

**ASSESSMENT OF THE INFLUENCES OF NEONICOTINOID SEED
TREATMENTS OF BT MAIZE UPON RESISTANCE MANAGEMENT
AND ENVIRONMENTAL RESIDUES**

by

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ABSTRACT

The western corn rootworm (WCR) *Diabrotica virgifera virgifera* Leconte is a major pest of maize in the United States. Currently, it is principally managed using Bt maize hybrids and neonicotinoid seed treatments (NSTs), which were concurrently introduced in the early 2000s. This simultaneous release, and subsequent rapid adoption, created a situation in which Bt maize hybrids were never assessed in the absence of NSTs, and vice versa. Consequently, neonicotinoids' influence on refuge function, primarily whether these insecticides aid or hinder the production of a sufficient population of susceptible beetles to delay resistance, has not been assessed. Moreover, a mounting suite of detrimental environmental effects of NSTs have been documented, lending some urgency to questions about their necessity.

To determine the influence of NSTs on refuge function, untreated and treated Bt maize fields were planted with 5% untreated refuge marked with ^{15}N . Throughout the field seasons of 2019 and 2020, adults were collected from these fields and analyzed to determine their natal host plant. Results documented that the numbers of refuge beetles produced by the 5% seed blend are likely insufficient to result in rates of mating to delay resistance development.

To determine if the effect of using NSTs in combination with Bt maize hybrids is additive, synergistic, or neutral at managing secondary soil pests in the Midwest, four 16 block fields were planted in 2018, 2019 and 2020 comparing four treatments (1. Untreated, Bt seed; 2. NST, Bt seed; 3. Untreated, non-Bt seed; 4. NST, non-Bt seed). Compact method sampling, root rating, and yield were used to document the presence of secondary soil pests. All three sampling years documented low abundance of white grubs and wireworms. There was minimal influence of NSTs on maize yield (2018: $p = 0.07$; 2019: $p = 0.62$; 2020: $p = 0.056$) and root damage (0-3 scale) (2018: $M = 0.0092$; 2019: $M = 0.0091$; 2020: $M = 0.0361$). These same fields were used to document the presence of NSTs in soil, as well as residues in nearby waterways. Results documented greatest soil and water clothianidin levels earlier in the season and declined as the season progressed.

CHAPTER 1. INTRODUCTION AND BACKGROUND

1.1 Introduction

The western corn rootworm (WCR) *Diabrotica virgifera virgifera* LeConte is a major pest of maize in the United States, with the overall rootworm complex, composed of the WCR as well as the Northern corn rootworm (*D. barberi*) and the Southern corn rootworm (*D. undecimpunctata howardi*), estimated to have potential to cause over \$1 billion in yearly economic damage in the United States (Gray et al., 2009). Of the three rootworm species in this complex, the WCR causes the most damage in the Midwest of the United States (Gray et al., 2009). Larval pruning of immature maize plant roots causes reduction of water uptake by the plant, in turn affecting nutrient uptake and accumulation, as well as increasing chance of disease and lodging (Verasan and Phillips, 1978; Kahler et al., 1985; Levine and Oloumi-Sadeghi, 1991). This feeding hinders the overall vigor of the plant, leading to a potential decrease in yield as well as increased expenses to control the pest (Levine and Oloumi-Sadeghi, 1991). Control of the WCR has historically been achieved using a range of tactics that include crop rotation, insecticides, genetically engineered seed and neonicotinoid seed coatings.

1.2 Western corn rootworm movement and mating behavior

The behavior of the WCR provides important insight for the development and evaluation of tactics employed to control the pest. Through the study of their movement, mating behavior and host-finding behavior, management of the WCR will continue to advance.

Movement patterns of the WCR are bimodal – occurring in the morning and the evening – with higher activity during cooler times of the day (Spencer et al., 2009). WCR activity, especially flight, is influenced by environmental conditions, with extreme environmental conditions such as temperature, darkness and windspeed restricting the flight activity of the WCR (Isard et al., 1999). Movement within or between fields will not occur if temperatures are below 15°C or exceeding 31°C (Isard et al., 1999). In addition, WCR flight will not occur during the nighttime hours. Furthermore, windspeeds exceeding 2.0 m s⁻¹ will also result in decreased flight activity (Isard et al., 1999). In addition to weather conditions, WCR activity and movement are influenced by the conditions of the soil. Greater bulk density, due to soil compaction, decreases the survival of the

WCR larvae, which in turn reduces root injury caused by the larvae (Ellsbury et al., 1994). Inter-row soil compaction, caused by the wheels of farm equipment during planting and field maintenance, inhibits larval movement to adjacent maize roots (Ellsbury et al., 1994). Adult dispersal may also be influenced by density conditions while in the larval stage. In a flight mill study conducted by Yu, Gassmann and Sappington (2019), adult WCR females reared in higher density conditions as larvae were found to have increased flight activity compared to those reared in less crowded conditions. Their results suggest that adults emerging from fields of greater larval densities are more likely to disperse than those from less crowded conditions (Yu et al., 2019). The WCR also exhibits long distance movement. Passing cold fronts may take up flying adults and transport them long distances, eventually dropping the beetle during downdrafts of the storm when passing over a large body of water. Some instances of storm transport result in large numbers of WCR beetles washing up on the southern shores of Lake Michigan (Grant and Seevers, 1989).

The WCR are a protandrous species in which the males emerge before females and must go through five to seven days of sexual maturation prior to mating. In contrast, the females are sexually mature upon emergence (Spencer et al., 2009). Following emergence from the soil, females travel up stalk of the maize plant to rest. Newly emerged females do not travel far from their site of emergence prior to mating, which typically does not occur before four hours post-emergence (Marquardt and Krupke, 2009). Females are completely sclerotized and no longer teneral after 12-24 h post-emergence (Cates, 1968). Field observations show that most mating females are teneral and, therefore, assumed to be newly emerged (Quiring and Timmins, 1990). Newly emerged females rarely travel to other portions of the field prior to mating. Proportions of unmated males moving between different portions of the field occurs at levels far greater than that of females (Spencer et al., 2013). Over half of all females exhibit calling behavior on the first day of emergence; this behavior is characterized by a fixed stance and display of the pheromone producing glands between the 7th and 8th abdominal segments (Hammack, 1995). Once exhibiting the calling behavior, the females will release a sex pheromone with the structure 8-methyldecan-2-yl propionate (Sun et al., 2018), which is also attractive to the Mexican corn rootworm *Diabrotica virgifera zea* Krysan and Smith (Krysan et al., 1980) and is structurally similar to the pheromones of the southern corn rootworm *Diabrotica undecipunctata howardi* Barber (Wilkin et al., 1986). Once emitted, the sex pheromone elicits a response from the male. The males use the pheromones released by females as a cue when searching for mates. Males have peaks in activity

in the morning and evening in response to sensing pheromones (Bartelt and Chiang, 1977). When seeking out the female, the male's antennae move quickly up and down and increase in speed as he nears her (Lew and Ball, 1979). The male will point his body in the direction of the pheromone source and fly into the plume toward the source (Lew and Ball, 1979). Upon reaching the source, the male hovers above the female and points his antennae toward her (Lew and Ball, 1979). Approaching the female from behind, the male touches her abdomen with his antennae and proceeds to engage in courtship behavior (Lew and Ball, 1979).

The mating process commences with the male climbing onto the female from the rear. He then latches onto the female's elytra with his first and second pair of legs (Lew and Ball, 1979). The female may reject the male and kick him off. If she does not reject the male, he will proceed with courtship behavior for 10 – 60 minutes prior to copulation (Lew and Ball, 1979). During this courtship period, the male may stroke the female with his antennae to relax her vaginal duct and allow the male to insert his aedeagus. Tapping of the male's antennae on the female's head calms the female, which eases the male's ability to insert his aedeagus (Lew and Ball, 1979). During copulation, the male grasps the female's abdomen to prevent her from escaping. Throughout this process, the male continues to occasionally stroke the female with his legs to calm her (Lew and Ball, 1979). The male then transfers a spermatophore into the bursa copulatrix of the female along with nutrients which are then passed on to the eggs (Murphy and Krupke, 2011). Mating lasts three to four hours during which the female is able to walk around and feed (Lew and Ball, 1979). Mating for one hour or more increases the development of eggs as well as the chance of oviposition, with a duration of two hours or more resulting in the transfer of sperm (Sherwood and Levine, 1993). According to research on likelihood of multiple matings done by Kang and Krupke (2009) in small lab enclosures, within the first ten days following their first mating, the WCR males mated an average of two times. A majority of males did not mate again for 11 to 20 days following mating (Kang and Krupke, 2009).

Following copulation, the WCR female will oviposit near roots of larval food source, primarily field corn (Spencer et al., 2009). The WCR are a univoltine species, having one generation of offspring per year (Meinke et al., 2009). One WCR female can lay over 1,800 eggs per season (Knodel et al., 2017). In late summer, females oviposit into moist soil at the base of the plant, but the adult beetles do not burrow. Instead, adult females take advantage of already existing paths through the soil. After periods of aridity, the WCR will use drought cracks in the soil to reach

moist soil for oviposition (Kirk, 1981). If the soil is too saturated, therefore eliminating drought cracks, females will use burrows produced by earthworms as pathways and sites for oviposition (Kirk, 1981). Earthworms plug the entrance of their burrows with casts during the day, but remove them at night when venturing outside the burrow. The WCR are unable to remove the casts sealing the earthworm burrow, consequently forcing them to lay their eggs at night when the burrow is opened (Kirk, 1981). The WCR eggs are characterized by their football shape and white exterior and are approximately 0.1016 mm long (Wright et al., 1999).

Oviposition into the soil occurs in late summer and eggs overwinter. There are two phases of overwintering: obligate diapause and facultative diapause (Meinke et al., 2009). Obligate diapause occurs at a fixed time and is not influenced by varying environmental conditions, while facultative diapause is optional and is prompted by extreme environmental conditions (Gullan and Cranston, 2014a). Development of the egg begins shortly after oviposition, but development is paused when it enters diapause after approximately 11 days at 20°C (Meinke et al., 2009). This initial diapause is obligate; therefore, its termination is not governed by environmental cues, it is instead ended after a fixed period that ranges from 78 to 163 days and is affected by the latitude of the location (Meinke et al., 2009). Following the phase of obligate diapause, the eggs enter facultative diapause until the soil is warmed above 11°C (Meinke et al., 2009), after which, the egg resumes development. Both egg and larval development occur more rapidly when exposed to varying temperatures. In contrast, development occurs more slowly if the eggs or larvae are exposed to a constant temperature (Wilstermann and Vidal, 2013).

The following spring, larvae hatch from the eggs and orient themselves toward the source of CO₂ being released from the maize roots. First instar larvae do not burrow; instead, they travel through pores in the soil to reach the maize roots (Spencer et al., 2009). The larval stage is composed of three larval instars (Toepfer et al., 2006). A newly hatched WCR larva is approximately 3.175 mm in length and is characterized by a white worm-like appearance. In addition to a white body, the larvae have a brown head capsule as well as three miniscule sets of legs (Wright et al., 1999). A majority of damage to the maize plant host is caused during the larval stages, with the greatest amount of damage caused by the third instar (Urias-Lopez et al., 2000). Adult WCR emerge from the soil in July and August and primarily feed on maize silk and pollen, as well as pollen from surrounding plants (Wright et al., 1999).

1.3 Management of the western corn rootworm

Integrated Pest Management (IPM) is a strategy for pest control that integrates a range of tactics to assess and control pests and maintain their populations below economic injury level (Gullan and Cranston, 2014b). The management of the WCR, using an IPM approach, aims to utilize cultural control, genetic modification of hosts, and chemical strategies to control populations.

1.3.1 Cultural control of the western corn rootworm

The system of annual crop rotation for agronomic benefits and management of corn rootworms was first introduced in the early 1900s and remained a successful practice for WCR management until the mid-1990s, when its effectiveness declined as a result of selection for rotation-resistant individuals and adaptation to this form of control (Levine et al., 2002). Crop rotation was introduced as a form of pest control for rootworm beetles because it took advantage of WCR reproductive behavior. WCR beetles are a univoltine species and have a strong fidelity to oviposition in maize fields (Spencer et al., 2009). As previously mentioned, in late summer the adult females lay their eggs in moist soil near the roots of maize. The overwintering eggs remain in diapause in the soil until the following spring, when larvae hatch and feed upon maize roots, if available (Spencer and Levine, 2008). Atypically late planting, or saturated soils may hinder host finding and increase mortality (Tinsley et al. 2008). Larval feeding on maize roots hinders the overall success of the plant; increasing the chance of disease, reducing its ability to uptake water and nutrients, and increasing the likelihood of lodging (Spencer et al., 2009). This in turn may cause a decrease in yield. Maize is the primary host-plant of WCR beetles and their larvae can only survive and develop if feeding on maize roots and some grasses (Levine and Oloumi-Sadeghi, 1991). Annual crop rotation between maize, a host plant, and soybeans, a non-host plant, prevents any development of the WCR larvae because they hatch in a soybean field the following spring and are unable to survive when feeding upon soybean roots, eventually leading to starvation. This tactic of crop rotation ultimately led to selection pressure for rotation resistant WCR (Mabry and Spencer, 2003). Soybeans produce cysteine protease inhibitors (CystPIs) that are released in response to herbivory. The ingestion of CystPIs inhibits the digestive functions of wild-type WCR by impeding proteolysis (Chu et al., 2015). Rotation resistant (RR) WCR have adapted their gut

physiology to ingesting soybeans (Curzi et al., 2012). RR WCR can't survive solely off of consuming soybeans; they must periodically switch back and forth from soybeans to maize (Mabry and Spencer, 2003). Beetles feeding solely on soybeans for two days can return to peak vigor after two days of feeding on maize (Mabry and Spencer, 2003). RR WCR have adapted to have a higher tolerance of CystPIs in soybeans through greater activity and expression of cathepsin L protease (Curzi et al., 2012). The RR WCR now inhabit and feed on non-host soybeans long enough to oviposit in the soil, allowing the larvae to hatch the following spring into a rotated maize field and feed on maize roots (Curzi et al., 2012). Oviposition in soybean fields is advantageous to the beetles because, under the typical crop rotation system in the upper Midwest, eggs laid in soybean fields would hatch in maize fields the following year (Spencer and Levine, 2008). This advantage led to selection for females with reduced ovipositional fidelity to maize fields and eventual adaptation and resistance to crop rotation (Spencer and Levine, 2008). Other tactics of IPM were then employed to combat the rotation-resistant WCR populations.

1.3.2 Genetically modified maize for control of the western corn rootworm

In addition to cultural control, the genetic modification of maize seeds is the principal strategy used in the current suite of IPM approaches managing WCR. Bt maize hybrids are genetically modified maize hybrids that produce the insecticidal Cry proteins that naturally occur in the bacterium *Bacillus thuringiensis* (Cullen et al., 2008). There are multiple strains of Bt, each targeting a specific insect pest (Cullen et al., 2008). There are six Cry proteins, which target corn rootworm larvae (Prasifka et al., 2013). Use of a “trait pyramid,” in which multiple Cry proteins are expressed in a seed, aids in delaying the pest's resistance to one trait (Prasifka et al., 2013), and is now the dominant approach. In addition to delaying resistance to a trait, pyramiding the proteins provides better root protection from the WCR larvae (Prasifka et al., 2013).

When planting Bt maize, the Environmental Protection Agency (EPA) requires that a plan of Insect Resistance Management (IRM) be employed to delay the development of pest resistance to the transgenic maize (US EPA, 2017). The IRM plan is applied by planting refuge maize seed, which lacks the Bt trait, when also planting the Bt maize. Refuge maize lacks the Bt insecticidal proteins found in transgenic Bt maize (Cullen et al., 2008). The EPA requires that a 5% refuge be planted along with Bt maize hybrids targeting coleoptera (US EPA, 2017). The refuge can be planted either adjacent to- or within the Bt maize field and can be planted in a block, in strips

within in the field, or in a seed blend incorporating both the Bt and refuge seeds (US EPA, 2017). The purpose of the IRM plan and planting a refuge is to provide a habitat for larval WCR to feed without exposure to the insecticidal Cry proteins (Cullen et al., 2008). This habitat will give rise to non-resistant, Bt-susceptible insects that will mate with potentially Bt-resistant insects and produce heterozygous offspring who are, theoretically, still susceptible to the Bt toxin, thus, delaying the development of resistance to the Bt toxin (Cullen et al., 2008). The potential for heterozygous recessive offspring is based on an assumption that the dose of toxin is high enough to kill heterozygous dominant offspring, making resistance functionally recessive. However, the Bt toxins targeting WCR are not high dose (Storer et al., 2006; Gassmann 2012) and, therefore, inheritance of resistance may be non-recessive (Andow et al., 2016). This IRM strategy also assumes random mating of individuals from refuge and Bt host plants, but this assumption is flawed (Kang and Krupke, 2009; Spencer et al., 2013; Taylor and Krupke, 2018). In reality, mating opportunities between individuals from refuge and Bt plants is not random due to several factors, including spatial separation in fields (Marquardt and Krupke, 2009), delayed emergence of adult from Bt plots (Storer et al., 2006) and differences in size of adults between Bt and refuge plots, which in turn influences mate choice (Taylor et al., 2016). The assumption that mating between Bt-resistant individuals is rare has been disproved in previous field studies (Taylor et al., 2016). Beetles emerging from strip refuge rarely disperse outside of the refuge boundary (Taylor and Krupke, 2018). In contrast, the use of seed blends produces distributions of refuge individuals and Bt-resistant individuals across a field and may better facilitate mating between the two populations to produce heterozygous-susceptible offspring (Hughson and Spencer, 2015). Though the use of seed blends produces a greater chance of mixed mating, the use of a 5% refuge is not likely to produce sufficient numbers of Bt-susceptible individuals to effectively delay resistance (Taylor and Krupke, 2018). In addition, the previously mentioned timeline of mating (Kang and Krupke, 2009), paired with the three-to-seven-day delay of adult emergence from Bt maize compared to refuge maize (Storer et al., 2006), may have an effect on WCR resistance management. Due to this timeline, females emerging from Bt maize may be more likely to mate with younger males emerging from Bt maize than with earlier-emerging refuge-fed males that will likely already have mated (Kang and Krupke, 2009). My research will help determine if NSTs influence the refuges' ability to produce a sufficient population of Bt-susceptible beetles to delay resistance to Bt maize.

In-field WCR resistance to *Cry* proteins was documented over a decade ago and has increased since. By 2009, just six years after its introduction by the EPA, resistance to Cry3Bb1 was discovered in Iowa and, in recent years, there has been an increase in WCR resistance to a majority of the other currently available *Cry* proteins (Gassmann et al., 2020). A possible key contributing factor is that the current *Cry* insecticidal proteins do not produce a high enough dose of the toxin (Gassmann, 2021). Resistance is not restricted to the fields where it first arises: a study conducted in Iowa found evidence of movement of genetic resistance to Cry3Bb1 occurring between fields of high WCR abundance and neighboring fields of lower WCR abundance (St. Clair et al., 2020). Those surrounding fields employed different management tactics than the focal fields which would typically lead to lower resistance levels, but these surrounding fields still had resistance to Cry3Bb1 (St. Clair et al., 2020). As resistance to Bt toxins increases, alternative practices become more important. Crop rotation of maize and soybeans can aid in decreasing effects of Bt-resistant WCR. Despite some occurrences of the previously-mentioned rotation resistant WCR, crop rotation is still a viable management tactic and has increased success when paired with additional tactics, such as pyramiding Bt traits (Carriere et al., 2020). Additionally, the insecticidal protein *mMpp75Aa* from the bacterium *Brevibacillus laterosporus* has shown to lead to reduction in emergence of both susceptible and resistant WCR adults (Bowen et al., 2021). More recently, genetically modified maize expressing the RNA interference (RNAi) trait DvSnf7 (stacked with Cry3Bb1 and Cry34Ab1/Cry35Ab1) has been approved for planting in 2022. Once ingested by the larvae during root feeding, this trait uses small interfering RNAs (siRNAs) as a guide to target mRNA, which is then suppressed, leading to mortality of the targeted insect (Bolognesi et al., 2012; Velez et al., 2020). Both mMpp75Aa and DvSnf7 have the potential to be a useful new tool managing WCR already resistant to currently available *Cry* proteins (Bolognesi et al., 2012; Head et al., 2017; Bowen et al., 2021). However, like the currently available *Cry* proteins, DvSnf7 is not a high dose toxin and may have a similar outcome of resistance evolution (Head et al., 2017).

1.3.3 Chemical control of the western corn rootworm

The use of chemical control by means of neonicotinoid seed treatments (NSTs) is also ubiquitous in WCR management. Neonicotinoid insecticides were first developed in the 1980s and introduced commercially in the 1990s (Goulson, 2013). Their use increased dramatically in 2003

with the introduction of neonicotinoid seed treatments (NSTs), which are applied as a seed coat prior to planting to protect seeds against early-season secondary soil pests, such as white grubs and wireworms (Douglas and Tooker, 2015; Jordan et al., 2012; Wilde et al., 2004). This increase occurred alongside the introduction of Bt maize hybrids targeting WCR (Douglas and Tooker, 2015). NSTs act systemically as the plant develops, potentially protecting the entire plant from pests (Goulson, 2013). In field maize, this systemic protection is present in the plant tissues for a maximum of two to three weeks after planting (Alford and Krupke 2017). Neonicotinoids attack the central nervous system (CNS) of insects by binding to and stimulating nicotinic acetylcholine receptors through agonism, leading to paralysis, or possibly death at higher concentrations (Goulson, 2013). The principal NSTs used in maize are clothianidin and thiamethoxam (Goulson, 2013).

Concerns around neonicotinoids have risen as knowledge of their detrimental environmental effects has come to light. Neonicotinoids are water soluble, which allows them to leach into surrounding waterways. Studies find higher concentrations of neonicotinoids appear in waterways after precipitation events post-planting and taper off as the growing season progresses (Hladik et al., 2014; Alford and Krupke, 2019). A study conducted in Pennsylvania analyzed water samples collected from no-till crop field lysimeter plots and found thiamethoxam concentration levels were highest following planting and decreased as the study progressed, while clothianidin levels persisted throughout the study (Frame et al., 2020). In addition to their water solubility, neonicotinoids are persistent, with repeated applications leading to accumulation in soils (Hladik et al., 2018). Approximately 2% of the neonicotinoid seed coating is actually absorbed by the plant, leaving the remaining 98% to either accumulate in the soil or run off into surrounding waterways (Alford and Krupke, 2017). Moreover, the water-soluble nature and systemic activity of neonicotinoids also affects non-pest arthropods (Goulson, 2013). When exposed to sublethal clothianidin concentrations, aquatic invertebrates, including water bugs (*Belostoma flumineum*) and crayfish (*Orchonectes propinquus*), experienced altered behavior, causing reductions in prey consumption and response to external stimuli, respectively (Miles et al., 2017). Non-crop plants surrounding agricultural fields also contain neonicotinoids in their pollen and nectar, having a negative impact on pollinators (Krupke and Long, 2015). In addition to non-pest arthropods, neonicotinoids can cause both sublethal and lethal effects in vertebrates, such as birds and mammals (Goulson, 2013; Roy and Coy, 2020). Spills of neonicotinoid treated seeds are consumed

by birds and mammals and provide a large enough number of seeds to cause sublethal effects (Goulson, 2013; Roy and Coy, 2020).

The use of neonicotinoids may violate a key principle of IPM, which is to first assess the populations of pests before applying an insecticide (Furlan and Kreutzweiser, 2015). Insecticide treatments should not be applied unless there is evidence of pest levels exceeding economic threshold, which, in the case of WCR management, is quantified by measuring root injury on a 0 – 3 point node injury scale (Oleson et al., 2005). In this scale, the number preceding the decimal denotes the number of full nodes eaten and the number following the decimal denotes the percentage of the next node eaten (Oleson et al., 2005). The minimum node injury score that causes economic damage can be calculated by removing I (root injury) from the Economic injury level (EIL) equation ($EIL = \frac{C}{V \times I \times D \times K}$) so that the equation to find the minimum node injury score to cause economic damage = $\frac{C}{V \times D \times K}$, where C is management cost, V is market value per unit of product, D is yield loss per unit number of insects and K is the reduction of insect population due to control measures (Oleson et al., 2005). Monitoring for early season root feeders, such as white grubs and wireworms, is typically conducted the previous season, if it is done at all. This task can be difficult and time consuming, leading most growers to instead take preventive measures, including planting seed treated with NSTs (Sappington et al., 2017). In contrast to monitoring, NSTs are applied to the seed before it is even planted, without any knowledge of infestation (Furlan and Kreutzweiser, 2015). Research conducted by Alford and Krupke (2018) found that NSTs can perform similarly, in terms of WCR management, with other insecticides and Bt hybrids, and also have a comparable probability of recovering costs of growers. With this in mind, Alford and Krupke (2018) proposed the notion of offering NSTs as an optional form of protection from WCR feeding, to incorporate into a grower's insecticide rotation. Making the use of NSTs optional to growers could reduce their expenses (i.e. reduce costs associated with Bt maize hybrids), as well as decrease the chance of deleterious environmental effects and delay the resistance of pests to both NSTs and Bt toxins (Alford and Krupke, 2018). My research will help determine if there is a benefit to combining both NSTs and Bt maize hybrids in managing WCR. If this combination of tactics is successful in achieving increased killing, in which beetles resistant to one of the tactics are susceptible to the other, employing them in tandem would be beneficial. However, if Bt hybrids alone are sufficient in preserving yield, the limited early protection from NSTs, as well as their

extra expenses, may have little use in WCR management. In addition to this, my research will help measure neonicotinoid insecticide residue levels found in both agricultural and non-agricultural soil and waterways in Indiana.

1.4 Management of secondary soil pests

Prior to the introduction of Bt hybrids, WCR were managed using a range of soil-applied insecticides which also aided in managing soil-dwelling secondary pests. However, with the release of WCR-targeting Bt hybrids, the addition of NSTs aided in management of soil pests not targeted by the Bt insecticidal proteins (Douglas and Tooker, 2015). Although they are used on all maize, the use of NSTs in secondary soil pest management is only necessary in regions with economically significant levels of pest pressure and damage. In general, there is low pressure and economic damage associated with sporadic pests across the Midwest, including wireworms or white grubs (Sappington et al., 2017). While NSTs can help protect yield in fields that exceed the white grub threshold (Jordan et al., 2012), environments which lack damaging populations of secondary soil pests do not exhibit significant differences in yield as a result of NST use (Wilde et al., 2007; Labrie et al., 2020). Their sporadic presence and inconsistent levels of damage may be better managed by focusing on monitoring for pest presence, rather than a prophylactic approach.

My research will aid in determining if the effect of using NSTs in combination with Bt maize hybrids is additive, synergistic or neutral at managing secondary soil pests in the Midwestern United States. If additive, the sum of the effect of a neonicotinoid seed treatment on a Bt seed will be equal to the individual effects of NSTs and Bt seeds combined. If synergistic, the sum of the effect of a neonicotinoid seed treatment on a Bt seed will be greater than the individual effects of NSTs and Bt seeds combined, as measured by improved preservation of yield potential. A neutral effect will result in the sum of the effects, and therefore the yield, being no different than using one treatment alone.

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CHAPTER 2. ASSESSMENT OF THE EFFECTS OF NEONICOTINOIDS ON RESISTANCE MANAGEMENT OF THE WESTERN CORN ROOTWORM IN BT MAIZE

2.1 Introduction

Neonicotinoid insecticides were first developed in the 1980s and introduced commercially in the 1990s (Goulson, 2013). Their adoption increased dramatically in 2003 with the introduction of neonicotinoid seed treatments (NSTs) on maize seeds (active ingredient clothianidin), applied as a seed coat prior to planting to protect seeds against early-season secondary soil pests, such as white grubs and wireworms (Douglas and Tooker, 2015; Jordan et al., 2012; Wilde et al., 2004). This increase occurred alongside the introduction of Bt maize hybrids targeting WCR (Douglas and Tooker, 2015). NSTs act systemically as the plant develops, protecting the entire plant from pests (Goulson, 2013). In maize, this systemic protection is present in the plant tissues for only two to three weeks after planting (Alford and Krupke, 2017). Concerns surrounding the non-target effects of widespread neonicotinoid use, such as pollution of waterways and negative impacts upon non-pest arthropods, have increased as knowledge of their detrimental environmental effects has come to light (Hladik et al., 2014; Goulson, 2013).

Management of WCR larvae using genetically modified maize began in 2003 when the Environmental Protection Agency (EPA) registered Bt maize hybrids that produce the insecticidal protein Cry3Bb1 (US EPA, 2017). These transgenic seeds were introduced concurrently with NSTs; there are presently no transgenic corn seeds targeting the RW complex available commercially without this treatment. When using Bt maize hybrids, the EPA requires an Insect Resistance Management (IRM) plan be employed to delay the development of pest resistance to the transgenic maize (US EPA, 2017). This IRM plan consists of planting 5% refuge maize seed, which lacks the Bt insecticidal trait (but does include NST), when planting Bt maize (US EPA, 2017). The purpose of the refuge plants is to provide a habitat for larval WCR to feed without exposure to the insecticidal Cry proteins and produce Bt-susceptible insects that will (theoretically) mate with Bt-resistant insects to produce Bt-susceptible heterozygous offspring, delaying the development of resistance (Cullen et al., 2008). An IRM strategy using 5% refuge assumes random mating between Bt-susceptible and Bt-resistant beetles, but some data suggest that mating opportunities between these beetles are not actually random (Kang and Krupke, 2009; Spencer et

al., 2013; Taylor and Krupke, 2018) and that mating between Bt-resistant beetles is not as infrequent as originally hypothesized (Taylor et al., 2016). These mating opportunities are instead influenced by beetle fitness and movement (Taylor et al., 2016; Taylor and Krupke, 2018). WCR resistance to Cry proteins has been documented repeatedly in the field. By 2009, just six years after its introduction by the EPA, resistance to Cry3Bb1 was discovered in Iowa and, in recent years, there have been increases in WCR resistance to a majority of the other currently available Cry proteins (Gassmann et al., 2020).

Although the adoption of the Bt hybrid + NST approach has been almost ubiquitous for over a decade, the role of NSTs in refuge function is unclear – whether they facilitate or hinder the larger goal of generating susceptible beetles from refuges has not been answered in a field setting. The objective of this chapter was to quantify the effects of NSTs on the performance of refuges in delaying resistance evolution in Bt maize fields by determining if the presence of NSTs affects the population sizes and mating rates of Bt-fed and refuge-fed insects. By removing NSTs from all refuge seeds, this study maximizes the opportunities for refuge-feeding larvae to survive to the adult stage. My study then uses field observations of adult emergence ratios and mating between resistant and susceptible beetles to determine if a 5% seed blend is sufficient for delaying resistance in the field. I hypothesize that, since only a small fraction of beetles collected from 5% refuge plots actually emerge from refuge plants (Taylor and Krupke, 2018), there will be a greater population of refuge beetles from untreated plots than treated plots, but overall, an insufficient population of refuge beetles to mate with Bt-emerged beetles.

2.2 Materials and Methods

The primary focus of this objective was to determine whether neonicotinoid seed treatments (NSTs) affect the efficiency of refuges in producing Bt-susceptible beetles in Bt maize targeting the western corn rootworm. I replicated trials at two field locations in Indiana for two growing seasons. Fields were located at Throckmorton-Purdue Agricultural Center (TPAC) in Lafayette, Indiana (40°17'48''N, 86°54'13''W) and Pinney Purdue Agricultural Center (PPAC) in LaPorte Co. (41°26'35''N, 86°55'49''W).

Treatments included: Bt seed + NST, Bt seed untreated, and refuge seed untreated. Seeds were purchased from Bayer Crop Science (Dekalb; Bayer Crop Science, St. Louis, MO, USA). Bt + NST hybrid seeds (Genuity SmartStax RIB Complete® (DKC 62-08 P1250) with glyphosate and

glufosinate tolerance) and untreated Bt seeds (Genuity SmartStax RIB Complete[®] (DKC 62-08) with glyphosate and glufosinate tolerance) (Dekalb; Bayer Crop Science, St. Louis, MO, USA) expressed the *Cry3Bb1* + *Cry34/35Ab1* proteins targeting corn rootworm, while refuge seeds (untreated Spectrum Non GMO 6334) did not express those insecticidal proteins, nor did they express any herbicide tolerance traits. The neonicotinoid-treated seeds were purchased with a rate of 0.25 mg a.i./seed applied (active ingredient clothianidin, trade name: Acceleron[®] Basic) (Bayer Crop Science, St. Louis, MO, USA) and an additional 1.00 mg a.i./seed was applied by the seed treatment manufacturer (Bayer CropScience in 2018 and BASF in 2019, Research Triangle Park NC, USA) for a total clothianidin rate of 1.25 mg a.i./seed. Seeds used in untreated Bt plots in 2020 were washed to remove the seed coating using a protocol developed by Dr. Joseph Spencer at the University of Illinois (personal communication). Seed coatings were removed as follows: seeds were partitioned into lots of 5,000 seeds and added to an 18.95 L bucket with 5 L DiH₂O and 20 mL dish soap (Ultra Palmolive Original; Colgate-Palmolive Company, New York, NY, USA). Seeds were agitated by manually stirring for 20 minutes. After stirring, the seeds were poured into a sieve and rinsed four times with DiH₂O. This washing and rinsing procedure was repeated two more times. Afterwards, the seeds were blotted with paper towels and dried for 12 h and subsequently soaked in 10% bleach, to prevent mold and bacterial growth, for 1 h, agitating every 15 minutes. The seeds were then removed from the bleach solution and rinsed 10 times in DiH₂O. After rinsing, the seeds were blotted with paper towels and dried for 24 h. All washed seed was refrigerated until planting to prevent premature germination.

2.2.1 Field layout and plot dimensions

To assess the effect of NSTs on refuge performance in Bt maize, two treatments were compared: (1) Untreated Bt seeds with 5% untreated refuge and 2) Neonicotinoid treated Bt maize seeds at 1.25 mg/kernel clothianidin with 5% untreated refuge. These treatments were replicated at two locations in Indiana. Plots were located approximately 1.25 km apart at TPAC and 1.4 km apart at PPAC and were adjacent to non-experimental maize or soybeans with a 2 m buffer. Experimental plots were 30 x 30 m (100 x 100 ft) wide (**Figure 2.1**). Maize was planted on 76-cm (30-inch) row spacing at a population of 68,419 seeds per hectare (27,000 seeds per acre). Plots were machine-planted with 100% Bt seed using a four-row planter (JD MaxEmerge 7200; John Deere, Moline, IL, USA). Immediately following planting, 345 randomly chosen locations (5% of

total planting) within each plot were handplanted with two untreated refuge seeds and flagged. Plots were planted on 4-6 June and 18 June in 2019; and 26 May and 2 June in 2020. At the V2 growth stage (Corn and Soybean Field Guide, 2019), approximately 15 days after planting, three randomly chosen plants per row were tested to confirm expression of the Cry3Bb1 protein using EnviroLogix (Envirologix Inc., Portland, ME) gene-check strips. At the same time, the smaller of the two refuge plants and the nearest Bt plant at each location were removed.

2.2.2 Stable Isotope Labeling

To assess refuge performance, refuge plants were labeled with ammonium nitrate ^{15}N , a stable isotope of nitrogen (Cambridge Isotope Laboratories, Inc. Andover, MA). The nitrogen stable ^{15}N isotope is systemically distributed throughout the plant tissue and is effectively transferred to feeding WCR larvae (Taylor et al. 2016). Marking insects with ^{15}N has minimal effect on their behavior or fitness (Hood-Nowotny and Knols, 2007) and the mark is readily detectable in WCR adults (Taylor et al. 2016, Taylor and Krupke, 2018). After emergence, adult beetles were collected and analyzed for ^{15}N excess using stable isotope analysis (details below). Captured beetles with an excess of the stable isotope of nitrogen were assumed to have fed primarily on refuge plants as larvae.

Refuge plants were labeled with ammonium nitrate ^{15}N as outlined in Taylor et al. (2016), as follows: at the V2 stage, which is characterized by visibility of the second leaf collar, an aqueous solution of ammonium nitrate ^{15}N (~98% ^{15}N) and distilled water was applied to refuge plants at a rate of 0.6125 g ammonium nitrate per liter of dH₂O. Using a CO₂-pressurized backpack sprayer (R & D Sprayers, Opelousas, LA, USA), 10 mL of the aqueous solutions was applied as a soil drench to a 1 cm deep hole at the base of each refuge plant.

To determine if ^{15}N is leaching to surrounding unenriched plants, root tissue from ^{15}N -enriched and unenriched maize plants was sampled approximately four weeks after ^{15}N enrichment in 2020 and analyzed for ^{15}N concentration. Five enriched refuge plants were sampled from each plot and six unenriched plants surrounding each enriched plant were also sampled (within row: two plants ahead and two behind; adjacent row: one plant to the left and one to the right), for a total of 35 plants sampled per plot (five enriched and 30 unenriched). Plants were removed whole and washed of soil particles. Plant tissue was dried in a laboratory oven at 90°C for 24 h. Approximately 5 mg of dried root tissue was removed, ground into a fine powder and weighed

into tin capsules (Costech Analytical Technologies, Inc., Valencia, CA, USA) for mass spectrometry performed by the Virginia Tech Department of Geosciences Stable Isotope Laboratory.

2.2.3 Beetle Collection

A team of three individuals scouted eight random rows of each experimental plot for rootworms. Sampling occurred two to three times per week beginning at the appearance of the first adult male rootworm beetle in each location, which was monitored using pheromone lures (CSALOMON®, Plant Protection Institute, Hungarian Academy of Sciences, Budapest, Hungary) on a yellow sticky trap (Trece Pherocon AM No-Bait trap, Trece Inc. Adair, OK, USA). Sampling occurred during peak rootworm mating hours, between 0800-1100 h. Plots and rows were sampled in random order by alternating order of field sites and observers between visits. Every adult rootworm found along each of those eight rows was collected into a 30 mL amber glass vial (Brockway Glass Co., Inc., Brockway, PA) with a funnel attachment at the mouth of the vial and labeled with the date and location. Mating pairs were collected, labeled and stored together in the same vial. Collected beetles were stored at -20°C until processing.

Sex was determined for each collected beetle by looking for a planar patch on the tarsomere, which indicates a male (Hammack and French, 2007). Head capsule widths and dry weights were measured to determine fitness (Murphy and Krupke, 2011). Beetle head capsule size was measured using a Leica stereo microscope (Leica M125, Leica Microsystems, Wetzlar, Germany) with a digital camera attachment (Leica EC3, Wetzlar, Germany) and AnalySIS Microsuite imaging software. Using the camera and imaging software, an image was recorded and the distance between the outer edge of each eye was measured to the nearest 0.01 mm. Following sex determination and head capsule measurements, beetles were dried in a laboratory oven (Grieve-Hendry Co., Inc., Chicago, IL) at 90°C for 24 h to remove moisture. Once dried, the dry mass was measured to the nearest 0.01 mg (Mettler Toledo, MT5, Columbus, Ohio, USA).

¹⁵N levels were measured using stable isotope testing. A random subsample was taken from each location and collection date to measure ¹⁵N levels and determine the relative proportions of Bt-fed and refuge-fed beetles. Only the beetles' elytra and head capsules were used for the isotope analysis to minimize contamination by nitrogen present in the digestive tract, as well as nitrogen transferred to females from males via the spermatophore, which has also been shown to retain the

^{15}N label (Murphy and Krupke, 2011). Beetles were prepared for mass spectrometry using an adaptation of the protocol outlined in Taylor et al. (2016). Samples were prepared for stable isotope testing, as follows: Dried elytra and head capsules were removed and placed into a 4x6 mm tin capsule (Costech Analytical Technologies, Inc., Valencia, CA, USA), weighed to the nearest 0.001 mg, and crushed within the capsule. The capsule was then cinched, rolled into a ball and placed into a sterile 96-well plate (Falcon, REF 353075, Corning, NY, USA). The workspace and all instruments were cleaned with >70% ethanol between samples. The Virginia Tech Department of Geosciences Stable Isotope Laboratory performed all mass spectrometry testing. Baseline constants were used to adjust the percentage of ^{15}N and determine the fraction of ^{15}N that is in excess of environmental background levels. A percentage of ^{15}N that is in excess of three times the baseline constant was used as a threshold to discriminate between beetles that, as larvae, fed primarily on labeled plants vs. larvae that fed minimally, or not at all, on labeled plants. These adjustments were conducted based on calculations by Taylor and Krupke (2018).

Arthropod (~0.5 mg) and plant (~4 mg) tissue were ground, homogenized and weighed into tin cups for isotopic and elemental analysis. Stable nitrogen isotope values ($\delta^{15}\text{N}$) and %N were determined on an Isoprime 100 isotope ratio mass spectrometer (IRMS) coupled with a vario ISOTOPE elemental analyzer (EA) in the Geosciences Department at Virginia Tech. Stable nitrogen isotope compositions were calibrated relative to the AIR scale with USGS25 and USGS26 via a two-point linear calibration ($\delta^{15}\text{N} = -30.41\text{‰}$ and 53.75‰ , respectively). The technicians conducting the analysis monitored and corrected for linearity and drift when appropriate using a suite of commercial standards (Elemental Microanalysis *urea*, $\delta^{15}\text{N} = -0.30\text{‰}$; *protein*, $\delta^{15}\text{N} = 5.94\text{‰}$; and *wheat flour*, $\delta^{15}\text{N} = 2.85\text{‰}$) interspersed throughout the analytical runs. Analytical uncertainty (1σ), based on the standard deviation of 414 commercial standards, is less than 0.1% for $\delta^{15}\text{N}$.

2.3 Results

2.3.1 Adult Beetle Population

Rootworm collections occurred from 23 July through 5 September in 2019 and 16 July through 20 August in 2020. Collections began at the first rootworm sighting on yellow sticky trap (Trece Pherocon AM No-Bait trap, Trece Inc. Adair, OK, USA) baited with a pheromone lure

(CSALOMON®, Plant Protection Institute, Hungarian Academy of Sciences, Budapest, Hungary) at each location. The total number of rootworms collected was 1028 in 2019, and 2901 in 2020. A representative subsample of 493 beetles was taken in 2019 and 594 beetles in 2020 in the interests of time and materials needed for processing samples. In 2019, emergence peaked at TPAC on 15 August when 195 adults were collected and at PPAC on 2 August when 11 adults were collected, and again on 20 August when 11 adults were collected. In 2020, emergence peaked at TPAC on 31 July when 442 adults were collected, and at PPAC on 30 July when 83 adults were collected. A total of 27 mating pairs were collected in 2019 and 92 mating pairs were collected in 2020. In Indiana, labeled and unlabeled refuge beetles had a mean percent excess ^{15}N of 4.67% (SE \pm 0.46) and 0.32% (SE \pm 0.02) respectively, in 2019. In 2020, labeled and unlabeled atom percent excesses ^{15}N were 11.31% (SE \pm 3.24) and 0.19% (SE \pm 0.02), respectively.

Beetle emergence by sampling date and treatment is summarized in **Figure 2.2**. In 2019, Bt beetle emergence from NST plots began on 23 July and continued until 5 August. Two refuge+ beetles were collected when a single beetle was recovered on 5 August and again on 7 August. Bt+ beetle emergence peaked at TPAC on 15 August and at PPAC on 14 August, when 37 and seven beetles were recovered at each location, respectively. A total of two refuge+ and 217 Bt+ beetles were collected in 2019. A total of eight refuge- and 266 Bt- beetles were collected from non-NST plots. Bt- beetle emergence occurred from 24 July until 5 September and refuge- beetle emergence occurred from 24 July until 13 August. Bt- beetle emergence peaked at TPAC on 15 August, when 158 beetles were captured. At PPAC, there were three notable peaks in Bt- beetle emergence, occurring on 30 July, 2 August, and 20 August, when seven, nine, and eight beetles were collected each day, respectively. Peak emergence of refuge- beetles occurred at TPAC on 31 July, when three beetles were captured. No refuge- beetles were recovered from PPAC in 2019.

In 2020, Bt beetle emergence from NST plots occurred from 16 July to 18 August. Two refuge+ beetles were collected when a single beetle was recovered on 20 July and another on 29 July. At TPAC, two notable peaks in Bt+ beetle emergence occurred on 16 July and 31 July when 56 and 50 beetles were collected each day, respectively. Bt+ beetle emergence peaked at PPAC on 17 July and 23 July, with 17 and 11 were collected each day, respectively. A total of two refuge+ and 68 Bt+ beetles were collected in 2020. In non-NST plots, Bt- beetle emergence occurred from 16 July to 20 August. Refuge- beetle emergence occurred from 16 July to 29 July, with an additional single beetle collected on 6 August. Beetle emergence peaked at TPAC on 31 July when

392 beetles were collected and at PPAC on 30 July when 76 beetles were collected. Refuge- beetle emergence peaked at TPAC on 16 July when ten beetles were recovered. Two refuge- beetles were collected at PPAC when a single beetle was recovered on 17 July and another on 6 August. A total of 21 refuge- beetles and 503 Bt- beetles were collected in 2020.

The proportions of treated Bt host plant-emerged (herein “Bt+”) and refuge host plant-emerged beetles (herein “refuge- (Bt+ plot)”) and untreated Bt (herein “Bt-“) and refuge (herein “refuge-“) beetles did not statistically differ in both 2019 and 2020 (Fisher’s exact test: 2019, $P = 0.1969$; 2020, $P = 1$). A total of two refuge- (from Bt+ plots) beetles and eight refuge- (from Bt- plots) beetles were collected in 2019. In 2020, a total of two refuge- (from Bt+ plots) beetles and twenty-one refuge- (from Bt- plots) beetles were collected. In 2019, the overall subsample of collected adults was composed of 2.03% refuge beetles, while the subsample from Bt- fields was composed of 2.93% refuge beetles and Bt+ fields were 0.91% refuge beetles. In 2020, the overall subsample of collected adults was composed of 3.87% refuge beetles, while 4.01% of beetles collected from Bt- fields were refuge and 2.86% of beetles collected from Bt+ fields were refuge beetles. In both 2019 and 2020, the overall collected refuge beetles had female-biased sex ratios (2019, 60%; 2020, 65.22%). In 2019, 50% of refuge beetles collected from Bt- fields were female, while 100% of refuge beetles collected from Bt+ fields were female. In 2020, 66.67% of refuge beetles collected from Bt- fields were female, while 50% of refuge beetles from Bt+ fields were female. In 2019, 100% of the mating pairs collected from Bt+ fields were between two beetles that fed on Bt natal host plants, while 4.17% of mating pairs collected from Bt- fields were between one refuge-fed and one Bt-fed beetle and the remainder of mating pairs were between two Bt-fed beetles. In 2020, 7.7% of the mating pairs collected from Bt+ fields were composed of one refuge-fed and one Bt-fed beetle, with the remainder of the mating pairs between two Bt-fed beetles. Of the mating pairs collected from Bt- fields, 88.6% were composed of two Bt-fed beetles, 10.1% of pairs were between a refuge beetle and a Bt beetle and 1.3% of pairs were between two refuge beetles.

2.3.2 Head capsule width and dry weight

Beetle head capsule width and dry weight are summarized in **Figure 2.3**. Due to a low number of collected refuge beetles, a t-test between neonicotinoid treated and non-neonicotinoid treated beetles was used to determine whether insecticide treatment had an effect on beetle head

capsule width and dry weight. Beetle dry weight data were non-normally distributed and had a positive skew in both 2019 and 2020.

In 2019, there was a significant difference in mean head capsule width ($t = 3.4097$, $df = 436.9$, $p\text{-value} = 0.0007$) between beetles who fed on insecticide-treated natal host plants ($M = 1.12$ mm) and untreated natal host plants ($M = 1.11$ mm). There was also a significant difference in mean dry weight measurements in 2019 ($t = 9.9681$, $df = 394.58$, $p\text{-value} < 0.001$) between beetles feeding on insecticide-treated natal host plants ($M = 4.19$ mg) and untreated host plants ($M = 2.96$ mg). These significant differences between beetles fed from untreated and treated natal host plants in both head capsule width and dry weight may be due to a smaller sample size of beetles collected from treated plots.

In 2020, a t-test showed no significant difference in mean head capsule width ($t = -1.7286$, $df = 77.684$, $p\text{-value} = 0.0879$) between beetles who fed on treated natal host plants ($M = 1.16$ mm) and those who fed on untreated host plants ($M = 1.17$ mm). Results showed no significant difference in mean dry weight ($t = 0.2009$, $df = 85.144$, $p\text{-value} = 0.8412$) between beetles that fed on treated host plants ($M = 3.53$ mg) and beetles that fed on untreated host plants ($M = 3.50$ mg).

2.4 Discussion

Management of western corn rootworms using Bt maize hybrids is currently deployed with a mandatory 5% non-Bt refuge as a seed mix. Results from my study documented low numbers of refuge beetles produced by this 5% seed blend in the field. This data further supports Taylor and Krupke's (2018) notion that a 5% refuge seed blend may not produce sufficient populations of Bt-susceptible, refuge-fed beetles to delay rootworm resistance to Bt hybrids. In their study, Taylor and Krupke (2018) compared different refuge structures (i.e. strip and blend) and varying percentages of refuge (i.e. 5% and 20%), with all seed treated with NSTs. My work expands on that study by comparing mating in both neonicotinoid-treated Bt and untreated Bt plots, but providing beetles with a 5% refuge that is *not* treated with neonicotinoids. Results from my study suggest that, even when using an insecticide-free refuge, a 5% refuge seed blend is not sufficient to produce an adequate population of susceptible beetles to delay resistance. These results are based on the determination of beetle natal host plants indicated by ^{15}N concentration levels of adults and do not indicate resistance. Beetles were not tested for resistance to Bt toxins and there is currently no documentation of field resistance in Indiana. Over the course of this study, the

highest percentage of refuge beetles collected from a field was just 4.55%, which is unlikely to result in sufficient mixed matings to delay resistance evolution. Western corn rootworm resistance evolution to Bt maize hybrids began early on in the use of the toxins. This may be partly due to the use of block refuges as opposed to seed blends and the reluctance of some growers to plant refuges (Reisig, 2017). Western corn rootworms have limited dispersal prior to mating (Marquardt and Krupke, 2009). Refuges structured in nearby blocks limit mating opportunities between Bt-emerged and refuge-emerged beetles and decrease the refuge's ability to delay resistance. From this standpoint, the seed mix refuge is a superior choice (Murphy et al. 2011), although it is clear from my work and others cited here that larger refuges would facilitate a far greater degree of mixed matings.

In current GM maize hybrids targeting insect pests, Bt traits are typically “pyramided”, or “stacked”, and paired with refuge to delay resistance. When multiple toxins are pyramided within a hybrid, any insects resistant to one of the toxins are theoretically killed by the other toxin within the pyramid (Roush, 1998). However, if there is an evolution of resistance to one of the toxins, or cross resistance between the toxins, which takes place when resistance to one of the toxins occurs due to exposure and resistance to a similar toxin, the delay of resistance significantly decreases (Gressel et al., 2017). One example of WCR cross resistance to Bt toxins, and subsequent decrease in the expected time for resistance development in the field, is the cross resistance between Cry3Bb1 and mCry3A toxins (Gassmann et al., 2014). Western corn rootworm resistance was first observed in 2009 in Iowa, in which feeding injury occurred in maize producing the Bt toxin Cry3Bb1 (Gassmann et al., 2011). In the following years, continued observations of feeding injury occurred in Iowa. Bioassays later discovered resistance to mCry3A and eCry3.1Ab in addition to Cry3Bb1, reducing the likelihood of success with pyramiding traits (Gassmann et al., 2014; Jakka et al., 2016).

In contrast, Bt maize has shown durable, long-term efficacy in managing European corn borers (*O. nubilalis*). Corn borers are currently managed through the Bt maize high-dose refuge strategy and have retained susceptibility to current Cry toxins used for their management (Siegfried et al., 2007). Since their initial release in the late 1990s, there have been only isolated cases of reduction in corn borer susceptibility to Bt toxins. In 2001, a field collected population in Minnesota was found to exhibit <99% mortality to the Cry1Ab toxin in a bioassay (Siegfried et al., 2007). Similarly, in 2004, a field collected population in Iowa showed reduced mortality to the

Cry1F toxin (Siegfried and Hellmich, 2012), and more recently, Smith et al. (2019) reported practical resistance to the Cry1F toxin in Nova Scotia, Canada. Though there have been some instances of reduced mortality, it appears that the high-dose refuge strategy is continuing to delay overall resistance of corn borers to Bt hybrids (Siegfried and Hellmich, 2012). In contrast, of the Bt traits currently available for the management of western corn rootworm, none produce what is considered a high dose of toxin, because of this, inheritance of resistance traits may be non-recessive (Andow et al., 2016). Similarly, Bt maize hybrids targeting lepidopteran insects are not high dose for corn earworm (*Helicoverpa zea*) (Siegfried and Hellmich, 2012), and resistance to Bt maize toxins has also been reported in this species (Reay-Jones et al., 2020). Bt maize targeting corn borers must be planted with a 20% non-Bt refuge to provide a sufficient population of susceptible moths to mate with Bt-resistant moths (Siegfried and Hellmich, 2012). This is compared with the 5% non-Bt refuge required for Bt maize targeting western corn rootworms, which Taylor and Krupke (2018) have previously stated is insufficient to provide susceptible beetles. They found a 20% seed blend refuge (which is experimental only and not an option for commercial corn planting) to be more successful in producing refuge beetles. The high-dose refuge strategy is also successful for corn borer management because both males and females frequently travel 0.8 km or more prior to mating (Showers et al., 2001), compared to the very limited pre-mating dispersal of western corn rootworm females (Marquardt and Krupke, 2009). Western corn rootworm females often do not move at all before mating, with teneral females typically mating close to their natal host plant (Spencer et al., 2013). Of those females who do disperse before mating, Spencer et al. (2013) found the maximum dispersal distance to be less than 30 m. While western corn rootworm males have a greater likelihood of dispersing before mating than females, their dispersal distances are similar (Spencer et al., 2013). The dispersal of corn borers prior to mating allows for a greater chance of random mating between refuge-fed and Bt-fed insects.

Success of Bt maize in managing European corn borer may also be due to fitness costs associated with the toxins, causing incomplete resistance in resistant homozygous insects (Gassmann et al., 2009). These fitness costs, including slower development and fewer offspring, slow down the rate of resistance evolution, delaying resistance (Gassmann et al., 2009). My results in 2019 included a significant difference in mean head width and dry weight between beetles who fed on insecticide-treated natal host plants and untreated natal host plants, with NST-fed beetles having greater mean head capsule widths and dry weights than those from untreated plots, although

these results may be skewed due to a relatively small number of beetles collected ($n = 219$) from neonicotinoid-treated plots, and possibly do not fully reflect the field population. This apparent lack of fitness costs associated with NST exposure may also explain the increase in western corn rootworm resistance evolution. Fitness costs related to Bt resistance are adverse consequences, such as decreased weight, effectively making resistant beetles less fit than susceptible beetles (Gassmann et al., 2009). Male WCR are more likely to mate with heavier females, indicating non-random mating (Kang and Krupke, 2009). If these observations are carried to the population level, this preference for females with a greater weight may lead to a greater likelihood of mating between NST-fed beetles and an increase in resistance evolution.

Results from my study suggest that the current IRM strategy of deploying a 5% refuge seed blend with Bt maize is insufficient in producing an adequate proportion of susceptible beetles, even when provided with ideal survival conditions of an insecticide-free refuge. While this objective of the study has suggested NSTs to be unhelpful in WCR resistance management, the subsequent objective determines if the addition of NSTs aids in optimizing maize yield potential.

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2.6 Tables and Figures

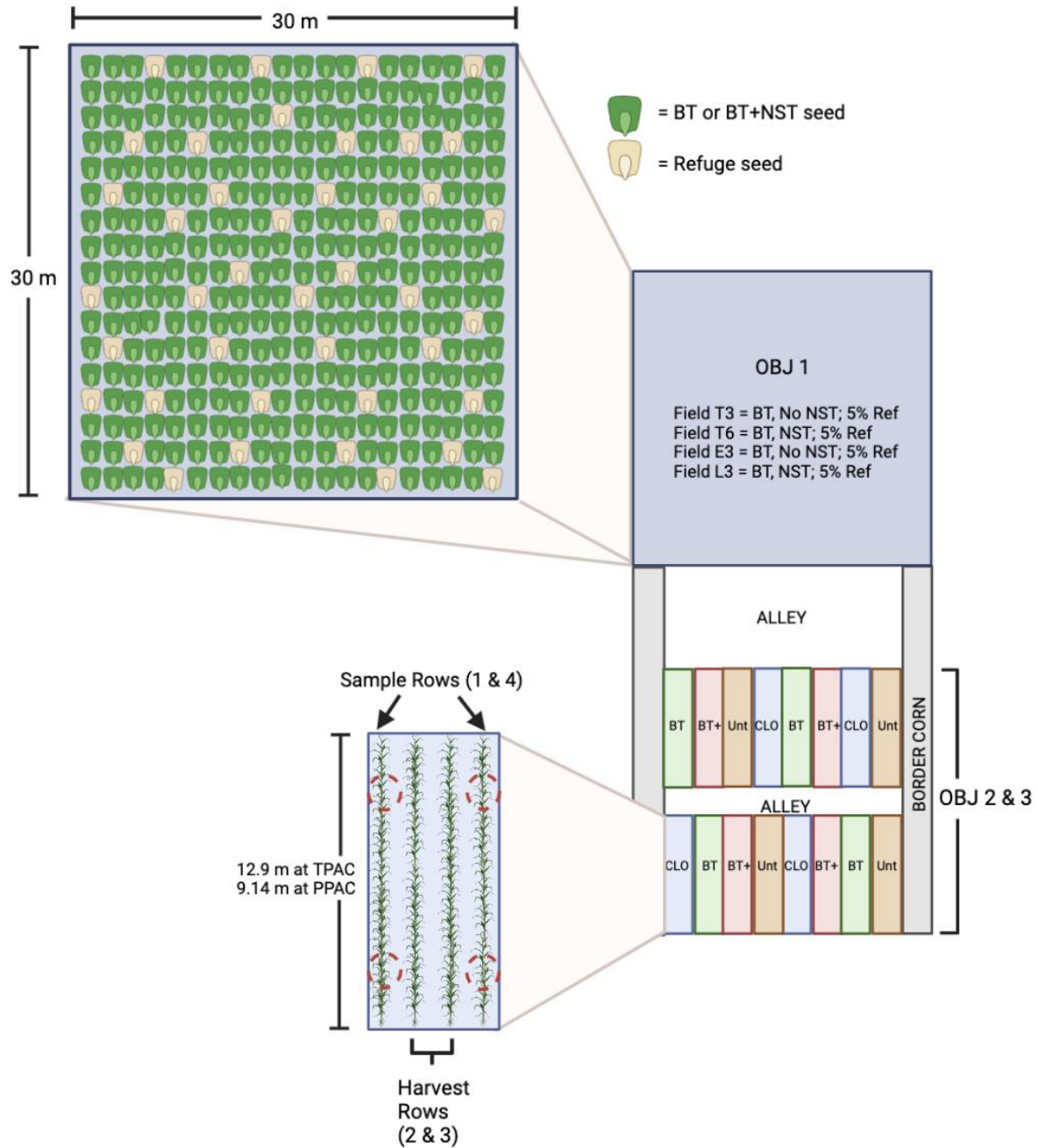


Figure 2.1. Schematic showing the placement of refuge/Bt seed (Objective 1) (seed placement per meter is not to scale), arrangement of treatments within plots (Objective 2 and 3), and soil sampling locations (Objective 3).

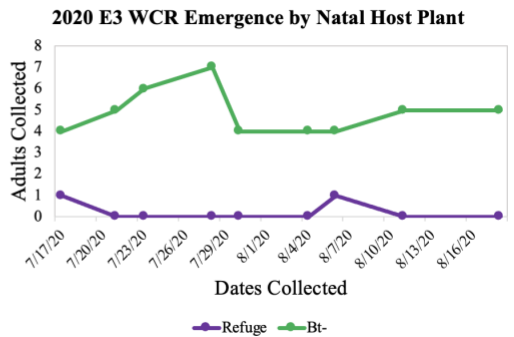
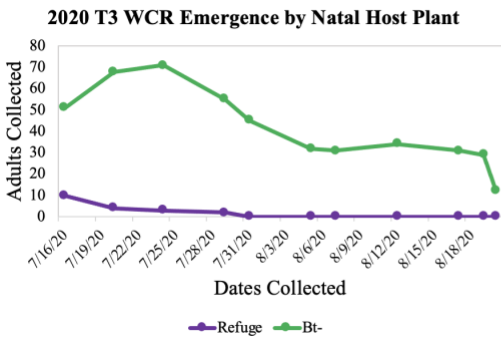
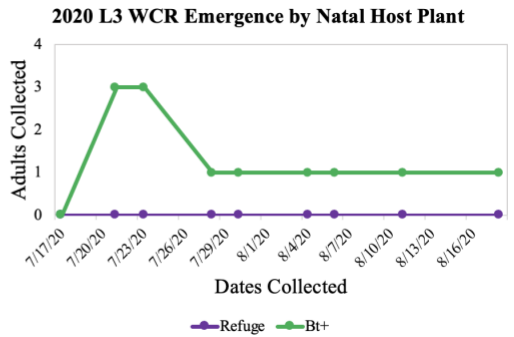
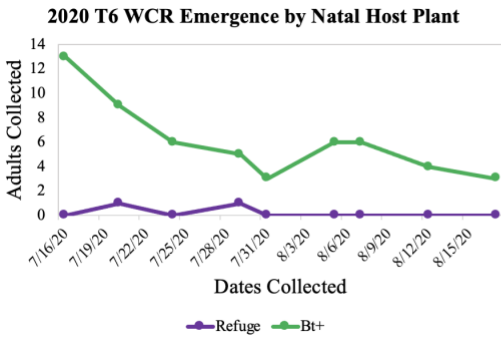
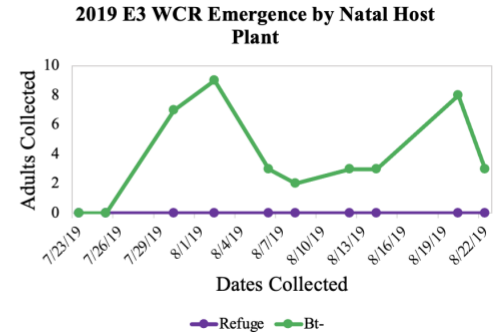
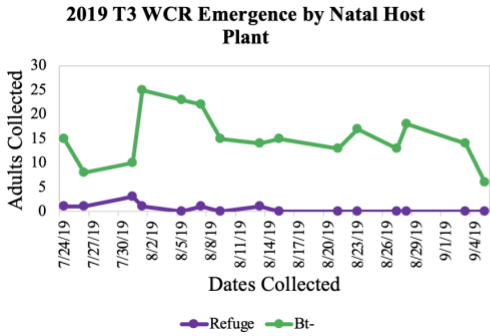
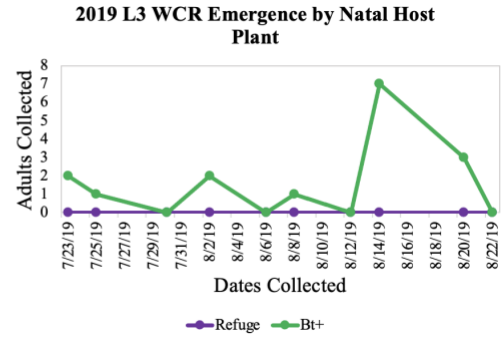
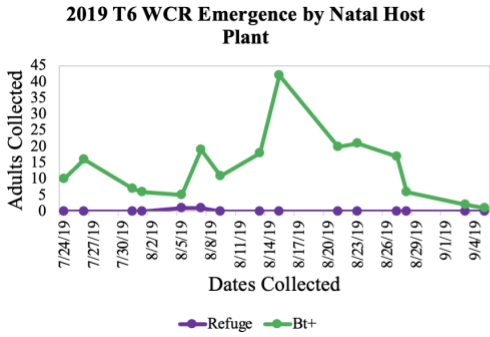


Figure 2.2. 2019 and 2020 western corn rootworm emergence by natal host plant.

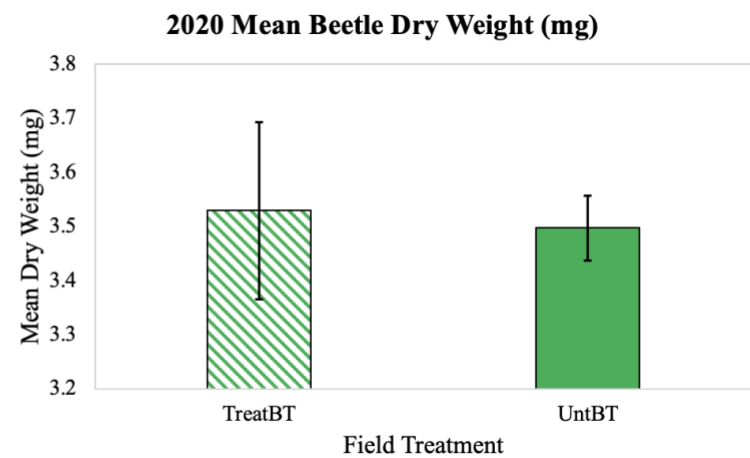
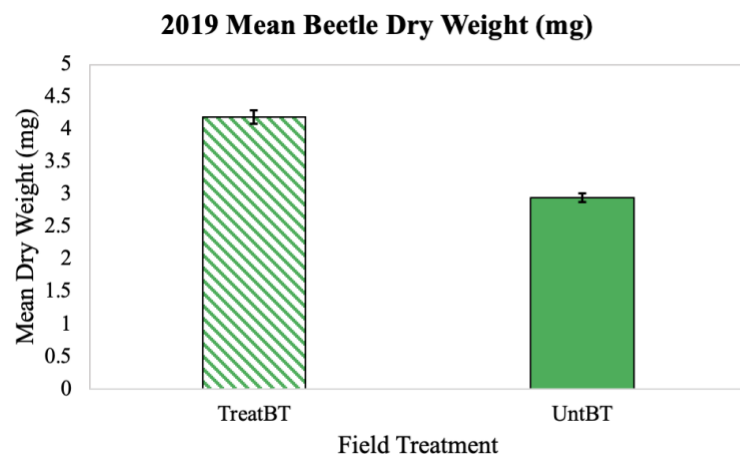
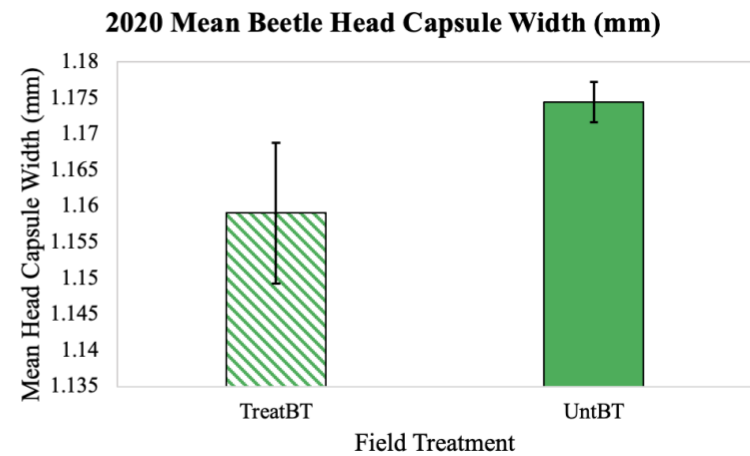
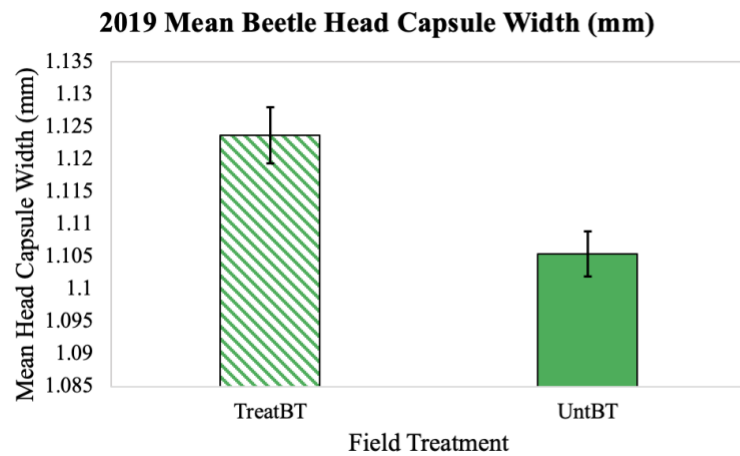


Figure 2.3. 2019 and 2020 mean (\pm SEM) beetle head capsule width (mm) and dry weight (mg).

CHAPTER 3. ASSESSMENT OF NEONICOTINOID SEED TREATMENTS IN COMBINATION WITH BT MAIZE FOR WESTERN CORN ROOTWORM AND SECONDARY SOIL PEST MANAGMENT

3.1 Introduction

Prior to the introduction of Bt hybrids, WCR was managed using a range of soil-applied insecticides which also aided in managing soil-dwelling secondary pests. However, with the release of WCR-targeting Bt hybrids, the addition of NSTs assisted in management of soil pests not targeted by the Bt insecticidal proteins (Douglas and Tooker, 2015). Although they are used on all maize, the use of NSTs in secondary soil pest management is only necessary in regions with high levels of pest pressure and damage (Alford and Krupke 2018). In general, there is low pressure and economic importance in the Corn Belt from sporadic pests, such as wireworms or white grubs (Sappington et al., 2017). While NSTs can preserve yield in fields that exceed the white grub threshold (Jordan et al., 2012), the far more common environments are those which lack damaging populations of secondary soil pests. These environments do not exhibit a significant increase in preservation of yield as a result of NST use (Wilde et al., 2007; Krupke et al. 2017, Labrie et al., 2020). Their sporadic presence and inconsistent levels of damage may be better managed by focusing on monitoring for pest presence, rather than a prophylactic approach.

The objective of this chapter is to quantify the benefit of using NSTs in combination with Bt maize hybrids, determining if it is additive, synergistic, or neutral at managing WCR and secondary soil pests in the Midwestern United States. If additive, the sum of the effect of a neonicotinoid seed treatment on a Bt seed will be equal to the individual effects of NSTs and Bt seeds combined. If synergistic, the sum of the effect of a neonicotinoid seed treatment on a Bt seed will be greater than the individual effects of NSTs and Bt seeds combined, as measured by improved preservation of yield potential. A neutral effect will result in the sum of the effects, and therefore the yield, being no different than using one treatment alone. I hypothesized that the use of NSTs in combination with Bt insecticidal proteins will result in higher mortality of WCR larvae and secondary soil pests when compared to planting with Bt traits alone, resulting in improved plant stands and root protection.

3.2 Materials and Methods

The primary focus of this objective was to evaluate the benefit of using NSTs in combination with Bt maize hybrids for management of WCR and secondary soil pests by comparing several estimates of below-ground pest insect abundance and damage, including stand counts, root injury rating (Oleson et al. 2005) and yield between seed-treated and non-seed-treated Bt and non-Bt plants. Trials were replicated during the 2018, 2019 and 2020 growing seasons in two fields at Throckmorton Purdue Agricultural Center (TPAC) in Lafayette, Indiana (40°17'48''N, 86°54'13''W) and two fields at Pinney Purdue Agricultural Center (PPAC) in LaPorte Co., Indiana (41°26'35''N, 86°55'49''W). Fields were located approximately 1.25 km and 1.4 km apart, respectively.

3.2.1 Field and plot layout

Four treatments were compared: 1) untreated, Bt seed (herein referred to as “Bt”) (Genuity SmartStax RIB Complete® (DKC 62-08) expressing Cry3Bb1 + Cry34/35Ab1 with glyphosate and glufosinate tolerance); 2) NST, Bt seed (herein referred to as “Bt+”) (Genuity SmartStax RIB Complete® (DKC 62-08, Poncho 1250) expressing Cry3Bb1 + Cry34/35Ab1 with glyphosate and glufosinate tolerance); 3) untreated, non-Bt seed (herein referred to as “refuge”) (DKC 62-05); 4) NST, non-Bt seed (herein referred to as “refuge+”) (DKC 62-05, Poncho 1250). Seeds used in untreated plots in 2020 were washed to remove the seed coating using the previously described protocol developed by Dr. Joseph Spencer at the University of Illinois. Seeds were purchased from Bayer Crop Science (Dekalb; Bayer Crop Science, St. Louis, MO, USA). The neonicotinoid-treated seeds were purchased with a rate of 0.25 mg a.i./seed applied (active ingredient clothianidin, trade name: Acceleron® Basic) and an additional 1.00 mg a.i./seed was applied by the seed treatment manufacturer (Bayer Crop Science in 2018 and BASF in 2019, Research Triangle Park NC, USA) for a total clothianidin rate of 1.25 mg a.i./seed. These treatments were planted in a randomized 16-plot block comprised of four replicates of each of the four treatments (**Figure 3.1**). Plots were planted on 76-cm (30-inch) row spacing with a plant population of 68,419 seeds per hectare (27,000 plants per acre). Individual plots were four rows wide and 12.19 m (i.e. 40 ft) long at TPAC and 9.14 m (i.e. 30 ft) long at PPAC. Plots were machine-planted on 17 May, 22 May and 4 June in 2018; 4-6 June and 18 June in 2019; and 26 May and 2 June in 2020.

3.2.2 Soil Pests and Stand Counts

Approximately one to two weeks following plant emergence, the presence of secondary soil pests (i.e. wireworms (*Agriotes*, *Limonius*, *spp.*) and white grubs (*Phyllophaga*, *Popilla*, *Maladera*, *spp.*)) and their damage were assessed by taking stand counts of one of the two center rows in each plot. Stand counts were taken by counting the numbers of plants emerged within 9.14 m (i.e. 30 ft) of row length. The presence of secondary soil pests was determined by taking four soil samples from the outer rows of each plot using the compact method (CM), in which a 20 cm x 20 cm square x 15 cm deep hole was dug and sifted for non-WCR insect larvae (Jordan et al., 2012). The collected larvae were identified to species. Stand counts and sampling for soil pests occurred on 30 May, 12 June and 25 June in 2018; 27-28 June and 1 July in 2019; and 19 June, 22 June and 25 June in 2020.

3.2.3 Root Damage Ratings

Root damage was measured in late July or early August by removing six randomly chosen plants per plot (three from row one and three plants from row four). Plant stalks were removed, and root systems were soaked and rinsed with water to remove soil. Root damage was assessed using the 0-3 node injury scale (Oleson et al., 2005). Root ratings were taken on 26-27 July and 30 July in 2018; 12-13 August and 20 August in 2019; and 5-6 August in 2020.

3.2.4 Yield

Grain yield and moisture were estimated at harvest from the two center rows using a Kincaid® 8-XP plot combine equipped with a HarvestMaster GrainGage®. Yield was calculated as bushels grain at 15.0 percent moisture. Plots were machine-harvested on 29 October in 2018; 4-5 November in 2019; and 3 and 11 November in 2020.

3.3 Results

3.3.1 Secondary Soil Pests

All three sampling years documented low abundance of wireworms and white grubs per sample (2018: 0.0898 white grubs/sample, 0 wireworms/sample; 2019: 0.1133 white grubs/sample,

0.0039 wireworms/sample; 2020: 0.0820 white grubs/sample, 0.0430 wireworms/sample). Wireworms have an economic threshold of an average of one wireworm/bait trap (Labrie et al., 2020).

3.3.2 Stand counts (*plants per hectare*)

Plant populations were analyzed separately by year. The data fit a normal distribution. Treatment means for each year were compared using a two-way analysis of variance (ANOVA), using plant population as the response variable, treatment as a fixed effect and block as a blocking effect. The mean plant populations were 66,752 plants per hectare (pph), 65,322 pph and 63,752 pph for 2018, 2019 and 2020 respectively. There was a significant effect of treatment on plant population in 2018, 2019 and 2020 (**Table 3.1**). A Tukey post hoc test (Tukey HSD) was used to compare treatment means for each year. In 2018, plant population was significantly higher in Bt, Bt+, and refuge+ compared to that of refuge. There was no significant difference between Bt, Bt+ and refuge+. In 2019, a Tukey HSD revealed no significant difference between treatments. Of the four treatments, Bt+ had the greatest mean plant population, but was not significantly greater than Bt, refuge+ or refuge. In 2020, the plant population of Bt was significantly lower than Bt+, refuge+ and refuge. Refuge was also significantly greater than Bt+ (**Table 3.2**).

3.3.3 Yield

Plot yields were analyzed separately by year. Treatment means were compared using a two-way ANOVA, using metric tons per hectare (mtha) as the response variable, treatment as a fixed effect and block as a blocking effect. Yield values were standardized by using a correction factor which accounted for variable plant populations in each plot. Mean yields (mtha) were 12.51 mtha, 11.45 mtha, and 13.03 mtha for 2018, 2019, and 2020 respectively (**Table 3.2**). There was no significant effect of treatment on yield in 2018, 2019, or 2020 (**Table 3.1**).

3.3.4 Root ratings

Root damage ratings were analyzed separately by year. Treatment means were compared using a two-way ANOVA, using rating as the response variable, treatment as a fixed effect and block as a blocking effect. Mean root ratings were 0.0092, 0.0091, and 0.0361 for 2018, 2019, and

2020 respectively. There was a significant effect of treatment on root rating in 2018, 2019, and 2020 (**Table 3.1**). Tukey HSD was used to compare treatment means for each year. In 2018, the refuge root rating was significantly greater than Bt. There were no other significant differences between the other treatments. In 2019, refuge root rating was significantly greater than Bt and Bt+. Refuge+ was significantly greater than Bt+. There was no significant difference between the root rating of refuge and refuge+. In 2020, refuge had the greatest root rating mean, although narrowly missing the 0.05 significance threshold when compared to Bt+ and was not significantly different from Bt or refuge+ (**Table 3.2**).

3.4 Discussion

Results from the soil survey component of my study, complemented by stand counts, both indicated a low abundance of both wireworms and white grubs. It should be addressed that, in comparison to the overall volume of my plots, a very small volume of soil was sampled when surveying soil pest abundance. Using the Compact Method described by Jordan et al. (2012), a single soil sample, with the volume of 0.006 m^3 , was taken from four locations in each of the 16 plots per field. In total, 0.384 m^3 of soil was sampled from each field, which is just 0.43% and 0.57% of total field soil volume at 15cm in depth at TPAC and PPAC, respectively. This small volume, in addition to the typically patchy distribution of white grubs and wireworms (Corn and Soybean Field Guide, 2019), may have limited the sampling power of the study and the conclusions we can draw from it. It is important to note however, that our sampling regime is likely to mimic that of commercial growers (Krupke, pers. Comm.) Though I sampled a small volume of my fields, my findings do overlap with that of others who have documented low pressure from these secondary soil pests in the Corn Belt and have found these pests to be generally uncommon and to cause little economic damage (Labrie et al., 2020; Royer et al., 2004; Sappington et al., 2017; Smith et al., 2020). Both wireworms and white grubs are sporadic pests in this region and, when present, occur in April to mid- to late June (Corn and Soybean Field Guide, 2019; Royer et al., 2004). Their early season presence coincides with the window of greater neonicotinoid insecticide concentration within the maize plant's tissues (Alford and Krupke, 2017). Bt maize hybrids do not target secondary pests, such as wireworms and white grubs, therefore the use of neonicotinoid seed treatments (NSTs) in addition to Bt hybrids are complementary at protecting against both western corn rootworm and secondary soil pests if both are present (Douglas and

Tooker, 2015). Consequently, NSTs have been found to have no significant effect on yield in areas with low abundance of these secondary pests (Labrie et al., 2020; Smith et al., 2020) and would instead be of greater use in areas of higher early season secondary pest populations (Wilde et al., 2007).

My results found no significant effect of treatment on yield for each season of this study. Previous studies have found NSTs to produce comparable yield to that of untreated Bt hybrids (Alford and Krupke, 2018) and are typically of greatest utility in areas with high pressure from early season secondary pests (Wilde et al., 2007).

In each of the three field seasons of my study, I found very low root damage ratings, indicating that economically significant levels of pressure from WCR larval feeding was not present in this study, indicating a low presence of WCR. This study, and conclusions drawn from it, may be limited by the low density of WCR in the plots. With a greater presence, the yield results may have shown a significant difference when comparing treatments, with one treatment more successful at preserving yield than others. Additionally, NST protection against WCR has been shown to be inconsistent (Alford and Krupke, 2018). The average treatment root damage rating on the 0-3 scale for each year was 0.0177 for refuge in 2018, 0.0177 for refuge in 2019, and 0.0958 for refuge in 2020. Minimum node-injury scores that cause economic damage at moderate and high environmental stress levels can be as low as 0.38 and 0.13, respectively (Oleson, 2005). Considerable economic damage is likely to occur at a rating of 1.0 (Agronomy Spotlight), which far exceeds the average ratings documented in my study. In each of the three years of my study, the average root rating of refuge plots was greater than that of Bt, refuge+, and Bt+, with refuge having a significantly greater rating than Bt in 2018 and Bt and Bt+ in 2019. Although refuge plants had a greater rating, the average ratings were still relatively low and we rated very few individual plants with damage above a 1.0. This overall low root damage indicates a low presence of WCR in the plots, which corresponds with declining WCR densities observed in Illinois soybean fields (Tinsley et al., 2018). This decline in WCR density, in combination with low presence of secondary soil pests and no difference in yield, should be considered by growers when choosing pest management tactics in maize. With such low pest presence in our region, it would be advisable for growers to use untreated transgenic maize, which performs similarly to that of NST maize (Alford and Krupke, 2018). Planting untreated transgenic maize is beneficial to growers both short-

term, through decreased expenses, and long-term, by reducing the risk of resistance evolution (Alford and Krupke, 2018).

3.5 References

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3.6 Tables and Figures

Table 3.1. Degrees of freedom (df), F-values, and p-values for two-way analysis of variance (ANOVA) model describing plant population (pph), yield (mtha), and root rating by year.

Sample Type	Year	Factor	df	F-value	p-value
Plant population (pph)	2018	Treatment	3	6.674	< 0.001*
	2019	Treatment	3	3.096	0.0362*
	2020	Treatment	3	11.352	< 0.001*
Yield (mtha)	2018	Treatment	3	2.566	0.0665
	2019	Treatment	3	0.602	0.617
	2020	Treatment	3	2.715	0.0558
Root Rating (0-3)	2018	Treatment	3	3.211	0.0317*
	2019	Treatment	3	6.572	< 0.001*
	2020	Treatment	3	2.953	0.0425*
*P<0.05					

Table 3.2. Mean and standard deviation (SD) of plant population (pph), yield (mtha), and root rating by year and treatment.

Year	Treatment	pph Mean	pph SD	mtha Mean	mtha SD	Root Mean	Root SD
2018	Bt	70547	5795	13.2	3.04	0.0057	0.0148
	Bt+	67206	5691	12.1	2.94	0.0068	0.0063
	Ref	61568	8712	12.3	2.50	0.0177	0.0206
	Ref+	67363	8283	12.4	2.70	0.0068	0.0115
2019	Bt	66376	8745	11.4	3.04	0.0047	0.0052
	Bt+	67901	8114	11.3	4.04	0.0010	0.0029
	Ref	59380	12367	12.0	4.81	0.0177	0.0152
	Ref+	67632	6930	11.1	3.23	0.0130	0.0175
2020	Bt	51935	13708	12.5	2.70	0.0167	0.0152
	Bt+	61981	10601	13.2	3.93	0.0094	0.0085
	Ref	72206	7025	12.6	2.65	0.0958	0.1930
	Ref+	68888	9167	13.8	3.12	0.0224	0.0245

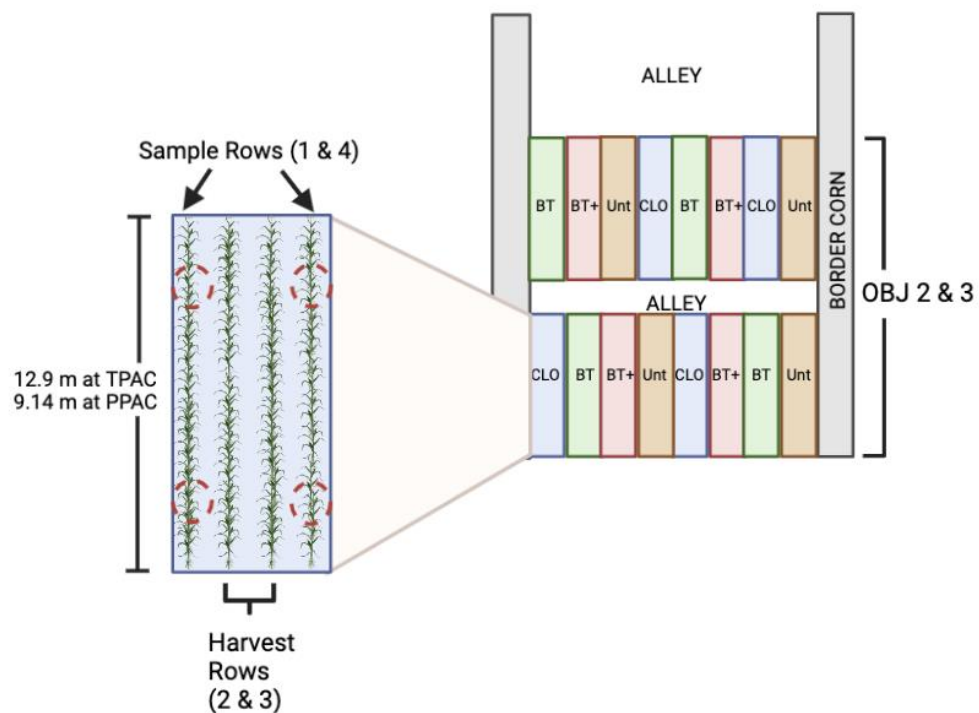


Figure 3.1. Schematic showing the arrangement of treatments within plots, harvest rows, and sampling locations. Secondary soil pest (white grub and wireworm) sampling, as well as root digs for rating (0-3 scale), were sampled from rows one and four of each plot. Each plot was harvested from rows two and three.

CHAPTER 4. MEASUREMENT OF NEONICOTINOID RESIDUES IN AGRICULTURAL SOIL AND SURROUNDING WATERWAYS

4.1 Introduction

Neonicotinoid insecticides were first developed in the 1980s and introduced commercially in the 1990s, with the first widely-used compound being imidacloprid (Goulson, 2013). This release was followed by a dramatic increase in neonicotinoid use in 2003 with the introduction of neonicotinoid seed treatments (NSTs), which are applied as a coating to seeds of large acreage annual crops, prior to planting (Douglas and Tooker, 2015). Two of the more common neonicotinoid compounds used as seed coatings are clothianidin and thiamethoxam, which are the focus of this study, and are primarily used on maize and soybeans, respectively. Advantages of neonicotinoids, which led to their increase in use, include their water solubility and high uptake into plant tissues, selectively higher toxicity for invertebrates and minimal toxicity to vertebrates (Tomizawa and Casida, 2005), as well as the simplicity of planting a pre-treated seed, possibly minimizing future needs for monitoring and spraying.

Although neonicotinoids provide a theoretically safer and simpler form of insect management, their propensity to dissolve in water and accumulate in soil has led to detrimental effects on the environment (Goulson, 2013). Due to their water-soluble nature, an increase in moisture causes the seed to swell and absorb the neonicotinoid which has dissolved in the water (Goulson, 2013). While it aids in absorption of the compounds by plant tissues, the high water-solubility of NSTs (Hladik et al., 2018) facilitates runoff into surrounding waterways, with higher concentrations of NSTs appearing in waterways after precipitation events following planting (Hladik et al., 2014; Alford and Krupke, 2019). In addition to their water solubility, neonicotinoids are persistent in soil, with repeated applications leading to accumulation (Hladik et al., 2018). The water-soluble nature, tendency to accumulate, and systemic activity of neonicotinoids negatively affect both aquatic and terrestrial non-pest invertebrates (Krupke et al., 2012; Goulson, 2013; Krupke and Long, 2015; Sanchez-Bayo et al., 2016; Miles et al., 2017; Raby et al., 2018).

The objective of this chapter is to quantify the residue levels of NSTs, specifically the compounds clothianidin and thiamethoxam, in agricultural soil and surrounding waterways, as well as their presence throughout the field season, by measuring the levels of neonicotinoid insecticide residue found in soil and waterways surrounding plots planted with and without NSTs.

I hypothesize that, due to the solubility of neonicotinoid insecticides, soil and waterways surrounding plots containing NSTs will yield higher NSTs residues compared to plots not containing NSTs. Documenting the presence of these compounds will aid in determining if the advantages of neonicotinoid use is outweighed by potential for negative non-target effects.

4.2 Materials and Methods

Trials were replicated during the 2018, 2019 and 2020 growing seasons in two fields at Throckmorton Purdue Agricultural Center (TPAC) in Lafayette, Indiana (40°17'48''N, 86°54'13''W) and two fields at Pinney Purdue Agricultural Center (PPAC) in LaPorte Co. (41°26'35''N, 86°55'49''W). Fields were located approximately 1.25 km and 1.4 km apart, respectively. This objective was conducted in the same field plots as in Chapter 3. A layout of the plots is shown in **Figure 4.1**. Four treatments were compared: 1) Untreated, Bt seed (Genuity SmartStax RIB Complete® (DKC 62-08) expressing Cry3Bb1 + Cry34/35Ab1 with glyphosate and glufosinate tolerance); 2) NST, Bt seed (Genuity SmartStax RIB Complete® (DKC 62-08, Poncho 1250) expressing Cry3Bb1 + Cry34/35Ab1 with glyphosate and glufosinate tolerance); 3) untreated, non-Bt seed (DKC 62-05); 4) NST, non-Bt seed (DKC 62-05, Poncho 1250). Seeds were purchased from Bayer Crop Science (Dekalb; Bayer Crop Science, St. Louis, MO, USA). The neonicotinoid-treated seeds were purchased with a rate of 0.25 mg a.i./seed applied (active ingredient clothianidin, trade name: Acceleron® Basic) and an additional 1.00 mg a.i./seed was applied by the seed treatment manufacturer (Bayer CropScience in 2018 and BASF in 2019, Research Triangle Park NC, USA) for a total clothianidin rate of 1.25 mg a.i./seed. Seeds used in untreated plots in 2020 were washed to remove the seed coating using a protocol developed by Dr. Joseph Spencer at the University of Illinois (personal communication), which is described in detail in the methods of Chapter 3.

These treatments were planted in a randomized 16-plot block comprised of four replicates of each of the four treatments (Figure 1). Plots were planted on 76-cm (30-inch) row spacing with a plant population of 68,419 seeds per hectare (27,000 plants per acre). Individual plots were four rows wide and 12.19 m (i.e. 40 ft) long at TPAC and 9.14 m (i.e. 30 ft) long at PPAC. Plots were machine-planted on 17 May, 22 May and 4 June in 2018; 4-6 June and 18 June in 2019; and 26 May and 2 June in 2020. Neonicotinoid residue levels were measured in soil and water at a variety of sites summarized in **Table 4.1** and **Table 4.2**.

Soil samples were collected from four random locations per plot, two samples from row one and two from row four. Soil samples were collected using a 10 cm diameter x 15 cm soil core and samples were combined in an 18.95-liter bucket. A subsample (~ 250g) was taken from the mixture of soil and stored in paper bags labeled with location and date. Samples were stored at -20°C until analysis. Surface water samples were collected from ditches and waterways adjacent to fields and stored in 500 mL Thermo Scientific Nalgene plastic amber bottles labeled with location and date and stored at -20°C until analysis. Soil samples were weighed to 3.00 g while water samples were 40 mL in volume. All samples were analyzed using a modified version of the QuEChERS protocol (adapted by Bindley Bioscience Center at Purdue University). Using the QuEChERS protocol, clothianidin and thiamethoxam (the primary neonicotinoid seed treatments used in corn and soybeans) were extracted by first centrifuging the soil samples with 15 mL acetonitrile, 15 mL water, 10 uL internal standards and the salts magnesium sulfate (6.00 g) and sodium acetate (1.50 g). After centrifuging, the supernatant (10 mL) was transferred to a BondElut tube (Santa Clara, CA) and centrifuged. From this, the supernatant (6 mL) was transferred to a 15 mL centrifuge tube and dried down in a SpeedVac (Thermo Scientific Savant SpeedVac AES2010, Waltham, MA). Extraction of water samples were conducted by first spiking each sample with 40 uL of 1 ng/uL internal standard mix (containing thiamethoxam and clothianidin) and mixing by gently shaking. Using a Preppy vacuum manifold (Sigma-Aldrich, St. Louis, MO, USA), the water sample was poured into a 6cc PRiME HLB cartridge (Waters, Milford, MA, USA), installed onto the port, and drained into the waste jar until all water was eluted. Three 1 mL aliquots of ACN were then poured into the cartridges and collected into a 5 mL tube and dried down in a SpeedVac. The remaining concentrated solvent of the soil and water samples was analyzed by liquid chromatography/mass spectrometry utilizing an Agilent 1200 LC interfaced to a Thermo-LTQ mass spectrometer. All QuEChERS extractions and analyses were performed at Purdue University's Bindley Bioscience Center (West Lafayette, IN).

4.3 Results

4.3.1 Neonicotinoid residues in soil

Residues in soil of the neonicotinoid seed treatments clothianidin and thiamethoxam were analyzed separately by year. Treatment means for each year were compared using two-way

repeated measure analysis of variance (ANOVA), using clothianidin concentrations and thiamethoxam concentrations as response variables, sampling week and treatment as fixed effects, and block as a blocking effect.

Outliers were identified using the “identify_outliers” function in the R package rstatix version 0.7.0 (Kassambara, 2021). Using the interquartile range (IQR), data points below $Q1 - 1.5 \times IQR$ or above $Q3 + 1.5 \times IQR$ were considered outliers and were removed. The identification of outliers using the interquartile range is described by Dunn and Clark (2009: 54 – 55). Research conducted by Hladik et al. (2014) found median clothianidin levels in water to be around 8 ng/L (0.008 ppb) and median thiamethoxam levels at <2 ng/L (<0.002 ppb).

In 2018, the values of clothianidin concentration (ppb) in soil did not fit a normal distribution and contained outliers. After the removal of outliers, the data were log transformed to fit the normal curve. The results of a two-way repeated measures ANOVA found a significant difference in clothianidin concentration based on week of sampling and treatment type (**Table 4.3**). A Tukey post hoc test (TukeyHSD) was used to compare sampling week means, treatment means, and the interaction between sample week and treatment means. Clothianidin concentrations (ppb) found in soil three weeks post planting were significantly greater than concentrations at pre-planting, post-planting, one week, six weeks, and 12 weeks post-planting. Soil clothianidin concentrations one week after planting were significantly lower than post-plant, three weeks, six weeks, and 12 weeks after planting. A comparison of clothianidin concentrations by treatment showed Bt+ and refuge+ soil concentrations to be significantly greater than Bt, refuge, and forested control sites. Control site residue concentrations were also significantly lower than Bt and refuge field sites. There was no significant difference between Bt+ and refuge+ concentrations, as well as no significant difference between Bt and refuge (**Table 4.4**). There was a significant interaction between sample week and treatment in 2018. There were no significant interactions between treatments of soil samples collected prior to planting. Of the soil samples collected after planting, the mean clothianidin concentration of samples collected from the forested control sites was significantly lower than that of refuge, Bt, Bt+, and refuge+ plot concentrations. The mean clothianidin concentration of forested control soil samples collected one week after planting were also significantly lower than that of refuge, Bt, Bt+, and refuge+ plots. Three weeks after planting, the mean clothianidin concentrations of refuge, Bt, Bt+, and refuge+ plots were all significantly greater than the concentration of samples collected from the forested control sites. Additionally,

both Bt+ and refuge+ soil sample mean clothianidin concentrations were significantly greater than that of Bt and refuge plots. Of the soil samples collected six weeks after planting, the mean clothianidin concentration of forested control samples was significantly lower than that of refuge, Bt, refuge+, and Bt+ plots. Both refuge+ and Bt+ soil mean concentrations were also significantly greater than untreated refuge and Bt six weeks after planting. Twelve weeks after planting, the mean clothianidin concentration of soil from refuge+ plots was significantly greater than concentrations of refuge, Bt, Bt+, and forested control sites. Mean Bt+ concentration was also significantly greater than Bt and refuge plots.

In 2019, the clothianidin concentration (ppb) data did not fit a normal distribution and contained outliers. After the removal of outliers, the data was log transformed to fit the normal curve. The results of a two-way repeated measures ANOVA found a significant difference in clothianidin concentration based on week of sampling and treatment type (**Table 4.3**). Tukey HSD was used to compare sampling week means and treatment means (**Table 4.4**). Clothianidin concentrations (ppb) found in soil after planting and one week after planting were significantly greater than concentrations before planting, six weeks after and 12 weeks after planting. Additionally, concentrations at three weeks after planting were significantly greater than before planting and 12 weeks after planting. Pre-planting clothianidin concentrations were also significantly lower than six weeks after planting. A comparison of clothianidin concentrations in soil based on treatment showed Bt+ to be significantly greater than forested control sites, refuge, Bt and refuge+. Refuge+ was significantly greater than Bt, refuge, and control sites. Clothianidin concentrations at forested control sites were also significantly lower than Bt and refuge field sites. There was no significant difference between refuge and Bt. There was a significant interaction between sample week and treatment in 2019. Of the soil samples collected prior to planting, the mean clothianidin concentration of the forested control soil was significantly lower than that of refuge, Bt, refuge+, and Bt+ plot samples. The mean concentration of the control soil was also significantly lower than that of refuge, Bt, refuge+, and Bt+ after planting. Additionally, after planting, the mean clothianidin concentrations of refuge+ and Bt+ soil samples were significantly greater than refuge and Bt. These same significant interactions also occurred one week and three weeks after planting. Additionally, three weeks after planting, Bt+ mean clothianidin concentration was significantly greater than refuge+. Of the soil samples collected six weeks after planting, the mean clothianidin concentration of Bt+ plots was significantly greater than that of the control,

refuge, Bt, and refuge+ soil. Refuge+ was also significantly greater than the forested control soil clothianidin concentration. Twelve weeks after planting, Bt+ was significantly greater than the control, refuge, and Bt, while refuge+ was only significantly greater than the control.

In 2020, the soil clothianidin data did not fit a normal distribution and contained outliers. After removal of outliers, the data was log transformed to fit a normal distribution. The results of a two-way repeated measures ANOVA found a significant difference in clothianidin concentration based on week of sampling and treatment type (**Table 4.3**). Mean clothianidin concentrations (**Table 4.4**) were compared between sampling weeks and treatment type using a Tukey post hoc test (TukeyHSD). Pre- and post- plant clothianidin concentrations were significantly greater than one week, three weeks, six weeks, and 12 weeks after planting. Mean concentration 12 weeks post planting was also significantly lower than one week post planting. A comparison of clothianidin concentrations by treatment type (TukeyHSD) showed that Bt+ and refuge+ were significantly greater than forested control sites, refuge, and Bt. Bt+ was significantly greater than refuge+. Control sites were also significantly lower than refuge and Bt. There was no significant difference between refuge and Bt. There was a significant interaction between sample week and treatment in 2020. Prior to planting, mean soil clothianidin concentration from Bt plots was significantly greater than that of the control site. Of the soil samples collected after planting, the mean clothianidin concentration of the Bt+ plots was significantly greater than that of refuge, Bt, refuge+, and control. The soil collected from the refuge+ plots after planting was also significantly greater than the control site. One week after planting, the soil collected from Bt+ plots had a mean concentration that was significantly greater than refuge and control samples. Three weeks after planting, the mean clothianidin concentration of the Bt+ plots was significantly greater than that of soil collected from refuge, Bt, and control sites. Bt+ was also significantly greater than those sites six weeks after planting. Additionally, the mean clothianidin concentrations of Bt and refuge+ plots six weeks after planting were significantly greater than the control site. Twelve weeks after planting, Bt+ was significantly greater than refuge, Bt, and the control, as well as refuge and refuge+ being significantly greater than the control, too.

The soil thiamethoxam concentrations (ppb) concentrations for 2018, 2019, and 2020 were analyzed separately by year. The data did not fit a normal distribution and contained outliers. After the removal of outliers, each year's data was log transformed to fit a normal distribution.

In 2018, the results of a two-way repeated measures ANOVA found a significant effect of sample week and treatment on thiamethoxam concentration (**Table 4.3**). Mean thiamethoxam concentrations (**Table 4.4**) were compared between sampling weeks and treatment using TukeyHSD. Pre-plant thiamethoxam concentrations were significantly greater than post-plant one week, three weeks, six weeks, and 12 weeks after planting. A comparison of thiamethoxam concentrations based on treatment type (TukeyHSD) showed that forested control sites residue concentrations were significantly lower than Bt+, refuge+, and refuge. Bt+ and refuge+ were also significantly greater than Bt. There was a significant interaction between sample week and treatment in 2018. Of the soil collected before planting, the mean thiamethoxam concentrations of refuge+ and Bt+ were significantly greater than that of refuge and Bt. There were no other significant interactions between sample week and treatment in the remaining sampling weeks.

In 2019, the results of a two-way repeated measures ANOVA found a significant effect of sample week and treatment on thiamethoxam concentration in soil (**Table 4.3**). Tukey HSD was used to compare sampling week and treatment mean thiamethoxam concentrations (**Table 4.4**). Pre-plant, post-plant, and one week post-plant thiamethoxam concentrations were significantly greater than three weeks, six weeks, and 12 weeks after planting. There was no significant difference between concentrations at pre, post, and one week after planting. A comparison of thiamethoxam concentrations based on treatment (TukeyHSD) showed the mean thiamethoxam concentration at forested control sites was significantly lower than at Bt+, refuge+, Bt, and refuge plots. Refuge was also significantly greater than refuge+. There was a significant interaction between sample week and treatment in 2019. One week after planting, soil collected from untreated refuge plots had a mean thiamethoxam concentration significantly greater than plots planted with Bt treated with clothianidin, refuge treated with clothianidin, and the control site. There were no other significant interactions between sample week and treatment in the other sampling weeks.

In 2020, the results of a two-way repeated measures ANOVA found a significant effect of sample week on soil thiamethoxam concentration (ppb) (**Table 4.3**). A comparison of thiamethoxam concentrations (**Table 4.4**) based on sampling week (TukeyHSD) showed concentrations at pre-plant and post-plant to be significantly greater than three weeks, six weeks, and 12 weeks after planting. Pre-planting soil concentrations were also significantly greater than post-plant and one week after planting. There was no significant effect of treatment on thiamethoxam concentration in soil. There was a significant interaction between sample week and

treatment in 2020. Of the soil samples collected prior to planting, the mean thiamethoxam concentration of the Bt plots was significantly greater than that of the Bt plots treated with clothianidin. There were no other significant interactions between sampling week and treatment for thiamethoxam concentrations in 2020.

4.3.2 Neonicotinoid residues in water

Neonicotinoid seed treatment (clothianidin and thiamethoxam) residues in water were analyzed separately by year. Treatment means for each year were compared using two-way repeated measure analysis of variance (ANOVA), using clothianidin and thiamethoxam concentrations as response variables, sampling week and treatment as fixed effects, and water source location as a blocking effect.

In each year, the clothianidin concentration (ppb) data did not fit a normal distribution. The data were transformed to fit a normal distribution using the logarithmic transformation. In 2018, the results of a two-way repeated measures ANOVA found a significant effect of sample week on water clothianidin concentration (**Table 4.3**). A comparison of water clothianidin concentration means (**Table 4.4**) based on sampling week (TukeyHSD) found samples taken pre-plant and one week after planting were significantly greater than 12 weeks after planting. There was no significant effect of treatment on water clothianidin concentration.

In 2019, the results of a two-way repeated measures ANOVA found a significant effect of sample week on water clothianidin concentration (**Table 4.3**). TukeyHSD comparison of sampling weeks means (**Table 4.4**) showed clothianidin concentrations six weeks after planting were significantly greater than pre-planting and one week after planting. There was no significant effect of treatment on clothianidin concentration.

In 2020, the results of a two-way repeated measures ANOVA found a significant effect of sample week and treatment on water clothianidin concentration (**Table 4.3**). A comparison of mean clothianidin concentration (**Table 4.4**) based on sampling week (TukeyHSD) showed no significant difference between water clothianidin concentrations. TukeyHSD comparison of treatment means showed clothianidin concentrations in control streams were significantly lower than agricultural streams and control ponds.

In all three years, thiamethoxam concentration (ppb) data did not fit a normal distribution. The data were transformed to fit a normal distribution using the square root transformation. In

2018 and 2020 there was no significant effect of sample week or treatment on thiamethoxam residue concentration in water (**Table 4.3**). In 2019, the results of a two-way repeated measures ANOVA found a significant effect of sample week on thiamethoxam concentration in water. TukeyHSD comparison of sampling week means showed thiamethoxam concentrations collected after planting were significantly greater than pre-planting, one week, three weeks, and six weeks after planting. There was no significant effect of treatment on thiamethoxam concentration.

4.4 Discussion

Each year of the study revealed a different sampling week exhibiting greatest clothianidin concentrations in soil, but ultimately each year found clothianidin levels to be greatest earlier on in the season and to decline as the season progressed (**Figure 4.2**). These findings further support previously documented increases in soil neonicotinoid concentrations after planting (Schaafsma et al., 2015). As previously mentioned, my study was inconsistent in revealing the sampling week with the greatest soil clothianidin concentrations. This difference between years may be due to rain events, which can cause leaching from the soil and into nearby waterways (Hladik, 2014). In each of the three field seasons of my study, I found soil clothianidin concentrations at both Bt+ and refuge+ plots to be significantly greater than that of soil at forested control sites, refuge and Bt plots.

Although I did not plant seeds treated with thiamethoxam, levels of this neonicotinoid were detected in my soil samples. In each of the three years of the study, thiamethoxam concentrations in soil were greatest earlier in the season and decreased as the season progressed. My results coincide with other findings that thiamethoxam concentrations in soil were greatest earlier in the season following planting and declined throughout the season (Frame et al., 2021). The reappearance of thiamethoxam in the plots each year may be due to drift from nearby planting, or from reworking the ground prior to planting each year (Krupke et al., 2017). In any event, the source is likely to be from nearby crop planting; in both 2018 and 2019, my results revealed soil thiamethoxam concentrations at forested control sites to be significantly lower than that of the four field treatments.

Results from my study revealed the greatest mean concentration of clothianidin in water to occur before planting in 2018 and occurring post planting and one week post in 2020. In 2018, clothianidin concentrations before planting were significantly greater than concentrations in water

12 weeks after planting. Similarly, thiamethoxam concentrations in the water samples were greatest earlier in the season and decreased throughout the season, with the exception of a decline and then slight increase at 12 weeks post planting in 2019. These results coincide with the “spring flush” in which pesticide concentrations in water are found to greatly increase in May through June, accompanying increased application during time of planting (Thurman et al., 1992; Alford and Krupke, 2019). However, these results do not align with previous findings by Frame et al. (2021), in which clothianidin levels persisted throughout the study. This difference in results may be due to differing sampling techniques, in which Frame et al. (2021) collected their water samples from lysimeter plots after each rainfall event. Raby et al. (2018) found that repeated surges of neonicotinoids in water, similar to what occurs during the “spring flush” and applications afterwards, led to toxic effects on sensitive invertebrates, such as the midge *C. dilutus*, which had an LC50 <65 ug/L (65 ppb), and the mayfly *N. triangulifer* with an LC50 as low as 1.7 ug/L (1.7 ppb). Additionally, the LC50 of the mayfly *E. longimanus* has been reported as 2.1 ppb at 24 h and fall to 0.65 ppb at 96 h (Alexander et al., 2007). Results from my study documented instances in which mean water clothianidin concentrations exceeded the LC50 values of the aforementioned mayfly species. This included an average clothianidin concentration of 5.91 ppb in the pre-planting sampling period in 2018 as well as an average concentration of 3.18 ppb six weeks after planting in 2019. Though a majority of water sample concentrations remained lower, these concentrations could still have a negative effect, with lower doses still capable of evoking sublethal effects, such as reductions in feeding, movement, and reproduction (Goulson, 2013). Additionally, the constant presence of low concentrations of neonicotinoids in water has the potential to cause an eventual evolved resistance to the insecticide (Alford and Krupke, 2018).

A decline in water neonicotinoid concentrations as the season progresses may be due to fewer rain events later on in the season, or due to the uptake of neonicotinoids by plants (Hladik, 2014; Alford and Krupke, 2019). In contrast, my results in 2019 showed clothianidin concentrations in water to be greatest six weeks after planting. This late season increase may be due to a rain event that followed a dry period. In both 2018 and 2019, there was no significant effect of sampling site, and therefore treatment, on the clothianidin concentration in water. This further proves other documentation that neonicotinoids are widespread across our region, with even forested waterways being contaminated with neonicotinoid residues.

4.5 References

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4.6 Tables and Figures

Table 4.1. List of sampling sites at Throckmorton Purdue Agricultural Center (TPAC) sampled and monitored for clothianidin and thiamethoxam concentrations in Objective 3, in addition to each site's corresponding crop type, theoretical potential for environmental loading of neonicotinoids, and frequency of site sampling.

Site ID	Sampling Site	Site Location	Sample Type	Crop Type	Neonicotinoid load potential	Frequency of Sampling	Total Number of Samples
Forested Reference Site	-Soil in Martel Forest (40°25'53"N, 87°02'16"W	Soil	None	-/-	Pre-plant, at planting, 1, 3, 6, 12 weeks post-planting	4 per time period/matrix
	-Water from Martell Pond	40°26'03"N, 87°02'13"W	Surface water				
Field plots	-Fields T3 and T6 -Trt 2&4	40°17'48"N, 86°54'13"W	Soil	NSTs on continuous corn	+/-	Pre-plant, at planting, 1, 3, 6, 12 weeks post-planting	4 per time period/matrix
Ditches and waterways	-TPAC ditch	40°18'04"N, 86°53'41"W	Surface water	NSTs on continuous corn	-/+	Pre-plant, at planting, 1, 3, 6, 12 weeks post-planting	1 per time period/matrix
Field plots	-Fields T3 and T6 -Trt 1&3	40°17'48"N, 86°54'13"W	Soil	Untreated continuous corn	-/-	Pre-plant, at planting, 1, 3, 6, 12 weeks post-planting	4 per time period/matrix
Ditches and waterways	-Martell Indian Creek	40°25'53"N, 87°02'16"W	Surface water	Untreated continuous corn	+/+	Pre-plant, at planting, 1, 3, 6, 12 weeks post-planting	1 per time period/matrix

Table 4.2. List of sampling sites at Pinney Purdue Agricultural Center (PPAC) sampled and monitored for clothianidin and thiamethoxam concentrations in Objective 3, in addition to each site's corresponding crop type, theoretical potential for environmental loading of neonicotinoids, and frequency of site sampling.

Site ID	Sampling Site	Site Location	Sample Type	Crop Type	Neonicotinoid load potential	Frequency of Sampling	Total Number of Samples
Forested Reference Site	- <u>Soil</u> in Pinney woodlands	41°26'14"N , 86°55'20"W	Soil	None	-/-	Pre-plant, at planting, 1, 3, 6, 12 weeks post-planting	4 per time period/matrix
	- <u>Water</u> from pond in Pinney woodlands	41°26'14"N , 86°55'20"W	Surface water				
Field plots	-Fields L3 and E3 -Trt 2&4	41°26'35"N, 86°55'49"W	Soil	NSTs on continuous corn	+/-	Pre-plant, at planting, 1, 3, 6, 12 weeks post-planting	4 per time period/matrix
Ditches and waterways	-E3 ditch	41°26'42"N , 86°56'14"W	Surface water	NSTs on continuous corn	-/+	Pre-plant, at planting, 1, 3, 6, 12 weeks post-planting	1 per time period/matrix
Field plots	-Fields L3 and E3 -Trt 1&3	41°26'35"N, 86°55'49"W	Soil	Untreated continuous corn	-/-	Pre-plant, at planting, 1, 3, 6, 12 weeks post-planting	4 per time period/matrix
Ditches and waterways	-Valpo ditch	41°26'04"N , 87°03'51"W	Surface water	Untreated continuous corn	+/+	Pre-plant, at planting, 1, 3, 6, 12 weeks post-planting	1 per time period/matrix

Table 4.3. Degrees of freedom (df), F-values, and p-values for two-way repeated measure analysis of variance (ANOVA) model describing clothianidin and thiamethoxam residue in soil and water by year based on sampling week and treatment and the interaction between sampling week and treatment.

Sample Type	Year	NST	Factor	df	F-value	p-value
Soil	2018	Clothianidin	Sample week	5	14.494	< 0.001*
			Treatment	4	103.321	< 0.001*
			Sample week : Treatment	19	7.412	< 0.001*
		Thiamethoxam	Sample week	5	43.278	< 0.001*
			Treatment	4	6.377	< 0.001*
			Sample week : Treatment	19	3.846	< 0.001*
	2019	Clothianidin	Sample week	5	15.179	< 0.001*
			Treatment	4	134.935	< 0.001*
			Sample week : Treatment	20	5.53	< 0.001*
		Thiamethoxam	Sample week	5	18.454	< 0.001*
			Treatment	4	6.736	< 0.001*
			Sample week : Treatment	20	1.622	0.046*
	2020	Clothianidin	Sample week	5	48.266	< 0.001*
			Treatment	4	62.875	< 0.001*
			Sample week : Treatment	20	4.377	< 0.001*
		Thiamethoxam	Sample week	5	26.171	< 0.001*
			Treatment	4	1.371	0.2439
			Sample week : Treatment	20	1.886	0.0129*
Water	2018	Clothianidin	Sample week	5	4.143	0.0203*
			Treatment	2	2.658	0.1108
			Sample week : Treatment	8	1.016	0.4731
		Thiamethoxam	Sample week	5	1.11	0.405
			Treatment	2	0.966	0.408
			Sample week : Treatment	8	0.864	0.57
	2019	Clothianidin	Sample week	5	4.216	0.0254*
			Treatment	2	0.438	0.6571
			Sample week : Treatment	10	0.69	0.7157
		Thiamethoxam	Sample week	5	6.014	0.0080*
			Treatment	2	1.837	0.2091
			Sample week : Treatment	10	7.845	0.0016*
	2020	Clothianidin	Sample week	5	3.767	0.0208*
			Treatment	2	8.04	0.0042*
			Sample week : Treatment	10	0.827	0.6106
		Thiamethoxam	Sample week	5	1.211	0.351
			Treatment	2	1.068	0.369
			Sample week : Treatment	10	0.931	0.533

*P<0.05

Table 4.4. Mean and standard deviation (SD) of clothianidin and thiamethoxam concentrations (ppb) in soil and water by year, sample week, and treatment. Means followed by a different letter are statistically different in each category.

Sample Type	Year	NST	Factor		Mean Conc. (ppb)	SD
Soil	2018	Clothianidin	Sample Week	Pre-planting	4.76ab	2.17
				Post-planting	7.68b	8.36
				1 week Post	4.16a	1.44
				3 weeks Post	12.9c	15.2
				6 weeks Post	8.21b	8.24
				12 weeks Post	7.81b	7.86
			Treatment	Bt	4.32b	2.11
				Bt+	10.4c	11.8
				Ref	4.53b	1.84
				Ref+	12.3c	11.7
				Control	0.521a	0.597
		Thiamethoxam	Sample Week	Pre-planting	0.737d	0.78
				Post-planting	0.258c	0.18
				1 week Post	0.304c	0.307
				3 weeks Post	0.202bc	0.131
				6 weeks Post	0.128ab	0.0749
				12 weeks Post	0.0719a	0.066
			Treatment	Bt	0.207ab	0.198
				Bt+	0.376c	0.648
				Ref	0.247bc	0.202
				Ref+	0.338c	0.449
				Control	0a	0
	2019	Clothianidin	Sample Week	Pre-planting	5.72a	4.02
				Post-planting	25.5d	52.2
				1 week Post	35.4d	73.5
				3 weeks Post	18.9cd	29.2
				6 weeks Post	16.2bc	25
				12 weeks Post	9.02ab	10.8
			Treatment	Bt	4.9b	3.24
				Bt+	50.3d	73.1
				Ref	5.03b	2.41
				Ref+	15.7c	16
				Control	0.401a	0.174
		Thiamethoxam	Sample Week	Pre-planting	0.166b	0.16
				Post-planting	0.197b	0.122
				1 week Post	0.21b	0.274
				3 weeks Post	0.0703a	0.0506
				6 weeks Post	0.0698a	0.0509
				12 weeks Post	0.0889a	0.0796

Table 4.4 continued

				Treatment	Bt	0.142bc	0.158
					Bt+	0.117bc	0.104
					Ref	0.172c	0.219
					Ref+	0.113b	0.0984
					Control	0a	0
2020	Clothianidin	Sample Week	Pre-planting	14c	15.9		
			Post-planting	22.7d	30.5		
			1 week Post	6.13b	4.53		
			3 weeks Post	5.07ab	2.72		
			6 weeks Post	5.53ab	4.28		
			12 weeks Post	4.21a	2.12		
			Treatment	Bt	7.21b	11.5	
		Bt+		17d	24.7		
		Ref		5.28b	5.19		
		Ref+		9.76c	12.9		
		Control		1.91a	1.84		
		Thiamethoxam		Sample Week	Pre-planting	0.169c	0.228
					Post-planting	0.0887b	0.0817
			1 week Post		0.0516ab	0.0332	
	3 weeks Post		0.0347a		0.0364		
	6 weeks Post		0.0265a		0.0281		
	12 weeks Post		0.0214a		0.0322		
	Treatment		Bt		0.0745a	0.161	
		Bt+	0.0462a	0.0641			
		Ref	0.0641a	0.0878			
		Ref+	0.0682a	0.113			
Control		0.0549a	0.0685				
Water	2018	Clothianidin	Sample Week	Pre-planting	5.91b	13.5	
				Post-planting	0.508ab	NA	
				1 week Post	1.18b	1.6	
				3 weeks Post	0.221ab	0.12	
				6 weeks Post	0.487ab	0.84	
				12 weeks Post	0.0955a	0.0747	
				Treatment	Control Stream	3.21a	9.27
		Untreated Ag Stream	0.535a		0.661		
		Treated Ag Stream	0.204a		0.149		
		Thiamethoxam	Sample Week		Pre-planting	0.89a	2.22
					Post-planting	0.167a	NA
					1 week Post	0.0386a	0.0434
					3 weeks Post	0.00716a	0.00617
				6 weeks Post	0.00464a	0.0051	
	12 weeks Post			0.00334a	0.00423		
	Treatment			Control Stream	0.431a	1.52	

Table 4.4 continued

			Untreated Ag Stream	0.016a	0.0228
			Treated Ag Stream	0.0183a	0.0418
2019	Clothianidin	Sample Week	Pre-planting	0.0866a	0.104
			Post-planting	0.13ab	0.13
			1 week Post	0.0732a	0.05
			3 weeks Post	0.279ab	0.372
			6 weeks Post	3.18b	3.52
			12 weeks Post	0.523ab	0.494
		Treatment	Control Stream	0.938a	2.2
			Control Pond	0.198a	0.176
			Treated Ag Stream	0.811a	1.85
	Thiamethoxam	Sample Week	Pre-planting	0.00546a	0.00486
			Post-planting	0.0357b	0.0643
			1 week Post	0.00462a	0.00704
			3 weeks Post	0.00509a	0.00458
			6 weeks Post	0.00239a	0.00165
			12 weeks Post	0.00787ab	0.00941
		Treatment	Control Stream	0.00542a	0.00688
			Control Pond	0.0207a	0.0523
			Treated Ag Stream	0.00704a	0.00652
2020	Clothianidin	Sample Week	Pre-planting	0.619a	0.83
			Post-planting	1.15a	1.22
			1 week Post	1.65a	3.07
			3 weeks Post	0.242a	0.208
			6 weeks Post	0.074a	0.106
			12 weeks Post	0.106a	0.16
		Treatment	Control Stream	0.0818a	0.096
			Control Pond	1.16a	2.24
			Treated Ag Stream	0.677a	0.877
	Thiamethoxam	Sample Week	Pre-planting	0.0135a	0.00926
			Post-planting	0.0135a	0.0141
			1 week Post	0.413a	0.0984
			3 weeks Post	0.163a	0.332
			6 weeks Post	0.00357a	0.00265
			12 weeks Post	0.00352a	0.00442
		Treatment	Control Stream	0.00589a	0.00633
			Control Pond	0.219b	0.694
			Treated Ag Stream	0.0805b	0.239

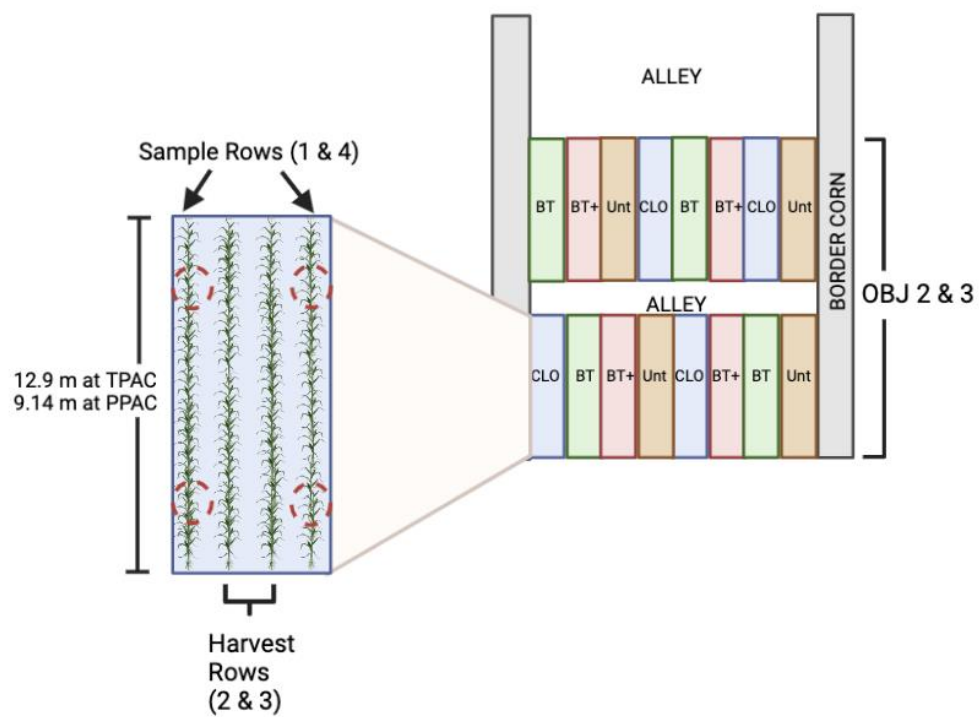


Figure 4.1. Schematic showing the arrangement of treatments within plots and soil sampling locations. In-plot soil samples were sampled from rows one and four of each plot.

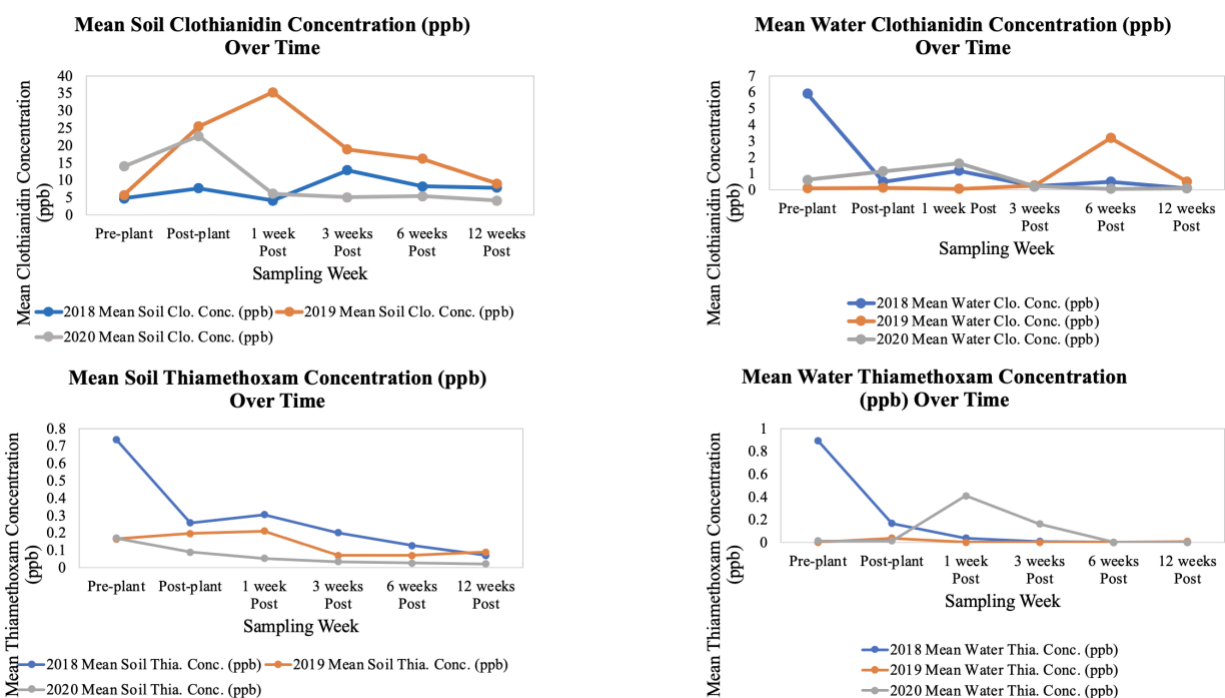


Figure 4.2. Mean clothianidin and thiamethoxam concentrations in soil and water over time for 2018, 2019, and 2020.

CHAPTER 5. CONCLUSION

Based on the results of my three objectives, it is evident that neonicotinoid seed treatments are likely not providing a pest management benefit for primary or secondary pests in the Midwestern region of the United States and should clearly be offered as an optional treatment for growers. This is supported by results of my first objective, in which I strove to quantify the effects of NSTs on the performance of refuges in delaying resistance evolution in Bt maize fields by determining if the presence or absence of NSTs affects the population sizes and mating rates of Bt-fed and refuge-fed insects. My results suggest that the current IRM strategy of deploying a 5% refuge seed blend with Bt maize is insufficient in producing an adequate proportion of susceptible beetles. The current and future IRM strategies could potentially be improved by both increasing refuge size or by not dressing refuge seeds with neonicotinoid seed treatments.

The second objective of this study, in which I quantified the benefit of using NSTs in combination with Bt maize hybrids in managing WCR and secondary soil pests in the Midwest, documented a low presence and, consequentially, low damage by both WCR and secondary soil pests. With such low secondary pest presence in our region, it would be advisable for growers to use untreated transgenic maize, which performs similarly at preserving yield to that of neonicotinoid treated maize. Planting untreated transgenic maize beneficial to growers both short-term, through decreased expenses, and long-term, by reducing the risk of resistance evolution to the insecticides.

Lastly, the third objective of this study, in which I quantified the residue levels of NSTs in agricultural and non-agricultural soils and surrounding waterways, as well as their presence throughout the field season, documented a widespread presence of neonicotinoids across our region, with levels decreasing as the season progresses. With such a widespread presence, the risk of resistance evolution to the insecticide is even greater. Providing growers with the option to plant neonicotinoid treated seeds will allow them to incorporate this tactic into their integrated pest management plan, only using it when necessary. This decreased use will in turn decrease environmental residue levels and associated negative effects upon non-target organisms, while reducing the selection pressure for resistance development among pest species.