MITIGATING RISK OF BUTTERFLY HABITAT CONTAMINATION BY PESTICIDES ON AGRICULTURAL LAND

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Abstract

Butterfly declines have been documented with long-term data sets in Europe and in the US. Analyses have shown that habitat loss and pesticide use are major drivers of the declines. Agricultural field margins have been suggested as opportune locations for habitat installation, but their use coincides with risk of pesticide contamination of host plants. Neonicotinoids have become the pesticide of choice for various crops like corn and soy, but are especially concerning for butterflies because of their systemic mode of action. There isn't enough research on habitat contamination in working landscapes or toxicity of neonicotinoids to butterflies to determine if installing habitat in agriculture is beneficial for butterfly abundance and diversity. To learn more, we conducted a field experiment to measure concentrations of Thiamethoxam (TMX) taken up by butterfly host plants (such as milkweed and sunflowers) downslope of plots planted with pesticide-coated corn seeds. We also carried out a lab experiment to measure toxicity of Thiamethoxam to painted lady butterfly larvae in order to interpret the risk of TMX concentrations found in sunflowers. The highest concentration of TMX found in the field was about 40 ppb, which was lower than the concentration at which we found significant sublethal and lethal effects in the lab, between 25,000 ppb and 30,000 ppb. We also tested if buffer strips and cover crops could reduce the concentration taken up by butterfly host plants. Neither treatment had a significant effect on concentration. More research needs to be completed to fill this gap in knowledge, and to create habitat that has positive conservation value.

Introduction

Butterfly declines and significance

With increasing evidence of pollinator declines in Europe and North America (Fox et al 2015; Hallmann et al 2017; Leather, S. R. 2017; Macgregor et al 2019; Goulson et al 2015; Potts et al 2010; Dirzo et al 2014), it is becoming more important to study and mitigate the possible causes of decline including habitat loss, competition with invasive species and battling their foreign pathogens, climate change, and pesticide use (Goulson et al 2015; Habel et al 2019; Thomas et al 2019). Pollination is a valuable ecosystem service- without it, our diets as humans would be lacking nutritionally and culturally (Klein et al 2007; Chagnon et al 2015). The FAO reports that globally, three out of four fruiting or seeding crops for human food depend on pollinators (FAO). In order to feed a growing human population, diverse and resilient pollinator communities are needed to pollinate agricultural landscapes (Klein et al 2007; Kremen 2008).

Included in resilient pollinator communities, are butterflies. There is evidence of a butterfly decline in Europe: the UK Butterfly Monitoring Survey (2019) found that about a third of butterfly species have shown a significant long-term decline since 1976, and in south-western Germany, Habel et al (2019) found significant declines for the majority of butterfly species. Literature suggests that the US could be experiencing similar declines: In Ohio, butterfly abundance has declined by a third in the past 20 years (Wepprich et al 2019) and Forister et al. (2011) found declines in the majority of species in the Central Valley of California. However, butterfly populations in the US have not been studied to the same extent as populations in Europe due to the lack of a comprehensive long-term pollinator population monitoring program.

One species of butterfly is often looked to as a flagship of all butterflies and studied in depth: the monarch butterfly (*Danaus plexippus*, Linnaeus 1758) has been instrumental in

bringing people together to support conservation of pollinators with its natural beauty and cultural significance (Guiney and Oberhauser 2008). The US Fish and Wildlife Service was petitioned to list the monarch butterfly under the Endangered Species Act after severe declines (Brower et al 2012); Semmens et al. (2016) modelled an 84% decline in two decades. The greatest threat identified to monarchs is loss of habitat (Belsky and Joshi 2018; Thogmartin et al 2017 b), however, extreme weather conditions (Brower et al 2012), protozoan parasites (Altizer and Oberhauser 1999), and pesticides (Pecenka and Lundgren 2015; Krischik et al 2015) have also been implicated in the decline (Belsky and Joshi 2018; Thogmartin et al 2017 b). Habitat loss is an issue in both overwintering and breeding grounds for monarchs: Overwintering habitat in Mexico has been reduced due to illegal logging (Thogmartin et al 2017 b); and summer breeding habitat in the north-central US, especially around agricultural landscapes, has been decimated by herbicides like glyphosate (Pleasants and Oberhauser 2013).

Monarch larvae are monophagous and can only feed on milkweed plants (*Apocynaceae*) until they pupate and emerge as adults. It is essential that more milkweed be planted throughout the north-central US to support monarch reproduction (Thogmartin et al 2017 a; Pleasants and Oberhauser 2013). Milkweed populations have been declining with more than 861 million stems disappearing since 1999 (Pleasants 2017), with 98% of those lost from corn and soy fields (Pleasants 2017). Furthermore, corn and soy fields cover such vast expanses of land (USDA-NASS 2013), that their inclusion is integral to reaching the 1.3-1.6 billion additional milkweed stems goal of the USFWS to maintain a resilient population of monarchs (Thogmartin et al 2017 b).

Monarch butterflies serve as a symbol of pollinator conservation and inspire habitat creation across the country, and that habitat is needed to buttress the butterfly community as a

whole. However, butterfly host plants in agricultural field margins have higher risk of contamination with pesticides. This risk is highlighted in the literature with Gilburn et al (2015) finding correlations between butterfly declines and acres of farmland with neonicotinoid pesticide use in the UK, as well as Forister et al (2016) finding correlations between butterfly declines in northern California and increasing neonicotinoid use. Planted unthoughtfully, butterfly habitat in agricultural areas could be counterproductive to conservation goals.

To determine if butterfly habitat can be planted on agricultural land, we need to know the uptake of pesticides by butterfly host plants, and toxicity to butterfly caterpillars. Knowing milkweed uptake of pesticides and sensitivity of monarch larvae cannot predict the uptake of other host plants and the sensitivity of other butterfly species' larvae, so each host plant and butterfly larvae pair needs to be studied to understand risk to the butterfly community as a whole. Sunflowers are common host plants to various butterflies; studying uptake of pesticides by sunflowers is necessary to understand exposure to many butterflies. Painted lady butterflies (*Vanessa cardui*, Linnaeus 1758) use sunflowers as a host plant, and studying sensitivity of painted lady larvae to pesticides in their diet contributes to the body of knowledge of butterfly larval toxicity to pesticides, and can represent what levels of toxicity might be dangerous for other sunflower-feeding butterfly species until toxicity research is published on those species.

Pesticides and Transport

There are five major groups of pesticides: organophosphates, carbamates, phenyl-pyrazoles, pyrethroids, and neonicotinoids (Simon-Delso et al 2015). Neonicotinoids were developed in the 1980's and made popular among agriculturalists after many insect pests developed resistance to pesticides belonging to most of those other groups (Georghiou and

Mellon 1983; Denholm et al 1998; Alyokhin et al 2008; Simon-Delso et al 2015). Neonicotinoids were promoted for their selective toxicity to arthropods, long persistence, systemic treatment within plants, various application methods, and high solubility in water (Simon-Delso et al 2015; Maienfisch et al 2001). Neonicotinoids mainly target sucking and chewing pests such as Aphidae, Aleyrodidae, Cicadellidae, Chrysomelidae, Elateridae, Fulgoroidea, Psuedococcidae, and phytophagous mites (Jeschke et al 2011; Maienfisch et al 2001; Simon-Delso et al 2015). The most popular method of application is seed coating, especially for staple crops like cotton, wheat, soybean, and corn (Jeschke et al 2011), and for this, Thiamethoxam (TMX) (Syngenta) or its metabolite Clothianidin (CLO) (Sumitomo and Bayer CropScience) (Nauen et al 2003) are the preferred chemicals (Simon-Delso et al 2015) (Figure 1). The seed coating saturates the soil with a "cocktail" of herbicides, fungicides, and pesticides, and when it rains, the chemicals dissolve in the water, are taken up by plants through the roots, and distributed to all their tissues via the xylem, providing long-term protection from pests (Jeschke et al 2011). 60% of neonicotinoids are used as seed coatings for these staple crops (Jeschke et al 2011). Corn alone covered 97.4 million hectares of land across the US in 2013 (USDA-NASS 2013; Simon-Delso et al 2015). Because seed coatings are popular among common crops, the chemicals TMX and CLO are widespread across agricultural landscapes. In an ideal situation, all of the pesticides from the seed coating would be taken up by crops and remain in the agricultural fields, however, crops only take up 2-20% of the pesticide in the seed coatings (Sanchez-Bayo and Goka 2014), and the rest is available for transport into the surrounding environment. Researchers have shown concern that neonicotinoids' broad application and lack of containment could impact the environment throughout our country's

agricultural landscapes and beyond (Sánchez-Bayo and Goka 2014; Pisa et al 2015; Chagnon et al 2015; Goulson 2014).

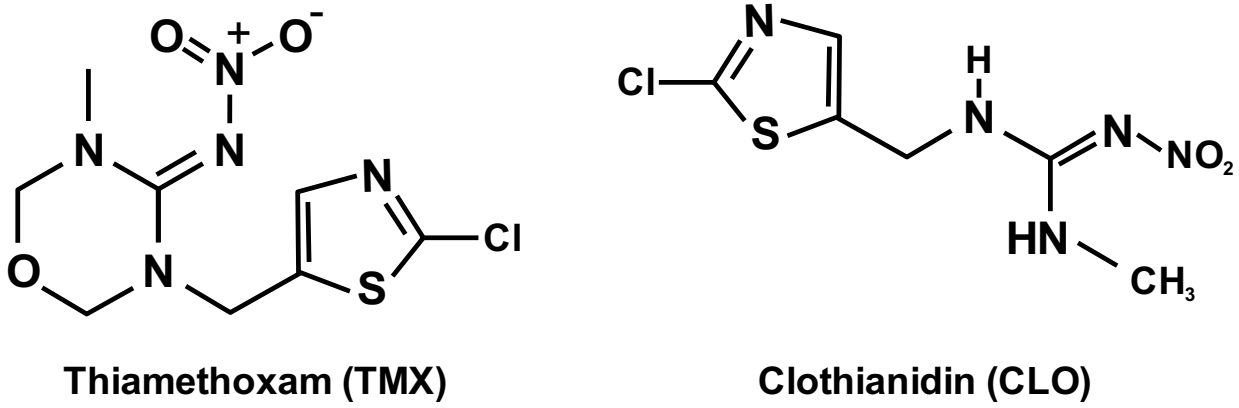

Figure 1. Chemical structure of Thiamethoxam and its metabolite Clothianidin.

There are many variables that impact the amount of TMX in the soil column, the distance that TMX is transported via subsurface flow, the amount taken up by plants, and the amount ingested by soil invertebrates. The concentration of TMX in the soil column decreases over time due to abiotic and biotic degradation, but at very different rates depending on soil conditions as enumerated below (Bonmatin et al 2015; Jones et al 2014). TMX can have a half-life between 7 and 353 days in the soil depending on these conditions (Goulson 2014). In several agricultural fields in England, TMX was found in soils 75 to 109 days after application (Jones et al 2014). A previously private study funded by Syngenta and written by Hilton et al. (2015) revealed a half-life or DT50 for TMX between 7.1 and 92.3 days, with a geometric mean of 31.2 days. Some neonicotinoids have the potential to accumulate in soils with annual coated-seed sowing, but degradation limits the accumulation after 2-6 years (Wood and Goulson 2017). These studies show that there is much variation in degradation rates, and there is not enough research yet to explain that variation.

Degradation in soils varies depending on soil texture, soil organic matter, moisture, temperature, and pH (Bonmatin et al 2015). Microbial degradation is the most important factor in decomposing neonicotinoids in the soil column (Liu et al 2011); many of the environmental factors listed above are related to microbial productivity. For example, degradation rates increased with organic carbon content in soils and moisture content (Li et al 2018). Soil composition, saturation, and microbiotic community influence TMX concentrations in the soil column, and some of the same variables that determine degradation rates also influence the transportability of TMX into and out of the soil column. Adsorption to soil particles affects the availability of TMX for transport, and there are strong positive correlations between soil adsorption and organic carbon content (Campbell et al 2005), and pH (Li et al 2018). However, adsorption is weak for TMX and CLO, and this increases their mobility.

Neonicotinoids are effective in protecting crops from pests because of their high water solubility and long persistence, and it is these same qualities that make them transportable through the subsurface environment (Bonmatin et al 2015). This is problematic because pesticides could leach from agricultural fields and contaminate the natural environments surrounding them. Bonmatin et al. (2015) describe TMX with a high leaching potential (Groundwater Ubiquity Score of 3.82 on a scale of -2 to 6 based on soil hydraulic conductivity), and low soil adsorption. In one experiment, 54.1-48.9 % of applied TMX leached from a soil column with field-realistic moisture levels over 90 days, and 66-79% of applied TMX leached from a soil column submerged in water for 90 days, with no residues found in the soil (meaning that the remaining percentage degraded to metabolites, Gupta et al 2008). While the number of pesticide molecules available for transport in the soil column is changing over time via degradation and adsorption to soil particles, (Bonmatin et al 2015), the location of pesticide

molecules also change over time due to dispersion and velocity of subsurface flow, therefore changing concentrations in the soil column. Some variables that might impact velocity and dispersion are precipitation, slope, soil texture and structure, and soil water saturation. Radolinski et al. (2018) affirmed the expectation that coarse-textured soils will transport more solutes than fine-textured soils in their 20 cm column experiment, however in their 60 cm column experiment, the loam soil column transported more TMX than both the clay (fine-textured) and the sand (coarse-textured) columns, regardless of particle size. Radolinski et al. (2018) suggest that heterogeneity and preferential flow in the loam column could be the cause for the discrepancy, and discuss the importance of soil structure perhaps above that of texture. Radolinski and co-authors (2018) also point out drastically increased transport of TMX during a simulated intense rainstorm. Because there are so many variables involved in solute transport, modelling is the best method to predict the location of potentially dangerous pesticide concentrations for pollinators on a landscape at a given time. Modelling transport of pesticides through subsurface flow and associated risk to invertebrates will be important to inform agricultural land management and conservation in the future, when we have more of the required data.

Plant Uptake of Neonicotinoids (ng/g ≈ ppb)

Once pesticides have been transported off of the agricultural field and into the surrounding environment, they can be taken up by nearby plants. Wood and Goulson et al. (2017) report average levels of neonicotinoids in wild plants: from 1.0 to 7.2 ng/g in whole flowers, 0.4 to 13.5 ng/g in foliage, <0.1 to 1.5 ng/g in nectar and <0.04 to 14.8 ng/g in pollen. These estimates are insightful, but various studies show much variation in plant uptake

depending on transport and plant species, so these data cannot be used to represent concentrations taken up by all wild plants bordering all crop fields.

To evaluate variation in uptake across plant types, Botías et al. (2016) collected leaf samples of 45 plant species adjacent to five oilseed rape fields in East Sussex, England. In previous years, seeds had been planted at the sites with CLO coatings, but the year leaf samples were collected, the seeds were coated in TMX. Leaf samples were taken from oilseed rape in the fields 10 months after sowing, but also from a two-meter buffer of herbaceous plants between the fields and woody hedges. The oilseed rape foliage contained on average 1.04 ng/g TMX and 2.92 ng/g CLO, with maximum concentrations 2.3 ng/g TMX and 8.7 ng/g CLO. TMX was detected in all oilseed rape leaf samples. Imidacloprid (IMD), another neonicotinoid, was also detected in these samples with a maximum concentration of 3.1 ng/g although it had not been applied to the field in at least three years. In wild plants in the buffer, foliage concentrations were higher than that of the oilseed rape for TMX (8.71 ng/g), and lower for CLO (0.51 ng/g). There was more variation and maximums were much higher in the buffer plants: the highest concentration of TMX detected was in *Cirsium vulgare* at 106 ng/g and the highest concentration of CLO was in *Rubus fruticosus* at 11 ng/g. Neonicotinoids were detected in 52% of wild plant foliage in this buffer. Botías et al. (2016) also found differences in concentrations depending on plant life history: TMX was found in higher concentrations in perennial and biennial plants than annuals, and CLO was found in higher concentrations in annual plants than perennials. Traces of thiacloprid and acetamiprid (two additional neonicotinoids) were also found in wild plant foliage although they were never applied at the field sites. This study is important because it suggests that there is considerable variation in plant uptake, wild plants bordering crop fields can have higher concentrations of pesticides than the crop plant itself, and some neonicotinoids are

persistent in the environment or within plant tissues- allowing plants to contain molecules of more than one compound at a time, despite seasonal application of just one.

There are few studies observing pesticide uptake of butterfly host plants: Basley and Goulson (2018) found that the leguminous host plants of butterfly *Polyommatus icarus* growing in field margins of wheat treated with CLO contained 0-48 ppb concentrations in foliage, between 10 and 21 months after wheat seed set. Accompanied by laboratory experiments, the authors resolved that these concentrations were high enough to cause sublethal effects in butterfly larvae, such as reduced size. TMX was also found in the samples collected 10 months after seed set with no history of its application on the farm; this could be because the pesticide was transported from a nearby field using pesticides. This study demonstrates the need for more studies in host plant uptake and toxicity to butterflies.

An example of a study on a common butterfly host plant, Mogren and Lundgren (2016) collected leaf samples from wild plants bordering CLO-treated corn seed plots and found concentrations in sunflower (*Helianthus annuus*), ranging from 0-81 ppb CLO. *Helianthus* is a host plant for many butterflies, and these foliar concentrations could be lethal to larvae (Peterson, Shaw, and Smith 2019). Ideally, studies similar to these would be carried out for various butterfly host plant species, to understand how neonicotinoids might impact the abundance and diversity of the butterfly community near agricultural areas.

In the context of specialized butterflies, milkweeds have been found to take up enough neonicotinoids to cause sublethal effects in monarch butterflies. Pecenka and Lundgren (2015) collected milkweed leaf samples from within 2m of corn field edges in Brookings County, SD recently after seed planting, and discovered concentrations of mean 0.58 ppb and maximum 4.02 ppb. They found through lab studies that 1 ppb of CLO causes sublethal effects in monarch

larvae such as reduced head capsule width, body length, weight, and duration of the 2nd instar, and 30.7 ppb CLO kills 90% of butterflies. However, there is much variation of uptake within and among studies with milkweeds, which may mediate exposure of sublethal or lethal concentrations to monarch butterflies.

For example, Olaya-Arenas and Kaplan (2019) collected field-adjacent common milkweed leaves in June, July, and August of 2015 and 2016. In the first year, CLO was found in 4.6 % of leaves, with a mean concentration of 0.71 ng/g and a maximum concentration of 56.5 ng/g. In the second year, CLO was found in 8.1% of leaves, with a mean of 0.48 ng/g and a maximum of 28.5 ng/g. For TMX, the percent detection in 2015 was only 1.8, with mean concentration of 0.19 ng/g but a maximum concentration of 94.8 ng/g. In the last year, TMX was found in 75.4 % of leaves, with a mean concentration of 1.87 ng/g and a maximum of 151.3 ng/g. They also found concentrations in leaves decreased with distance from the crop field. This study shows the incredible variation in uptake of milkweeds in the field, and the potential for lethality of monarch butterfly larvae.

All of these summarized studies contribute to the body of knowledge that describes plant uptake of pesticides. However, until uptake can be modeled to account for variation due to different soils, slope and topography, weather and environmental conditions, other variables affecting transport, time, species-specific plant uptake, and plant community characterization, butterfly host plants in field-adjacent buffers need to be sampled locally and with appropriate timing to identify exposure of butterflies to pesticides. Also needed to clarify exposure, is distribution of pesticide throughout plant tissues and accumulation and degradation of pesticide over time within those tissues, but there is even less published research on these subjects.

Neonicotinoid Toxicity to Butterflies

Neonicotinoids are dangerous to insect pollinators when they ingest contaminated plant material because they work as agonists on neuro-acetylcholine receptors (nAChRs) opening cation channels (Simon-Delso et al 2015). There are different structures of nAChRs in vertebrates and invertebrates, and neonicotinoids can only bind to the structure in invertebrates, which makes neonicotinoids safer for humans. There are also nAChR subunits within invertebrates, and different neonicotinoids target different subunits. When the neonicotinoid binds to the receptor, the neuron experiences continuous excitation and holds open the cation channel. This causes cell energy exhaustion and eventually paralysis of the insect (Simon-Delso et al 2015). Effects on the insect vary due to inconsistent sensitivity of receptors and their subunits (Simon-Delso et al 2015). This means that the toxicity of neonicotinoids is likely different to every species and it is important to examine toxicity to a variety of species to develop a range of impacts on the insect community.

Despite their cultural significance and well-documented decline, few papers have discussed sublethal effects and mortality of monarch butterflies due to neonicotinoids, even fewer describing effects on other butterfly species. Krischik et al. (2015) reared both monarch and painted lady larvae on Mexican milkweed and Globe thistle plants grown in pots of soil treated with 0 mg IMD, 300 mg IMD, or 600 mg IMD. Leaf concentrations were not quantified, but whole flower concentrations ranged 6.03 ppm to 45.89 ppm IMD. Almost all monarch larvae on treated plants died within 7 days. After 14 days, painted lady larval survival was 40% on control plants, and about 20% on treated plants. Twenty-two percent of painted lady larvae pupated in the control groups, 2.5% pupated in the lowest treatment group, and 0% pupated in the highest treatment group. This study exemplifies that toxicity varies across butterfly species.

Peterson, Shaw, and Smith (2019) found that at 5 ug/g CLO in artificial diet, painted lady larvae had slower development time and smaller mass as adults. Finally, Basley and Goulson (2018) found that host plants watered with 50 ppb CLO fed to larvae of *Polyommatus icarus* reduced their larval size, and host plants watered with 500 ppb CLO reduced survival of larvae. These studies are helpful in understanding toxicity of various species, but they are not easily applicable to field scenarios. More research is needed to characterize toxicity for common farmland butterflies, and host plant leaf sampling along field edges should be carried out to ensure that a butterfly decline is not caused by pesticides.

Pesticide Containment and Pollinator Habitat

Ecological intensification is the practice of embracing more wildness in agriculture that enhances ecosystem function and crop production (Pywell et al 2015). These tactics can be used in agriculture to protect or create butterfly habitat as well as provide a variety of ecosystem services to sustain crop yields (Pywell et al 2015). For example, buffer strips downslope of field margins are commonly used to reduce pesticides escaping into the surrounding environment (Reichenberger et al 2007; Carluer et al 2011), as well as reducing nitrogen, phosphorus, and sediment (Collins et al 2009) in runoff and subsurface flow. Buffer strips achieve this by decreasing runoff velocity, enhancing infiltration of pollutants, increasing microbial community diversity which facilitates degradation of pesticides, increasing pesticide adsorption to soil particles, and increasing plant uptake of pesticides (Lerch et al 2017). For these benefits, buffers have been made mandatory in some countries of the EU as a part of their agri-environment schemes (Batáry et al 2015; Cordeau, Reboud, and Chauvel 2011), and over 29,000 ha of buffers have been implemented in the UK alone (Blake et al 2011). The buffers seem to be effective in

reducing concentrations of pesticide (Popov and Cornish 2006; Carluer et al 2011; Reichenberger et al 2007), however percent reduction varies widely due to factors related to pesticide application rates and field size, factors that affect subsurface transport, as well as width, vegetation type, and vegetation cover of the buffer (Collins et al 2009; Lerch et al 2017).

Vegetative strips have been highlighted as a possible measure to increase pollinator habitat in addition to their other ecological benefits (Wratten et al 2012; Cole et al 2015). Blake et al. (2011) made recommendations for planting wildflowers in grass buffer strips to benefit butterflies. Including flowers and host plants necessary for a thriving insect community could bring benefits to farmers by increasing pollination of their crops and increasing pest predator populations (Carvalheiro et al 2012; Cordeau, Reboud, and Chauvel 2011). A review of buffer strips for insect conservation (Haaland et al 2011) shows that insect abundance and diversity is greater in wildflower strips than grass strips or naturally regenerated strips, but that they typically only increase prevalence of common species; though it could be argued that less common but highly mobile specialist butterflies (like monarchs) might find host plants in such buffer strips and see an increase in populations too. Semmens and Ancona (2019) recommend buffer strips be planted with milkweed to provide more host plants for monarch butterflies. Buffer strips create the potential to maintain uncontaminated habitat to combat pollinator declines for species such as the monarch butterfly and support abundance for species such as the painted lady butterfly. However, if butterfly host plants take up contaminated subsurface flow from the agricultural fields they border, the buffer strips could create an “ecological trap”.

Host plants can also vary in the amount of pesticide they take up: Mogren and Lundgren (2016) found that a buffer strip intended to alleviate stresses of agriculture on honey bees accumulated pesticides: Sunflowers had the highest uptake of CLO up to 81 ppb in their leaves,

whereas buckwheat and phacelia had lower max concentrations of 52 ppb and 33 ppb. Concentrations of CLO were found in nectar with mustard accumulating the highest concentration of about 1.6 ppb, and also in the bees' honey at 6.61 ppb. All of those maximum leaf concentrations are higher than the CLO LC90 for monarch larvae Pecenka and Lundgren (2015) report, and are above the rate at which Peterson, Shaw, and Smith (2019) found sublethal effects in painted lady larvae. This information validates the concern that pollinator habitat could be contaminated by pesticides if installed adjacent to agricultural fields regardless of buffer strip installation, and could work against conservation efforts for the monarch butterfly and abundance and diversity goals for common butterflies like the painted lady.

Cover crops also provide ecosystem services: cover crops can increase soil organic matter (Wander et al 1994), increase nitrogen recovery (Weinart et al 2002), and reduce erosion (Pimentel et al 1995). More research needs to be done to determine their effect on pesticide transport (Holvoet et al 2007), but they have also been recommended as a solution for installing pollinator habitat on agricultural lands (Ellis and Barbercheck et al 2015; Wratten et al 2012).

However, the same threat persists that any pollinator habitat on agricultural land could be contaminated by pesticides. For example, Bredeson and Lundgren found in 2019 that cereal rye and hairy vetch planted between rows of seed-treated corn took up pesticides at concentrations as high as 0.33 ng/g TMX and 2.61 ng/g CLO, and 0.51 ng/g TMX and 9.73 ng/g CLO respectively, in their leaves. Pecenka and Lundgren (2015) found an LC10 for monarch larvae below 9.73 ng/g CLO after an exposure period only lasting until the 3rd instar, and found sublethal effects on monarchs as low as 1 ng/g. If milkweeds took up similar concentrations, it could lead to monarch mortality instead of helping conservation efforts. Peterson, Shaw, and Smith 2019 found sublethal effects on painted lady larvae below 9.73 ng/g CLO. Contaminated

cover crops could also contribute to a loss of butterfly abundance and diversity. Pollinator conservation and agricultural land management strategies could work hand in hand if buffer strips and cover crops could be planted in such a way to prevent exposure to high concentrations of pesticides.

Pesticides can leach out of agricultural fields via subsurface flow and contaminate pollinator resources downslope (Figure 1). It is important to explore the ability of cover crops and buffer strips to take up these pesticides because they provide a potential opportunity to reduce pesticide leaching and also create pollinator habitat. However, it must be elucidated whether the pesticides taken up by the plants could pose a risk to pollinator survival and development i.e. an ecological trap.

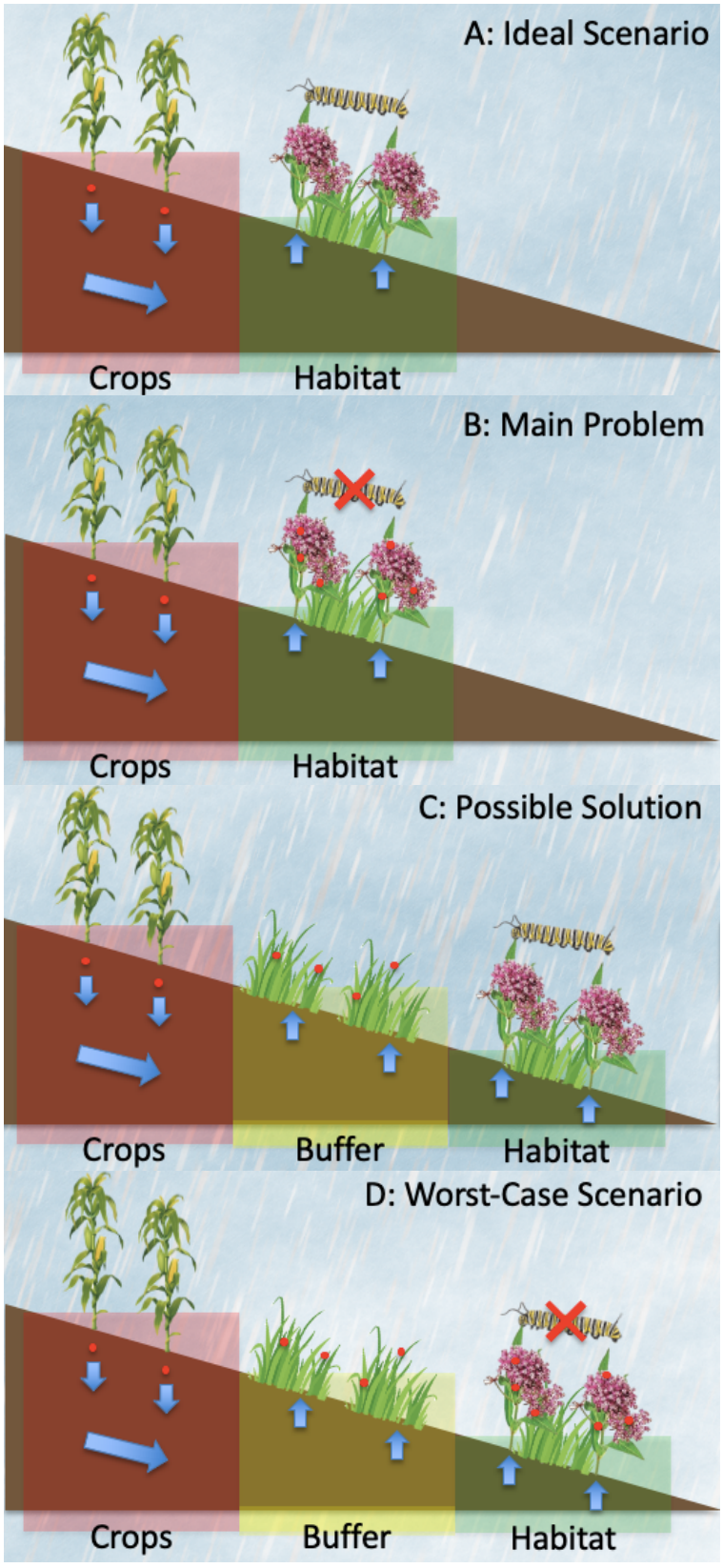

Figure 1. Pesticides applied as seed coatings that are not taken up by crops can be transported via subsurface flow and taken up by plants in buffer strips and pollinator habitat adjacent to agricultural fields. Box A represents the ideal scenario, in which pesticides are not transported and habitat is not contaminated, while Box B represents the problem found in the literature, that pesticides are transported and contaminating margins with habitat. Box C represents a possible solution, planting buffers to take up some pesticides before they reach habitat, and Box D represents the worst-case scenario, in which the land managers installed buffers to prevent contamination of habitat, but the strategy is not successful in capturing all the pesticides, and the habitat is contaminated.

Objectives

This project, a collaboration by Ryan Stewart and Kang Xia at Virginia Tech and T'ai Roulston at the University of Virginia, a) planned to model transport of TMX via runoff and subsurface flow; b) evaluated the ability of buffer strips and cover crops to capture contaminated runoff and reduce pesticide concentrations in flow; and c) examined the risk of this pesticide exposure pathway to butterflies. The project was based in Orange County, VA at the Northern Piedmont Research Center (NPRC) managed by Virginia Tech, where three kinds of buffer strips (fescue, forbs, and both forbs and grasses) were planted downhill of corn plots with and without cover crops (this study only used the grass strips and grass and forb strips). Lysimeters were installed below the rooting zone throughout the buffer strips to collect subsurface flow, and runoff collectors were installed at the downslope border of each buffer strip to collect runoff. Water sampling devices determined if the buffer strips and cover crops reduced concentrations in the flow and which type of buffer strip was the most effective. In particular, my thesis was centered on plant uptake and risk of mortality and sublethal effects to butterfly larvae.

My main questions were:

1) What concentrations of TMX are in butterfly host plants adjacent to seed-coated corn fields? (Field Study)
2) Can buffer strips reduce the concentration of TMX in host plants near seed-coated corn fields? (Field Study)
3) Can cover crops reduce the concentration of TMX in host plants adjacent to seed-coated corn fields? (Field Study)
4) Are TMX concentrations found in host plants in the field dangerous to butterfly larvae? (Lab Trials)

5) What concentrations of TMX would be enough to cause mortality during development? (Lab Trials)
6) What concentrations of TMX would be enough to cause sublethal effects during development? (Lab Trials)

To answer these questions, I first quantified uptake by milkweeds and sunflowers downslope of corn plots and determined if buffer strips and cover crops have an effect on their uptake at the NPRC. Then, I investigated sublethal and lethal effects of TMX at various concentrations in artificial diet to painted lady butterflies in the lab at Blandy Experimental Farm. I predicted that sublethal and lethal thresholds will exceed concentrations taken up by milkweed and sunflower plants at the NPRC.

Methods

Field Study:

The field study determined what concentrations of TMX were in butterfly host plants adjacent to seed-coated corn fields, and if buffer strips or cover crops could reduce those concentrations to alleviate habitat contamination (Main Questions 1-3).

Description of Field Site

All fieldwork was conducted at the Northern Piedmont Research Center. The NPRC is located in Orange County, Virginia at 38° 13' 26.10" N, 78° 07' 13.08"W. Elevation at the NPRC ranges from 146 m to 167 m (Fig 2). The climate over the summer in the months of May, June, and July (when measurements were taken) is characterized by an average maximum temperature of 86.4*F, and an average minimum temperature of 52.1*F (Virginia Tech, see Appendix A for

more details). Soils are typically Davidson clays, Dyke loams, and Starr silt loams (Fig 5, see Appendix B for more details).

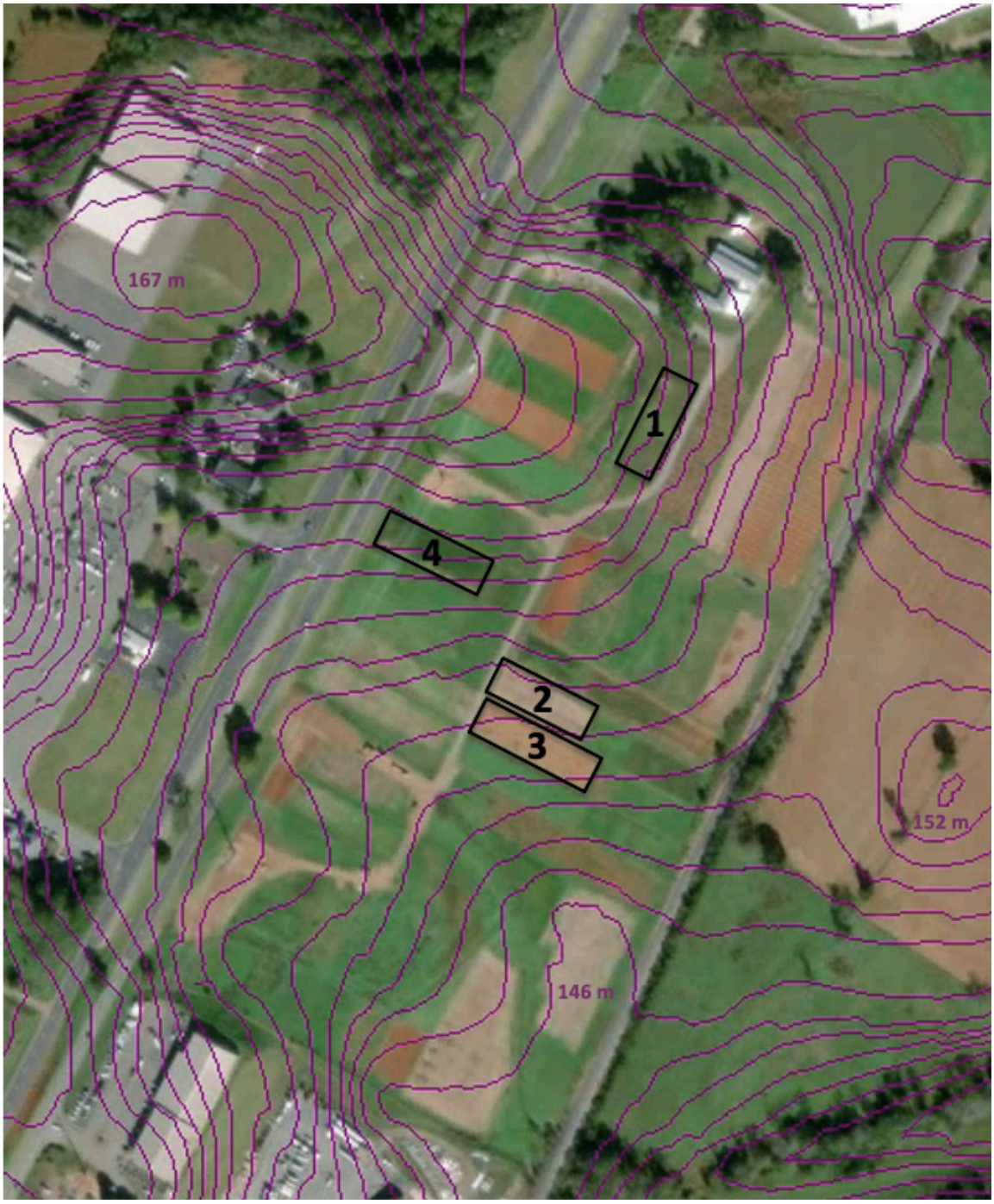

Figure 2. Topographic map of NPRC. Highest point of elevation is in the top left of the image at 167 m, and the lowest point of elevation is in the bottom right of the image at 146 m. The four blocks are discussed in the experimental design.

Experimental Design, Setup, and Sampling

At the field site, buffer strips and cover crops were installed in a split-plot experimental design to examine differences in pesticide transport and uptake by host plants: The whole plot (n=8, 4 replicates per treatment) comprised a strip of grass buffer or grass and forb habitat, and contained two TMX seed-coated corn subplots, one with cover crops over the winter and the other without, upslope from the buffer strip (Figure 3). Whole plots were organized into four spatially separated blocks (one habitat whole plot and one buffer strip whole plot in each, Figure 4). Whole plots measured 21.03 m by 15.24 m. The habitat or buffer strip along the bottom was 15.24 m long and 4.27 m wide. The corn plots above were 6.71 m wide and 16.76 m long, with a break of 1.83 m in between them (Figure 3).

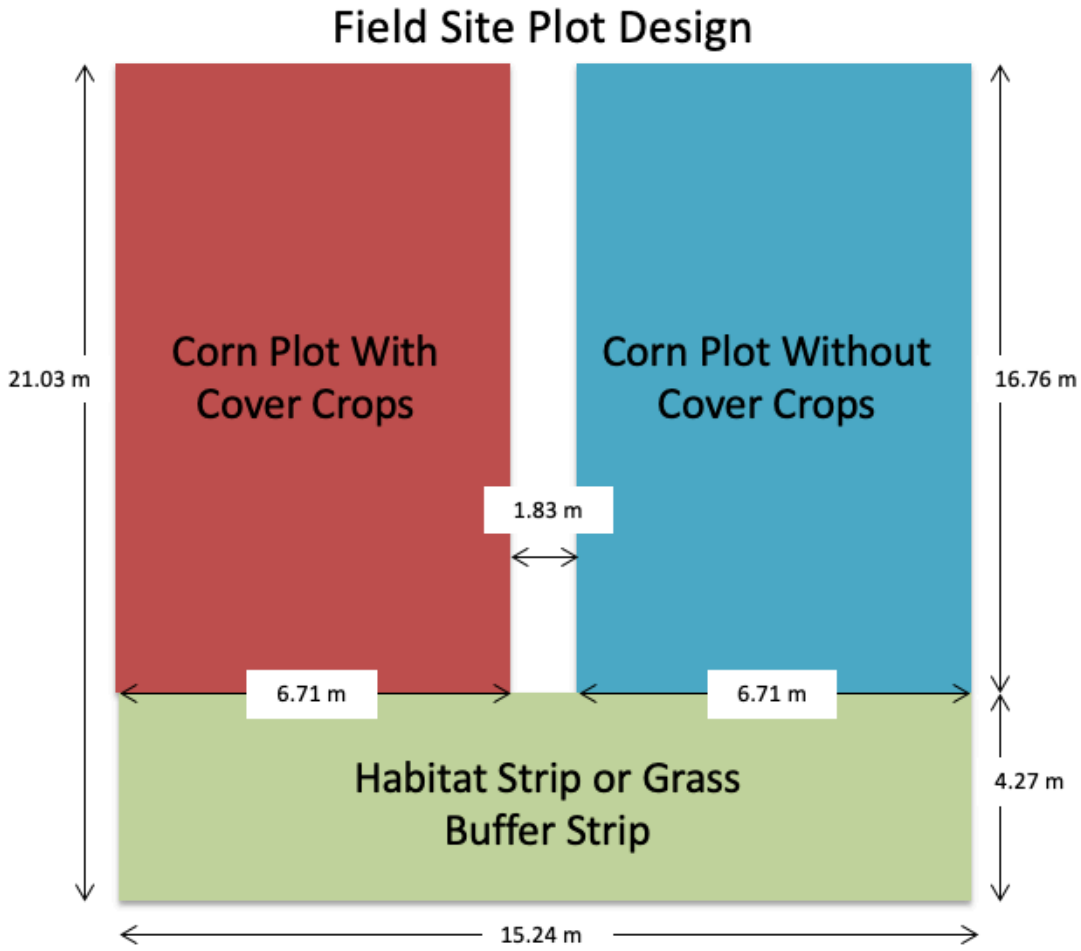

Figure 3. Measurements of whole plots.

Figure 4. Whole plots randomly distributed into four blocks at the NPRC. Whole plots with green strips were grass buffers, whole plots with lavender strips were habitat strips, and whole plots with yellow strips were excluded.

To determine the effectiveness of cover crops at reducing TMX concentrations, leaf samples were taken from host plants transplanted into the upslope boundary of the buffer/habitat strips compared across subplots. To determine the effectiveness of the grass buffer, differences in leaf sample concentrations from host plants in the upslope and downslope boundaries of the buffer/habitat strips were compared within whole plots and replicated across whole plots.

In June 2020, pesticide-coated corn seeds (CruiserMaxx 1250, Syngenta, United States) (active ingredients: Thiamethoxam, Mefenoxam, Fludioxonil) were sown in 24 plots at the NPRC, each plot with a downslope grass buffer strip (mostly *Bouteloua curtipendula* and *Schizachyrium scoparium*, but see full list in Appendix C) or habitat strip of grasses and forbs (mostly *Echinacea purpurea*, *Heliopsis helianthoides*, and *Chamaecrista fasciculata*, see full list in Appendix D). More forb seeds were broadcast (*Chamaecrista fasciculata* and *Rudbeckia hirta*) to encourage forb establishment in the habitat strips in April 2021, and a second round of treated corn seeds were planted in May 2021. With treated corn seeds planted in years 2020 and 2021, we expected there to be active TMX in the soil column in the corn plots available for transport during the summer of 2021. 180 common milkweed (*Asclepias syriaca*) and 144 sunflower (*Helianthus maximiliani*) seeds were grown from seed in the greenhouse at Blandy in the fall of 2019, and transplanted one meter apart along the top and bottom borders of the buffer and habitat strips at the NPRC in May and October of 2020 respectively (Figure 5). These plants are host plants to monarch and painted lady butterflies, and as such were sampled throughout the summer to examine uptake of pesticides leached and transported from the treated

corn seeds to better understand risk of contamination of butterfly host plants.

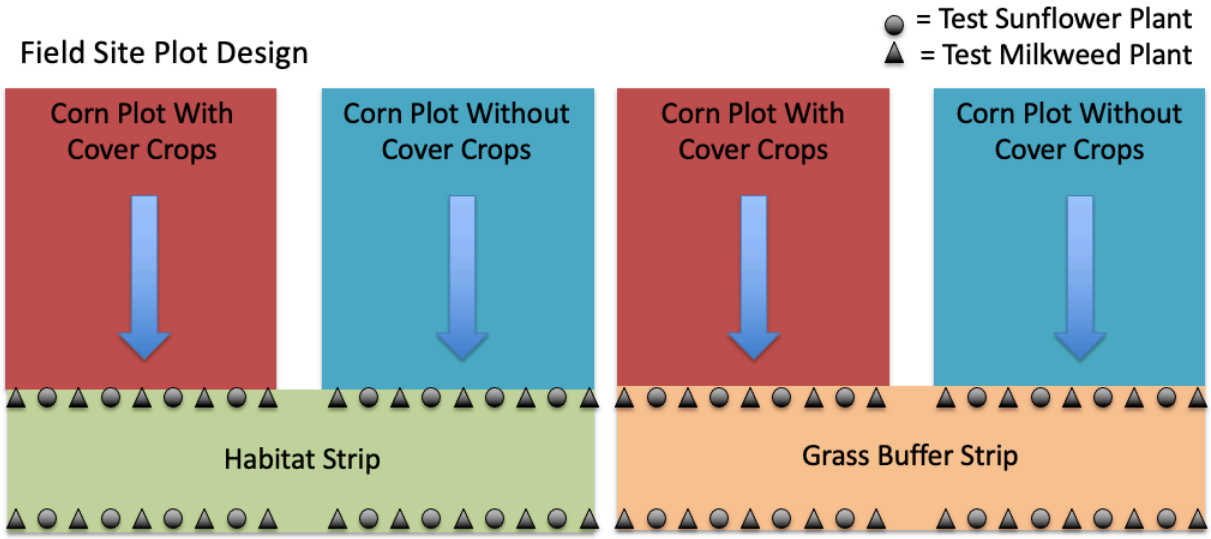

Figure 5. Field site plot design: Whole plots determined by buffer or habitat strip, subplots determined by cover or no cover crops, and milkweeds and sunflowers planted into the top and bottom of the strip.

Within the first couple days after the first heavy rainstorm of May and June at the NPRC in the summer of 2021, leaf samples were taken. Sampling was event-based because Radolinski et al (2018) found that leaching of TMX occurs at the greatest rate during and immediately after heavy rainstorms. Whole leaves were collected from near the top of the stems of milkweeds and sunflowers in all habitat and buffer strips and contained in plastic Ziploc® bags labeled with sharpie inside a cooler with dry ice to preserve TMX levels at the time of sampling. Leaf samples were weighed on a scale inside their Ziplocs (an average Ziploc baggie weight was subtracted from the weight) and kept in the -80*C freezer until they could be pooled and transported to Virginia Tech for analysis. Leaf samples were pooled to reduce costs of analysis (Analysis for TMX costs 31$ per sample) and to increase mass of samples that were too small for analysis on

their own. Efforts were made to have two pooled replicates for each treatment (block x cover/nocover x habitat/buffer x top/bottom) at each sampling time (Figure 6).

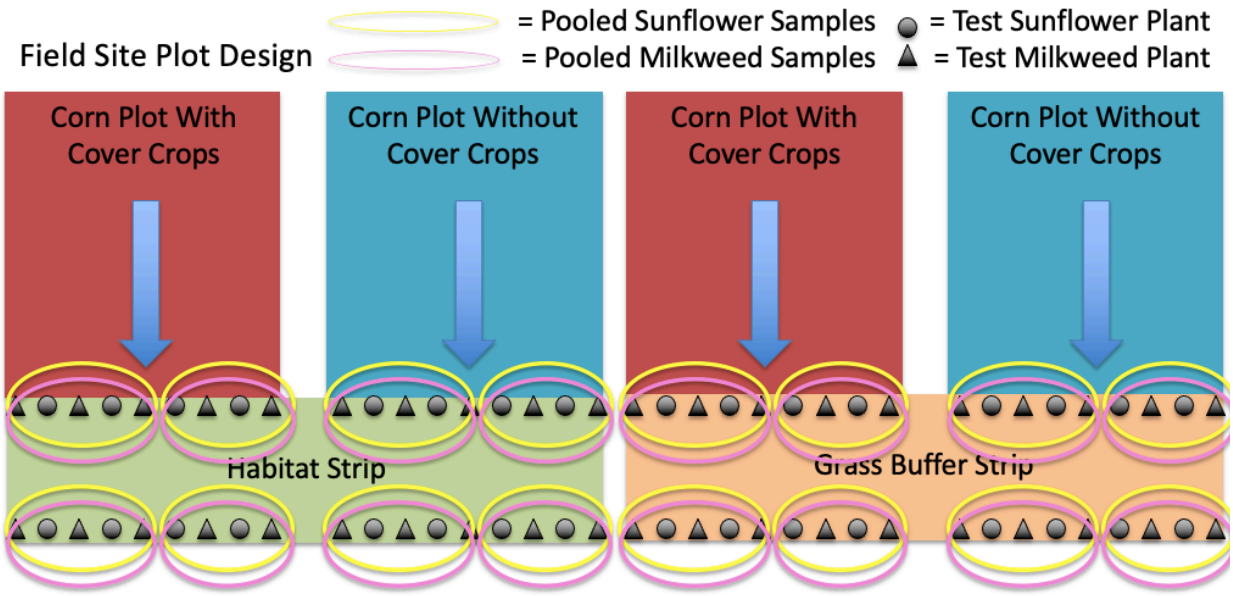

Figure 6. Leaf samples were pooled in two groups per treatment (block x cover/nocover x habitat/buffer x top/bottom).

Samples were sent to the Virginia Tech lab for analysis using HPLC (High Performance Liquid Chromatography). There, the leaf samples were freeze-dried, ground, extracted, and concentrated into liquid samples that can be run through the HPLC. The HPLC detects and quantifies various compounds as they pass from the pressurized solvent and through the adsorbent at different times, and the peaks and times are compared with that of the standards to determine the concentration (Jyot and Singh 2017). This process yielded TMX concentrations per unit weight of freeze-dried leaf material, from which I calculated the wet leaf concentration, which told us how much pesticide was available to be consumed by a butterfly larva.

Lab Study:

Uptake information is vital, but needs accompanying toxicity information to determine if the concentrations in leaves are dangerous to butterfly larvae. My lab experiments examined development of painted lady butterfly larvae feeding on contaminated artificial diet spanning the range of concentrations found in leaf samples from the field site, as well as identified lethal and sublethal concentrations of TMX (Main Question 4).

Setup

This part of the study was performed in the lab at Blandy Experimental Farm, in Boyce, Virginia. The lab was kept around 22*C most of the experiment. My lab bench was next to big windows that let in lots of natural light, and small light fixtures were hung and kept on during the day to evenly distribute the light among caterpillars (the cups were shuffled every other day to prevent bias from lighting).

Thiamethoxam powder was ordered from Sigma Aldrich (PESTANAL, analytical standard). TMX stock solution (1 mg/L) was prepared ahead of time and kept in the refrigerator to minimize degradation.

We chose to use an artificial diet to administer the pesticide because the chemical is homogenized throughout the diet, like the pesticide would be in a plant contaminated with a systemic pesticide. Other studies spray leaves with pesticide solutions, which results in residues concentrated on the surface of the leaf resulting in highly variable amounts of chemical ingested per caterpillar bite and extrinsic contact exposure between the pesticide and the caterpillar's integument. Powdered artificial diet was purchased for painted ladies from Ward's Science (Stonefly *Heliothis* Diet). The artificial diet powder was combined with white vinegar and

diluted TMX stock solution to reach the desired treatment concentration (TMX solution instead of water required by the package recipe). The final product resembled cookie dough consistency.

Painted lady eggs were ordered from Carolina Biological Supply Company. Once they arrived, 0.5 grams of diet was measured out into 12 oz clear plastic cups and one painted lady egg was placed in each cup near the food. The cups had rounded lids with no straw-hole in the top. Holes were poked in the top of the lids to allow some air exchange. The cups were kept by the window under the lights in trays (Figure 7). The eggs hatched within a day, and the caterpillars started eating. The diet was replaced every other day to ensure that it didn't dry out and become inedible. There were 20 caterpillars per treatment (n = 20, N=100 per trial).

Figure 7. Cups with diet and larvae.

Rearing and Data Collection

Every day during a lab trial, mortality was recorded and the caterpillars were checked for qualitative sublethal effects such as discoloration and sluggishness. When they pupated,

development time from hatched egg to pupa was recorded. Around 24 hours after pupating (as weights change drastically with time throughout the pupal stage), they were collected by pulling the cremaster away from the foot with forceps, careful not to put any pressure on the cocoons. Any remaining silk attached to the cremasters was removed with scissors, the pupae were weighed on a scale, and the chrysalises from the second trial were sexed under the dissecting scope. The cocoons were sexed by checking for the genital opening near the cremaster (Monarch Watch). The cocoons were returned to their cups by hot gluing the cremaster to a piece of paper, and hot gluing the other side of the paper to the top of the cup. The paper gave the newly emerged butterfly something to hold on to as they hung upside down and dried their wings. Percent survival through the larval stage was recorded for each treatment after the last caterpillar pupated or died.

The cocoons were left alone until adult butterflies emerged, at which point, development time was recorded for the pupal stage. Adult butterflies were observed for crinkles or folds in their wings, their ability to hold themselves upright, and for any leaking fluids. Butterflies were held by the wings between the knuckles of the middle finger and pointer finger to prevent them from squirming and getting hurt. They were transferred into pre-weighed glassine envelopes to collect adult weight data, and wing measurements were taken using a caliper. After all butterflies emerged or their cocoons withered and turned black, percent survival was recorded for each treatment.

This process was repeated two times for painted ladies, each time using different concentrations of pesticide in the artificial diet, to create bookends containing the lethal limit and the sublethal limit, and also to test the concentrations likely to be found in sunflowers' foliage in the field (based on literature from the introduction). On the first trial of painted ladies, we used

concentrations of 0 ppb, 25,000 ppb, 50,000 ppb, 75,000 ppb and 100,000 ppb to make a first guess at the sublethal limit (based on results from Petersen et al 2019) and the lethal limit (based on results from Krischik et al 2015). A practice trial showed us that the 100,000 ppb treatment was above the lethal limit, and the lowest treatment was similar to the controls.

After obtaining results from the first trial, I lowered the highest treatment to try to make the smallest bookends for the lethal limits as possible, and added an even smaller dose treatment to represent field-realistic levels of pesticides. The concentrations for my second trial were 50 ppb, 500 ppb, 3,000 ppb, and 30,000 ppb compared against a control group. Adult butterflies from the first trial were kept in a tent at Blandy with signage about their biology, development, and the threat of pesticides for community engagement and education (Figure 8). Butterflies from the second trial were humanely put down in the freezer.

Figure 8. Tent that housed the surviving adult butterflies from trial one with signage.

Statistical Analysis

The independent variables for the field experiment were the three dates that host plants were sampled after sowing the corn seeds, the strip treatment with forbs and grasses or just grasses, the presence or absence of cover crops over the winter, the position of the host plants in the top or bottom of the strip, and the species, sunflower or milkweed. The dependent variables were the concentrations of TMX and CLO found in host plant leaves as ordered categories:

Concentration data were highly left-skewed, and the mode for both TMX and CLO was zero, so we decided to make the data categorical instead of numeric. We split the data into three ordered categories: “None” for zero TMX or CLO detected, “Trace” for TMX or CLO detected in the sample but not quantified because it was below the lowest standard concentration used in the analysis, and “Measured” for measured concentrations above the lowest standard concentration. The lowest standard concentration was 0.0857 for TMX and 0.0770 for CLO.

Once the data was reconfigured into categories, we used an ordinal logistic regression to perform the analysis. We used the “clmm” or “Cumulative Link Mixed Models” function in the “ordinal” package version 2019.12-10, developed by Rune Haubo B Christensen. The model was run with all independent variables and one random effect for block. Analyzing the data with ordered categories allow the main effects to be tested for significance without the structure of the split-plot-within-a-split-plot experimental design.

In the lab trials, the independent variables were the pesticide treatments and butterfly sex, and the dependent variables were the development time in the larval and pupal stages, pupal weight and adult weight, chrysalis length and several adult wing length parameters, and mortality.

Mortality is a binomial dependent variable (alive/dead), and was analyzed using a Firth's Bias-Reduced Logistic Regression ("logistf" function and package version 1.24, developed 2020-09-10, by Georg Heinze, Meinhard Ploner, Daniela Dunkler, Harry Southworth, and Lena Jiricka), because this version cooperated with data that had complete separation in some cases e.g. full mortality in high treatments and full survival in controls. The sole independent variable was treatment concentration; sex could not be included in the model because sex could not be determined on butterfly larvae.

I found that data from both trials could be used when analyzing mortality data, but that doses from the first trial were not as relevant for sublethal variables for several reasons. The first reason is that the sublethal variable data were collected in the pupal and adult stages, and the first trial was testing treatments that were so high that, except for the 25,000 ppb treatment and the control group, the larvae often didn't make it to the pupal and adult stages. The second reason is that the 25,000 ppb treatment was similar to the 30,000 ppb treatment in the second trial. The third reason is that the larvae that did survive to adulthood from the first trial were not sexed, and we wanted to include this variable in the model for the sublethal variables related to size (It is suggested that male painted ladies are slightly smaller than females). The final reason is that there was a significant difference in development time from egg to adult for control butterflies between trial one and trial two (Kruskal-Wallis, chi-squared = 32.85, df = 1, p-value = 9.957×10^{-9}), likely due to temperature differences in the lab environment. In all, it seemed that including the trial one data would contribute more noise to my dataset than clarity.

Sublethal variables (Development times, weights, and lengths) are numeric continuous variables. I first determined whether or not sex had an effect on each variable by testing just the control group, which had the greatest survival and therefore the greatest sensitivity to detect the

effect; I only included sex as a factor in the full analysis if it was significant for the controls. The wing length parameters were the only dependent variables for which we kept sex in the model. We checked for normal distribution with a Shapiro-Wilk test (most of my variables were non-normal and transformations didn't help achieve normality). Then each variable was checked for homogeneity of variances with a Levene's test. Usually conditions for an ANOVA were not met, and we proceeded with a Kruskal-Wallis test. The Kruskal-Wallis test told us if sex had an effect or not. Then, we could proceed with the actual analysis: we ran the Shapiro Wilk and Levene's tests on the variables with all trial two data, performed an ANOVA when appropriate and conducted a Kruskal-Wallis otherwise. After the ANOVA, we ran a Tukey HSD for the post-hoc test, and after the Kruskal-Wallis test, we ran a Dunn test (with a Bonferroni correction) to determine which treatments are different. In the case that sex mattered and we needed to run the analysis with both sex and treatment, we used a Scheirer-Ray-Hare test, followed by the Dunn test.

Results

Field Results

We analyzed 290 samples total for TMX and CLO. Of those samples, 131 were below the detectable limit for TMX, 97 were detectable in trace amounts (but below the lowest standard concentration of TMX at 0.752 ppb, and therefore could not be confidently quantified), and 62 samples had quantifiable concentrations of TMX. Of the 290 samples, 235 were below the detectable limit for CLO, 36 were detectable, but below the lowest standard concentration of CLO at 0.077 ppb, and 19 samples had quantifiable concentrations of CLO. The largest concentration of TMX detected was 41.652 ppb; that sample was collected from two sunflower plants on the first sampling date (6/12/21), from the third block, in the bottom of the buffer, where there were grasses and forbs planted in the buffer, and where there were cover crops grown over the winter. The second highest concentration of TMX was 38.112; that sample was collected from two sunflower plants on the first sampling date (6/12/21), from the first block, in the bottom of the buffer, where there were grasses and forbs planted in the buffer, and where there were cover crops grown over the winter. The highest CLO concentration detected was 1.628 ppb in sunflower. All concentrations captured in the field were below our lowest TMX concentration tested in the lab trials.

The ordinal logistic regression revealed that the none of the independent variables were significant for TMX or CLO except for the date (Figure 9 and 10 respectively). The concentrations taken up by host plants decreased over the three sampling dates.

Table 9. Shows output from the ordinal logistic regression for TMX.

	Estimate	Std. Error	z value	Pr(>|z|)	
Date2021-07-05	-2.5418	0.3115	-8.159	3.37E-16	***

Date2021-07-23	-1.9999	0.3168	-6.312	2.75E-10	***
SpeciesSUN	-0.2116	0.2541	-0.833	0.405	
Buffer2	0.0639	0.2456	0.26	0.795	
PositionTOP	-0.2135	0.2438	-0.875	0.381	
Cover2	-0.2974	0.2439	-1.219	0.223	

Table 10. Shows output from the ordinal logistic regression for CLO.

	Estimate	**Std. Error**	**z value**	**Pr(>|z|)**	
Date2021-07-05	-2.0379	0.4464	-4.565	4.99E-06	***
Date2021-07-23	-1.9345	0.485	-3.989	6.65E-05	***
SpeciesSUN	0.5288	0.3465	1.526	0.127	
Buffer2	-0.4814	0.3239	-1.486	0.1372	
PositionTOP	0.4927	0.3325	1.482	0.1385	
Cover2	-0.5661	0.3375	-1.677	0.0935	.

Lab Results

Mortality data:

Survival from egg to adulthood ranged from over 90% for the lowest treatment concentrations of TMX to 0% for treatment concentrations of 75,000 ppb and over (Figure 11).

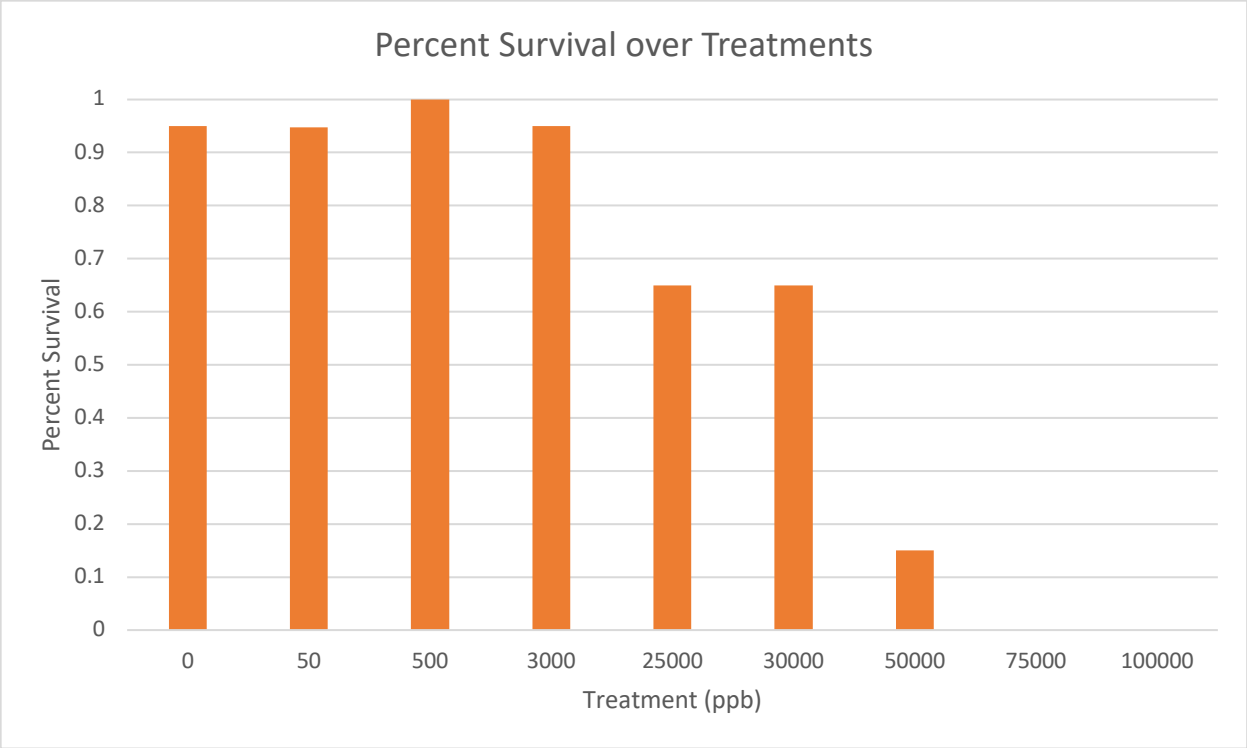

Figure 11. Reveals percent survival response to various treatment doses.

We found that all treatments from both trials equal to or above 25,000 ppb had statistically greater overall mortality than the controls, and that all treatments below that threshold had no difference from the controls (Table 1). (Likelihood ratio test=148.5551 on 8 df, p=0, n=199, Wald's test = 55.66322 on 8 df, p = 3.280505e-09, Method = 2-Profile penalized log likelihood).

Table 1. Results of logistic regression of overall mortality data.

	coef	se(coef)	lower 0.95	upper 0.95	Chisq	p	method
(Intercept)	2.7343675	0.6607748	1.647943	4.3171481	38.0072975	7.05E-10	2
TREAT50	-0.2220619	1.0932871	-2.310634	2.2201226	0.04226225	8.37E-01	2
TREAT500	0.9792045	1.6086773	-1.590682	5.9243451	0.46713117	4.94E-01	2
TREAT3000	-0.1694182	1.0910864	-2.255433	2.2710657	0.0247655	8.75E-01	2
TREAT25000	-2.1465808	0.8089507	-3.921303	-0.6884959	8.57906835	3.40E-03 **	2
TREAT30000	-2.1465808	0.8089507	-3.921303	-0.6884959	8.57906835	3.40E-03 **	2
TREAT50000	-4.3438054	0.8925376	-6.302129	-2.7916118	40.2167824	2.27E-10 ***	2

TREAT75000	-6.4479395	1.6086773	-11.406282	-4.0764937	58.2210662	2.34E-14 ***	2
TREAT1e+05	-6.4479395	1.6086773	-11.406282	-4.0764937	58.2210662	2.34E-14 ***	2

This does not mean that the treatments jump from no mortality to full mortality. We used the same analysis with treatments 25,000 and above (no control group), to show that the 25,000 ppb treatment was significantly different from 50,000, 75,000, and 100,000 ppb (Table 2). (Likelihood ratio test=31.54073 on 3 df, p=6.539748e-07, n=80, Wald test = 18.31445 on 3 df, p = 0.000378812) This confirms that there is a mortality curve at this scale of concentrations.

Table 2. Results of logistic regression of treatments 25,000 ppb and higher.

	coef	se(coef)	lower 0.95	upper 0.95	Chisq	p	method
(Intercept)	0.5877867	0.4666667	-0.281431	1.534367	1.738406	1.87E-01	2
TREAT50000	-2.1972246	0.760117	-3.786311	-0.8268406	10.393857	1.26E-03 **	2
TREAT75000	-4.3013587	1.5391547	-9.198811	-2.0826136	21.367745	3.79E-06 ***	2
TREAT1e+05	-4.3013587	1.5391547	-9.198811	-2.0826136	21.367745	3.79E-06 ***	2

The stage of development when the butterflies died varied depending on treatment (Figure 12). For example, the most common stage of death for the 100,000 ppb treatment was the first instar, and the most common stage of death for 25,000 ppb - 50,000 ppb treatments was the pupal stage.

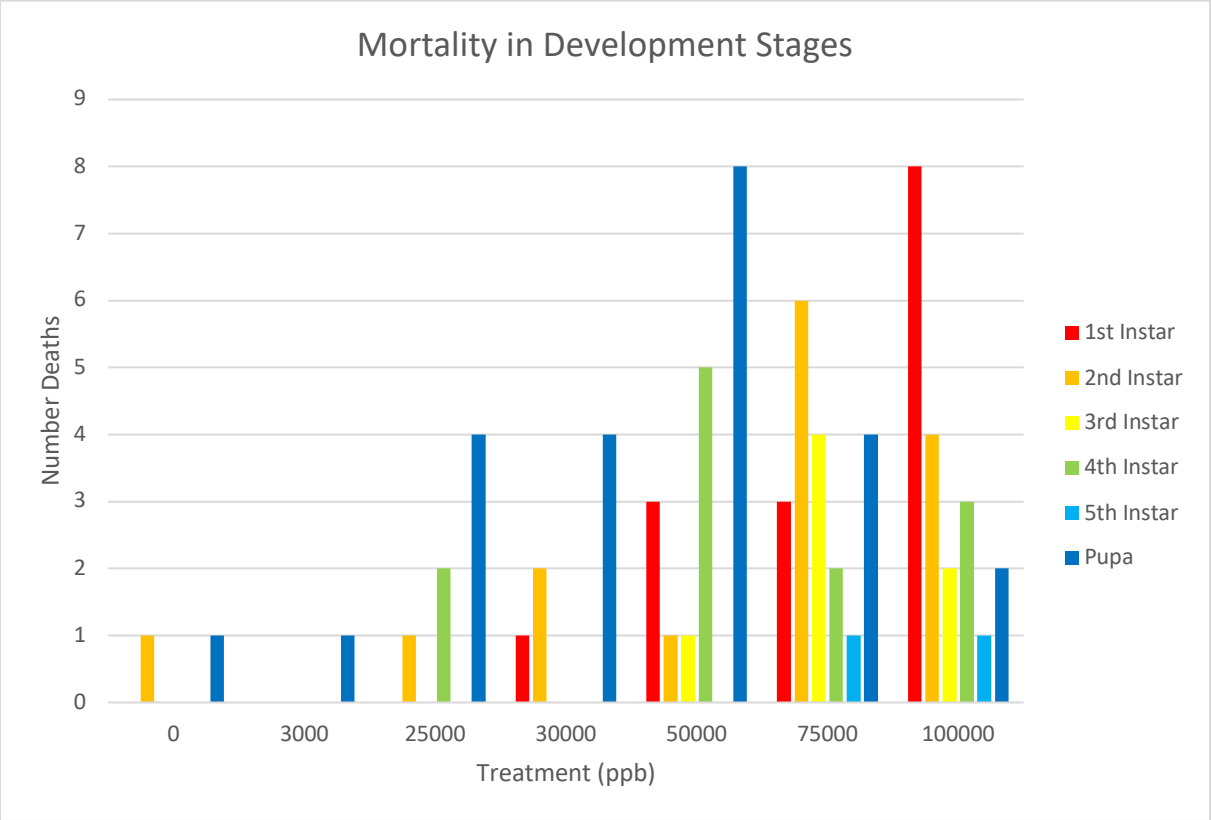

Figure 12. Shows mortality due to pesticide treatment across the stages of development.

Sublethal pupal data:

Pupation time was affected by TMX concentration (Kruskal-Wallis, $p = 2.61 \times 10^{-6}$), but only at concentrations higher than 3,000 ppb. Pupae in the 30,000 treatment took longer to develop. (Table 3, Figure 13).

Table 3. Results of pairwise comparisons in Dunn test.

Comparison	Z	P.unadj	P.adj	
0 - 3000	-0.6240442	5.33E-01	1.00E+00	
0 - 30000	-4.8326116	1.35E-06	1.35E-05	***
3000 - 30000	-4.2786941	1.88E-05	1.88E-04	***
0 - 50	-0.7435017	4.57E-01	1.00E+00	
3000 - 50	-0.1275099	8.99E-01	1.00E+00	
30000 - 50	4.1215255	3.76E-05	3.76E-04	***
0 - 500	0.2766382	7.82E-01	1.00E+00	
3000 - 500	0.9006824	3.68E-01	1.00E+00	
30000 - 500	5.0781626	3.81E-07	3.81E-06	***

50 - 500	1.0165701	3.09E-01	1.00E+00

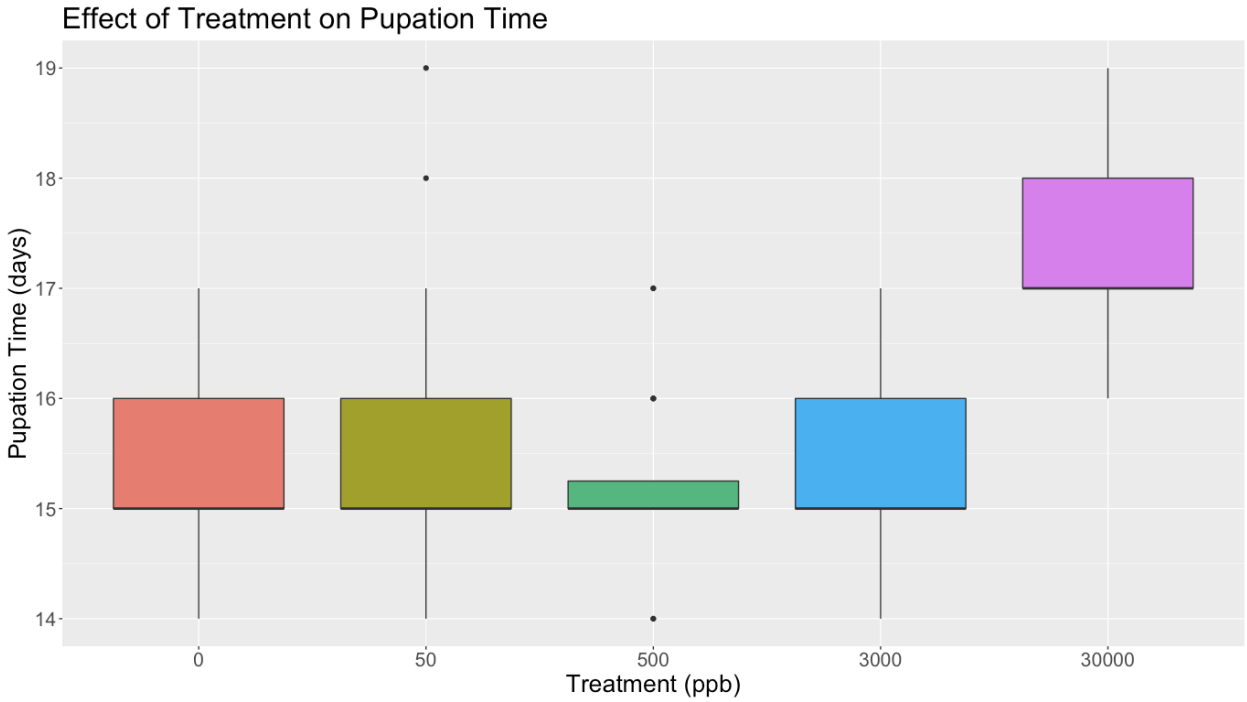

Figure 13. Shows the effect of treatment on pupation time.

An ANOVA found that there was a significant difference in **pupal weight** across treatments (Table 4). We found that pupae in the 30,000 ppb treatment took longer to develop than the rest, including pupae in the control group (Table 5, Figure 14).

Table 4. Output of ANOVA to determine the effect of treatment on pupal weight.

	Df	Sum Sq	Mean Sq	F value	Pr(>F)	
trialtwo$treatf	4	0.3147	0.07868	26.96	1.47E-14	***
Residuals	87	0.2539	0.00292			

Table 5. Output of Tukey HSD to determine the effect of treatment on pupal weight.

Treat	diff	lwr	upr	p adj	
50-0	-0.0265474	-0.0747573	0.02166254	0.5434545	
500-0	-0.0159	-0.0634878	0.03168782	0.8841806	
3000-0	-0.007895	-0.0554828	0.03969282	0.9904625	
30000-0	-0.1782923	-0.2319048	-0.1246798	0	***

500-50	0.01064737	-0.0375625	0.05885728	0.972306	
3000-50	0.01865237	-0.0295575	0.06686228	0.8174208	
30000-50	-0.1517449	-0.2059104	-0.0975795	0	***
3000-500	0.008005	-0.0395828	0.05559282	0.9899486	
30000-500	-0.1623923	-0.2160048	-0.1087798	0	***
30000-3000	-0.1703973	-0.2240098	-0.1167848	0	***

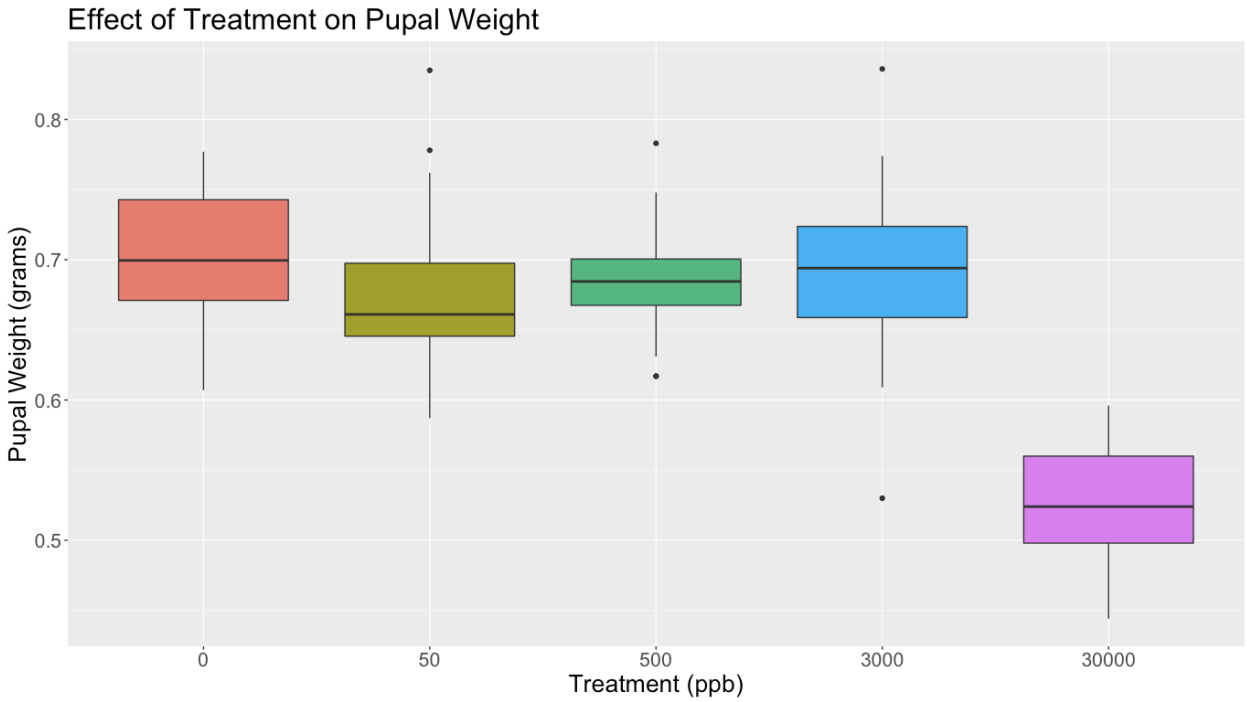

Figure 14. Shows effect of treatment on pupal weight.

An ANOVA found that treatment did have an effect on **pupal length**. A Tukey HSD revealed that pupae in the 30,000 ppb treatment weighed less than all the rest (Table 7), and the other treatments were the same as the control (Table 7, Figure 15).

Table 6. Output of ANOVA to determine the effect of treatment on pupal length.

	Df	Sum Sq	Mean Sq	F value	Pr(>F)	
trialtwo$treatf	4	50.28	12.571	7.581	2.75E-05	***
Residuals	87	144.27	1.658			

Table 7. Output of Tukey to determine the effect of treatment on pupal length.

Treat	diff	lwr	upr	p adj	
50-0	0.04736842	-1.101909	1.1966459	0.99996	
500-0	-0.315	-1.449447	0.8194474	0.9375591	
3000-0	-0.48	-1.614447	0.6544474	0.7634555	
30000-0	-2.2307692	-3.508839	-0.9526991	0.0000494	***
500-50	-0.3623684	-1.511646	0.786909	0.9041151	
3000-50	-0.5273684	-1.676646	0.621909	0.70512	
30000-50	-2.2781377	-3.569389	-0.986886	0.0000402	***
3000-500	-0.165	-1.299447	0.9694474	0.9942308	
30000-500	-1.9157692	-3.193839	-0.6376991	0.000658	***
30000-3000	-1.7507692	-3.028839	-0.4726991	0.0023005	**

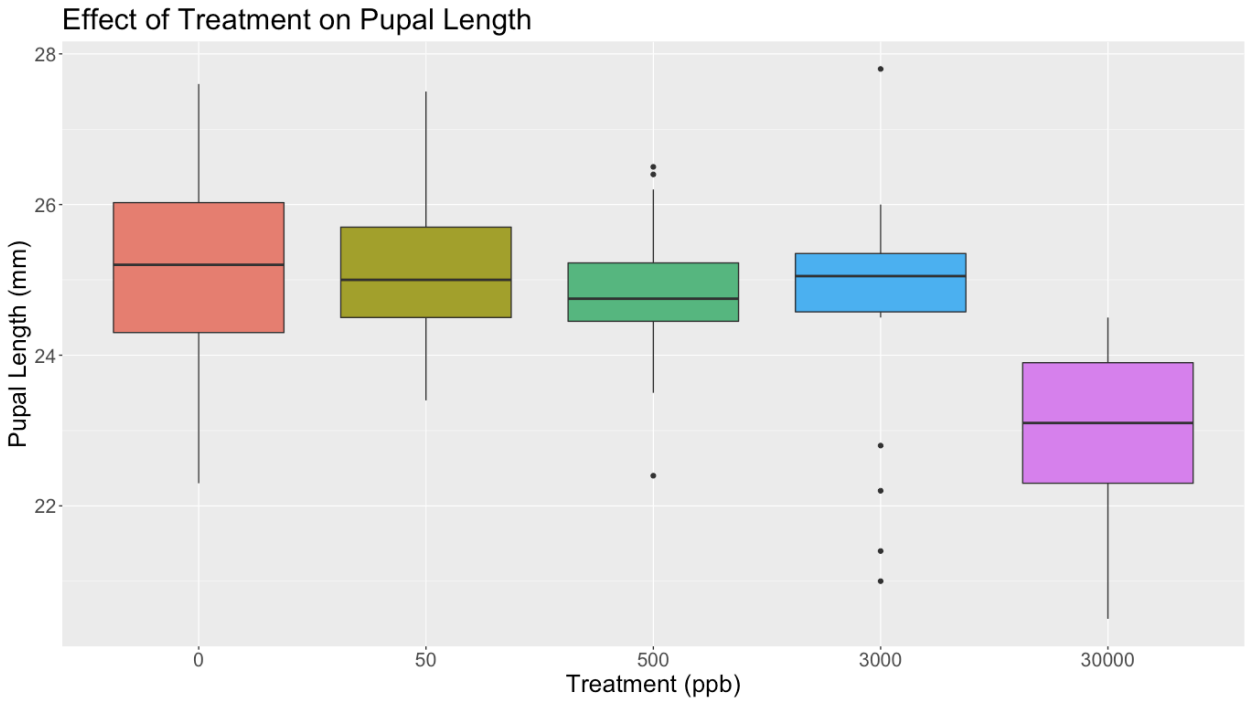

Figure 15. Shows the effect of treatment on pupal length.

Sublethal adult data:

Treatment did have an effect on **development time** (Kruskal-Wallis, $p = 3.023 \times 10^{-7}$, Table 8). Butterflies in the highest treatment of 30,000 ppb had longer development time compared to butterflies the other treatments and the control (Dunn test, Table 8, Figure 16).

Table 8. Output from Dunn test describing the effect of treatment on development time.

Comparison	Z	P.unadj	P.adj	
0 - 3000	-0.2967511	7.67E-01	1.00E+00	
0 - 30000	-4.9139861	8.92E-07	8.92E-06	***
3000 - 30000	-4.6464983	3.38E-06	3.38E-05	***
0 - 50	-0.9168708	3.59E-01	1.00E+00	
3000 - 50	-0.6284806	5.30E-01	1.00E+00	
30000 - 50	3.9698102	7.19E-05	7.19E-04	***
0 - 500	0.7136157	4.75E-01	1.00E+00	
3000 - 500	1.0103668	3.12E-01	1.00E+00	
30000 - 500	5.5572306	2.74E-08	2.74E-07	***
50 - 500	1.6103806	1.07E-01	1.00E+00	

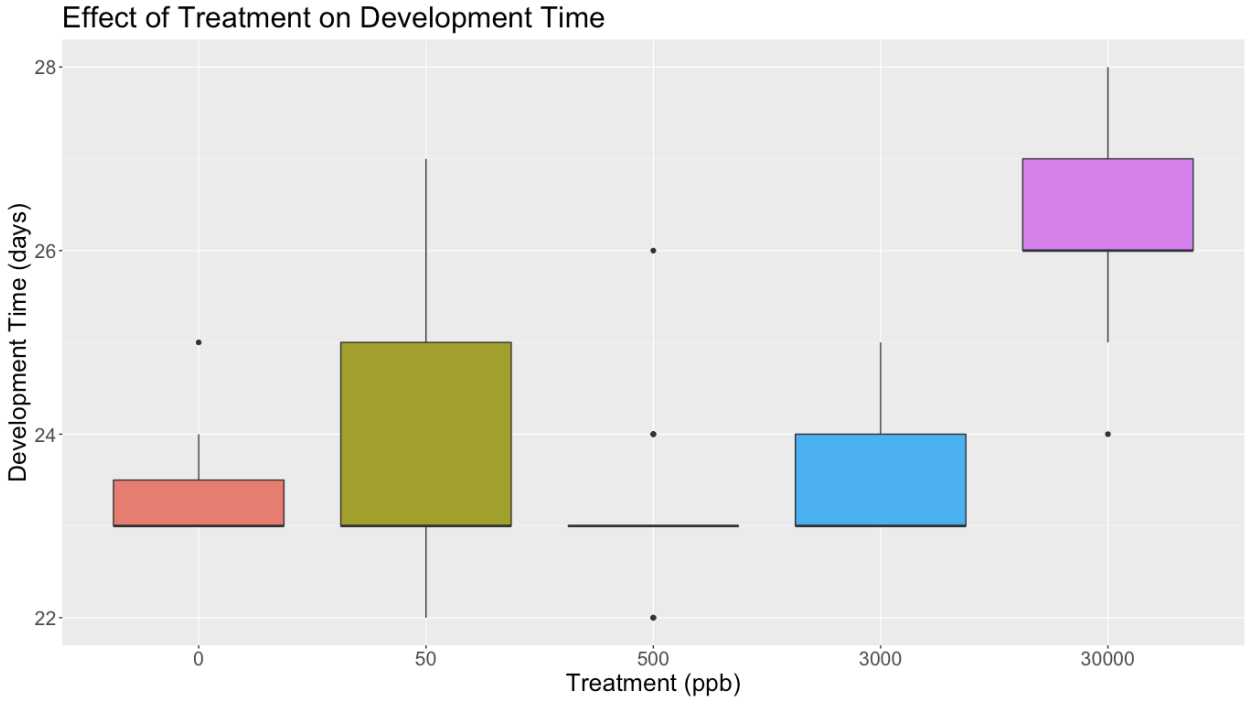

Figure 16. Shows the effect of treatment on development time.

Treatment did have an effect on **adult weight** (ANOVA, $p = 9.54 \times 10^{-15}$, Table 9), and butterflies in the highest treatment of 30,000 ppb TMX/artificial diet weighed less than than the other treatments and the control (Tukey HSD, Table 10, Figure 17).

Table 9. Output of ANOVA determining the effect of treatment on adult weight.

	Df	Sum Sq	Mean Sq	F value	Pr(>F)	
data$treatf	4	0.09763	0.024407	28.22	9.54E-15	***
Residuals	82	0.07092	0.000865			

Table 10. Output from Tukey HSD test describing the effect of treatment on adult weight.

	diff	lwr	upr	p adj	
50-0	-0.0061331	-0.0335201	0.0212538	0.9707075	
500-0	-0.0024737	-0.029089	0.02414162	0.9989854	
3000-0	-0.0020526	-0.0286679	0.02456267	0.999514	
30000-0	-0.0963684	-0.1258955	-0.0668414	0	***
500-50	0.00365944	-0.0237275	0.03104637	0.9958136	
3000-50	0.0040805	-0.0233064	0.03146742	0.9936328	
30000-50	-0.0902353	-0.1204597	-0.0600109	0	***
3000-500	0.00042105	-0.0261943	0.02703636	0.9999991	
30000-500	-0.0938947	-0.1234218	-0.0643677	0	***
30000-3000	-0.0943158	-0.1238428	-0.0647888	0	***

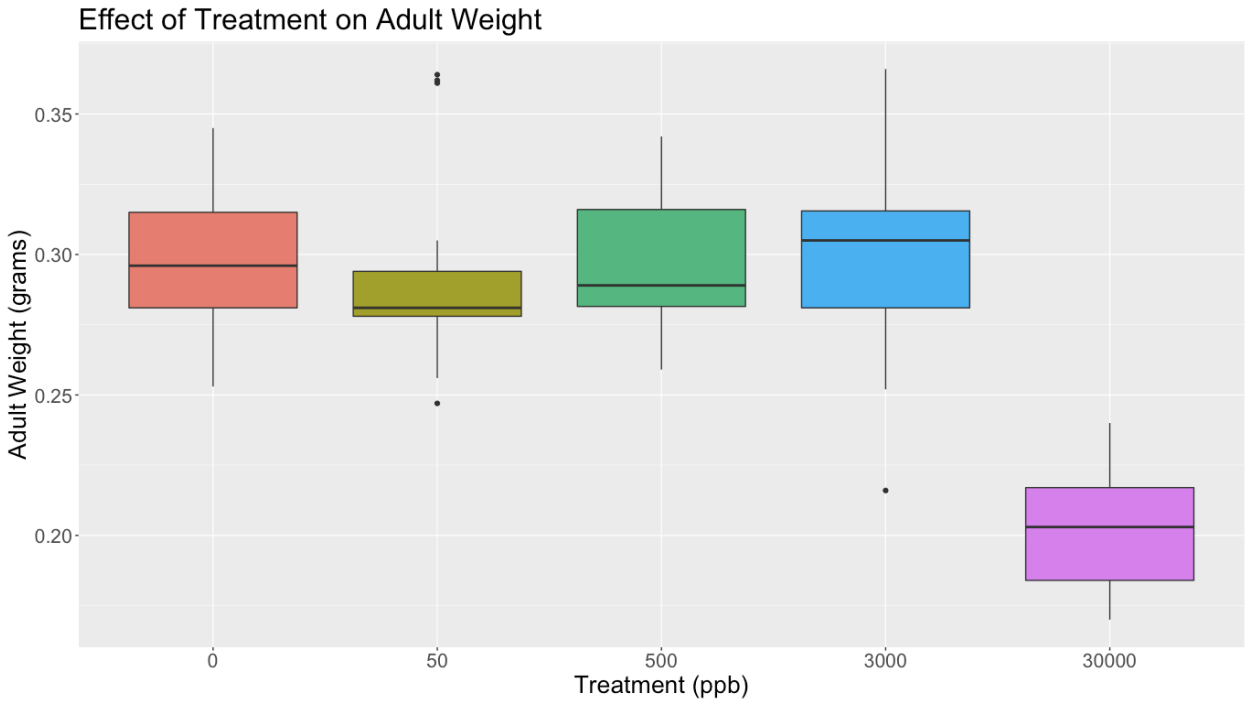

Figure 17. Shows the effect of treatment on adult weight.

The first wing measurement variable is **forewing length**. There was a significant difference between sexes in forewing length (Kruskal-Wallis, $p = 3.928x\ 10^{-5}$) and sex was kept in the model. Treatment did have an effect on forewing length (Scheirer-Ray-Hare, Table 11).

Butterflies in the 30,000 ppb treatment had shorter forewing lengths than the rest except for butterflies in the 50 ppb treatment (Table 12, Figure 18).

Table 11. Output of Scheirer-Ray-Hare test in determining the effect of treatment and sex on forewing length.

	Df	Sum Sq	H	p.value	
treatf	4	14673.6	23.0298	0.00012	***
sexf	1	10568.9	16.5876	0.00005	***
treatf:sexf	4	1741.4	2.7331	0.60343	
Residuals	77	27811.5			

Table 12. Output of Dunn test in determining the effect of sex and treatment on forewing length.

Comparison	Z	P.unadj	P.adj	
0 - 3000	-1.5970237	1.10E-01	1.00E+00	
0 - 30000	3.0040686	2.66E-03	2.66E-02	*
3000 - 30000	4.4436064	8.85E-06	8.85E-05	***
0 - 50	0.1857143	8.53E-01	1.00E+00	
3000 - 50	1.7377424	8.23E-02	8.23E-01	
30000 - 50	-2.7664758	5.67E-03	5.67E-02	
0 - 500	-1.1632245	2.45E-01	1.00E+00	
3000 - 500	0.4337992	6.64E-01	1.00E+00	
30000 - 500	-4.0525851	5.07E-05	5.07E-04	***
50 - 500	-1.3161654	1.88E-01	1.00E+00	

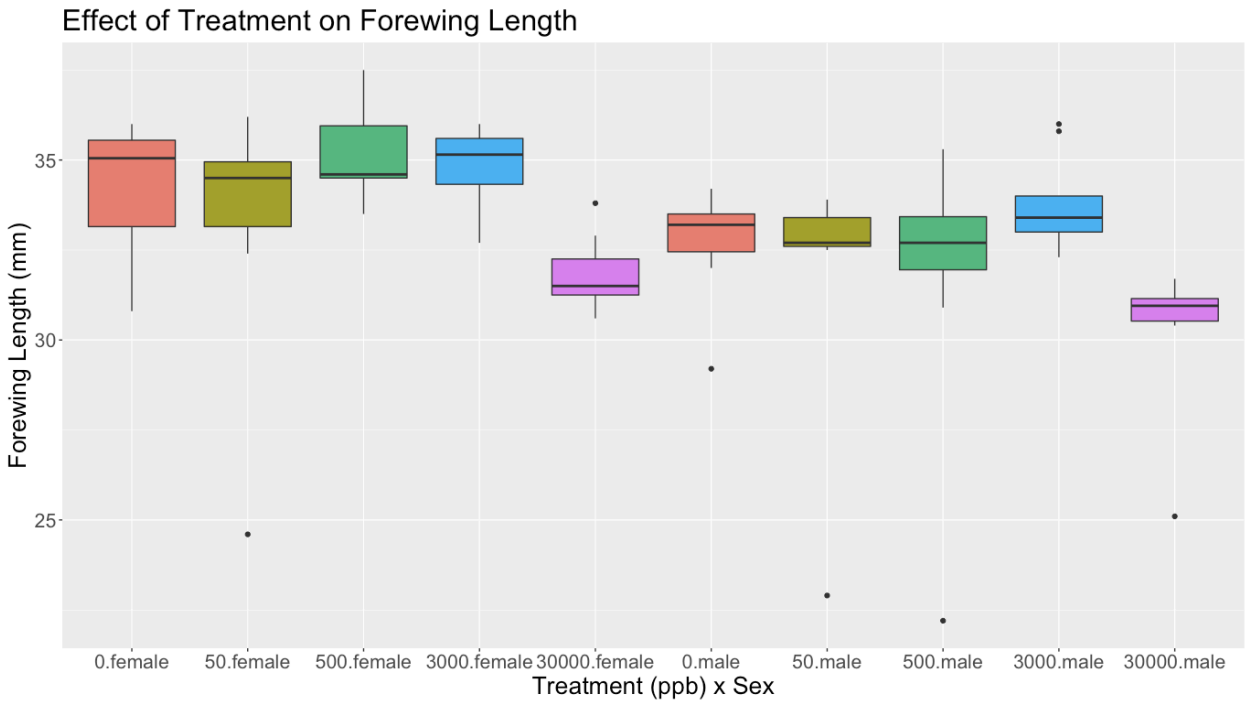

Figure 18. Shows the effect of sex and treatment on forewing length.

Forewing height did have a significant difference between sexes (Kruskal-Wallis, p = 0.0003) and sex was kept in the model. Treatment did have an effect on forewing height (Scheirer-Ray-Hare test, Table 13). Butterflies in the 30,000 ppb treatment had shorter forewing lengths than all the other butterflies (Table 14, Figure 19).

Table 13. Output of Scheirer-Ray-Hare test in determining if treatment and sex have an effect on forewing height.

	Df	Sum Sq	H	p.value	
treatf	4	15886.1	24.9223	0.00005	***
sexf	1	8167.1	12.8127	0.00034	***
treatf:sexf	4	2548.7	3.9985	0.40621	
Residuals	77	28216.6			

Table 14. Output of Dunn test in determining the effect of treatment and sex on forewing height.

Comparison	Z	P.unadj	P.adj	
0 - 3000	-1.2432968	2.14E-01	1.00E+00	
0 - 30000	3.0985558	1.94E-03	1.94E-02	*
3000 - 30000	4.2192484	2.45E-05	2.45E-04	***
0 - 50	-0.9827388	3.26E-01	1.00E+00	
3000 - 50	0.2255285	8.22E-01	1.00E+00	

30000 - 50	-3.917541	8.95E-05	8.95E-04	***
0 - 500	-1.5131597	1.30E-01	1.00E+00	
3000 - 500	-0.2698629	7.87E-01	1.00E+00	
30000 - 500	-4.4624995	8.10E-06	8.10E-05	***
50 - 500	-0.4877881	6.26E-01	1.00E+00	

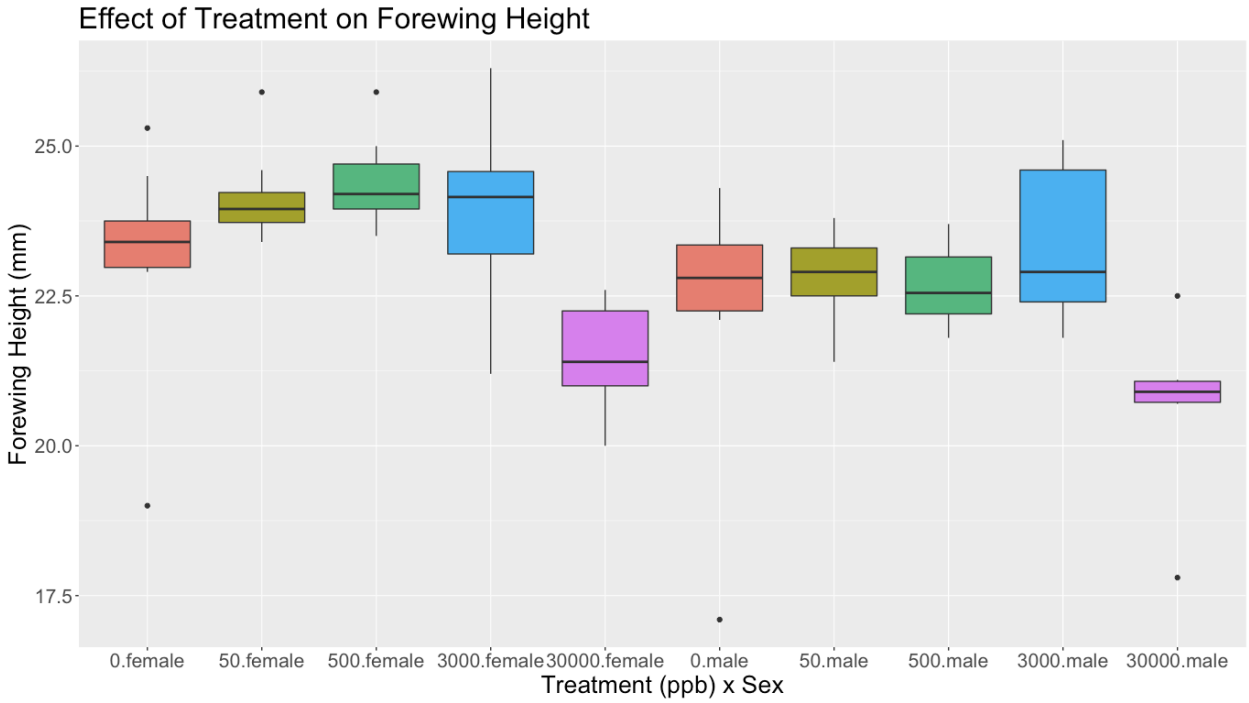

Figure 19. Shows the effect of treatment and sex on forewing height.

Hindwing length did have a significant difference between sexes (Kruskal-Wallis, p = 0.003) and sex was kept in the model. I found that the variances were distributed homogeneously (Levene's test, p = 0.819), and that the data was not normally distributed (Shapiro's test, p = 2.23 x 10^{-9}). However, there was an outlier (as can be seen in QQ Plot Figure 20), and when I removed the outlier, the Levene's test yielded a p-value of 0.167, and the Shapiro's test yielded a p-value of 0.276, meaning that the data without the outlier was then normally distributed (Figure 21).

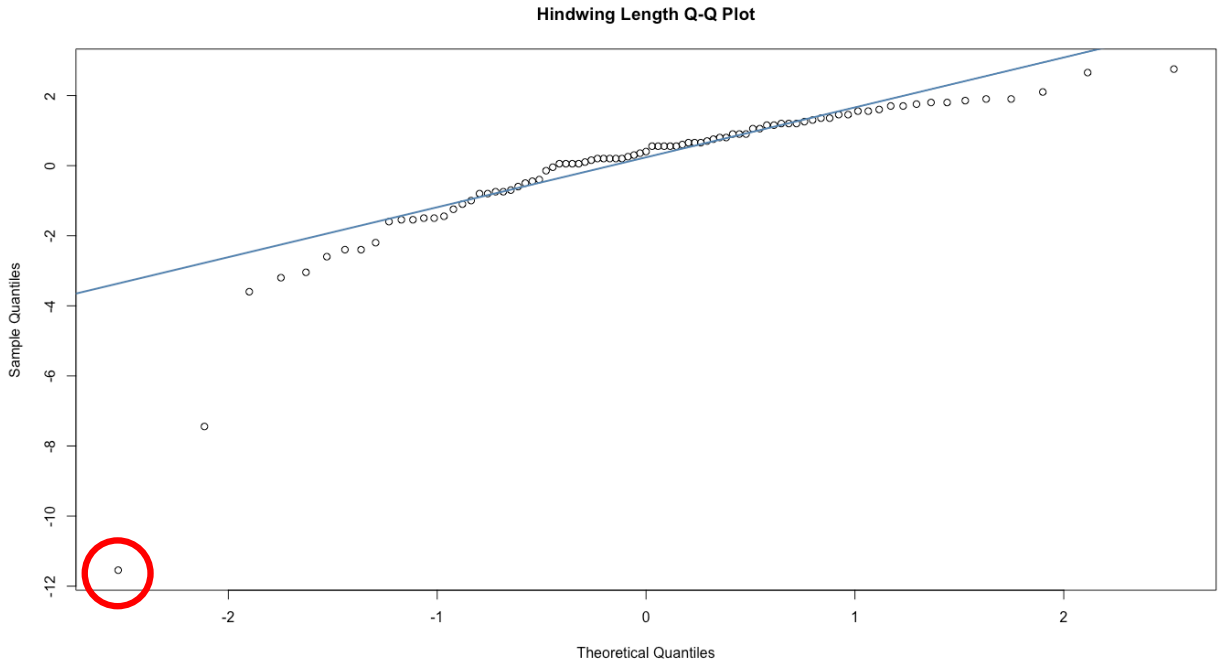

Figure 20. Hindwing length Q-Q Plot with the outlier.

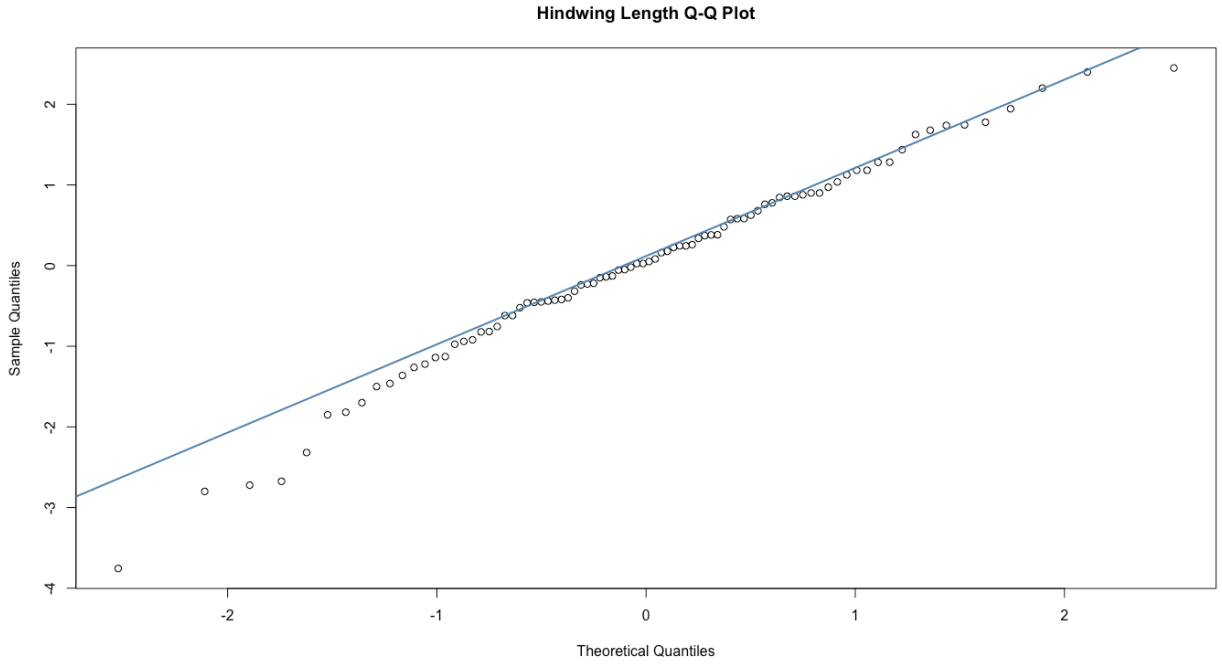

Figure 21. Hindwing length Q-Q Plot without the outlier.

Treatment and sex did have an effect on hindwing length (Table 15). Butterflies in the 30,000 ppb treatment had shorter hindwing lengths than butterflies in the other treatments and the control (Table 16, Figure 22).

Table 15. Output of ANOVA in determining if treatment and sex have an effect on hindwing length.

	Df	Sum Sq	Mean Sq	F value	Pr(>F)	
dat$treatf	4	55.92	13.981	8.44	1.09E-05	***
dat$sexf	1	24.24	24.245	14.636	0.000266	***
dat$treatf:dat$sexf	4	5.94	1.485	0.896	0.470384	
Residuals	76	125.9	1.657			

Table 16. Output of Tukey HSD in determining the effect of treatment and sex on hindwing length.

$`dat$treatf`

	diff	lwr	upr	p adj	
50-0	-0.0124183	-1.2287584	1.2039218	0.9999998	
500-0	0.2111111	-0.9718474	1.3940696	0.9872561	
3000-0	0.4690058	-0.7139527	1.6519644	0.8019027	
30000-0	-2.0196581	-3.3287033	-0.710613	0.0004522	***
500-50	0.2235294	-0.9771628	1.4242216	0.985066	
3000-50	0.4814241	-0.7192681	1.6821164	0.7953002	
30000-50	-2.0072398	-3.3323323	-0.6821473	0.0005973	***
3000-500	0.2578947	-0.9089684	1.4247578	0.9718607	
30000-500	-2.2307692	-3.5252876	-0.9362508	0.0000705	***
30000-3000	-2.488664	-3.7831824	-1.1941456	0.000008	***

$`dat$sexf`

	diff	lwr	upr	p adj	
male-female	-1.057016	-1.610007	-0.5040262	0.000283	***

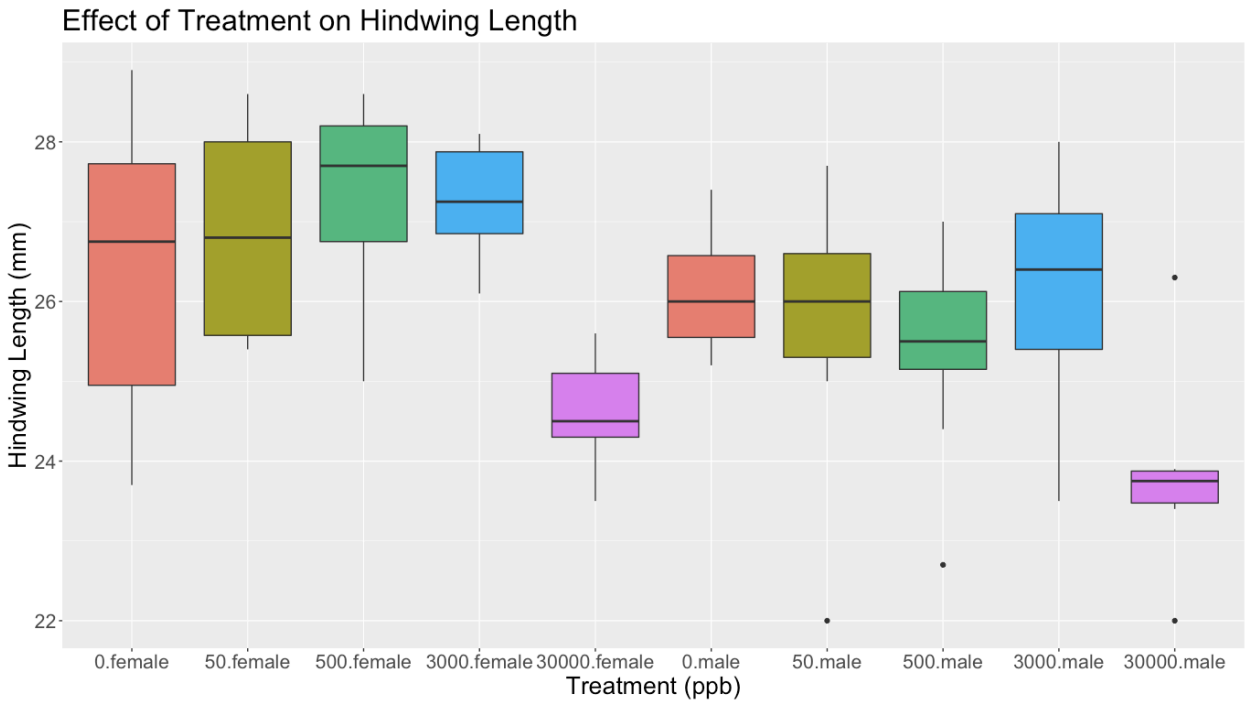

Figure 22. Shows the effect of treatment and sex on hindwing length. This plot was made without the outlier.

Hindwing height did have a significant difference between the sexes (Kruskal-Wallis, p = 0.003) and sex was kept in the model. A Schierer-Ray-Hare test revealed that both sex and treatment mattered, but not the interaction of the two (Table 17). Butterflies in the 30,000 ppb treatment had shorter hindwing heights than the rest of the butterflies (Table 18, Figure 23).

Table 17. Output of Schierer-Ray-Hare test to determine if sex and treatment have an effect on hindwing height.

	Df	Sum Sq	H	p.value	
treatf	4	20130.7	31.5995	0	***
sexf	1	5348.2	8.3952	0.00376	**
treatf:sexf	4	705.2	1.107	0.89316	
Residuals	77	28602.8			

Table 18. Output of Dunn test for hindwing height.

Comparison	Z	P.unadj	P.adj	
0 - 3000	-1.9795633	4.78E-02	4.78E-01	
0 - 30000	3.52793208	4.19E-04	4.19E-03	**
3000 - 30000	5.31228629	1.08E-07	1.08E-06	***

0 - 50	-0.0659512	9.47E-01	1.00E+00	
3000 - 50	1.85783853	6.32E-02	6.32E-01	
30000 - 50	-3.506291	4.54E-04	4.54E-03	**
0 - 500	-1.2115184	2.26E-01	1.00E+00	
3000 - 500	0.76804483	4.42E-01	1.00E+00	
30000 - 500	-4.61998	3.84E-06	3.84E-05	***
50 - 500	-1.1114331	2.66E-01	1.00E+00	

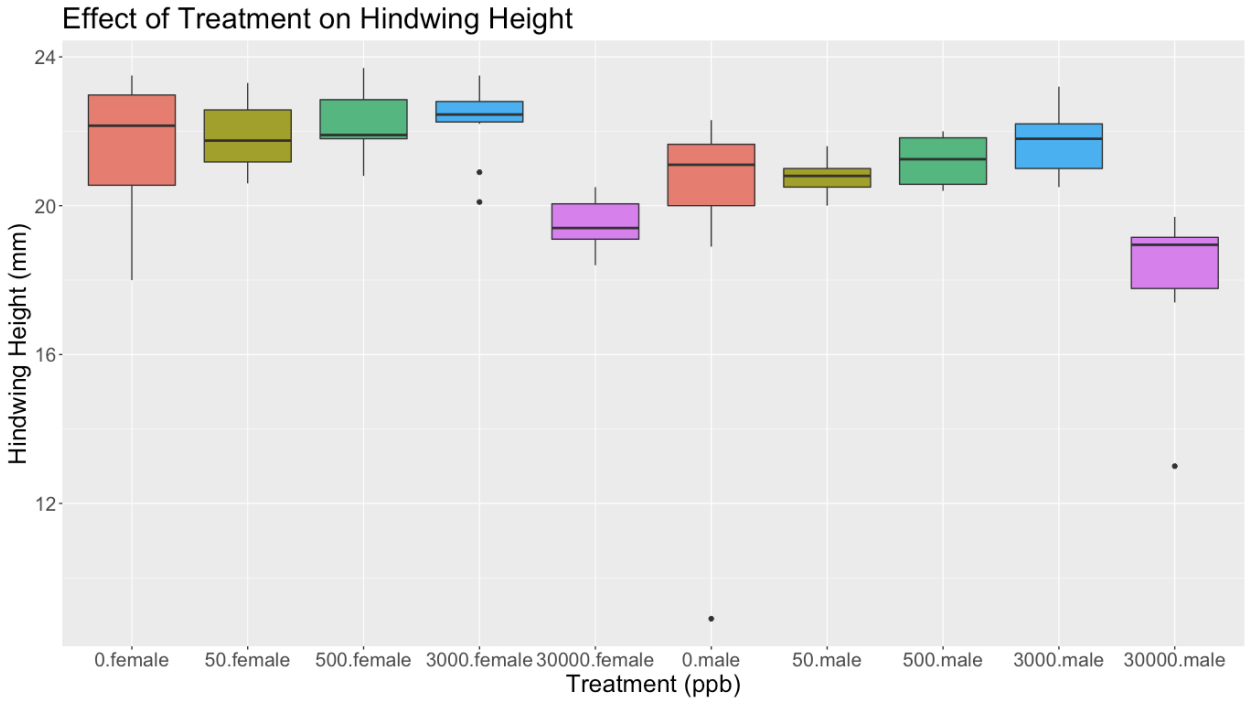

Figure 23. Shows the effect of treatment and sex on hindwing height.

Discussion:

Altogether, my thesis data indicated that host plants planted nearby plots planted with corn coated with TMX did not accumulate enough pesticides to be harmful to painted lady caterpillars. The highest concentration detected from the field was 42 ppb TMX in sunflower, and we found that 50 ppb TMX in artificial diet for painted ladies yielded mortality similar to controls with no statistically significant sublethal effects. This does not mean that these pesticides are absolved of risk to painted ladies or other butterflies in working landscapes. We did not test for any other pesticides in the leaf samples from the field site, where there might have been additional insecticides and fungicides from the seed coatings or remainder in the plots. Herbicides were also used nearby some of our plots that might have drifted to soil and host plants. Several chemicals interacting on a working landscape can produce synergistic toxicity to pollinators- fungicides and insecticides especially can be a dangerous combination.

The field experiment yielded low concentrations of TMX and CLO. Similar field studies found up to 106 ppb of TMX (Botías et al 2016). There are several reasons that for the low concentrations: the pesticide might have degraded faster than expected, the pesticide might have been taken up by grasses between the corn rows, there might not have been enough rainfall to create substantial runoff and subsurface flow to transport the chemicals into the strips (Appendix E), the chemicals might have been taken up by the plants but the concentration diluted by the growth and water uptake of the plants, the chemicals could have degraded in the plants before leaves were collected, leaf samples were frozen and thawed several times while fixing labeling and weighing and this might have degraded the pesticide… There are many possibilities with such a complex system.

Jacob Maris, a masters student at Virginia Tech, collected soil samples and analyzed them for concentrations of TMX and CLO from the field site the same summer. Jacob was going to collect water samples with lysimeters and runoff collectors, but both failed to collect enough water to sample. He found soil TMX concentrations below 4 ppb with the occasional spike of TMX to around 40 ppb- very similar to my data. This gives a window into what concentrations might have been present in subsurface flow between rain events (when the dissolved chemicals are moving less quickly) that was taken up by the plants.

Sometimes pesticides can stay in soils for several years, but our conditions could have been conducive to degradation, because our data showed the highest concentrations of TMX and the most samples with measurable concentrations on the sampling date nearest seed planting (Figure 24).

	Date		
CatTMX	2021-06-12	2021-07-05	2021-07-23
None	16	73	42
Trace	53	19	25
Measured	46	10	6

Figure 24. Shows the number of leaf samples with “None”, “Trace”, and “Measured” concentrations of TMX for each sampling date. Jacob found that pesticides were still detected at very low concentrations until September of that year in the soil.

The highest concentration of CLO found in milkweed was 1.085 ppb. Monarch butterfly toxicity has been studied more in depth than all other butterflies, however there is still uncertainty about what concentrations of neonicotinoids are safe:

Pecenka and Lundgren (2015) observed lethal and sublethal effects of CLO on early instar monarch larvae by spraying swamp milkweed leaves with various concentrations of aqueous CLO. They found the LC10 to be 7.72 ppb, LC50 to be 15.63 ppb, and the LC90 to be

30.70 ppb (concentration for 10, 50, 90 percent of the larvae to die). Although this was a landmark study, this often-cited paper has lower LCs than some other papers suggest.

In particular, a study by Bargar, Hladik, and Daniels (2020) examined the effects of granular CLO soil application rate in residential or commercial landscapes when applied to milkweeds fed to developing monarch larvae. They found a much higher LC50 of monarch larvae consuming leaves of 47-205 ppb CLO and sublethal effects such as reduced larval growth at 177 ppb CLO. This suggests that the larvae are less sensitive to CLO than Pecenka and Lundgren describe, and concentrations found in the field would not come close enough to these limits to contribute to the monarch's population decline.

Similarly, Olaya-Arenas et al. (2020) also observed effects on adult monarchs, but after larval exposure to CLO through spraying solution on milkweed leaves. However, they only found marginally significant decreases in survival at 15-56 ppb.

The highest concentration of TMX found in milkweed was 13.533 ppb. The effects of TMX on monarchs were not elucidated until the year 2020 by Krishnan et al. They examined mortality of larvae in 2^{nd},3^{rd}, and 5^{th} instars for both cuticular exposure and dietary exposure by spraying tropical milkweed with pesticide solutions. The cuticular LC50 ranged from 0.13 to 4.2 ug/cm^2 per larva depending on the instar. The dietary LC50 ranged from 3.5 to 33 ppb depending on the instar. Larval mass typically decreased with increasing TMX concentration.

While informative, it is difficult to infer risk of death or sublethal effects of monarch larvae on contaminated milkweed for conservation efforts because each study uses different neonicotinoids, application methods, milkweed species, exposure times, and reports lethal concentrations in different units and for different instars or the larval or adult stage as a whole. In addition, all of these studies are performed in a laboratory (or otherwise protected) setting,

without field context. More research needs to be done in the lab to explain some of the variation surrounding the LC50 of monarch larvae among these studies, and more research needs to be done in the field to understand potential risk of exposure.

Understanding monarch sensitivity is important for farmland management decisions and conservation of the species, but this doesn't represent pesticide toxicity of all butterfly species. For example, monarchs are a relatively large butterfly, whereas smaller butterflies are thought to have greater sensitivity to pesticides. Different management strategies might need to be implemented to protect butterfly diversity on farmland depending on the range of uptake by all host plants and range of sensitivity of all butterfly larvae.

There are fewer toxicity studies for painted ladies, so the lab trials were designed to supplement that gap in knowledge and better interpret the risk posed by the field concentrations of neonicotinoids. I found that the LC50 was between 25,000 and 50,000 ppb, under the conditions of this lab experiment. Krischik et al (2015) found whole flower concentrations ranged 6,030 ppb to 45,890 ppb IMD (Imidacloprid) in their potted host plant experiment and painted lady larvae survival was reduced to 20% after 14 days (however, survival in the control group was 40% at this time). My result was similar that less that 20% of larvae survived to adulthood feeding on 50,000 ppb TMX diet.

The only other study of painted lady butterfly toxicity of neonicotinoids was Peterson, Shaw, and Smith (2019) that found sublethal effects such as slower development time and smaller adult mass at 5,000 ppb CLO in diet. At my test concentration of 3,000 ppb TMX, we didn't find any significant sublethal results; but at 30,000, development time and adult weight were significantly affected in addition to pupal weight, larval development time to pupa, pupal length, and adult wing length parameters. It was interesting that there was no significant

difference between the 30,000 ppb and 50 ppb treatments for forewing length; but it was unclear if there were similar effects from the two concentrations or if the lack of a difference was a statistical artifact of small sample sizes. Sublethal effects are important to measure, because effects like reduced adult weight might make migration more challenging with less fat stores to burn. Sublethal effects can have lasting impacts on individuals and populations.

Another interesting piece of information to note, is that Krishnan et al (2020) found that neonicotinoids, more than any other pesticide class, caused arrested ecdysis (pupae never emerge from the chrysalis). They believe that there are two distinct ways neonicotinoids can cause mortality during monarch development, one targeting larvae, and the other resulting in arrested ecdysis. I observed in Figure 12 that at my highest tested concentrations, larvae could not survive past the first instar. However, at lower concentrations, the most common death was during pupation. The pupal stage might be a vulnerable time for developing butterflies, because I observed many caterpillars, especially in the 25,000 ppb and higher treatments, undergo transformation from caterpillar to chrysalis without completion. I described this as “Helmet” in my notes because the chrysalis was only formed at the bottom of the J-shape, looking almost like the caterpillar put on a helmet (Figure 25).

Figure 25. Photograph of caterpillar with the “helmet” phenomenon.

If caterpillars are found near an agricultural field with “helmets”, this might indicate that neonicotinoids have leached from the field, have contaminated host plants, and are harming butterflies.

Answering my main questions, concentrations of TMX in butterfly host plants adjacent to seed-coated corn plots ranged from 0 ppb to about 42 ppb, and CLO concentrations ranged from 0 ppb to about 2 ppb (Main Question 1). Strips of grasses or grasses and forbs did not significantly reduce the concentrations taken up by host plants (Main Question 2). It was interesting that the two highest concentrations were from whole plots with both grasses and forbs in the strip, however we found it was extremely difficult to prevent forbs from growing in the grass strips, and keep forbs growing in the mixed strips, so this result is likely serendipitous. The presence or absence of cover crops had no effect on concentrations taken up by host plants (Main Question 3). It was interesting that the two highest concentrations of TMX detected were both in subplots with cover crops over the winter- this is the opposite of what we were expecting. Part of the reason why cover had no effect might be that the cover crops did not grow to be very big over the winter like we had hoped. If they had grown more, perhaps they might have taken up more pesticides from the year before.

There was mortality in all treatments and the control group in the lab study- this was unavoidable, as not all larvae survive in the wild, let alone in lab conditions. However, there was mortality significantly different from the control group at concentrations 25,000 ppb and higher during development (Main Question 4). We also found that all sublethal effects measured were significantly different from the control group at 30,000 ppb (Main Question 5). We did not test if sublethal effects were significant at the 25,000 ppb concentration because that treatment was a part of a different trial with considerably different circumstances.

This thesis first and foremost contributes to literature investigating concentrations of neonicotinoids taken up by plants intended to benefit pollinators. The data collected contributes to a body of knowledge describing concentrations in foliage and used to determine if pollinator habitat can be installed on agricultural land (as it is badly needed) without contamination that could counter the benefits of habitat creation. More research needs to be conducted to determine if strips and/or cover crops can be used this way. Our study dealt with small plots of corn that probably didn't supply as much pesticide as typical agricultural corn fields, and our plots were only treated with pesticide-coated seeds for two summers, when in reality, corn fields are sown every year for generations, contributing more pesticides. So, our host plants didn't detect enough pesticides to be sensitive to changes like a buffer strip or cover crops. Future research should be done at the same scale as intensive agriculture. Though, our work might indicate that these strategies are not easy to implement, and might not be ideal solutions for farmers. There need to be many more toxicity studies to determine safe concentrations and applications of pesticides, but this will take a long time. Until then we should be employing the Precautionary Principle, using utmost caution when applying pesticides in case of harm to our pollinators and other beneficial invertebrates.

Conclusions

Lepidopteran conservation requires habitat installation within agricultural landscapes, but habitat could be contaminated by pesticides which can be transported from seed coatings by subsurface flow into surrounding environments. I conducted a field experiment to measure concentrations of Thiamethoxam taken up by butterfly host plants such as milkweed and sunflowers downslope of plots planted with pesticide-coated corn seeds. I carried out a lab experiment to measure toxicity

of Thiamethoxam to painted lady butterfly larvae to interpret the risk of TMX concentrations found in sunflowers. I found that concentrations taken up by host plants in the field were not dangerous to painted lady larvae, and I found that effects of TMX in artificial diet began significantly affecting caterpillars (both sublethal and lethal) between 25,000 ppb and 30,000 ppb. Buffer strips and cover crops did not reduce the concentration of TMX taken up by host plants in the field. Butterflies are in decline, and habitat installed on working landscapes can be a part of the solution. More research is necessary to elucidate concerns for habitat contamination, and to document toxicity of pesticides to butterflies.

Appendices:

Appendix A. Monthly temperature and precipitation averages at the NPRC (Virginia Tech).

MONTH	MIN. TEMP (F)	MAX. TEMP (F)	AVG. TEMP (F)	AVG. PRCP (IN)	AVG. SNOW (IN)
Jan	22.0	42.5	32.3	2.80	7.5
Feb	24.6	45.6	35.1	2.74	7.2
Mar	33.2	56.0	44.6	3.42	3.2
Apr	42.5	66.2	54.3	3.12	0.3
May	52.1	74.9	63.5	4.43	0.0
Jun	60.7	82.8	71.8	3.42	0.0
Jul	65.0	86.4	75.7	4.47	0.0
Aug	63.8	85.0	74.4	4.28	0.0
Sep	56.8	78.8	67.8	3.54	0.0
Oct	44.5	68.2	56.4	4.02	0.1
Nov	36.0	58.0	47.0	3.63	1.0
Dec	26.8	46.7	36.7	3.04	4.2

Appendix B. Soil types at the NPRC (Web Soil Survey).

Soil types at the NPRC as identified in Web Soil Survey. See soil type descriptions below.

Orange County, Virginia (VA137)			
Map Unit Symbol	Map Unit Name	Acres in AOI	Percent of AOI
DaB2	Davidson clay loam, 2 to 7 percent slopes, eroded	6.3	5.6%

DaC2	Davidson clay loam, 7 to 15 percent slopes, eroded	11.8	10.4%
DdB3	Davidson clay, 2 to 7 percent slopes, severely eroded	18.2	16.1%
DdC3	Davidson clay, 7 to 15 percent slopes, severely eroded	35.2	31.1%
DkB2	Dyke loam, 2 to 7 percent slopes, eroded	15.7	13.9%
Ee	Elbert silt loam, overwash	1.2	1.1%
MyB2	Myersville silt loam, 2 to 7 percent slopes, eroded	0.5	0.4%
RaC2	Rabun clay loam, 7 to 15 percent slopes, eroded	5.1	4.5%
SrC	Starr silt loam, 2 to 10 percent slopes	17.8	15.7%

W	Water	1.5	1.3%
Totals for Area of Interest		113.4	100.0%

Appendix C. Full list of grasses planted in grass buffer strips at the NPRC.

% of Mix	Latin Name	Common Name	Cultivar/ Ecotype
50.0	*Bouteloua curtipendula*	Side Oats	Any
50.0	*Schizachyrium scoparium*	Little Bluestem	FIG (PA) or Camper
100	Total		

Appendix D. Full list of forbs planted in habitat strips at the NPRC.

% of Mix	Latin Name	Common Milkweed	Cultivar/ Ecotype
2.0	*Asclepias syriaca*	Common Milkweed	PA
26.7	*Chamaecrista fasciculata*	Partridge Pea	PA
26.6	*Echinacea purpurea*	Purple Coneflower	Any
6.7	*Helianthus maximiliani*	Maximilian's Sunflower	Any
13.3	*Heliopsis helianthoides*	Ox-Eye Sunflower	PA
2.7	*Monarda fistulosa*	Wild Bergamot	FIG (PA)
2.0	*Pycnanthemum tenuifolium*	Narrow Leaved Mountain Mint	PA
20.0	*Rudbeckia hirta*	Black Eyed Susan	Any

100	Total		

Appendix E. Rainfall in Orange, VA.

Date	Station Id	Air Temperature Maximum (degF)	Air Temperature Minimum (degF)	Precipitation Increment (in)
7/26/21	2039	88	69	0.06
7/25/21	2039	87	68	0.48
7/24/21	2039	84	65	0.3
7/23/21	2039	83	63	0
7/22/21	2039	81	63	0
7/21/21	2039	88	66	0
7/20/21	2039	89	65	0
7/19/21	2039	83	66	0
7/18/21	2039	83	66	0
7/17/21	2039	89	69	0.42
7/16/21	2039	90	67	0
7/15/21	2039	89	66	0.24
7/14/21	2039	89	68	1.02
7/13/21	2039	91	71	0
7/12/21	2039	91	71	0
7/11/21	2039	88	67	0.02
7/10/21	2039	85	65	0
7/9/21	2039	88	63	0
7/8/21	2039	81	67	0.08
7/7/21	2039	90	69	0
7/6/21	2039	91	65	0
7/5/21	2039	88	64	0
7/4/21	2039	83	57	0.02
7/3/21	2039	78	58	0
7/2/21	2039	79	61	0.4
7/1/21	2039	87	68	0.16
6/30/21	2039	93	68	0.02
6/29/21	2039	90	68	0
6/28/21	2039	89	68	0

6/27/21	2039	87	68	0
6/26/21	2039	83	67	0.05
6/25/21	2039	79	52	0
6/24/21	2039	75	50	0
6/23/21	2039	74	55	0
6/22/21	2039	78	58	0.47
6/21/21	2039	91	70	0
6/20/21	2039	86	66	0
6/19/21	2039	86	67	0
6/18/21	2039	84	51	0
6/17/21	2039	78	54	0
6/16/21	2039	78	60	0
6/15/21	2039	79	62	0
6/14/21	2039	86	66	0
6/13/21	2039	80	65	0.01
6/12/21	2039	72	61	0.05
6/11/21	2039	69	62	0.45
6/10/21	2039	85	68	0.73
6/9/21	2039	84	67	0.05
6/8/21	2039	88	70	0.15
6/7/21	2039	85	68	0
6/6/21	2039	89	64	0
6/5/21	2039	87	61	0
6/4/21	2039	83	64	0.13
6/3/21	2039	82	65	0.92
6/2/21	2039	79	60	0
6/1/21	2039	76	49	0
5/31/21	2039	72	47	0
5/30/21	2039	59	47	0.01
5/29/21	2039	63	47	0.2
5/28/21	2039	77	63	0.22
5/27/21	2039	86	66	0
5/26/21	2039	91	65	0
5/25/21	2039	80	57	0
5/24/21	2039	76	57	0.03
5/23/21	2039	90	69	0
5/22/21	2039	87	53	0
5/21/21	2039	81	51	0
5/20/21	2039	86	56	0
5/19/21	2039	81	50	0

5/18/21	2039	75	47	0
5/17/21	2039	71	45	0
5/16/21	2039	60	44	0
5/15/21	2039	75	44	0
5/14/21	2039	69	47	0
5/13/21	2039	67	46	0
5/12/21	2039	63	46	0
5/11/21	2039	68	49	0
5/10/21	2039	69	50	0
5/9/21	2039	69	43	0
5/8/21	2039	62	37	0
5/7/21	2039	58	39	0.05
5/6/21	2039	63	42	0
5/5/21	2039	80	51	0.01
5/4/21	2039	85	59	0.31
5/3/21	2039	74	61	0.06
5/2/21	2039	82	47	0
5/1/21	2039	69	39	0

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