67-72 and 67-44 (PROC NPAR1WAY, SAS Institute 2012). There was no significant difference in total area of damage between 67-72 (2.71 cm^2 ± 0.19 SEM) and 67-44 VT2P (2.37 cm^2± 0.19 SEM), Z=0.45, P = 0.65. Therefore, the data from 67-72 and 67-44 VT2P were pooled.

To analyze the effects of Bt corn on ear damage, results were analyzed using a generalized linear mixed model, modeled as a normal distribution using the identity link function (PROC GLIMMIX, SAS Institute 2012) where average area of damage per ear (cm^2) for each hybrid per plot was coded as the dependent variable and the presence of Bt, year sampled, and their interaction as the independent variables. Site location was included as a random effect. To satisfy the normality assumption, ear damage was ln(x+1) transformed. Means were separated using Tukey's Honestly Significant Difference test, α=0.05.

To analyze larval development in the ear, a similar generalized linear mixed model (PROC GLMMIX, SAS Institute 2012) was constructed with proportion of ears sampled with ≥

1 larval exit hole per sample as the dependent variable and the presence of Bt, year sampled, and their interaction as the independent variables. Site location was included as a random effect. Means were separated using Tukey's Honestly Significant Difference test, α=0.05.

Because our study includes multiple sample sites at varying distances from each other, there is the potential for spatial autocorrelation to occur among the sites, which would violate the assumption of independence in statistical models. To test for spatial autocorrelation, Moran's test was conducted in R version 3.6.0 using the *ape* package (R Core Team 2019) was conducted (Overmars et al. 2003). Using ear damage as the predictor, there was significant autocorrelation among sample sites ($P = 0.01$), indicating that sample locations close to one another have similar amounts of damage. A spherical spatial covariance structure was included to account for spatial autocorrelation among sample sites.

To relate the average area of ear damage (cm^2) and the proportion of larval exit holes per sample to the area of Bt expressing crops (corn and cotton) surrounding sample sites in the 1 km buffer radius, two generalized linear mixed models, modeled as a normal distribution using the identity link function (PROC GLIMMIX, SAS Institute 2012), were constructed (full models)(Table S2.1; Table S2.4). Both models used the same independent variables which included the proportional area of corn during the year prior to sampling (up to the 1 km buffer radius), the proportional area of cotton during the year prior to sampling (up to the 1km buffer radius), the proportional area of soybean during the year prior to sampling (up to a 1km buffer radius), the presence of Bt, and year sampled. Interactions among the independent variables were also included. Using backward elimination of non-significant terms at 0.05 level, the fixed effects in the ear damage model were proportional area of corn during the previous year (up to a 1 km buffer radius), the proportional area of cotton during the previous year (up to a 1 km buffer radius), the presence of Bt, and the year sampled were included in this reduced ear damage model (Table S2.2). The same fixed effects were included in the reduced larval exit hole model; even though the fixed effect of the proportion of cotton within the 1 km buffer radius during the previous year and year sampled was not significant, we left those terms in the model to illustrate their effects relative to the reduced ear damage model (Table S2.5). Soybean was included in each full model because it is also an important late-season host and can influence populations, although it does not express Bt. However, since it was not significant in either model, it was not included in our reduced models. No interaction terms were significant. Because the main effects in both models of the proportional areas of corn and cotton during the previous year were additive in both models, we combined them into a single term as final models (Table S2.3; Table S2.6). Finally, because the effect of year was significant for ear damage, we analyzed each year individually (final yearly model). In contrast, because the effect of year was not significant for larval exit hole, we did not analyze each year individually. Intercept for each site was included as a random effect in each model. A spherical spatial covariance structure was included to account for spatial autocorrelation among sample sites. To satisfy the normality assumption, ear damage was ln(x+1) transformed.

To understand how the proportional area of corn surrounding sample sites in a 1 km buffer radius during the sample year relates to the amount of damage and proportions of larval exit holes present, we tested similar generalized linear mixed models (PROC GLIMMIX, SAS Institute 2012). The area of damage (cm^2) per sample and the proportion of larval exit holes was coded as the dependent variable in each model and the proportional area of corn during the current sampling year surrounding sample sites in a 1 km buffer radius, the presence of Bt, and the year sampled coded as the independent variables. Cotton and soybean were excluded from this analysis because they are not attractive as hosts at the time second-generation *H. zea* are ovipositing on silking-corn (Johnson et al. 1975). A spherical spatial covariance structure was included to account for spatial autocorrelation among sample sites. To satisfy the normality assumption, ear damage was ln(x+1) transformed.

Based on the assumptions that damage levels in Bt corn will be greater where Bt resistant *H. zea* are most abundant and that differences in damage or larval exit holes between Bt and non-Bt corn reflect levels of Bt resistance, we hypothesized that these differences would be positively related to the proportional area of Bt crops (corn and cotton) in the surrounding landscape during the preceding year. To test this hypothesis, we conducted multiple linear regressions by taking the ratio of the average area of ear damage (cm^2) in Bt to that non-Bt and the ratio of the total number of larval exit holes in Bt to that in non-Bt and related them to the area of corn from the previous year (up to a 1 km buffer radius), the area of cotton from the previous year (up to a 1 km buffer radius), the area of soybean from them previous year (up to a 1km buffer radius), and the year sampled. Similar to previous models, soybean was not a significant variable; therefore, it was not included in this analysis. In sample sites that had more than one Bt variety planted (67-72 and 67-44 VT2P), the average damage or larval exit holes between the two varieties were used in the model. Multiple linear regression statistics were run in R version 3.6.0 using the *lm* base package (R Core Team 2019).

3 Results

A total of 4117 Bt and 3504 non-Bt ears of corn across 139 sample locations from 2017 to 2019 were measured for *H. zea* feeding damage (cm^2) and the presence of larval exit holes. The amount of damage and proportion of ears with larval exit holes varied per sample during 2017 through 2019. Average area of damage per ear (out of 25-35 ears per plot) ranged from zero cm^2 to > 37 cm^2 in both Bt and non-Bt plots (Fig. 2.1). Similarly, percent of ears with larval exit holes per sample ranged from a low of 0% in both Bt and non-Bt plots to 92% in Bt plots and 100% in non-Bt plots (Fig 2.2).

3.1 Bt toxin effects on damage

The main effect of toxin on area of kernels damaged was highly significant (F = 53.56; df = 1, 161; P < 0.0001) as was the main effect of year (F=49.79; df = 2, 161; P < 0.0001). Bt corn ears had significantly less damage per ear (5.65 cm^2±0.58 SEM) than non-Bt corn ears (8.59 cm^2±0.86). The toxin by year interaction was not significant (F =1.32; df = 2, 161; P = 0.27) indicating that the effect of Bt on the amount of damage did not differ across years (Table 2.1).

3. 2 Bt toxin effects on larval development

The proportion of ears with larval exit holes per sample was significantly lower in Bt than non-Bt corn (0.19 ± 0.01 SEM vs 0.31 ± 0.02, respectively, F = 66.68; df = 1, 160; P < 0.0001). Comparing the proportion of larval exit holes per sample in Bt and non-Bt, there was no significant interaction between toxin and year (F = 2.86; df = 2, 160; P = 0.06), indicating that the effect of Bt on proportion of larval exit holes per sample did not differ across years. The effect of year on the proportion of larval exit holes per Bt and non-Bt hybrids in each sample was not significant (F = 0.20; df = 2, 160; P = 0.82)(Table 2.2).

3.3 Effect of the previous year's crop composition on damage and larval exit holes

There was a significant negative relationship between ear damage and the proportional area of corn and cotton in the 1 km buffer during the preceding year (F = 27.03; df = 1,161; P < 0.0001) (Fig 2.3; Table 2.3; Table 2.4). Additionally, Bt corn ears had significantly less damage than non-Bt ears (F = 52.11; df = 1,161; P < 0.0001). The effect of year was significant (F = 55.82; df = 2, 161; P < 0.0001), reflecting higher infestation levels in 2017, but the year by toxin interaction effect was not significant. We analyzed each year individually (Table 2.5). Across all years, as the proportional area of corn and cotton during the previous year in the 1 km buffer increased, ear damage per sample significantly decreased (2017 (F = 6.68; df = 1,38; P = 0.01), 2018 (F = 10.56; df = 1, 75; P = 0.001), 2019 (F = 9.12; df = 1, 46; P = 0.004)). Additionally, Bt corn ears had significantly less damage than non-Bt ears across all years (2017 (F = 14.86; df = 1, 38; P < 0.001), 2018 (F = 28.99; df = 1, 75; P < 0.0001), 2019 (F = 9.10; df = 1, 46; P < 0.001)).

There was a significant negative relationship in the proportion of larval exit holes per sample to the proportional area of corn and cotton in the 1 km buffer during the previous year (F = 15.26; df = 1,160; P < 0.0001)(Fig. 2.3; Table 2.6; Table 2.7). Moreover, Bt corn had significantly fewer larval exit holes than non-Bt corn (F = 64.39; df = 1,160; P < 0.0001). Year did not have a significant effect on the proportion of larval exit holes (F = 0.01; df = 2,160; P = 0.91).

3.4 Effect of the current year's corn composition on damage and larval exit holes

Similar to the ear damage and larval exit hole models in relation to the proportional area of corn and cotton in a 1 km buffer radius during the previous year, average ear damage (cm^2) per sample significantly decreased as the proportional area of corn in a 1 km buffer radius during the current year increased (F = 15.54; df = 1, 161; P = 0.0001)(Fig 2.5; Table 2.8). Additionally, Bt ears had significantly less damage that non-Bt ears (F = 52.47; df = 1, 161; P < 0.0001). Since the effect of year sampled was significant (F = 56.56; df = 2, 161; P < 0.0001)(Table 2.9), we analyzed each year individually. In 2017 and 2019, there was a significant negative relationship between the proportional area of corn in the 1 km buffer radius during the current year and the average area of ear damage per sample (F = 5.47; df = 1, 38; P = 0.02 and F = 9.32; df = 1, 45; P

= 0.003, respectively). In 2018, the relationship was not significant (*F* = 2.96; df = 1, 75; *P* = 0.09). Across all years, Bt ears had significantly less damage than non-Bt ears (2017 (*F* = 14.80; df = 1, 38; *P* < 0.001), 2018 (*F* = 29.25; df = 1, 75; *P* < 0.0001), 2019 (*F* = 9.10; df = 1, 45; *P* < 0.001)).

Similarly, the proportion of larval exit holes per sample significantly decreased as the proportional area of corn in a 1 km buffer radius during the current year increased (*F* = 11.27; df = 1, 160; *P* = 0.001)(Fig 2.6;Table 2.10). Additionally, Bt ears had significantly less larval exit holes than non-Bt ears (*F* = 64.76; df = 1, 160; *P* < 0.0001). Year sampled did not have an effect on the proportion of larval exit holes (*F* = 0.21; df = 1, 160; *P* = 0.81).

3.5 Landscape effects in damage ratio between Bt and non-Bt

The ratio of the area of kernel damage between Bt and non-Bt varieties was not significantly affected by the proportional area of corn (*F* = 0.11; df = 1, 129; *P* = 0.74), cotton (*F* = 0.005; df = 1, 129; *P* = 0.94) during the previous year (up to a 1 km buffer radius), or the year sampled (*F* = 1.67; df = 2, 129; *P* = 0.17)(Fig 2.7; Table 2.11).

3.6 Landscape effects on exit hole ratio per sample between Bt and non-Bt

The ratio of larval exit holes between Bt and non-Bt varieties per sample was not significantly affected by the proportional area of corn (*F* = 0.757; df = 1, 129; *P* = 0.38), cotton (*F* = 0.858; df = 1, 129; *P*= 0.36) during the previous year (up to a 1 km buffer radius), or the year sampled (*F* = 2.15; df = 2, 129; *P* = 0.12)(Fig. 2.8; Table 2.12).

4 Discussion

Multiple studies have evaluated the performance of Bt corn compared to non-Bt corn in management of *H. zea* at a limited number of geographical locations (Reay-Jones and Reisig 2014, Reisig and Reay-Jones 2015, Dively et al. 2016). However, no study has comprehensively evaluated *H. zea* damage in paired Bt and non-Bt corn plots across a broad geographical region with such a high density of trials. Because of the inter-annual lifecycle of *H. zea*, our study analyzed the effects of both the previous year's Bt crop (corn and cotton) composition as well as the current year's corn composition within 1 km radius of sample fields to understand how the abundance of these Bt expressing crops relate to ear damage and larval exit holes present in individual corn fields. We found that the temporal and spatial crop composition surrounding sample sites influenced the amount of ear damage and presence of larval exit holes in corn. Although soybean is another important late season host in the region, our results indicated that the proportional area of soybean surrounding sample sites in the previous year had no effect on the amount of kernel damage and larval exit holes present in corn. This suggests that successful overwintering of *H. zea* completing development in soybean the previous year did not significantly influence the size of the larval infestation in corn in the current year. Because annual *H. zea* generations cycle through crops in a predictable sequence (Jackson et al. 2008, Head et al. 2010), understanding how the temporal and spatial composition of host crops will help us understand how they affect abundance and resistance selection, and how Bt corn is managing populations and contributions of resistant individuals to subsequent generations.

Our results demonstrate that Bt corn expressing Cry1A.105 + Cry2Ab2, compared to non-Bt corn, reduced ear damage from *H. zea*, indicating that resistance was incomplete across North Carolina and South Carolina (Tabashnik et al. 2014). However, Bt corn reduced damage by only 35% ± 0.02 SEM (min. 0; max. 100%) compared to previous studies in Bt corn expressing the same toxins as the varieties used here in which reductions of 90 to 95% were reported in 2010 (Dively et al. 2016) and 2012 (Reisig and Reay-Jones 2015). Our study differed from these previous studies in several important ways. In addition to evaluating hybrid kernel damage across a broad geography, through our exit hole analysis, we demonstrate that almost 20% of *H. zea* completed development in Bt ears and successfully exited the ear to pupate. This finding suggests that Bt corn is likely contributing Bt resistant *H. zea* that may later infest Bt cotton (assuming they complete pupation in the soil and are fit enough to disperse and mate). Furthermore, not all *H. zea* completing development in an ear create an observable exit hole in the husk, as some may exit the ear through the silk channel (Quaintance and Brues 1905). Therefore, our larval exit hole data may underestimate the number of larvae completing development in the ear.

Contrary to our hypothesis that greater damage and larval exit holes in Bt corn would occur in landscapes containing a relatively high proportional areas of corn and cotton due to increased selection pressure, our study revealed decreased ear damage and larval exit holes in both Bt and non-Bt corn in landscapes of high proportional areas of corn and cotton prior to the sampling year in a 1 km buffer radius. Therefore, crop composition during the previous year surrounding sample sites likely had an effect on previous year's *H. zea* populations in terms of magnitude of resistance and abundance. Moreover, our results show that there is not an association in the reduction of damage and ears with larval exit holes in the Bt corn hybrid compared to a non-Bt near isoline and the proportional area of corn and cotton during the previous year in a 1 km buffer radius; this suggests that local abundance of Bt-crop hosts (corn and cotton) and non-Bt crop host (soybean) during the previous year did not influence the level of resistance as measured by amount of ear damage or larval survival in Bt corn at out sample sites. Furthermore, we found that the proportional abundance of corn in a 1 km buffer radius surrounding our sample sites during the current year was negatively related to ear damage and the proportion of ears with larval exit holes. Because cotton and soybean fields during the sampling year are not competing hosts for the generation of *H. zea* that infests silking-stage corn, we assumed their presence would not affect the amount of damage and larval exit holes present in corn when evaluating how the current year's corn composition influenced ear damage and larval exit holes. Collectively, these results suggest that landscape composition of Bt crops (relative to the proportional area of corn and cotton) may not have as significant of an effect on Bt resistance as previously assumed (ie., in Storer 2003 a & b), but that the differences we see in damage across the landscape is probably due to abundance of *H. zea* present in corn fields. In combination, these results indicate that the presence of resistance to Bt manifests as a reduction in efficacy, but not complete loss of control. Importantly, these results illustrate how the previous year's Bt crop (corn and cotton) composition and the current year's corn composition influenced ear damage and larval exit holes in corn.

In Chapter 1, we demonstrated that the proportional area of cotton during the previous year had a significant positive effect on the level of Cry1Ac resistant larvae collected in non-Bt corn ears. Here, we demonstrated that the increase in Cry1Ac resistance, as measured in bioassays, did not affect the damage levels or proportion of ears in which larvae completed development in Bt corn expressing Cry1A.105 + Cry2Ab2. Additionally, this chapter's results illustrate that as the proportional area of corn and cotton in a 1 km buffer radius during the previous year increased, the level of ear damage and larval exit holes decreased. This suggests that the increase of resistance to Cry1Ac from the previous year's cotton was not (yet) sufficient to affect a loss of efficacy of the Cry1A.105 + Cry2Ab2 toxins expressed in corn (as indicated by no effect on Bt: non-Bt damage ratio), or a detectable increase in *H. zea* populations infesting corn (Bt and non-Bt). Because Cry1Ac and Cry1A.105 are highly similar (US EPA 2010), this indicates that Cry2Ab2 is likely affecting *H. zea* feeding, which puts greater selection pressure for resistance against Cry2Ab2.

Multiple studies have documented *H. zea* resistance to Cry1 and Cry2 proteins (Dively et al. 2016, Reisig et al. 2018, Bilbo et al. 2019, Kaur et al. 2019). In Maryland, Dively et al. (2016) documented increased ear damage from *H. zea* in sweet corn expressing Cry1A.105 and Cry2Ab2 from 2010 to 2016. Similarly, in Louisiana, field corn expressing Cry1A.105 and Cry2Ab2 failed to manage *H. zea* due to resistance (Kaur et al. 2019). Unlike previous studies that examine *H. zea* resistance to Bt through field damage and lab-based bioassays, our study related the Bt crop (corn and cotton) composition in the local landscape across a broad geographic area to the amount of ear damage present in Bt and non-Bt corn, and the contribution of putatively resistant *H. zea* to the population (using larval exit holes in Bt hybrids relative to non-Bt hybrids as a proxy for contribution to the next generation). Because Bt resistance selection occurs only in crops expressing Bt (corn and cotton), we hypothesized that areas of relatively high proportional areas of corn and cotton production would result in increased ear damage and larval exit holes in Bt corn, compared to areas of relatively low corn and cotton production, due to increased resistance selection. This hypothesis was further supported by modeling results from Storer et al. (2003a & b). However, we saw the opposite effect. Here, we present three hypotheses that may explain our results: 1) pest dilution and crowding; 2) pest suppression through increased plantings of Bt crops; and 3) refuge compliance.

Pest dilution occurs when pest densities (number of pests per plant) decrease with an increasing abundance of a pest's host and crowding occurs when pest densities increase as the pest's host abundance decreases because the population is distributed over greater or lesser abundance of suitable hosts (Bosem Baillod et al. 2017). In this study, we do not know the overall abundance of *H. zea* populations in each landscape. However, if we assume populations are equally distributed across all landscapes, infestations of *H. zea* in individual corn fields within the landscape would be expected to be relatively less dense in landscapes where the proportional area of corn in the landscape is high, reducing the probability that a sampled ear of corn would have an *H. zea* egg oviposited. Several studies have observed these effects. For example, Ricci et al. (2009) observed lower trap catches of *Cydia pomonella* L. as the proportional area of pomefruit orchards increased in a 500 m buffer. Similarly, Zaller et al. (2008) observed decreased damage from *Ceutorhynchus assimilis* and *Dasineura brassicae* in winter oilseed rape (*Brassica napus* L.) fields as the proportional area of winter oilseed rape fields in a 2 km buffer increased. Without pest dilution, individual corn fields may have experienced a relatively higher density of infestation and an associated higher probability of sampling a damaged ear compared with sampling sites in which the current year's corn is proportionately more abundant. In our study, we observed a negative relationship between the proportional area of corn in a 1 km buffer radius during the current year and the amount of ear damage and the proportion of ears with a larval exit hole. Although cotton and soybean are both primary hosts, corn is more of an attractive host to the second generation of *H. zea* (the generation that typically infests silking-stage corn) (Johnson et al. 1975); therefore, the proportional area of cotton and soybean in a 1 km buffer were not included the current year's analysis. Consequently, the spatial distribution of corn during the current year, as well as the crop's overall attractiveness to ovipositing moths compared to other hosts (Yu et al. 1992), likely has an effect on the magnitude and distribution of the *H. zea* population in corn fields; therefore, the amount of damage and larval exit holes present in corn fields. Likewise, we see a similar relationship in the previous year's proportional area of corn and cotton. We hypothesized that the proportional area of corn and cotton from the previous year would affect resistance; however, our data do not support that, indicating their presence potentially affects the abundance of the overwintering population.

Regional suppression of lepidopteran pests can be attributed to widespread planting of Bt crops in which Bt crops act as a trap crop, which has been demonstrated in both *O. nubilalis* and *H. zea* (Wu et al. 2008, Hutchison et al. 2010, Dively et al. 2018). In our study, we observed less damage and fewer larval exit holes in both Bt and non-Bt corn in landscapes planted to relatively higher proportional areas of corn and cotton during the previous year and current year's corn composition, aligning with results from previous studies that demonstrate pest suppression. In landscapes of relatively high proportional areas of Bt crops (corn and cotton) during the previous year, these crops could be acting as trap crops, with corn suppressing the population that would infest cotton and soybean, and cotton suppressing the overwintering population. Furthermore, because *H. zea* can sometimes infest whorl stage corn (Kennedy and Storer 2000), the current year's corn composition could also act as a trap crop, further suppressing *H. zea* populations that would later infest silking-stage corn; therefore, reducing damage and larval exit holes in corn. Because *H. zea* prefer flowering non-crop hosts versus whorl-stage corn, a large portion of first-generation *H. zea* are probably completing development on non-crop hosts than whorl-stage corn (Neunzig 1963, Stadelbacher 1980). Therefore, as the abundance of corn decreases in the landscape, a larger portion of first generation *H. zea* develop on non-crop hosts, increasing the unexposed population which would then oviposit on silking-corn, resulting in higher infestations than areas of relatively more abundant corn (because whorl-stage Bt corn would be suppressing first generation *H. zea*).

This dilution effect could erode as the rate of *H. zea* resistance increases. In our analysis, we did not find a significant association between damage or larval exit hole ratios in Bt and non-Bt corn and the proportional area of corn and cotton during the previous year. Consequently, variation in the amount of damage and larval exit holes across the different landscapes most likely reflects variation in *H. zea* abundance rather than variation in resistance levels. This result suggests that Bt resistant *H. zea* populations are widely distributed across the region. Studies that relate intense insecticide use at the local landscape level to resistance are often found in insects that are specialist herbivores or polyphagous species that have lower dispersal capabilities (Huseth et al. 2015, Dunbar et al. 2016, Huseth et al. 2018, Reinders et al. 2018).

Finally, refuge compliance could play an important role in the amount of damage present in Bt corn. In the Southeast, corn growers are required to plant a 20% spatially structured refuge consisting of non-Bt corn plants (US EPA 2018). Since our study found no difference in area of damage in Bt corn relative to non-Bt corn at each location in relation to the amount of corn and cotton in the landscape, it is likely that refuge compliance at a local level had a minimal effect on Bt susceptibility on *H. zea* populations. However, without knowing refuge compliance surrounding each sample site or across a larger area, it is impossible to make this assumption.

In conclusion, we demonstrated that Bt corn expressing Cry1A.105 + Cry2Ab2 reduced ear damage from *H. zea* relative to non-Bt corn; however, the suppression level was lower than reported in previous studies, suggesting higher levels of resistance may negate the suppression effect. Furthermore, our larval exit hole analysis illustrated that Bt corn is likely contributing Bt resistant *H. zea* to the landscape. Finally, when coupled with our landscape analysis, we demonstrated that the previous year’s crop and current year’s crop composition influenced ear damage and larval exit holes in corn. Importantly, our study demonstrated that although levels of damage to Bt corn are greater than previously reported, Bt corn (toxins) are continuing to provide reductions in ear damage and suppression of the overall population of *H. zea* at a localized level, even though *H. zea* are known to be highly mobile.

Our findings could change resistance management for *H. zea* through the planting of non-Bt corn varieties in landscapes that are at a reduced risk for ear damage. This could be a substantial seed cost saving to the grower as well as reducing selection for resistant *H. zea*.

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TABLES

Table 2.1. Average ear damage (cm²) across all locations in Bt and non-Bt by year.

Toxin	Average damage (cm²) across all sites (± SEM)		
	2017	2018	2019
Bt	13.93 ± 1.54	3.18 ± 0.48	2.87 ± 0.38
Non-Bt	19.02 ± 1.95	4.78 ± 0.67	3.69 ± 0.48

Table 2.2. Proportion of larval exit holes across all locations in Bt and non-Bt by year.

Toxin	Proportion of larval exit holes per sample across all sites (± SEM)		
	2017	2018	2019
Bt	0.20 ± 0.03	0.17 ± 0.02	0.21 ± 0.03
Non-Bt	0.34 ± 0.08	0.31 ± 0.04	0.27 ± 0.03

Table 2.3. Summary of the generalized linear mixed model (reduced model) results of area of ear-damage for years 2017-2019 with individual terms for area of corn and cotton from the previous year in a 1 km buffer radius.

Response Variable	Model parameters	Coefficient estimate	Standard error	t value	*P* value
Average damage (cm²) per corn plot (ln(x+1) transformed)	Intercept	3.14	0.142	22.20	<.0001
	Corn	-2.032	0.547	-3.71	0.0003
	Cotton	-2.559	1.041	-2.46	0.015
	Toxin (Bt)	-0.273	0.038	-7.21	<.0001
	Year (2018)	-1.362	0.152	-8.99	<.0001
	Year (2019)	-1.313	0.154	-8.52	<.0001
	Corn*cotton	-0.314	7.75	0.04	0.9677

Table 2.4. Summary of the generalized linear mixed model (final model) results of area of ear-damage for years 2017-2019 with the single term for combined area of corn and cotton from the previous year in a 1 km buffer radius.

Response Variable	Model parameters	Coefficient estimate	Standard error	t value	*P* value
Average damage (cm^2) per corn plot (ln(x+1) transformed)	Intercept	3.165	0.134	23.55	<.0001
	Corn and cotton	-2.189	0.421	-5.20	<.0001
	Toxin (Bt)	-0.273	0.038	-7.22	<.0001
	Year (2018)	-1.386	0.145	-9.54	<.0001
	Year (2019)	-1.336	0.149	-8.99	<.0001

Table 2.5. Summary of the generalized linear mixed model (final yearly model) results for area of damaged ears by year.

Year	Response variable	Model parameters	Coefficient estimate	Standard error	t value	*P* value
2017	Average damage (cm^2) per corn plot (ln(x+1) transformed)	Intercept	3.16	0.179	15.78	<.0001
		Corn and cotton	-2.014	0.779	-2.58	0.0137
		Toxin (Bt)	-0.336	0.087	-3.85	0.0004
2018	Average damage (cm^2) per corn plot (ln(x+1) transformed)	Intercept	1.83	0.179	8.65	<.0001
		Corn and cotton	-2.45	0.755	-3.52	0.0017
		Toxin (Bt)	-0.292	0.054	-5.38	<.0001
2019	Average damage (cm^2) per corn plot (ln(x+1) transformed)	Intercept	1.741	0.174	9.99	<.0001
		Corn and cotton	-1.976	0.654	-3.02	0.0041
		Toxin (Bt)	-0.192	0.064	-3.02	0.0042

Table 2.6. Summary of the generalized linear mixed model (reduced model) results for the proportion of larval exit holes for years 2017-2019 with the individual terms for proportional area of corn and cotton in a 1 km buffer radius.

Response Variable	Model parameters	Coefficient estimate	Standard error	t value	*P* value
Proportion of larval exit holes	Intercept	0.426	0.047	9.18	<.0001
	Corn	-0.545	0.177	-3.08	0.0024
	Cotton	-0.516	0.336	-1.53	0.1270
	Toxin (Bt)	-0.109	0.013	-8.02	<.0001
	Year (2018)	-0.022	0.049	-0.46	0.6472
	Year (2019)	-0.015	0.050	-0.32	0.7524
	Corn * cotton	0.08	2.506	0.03	0.9738

Table 2.7. Summary of the generalized linear mixed model (final model) results for proportion of larval exit holes with single term for combined proportional area of corn and cotton in a 1 km buffer radius for years 2017-2019.

Response variable	Model parameters	Coefficient estimate	Standard error	t value	*P* value
Proportion larval exit holes	Intercept	0.424	0.044	7.13	<.0001
	Corn and cotton	-0.531	0.136	-3.91	0.0001
	Toxin (Bt)	-0.109	0.014	-8.02	<.0001
	Year (2018)	-0.021	0.047	-0.44	0.6601
	Year (2019)	-0.014	0.048	-0.30	0.7672

Table 2.8. Summary of the generalized linear mixed model (final model) results for the average area of damage (cm^2) to the proportional area of corn from the current year in a 1 km buffer radius surrounding sample sites for years 2017-2019.

Response variable	Model parameters	Coefficient estimate	Standard error	t value	*P* value
Average damage (cm^2) per corn plot (ln(x+1) transformed)	Intercept	3.0755	0.1391	20.13	<.0001
	Corn	-1.5911	0.4037	-3.94	0.0001
	Toxin (Bt)	-0.2742	0.03785	-7.24	<.0001
	Year (2018)	-1.4336	0.1514	-9.47	<.0001
	Year (2019)	-1.4137	0.1535	-9.21	<.0001

Table 2.9. Summary of the generalized linear mixed model (final yearly model) results for the average area of damage (cm^2) to the proportional area of corn from the current year in a 1 km buffer radius surrounding sample sites by year.

Year	Response variable	Model parameters	Coefficient estimate	Standard error	t value	*P* value
2017	Average damage (cm^2) per corn plot (ln(x+1) transformed)	Intercept	3.058	0.1546	17.61	<.0001
		Corn	-1.3428	0.5607	-2.39	0.0217
		Toxin (Bt)	-0.3359	0.08729	-3.85	0.0004
2018	Average damage (cm^2) per corn plot (ln(x+1) transformed)	Intercept	1.654	0.1943	7.00	<.0001
		Corn	-1.5859	0.9220	-1.72	0.0896
		Toxin (Bt)	-0.2927	0.05412	-5.41	<.0001
2019	Average damage (cm^2) per corn plot (ln(x+1) transformed)	Intercept	1.677	0.1559	9.52	<.0001
		Corn	-1.8743	0.6139	-3.05	0.0038
		Toxin (Bt)	-0.192	0.0637	-3.02	0.0042

Table 2.10. Summary of the generalized linear mixed model (final model) results for the proportion of larval exit holes per sample to the proportional area of corn from the current year in a 1 km buffer radius surrounding sample sites for years 2017-2019.

Response variable	Model parameters	Coefficient estimate	Standard error	t value	*P* value
Proportion of larval exit holes per sample	Intercept	0.4085	0.044	9.28	<.0001
	Corn	-0.4498	0.134	-3.36	0.001
	Toxin (Bt)	-0.11	0.01367	8.05	<.0001
	Year (2018)	-0.028	0.04799	-0.59	0.5593
	Year (2019)	-0.02659	0.04886	-0.54	0.5871

Table 2.11. Summary of multiple linear regression model (final model) results for Bt: non-Bt damage per site in relation to the proportional area of corn and cotton during the previous year in a 1 km buffer radius surrounding sample sites for year 2017-2019.

Response variable	Model parameters	Coefficient estimate	Standard error	t value	P value	r^2
Average Bt: non-Bt damage per plot	Intercept	0.8801	0.1367	6.44	<.0001	0.027
	Corn	-0.2905	0.4501	-0.645	0.520	
	Cotton	0.1712	0.5505	0.311	0.756	
	Year (2018)	-0.1986	0.1319	-1.506	0.135	
	Year (2019)	0.0047	0.1340	-0.035	0.972	

Table 2.12. Summary of multiple linear regression model (final model) for Bt: non-Bt larval exit holes per site in relation to the proportional area of corn and cotton during the previous year in a 1 km buffer radius surrounding sample sites for year 2017-2019.

Response variable	Model parameters	Coefficient estimate	Standard error	t value	*P* value	r^2
Total larval exit holes Bt: non-Bt per plot	Intercept	0.8269	0.1780	4.645	<.0001	0.044
	Corn	-0.6951	0.5790	-1.201	0.232	
	Cotton	-0.4409	0.7071	-0.624	0.534	
	Year (2018)	-0.2313	0.1707	-1.356	0.178	
	Year (2019)	0.0775	0.1727	0.449	0.655	

FIGURES

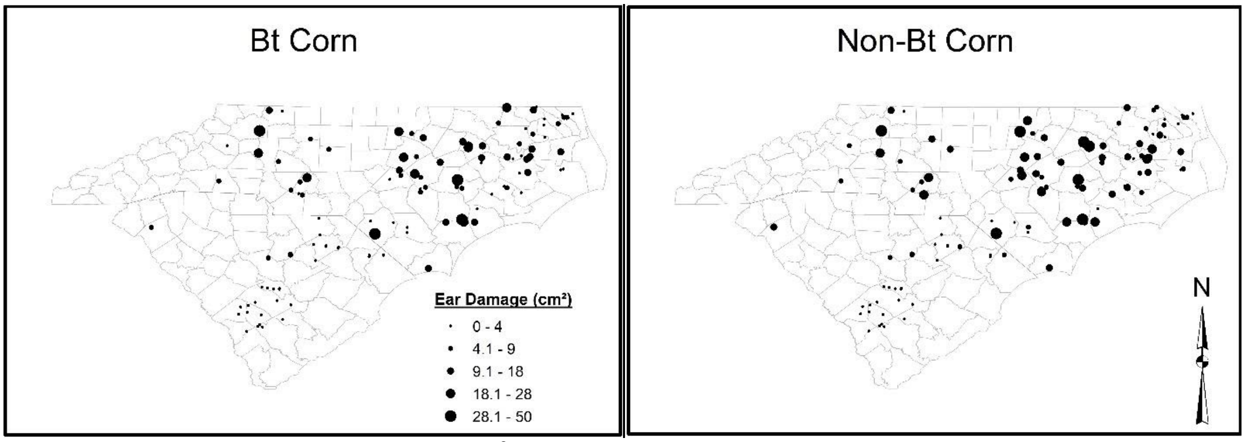

Figure 2.1. Average ear damage (cm^2) in Bt and non-Bt corn at each sample site during 2017-2019. Each dot represents a sample site. Dot size in each figure represents average ear damage (cm^2) per plot.

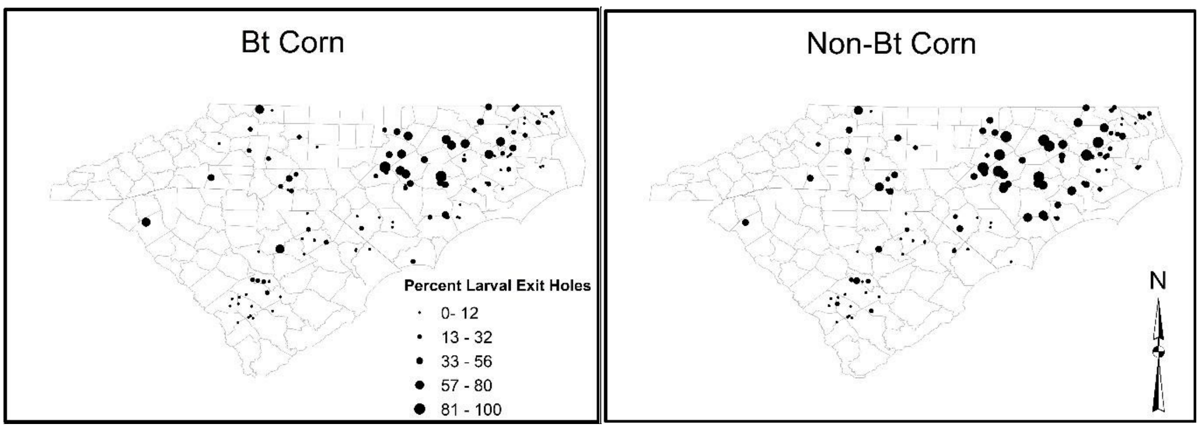

Figure 2.2. Percent larval exit holes in Bt and non-Bt corn at each sample site during 2017-2019. Each dot represents a sample site. Dot size in each figure represents percent larval exit holes per sample.

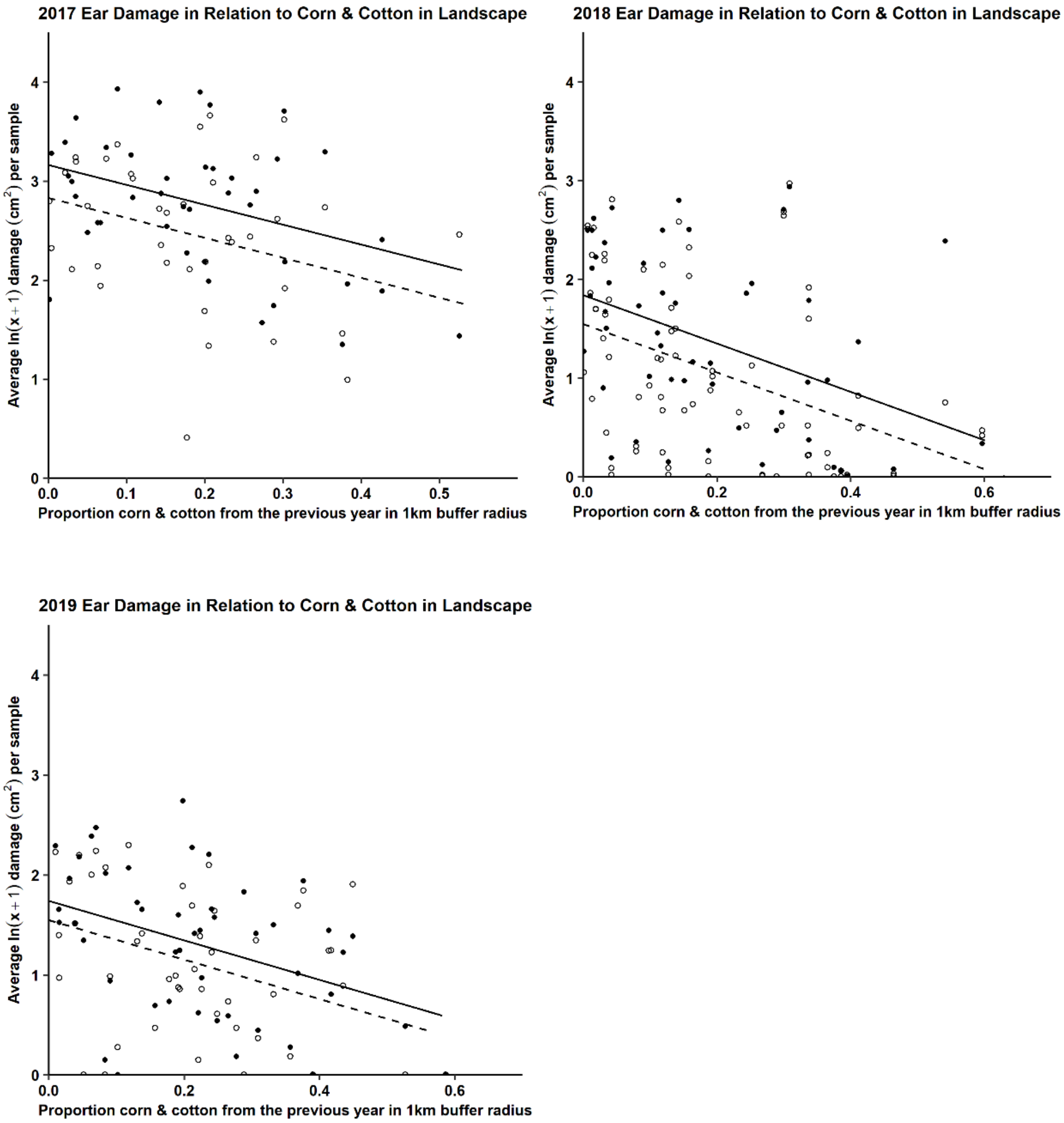

Figure 2.3. Relationship of average ln(x+1) area of ear damage (cm^2) per plot to the proportional area of corn and cotton during the previous year in a 1 km buffer radius. Open circle dots represent average ln(x+1) damage per sample in Bt corn and solid dots represent average ln(x+1) damage per sample in non-Bt corn. Dashed lines represent the regression estimates from a generalized linear mixed model for Bt corn and solid lines represent the regression estimates from a generalized linear mixed model for non-Bt corn.

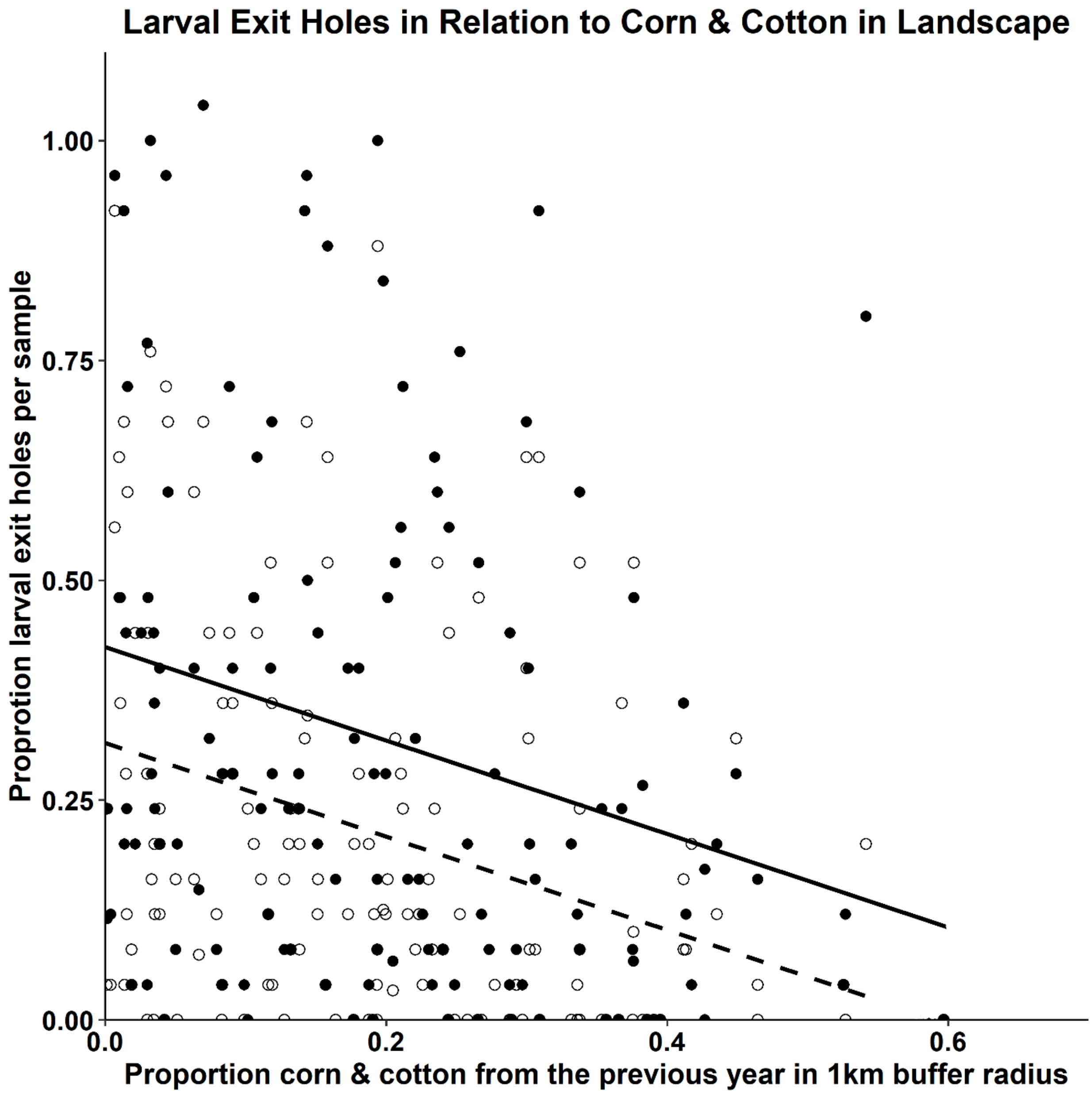

Figure 2.4. Relating the proportion of larval exit holes per sample to the proportional area of corn and cotton during the previous year in a 1 km buffer radius during 2017-2019. Open circle dots represent proportion of larval exit holes per sample in Bt corn and solid dots represent the proportion of larval exit holes per sample in non-Bt corn. Dashed line represent the regression estimates from a generalized linear mixed model for Bt corn and solid line represent the regression estimates from a generalized linear mixed model for non-Bt corn.

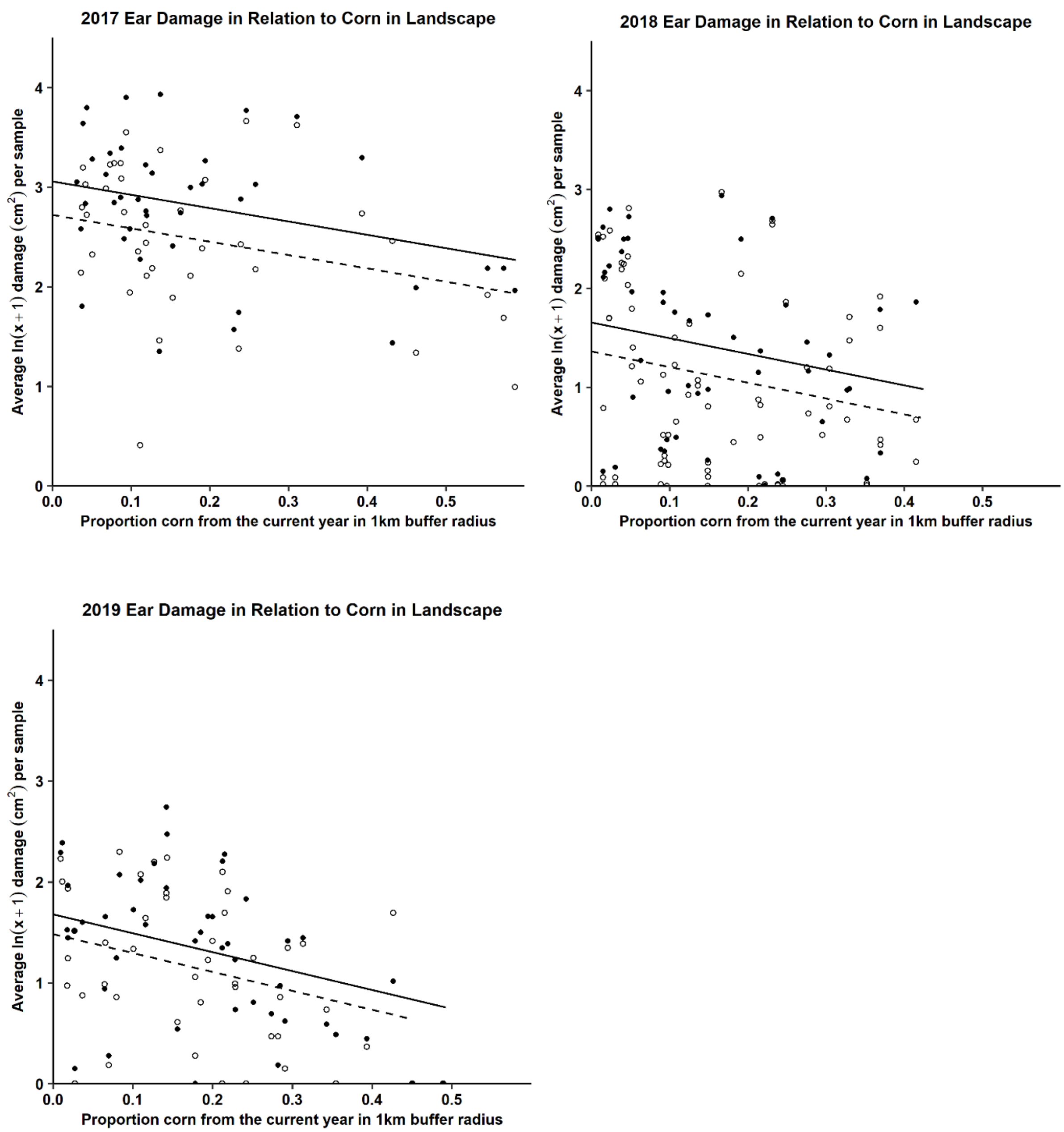

Figure 2.5. Relating average ln(x+1) area of ear damage (cm^2) per plot to the proportional area of corn during the current year in a 1 km buffer radius. Open circle dots represent average ln(x+1) damage per sample in Bt corn and solid dots represent average ln(x+1) damage per sample in non-Bt corn. Dashed lines represent the regression estimates from a generalized linear mixed model for Bt corn and solid lines represent the regression estimates from a generalized linear mixed model for non-Bt corn.

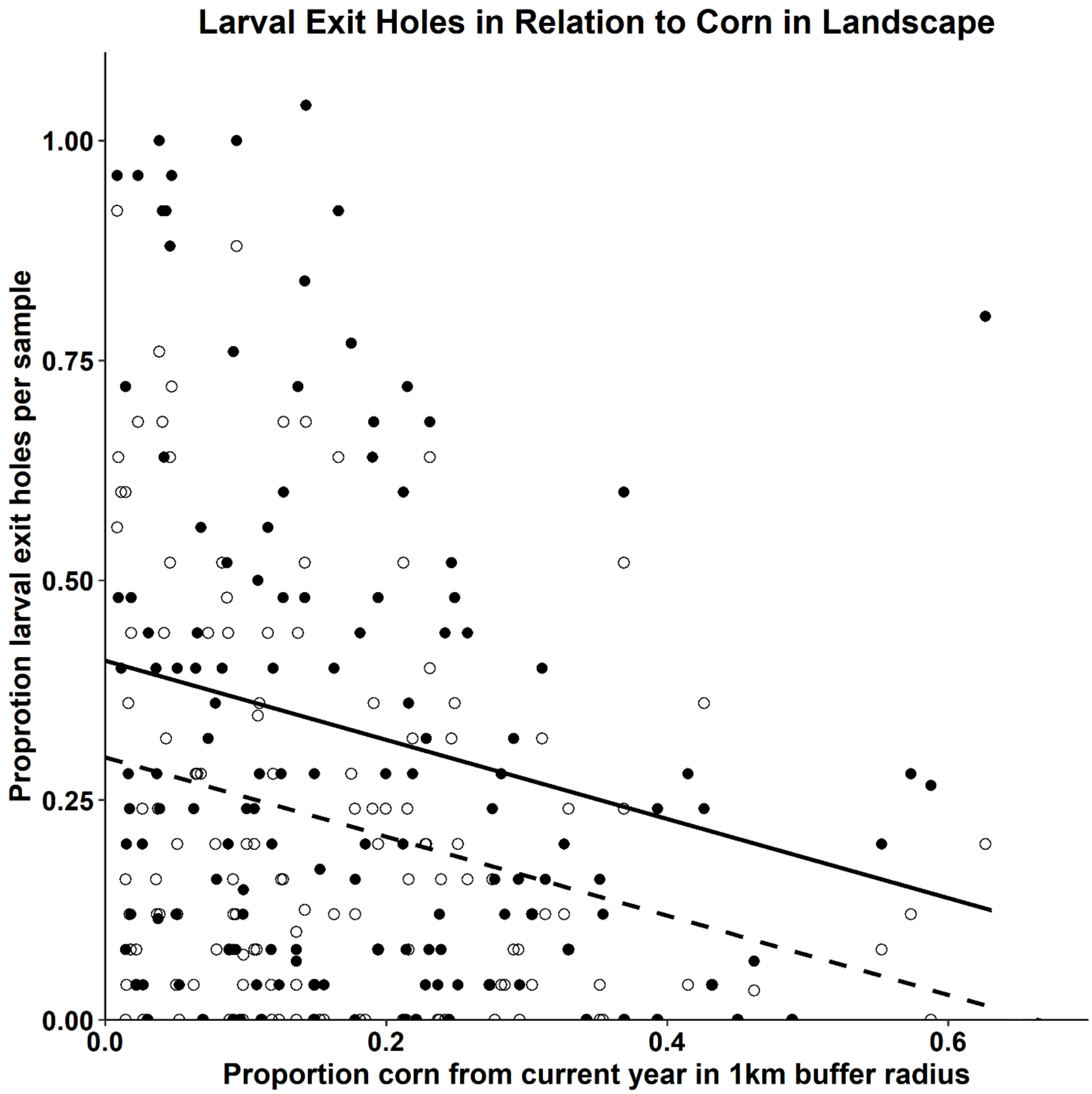

Figure 2.6. Comparing the proportion of larval exit holes per sample to the proportional area of corn during the current year in a 1 km buffer radius during 2017-2019. Open circle dots represent proportion of larval exit holes per sample in Bt corn and solid dots represent the proportion of larval exit holes per sample in non-Bt corn. Dashed line represent the regression estimates from a generalized linear mixed model for Bt corn and solid line represent regression estimates from generalized linear mixed model for non-Bt corn.

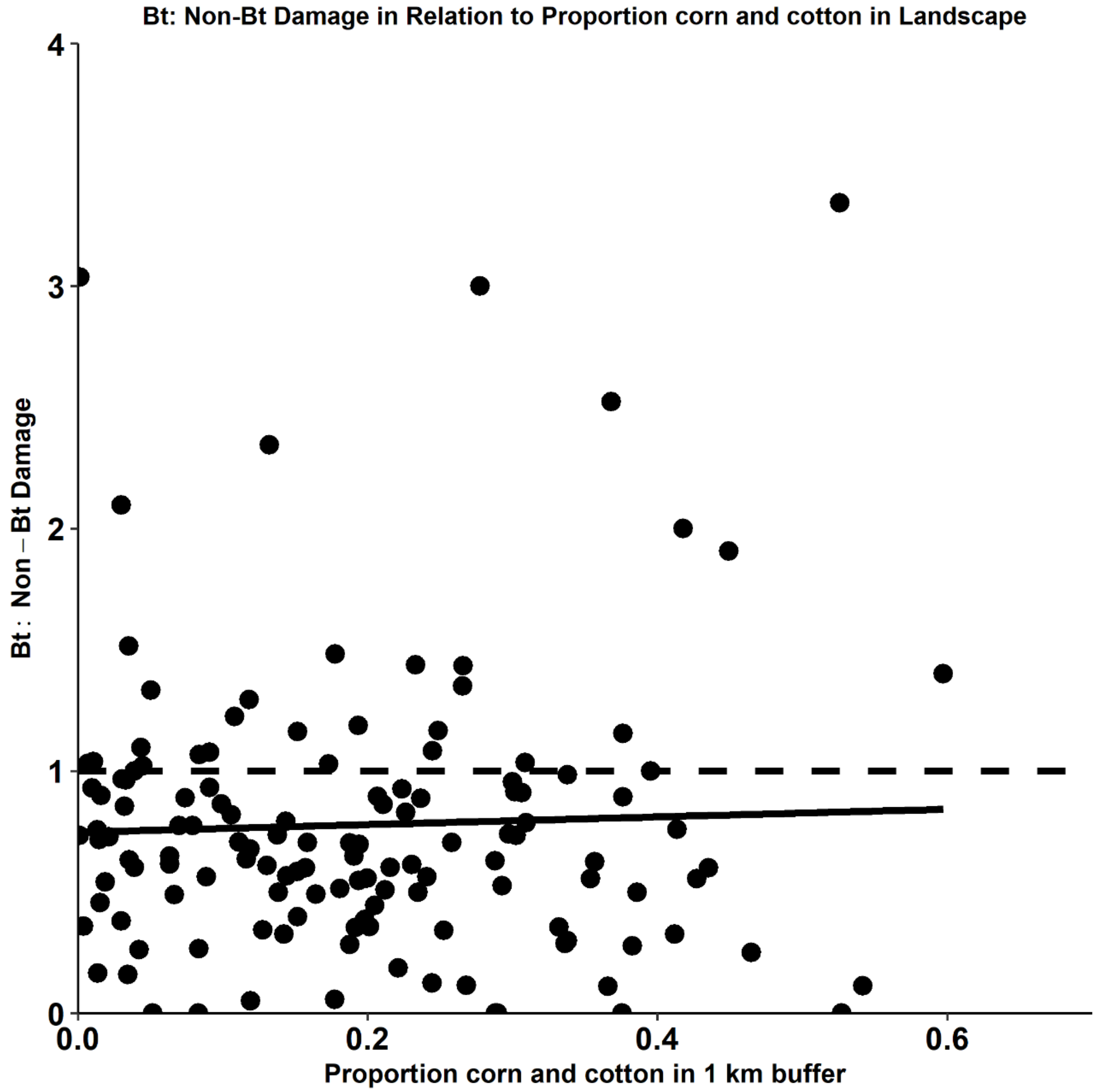

Figure 2.7. Relationship between the average damage ratio between Bt and non-Bt to the proportional area of corn and cotton during the previous year in a 1 km buffer surrounding sample sites from 2017-2019. Dots on the dashed line represent locations where damage between Bt and non-Bt were equal. Dots below the dashed line represent locations where damage in Bt was less than damage in non-Bt and dots above dashed line represent locations where damage in Bt was greater than damage in non-Bt.

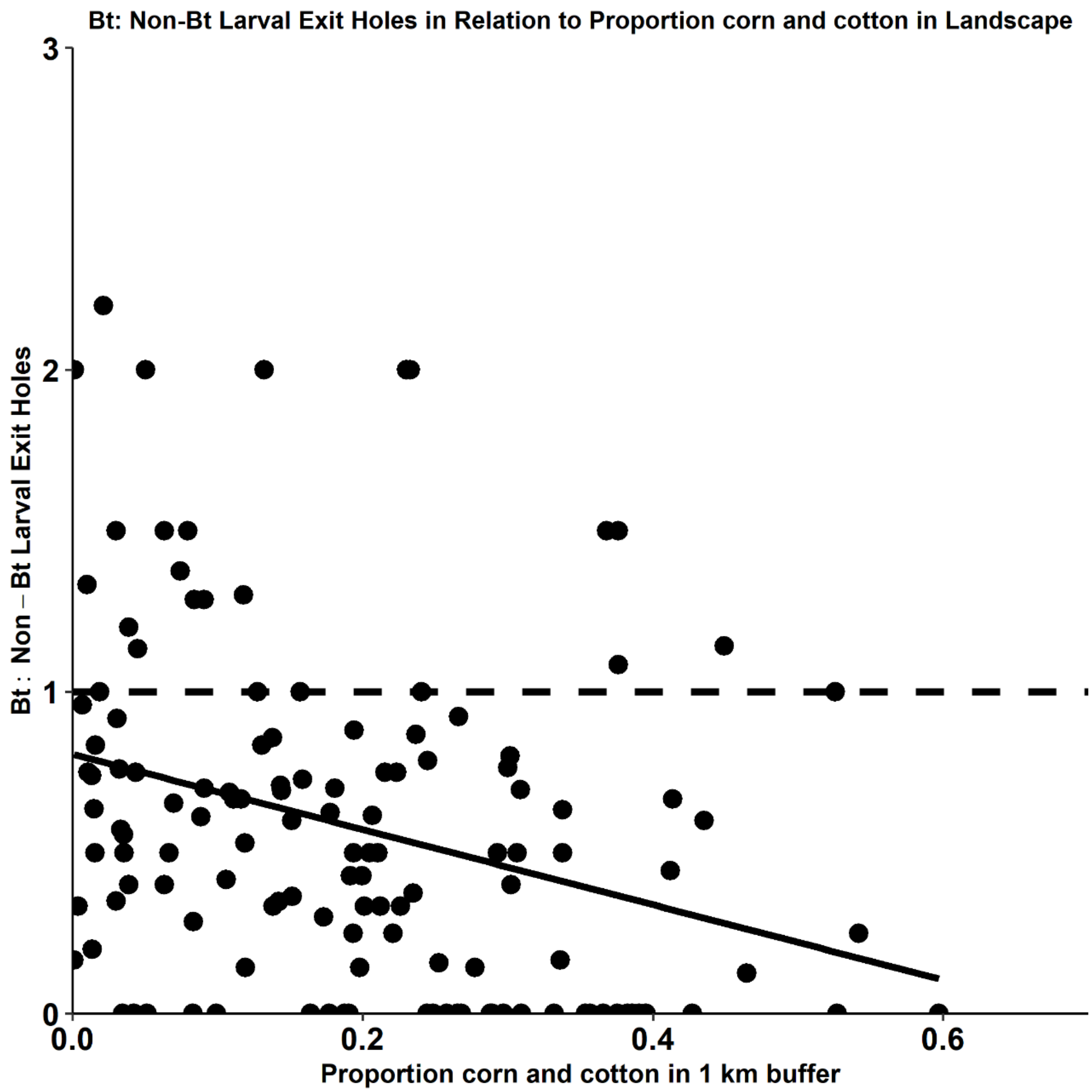

Figure 2.8. Relationship between the ratio of total larval exit holes per plot between Bt and non-Bt to the proportional area of corn and cotton during the previous year in a 1 km buffer surrounding sample sites from 2017-2019. Dots on the dashed line represent locations where larval exit holes between Bt and non-Bt were equal. Dots below the dashed line represent locations where larval exit holes in Bt was less than larval exit holes in non-Bt and dots above dashed line represent locations where larval exit holes in Bt were greater than larval exit holes in non-Bt.

SUPPLEMENTAL INFORMATION

Table S2.1. Type III effects of area of ear-damage full model

Parameter	Num. df	Denom. df	F- value	Pr>F
Previous year's corn	1	158	1.62	0.2049
Previous year's cotton	1	158	5.40	0.0214
Previous year's soybean	1	158	0.07	0.7984
Corn*cotton	1	158	1.91	0.1689
Corn*soybean	1	158	0.47	0.4940
Cotton*soybean	1	158	1.31	0.2541
Corn*cotton*soybean	1	158	1.72	0.1913
Toxin	1	158	51.21	<0.0001
Year	2	158	48.25	<0.0001
Toxin*year	2	158	1.20	0.3026

Table S2.2 Type III effects of area of ear-damage reduced model

Parameter	Num. df	Denom. df	F- value	Pr>F
Previous year's corn	1	161	13.79	0.0003
Previous year's cotton	1	161	6.05	0.0150
Corn*cotton	1	161	0.00	0.9677
Toxin	1	161	52.03	<0.0001
Year	2	161	49.27	<0.0001

Table S2.3 Type III effects of area of ear-damage final model

Parameter	Num. df	Denom. df	F- value	Pr>F
Previous year's corn + Previous year's cotton	1	161	27.03	<0.0001
Toxin	1	161	52.11	<0.0001
Year	2	161	55.82	<0.0001

Table S2.4 Type III effects of larval exit hole full model

Parameter	Num. df	Denom. df	F- value	Pr>F
Previous year's corn	1	157	1.44	0.2321
Previous year's cotton	1	157	2.29	0.1323
Previous year's soybean	1	157	0.17	0.6779
Corn*cotton	1	157	0.19	0.6614
Corn*soybean	1	157	0.31	0.5808
Cotton*soybean	1	157	0.72	0.3964
Corn*cotton*soybean	1	157	0.10	0.7551
Toxin	1	157	64.34	<0.0001
Year	2	157	0.02	0.9846
Toxin*year	2	157	2.69	0.0709

Table S2.5 Type III effects of larval exit hole reduced model

Parameter	Num. df	Denom. df	F- value	Pr>F
Previous year's corn	1	160	9.48	0.0024
Previous year's cotton	1	160	2.35	0.1270
Corn*cotton	1	160	0.00	0.9738
Toxin	1	160	64.39	<0.0001
Year	2	160	0.11	0.8981

Table S2.6 Type III effects of larval exit hole final model

Parameter	Num. df	Denom. df	F- value	Pr>F
Previous year's corn + Previous year's cotton	1	160	15.26	0.0001
Toxin	1	160	64.39	<0.0001
Year	2	160	0.10	0.9056

Chapter 3

Magnitude and Association of Whorl-Stage Bt and Non-Bt Corn Feeding by *Helicoverpa zea* and *Spodoptera frugiperda* (Lepidoptera: Noctuidae) Across North Carolina

Abstract

Helicoverpa zea (Boddie) is a common pest of corn, cotton, and soybean, but is not considered a yield-limiting pest in corn. Both corn and cotton can express *Bacillus thuringiensis* (Bt), which has moderate activity on *H. zea*. Multiple studies have documented *H. zea* resistance to Bt toxins expressed in corn and cotton, allowing for increased survival in these crops. Early generations of *H. zea* can feed on whorl-stage corn where potentially strong Bt selection can occur due to the high levels of Bt expression in the leaves relative to ear tissue. The objective of our study was to determine the magnitude of whorl-stage feeding in early generations of *H. zea* and to see if the amount of whorl-stage damage was associated with ear-stage damage. Additionally, we were interested to see if the composition of late-season hosts (cotton and soybean that could harbor overwintering pupae) during the preceding year influenced the amount of whorl-stage damaged corn plants. To accomplish this, we measured whorl-stage and ear-stage damage in 30 paired Bt/non-Bt corn plots in corn-growing counties across North Carolina in 2019. Our results indicated that whorl-stage damage is caused by both *H. zea* and *Spodoptera frugiperda* (J.E. Smith), is rare in non-Bt whorl-stage corn, and is even rarer in Bt whorl-stage corn. However, ear-stage damage by *H. zea* is common in both Bt and non-Bt corn. We found no associations between the amount of whorl-stage damage and the amount of ear-stage damage. Finally, we found no relationship in the abundance of late-season hosts during the preceding year and the amount of whorl-stage corn damage.

1 Introduction

Helicoverpa zea (Boddie) is a common pest of corn (*Zea mays* L.), cotton (*Gossypium hirsutum* L.) and soybean (*Glycine max* L.) (Fitt 1989, Kennedy and Storer 2000), as well as other crops. Of these primary hosts in the United States, most commercially planted corn hybrids and cotton varieties express *Bacillus thuringiensis* (Bt) Berliner toxins (USDA 2018) that have activity on *H. zea*; however, these Bt toxins are expressed at only a moderate dose and complete control is not achieved (Storer et al. 2001, Reay-Jones and Reisig 2014, US EPA 2018). Moreover, multiple studies have illustrated *H. zea* resistance to Cry1 and Cry2 toxins expressed in both Bt corn and Bt cotton (Reisig et al. 2018, Bilbo et al. 2019, Kaur et al. 2019). The widespread planting of Bt crops has multiple benefits such as reduced pesticide use, regional suppression of certain pests, and savings to growers (Hutchison et al. 2010, Dively et al., 2018). However, the widespread planting Bt crops also increases the selection for resistance (Tabashnik 1994, Gould 1998).

In North Carolina, *H. zea* is multivoltine and capable of completing at least four generations per year (Neunzig 1969, Kennedy and Storer 2000). The first generation, which emerges from overwintering pupae in May (Logan et al. 1979), typically infests whorl-stage corn and other non-crop flowering hosts (Quaintance and Brues 1905, Neunzig 1963, Fitt 1989, Kennedy and Storer 2000). Second-generation adult emergence coincides with silking-stage corn, the preferred host for oviposition (Barber 1943, Hardwick 1965, Johnson et al. 1975). Finally, third generation *H. zea* adults oviposit on later season flowering crops such as cotton and soybean (Kennedy and Storer 2000), where a proportion of the larvae that develop on those hosts will overwinter as pupae. Because both corn and cotton express similar Bt toxins (Welch et al. 2015), it is possible that selection for Bt resistance can occur in each of the first three generations in landscapes where Bt crops are common. Although ear damage in timely planted corn does not cause yield losses (Bowen et al. 2014, Reay-Jones and Reisig 2014, Bibb et al. 2018), *H. zea* can cause economic damage to reproductive bolls in cotton. Reisig et al. (2018) documented field-evolved resistance in North Carolina cotton fields planted to dual Bt-toxin varieties, resulting in increased foliar insecticide sprays for *H. zea*.

In addition to damage from *H. zea*, whorl-stage corn is also susceptible to damage from *Spodoptera frugiperda* (J.E. Smith) (Reay-Jones 2019). However, *S. frugiperda* does not overwinter in areas north of southern Florida and Texas (Sparks 1979), and annual dispersion occurs throughout much of the U.S. (Nagoshi et al. 2017). Prevailing winds from the south aid in northward dispersal. Typically, *S. frugiperda* arrive in North Carolina late May to June (Sparks 1979). *Spodoptera frugiperda* resistance to Cry1F has been documented in the southeastern U.S. (Huang et al. 2014), although Cry1F resistant alleles are relatively rare in North Carolina (Li et al. 2016). Additionally, these studies revealed that Cry1F resistant *S. frugiperda* also are cross-resistant to Cry1A.105, but remain susceptible to Cry2Ab2. Because certain corn hybrids express Cry1A.105 + Cry2Ab2, these hybrids can increase selection pressure for resistant *S. frugiperda* to Cry2Ab2, especially if those populations are resistant to Cry1A.105. More recently, Bilbo et al. (2020) found relatively little damage in South Carolina Bt corn plots that included hybrids expressing Cry1F and Cry1A.105, indicating levels of resistance vary in immigrating source populations. Nonetheless, given the dispersal capabilities of *S. frugiperda*, it is likely that resistant populations can spread over a large geographical area.

Managing resistance to Bt relies on two main tactics. The first is the high-dose refuge strategy, in which toxic plants are grown alongside, or near-by, non-toxic plants (Gould 1998). The high-dose refuge strategy allows susceptible insects that develop on non-toxic plants to mate with rare (assumed) resistant insects that develop on toxin plants (Gould 1998). In North Carolina, corn growers that plant Bt corn are required to plant a 20% refuge of non-Bt corn of their total acreage (US EPA 2018); however, refuge compliance among growers has been low (Reisig 2017). In addition to the high-dose refuge strategy, some Bt crop varieties express multiple toxins (pyramided) to slow the evolution of resistance (Carrière et al. 2015). These crops are more effective at delaying resistance if each toxin has a unique pathway to kill the insect such that each toxin acts independently on the insect (Greenplate et al. 2003, Carrière et al. 2015). However, cross-resistance can be an issue across similar certain toxins expressed in corn and cotton (Welch et al. 2015, US EPA 2018). Because early generations of *H. zea* typically develop on corn and later generations on cotton (both Bt crops) (Jackson et al. 2008, Head et al. 2010), there is significant potential multiple selection events to occur across multiple generations throughout the growing season which could accelerate resistance evolution (Reisig and Kurtz 2018).

In our study, we were interested in the dynamics of the generation of *H. zea* that emerges from overwintering pupae and infests whorl-stage corn. Bt toxin levels in corn can vary by the type and age of the tissue, with younger tissues typically expressing higher concentrations of Bt (Dongyan et al. 2004, Bilbo et al. 2019). Furthermore, toxin levels expressed in corn leaves are higher than levels expressed in silks and kernels (US EPA 2010). For example, Cry1A.105 is expressed 2.7 times more in leaves than silks and over 12 times more than the grain (US EPA 2010). Similarly, Cry2Ab2 is expressed 1.8 times more in leaves than silks and 100 times more than the grain (US EPA 2010). Accordingly, Pilcher et al. (1997) found no *H. zea* larval survival on whorl-stage Bt corn expressing Cry1Ab compared to a 20% survival on non-Bt plants. However, since field-evolved resistance has been documented in ear-stage Bt corn and cotton

(Reisig et al. 2018, Bilbo et al. 2019), we hypothesized that selection in Bt corn during the preceding year may lead to an increased level of resistance that allows survival on Bt whorl-stage corn.

The objectives of our study were to determine (1) the magnitude of damage to whorl-stage corn plants in both Bt and non-Bt hybrids; (2) if there is potential selection for Bt resistance in whorl-stage corn (using larval-damaged whorls as a proxy); (3) if damage levels in whorl-stage corn are associated with damage levels in ear-stage corn; and (4) if late-season host abundance (*i.e.,* cotton and soybean that could harbor overwintering pupae) during the previous year influenced infestation levels in whorl-stage corn. As mentioned previously, we hypothesized that resistance levels in *H. zea* have evolved to such a level that larvae can successfully feed and complete development on Bt whorl-stage corn and that damage levels in whorl-stage corn were associated with levels of ear-stage damage. Lastly, we hypothesized that the relative abundance of nearby late-season hosts (cotton and soybean) during the previous year were associated with damage in whorl-stage corn.

2 Methods

2.1 Corn Plots

In 2019, 31 paired Bt and non-Bt corn plots were planted in corn-growing counties across North Carolina. The Bt hybrid used was Dekalb 67-72 VT2P, Genuity VT Double Pro (VT2P) (Bayer CropScience, St. Louis, MO, USA). The non-Bt hybrid used was Dekalb 67-70RR (Bayer CropScience). All corn plots were four to twelve rows wide and > 40 m long. Larger plots were preferred and planted when possible to minimize cross-pollination among other hybrids. Not all plots had Bt and non-Bt hybrids planted adjacent to each other but all were planted at least within 50 m of each other. Plots were planted using commercial corn planters at various seed populations depending on soil type, fertility, and other planting conditions. Planting dates ranged from late March to early June depending on planting conditions at each site. Corn plots at each location were maintained using agronomic recommendations from their respective Cooperative Extension Services. To confirm that a corn plot was Bt or non-Bt, an ELISA strip test was performed following manufacturer's instructions using one composite leaf sample from three randomly selected plants in each plot (Quickstix Kit for Cry1Ab Corn Leaf and Seed, Envirologix Inc., Portland, ME). GPS coordinates were recorded at each location using a cellular phone.

2.2 Whorl-stage sampling

When corn plants were at the V4-V6 growth stage (approximately 4-6 weeks after planting), they were visually inspected for leaf damage and the presence of larvae. Whorl-stage sampling was done from mid-May to the end of June. Leaf damage was confirmed by circular holes chewed through inner leaves and the presence of frass (Fig 3.1). Inspections for damage were conducted by walking rows of the entire corn plot and recording the number of damaged plants and carefully dissecting plants in the field for larval identification. All plots with larvae present had *H. zea* present. However, *S. frugiperda*, which damages corn plants similar to *H. zea* (Fig. 3.2), was also present in some plots. Total number of plants inspected was estimated by calculating the average number of total plants in 15 m in 3 random places within the plot and then multiplying that value by total length (m) of plot and number of rows sampled.

2. 3 Ear-stage damage sampling

To verify what species damaged ear-stage corn, all plots were sampled for larvae when corn plants were at the R2-R3 growth stage and larvae were at least third instars. After larvae had exited the ear, but before secondary pathogens had obscured the effects of injury, 25 mature ears from both Bt and non-Bt plots were sampled at random from each location and measured for ear damage. To reduce the probability of sampling cross-pollinated ears, ears were randomly sampled from the middle rows of each plot and at least 5 m inside the plot, avoiding previously sampled ears. Each ear was visually measured for total area damage (cm^2) using a 6 cm by 3 cm ruler.

2.4 Landscape Analysis

Landscape composition surrounding each sample site was determined using remotely sensed data from the USDA National Agricultural Statistics Service-Cropland Data Layer (CDL) (USDA-NASS 2019). Because cotton and soybean are late-season hosts, and *H. zea* are likely to overwinter in fields planted to such crops, total area of cotton and soybean, in a 1 km buffer radius surrounding each sample site during the previous year was quantified using ArcGIS (Version 10.1 ESRI 2018, Redlands, CA). A 1 km buffer was selected based on findings by Graham et al. (1978) that the majority of rubidium-marked *H. zea* emerging from cornfields were captured in traps 0.8 km from the source field.

2.5 Statistical analysis

To determine if the relative proportion of whorl-stage damaged corn plants were associated with the relative proportion of ear-stage damage by *H. zea*, a simple linear regression model was constructed with average area ear damage (cm^2) per plot coded as the dependent variable and percent damaged whorl-stage plants coded as the independent variable. Since only three sites had damage in whorl-stage Bt corn, Bt corn was excluded from this analysis. To satisfy the assumption of normality, ear damage was $\ln(x+1)$ transformed. Average ear damage (cm^2) per plot was used since *H. zea* damage in corn expressed as area of injured kernels is collinear with the proportion of infested ears (Reay-Jones et al. 2018). Because whorl-stage damage in most sites was from *H. zea*, we did not split the analysis up between the two species.

To determine if nearby late-season hosts during the preceding year were associated with whorl-stage damage, a multiple linear regression model was constructed with the proportion of damaged whorl-stage corn plants coded as the dependent variable and the proportional area of cotton and the proportional area of soybean surrounding sample sites during the previous year (up to a 1 km buffer radius) as the independent variables. Similar to the previous model, Bt corn was excluded from the analysis since only three sites had whorl-stage damage. To satisfy the normality assumption, the proportion of damaged whorl-stage corn plants was arcsine-transformed. All regression models were run in R version 3.6.0, using the *lm* base package (R Core Team 2019).

Because our study consisted of sample sites at varying distances from each other, there was the potential for spatial autocorrelation among the sample sites to occur, which violated the assumption of independence in statistical models. To test for this, we conducted a Moran's I test (Overmars et al. 2003). Using percent of damaged whorl-stage corn plants as the predictor, we found no spatial autocorrelation among sample sites ($P = 0.67$), indicating sample sites are independent of one another in terms of whorl-stage corn damage. Moran's test was conducted in R version 3.6.0, using the *ape* package (R Core Team 2019).

3 Results

A total of 30 paired Bt and non-Bt corn plots were inspected for whorl-stage damage and ear-stage damage by *H. zea* (Fig. 3.3) out of the 31 planted. Across all sample sites, whorl-stage damage was generally low. Only three sample sites had damage in Bt corn, with the highest site containing only 0.11% whorl-stage damaged plants. No larvae were recovered in Bt corn.

Nineteen non-Bt plots had whorl-stage damage, ranging from 0% to 1.1% whorl-stage damaged plants. Of the 19 sites with damage in non-Bt corn, larvae (*H. zea* and *S. frugiperda*) were found in nine of the sites. *Helicoverpa zea* larvae were found in all sites where larvae were present and *S. frugiperda* were found in five sites. In the sites where both *H. zea* and *S. frugiperda* were found, *H. zea* were more abundant in two of the sites, comprising 66% of the larvae found, and *S. frugiperda* were more abundant in three of the sites, comprising 80% of the larvae found. However, ear-stage damage was exclusively caused by *H. zea* feeding, since only *H. zea* larvae were found during this time.

There was no relationship between the percent of damaged whorl-stage non-Bt corn plants and the amount of ear damage present in the same corn plot ($F = 0.629$; df = 1, 27; $P = 0.435$)(Fig. 3.4; Table 3.1). Similarly, the proportional area of cotton and soybean during the previous year (up to a 1 km buffer radius) had no effect on the proportion of damaged non-Bt whorl-stage corn plants (cotton ($F = 3.51$; df = 1, 26; $P = 0.07$), or soybean ($F = 0.688$; df = 1, 26; $P = 0.41$)(Fig 3.5; Table 3.2).

4 Discussion

Whorl-stage damage in corn from *H. zea* and *S. frugiperda* larvae is minor, and if present, does not usually cause damage that will result in yield loss (Quaintance and Brues 1905, Reay-Jones 2019). However, quantifying whorl-stage damage in corn is important because whorl-stage corn can serve as a host that selects for Bt resistance. Our study found whorl-stage damage in at least one non-Bt corn plant in the majority of our plots (65%) sampled; however, the percentage of damaged plants was extremely low with an average of 0.27% damaged plants in the non-Bt plots. Whorl-stage damage in Bt corn plants was even more rare, since only 10% of the locations had damage, with an average of 0.06% of damaged plants (4.5 times lower than the average number of non-Bt plants with damage). If the larvae that damaged whorl-stage corn plants survived to an adult, then on average, Bt whorl stage corn would be producing 37 adults per hectare, compared to 168 adults per hectare on non-Bt whorl-stage corn (assuming an average plant population of 62,400 plants per hectare). Early studies illustrated that whorl-stage Bt corn is more toxic than ear-stage corn (Fearing et al. 1997, Dongyan et al. 2004) with minimal survival of *H. zea* larvae on whorl-stage corn plants (Pilcher et al. 1997) and our study observed similar results. Although we found little damage in Bt plants, the presence of damage could lead to survival to adult, allowing for an additional opportunity for selection in early generations of *H. zea* and *S. frugiperda* in whorl-stage Bt corn. Furthermore, if individuals that develop in whorl-stage Bt corn are selected for a higher proportion of resistance alleles compared to those that develop in non-Bt corn or wild hosts, the contribution of whorl-stage Bt corn to resistance management could be significant.

In our study, we hypothesized that percentage of whorl-stage damage would be associated with ear-stage damage in corn. We assumed that the emergence of *H. zea* that developed on whorl-stage corn would coincide with silking-stage corn, which is highly attractive to *H. zea* females (Johnson et al. 1975); therefore, locations with higher percentages of damaged whorl-stage plants would be associated with higher amounts of ear damage. However, we found no associations between the percent of damaged plants and amount of ear damage present in non-Bt corn, indicating that whorl-stage corn may be a poor host relative to other suitable hosts (flowering non-crop) in the system that produce adults to oviposit in ear-stage corn. Both Bt and non-Bt corn had low whorl-stage damaged plants, but ear damage was present in Bt corn in 26 sites and 29 sites had ear damage in non-Bt corn, with Bt corn having less damage (3.18 cm^2± 0.52 SEM) than non-Bt corn (4.24 cm^2 ± 0.59) over all sample locations, indicating higher levels of infestations in ear-stage corn. Therefore, it is likely that the majority of the first generation of *H. zea* develop on flowering non-crop hosts and the subsequent generation disperses to ear-stage corn, a more suitable host. Furthermore, overwintering success of *H. zea* pupae is low (Slosser et al. 1975, Caron et al. 1978); therefore, populations of *H. zea* that emerge from overwintering are probably low in abundance relative to generations later in the season.

Five out of 30 of our sample sites had damage caused by *S. frugiperda*. Unlike *H. zea*, *S. frugiperda* do not overwinter in North Carolina and rely on long distance migration, assisted by southern prevailing winds, to reach locations farther north (Sparks 1979). However, we discovered late-instar *S. frugiperda* larvae in whorl-stage corn in mid-May, indicating they arrive in North Carolina earlier than previously thought. In three of the sample sites, *S. frugiperda* were responsible for the majority of the damage compared to *H. zea*. A study that investigated intraguild interactions between *S. frugiperda* and *H. zea* found *S. frugiperda* typically had a competitive edge during interactions with *H. zea* (Bentivenha et al. 2017); therefore, the three locations with a predominance of *S. frugiperda* relative to *H.* zea could be a result of a competitive advantage in locations where they were present.

Cotton, soybean, and late-planted corn fields are late-season hosts that could serve as a location for overwintering *H. zea* pupae (Caron et al. 1978). To test our hypothesis that higher levels of damaged whorl-stage corn plants would be associated with a greater area of nearby cotton and soybean fields, we quantified the proportional area of cotton and soybean during the previous year within a 1km buffer radius. Our analysis revealed no associations. However, in Chapter 2, we found that in the second generation of *H. zea*, the amount of kernel damage and larval exit holes in Bt and non-Bt corn significantly increased as the area of corn and cotton surrounding sampling sites in a 1 km buffer during the previous year decreased. Although we found no associations between the amount of damaged whorl-stage corn plants from the first generation of *H. zea* and the proportional area of cotton and soybean during the previous year within a 1 km buffer, this suggests that there could be considerable population growth in the region as the growing season progresses, in both local production and, potentially, migrants. This is further supported by previous studies that indicated that overwintering survival of *H. zea* is extremely low (Slosser et al. 1975, Caron et al. 1978) and populations of *H. zea* have the potential to increase in population size exponentially as the growing season progresses (Fitt 1989, Kennedy and Storer 2000). Furthermore, late-planted corn can also serve a location for overwintering *H. zea* (Caron et al. 1978). In our study, we do not know the planting dates of all the corn fields surrounding our sample sites; therefore, we potentially overlooked late-planted corn fields as a suitable location for overwintering *H. zea* in our analysis. Moreover, multiple biotic and abiotic factors (soil type, soil temperature, and rainfall) could influence overwintering pupal survival in the soil that we did not take into account.

Data from our study illustrate that both *H. zea* and *S. frugiperda* are feeding in whorl-stage corn. Although we did not recover any larvae in damaged Bt whorl-stage corn plants, we cannot say if all feeding we observed was caused by *H. zea* or *S. frugiperda* or if larvae that damaged those corn plants survived to an adult. However, higher levels of survival on whorl-stage Bt corn could have implications for resistance management because of an additional generation of Bt resistance selection. As the season progresses, Bt resistant populations of these pests could increase dramatically; consequently, growers could experience increased damage in Bt ear-stage corn and cotton, causing economic loss and increased foliar insecticide sprays.

Our study set out to determine if whorl-stage damage in corn was associated with ear damage as well as to determine if nearby cotton and soybean fields during the preceding year affected whorl-stage damage in corn. However, we found no associations. Our study was unique as our sample sites spanned a broad geography in North Carolina. Overall, we found that the amount of damaged whorl-stage corn plants was very low. Additionally, we found that whorl-stage Bt corn is highly effective at managing whorl-stage damage in both *H. zea* and *S. frugiperda*. However, we did observe several locations that exhibited damage in whorl-stage Bt corn, suggesting that Bt resistant *H. zea* or *S. frugiperda* populations could be evolving to survive on the concentrations of toxins present in whorl-stage corn. Future studies should observe whorl-stage corn damage in addition to ear damage for resistance monitoring to understand spatially where whorl-stage selection may be occurring to improve management practices and preserve the efficacy of Bt crops.

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TABLES

Table 3.1. Summary of simple linear regression of area of damaged non-Bt corn ears related to percent whorl-stage non-Bt damaged plants.

Response variable	Model parameters	Coefficient estimate	Standard error	t value	*P* value	r^2
Average damage (cm^2) per non-Bt corn plot (ln(x+1) transformed)	Intercept	1.359	0.141	9.644	<.0001	0.023
	Percent damaged plants	0.3187	0.402	0.793	0.435	

Table 3.2. Summary of simple linear regression of area of the proportion of whorl-stage non-Bt damaged plants to the proportion of cotton and soybean from the previous year in a 1 km buffer radius.

Response variable	Model parameters	Coefficient estimate	Standard error	t value	*P* value	r^2
Proportion damaged whorl-stage non-Bt corn plants (arcsine-transformed)	Intercept	0.01984	0.0105	1.955	0.0614	0.139
	Cotton	0.10304	0.0569	1.809	0.0821	
	Soybean	0.03461	0.830	0.830	0.4142	

FIGURES

Figure 3.1. Whorl-stage damage by *H. zea* with characteristic damage in inner leaves and presence of frass. Photo credit: Benjamin Arends, North Carolina State University.

Figure 3.2. Whorl-stage damage by *S. frugiperda*. Photo credit: Benjamin Arends, North Carolina State University.

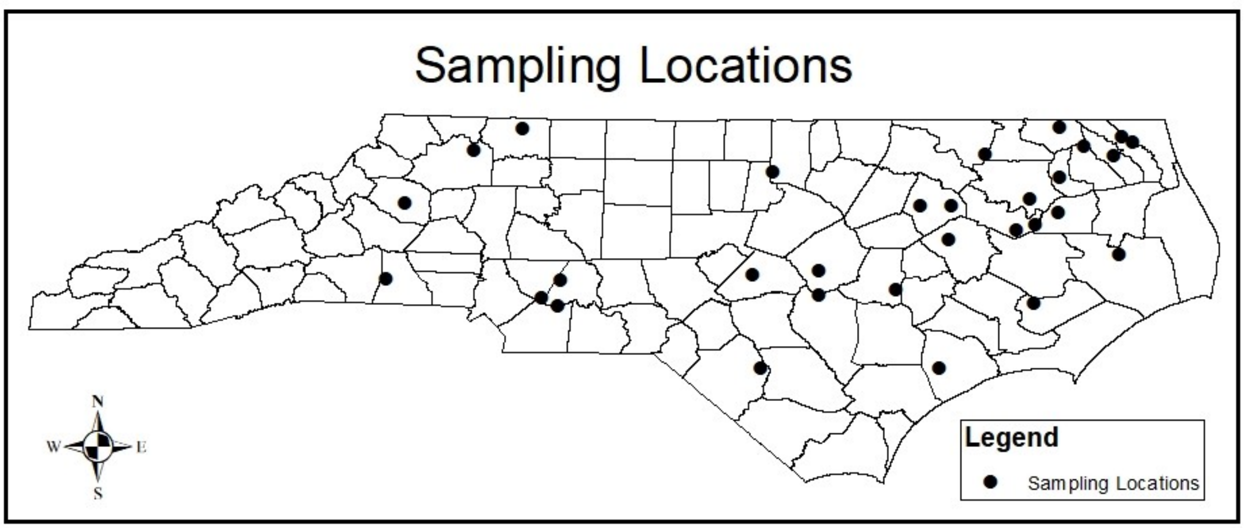

Figure 3.3. Map of sampling locations for whorl-stage damage and ear-stage damage.

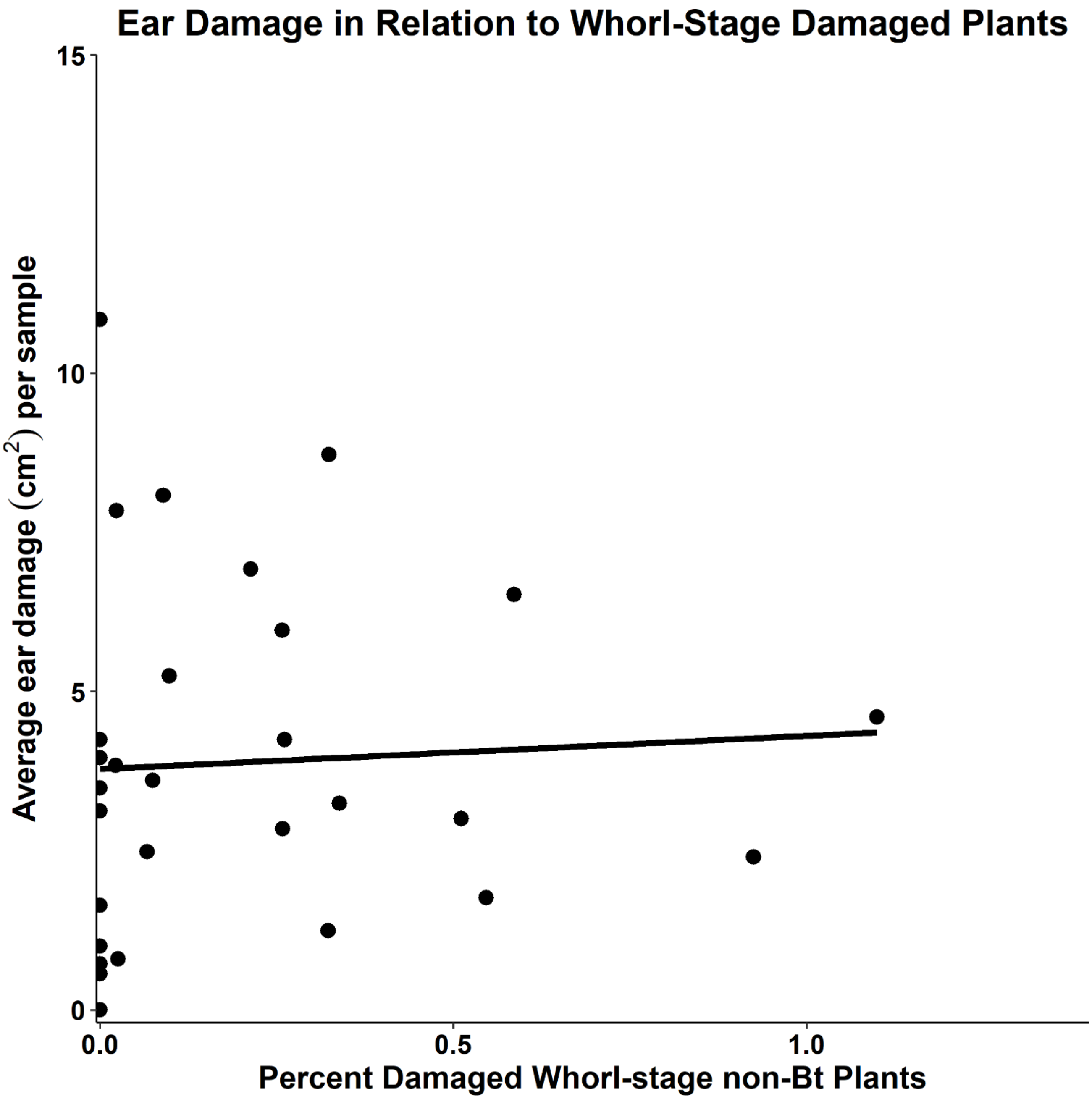

Figure 3.4. Relating the average damage (cm^2) per plot of non-Bt corn to the percent of damaged whorl-stage non-Bt corn plants per plot. Solid line represent regression estimates from the simple linear regression model.

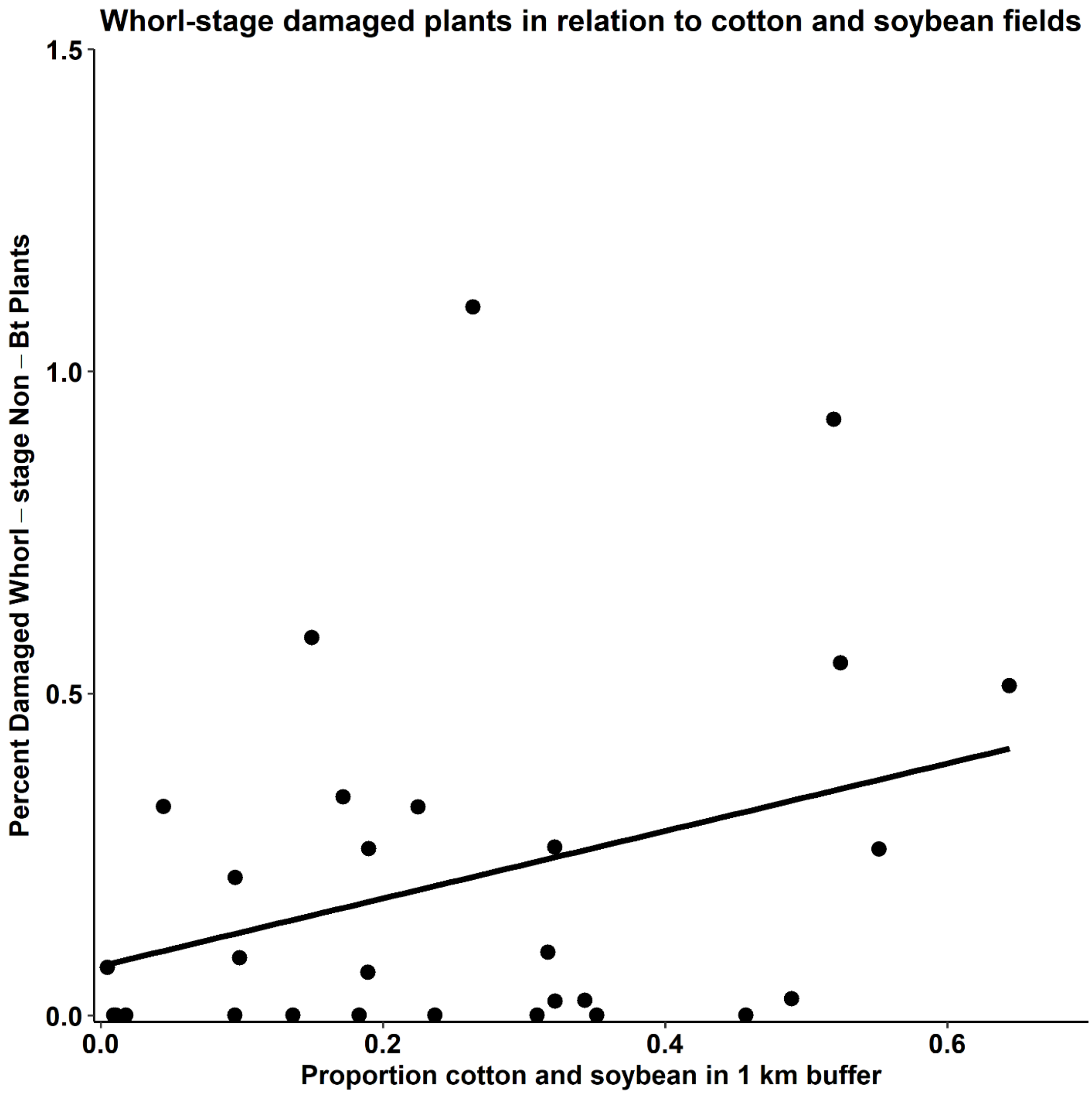

Figure 3.5. Percent of damaged non-Bt whorl-stage damaged plants relative to the proportion of cotton and soybean from the previous year in a 1 km buffer radius surrounding sample sites. Points in graph represent raw data. Solid line represent regression estimates from the multiple linear regression model.