PESTICIDE EXPOSURE RISK AND DEVELOPMENTAL CONSEQUENCES FOR MONARCH BUTTERFLIES IN AGRICULTURAL LANDSCAPES

by

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To my parents for encouraging me to be a successful and independent woman. To my friends at Purdue for their love, fun times and support during this PhD.

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ABSTRACT

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Chapter 1. Monarch butterflies are undergoing a long-term population decline, which has led to a search for potential causes underlying this pattern. One poorly studied factor is exposure to non-target pesticides on their primary host-plant, the common milkweed *Asclepias syriaca*, during larval development. This species frequently grows near agricultural fields in the Midwestern U.S., but the spectrum of pesticides encountered by monarch caterpillars on milkweed leaves is unknown. Further, it is unclear whether pesticide exposure can be avoided by isolating restored milkweed patches at sites far from cropland. Over 2 years, we analyzed 1,543 milkweed leaves across seven sites in northwestern Indiana for the presence and concentration of a range of commonly used agricultural insecticides, fungicides, and herbicides. Additionally, we tested the ability of local (i.e., nearest linear distance to crop field) and landscape-level (i.e., % of corn/soybean in 1km radius) variables to predict the presence of pesticides on focal milkweeds. Overall, we detected 14 pesticides−4 insecticides, 4 herbicides, 6 fungicides—on milkweeds that varied widely in their prevalence and concentration. The neonicotinoid clothianidin, the only pesticide for which toxicity data are available in monarchs, was detected in 15–25% of plants in June with nearly 60% of milkweeds at some sites testing positive (mean conc. = 0.71 and 0.48 ng/g in 2015 and 2016, respectively); however, no samples from July or August contained clothianidin. The related neonicotinoid thiamethoxam and the pyrethroid deltamethrin were detected in most (>75%) samples throughout the season, but only in the second year of the study. For thiamethoxam, isolating milkweeds 50–100m from the nearest corn or soybean field tended to decrease the concentration and likelihood of detecting residues, whereas landscape composition surrounding milkweed sites had comparatively weak predictive power. These data suggest that monarch caterpillars frequently consume a diversity of pesticides in their diet; the lethal or sublethal impacts of this exposure remain to be tested.

Chapter 2. Hundreds of recent studies have voiced concern over the negative impacts of non-target pesticides on pollinator health. However, pesticide loads are highly variable across agricultural landscapes and it is unclear whether pollinators exhibit behavioral responses (e.g., aversion) that mediate their exposure risk under realistic foraging environments. We tested whether monarch butterfly (*Danaus plexippus*) adults and larvae base their oviposition and foraging decisions, respectively, on the presence and concentration of pesticide residues on their milkweed host-plant, *Asclepias syriaca*. Using a two-year dataset that quantified pesticides on milkweeds bordering corn or soybean fields, we simulated field-realistic levels for six of the most commonly detected pesticides—one insecticide, two herbicides, and three fungicides—either alone or in combination. These laboratory and greenhouse manipulations experimentally paired an untreated control with the pesticides at their mean or maximum concentrations. Butterflies placed fewer eggs on milkweeds treated with a cocktail containing all six pesticides, but only when applied at their maximum-detected concentration, resulting in ca. 30% less oviposition compared to the untreated control. Neonate (1st instar) larvae also showed a preference for pesticide-free leaves in paired disc assays for most compounds tested, with feeding aversion observed at both mean and maximum concentrations. Later instars did not show a comparable behavior reaction to pesticide presence or concentration, but this could be partially due to the feeding-deterrent properties of the acetone solvent used. Our data provide evidence that monarchs are capable of adaptively adjusting their oviposition and foraging behaviors based on which pesticides are present on their host-plants. Yet, for gravid females, this impact was only observed at higher than average concentrations, meaning that in the field eggs are likely placed on milkweeds regardless of pesticide presence in most cases. Thus, it is unlikely that monarchs behaviorally regulate pesticide exposure risk by avoiding contaminated plants.

Finally, in Chapter 3 we used a no-choice experiment to evaluate the effects of continuous exposure to field-realistic pesticide concentrations on monarch butterfly larval and pupal development time, pupal weight, adult longevity and survival. Most monarch life stages were relatively unaffected by continuous exposure to the six pesticides tested. A negative effect in wing development and length was observed when larvae were exposed to the fungicides pyraclostrobin and trifloxystrobin and the mix of all six pesticides tested. Larval stage had higher mortality than the pupal stage and instars 2 and 5 were relatively more vulnerable. The negative effect on wing span and wing development could negatively impact migration, reproduction in the short-term and

population at long-term. Strategies to reduce contamination by pesticides of non-target plants and insects should be considered to protect diversity and maintain ecosystem integrity in the landscape.

CHAPTER 1. QUANTIFIYING PESTICIDE EXPOSURE RISK FOR MONARCH CATERPILLARS ON MILKWEEDS BORDERING AGRICULTURAL LAND

1.1 Introduction

Since 1960, agricultural intensification and a corresponding rise in pesticide use has been an environmental concern due to contamination of soil-water-air and movement of chemicals through the trophic chain (Carson, 1962; Krupke et al., 2007; Epstein, 2014; Douglas et al., 2015). Because broad-spectrum pesticides are, by nature, not specific to focal pests, they can affect non-target beneficial organisms (i.e., pollinators, parasitoids, predators) inhabiting crops, as well as unmanaged habitats neighboring agricultural land (Longley and Sotherton, 1997; Aktar et al., 2009). Routes of exposure are varied and challenging to track, but include direct contact with contaminated surfaces or spray droplets, residues remaining on the soil, and consumption via food resources such as leaves, nectar or pollen (Cilgi and Jepson, 1995; Longley and Stark, 1996). In many cases, only a small fraction of active ingredient makes contact with target pests, while the remainder is absorbed by the greater ecosystem. Pesticides applied by aircraft, for example, can reach as little as 50% of the target crop with the remainder moving to surrounding areas as far as 30km downwind (Pimentel and Levitan, 1986; Pimentel, 1995). As a result, a range of insect pests, from aphids to caterpillars, are estimated to contact <0.1% of insecticides applied for their control (Pimentel and Levitan, 1986). Even newer, more targeted technologies are vulnerable to this pesticide 'loss'; namely, seed treatments that were once touted for their limited off-site drift (Jeschke and Nauen, 2008). New data estimate that only 1.3% of initial seed treatment is recovered from corn plants exposed to the neonicotinoid clothianidin, with the remaining 98–99% of material leached into the environment (Alford and Krupke, 2017).

Off-site exposure to mobile insecticides is particularly a concern for pollinators, many of which inhabit agricultural landscapes and are undergoing long-term population declines. Several studies provide evidence of lower abundance and/or diversity of butterflies in the field margins of insecticide-treated crops compared with unsprayed controls (Rands and Sotherton, 1986; Dover et al., 1990; De Snoo et al., 1998). In most cases, it is unknown whether effects are caused by exposure to adults nectaring on flowering plants or larvae developing on contaminated leaves.

However, a field experiment exposing Pieris brassicae caterpillars at different distances downwind to spray drift from the insecticide diflubenzuron, showed higher mortality when developing on leaves of their host-plant up to 16 m from the field edge (Davis et al., 1991). Similarly, several studies illustrate that the nectar and/or pollen of wild flowering plants on crop field edges contain residues of neonicotinoid insecticides among other agrochemicals (Krupke et al., 2012; Botías et al., 2015, 2016; David et al., 2016; Mogren and Lundgren, 2016). Indeed, much of the recent focus of non- target impacts on pollinators centers on the neonicotinoids, due in large part to their widespread adoption in global agriculture (Douglas and Tooker, 2015). Although this work has primarily targeted bees, increasing evidence suggests that butterflies are also affected. Two recent time-scale analyses of reductions in butterfly diversity over the past several decades link these changes with the introduction and rise of neonicotinoids in the UK (Gilburn et al., 2015) and California (Forister et al., 2016). These correlative analyses were complimented by a few experimental lab studies showing strong negative effects on larval development for butterflies reared at field- realistic exposure levels for clothianidin (Pecenka and Lundgren, 2015) and imidacloprid (Whitehorn et al., 2018). Yet, due to the strong research emphasis on bees and pollen/nectar composition, we still lack field data on dietary exposure to pesticides for butterfly larvae developing on leaves of host-plants bordering cropland.

The common milkweed *Asclepias syriaca* L. is an abundant and opportunistic herbaceous plant growing in disturbed agricultural areas throughout the eastern United States (Woodson, 1954). It is notorious as being the primary larval food plant for the migratory monarch butterfly (Danaus plexippus L.) throughout its summer breeding range (Seiber et al., 1986; Wassenaar and Hobson, 1998). While Asclepias is a relatively diverse genus in North America and monarchs are capable of feeding on most, if not all, of these species, A. syriaca is by far the most widely available and used by monarchs in the Midwestern U.S. (Hartzler and Buhler, 2000; Zaya et al., 2017). Because A. syriaca grows in close proximity to corn and soybean fields and monarchs specialize on milkweed, this system offers a unique opportunity to examine the links between crop management, pesticide leaf concentrations, and butterfly development. Importantly, monarch populations have declined sharply over the last 20 years with censuses in overwintering sites reporting an 82% decrease in population size (Inamine et al., 2016; Semmens et al., 2016; Malcolm, 2018). Hypothesized contributors to this decline include: loss of overwintering forests in Mexico (Brower

et al., 2012); reductions in milkweed host-plants due to widespread use of the herbicide glyphosate (Hartzler, 2010; Pleasants and Oberhauser, 2013; Stenoien et al., 2016; Thogmartin et al., 2017a); urban development (Brower et al., 2012); severe weather events (Swengel, 1995; Brower et al., 2012); climate change (Oberhauser and Peterson, 2003; Flockhart et al., 2015; Saunders et al., 2017); and parasites (Altizer and Oberhauser, 1999; Altizer et al., 2004, 2015).

Although pesticides have been considered as a factor underlying the monarch decline (see Oberhauser et al., 2006; Krischik et al., 2015; Pecenka and Lundgren, 2015; Thogmartin et al., 2017a), it is difficult to evaluate this hypothesis because we lack data on field exposure during larval development. Interestingly, monarch declines have temporally coincided with the increase in use of neonicotinoids throughout agricultural regions in their summer breeding habitat, leading some to speculate whether this is a correlative or causal relationship (Stone, 2013). A recent petition by the U.S. Fish & Wildlife Service to protect monarchs under the endangered species act highlights this point: "*It is notable that the monarch decline has occurred during the same time period that the use of neonicotinoid insecticides in the key monarch breeding areas has dramatically increased, although, to our knowledge no one has tested the hypothesis that neonicotinoid use is a significant driver of monarch population dynamics.*"

A lab toxicity assay of monarch larvae exposed to different concentrations of clothianidin—the main neonicotinoid seed treatment applied to corn—showed lethal effects with an LC_{50} at 15.6 ng/g and sub-lethal effects at as little as 1 ng/g (Pecenka and Lundgren, 2015). Despite the lack of data on realistic field exposure, some have taken proactive measures to protect monarchs against potential harm. In 2014, for instance, the U.S. Fish & Wildlife Service phased out neonicotinoid insecticides on crops grown on National Wildlife Refuge System lands. Further, the U.S. Department of Agriculture developed a wildlife habitat evaluation guide and decision support tool for monarch butterfly restoration in which a 125-foot-wide pesticide-free buffer around restored milkweed habitat is advocated (USDA- NRCS 2016). To our knowledge, these buffers have not been "ground truthed" by quantifying actual pesticide residues on milkweed plants varying in their distance from the edge of agricultural fields. Such data are critical for defining the validity of nearest-distance thresholds used by land managers creating monarch habitat. Given that recent monarch population models estimate that 1.6 billion milkweed stems need to be added to the goals,

close Midwestern region to achieve future conservation proximity to agricultural land is unavoidable (Pleasants, 2017; Thogmartin et al., 2017b).

With this in mind, our primary aim in this study was to define and quantify the spectrum of pesticides exposed to potential consumption by monarch caterpillars on their host- plant, *A. syriaca*, in agricultural landscapes. Secondarily, we assessed how pesticide presence varies with linear distance between focal milkweeds and cropland. This was done to test the degree to which pesticide residues diminish with increasing spatial isolation at a local-scale, which is most relevant to land managers who often have some amount of flexibility over local habitat placement on their property. Pesticide-free buffers assume a proximity threshold beyond which exposure is minimal to non-existent. Last, we compared the effectiveness of nearest-distance buffer models with broader landscape- scale analyses of land use to determine which better predicts monarch exposure.

1.2 Methods

1.2.1 Study Areas

In 2015 and 2016, we sampled *A. syriaca* at seven sites across two counties—Tippecanoe and Newton—in northwestern Indiana, USA. Each site was separated from the nearest site by at least 2km with the farthest two sites ca. 100km apart. A site consisted of a patch of at least 30 milkweed plants growing in an area adjacent to a corn or soybean field. Although all milkweed patches were embedded within agricultural landscapes dominated by corn and soybean production (see Table 1.1 for land use data and SI Appendix, Figures S1 and S2 for reference GIS land use maps to visualize surrounding habitats for a representative agricultural and natural site, respectively), the local habitat varied widely from unmanaged crop field edges to large prairies used in restoration or conservation. As a result, the degree of isolation separating milkweeds from the nearest crop field varied widely, from 0 to >2km; however, most were within a 100m buffer zone of the field edge. Because we were constrained by the location of existing milkweeds and site configuration, we had little control over min/max distances, as well as other factors that could affect pesticide movement, e.g., soil type, direction of milkweed patch relative to crop field (upwind vs. downwind). Data on number of plants sampled per site/year, distance range separating milkweeds

from crop, size of neighboring crop field, and direction of milkweeds compared to crop are provided in Table 1.2. Sites included:

(i) Purdue Agronomy Center for Research & Education (ACRE), a 1,408 acre farm managed for row crop research, mainly corn and soybean. Within ACRE, we identified and sampled milkweed plants in the Peterson Prairie Plot, a 4 acre tall grass prairie restoration planting established in 2003.

(ii) Kankakee Sands, a 20,000 acre protected savannah-prairie owned and managed by The Nature Conservancy. Because of its large area, we identified two sites within this location; one directly abutting a soybean field named "Kankakee close" and another that was in the core area, at least 1,500m from the nearest agricultural land designated as "Kankakee far."

(iii) Meigs-Purdue Agricultural Center, a 145 acre research farm used primarily for fruit and vegetable production, but also including row crop agriculture. Because we could only identify 28 naturally growing milkweeds at this site, we supplemented by transplanting an additional 38 plants. Seedlings from two milkweed species (A. syriaca and A. incarnata) were transplanted in the field in April 2015 in six rows, each of which contained five plants along a distance transect from the corn field edge: 0, 5, 10, 20, and 30 m. Transects were separated by 10 m. An additional eight plants at 0m were placed along the northern and western borders of the field. It is unclear whether the lack of milkweed at this site, as well as site (vi) below, was due to the high local use of glyphosate or because these field margins were occasionally mowed, which likely reduced milkweed stand establishment.

(iv) Prophetstown State Park, a 900 acre restored prairie. Milkweeds in this area were within a grassland close to a large corn field.

(v) Purdue Wildlife Area (PWA), a 159 acre property that includes forest, wetlands, and early successional habitat. Milkweeds were adjacent to a corn field on the western border.

(vi) Throckmorton-Purdue Agricultural Center (TPAC), an 830 acre research farm managed for row crop research, mainly corn and soybean. Similar to the Meigs site described above, we used milkweed transplants along distance transects running perpendicular to the corn field edge. In 2015, 36 plants were placed around the corn field; four transects at 0, 5, 10, 20, and 30m along the western field edge, four transects at 0, 5, 10m along the eastern edge, and four individual plants at 0m along the north and south field edges.

1.2.2 Field sampling

Milkweed leaf samples: During June, July, and August in both years, leaf tissue was collected from milkweed plants for chemical analysis. On average, we sampled 48 plants per site each year, with 524 total milkweed plants sampled over the 2-year period across all sites. Within a given site, sampled plants were semi-randomly chosen to span a distance gradient along a transect extending out from the crop field edge. Each month, two leaves were removed to provide at least one-gram of tissue for analysis. The two leaves were located in the central portion of the plant, avoiding the new growth in the apical meristem and older senescent leaves at the bottom of the stem. This leaf position roughly coincides with where we often observe monarch larvae feeding in the field. Leaves were sealed in plastic bags and kept in a cooler with ice before they were transferred to a −80°C freezer in the laboratory. Because we collected whole-leaf samples, we do not know whether residues were on plant surfaces or inside of plant tissues. Similarly, due to the large number of pesticides measured and logistical challenges with sampling from multiple field sites over time, we did not attempt to control for variation in other factors that undoubtedly impact pesticide detection, e.g., rainfall, time since application, half-life. However, our sampling design over two years with several samples at different time points within a given year, using multiple sites, and a relatively large number of plant replicates per site, was in part intended to account for this inevitable background "noise" and provide a reasonable estimate for average exposure at a given time and place.

Plants were labeled with colored flagging tape to sample the same individuals in subsequent months and georeferenced to calculate the linear distance between focal milkweeds and the nearest corn/soybean field in the study area. To calculate the distance of each individual plant to the crop fields we used an ArcGIS model for each individual site. The tools used in the model include: "Project" that converts data from one coordinate system (WGS_1984) to another (NAD_1983_UTM_Zone_16N); "Near" which calculates the distance between the input feature (milkweeds) and the near feature (crop field); "Add field" that adds a new field to a table, in our case the distance from milkweeds to the crop; and "Calculate field" which calculates the values within the new field in the table (SI Appendix, Figure S3).

Soil samples: We collected five soil samples per site during June, July and August 2016, resulting in 15 total samples per site. Soil was collected from random locations in the same approximate area where milkweeds were growing at different distances from the crop (SI Appendix, Figure S4). To do so, we used a soil core (2 cm diameter), sampling the top ca. 18cm, although the sampling depth varied with soil compaction across sites. Because soil type plays an important role in the retention or degradation of pesticides, we identified the types of soils at each site using the USDA Soil Survey Geographic Database (SSURGO) map data, which contains information for 3,200 soil surveys (SI Appendix, Table S1).

Land use analysis: Although we measured the linear distance of each plant to a specific crop field within our study areas, distance alone may be a poor predictor of variation in pesticide residues. Thus, we quantified the area of corn and soybean in a 1km radius buffer around the milkweed sampling sites since most of the pesticide inputs are compounds applied to these two crops, which dominate land use in our region. To do so, we used the ArcGIS buffer geoprocessing tool with a 1 km radius, extracted by mask to obtain the crops just within the buffer and tabulated area to calculate the percent of corn, soybean and other crops as a fraction of total land use. Land use data were obtained from USDA NASS Cropland Data Layer for Indiana (www. indianamap.org).

We also estimated corn and soybean pesticide use at a broader geographical scale (county-level) to assess the relationship between pesticide inputs in those crops and the residues associated with our plants. We used the USGS pesticide database, which estimates pesticide applications per crop per state; we used Epest-low values, which are more conservative and tend to better match other estimates. To quantify the amount of pesticides applied in the two counties (Tippecanoe and Newton) where milkweeds were sampled, we first divided the total amount of each corn or soybean pesticide applied at the state-level (i.e., for Indiana only) by state-wide acreage to provide a per area use rate in each year. This approach assumes that state- wide averages are reflected in local grower practices, which may not always be the case. The per-unit rate was then multiplied by the area of corn or soybean planted per county in that year to estimate how much of each pesticide was applied near milkweed sites (SI Appendix, Table S2). Because USGS datasets stopped including seed treatments in their pesticide surveys after 2014, we unfortunately could not include neonicotinoids and some fungicides using this approach. However, virtually all corn (>90%) in

our area is seed treated with clothianidin and thus total corn acreage is a good proxy for neonicotinoid input (Douglas and Tooker, 2015). In addition to the information provided by USGS, a list of the pesticides applied during our sampling to corn or soybean close to the milkweeds was provided by the staff managers at the different sites (SI Appendix, Table S3).

1.2.3 Laboratory Analysis

Leaf pesticide residue analysis: QuEChERS (Quick-Easy-Cheap-Effective-Rugged-Safe) extraction method was used to identify pesticide residues associated with milkweed samples. We screened 65 commonly used pesticides following the approach by Long and Krupke (2016). Multiple leaves within a sampled plant/date were combined and chopped with scissors to obtain a roughly homogenized 1g sample. All plant tissues were processed with scissors and forceps, cleaned in a 70% alcohol solution before processing each sample and latex gloves were used to avoid contamination between samples. Each 1 g sample was transferred into a 7 ml homogenizer tube (Bertin-technologies) with 2 g of zirconium oxide beads (2mm diameter; Bertin-technologies). To homogenize the tissue, 2 ml of double deionized (dd) water was added to each tube, after which tubes were set in a Precellys 24 lysis homogenizer, which processed samples using four cycles at 5,000 rpm. Homogenized samples were transferred to 15 ml tubes, and 2 ml dd water and 4 ml of the extraction solvent acetonitrile were added. The 15 ml tubes contained the 1 g plant tissue, 4 ml dd water and 4 ml acetonitrile. Ten µl of an isotopically labeled internal standard mix containing the pesticides screened was added to the 15 ml tubes. The standards help in the quantification of the pesticides in the samples, because a calibration curve is then created to assign a concentration value to peaks obtained from the processed samples.

The anhydrous salts magnesium sulfate (1.2g) and sodium acetate (0.3 g) were added to enhance the extraction efficiency and induce phase separation with acetonitrile. Each 15ml tube was agitated for 1min with a S8220 Deluxe Mixer Vortex (Scientific Products) and shaken on a VWR W-150 Waver Orbital Shaker at speed 10 for 10 min. The tubes were centrifuged at 4° C, 2,500 rpm for 10 min, for phase separation. One ml of supernatant was added to 2ml Agilent dispersive

Solid Phase Extraction tubes (part no: 5982-5321), containing 25 mg PSA, 7.5 mg GCB and 150 mg $MgSO_4$, cleaning up the samples before the analysis by liquid chromatography. The dispersive SPE tubes with the 1ml supernatant were spun in a vortex (Labnet VX100) for 10min and centrifuged at 15,000 rpm for 5min in an Eppendorf Centrifuge 5424. The supernatant was then transferred into 2ml Eppendorf tubes, which evaporated overnight in a speed vacuum (SC250EXP, ThermoFisher Scientific). The dry residue at the bottom of the tubes was mixed with 100 µl of acetonitrile, spun for 10 min in a vortex, centrifuged for 5min, and the supernatant was transferred to liquid chromatography mass spectrometry (LC- MS) autosampler vials. The identification, quantification and separation of the pesticide residues were carried out in an Agilent 1200 rapid resolution liquid chromatography with a triple quadrupole mass spectrometry (Agilent 6460 series) and an Agilent Zorbax SB-Phenyl 4.6 × 150 mm, 5 µm column (Agilent technologies, Santa Clara, CA). Both the QuEChERS method modification and LC-MS analysis were performed at the Bindley Bioscience Center at Purdue University.

Soil pesticide residue analysis: QuEChERS extraction method was modified and used to identify pesticide residues in soil, similar to the above-described protocol. Seven grams of wet soil were weighed on a scale (Mettler Toledo model MS3001S). The samples were dried for 2 days at 105◦C in individual aluminum baking cups. The dry weight of each individual sample was recorded to calculate the pesticide concentration in ng/g per sample; dry weight varied between 5.04 and 6.96g. Dry soil was sieved and slowly added and mixed to avoid clumping with 5 ml dd water in a 50 ml falcon tube. The 50 ml tubes were agitated for 1 min, then 5 ml of acetonitrile (ACN) at 99% and acetic acid at 1% were added, followed by 10 µl of an isotopically labeled internal standards mix containing the 65 pesticides targeted for screening. The tubes were agitated in a vortex for 7 min and then 4 g of magnesium sulfate (MgSO4) and 1 g of sodium acetate (NaOAC) were added slowly, shaking regularly in a vortex to facilitate the incorporation of the salts with the soil and avoid clumps. Upon adding salts, tubes were agitated in a vortex for another 2min to dissolve any clumps. The samples were centrifuged for 5min at 4,000 rpm and 1.4 ml of supernatant was transferred into dispersive Solid Phase Extraction tubes (part no: 5982-5122), containing 50 mg PSA, 50 mg C18EC and 150 mg MgSO4, to clean up the samples before the analysis by liquid chromatography. The dispersive SPE tubes were spun for 5min and centrifuged at 5,000 rpm for 3 min; 1 ml of supernatant was then transferred to 2ml Eppendorf tubes and left to dry overnight

in a speed vacuum (SC250EXP, ThermoFisher Scientific). The next day, samples were resuspended in 100 µl of acetonitrile, spun for 5 min and centrifuged for 7 min at 13,000 rpm before transferring the supernatant into an LC-MS vial. Pesticide identification and quantification were carried out as described above for leaf samples.

1.2.4 Statistical Analysis

We only targeted pesticides for statistical analysis and figures if they were detected in >1% of milkweed samples with overall concentrations >1 ng/g. Pesticides that fell below these thresholds were considered either too sporadic or diffuse to cause significant ecological impacts on monarch populations.

The effects of year, month and site on pesticide presence in milkweed tissue were evaluated with a mixed model logistic regression, with binary data (SI Appendix, Table S4). When pesticide residues were found in association with milkweed tissue, we assigned a value of 1 and when pesticide residues were below the detection limit we gave a value of 0. Site was considered as a random factor, and year and month were fixed factors. For this analysis, we only used 0/1 data, rather than the actual concentrations due to the large number of samples below the detection limit. We used a correlation analysis to test the relationship between pesticide concentrations found in soil vs. corresponding values in milkweed leaves. To do so, we created a 5m buffer around the points where soil samples were collected and selected the plants inside the buffer (SI Appendix, Figure S4). These soil-plant samples were paired together as spatially co- occurring to test for a correlative pattern. In cases where multiple plants were within the soil buffer, we averaged the plant data to create a single mean value for each pesticide at that location.

To evaluate the effects of land use on pesticide residues associated with milkweed leaves we used a three-tiered approach, starting with local habitat placement and ending with landscape- scale crop pesticide use. For local habitat placement, we used a two-part hurdle model with logistic regression using binary data based on detection frequency, followed by a secondary analysis using the continuous concentration data with non-detections removed. For this analysis, we focused on the three insecticides— thiamethoxam, clothianidin, deltamethrin—since the impacts of fungicides/herbicides on monarchs at this point are unknown. Because distance to field is

confounded with site, we were unable to include both factors in the model. In working with naturally occurring milkweed patches we were constrained by existing plant distributional patterns (see Table 1.2), resulting in some sites with all milkweeds clustered relatively close to the field margin (0–30 m for 2015 TPAC) and other sites that were far further away (2,300–2,400 m for 2015 Kankakee far). Thus, we developed site-specific models that include the factors year (when appropriate; some pesticides were mostly detected one of the 2 years), month, and distance separating milkweed plants from the nearest agricultural field. This allows us to test the effects of spatial isolation, while controlling for temporal variation. We only analyzed sites in which the distance gradient spanned the 125 ft distance threshold proposed for milkweed restoration. Several of our sites (see Table 1.2) included milkweeds that far exceeded this distance threshold, even at the closest proximity, and, consequently, distance from nearest crop field is biologically less relevant in these cases.

Next, simple linear regressions per year and pesticide active ingredient were used to quantify the relationship between percent of corn and soybean planted in a 1 km radius around milkweed habitats and the frequency of milkweed leaves with pesticide residues. For this analysis, we took advantage of natural variation in land use surrounding our sites, which varied widely from no agriculture to ca. 80% cropland (see Table 1.1). Last, we used correlations to determine whether corn or soybean pesticides applied at the county-level reflected the frequency of residues associated with milkweed leaves. This analysis used Tippecanoe as the focal county since this housed the majority of our milkweed sites and has a similar agricultural backdrop to the other county (Newton) surveyed. Also, we focused this county analysis only on fungicides for two reasons: one, given the chemical and application differences across pesticide classes, we wanted to avoid directly comparing, for example, insecticides and herbicides; and two, fungicides had the most active ingredients- −6 compounds—which allowed us to make this comparison (i.e., we were unable to use a correlation with only 2 or 3 data points in the case of insecticides and herbicides).

All statistical analyses were conducted with R software 3.5.1 (R team core 2013) using the packages car, ggplot2, lmer4, and multicomp, except for local habitat use (i.e., distance from crop), for which we employed the Proc Genmod and Proc GLM functions in SAS, V. 9.4.

1.3 Results

1.3.1 Leaf pesticides

Across both years of the study, 14 pesticides commonly used in crops in Indiana were detected on milkweed leaves (Table 1.3). It is important to note, however, that this is not a comprehensive list. While we screened a relatively large number of pesticides, focusing on ones that we know are ubiquitous components of row crop pest management in our region, some compounds are difficult to detect due to factors such as high volatility (e.g., dicamba) or require a different, more specialized analytical approach for quantification (e.g., glyphosate).

Clothianidin, the insecticide that to date has received the most attention for potential non-target impacts on monarchs, was only detected in 4–8% of total samples; however, those values are somewhat misleading since it averages across all sites and dates. As a general pattern for both sampling years, we almost exclusively detected clothianidin in June, but not in July or August (Figures 1.1 A,D). During these early season samples, clothianidin was detected in ca. 15–25% of plants with nearly 60% of milkweeds at some sites testing positive. Interestingly, both thiamethoxam (neonicotinoid) and deltamethrin (pyrethroid) varied dramatically in their detection rates across years (SI Appendix, Table S4), with both compounds occurring at high frequencies in 2016 (75–99%) while being virtually absent from samples in 2015 (Figures 1.1 A,D). Imidacloprid (neonicotinoid) was only found in a small number of plants (0.2%) in the first year of this study.

Atrazine was the most commonly detected (80–87% on average, although some months approached 100% of samples) and occurred at the highest mean concentrations (6.84 and 37.0 ng/g) of any pesticide surveyed in either year, followed by s-metolachlor and acetochlor among the herbicides (Table 1.3). Notably, s-metolachlor displayed consistent within- season patterns in both sampling years whereby detection rates were several-fold higher early in the season before gradually declining in July and August (Figures1.1 B,E; SI Appendix, Table S4).

Overall, fungicides were the most omnipresent of pesticides detected on milkweed with 6 compounds consistently occurring on leaves. Several fungicides, most notably propiconazole (98% detection rate in 2016), were somewhat commonly detected, but only at trace (<1 ng/g)

amounts. The compounds that combined relatively high concentrations and detection rates included pyraclostrobin (31–55%) and trifloxystrobin (27–40%; Table 1.3). In contrast with the herbicide s-metolachlor, which decreased throughout the season, the two strobilurin fungicides showed the opposite pattern, gradually increasing from June to August in both years (Figures 1.1 C,F; SI Appendix, Table S4). Propiconazole detection frequency displayed a nearly 3-fold increase between years one and two, from 34 to 98% of samples.

1.3.2 Soil pesticides

We found 7 pesticides in soil across the sites sampled in 2016 (Table 1.4), which were a subset of the 14 pesticides recorded from milkweed leaves. Clothianidin was the only insecticide detected and it was found in all samples consistently throughout the summer (Figure 1.2 A), in contrast with leaf presence, which was restricted to only June. Thus, clothianidin was far more ubiquitous in the soil than leaves. Importantly, soil concentrations of clothianidin were highly correlated with levels in co-occurring milkweed leaves (Figure 1.3; $r = 0.763$, $p < 0.0001$). This was the only pesticide showing a soil- plant association.

We detected three herbicides—atrazine, s-metolachlor, acetochlor—and three fungicides—azyoxystrobin, pyraclostrobin, metalaxyl. Similar to clothianidin, soil concentrations of these compounds tended to be far more stable over time, i.e., leaf values fluctuated dramatically across months when the same soil values remained relatively constant (compare Figure 1.1 vs. Figure 1.2) even though half-life of pesticides vary with soil physical and chemical characteristics and our plants grow under different soil types (SI Appendix, Table S1).

1.3.3 Land use

Linear distance separating milkweed plants from agricultural fields was a strong predictor of thiamethoxam detection frequency at all of the sites evaluated (Table 1.5). However, distance frequently interacted with sampling month, resulting in variation in the nature of the relationship over time. In 6 of 9 cases (3 sites × 3 months), detection rates declined with increasing distance separating milkweed from crop field up to 150m, although the shape of this relationship varied (Figure 1.4). The other two insecticides either showed no spatial patterning (clothianidin; no

significant main or interactive effects of distance from crop) or were detected in nearly 100% of samples and thus did not have sufficient variation in detection frequency to statistically evaluate using binary data (deltamethrin in 2016, Table 1.3). When continuous concentration data were used after removing samples below the detection threshold, one of the three sites also showed a distance relationship involving thiamethoxam (Figure 1.5; distance x month, $F = 11.35$, $P < 0.0001$). Similar to detection data, concentrations were higher in milkweeds growing closer to field edges. As with binary data, no relationships were observed for clothianidin or deltamethrin.

Although we found substantial site-level variation in pesticide presence on milkweed, landscape composition—namely, amount of corn and soybean—within a 1km radius surrounding focal sites was a poor predictor of our data. Across both 2015 (Figure 1.6) and 2016 (Figure 1.7), only one pesticide— pyraclostrobin in 2015 (Figure 1.6 D; $F = 8.61$, $P < 0.05$)— showed a relationship between land use and detection frequency (SI Appendix, Table S5). In this case, percent of plants with measurable amounts of pyraclostrobin increased from ca. 40 to 70% when comparing the least to most agricultural sites.

Finally, at the county-level, which encompasses the broadest spatial scale employed (for reference, Tippecanoe Co. is ca. 1,300 km2), the total amount of fungicides applied to soybean had a marginally significant ($r = 0.85$, $P = 0.06$) effect on the percent detection frequency of fungicides for milkweed leaves in 2015 (Figure 1.8 B). However, other relationships were not significant (corn 2015, $r = 0.38$, $P = 0.28$; corn 2016, $r = 0.18$, $P = 0.77$).

1.4 Discussion

Our study clearly shows that the foliage of milkweed growing in prairies and unmanaged habitats neighboring cropland contains residues from a wide variety of agricultural pesticides, primarily those applied to corn and soybean. The actual risk of these pesticides, however, depends on how frequently milkweeds contain those levels in the field. Our data reveal strong spatiotemporal variation in pesticide occurrence across sites, months, and years, which means that the threat posed by these chemicals depends on if, when, and where they coincide with monarch colonization and phenology. Below we highlight the implications of these findings for each of the three major

pesticide classes and discuss whether pesticide exposure can be avoided based on local and landscape-level habitat placement.

1.4.1 Insecticides

Contamination of non-target plants by neonicotinoids used in agriculture is widely reported, but almost exclusively for pollen or nectar samples taken from flowers (Greatti et al., 2003; Krupke et al., 2012; Bonmatin et al., 2014; Botías et al., 2015, 2016; Mogren and Lundgren, 2016). Consistent with this literature, our study found neonicotinoid residues associated with milkweed leaves around farmland, specifically the active ingredients clothianidin and thiamethoxam. Although seed treatment data are no longer reported for U.S. row crops, most corn in our region is seed treated, primarily with clothianidin, and much of the soybean acreage also employs a seed treatment, mainly thiamethoxam (Douglas and Tooker, 2015). Corn and soybean dominate land use in the areas surrounding each of our milkweed sites, and thus it is not surprising that these two insecticides were among the ones most commonly detected.

Importantly, the leaf concentrations we recorded (up to 56.5 and 151.3 ng/g for clothianidin and thiamethoxam, respectively) are within the range previously reported from other studies. For example, a recent analysis of clothianidin on the leaves of plants used in pollinator strips bordering seed-treated corn fields reported values that were comparable to our milkweed data (Mogren and Lundgren, 2016), including sunflower (max. 81 ng/g), buckwheat (max. 54 ng/g), and phacelia (max. 33 ng/g). Interestingly, some milkweed concentrations were also roughly similar to those reported from the leaves of seed-treated crops such as corn (7–86 ng/g at 20–34 days post planting; Alford and Krupke, 2017) and soybean (105 ng/g in V1 stage and 1.7 ng/g in V4 stage after 17 and 56 days; Magalhaes et al., 2009). Perhaps most relevant to our study, Pecenka and Lundgren (2015) documented clothianidin in 36–64% of milkweed leaves surveyed in South Dakota at mean concentrations of 1.24 and 1.11 ng/g. By comparison, we detected clothianidin at a far lower rate (4.6 and 8.1%, overall, for the 2 years), but with comparable mean values (0.71 and 0.48 ng/g). Pecenka and Lundgren (2015) used dose-response curves for monarch larvae to clothianidin, which revealed the LC50 at 15.63 ng/g and sublethal effects at as little as 1 ng/g. Based on extrapolating these calculations to our field data, sublethal effects should be observed for monarchs on 5–8% of leaves surveyed (averaged across all sites, months, and years; risk varies seasonally),

whereas lethal effects (i.e., >LC50) are limited to 1.4% of samples. It is important to note that our assessment is based solely on clothianidin, for which data exist on monarch growth and survival. Our second sampling year revealed that thiamethoxam can be much more prevalent— detected in 75% of samples—but its toxicity to monarchs is unknown at present.

Another critical aspect of our neonicotinoid data is that during both years of the study, residues diminished dramatically over the course of the summer. We virtually only detected clothianidin in June, and thiamethoxam detection in year 2 dropped by ~50% from June-July to August. This within-season decline would be consistent with pesticide degradation from the putative time of exposure (i.e., when seed-treated fields are planted in late spring) to the timing of when milkweeds were sampled. More importantly, the data suggest that early-season monarchs are at greater risk from neonicotinoid exposure than subsequent generations occurring later in the season. Similarly, our data suggest strong annual fluctuations in risk, indicating that monarchs likely encounter a different suite of pesticides each year. Thiamethoxam and deltamethrin, for example, were more prevalent in the second sample year. This is likely a result of local or regional differences in pest management approaches employed by farms. Active ingredients for foliar sprays such as deltamethrin can vary greatly across years, depending on factors such as price and availability. Thiamethoxam is more likely to be a reflection of seed treatments, which vary with the relative acreage of corn vs. soybean in the landscape. Further, in corn/soy rotations, the insecticides used will change on an alternate year basis. Overlaying temporal variation in pesticide presence with the timing of non-target insect colonization and development is a key component to risk assessment that, to our knowledge, is rarely incorporated into such studies.

While we did not document the mechanism by which neonicotinoids moved from cropland to milkweeds in this study, for clothianidin we found a strong positive relationship between soil and leaf concentrations (Figure 1.3). This could be simply correlative (i.e., areas with high neonicotinoid deposition result in correspondingly higher concentrations both in soil and on plant surfaces), or indicative of systemic uptake from soil into nearby plants. In all cases, we analyzed whole tissue samples so, unfortunately, do not know whether pesticides are on the leaf surface or inside the plant, for systemic compounds. Overall, the clothianidin concentrations in our soil samples (range: 0.88–8.59 ng/g; mean: 1.75 ng/g) were comparable with those reported in other

studies of agricultural soils, i.e., 6.57 ng/g (Botías et al., 2015), 7.0 ng/g (Xu et al., 2016), 2.1 and 6.3 ng/g (Krupke et al., 2012).

Last, in 2016 we frequently detected the pyrethroid deltamethrin in milkweed samples. Although pyrethroids are considered highly toxic to lepidopterans in general, nothing is known specifically about the deltamethrin-monarch relationship. A few studies have found negative non-target effects of the related pyrethroids, permethrin, and resmethrin, used in mosquito control on monarch caterpillars (Oberhauser et al., 2006, 2009). Similarly, field applications of deltamethrin in the UK increased mortality of Pieris butterfly larvae developing in hedgerows bordering cereals (Cilgi and Jepson, 1995). Topical application of 20 ng was sufficient to kill 50% of P. brassicae individuals after 2 weeks of exposure (Cilgi and Jepson, 1995); however, host plants influence caterpillar susceptibility to deltamethrin (Tan and Guo, 1996), and thus it is difficult to extrapolate these values for milkweed.

1.4.2 Herbicides and Fungicides

Monarch decline is often attributed to an indirect effect from glyphosate reducing milkweed abundance (Hartzler, 2010; Pleasants and Oberhauser, 2013; Pleasants, 2017). Yet, the direct effects of herbicides on monarchs (i.e., those not merely caused by a reduction in milkweed availability) are unknown, and likely dismissed since herbicides are considered non-toxic to insects (but see Russell and Schultz, 2009; Stark et al., 2012). Potential non-target pathways could occur via herbicide exposure, either topically or orally, changing some aspect of caterpillar physiology or altering the milkweed-monarch interaction by e.g., interfering with or amplifying the induced defense pathways employed by milkweeds (Boutin et al., 2004, 2014). For instance, the herbicide 2,4D functions as a plant defense elicitor, resulting in resistance to herbivorous insects on plants exposed to low doses (Xin et al., 2012). Also, drift of the herbicide dicamba into field margins reduced pollinator visitation rates (Bohnenblust et al., 2016), impacted the abundance of several arthropods in the community (Egan et al., 2014), and decreased caterpillar development (Bohnenblust et al., 2016). Herbicides such as glyphosate can even act directly on pollinators by disrupting their gut microbiome (Motta et al., 2018).

Of the herbicides sampled, atrazine was the most commonly detected and at the highest concentrations. Much of what is known about atrazine's impacts on invertebrates comes from aquatic food webs where run-off into streams or lakes alters community structure (Dewey, 1986; Gruessner and Watzin, 1996). While these are mostly indirect effects via reductions in the population of algae or related macrophytes, direct effects of atrazine on insects are documented (Miota et al., 2000; Graymore et al., 2001), as well as their role in synergizing insecticides such as organophosphates (Anderson and Lydy, 2002).

For fungicides, the compounds we detected in milkweed leaves largely match those reported from pollen, honey, nectar, wax, and foliage of wildflowers or crops (Krupke et al., 2012; Sanchez-Bayo and Goka, 2014; David et al., 2016; Long and Krupke, 2016). Fungicides inhibiting ergosterol biosynthesis, like propiconazole, act as synergists for neonicotinoid insecticides, increasing their toxicity to bees by inhibiting cytochrome P450s that function in detoxification (Pilling and Jepson, 1993; Pilling et al., 1995; Johnson et al., 2013). There is also the potential for additive toxicity when insects are exposed to mixtures of pesticides. Propiconazole was detected in 98% of milkweed samples in 2016, in many cases co-occurring with insecticides like deltamethrin and thiamethoxam. The high frequency of the fungicides propiconazole, pyraclostrobin, and trifloxystrobin compared with metalaxyl and azoxystrobin could be related to the high use of these fungicides to increase yield in hybrid corn and soybean (Paul et al., 2011; Mahoney et al., 2015).

1.4.3 Land Use

For the neonicotinoid thiamethoxam, we found that detection frequency and concentrations tended to be higher on milkweeds growing in closer proximity to agricultural land. This suggests that spatially isolating milkweed restoration sites from crop fields could be an effective approach to reduce risk. To our knowledge, the proposed 125 ft buffer distance is a somewhat arbitrary value that is not based on specific criteria; however, our data nevertheless suggest that milkweed habitat restoration abiding by this rule would likely result in fewer plants containing thiamethoxam and at lower concentrations (see values >38.1 m on Figures 1.4, 1.5). What remains unclear is the degree to which these reductions result in enhanced survival and/or performance of monarch caterpillars, which is the ultimate goal. This would require experimentally rearing larvae on plants in the field

along a distance transect extending from a crop field edge. In fact, bypassing high quality monarch habitat on land that is relatively close to a corn or soybean field could have a net detrimental effect on monarch conservation if the benefits of additional milkweed stems exceed the detrimental impact of higher pesticide load; a scenario that is entirely plausible, depending on the factor(s) most limiting monarch fitness. This is particularly true for pesticides such as clothianidin that already occur at relatively low frequencies. Deltamethrin also occurred at frequencies and concentrations that were independent of distance to crop. This could be due to the fact that this insecticide is likely applied via aerially spraying, which may result in greater propensity for drift beyond the immediate surrounding of crop fields.

At the landscape level, amount of row crop production in the 1km radius around milkweed sites was generally a poor predictor of pesticide presence on milkweeds. Only one of the pesticides tested—pyraclostrobin—showed a significant relationship whereby prevalence increased on milkweeds with increasing agricultural intensity. That being said, several of the pesticides, including clothianidin in both years, were most prevalent at the most heavily agricultural site while showing the lowest occurrence at the least agricultural site. We suspect that the lack of statistical power due to low site replication (n = 6 and 5 sites in 2015 and 2016, respectively) played a role in these outcomes, especially for a coarse predictor variable like total crop acres that does not account for variation in local site factors. A similar conclusion was drawn from a recent study of pesticide residues on native bees; despite trends, land cover in a 1km radius around sites was non-significant, likely due to low site replication (Hladik et al., 2018). Our county-level analysis led to an analogous conclusion. Correlations suggested that greater use by farmers at the regional scale increased prevalence of fungicides on milkweeds, but statistical effects were equivocal (i.e., marginal significance) due to low replication (Figure 1.8).

1.5 Conclusions

Risk assessment evaluating the potential impacts of pesticides on monarchs entails a two-step process; first, documenting the chemicals that larvae and/or adults are exposed to in the environment, and second, experimentally testing those chemicals most commonly encountered to assess lethal and sub-lethal effects. Here, we take the first step in this process, documenting the spectrum of pesticides encountered by monarch larvae on the most critical host-plant in their

summer breeding range, *A. syriaca*. We strongly emphasize, however, that pesticide presence does not necessarily translate into impact. Unlike honeybees, for which LD50 data are widely available on most compounds, at present such information is only available for clothianidin in the monarch system. Clearly, a major emphasis of future research efforts should be to close this knowledge gap by quantifying monarch larval responses to a range of pesticides under controlled lab settings. Based on our field data, obvious starting points for these trials would be insecticides such as thiamethoxam and several of the ubiquitous fungicides that occur on milkweed leaves.

Assuming pesticide presence is undesirable for land managers focused on restoring milkweed for monarch conservation, our data secondarily point to local habitat placement—namely, site isolation—as an effective tool for reducing non-target exposure. Additional work to help refine these recommendations could focus on site-specific factors that contribute to off-site pesticide drift beyond simple linear distance, e.g., wind direction, slope, soil type.

Author contributions

PO-A wrote the manuscript, collected, and analyzed the data. IK guided the research, reviewed, and edited the manuscript.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo. 2019.00223/full#supplementary-material

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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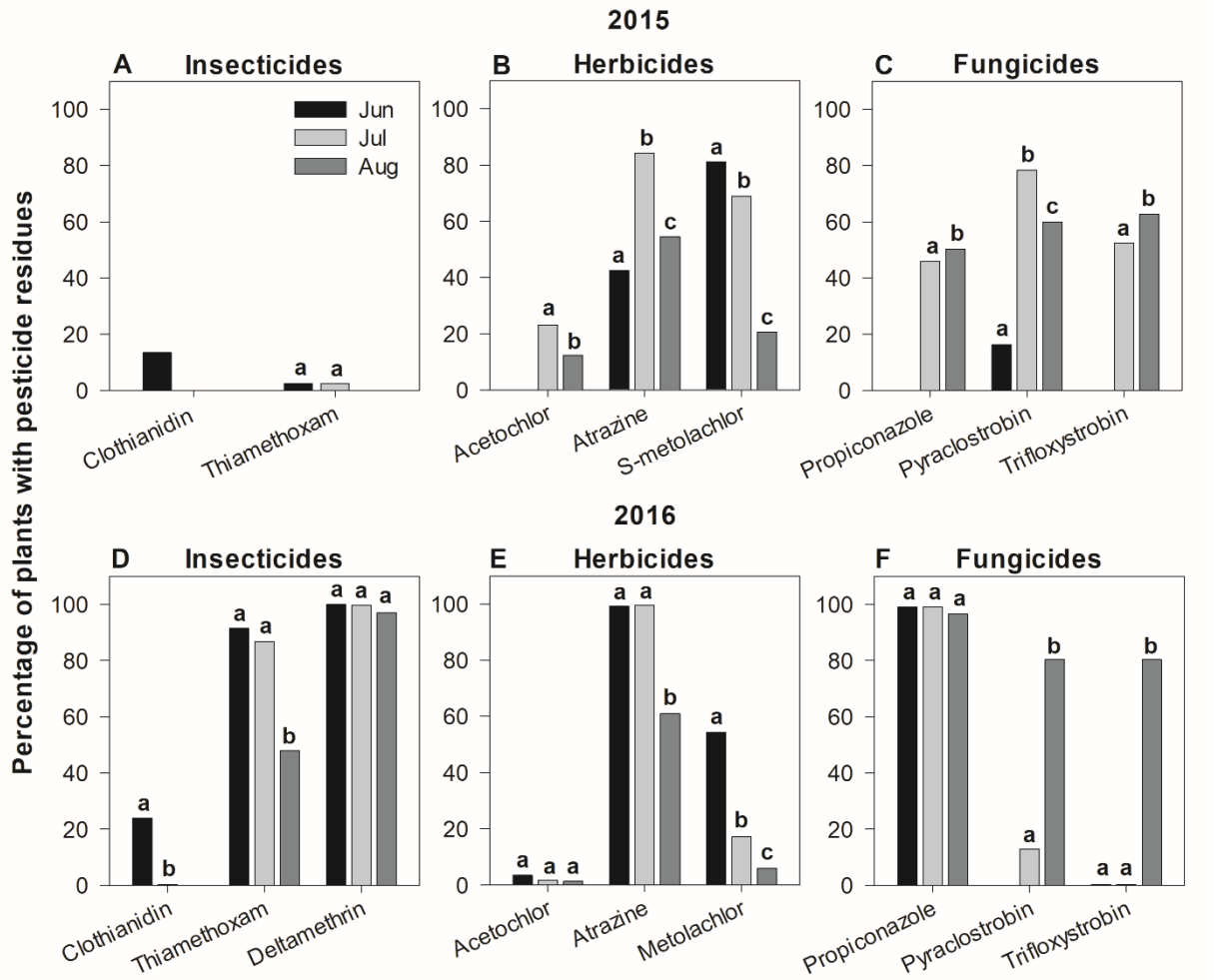

Figure 1.1. Frequency of pesticide residues detected from milkweed leaf tissue during June, July and August 2015 **(A–C)** and 2016 **(D–F)**. Percentages are calculated from samples summed across all study sites. Months with different letters, by pesticide, indicate significant differences ($P < 0.05$).

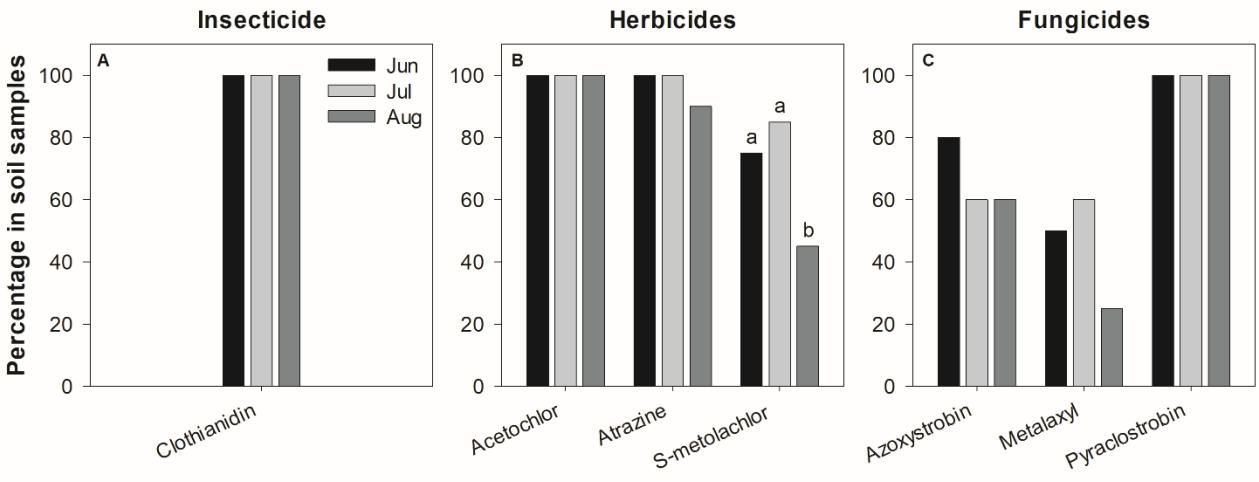

Figure 1.2. Frequency of pesticide residues detected in soil samples during June, July and August 2016 (**A–C**). Percentages are calculated from samples summed across all study sites. Significant differences were detected only in s-metolachlor ($P < 0.05$).

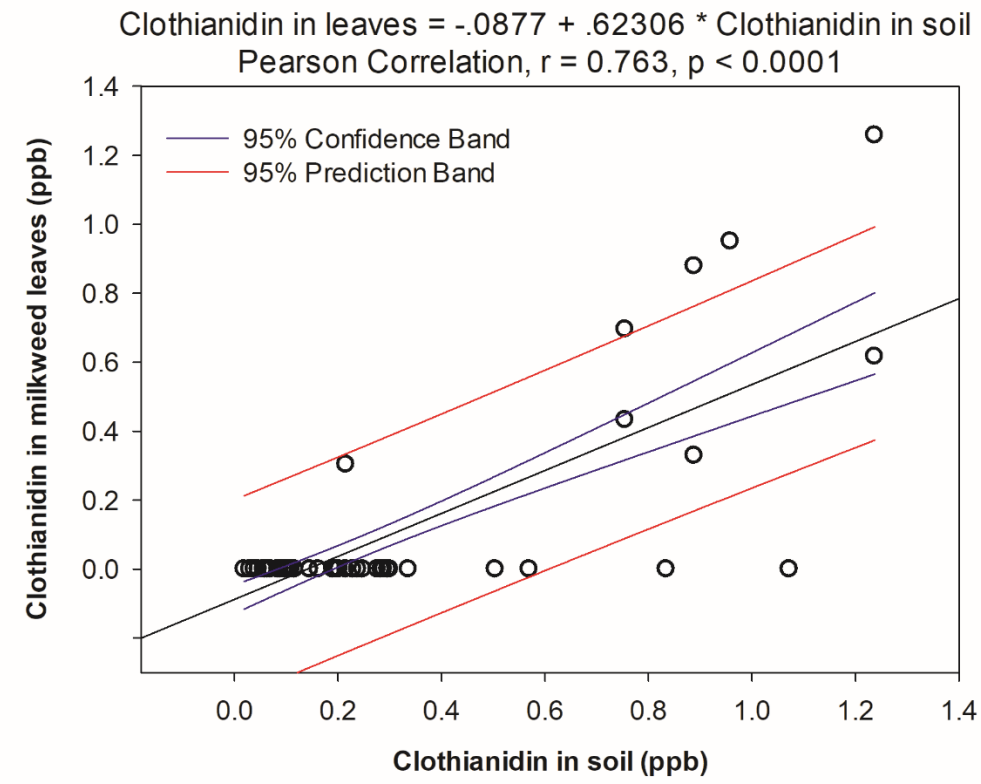

Figure 1.3. Relationship between clothianidin concentration detected in soil and leaves from adjacent milkweed plants. Data points represent a paired soil-leaf sample for a given site/month.

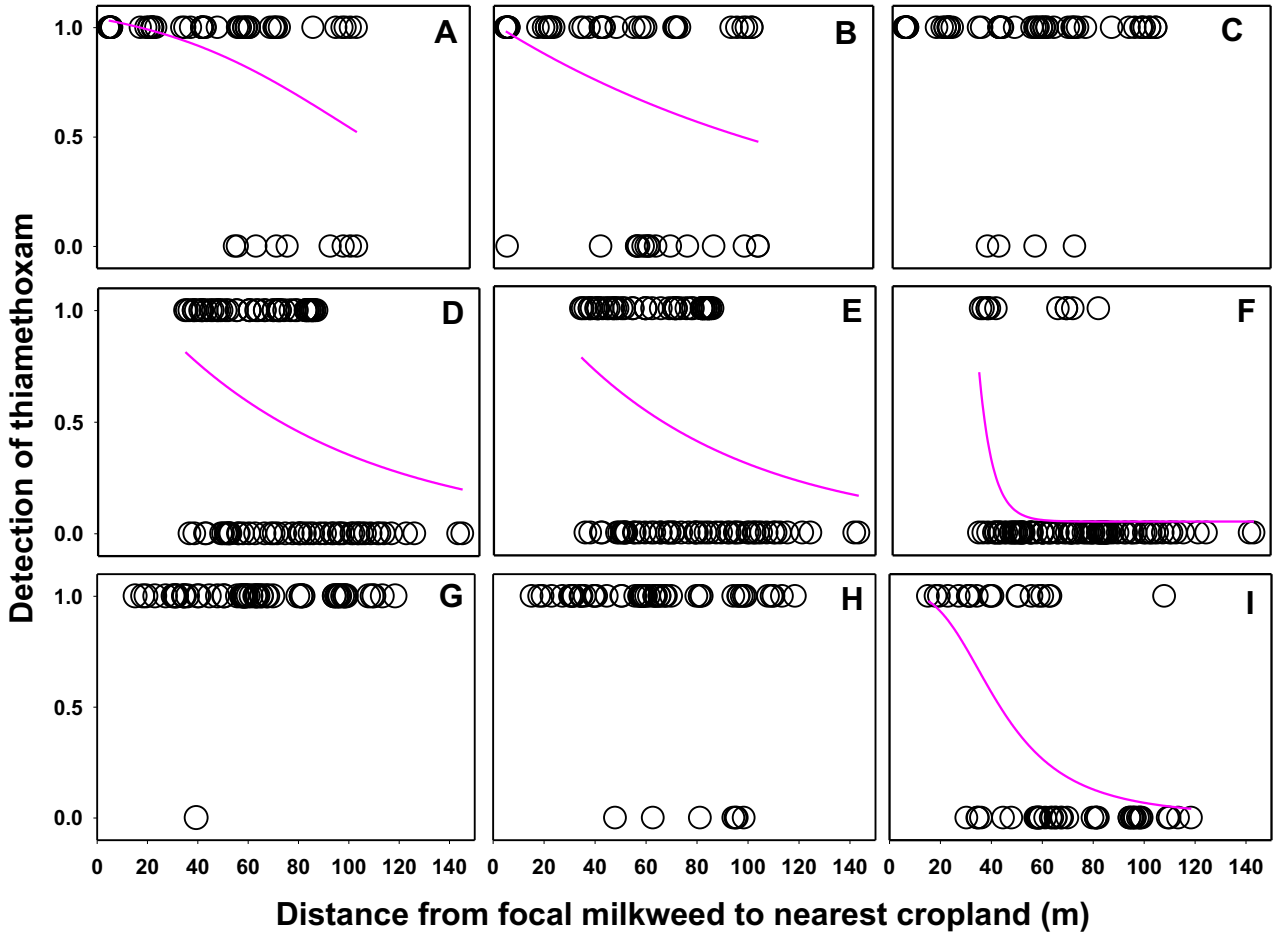

Figure 1.4. Relationship between distance separating focal plants from cropland and detection of the neonicotinoid thiamethoxam associated with milkweed leaves. Each data point is an individual plant sample with "1" values = thiamethoxam detected and "0" values = thiamethoxam not detected. Best fit curves plotted to the data using sigmoidal or exponential decay functions. Data are for June, July, and August, respectively, for three sites in Indiana: ACRE **(A–C)**, PWA **(D–F)**, and Prophetstown **(G–I)**.

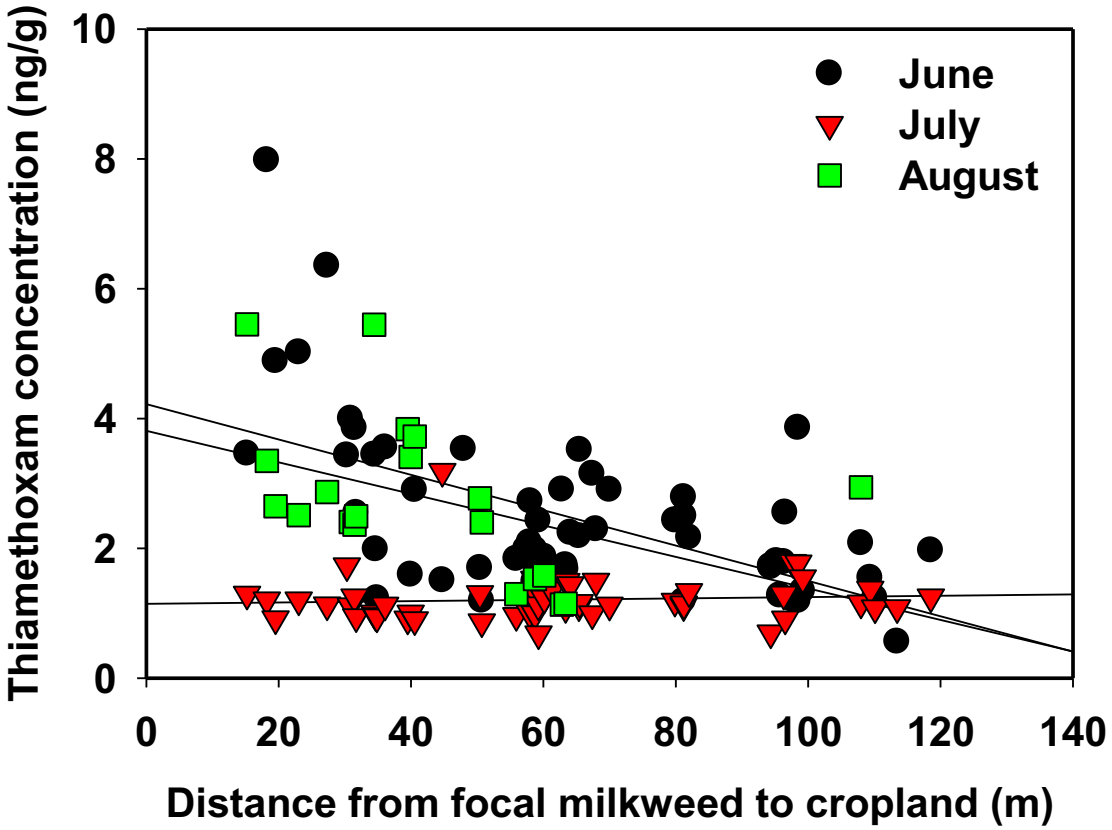

Figure 1.5. Relationship between distance separating focal plants from cropland and concentration of the neonicotinoid thiamethoxam associated with milkweed leaves at one of our sites, Prophetstown. Each data point is an individual plant sample for June, July, or August.

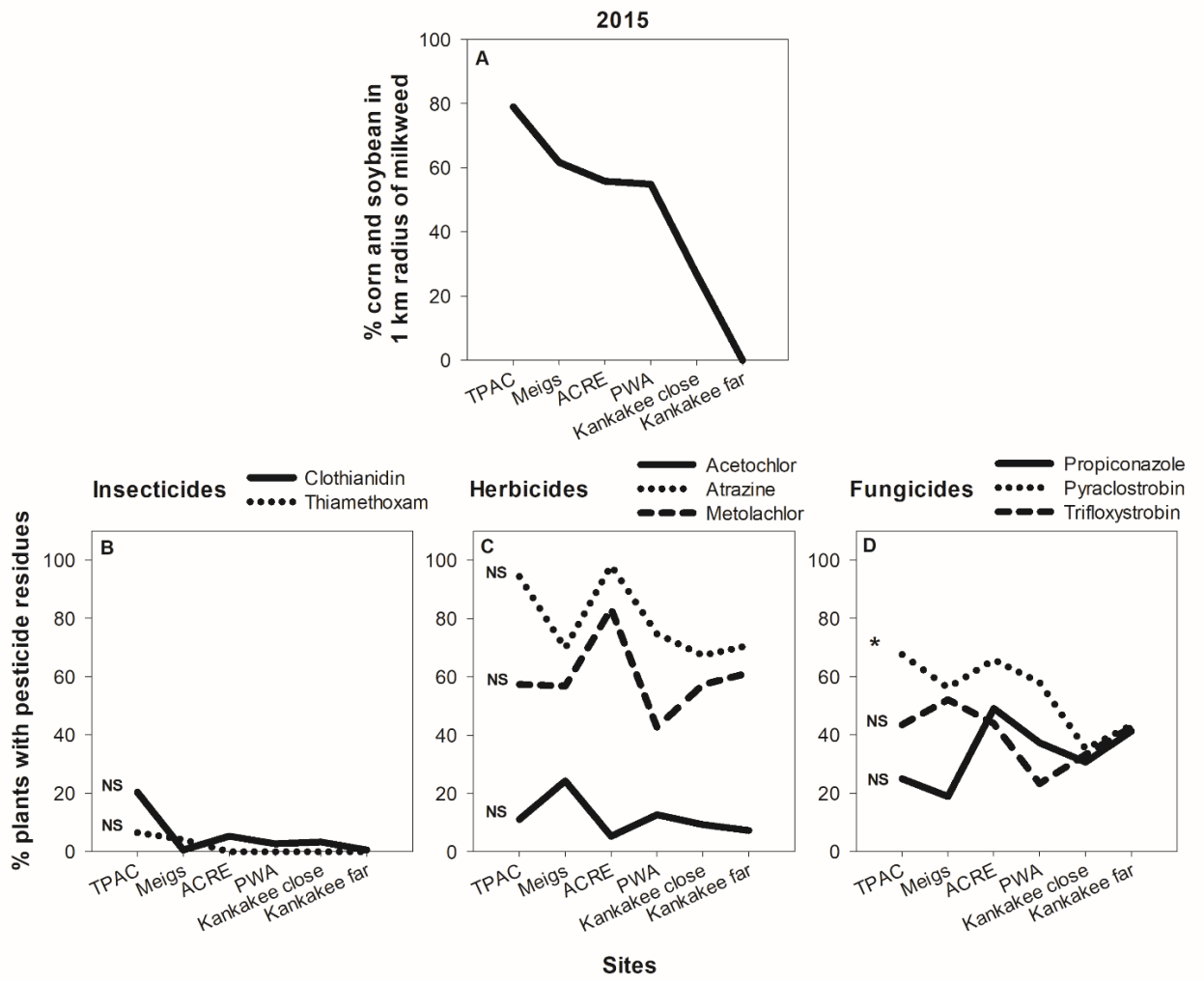

Figure 1.6. Area of corn and soybean, expressed as percent of total land use, in 1 km buffer surrounding the six milkweed sites sampled in 2015 **(A)** and corresponding site-level changes in insecticide **(B)**, herbicide **(C)**, and fungicide **(D)** residues associated with milkweed leaves. Statistical analyses compare, within each pesticide, the relationship between agricultural intensification and pesticide detection frequency. *$p < 0.05$, ns = not significant.

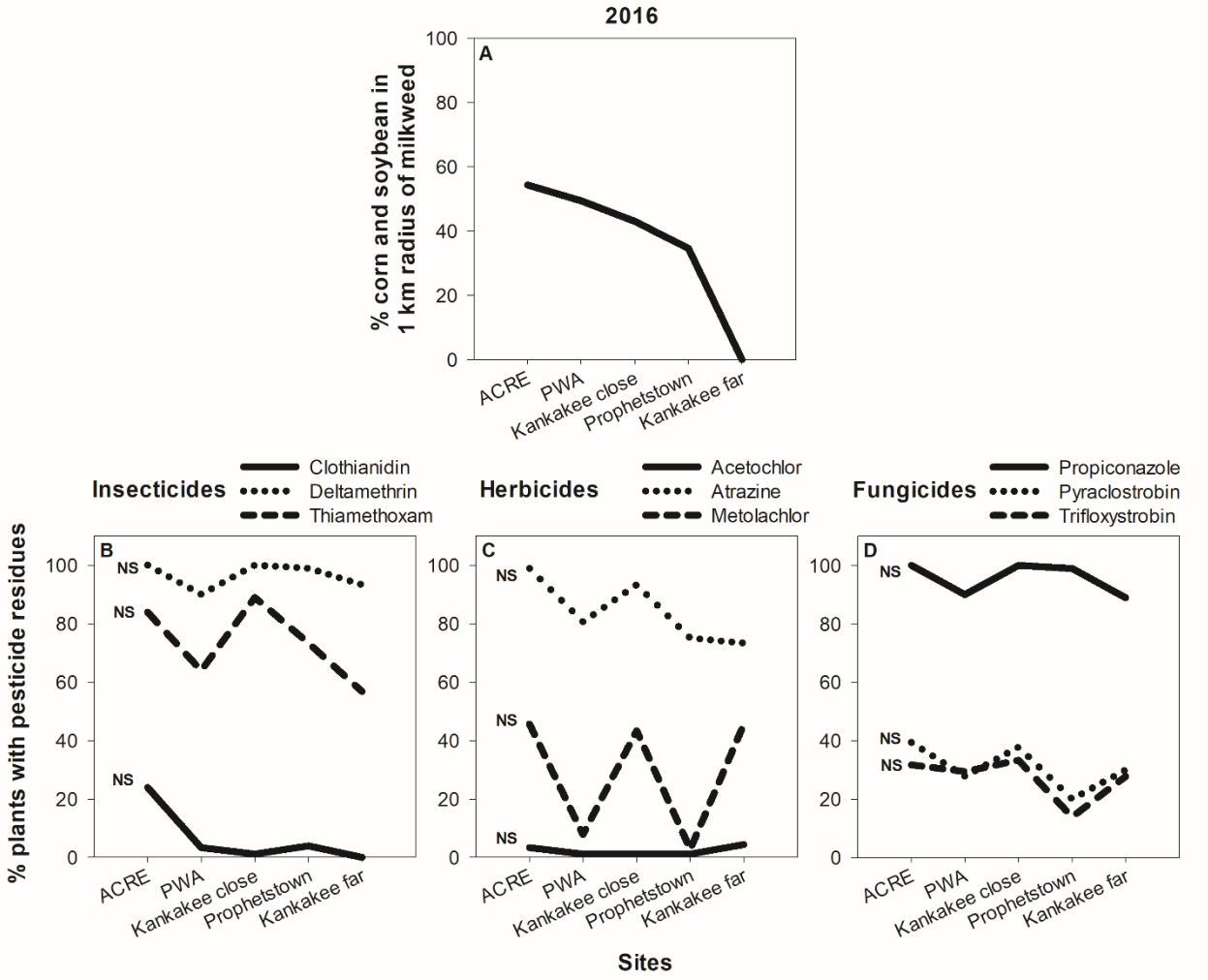

Figure 1.7. Area of corn and soybean, expressed as percent of total land use, in 1 km buffer surrounding the five milkweed sites sampled in 2016 **(A)** and corresponding site-level changes in insecticide **(B)**, herbicide **(C),** and fungicide **(D)** residues associated with milkweed leaves. Statistical analyses compare, within each pesticide, the relationship between agricultural intensification and pesticide detection frequency. ns = not significant.

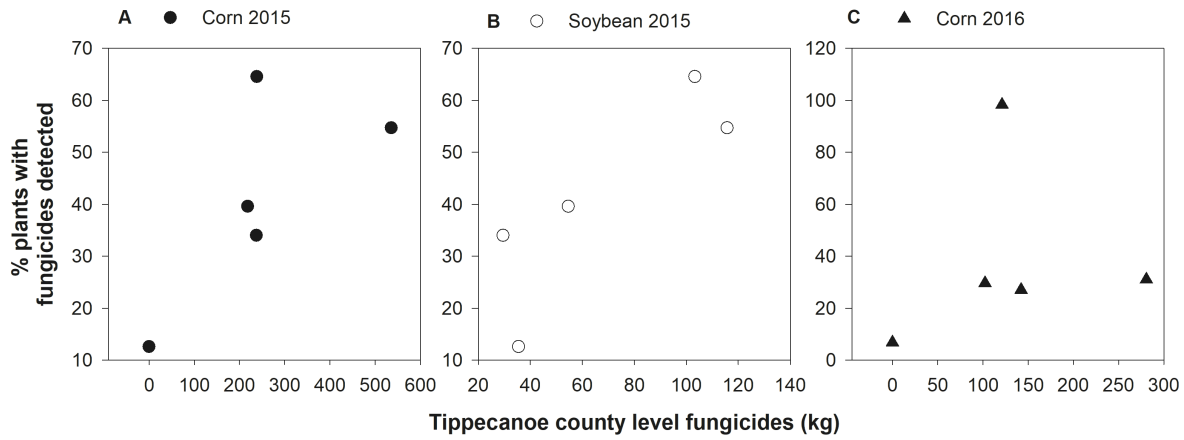

Figure 1.8. Relationship between amounts of corn **(A,C)** and soybean **(B)** fungicides used in Tippecanoe County in 2015 and 2016 vs. the percent of plants with detectable levels of fungicides. Data points show each of the five fungicides detected in our analysis.

Table 1.1. Area of corn and soybean, expressed as percent of total land use, planted in a 1 km radius around milkweed sites sampled in 2015 and 2016.

Year	Site	% Corn	% Soybean
2015	**ACRE**	29.6	26.2
	Kankakee close	12.4	14.4
	Kankakee far	0.0	0.0
	Meigs	24.8	36.9
	PWA	48.3	6.6
	TPAC	48.5	30.5
2016	**ACRE**	14.6	39.7
	Kankakee close	20.1	22.9
	Kankakee far	0.0	0.0
	Prophetstown	26.3	8.3
	PWA	27.2	22.2

Table 1.2. Local site characteristics and number of plant replicates for milkweeds sampled in 2015 and 2016.

Year	Site	No. milkweed plants sampled	Distance (min – max) from milkweed to nearest agricultural field (m)	Direction of milkweed relative to crop	Size of neighboring crop field (ha)
2015	**ACRE**	38	5 – 63	S	1.25
	Kankakee close	50	407 – 508	E	128.93
	Kankakee far	50	2,312 – 2,398	E	128.93
	Meigs	66	0 – 246	N,E,W	2.84
	PWA	50	36 – 143	W	74.73
	TPAC	36	0 – 30	N,S,E,W	4.26
2016	**ACRE**	60	5 – 105	S	1.25
	Kankakee close	30	62 – 74	E	96.78
	Kankakee far	30	1,641 – 1,714	E	96.78
	Prophetstown	60	15 – 119	E	43.38
	PWA	54	34 – 86	W	74.73

Table 1.3. Summary data for pesticides detected in milkweed leaf samples across both years of the study.

		2015 leaves (n=841)					2016 leaves (n=702)						
		% detection	mean (ng/g)	SE	median (ng/g)	max (ng/g)	% detection	mean (ng/g)	SE	median (ng/g)	max (ng/g)	LOD (ng/g)	% recovery
Insecticides	**Clothianidin**	4.6	0.71	0.15	<LOD	56.5	8.1	0.48	0.09	<LOD	28.5	1.060	107.4
	Thiamethoxam	1.8	0.19	0.12	<LOD	94.8	75.4	1.87	0.23	1.44	151.3	0.230	110.0
	Imidacloprid	0.2	0.01	0.01	<LOD	3.7	n.d.	n.d.	n.d.	n.d.	n.d.	0.640	93.5
	Deltamethrin	n.d.	n.d.	n.d.	n.d.	n.d.	98.9	3.78	0.47	1.91	248.5	0.420	109.8
Herbicides	**Atrazine**	79.7	6.84	0.61	0.52	238.7	86.6	37.00	4.36	4.73	1352.9	0.040	80.3
	Metolachlor	59.2	0.73	0.05	0.15	15.3	25.8	1.37	0.19	<LOD	58.1	0.040	99.4
	Acetochlor	10.1	0.26	0.06	<LOD	43.0	2.1	0.09	0.03	<LOD	11.6	0.126	99.5
	2-4D	0.2	0.002	<0.01	<LOD	1.0	n.d.	n.d.	n.d.	n.d.	n.d.	0.126	65.6
Fungicides	**Azoxystrobin**	64.6	6.80	1.33	0.01	245.8	29.6	0.21	0.03	<LOD	7.8	0.012	89.2
	Pyraclostrobin	54.7	4.66	0.56	0.11	211.7	31.1	11.44	1.37	<LOD	453.6	0.002	71.3
	Trifloxystrobin	39.6	1.77	0.32	<LOD	164.2	27.1	3.92	0.55	<LOD	151.4	0.012	69.9
	Propiconazole	34.0	0.41	0.05	<LOD	27.1	98.3	1.27	0.07	0.86	27.2	0.040	49.5
	Metalaxyl	20.0	0.019	<0.01	<LOD	2.2	5.3	0.02	<0.01	<LOD	1.3	0.012	93.7
	Difenoconazole	12.6	0.005	<0.01	<LOD	0.6	6.8	0.001	<0.01	<LOD	0.1	0.001	63.3

Table 1.4. Summary data for pesticides detected in soil samples collected from the field in 2016.

	2016 soil samples (n=75)				
Active Ingredient	**% detection**	**Mean (ng/g)**	**SE**	**Median (ng/g)**	**Max (ng/g)**
Clothianidin	100	1.75	0.25	1.19	8.60
Acetochlor	100	2.33	0.21	2.08	6.83
Atrazine	96.7	5.64	0.62	3.53	27.22
Metolachlor	68.3	1.54	0.23	0.80	8.01
Azoxystrobin	66.7	0.16	0.03	0.07	1.17
Pyraclostrobin	100	0.01	<0.01	0.01	0.07
Metalaxyl	45	0.29	0.05	<LOD	1.45

Table 1.5. The effects of month and distance separating focal plants from the nearest cropland on thiamethoxam detection associated with milkweed leaves at three sites (A–C) sampled in 2016.

	thiamethoxam detection			
A. ACRE	**Estimate**	**SE**	**Z**	***P***
intercept	4.7246	0.9683	4.88	<0.0001
distance	-0.0474	0.0129	-3.69	**0.0002**
month (June)	0	0	.	.
month (July)	-2.2309	1.076	-2.07	**0.0381**
month (Aug)	-1.8966	1.1915	-1.59	0.1114
distance*June	0	0	.	.
distance*July	0.0203	0.0147	1.38	0.1676
distance*Aug	0.0437	0.0149	2.93	**0.0034**
B. PWA				
Intercept	2.6371	0.6978	3.78	0.0002
distance	-0.0357	0.0087	-4.13	**<0.0001**
month (June)	0	0	.	.
month (July)	-0.2727	0.1585	-1.72	0.0854
month (Aug)	-1.6186	1.1439	-1.42	0.1571
distance*June	0	0	.	.
distance*July	0.0013	0.0013	1.02	0.3084
distance*Aug	-0.0149	0.0197	-0.76	0.4473
C. Prophetstown				
intercept	1.8445	1.1599	1.59	0.1118
distance	0.0441	0.0087	5.07	**<0.0001**
month (June)	0	0	.	.
month (July)	2.3034	1.59	1.45	0.1474
month (Aug)	1.0881	1.6329	0.67	0.5052
distance*June	0	0	.	.
distance*July	-0.0735	0.0154	-4.79	**<0.0001**
distance*Aug	-0.1072	0.0214	-5.01	**<0.0001**

1.7 Supplemental Information

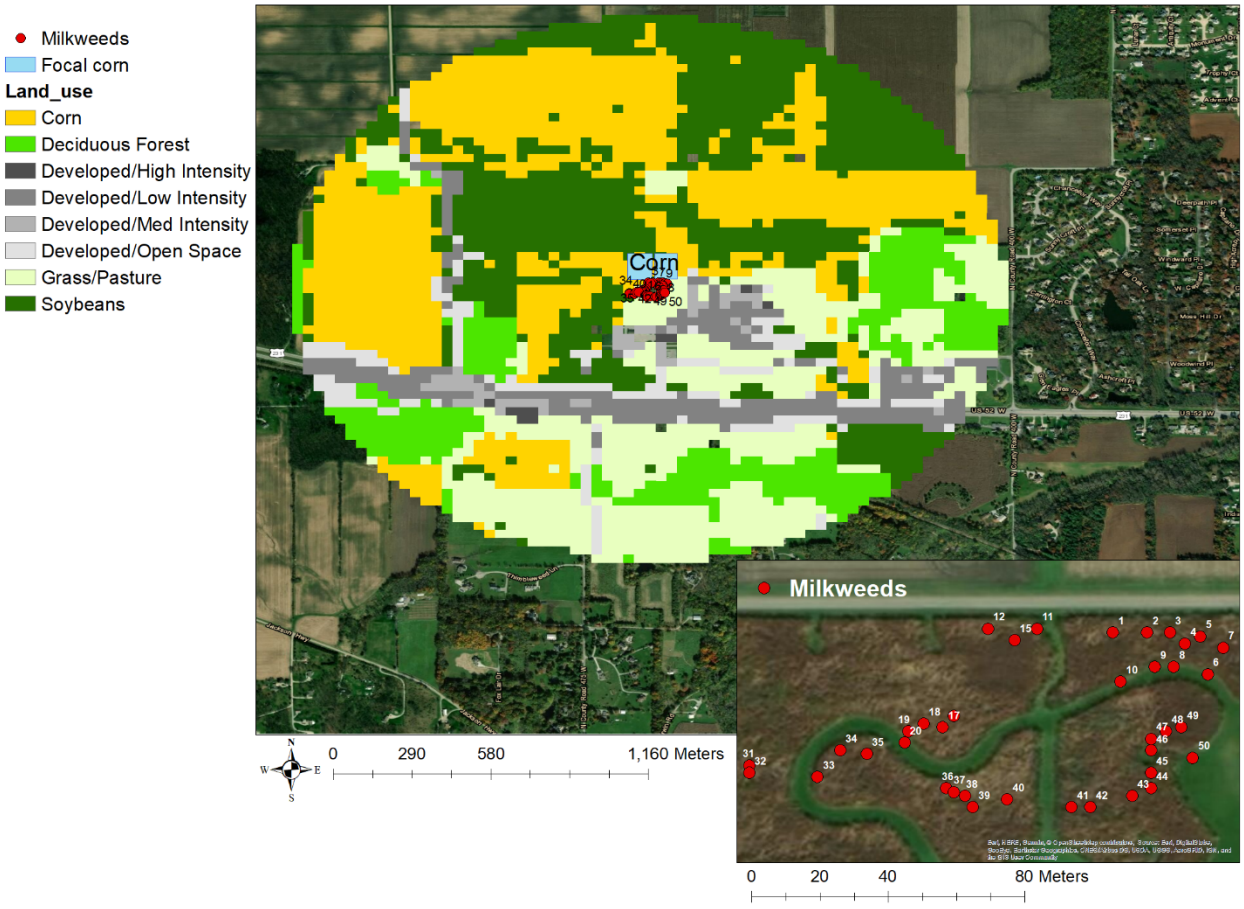

Figure S1. Distribution of *A. syriaca* relative to the focal corn field and local land use in 1 km radius around ACRE prairie.

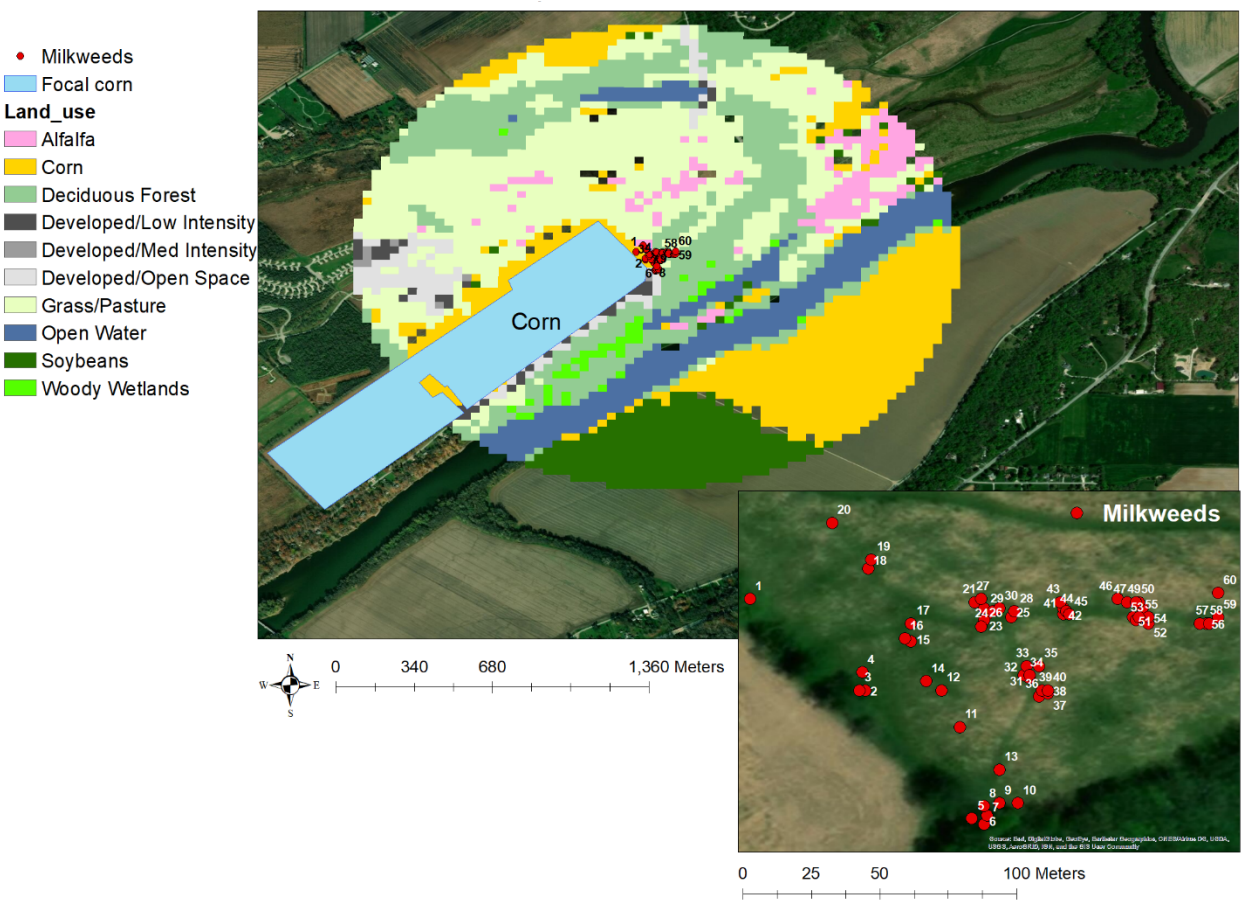

Figure S2. Distribution of *A. syriaca* relative to the focal corn field and local land use in 1 km radius around plants at Prophetstown State Park.

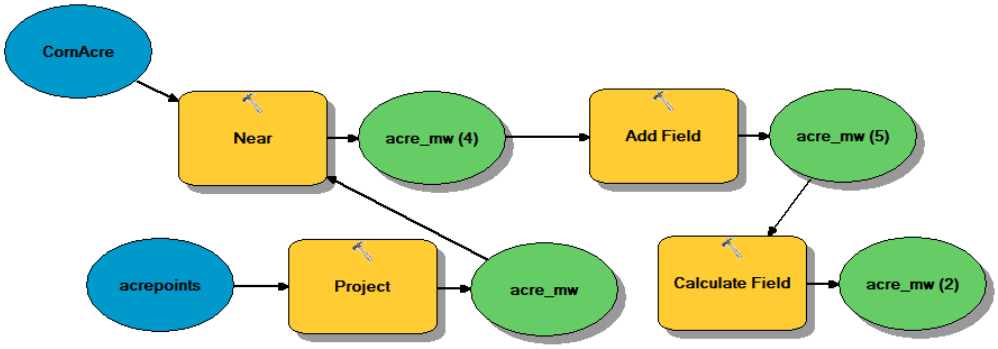

Figure S3. Model to calculate the linear distance between a corn field and milkweeds. Acrepoints represent the plants, CornAcre is the corn at which distances were calculated and acre_mw is the layer with a table with the calculated distances.

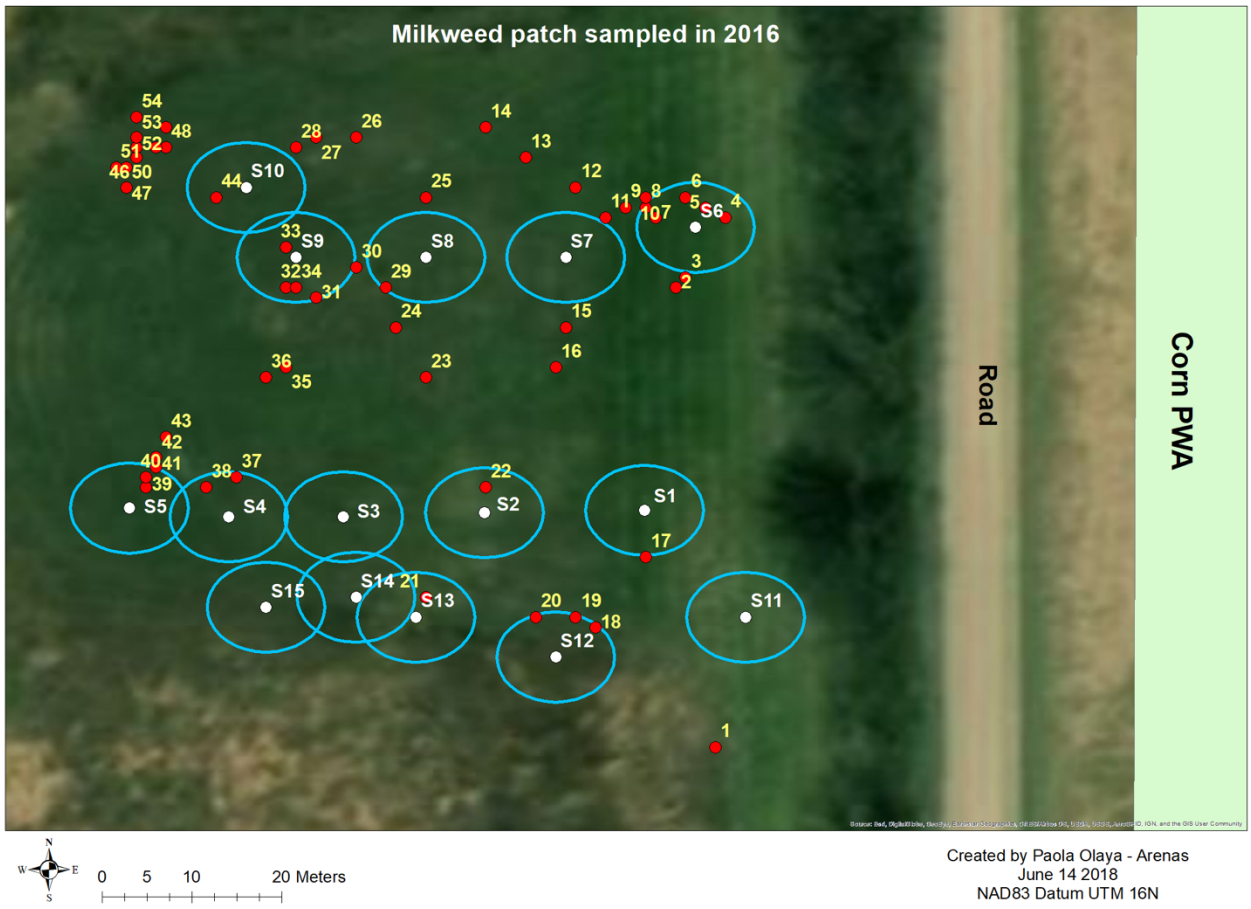

Figure S4. Milkweed plants (red dots) inside the buffers (blue circles) around soil sample points (white dots) to look for correlations between pesticides in soil and leaf tissue.

Table S1. Type of soil at milkweed sites sampled in 2016.

Site	Type of soil
Acre	Chalmers silty clay loam
Kankakee close	Tedrow loamy fine sand
Kanakakee far	Zaborosky fine sand
Prophetstown	Billet loam, gravelly substratum
Pwa	Starks-Fincastle

Table S2. Pesticides (kg) applied in corn and soybean in Tippecanoe and Newton counties.

Year	Pesticide type	Compound	Tippecanoe		Newton	
			Corn	Soybean	Corn	Soybean
2015	Insecticides	Deltamethrin	0.8	0.4	1.0	0.3
		Thiamethoxam	0.0	0.2	0.0	0.1
		Clothianidin	0.0	0.0	0.0	0.0
	Herbicides	Atrazine	38847.4	0.0	46536.0	0.0
		Acetochlor	23475.4	1146.8	28121.6	899.2
		S-metolachlor	14421.8	3465.6	17276.1	2717.5
	Fungicides	Pyraclostrobin	535.7	115.7	641.7	90.7
		Azoxystrobin	238.2	103.3	285.4	81.0
		Propiconazole	237.4	29.6	284.3	23.2
		Trifloxystrobin	218.0	54.6	261.2	42.8
		Difenoconazole	0.0	35.5	0.0	27.8
2016	Insecticides	Deltamethrin	0.0	*	0.0	8.0
		Thiamethoxam	0.0	*	0.0	0.0
		Clothianidin	0.0	*	0.0	0.0
	Herbicides	Atrazine	37454.5	*	42860.3	0.0
		Acetochlor	16244.1	*	18588.7	909.3
		S-metolachlor	15515.8	*	17755.2	3315.9
	Fungicides	Pyraclostrobin	280.7	*	321.2	218.7
		Azoxystrobin	102.3	*	117.1	126.4
		Propiconazole	121.2	*	138.7	52.4
		Trifloxystrobin	142.3	*	162.8	85.9
		Difenoconazole	0.0	*	0.0	17.1

***** *Data unavailable*

Table S3. Pesticides, and their active ingredients, applied to the crops immediately adjacent to milkweed sampling sites.

Sites	Trade name 2015	Active ingredients 2015	Trade name 2016	Active ingredients 2016
TPAC	Makaze	glyphosate	Site not sampled in 2016	Site not sampled in 2016
	Lexar	mesotrione, s-metolachlor, atrazine		
	Liberty	glufosinate		
Meigs	Bravo Weatherstik	chlorothalonil	Site not sampled in 2016	Site not sampled in 2016
	Previcur Flex	propamocarb, hydrochloride		
	Assail	acetamiprid		
	Asana	esfenvalerate		
	Champ Formula	copper hydroxide		
ACRE	Bicep II Magnum	atrazine, s-metolachlor	Bicep II Magnum	atrazine + s-metolachlor
	Round up	glyphosate	Round up	glyphosate
		2,4-D		2,4-D
	Liberty	glufosinate	Fusilade	fluazifop-p-Butyl
	Callisto	mesotrione	InterLock	adjuvant
	Princep	simazine		
	InterLock	adjuvant		
PWA	Round up	glyphosate	Round up	glyphosate
	Kamba master	2,4-D	Banvel	dicamba
Kankakee	Round-up	glyphosate	Touchdown	glyphosate
	Kamba Master	2,4-D	Salvan	2,4-D
	Ledger	S-metolachlor, Metribuzin	Ledger	s-metolachlor, Metribuzin
	Flexstar	fomesafen	Flextar	fomesafen
	Zidua	pyroxasulfone	Volunteer	clethodim
	Fusion	fluazifop-p-Butyl, Fenoxyprop	Zidua	pyroxasulfone
Prophetstown		Site not sampled in 2015	Data unavailable	

Table S4. Logistic regression mixed model results for differences in pesticide frequency between years and among months within the years.

Pesticide type	Active ingredient	year	Month	Estimate	Std. Error	Z	p value	
Insecticides	**Clothianidin**	**2015-2016 (a)**		0.94	0.29	3.22	0.0012	**
		2016	Jun-Jul			4.82	p<0.001	***
	Thiamethoxam	**2015-2016**		8.08	0.91	8.80	p<0.001	***
		2015	Jun-Jul	0.16	0.55	0.30	0.945	ns
		2016	Jun-Jul	0.42	0.26	1.58	0.25	ns
			Jun-Aug	2.64	0.26	10.10	p<0.001	***
			Jul-Aug	2.21	0.23	9.26	p<0.001	***
	Deltamethrin	**2016**		absent in 2015				
			Jun-Jul	24.91	341.34	0.07	1.00	ns
			Jun-Aug	26.98	341.33	0.08	1.00	ns
			Jul-Aug	2.07	1.09	1.90	0.11	ns
Herbicides	**Acetochlor**	**2015-2016**		-1.68	0.29	-5.80	p<0.001	***
		2015	Jul-Aug	1.59	0.29	5.56	p<0.001	***
		2016	Jun-Jul	0.71	0.62	1.15	0.48	ns
			Jun-Aug	1.00	0.68	1.47	0.30	ns
			Jul-Aug	0.29	0.77	0.38	0.92	ns
	Atrazine	**2015-2016**		0.50	0.24	2.11	0.0349	*
		2015	Jun-Jul	1.37	0.21	6.40	p<0.001	***
			Jun-Aug	2.75	0.32	8.60	p<0.001	***
			Jul-Aug	1.38	0.33	4.15	p<0.001	***
		2016	Jun-Jul	-0.70	1.23	-0.57	0.99111	ns
			Jun-Aug	4.74	0.74	6.41	p<0.001	***
			Jul-Aug	5.44	1.02	5.32	p<0.001	***
	S-metolachlor	**2015-2016**		-1.47	0.34	-10.64	p<0.001	***
		2015	Jun-Jul	1.57	0.26	6.09	p<0.001	***
			Jun-Aug	3.97	0.27	14.47	p<0.001	***
			Jul-Aug	2.39	0.21	11.54	p<0.001	***
		2016	Jun-Jul	2.62	0.28	9.28	p<0.001	***
			Jun-Aug	3.96	0.36	11.05	p<0.001	***
			Jul-Aug	1.34	0.35	3.88	0.00139	**
Fungicides	**Propiconazole**	**2015-2016**		6.13	0.44	13.90	p<0.001	***
		2015	Jul-Aug	-0.31	0.17	-1.76	0.0789	.
		2016	Jun-Jul	0.00	1.02	0.00	1	ns
			Jun-Aug	1.55	0.82	1.88	0.143	ns
			Jul-Aug	1.55	0.82	1.88	0.143	ns
	Pyraclostrobin	**2015-2016**		-1.11	0.16	-7.12	p<0.001	***
		2015	Jun-Jul	-3.03	0.23	-13.12	p<0.001	***
			Jun-Aug	-2.25	0.22	-10.47	p<0.001	***
			Jul-Aug	0.78	0.20	3.99	p<0.001	***
		2016	Jul-Aug	-3.81	0.31	-12.17	p<0.001	***
	Trifloxystrobin	**2015-2016**		-0.37	0.17	-2.12	0.033	*
		2015	Jul-Aug	-0.41	0.17	-2.38	0.0175	*
		2016	Jun-Jul	0.00	1.42	0.00	1	ns
			Jun-Aug	-8.31	1.11	-7.48	p<0.001	***
			Jul-Aug	-8.31	1.11	-7.48	p<0.001	***

**** p<0.001, ** p<0.01, * p<0.05, '.' P< 0.1, ns= not significant*

Table S5. Relationship between percent of corn and soybean planted in 1 km radius around field sites and percent of milkweed plants with pesticide residues.

Year	pesticide type	Active ingredient	Multiple R^2	Adjusted R^2	F(1,4)	p-value	significance
2015	Insecticides	Clothianidin	0.38	0.23	2.46	0.19	ns
		Thiamethoxam	0.47	0.34	3.54	0.13	ns
	Herbicides	Acetochlor	0.15	-0.06	0.73	0.44	ns
		Atrazine	0.34	0.18	2.09	0.22	ns
		Metolachlor	0.00	-0.25	0.01	0.94	ns
	Fungicides	Propiconazole	0.16	-0.05	0.77	0.43	ns
		Pyraclostrobin	0.68	0.60	8.61	0.04	*
		Trifloxystrobin	0.02	-0.23	0.08	0.79	ns
2016	Insecticides	Clothianidin	0.31	0.08	1.35	0.33	ns
		Deltametrhin	0.27	0.08	-0.22	0.64	ns
		Thiamethoxam	0.45	0.26	2.44	0.22	ns
	Herbicides	Acetochlor	0.35	0.14	1.65	0.29	ns
		Atrazine	0.50	0.33	2.95	0.18	ns
		Metolachlor	0.05	-0.26	0.17	0.71	ns
	Fungicides	Propiconazole	0.32	0.09	1.40	0.32	ns
		Pyraclostrobin	0.10	-0.21	0.31	0.61	ns
		Trifloxystrobin	0.05	-0.26	0.16	0.71	ns

** $p<0.05$, ns= not significant*

CHAPTER 2. DO POLLINATORS PREFER PESTICIDE-FREE PLANTS? AN EXPERIMENTAL TEST WITH MONARCHS AND MILKWEEDS

2.1 Introduction

Determining whether, and to what degree, pesticides impact pollinator health has become the focus of hundreds of studies over the past decade. However, the method of exposure to pesticides in such experiments strongly affects the interpretation of those data. Many studies, for example, employ no-choice assays that force individuals to develop at a fixed pesticide concentration, quantifying the lethal and sublethal effects of such exposure compared to a pesticide-free control. A benefit of this approach is that it allows for a relatively straightforward measure of individual and population-level impacts. The drawback is that under natural conditions, pollinators encounter highly variable pesticide loads over their lifetime and may simply choose to avoid foraging on pesticide-contaminated plants if given the option. Even within a limited foraging radius (i.e., several km), proximity to cropland will likely create a complex mosaic of floral resources that range from trace pesticide amounts to those exceeding the species' LD50 (Krupke et al., 2017; Tsvetkov et al., 2017; Olaya-Arenas & Kaplan, 2019).

An alternative approach is to conduct a more realistic choice study whereby pollinators are simultaneously presented with pesticide-treated vs. untreated plants. In this case, it would be expected that individuals preferentially forage on pesticide-free plants (i.e., avoiding poisonous food should be highly adaptive). Indeed, this expectation underlies the rationale, argued by some, that the detrimental effects of pesticides on bees are overestimated. The few controlled choice studies to date, however, report counterintuitive findings; namely, that honeybees and bumblebees *prefer* nectar containing neonicotinoid insecticides, as well as some herbicides and fungicides (Kessler et al., 2015; Liao et al., 2017). Yet, field experiments still report observations of pesticides deterring pollinators. For instance, fungicide application during cranberry bloom dramatically increased honeybee foraging on non-crop pollens (Jaffe, Lois & Guedot, 2018). Disentangling preference in social bees is further complicated by behavioral regulation and feedbacks that occur at the colony-level (Dolezal et al., 2015).

Unlike bees, pesticide-mediated food plant preferences are poorly studied in other pollinator groups such as butterflies, even though evidence is accumulating for the role of pesticides in butterfly declines (Gilburn et al., 2015; Muratet & Fontaine, 2015; Forister et al., 2016). This is particularly true for threatened or endangered species on wild host plants, since pesticide responsiveness is widely reported in crops for the many Lepidoptera that act as agricultural pests. Aside from sociality, butterflies ecologically differ from bees in several ways that could affect their behavioral response to pesticides. For one, choice can occur at two levels; initially, oviposition preference by gravid adult females (i.e., between plants), followed by larval feeding choices within and across leaves, which typically occurs on an individual plant. Either stage could result in behaviors leading to aversion or attraction. In monarch butterflies (*Danaus plexippus*), for example, preference for cardenolides—milkweed toxins that cure individuals from parasite infection—only occurs during oviposition (Lefèvre et al., 2012). Caterpillars do not display an analogous feeding preference, despite the medicinal benefits of consuming cardenolide-rich leaf tissue.

Monarchs are in midst of a long-term population decline and pesticides are one of several factors that are thought to be contributing to this decline (Inamine et al., 2016; Thogmartin et al., 2017; Agrawal & Inamine 2018; Malcolm, 2018). Milkweeds commonly grow on crop field margins, potentially exposing them to a range of agrochemicals such as neonicotinoid insecticides used in corn and soybean production (Pecenka & Lundgren, 2015; Olaya-Arenas & Kaplan, 2019).
Although numerous factors impact monarch oviposition, responses to pesticides are almost entirely unknown. In one of the only studies to date, oviposition by monarch females was unaffected by the pyrethroid insecticide, permethrin, used for mosquito control when sprayed on milkweeds, even though larval survival was reduced on permethrin-treated plants (Oberhauser et al., 2006).

In this study, we quantified the oviposition and feeding behaviors of monarch butterflies and caterpillars to variation in leaf pesticide residues on their primary host-plant in North America, the common milkweed *Asclepias syriaca*. To do so, we simulated field realistic pesticide residues using data from a two-year field survey that measured the presence and concentration of a diversity of pesticides on leaves of milkweeds bordering cropland (Olaya-Arenas & Kaplan, 2019). Our

central objective was to determine whether monarchs avoid pesticides on milkweed host-plants when given an option. Secondarily, we aimed to isolate the life stage (i.e., adult oviposition vs. larval foraging) during which preference occurs.

2.2 Methods

2.2.1 Plants and Insects

Milkweed used for all experiments were germinated from seed and seedling stems (Prairie Moon Nursery, Winona, MN) in a climate-controlled greenhouse (27-30ºC) on the Purdue University campus. Plants were cultivated in 366 ml pots using SunGro professional growing mix with two teaspoons of time-released NPK fertilizer (Scotts Osmocote Classic®). Monarch adults used in experiments were the offspring from a lab colony in Emory University provided by Jacobus De Roode and larvae were a combination of wild-captured on potted milkweeds in West Lafayette, Indiana and offspring of adults from the colony.

2.2.2 Adult Oviposition Preference

To evaluate the effects of pesticides (1 insecticide, 2 herbicides, 3 fungicides) on adult oviposition, we provided caged, mated females with the choice of plants randomly assigned to one of the following four treatments:

i) Pesticide-free control. Did not receive any experimental manipulation.

ii) Pesticide solvent and surfactant control. Plants were sprayed from a stock solution containing water (465.3ml), acetone (29.7ml) and tween (0.5ml). Acetone was used in pesticide dilutions. Tween is a surfactant that helps pesticides to be absorbed by plants; these are commonly added to pesticide mixtures for field application. This second or 'true' control was used to evaluate whether the non-pesticide components of the pesticide treatments contributed to observed effects on monarch behavior.

iii) Mean pesticide treatment. Plants were treated from a 480 ml solution containing six of the most commonly encountered pesticides on milkweeds in the field, combined into a single

treatment (Table 2.1). The target concentrations applied to the plants are based on field data collected in Olaya-Arenas & Kaplan (2019). We used a pesticide cocktail rather than individual compounds (unlike the larval trials described below) because it represents the actual blend that monarchs encounter in the field. For this treatment, we used the mean concentrations recorded for plants on which we detected each of the targeted pesticides.

iv) Max pesticide treatment. Same as treatment 3 above, except here we used the maximum concentrations observed for pesticides detected in the field (see Table 2.2 for targeted mean and max values). This was used as a 'worst-case scenario' for monarchs, while remaining within biologically plausible levels.

High-purity technical grade pesticides—clothianidin (99.0%), atrazine (98.1%), s-metolachlor (97.6%), azoxystrobin (99.5%), pyraclostrobin (99.9%), trifloxystrobin (99.4%)—were ordered from Sigma-Aldrich or Chem Service, Inc. Compounds were weighed, and an initial stock was prepared in 1 ml of acetone. From the stock, pesticides were individually diluted in acetone and water to mimic concentrations in the field. Each working dilution contained: V/V 93.9% water, 5.99% pesticide plus acetone, and 0.10% tween. To create the mix for treating plants, all six individual pesticides were mixed in a new 480 ml stock solution. Pesticide treatments were prepared the same day of application and kept in the refrigerator at 4ºC before use.

Potted milkweed plants of the same age and approximate size (mean height: 33.2 cm; weight range: 46-50 g) were sprayed with ca. 23 ml volume of solution on the abaxial and adaxial leaf surfaces to ensure complete coverage. Each plant was treated with the prepared solution using a hand sprayer (EquateTM spray bottle, 236 ml). Plants were sprayed within a cardboard box outside and left to dry overnight in the greenhouse. The next day, we combined four plants (i.e., one plant from each of the four treatments) in a rectangular mesh cage (314 cm3), resulting in 26 total cage replicates. The spatial positioning of treatments was randomly assigned within each cage, with plants placed in each corner to avoid contact between them. Two newly emerged monarch butterflies—one female and one male—were placed in cages. Males were included to ensure successful mating and oviposition behavior. We counted the number of eggs on each plant and recorded monarch survival on days two, four and seven after the pesticide was sprayed. Dead

individuals were replaced if noted during the experiment. Petri dishes containing sugar water (Gatorade), water, and fruit (slices of banana and tangerine) were provided as food. Cages were housed in a climate-controlled greenhouse for the duration of the experiment.

To corroborate that the pesticides added were in fact measurable on the pesticide-treated plants, we treated extra plants not used in the experiment for quantifying pesticide residues in response to spraying (n=3 mean; n=3 max). Because pesticide concentrations can decrease over time due to UV exposure or biotransformation, we collected two leaves from each plant at two, four and seven days after pesticide application, mirroring the timescale over which females were exposed in the oviposition trial. Leaves were stored at -80ºC and 1 g of each sample was analyzed for the six pesticides using liquid chromatography-mass spectrometry at the Metabolite Profiling Facility in the Bindley Bioscience Center at Purdue University (for methodological details see Olaya-Arenas & Kaplan, 2019).

2.2.3 Larval Feeding Preference

To test the effects of pesticides on larval feeding behavior, we measured the foraging preferences of early instar monarch caterpillars comparing pesticide-free vs. pesticide-treated milkweed leaves using a paired leaf disc assay enclosed in a series of Petri dishes. To do so, we paired untreated with pesticide-treated milkweed discs for each of the six pesticides tested in the above oviposition trial, except here we assessed each pesticide individually, rather than in combination. This was done each for the mean and maximum concentrations recorded, resulting in 12 comparisons (i.e., 6 pesticides x 2 concentrations). Using this set-up, we performed two separate trials. One trial used an untreated leaf disc as the control and 1st instars, while the second trial used acetone as the control, since pesticides were diluted in acetone, and 2nd instars. Both trials included 30 experimental replicates of each treatment combination except for mean concentrations in trial 1, which used only 25 replicates. Pesticides and concentrations are listed in Table 2.2. In trial 1, we did not include a mixed pesticide treatment, whereas we did so in trial 2.

Leaf cores (20 mm diameter) were taken from the leaves of potted milkweed plants using a cork borer. We avoided major leaf veins to provide a standardized amount of leaf tissue and avoid variation in leaf thickness. Cores were randomly assigned to treatments and treated with 20 □l of

each individual pesticide using an Eppendorf repeater pipette. Pesticides were applied to the underside of the leaf, which had fewer trichomes, resulting in less dispersion away from the disc surface. To standardize pesticide concentrations per unit leaf, we first calculated the weight of an average milkweed leaf disc (n=18 discs; 65.69 mg/disc) and adjusted accordingly through a process of serial dilutions of pure compounds in acetone as a solvent. Discs were treated inside of a fume hood and allowed to dry for 30 minutes, before moving them to Petri dishes.

Two discs—treated vs. control—were placed at opposite ends of a 9-cm diameter Petri dish on moistened filter paper. A single 1st (trial 1) or 2nd instar (trial 2) monarch larva was then placed at the center of the dish, equidistant between the two discs. Petri dishes were sealed with parafilm to avoid leaf desiccation and remained in the lab for 24 hours, after which we measured larval survival and the amount of leaf tissue consumed from each disc. As a measure of preference, we quantified monarch herbivory by counting the number of holes removed from each disc using a transparent grid placed over the top of leaves. These values were later confirmed by taking digital images and using ImageJ software to corroborate the area eaten from the two discs.

2.2.4 Statistical Analysis

Adult oviposition choice test: We used a generalized mixed model with a Poisson distribution using cage as a random effect to test the impact of pesticide treatment on the numbers of eggs per plant. Dunnett's test was then used to compare total eggs oviposited over plants treated with the solvent/surfactant (Acetone/Tween) solution, unsprayed (control), and plants treated with pesticides at their mean and maximum concentrations. Cages with no eggs (n=17) were removed from the analysis because females were either unmated or did not make a choice. Number of leaves per plant was included as a factor to control for plant size differences in oviposition, in the case that females choose to place more eggs on larger plants.

Larval feeding choice test: We used single linear models per treatment to test the effect of pesticides on monarch larval feeding preferences. The two trials were analyzed independently since they used different controls (untreated vs. acetone) and caterpillars varied in starting age (1st vs. 2nd instar). We tested the effect of position (right or left) of the control discs in the second trial. Petri dishes where larvae did not feed on either leaf disc were removed from the analysis.

R software 3.5.1 (R team core 2013) and the packages Car, lme4, multcomp, ggplot2 and Matrix were used for statistical analysis.

2.3 Results

2.3.1 Verification of Experimental Pesticide Treatments

Data from our LC-MS analysis showed variation in the efficacy of treatments designed to mirror those values recorded from the field. Specifically, whole-plant applications used for oviposition trials tended to have lower pesticide residues across both mean and maximum concentration treatments, compared with targeted applications on leaf discs using a pipette for larval choice (compare Tables 2.1 and 2.2). Whole-plant spray treatments were a fraction of their target values in most cases; for example, the mean targeted concentration for the fungicide pyraclostrobin (8.51 ng/g) was approximately 2-4 times higher than those recorded from sprayed plants (2.1 – 4.4 ng/g). However, concentrations in most cases remained relatively stable over the course of the experiment, with few showing evidence of substantial (>50%) degradation between days 2 and 7.

In all cases the maximum concentrations were far higher than mean concentrations, as intended. Unlike whole-plant assays, leaf disc treatments much more closely mimicked field-relevant concentrations, in many cases with the applied amounts showing <5% difference compared with the targeted field value (Table 2.2). For instance, we aimed for 15.28 and 56.55 ng/g clothianidin in the mean and maximum treatments; actual measured values from leaf discs simulating these values were 14.36 and 54.47 ng/g, respectively. Thus, leaf discs for larval choice closely matched the intended field dose.

2.3.2 Adult Oviposition Preference

There were no differences in the number of eggs on plants comparing those sprayed with the acetone/tween/water solution vs. the unsprayed control. Similarly, plants treated with the mean concentration of pesticides did not differ from the control, but we found fewer eggs laid on milkweeds treated with pesticides at their maximum concentration (Fig. 2.1, Table 2.3). Additionally, oviposition increased in relation to leaf number, especially on plants with 20-25 leaves (Fig. 2.2; $F_{(1,34)} = 5.57$, $p = 0.024$, $R^2 = 0.14$). However, the number of leaves did not vary

among treatments and thus this oviposition preference did not affect the experimental design ($F_{(3,32)}$ = 0.193, p=0.90; average number of leaves per treatment: control = 20.2, A/T = 20.5, mix max = 20.8, mix mean = 21.5).

2.3.3 Larval Feeding Preference

In the first trial using 1st instar larvae and untreated leaf disc controls, mean concentrations of clothianidin, s-metolachlor, azoxystrobin and trifloxystrobin significantly influenced caterpillar feeding preference; namely, there was reduced feeding on pesticide-treated leaf discs compared to untreated controls (Fig. 2.3 A, Table 2.4 A). Importantly, the untreated control was no different from the acetone control in this trial. When testing higher concentrations, we found even stronger negative effects of pesticides on larval feeding behavior for 4 of the 6 pesticides tested; however, control treatments with acetone alone also had a deterrent effect on feeding behavior (Fig. 2.3 B, Table 2.4 B).

In the second trial using 2nd instars, we similarly found deterrent effects of acetone treatments on larval feeding behavior at both mean (Fig. 2.4 A, Table 2.5 A) and maximum (Fig. 2.4 B, Table 2.5 B) concentrations. Because acetone was used as the control in this trial, virtually none of the pesticide treatments had an impact on foraging behavior, except a moderately stimulatory effect of the fungicide pyraclostrobin on feeding. All larvae survived in both trials under the two concentration levels tested.

2.4 Discussion

The experimental methodology employed here was largely successful in simulating pesticide residues occurring on field-grown milkweed plants. However, we noted important differences in adult and larval choice assays comparing sprayed plants vs. leaf discs, respectively. These differences can likely be explained as follows. First, the method of pesticide application was vastly different. In the larval experiment, leaf discs were precisely treated with a 20 µl droplet of pesticide solution at almost contact distance, whereas plants for the oviposition experiment were sprayed outside. Although we attempted to evenly coat the full plant across all leaves, this methodology is surely less accurate than pipetting onto small leaf discs where full coverage is ensured. We also

suspect there was biotransformation in the oviposition experiment due to processes such as plant and microorganism metabolism, and abiotic factors such as pesticide volatilization, or UV exposure and higher temperatures in the greenhouse compared to the lab. Nevertheless, pesticide values were relatively stable over the experiment and thus butterflies were exposed to approximately the same concentrations throughout, even if they were somewhat lower than field values, resulting in a more conservative evaluation for butterfly oviposition. The fact that maximum concentrations always produced substantially higher leaf values than mean concentrations, as intended, confirm the validity of this approach.

2.4.1 Adult oviposition preference

Contact with the pesticide-contaminated leaf surface could be an explanation for some of the mortality observed in the oviposition trial. Females and males touch the surfaces of milkweeds with their antennae, legs and abdomen to identify secondary metabolites (e.g., flavonoids) that stimulate oviposition (Haribal & Renwick, 1998). The death rate of females (65%) and males (54%) in cages with milkweeds treated with the pesticide cocktail is consistent with the findings in Oberhauser et al. (2006), in which 80-90% of females died in cages testing oviposition on milkweeds treated with permethrin. However, it is also possible that adults naturally died due to high temperature, age, pathogens, or related factors; the experiment was not designed to assess pesticide-induced mortality for adults.

Plants treated with pesticides at their maximum concentrations had a clearly deterrent effect on oviposition. The number of eggs placed on plants treated with a mix of pesticides at their maximum concentrations was 29.4% lower than plants receiving the solvent control. Oviposition deterrence behavior has been similarly studied in the lepidopteran pest *Plutella xylostella* when exposed to the insecticides gamma-cyhalothrin and spinetoram (Nansen, Baissac, Nansen, Powis & Baker, 2016) and extracts from different parts of the plant *Strychnos nux-vomica* (Loganiaceae) (Selvaraj, Kennedy & Suganthy, 2017). Chemosensory structures located in the tarsi and antennae of butterflies vary in their susceptibility to different concentrations of natural compounds used for host recognition, but monophagous species feeding exclusively on one plant family often detect stimulants or deterrent phytochemicals with great precision (Nishida, Ohsugi, & Fukami, 1990; Honda, 1995). Therefore, it is not surprising that monarch females, which are specialists on

milkweeds, detected the synthetic pesticides applied on milkweeds and adjusted oviposition behavior accordingly. However, the concern is that adults do not detect pesticides occurring at more typical, mean concentrations, and thus neonates will ultimately be exposed to these levels in the field.

2.4.2 Larval Feeding Preference

In general, young instars are more susceptible to deterrent/toxic compounds than later instars (Ahmad & Forgash, 1975; Parrot, Jenkins & McCarty, 1983; Hedin, Parrott & Jenkins, 1991). The increase in tolerance with age has been associated with higher levels of detoxification enzymes (e.g., microsomal oxidases, glutathione s-transferase and esterase) in older instars in Lepidoptera (Yu, 1983) and honey bees (Gilbert & Wilkinson, 1974). The increases in detoxification enzymes has also been related to larval size (Ahmad & Forgash, 1975), which changes through instars and since larger instars eat more this can account for less discrimination of what they are eating (Moreau & Bauce, 2003).

In our study, second instar monarch larvae were deterred by acetone, but pesticides did not affect their feeding preference. Unlike with second instars, four pesticides at mean concentrations (Fig. 2.3 A) and five pesticides at maximum concentrations (Fig. 2.3 B) had a deterrent effect on first instar larvae. Clothianidin and s-metolachlor acted as antifeedants under both concentrations, while azoxystrobin and trifloxystrobin only at mean concentrations, and pyraclostrobin and atrazine only at maximum concentrations. In three of the four larval groups tested, acetone acted as a feeding deterrent. This outcome is interesting because experiments testing effects of pesticides on bees (Taylor et al., 1987) and other Lepidoptera (Gist & Pless 1985; Bhattacharyya et al., 1995) using acetone as a control have not shown any negative effects. We used acetone because it dissolves all test materials and evaporates quickly, but the residues on (and within) the leaves can deter monarch feeding under certain conditions. One explanation is that acetone removes amino acids or secondary chemicals from the leaf surface that are important for monarch larvae; for example, flavonoids for which acetone is an excellent extraction solvent (Munhoz et al., 2014).

Clothianidin is a systemic insecticide that has negative effects on monarch larvae (Pecenka & Lundgren, 2015) and many other insects (Desneux, Decourtye & Delpuech, 2007). First instar

monarch larvae were highly susceptible to the mean concentration of clothianidin applied to leaf discs, as well as the maximum concentration of this insecticide. The maximum concentration of clothianidin used in this study exceeds the lethal concentration threshold when exposed during a 36-hour feeding trial (Pecenka & Lundgren, 2015). Sub-lethal effects of clothianidin on 1st instar larvae include stadium extension, lower weight and reduced body length. This last effect was also observed in 2nd instars (Pecenka & Lundgren, 2015). The lack of deterrence in 2nd instars could be related to instar-mediated variation in response to insecticides, which is consistent with other studies showing more susceptibility of early instars to clothianidin in *Agrotis ipsilon* (Hufnagel) (Lepidoptera: Noctuidae) (Ding, Zhao, Zhang, Xu & Mu, 2018) and to pyrethroids in *Pieris brassicae* (Linnaeus, 1758) (Lepidoptera: Pieridae) (Tan 1981). The susceptibility of young instars varies with both the insecticide and Lepidoptera species tested (Rodríguez-Saona et al., 2016).

2.5 Conclusions

Monarch females can prevent the exposure of their progeny to harmful xenobiotics by placing fewer eggs on plants contaminated with high concentrations of pesticides. Thus, variation in pesticide loads among milkweeds across the landscape places greater emphasis on correct oviposition decisions among females (assuming those pesticides impair larval development). We found evidence that monarch butterflies avoid pesticide-contaminated milkweeds, but there was only a moderately deterrent effect at the maximum concentrations recorded in our field survey. Based on this, we can conclude that pesticides can deter monarch adults, but only in the rare circumstances when concentrations are extremely high. In most instances, pesticide load will not affect monarch decision making, according to our oviposition experiment. This could be a concern because the mean concentration used for pesticides such as clothianidin (15.28 ng/g) is far beyond those levels resulting in negative developmental effects on monarch caterpillars (Pecenka & Lundgren, 2015). This implies that gravid monarch females are unable to differentiate among levels resulting in harm to their offspring.

As with adults, monarch larvae showed some evidence of deterrent effects of pesticides on foraging behavior, which could affect intra-plant distribution among leaves. Yet, the larval trials were somewhat inconclusive. Some of our data showed that early instars are deterred by a range of pesticides, with no corresponding effect of the acetone component of the mixture (Fig. 2.3 A).

This isolates the impact of the pesticide presence on monarch caterpillar behavior. However, later trials found deterrent effects of acetone itself, which confounds the interpretation of later instar foraging preferences (Fig. 2.4). Because 1st and 2nd instar experiments were conducted using different controls—untreated vs. acetone, respectively—it impossible to say conclusively whether the differences are due to developmental changes in larval preference. Documenting neonate survival and behavior on plants treated with pesticides in combination with differences in other natural attributes like trichomes, latex and cardenolides, will provide a better understanding of ecological pressures that pesticides place on this iconic butterfly. For example, monarch larvae grow slower on *A. syriaca* and *A. speciosa* than on *A. incarnata* and *A. faciculares* (Ladner & Altizer, 2005). It would be instructive for future studies to consider whether pesticide-mediated stress exacerbates the negative effects due to existing host-plant differences among milkweed species.

Authors' contributions

Paola Olaya-Arenas wrote the manuscript, collected and analyzed the data. Michael Scharf supervised the pesticide dilutions in the lab to match field concentrations and commented on the manuscript. Ian Kaplan conceived the ideas, designed the methods and reviewed the manuscript draft.

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Data accessibility

Data available from the Purdue University Research Repository (PURR), https://purr.purdue.edu/

2.6 References

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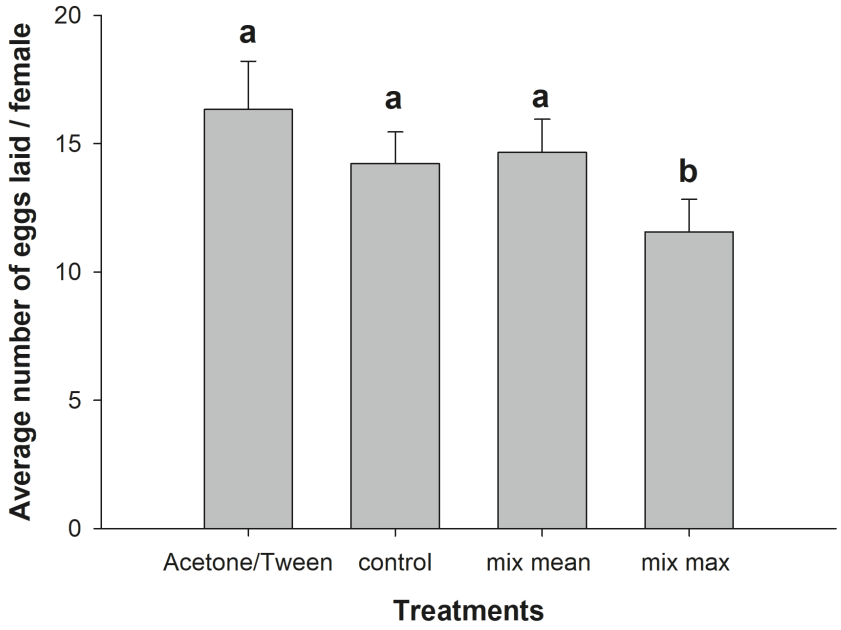

Figure 2.1. Mean (+SE) total eggs laid by monarch females per treatment. Control (unsprayed plants), mix mean (plants sprayed with a pesticide mix at mean concentrations) and mix max (plants sprayed with a pesticide mix at maximum concentrations). Mean and maximum concentrations refer to concentrations found in milkweed leaves in margins close to corn or soybean fields. Treatments with different letters indicate significant differences (P<0.05).

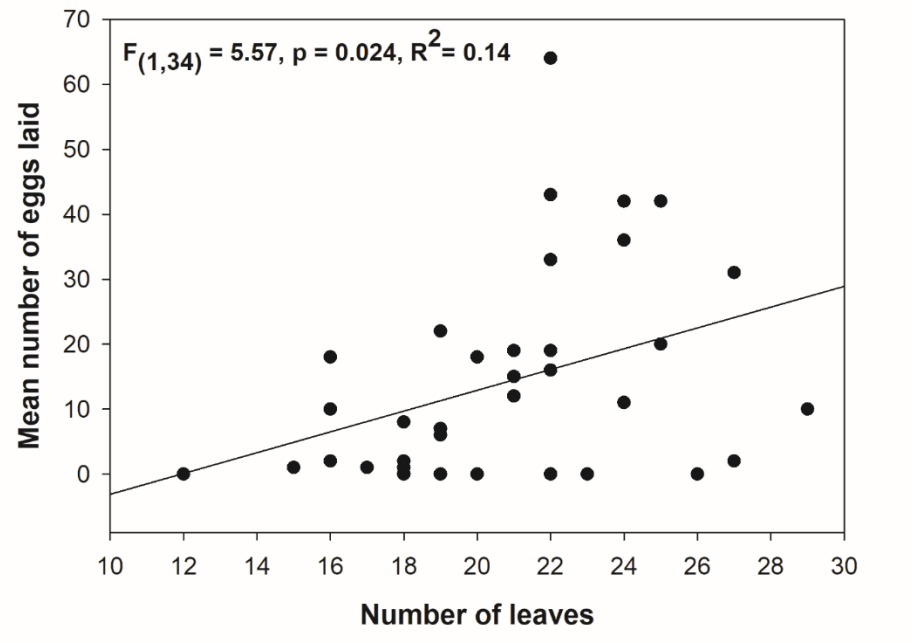

Figure 2.2. Association between the number of leaves per milkweed plant and the number of eggs laid by monarch females.

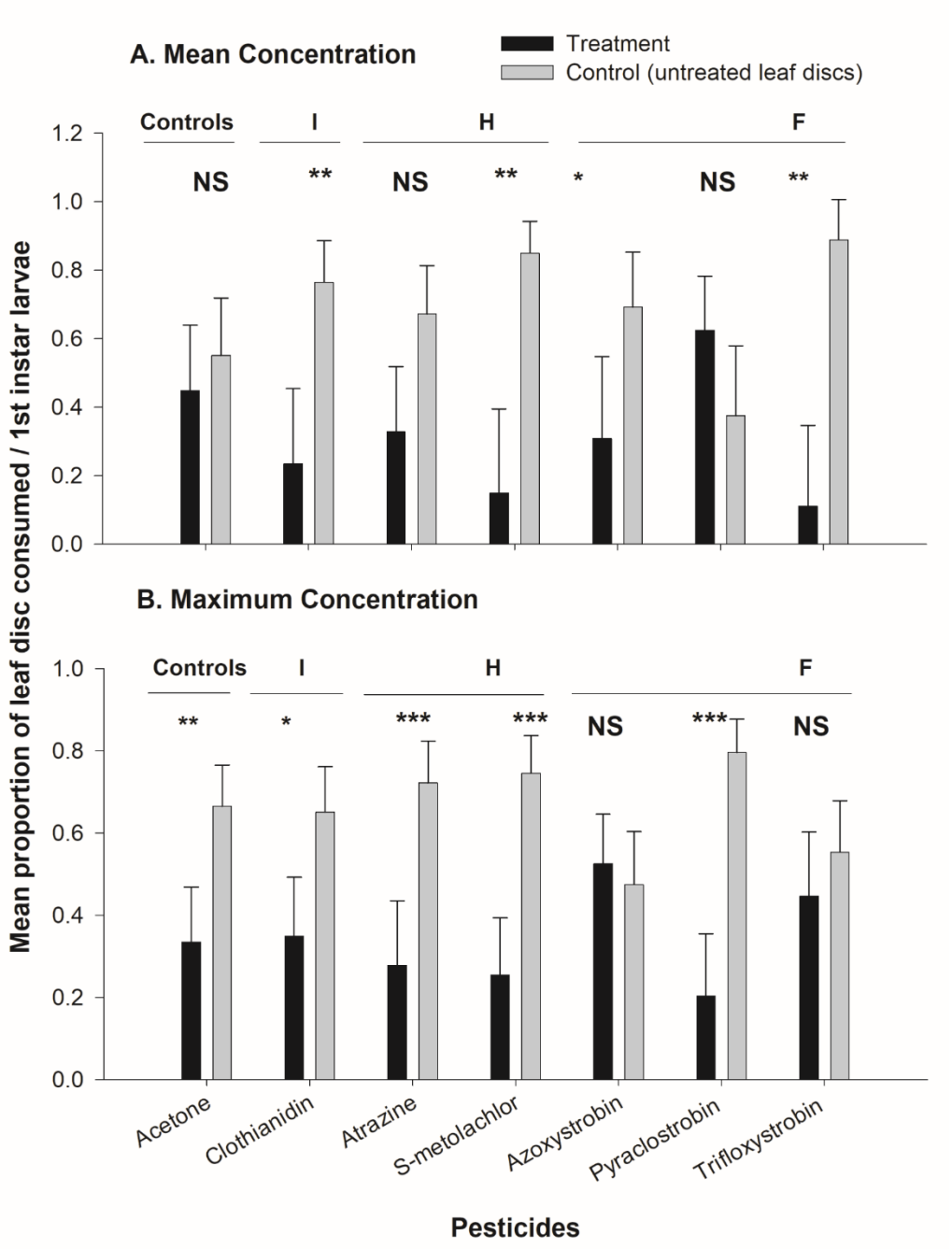

Figure 2.3. First instar larval feeding preferences (mean + SE) when exposed to the pesticides clothianidin (insecticide), atrazine and s-metolachlor (herbicides), and azoxystrobin, pyraclostrobin and trifloxystrobin (fungicides), at mean (A) and maximum (B) concentrations. The pesticide solvent (acetone) was also tested. NS = non-significant; * = significance at $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$. I = insecticides, H = herbicides, F = fungicides.

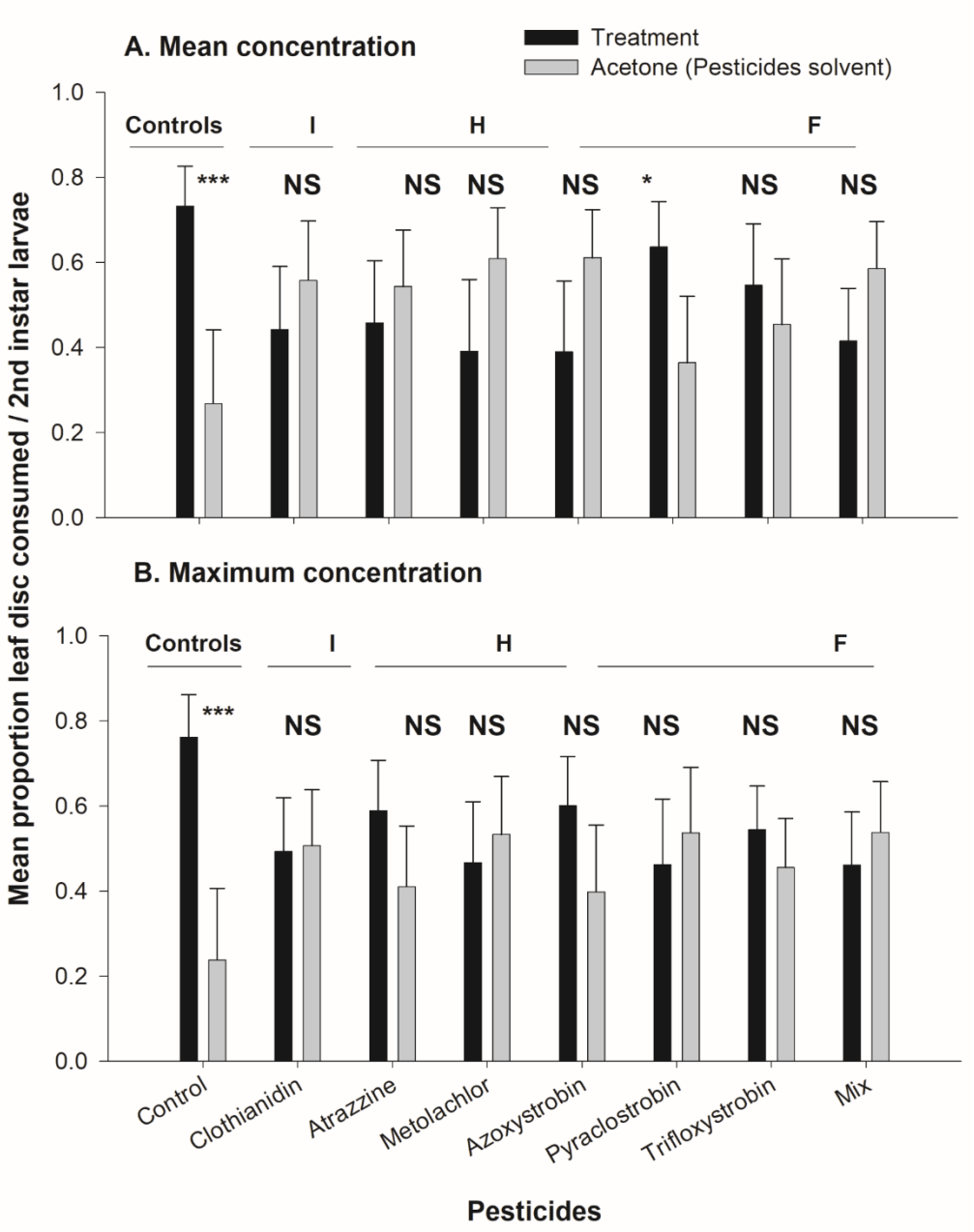

Figure 2.4. Second instar larval feeding preferences (mean + SE) when exposed to the pesticides clothianidin (insecticide), atrazine and s-metolachlor (herbicides), azoxystrobin, pyraclostrobin and trifloxystrobin (fungicides), or the mix of all of them (mix), at mean (A) and maximum (B) concentrations. Leaf discs treated with acetone were used as control and untreated leaf discs were the treatment. Significance at $p < 0.05$ (*), everything else was not significant (NS). I = insecticides, H = herbicides, F = fungicides.

Table 2.1. Pesticide concentrations measured on plants for the adult oviposition experiment. Values were recorded using QuEChERS method for pesticide extraction, followed by LC-MS for quantification.

Pesticide type	**Active ingredient**	Concentration (ng/g) in leaves **2 days** after spray		Concentration (ng/g) in leaves **4 days** after spray		Concentration (ng/g) in leaves **7 days** after spray	
		Mean	**Maximum**	**Mean**	**Maximum**	**Mean**	**Maximum**
Insecticide	Clothianidin	1.37	8.61	1.52	8.2	1.27	9.1
Herbicides	Atrazine	1.3	44.3	0.58	34.86	0.99	43.1
	S-metolachlor	0.11	0.52	0.13	0.25	0.08	0.16
Fungicides	Azoxystrobin	0.14	3.3	0.09	2.15	0.06	2.17
	Pyraclostrobin	2.46	95.22	2.14	110.8	4.43	204.7
	Trifloxystrobin	0.87	39.2	0.6	24.6	0.47	31.5

Table 2.2. Pesticide concentrations in 1 g of milkweed leaves collected in the field (Chapter 1), as well as leaf discs treated in the laboratory in leaf discs used to corroborate concentrations targeted for leaf discs.

Pesticide type	**Active ingredient**	**Concentration in the field (ng/g)**		**Concentration (ng/g) in leaf discs from experiment**	
		Mean	**Maximum**	**Mean**	**Maximum**
Insecticide	Clothianidin	15.28	56.55	14.36	54.47
Herbicide	Atrazine	8.59	238.7	9.7	329.5
	S-metolachlor	1.23	15.31	1.36	15.6
Fungicide	Azoxystrobin	0.67	31.06	0.48	23.2
	Pyraclostrobin	8.51	211.75	11.7	236.6
	Trifloxystrobin	4.48	164.25	6.17	157.9

Table 2.3. The effects of pesticide mixtures at their mean and maximum concentrations on monarch oviposition preference. Significance at $p < 0.001$ (***), $p < 0.01$ (**) and $p < 0.05$ (*).

Adult oviposition preference					
Fixed effects	**Estimate**	**SE**	**Z value**	***P***	
(Intercept)	2.0971	0.5281	3.971	<0.0001	***
Control	-0.1384	0.1204	-1.149	0.2504	
Mix max	-0.346	0.1276	-2.712	0.0067	**
Mix mean	-0.1076	0.1194	-0.901	0.3675	

Multiple Comparisons of Means: Dunnett Contrasts					
Linear hypotheses	**Estimate**	**SE**	**Z value**	***P***	
Control - A/T == 0	-0.1384	0.1204	-1.149	0.5311	
Mix max - A/T == 0	-0.346	0.1276	-2.712	0.0189	*
Mix mean - A/T == 0	-0.1076	0.1194	-0.901	0.7043	

Table 2.4. Effects of mean (A) and maximum (B) pesticide concentrations on 1st instar larval feeding preference. Significance at $p < 0.001$ (***), $p < 0.01$ (**) and $p < 0.05$ (*).

A. Mean concentrations

Treatments	**Estimate**	**Std. Error**	**t value**	***P***	
Acetone	-0.0182	0.0164	-1.1080	0.2765	
Clothianidin	-0.0505	0.0182	-2.7710	0.0092	**
Atrazine	-0.0102	0.0116	-0.8760	0.3882	
Metolachlor	-0.0488	0.0162	-3.0220	0.0051	**
Azoxystrobin	-0.0356	0.0134	-2.6490	0.0141	*
Pyraclostrobin	0.0087	0.0187	0.4640	0.6460	
Trifloxystrobin	-0.0226	0.0071	-3.1960	0.0056	**

B. Maximum concentrations

Treatments	**Estimate**	**Std. Error**	**t value**	***P***	
Acetone	-0.3304	0.1134	-2.9130	0.0054	**
Clothianidin	-0.3025	0.1239	-2.4420	0.0185	*
Atrazine	-0.4444	0.1208	-3.6790	0.0006	***
Metolachlor	-0.4900	0.1086	-4.5130	<0.0001	***
Azoxystrobin	0.0507	0.1245	0.4070	0.6850	
Pyraclostrobin	-0.5926	0.1010	-5.8700	<0.0001	***
Trifloxystrobin	-0.1083	0.1381	-0.7840	0.4370	

Table 2.5. Effects of mean (A) and maximum (B) pesticide concentrations on 2nd instar larval feeding preference. Significance at $p < 0.001$ (***), $p < 0.01$ (**) and $p < 0.05$ (*).

A. Mean concentrations

Treatments	**Estimate**	**Std. Error**	**t value**	***P***	
Control	0.4641	0.1168	3.9740	0.0003	***
Clothianidin	-0.1153	0.1432	-0.8050	0.4270	
Atrazine	-0.0850	0.1394	-0.6100	0.5460	
Metolachlor	-0.2189	0.1378	-1.5890	0.1210	
Azoxystrobin	-0.2200	0.1320	-1.6670	0.1040	
Pyraclostrobin	0.2737	0.1242	2.2040	0.0340	*
Trifloxystrobin	0.0933	0.1495	0.6240	0.5374	
Mix	-0.1711	0.1164	-1.4710	0.1510	

B. Maximum concentrations

Treatments	**Estimate**	**Std. Error**	**t value**	***P***	
Control	0.5237	0.1216	4.3070	0.0001	***
Clothianidin	-0.0139	0.1290	-0.1080	0.9150	
Atrazine	0.1756	0.1319	1.3320	0.1900	
Metolachlor	-0.0667	0.1398	-0.4770	0.6360	
Azoxystrobin	0.2030	0.1311	1.5480	0.1298	
Pyraclostrobin	-0.0745	0.1538	-0.4840	0.6320	
Trifloxystrobin	0.0887	0.1085	0.8170	0.4190	
Mix	-0.0771	0.1216	-0.6340	0.5300	

CHAPTER 3. TESTING CONSEQUENCES OF FIELD – RELEVANT INSECTICIDE EXPOSURE FOR MONARCH BUTTERFLY DEVELOPMENT ACROSS THEIR LIFE CYCLE.

3.1 Introduction

The intensification in agriculture has caused an extensive loss of habitat for pollinators and a concomitant increase in pesticide use has impacted their abundance and diversity in many regions around the world (Johansen 1977; Biesmeijer et al., 2006; Potts et al., 2010; Godfray et al., 2014). In particular, neonicotinoid insecticides have been rapidly adopted by farmers, becoming the most widely used insecticide over the past two decades (Douglas and Tooker 2015). Neonicotinoids are systemic insecticides easily absorbed by plants and moved through their tissues for crop protection. These insecticides impair the central nervous system in insects and affect their behavior and physiology at low doses but result in paralysis and death at higher doses (Goulson 2013). Herbicides, on the other hand, reduce the prevalence of wild plants in agroecosystems, while affecting plant phenology, morphology and chemistry (e.g., increased levels of toxic compounds). This can impact pollinating insects through a reduction in nectar sources for adults and leaf quality/quantity for larvae (in the case of butterflies), both critical for growth, development and reproduction (Dover et al., 1990; Longley and Sotherton 1997; Boutin et al., 2014; Hahn et al., 2014). Last, insecticides are commonly mixed with fungicides, which can increase their toxicity (Pilling and Jepson 1993; Pilling et al., 1995; Wade et al., 2019). The synergy between these two types of pesticides inhibits the activity of cytochrome P450 enzymes involved in detoxification of xenobiotics such as insecticides and plant chemicals (Després et al., 2007). Altogether, these inputs of diverse pesticide groups potentially harm pollinators through a variety of mechanisms and exposure routes.

Beneficial insects (i.e., pollinators, predators, parasitoids) are routinely exposed in the field to multiple pesticide classes applied in crops (Desneux et al., 2007). This includes natural areas adjacent to cropland that can be contaminated by pesticide drift, leaching and volatilization (van der Werf 1996). The effects of pesticides on insect behavior, development and reproduction are now well-documented (Haynes 1988; Thompson 2003; Desneux et al., 2007). This is especially true for the sublethal and lethal effects of several neonicotinoid insecticides and their interactions

with fungicides on bees such as *Apis mellifera* (Johnson et al., 2010; Brittain and Potts 2011), likely due to the fact that bees are important for the yield of pollinator-dependent crops (Calderone 2012; Klein et al., 2007). However, butterflies, along with other non-bee insects (i.e., beetles, flies, wasps), also play a central role in pollination (Krenn et al., 2010; Rader et al., 2015). Plant flowers are adapted to butterfly visitors, including species in the families Nymphalidae (monarch butterfly), Lycaenidae, Papilionidae and Pieridae and Hesperiidae (Reddi and Bai 1984). Although butterflies do not pollinate many species, their capacity to move pollen long distances allows for gene flow between highly isolated plant populations (Courtney et al., 1982). Recent studies report a correlative decline in the diversity and abundance of butterflies with the increased use of insecticides, mainly neonicotionids, in many regions (Godfray et al., 2014; Gilburn et al., 2015; Forister et al., 2016) and the effects of pesticides on butterflies was broadly discussed by Braak et al. (2018). Yet, comparatively few studies have experimentally tested the impacts of pesticide exposure for butterfly success.

In nature, adult butterflies and their larval caterpillars can be exposed to pesticide residues by contact or ingestion at the time of application or soon thereafter (Sinha et al., 1990; Davis et al., 1991; Cilgi and Jepson, 1995). Many studies describe the direct or indirect effects of pesticides on lepidopteran pests (Pisa et al., 2015), but there are few addressing the effects on non-target butterflies (Braak et al., 2018 and references therein; Mulé et al., 2017). Currently, four published studies have evaluated the impact of insecticides on monarch caterpillars, *Danaus plexippus* L., a species in decline throughout North America for which pesticides are thought to be a contributing factor. This includes two studies on neonicotinoids (Krischik et al., 2015; Pecenka and Lundgren, 2015) and two on pyrethroid insecticides involved in mosquito control (Oberhauser et al., 2006, 2009). These studies report a range of developmental consequences, such as reduced growth, survival, fecundity and oviposition. In general, however, there is still a lack of information related to the range of field realistic pesticide concentrations encountered by butterflies as caterpillars in their host plants inhabiting agricultural areas. For example, the work by Krischik et al. (2015) used milkweeds in home gardens where applicators used imidacloprid soil drenches, resulting in far higher concentrations than would be expected in milkweeds growing in natural areas. In addition, it is unknown how sensitive monarchs and other non-target butterfly species are to individual or combined pesticides ***during their entire life cycle***, and the response in different developmental

stages (e.g., larvae, pupae, adults) (Braak et al., 2018). Pecenka and Lundgren (2015), for instance, report strong negative consequences for monarch caterpillars ingesting the neonicotinoid clothianidin on milkweed leaves; however, this experiment was based on a brief 'pulsed' exposure over a 36 hour period. Longer-term life cycle studies are needed to more realistically assess the cumulative effects on butterfly development.

Here, we tested the lethal and sublethal effects of six pesticides—clothianidin (neonicotinoid insecticide); atrazine and metolachlor (photosynthetic and shoot inhibitor herbicides); azoxystrobin, pyraclostrobin and trifloxystrobin (QoI inhibitor fungicides)—and their combination on development of the iconic monarch butterfly, *D. plexippus*. All pesticides were experimentally tested in laboratory no-choice bioassays based on field-realistic concentrations measured from milkweed plants growing in margins around corn and soybean fields in Indiana. We evaluated monarch development time (larval and pupal stages), pupal weight, adult longevity, wing spam along with developmental malformations. These data will allow us to determine whether pesticide presence and identity on milkweeds are potentially causing harm to monarch populations and act as a contributing factor in their ongoing decline.

3.2 Methods

We used the six most frequent pesticides found in our field samples to develop a bioassay. For each, we tested the maximum and mean concentrations recorded from the field (Table 3.1). In the bioassay, neonate caterpillars (1st and 2nd instar larvae) were used to run two trials. The first trial was conducted from June 30 to July 17, 2017 and the second trial from September 11 to October 2, 2017. Milkweeds were maintained in the greenhouse (see below) and monarch eggs were the offspring from a wild-caught lab colony at Emory University provided by Jacobus De Roode.

3.2.1 Milkweed Plants

All experiments were performed using the common milkweed, *Asclepias syriaca*, the most prevalent milkweed host used by monarchs in their Midwestern summer breeding range. Seeds of *A. syriaca* were germinated following a procedure provided by Anurag Agrawal. Seeds were surface sterilized by soaking in a mixture of 95 mL tap water, 5 mL bleach, and a drop of dish soap

for 3-5 minutes. They were then rinsed in a strainer, first with tap water and then distilled water. Using a razor sterilized with a 10% bleach solution, we nicked the tip of each seed until a small amount of white internal tissue was exposed. Petri dishes were prepared by adding a circular coffee filter misted with DD water so that it was fully damp. Seeds were then placed in the petri dish using sterilized forceps in a 5x5 grid. Dishes were sealed with parafilm and covered with aluminum foil before being stored at 4°C for 1-2 weeks, after which they were moved to a growth chamber set at 28°C for 3-4 days. Dishes were then opened and checked for germinating seeds. If seeds had an observable root, they were removed and planted. If not, or the root was too small, dishes were re-covered and returned to the growth chamber for several more days, at which time they were re-examined.

Seeds with roots were planted in a mix of SunGro horticulture professional growing mix or germination mix and Osmocote 14-14-14 (ICL Specialty Fertilizers) that was watered until saturation. Fertilized soil was added to germination flats with 36 individual cells (5.66 cm L x 4.92 cm W x 5.66 cm D) and firmly packed. Small indentations were made in the middle of each container and a seed was placed in each indentation and covered slightly with soil, so a small amount of the root was still visible. Germination flats were placed over a propagation tray (64.03 cm L x 27.4 cm W x 3.27 cm D) from which water was added every other day. Seedlings were kept in a growth chamber (28°C, Relative Humidity = 65%, 12:14 LD) until they were sufficiently large to move to the greenhouse.

Seedlings were watered daily or as needed and checked to make sure their seed coat fell off. To control pests (mainly thrips and aphids), plants were inspected daily for damage and pest presence. Lacewing (*Chrysopa carnea*) larvae and predaceous mites (*Amblyseius cucumeris*) were added every two weeks to serve as a biocontrol. Occasionally, we added *Steinernema feltiae* nematodes to the soil to control fungus gnats. Any aphids found on plants were physically removed, and if a pest outbreak occurred a mixture of insecticidal soap, mineral oil, and water was applied to the plants. Milkweeds were moved to 366 ml pots filled with SunGro professional growing mix with two teaspoons of time-released NPK fertilizer (Scotts Osmocote Classic®) and kept in large (86.36 cm L x 86.36 cm W x 86.36 cm D) cages with PVC frames to exclude pests. In addition, yellow sticky cards were placed around and inside as an extra control for thrips and fungus gnats.

3.2.2 Pesticide Treatments

Leaves were collected from potted milkweed plants in the greenhouse and used for experimental pesticide exposure. After cutting at the base of the petiole, we washed the leaves first in tap water and then rinsed them with DD water, ensuring that any dirt and dry latex was removed. We then dried leaves completely with a paper towel. Leaf position was not controlled such that caterpillars in each treatment were exposed to a random variety of leaf ages over their full larval development. Leaf cores (20 mm diameter) were taken from leaves using a cork borer, sterilized with 70% EtOH prior to each use. We avoided major leaf veins except at the distal portion of the leaf where it is approximately the same thickness as other minor veins. Leaf discs were collected in 5.5 oz. plastic deli containers and covered with a paper towel to absorb excess moisture, then temporarily stored at 4°C. Leaf discs were collected daily to avoid wilting and degradation.

Pure pesticides—clothianidin (99.0%), atrazine (98.1%), s-metolachlor (97.6%), azoxystrobin (99.5%), pyraclostrobin (99.9%), trifloxystrobin (99.4%)—were ordered from Sigma-Aldrich or ChemService, Inc. Compounds were weighed on an analytical balance (Mettler Toledo XS64), and an initial stock was prepared in 1 ml of acetone. From the stock, pesticides were individually diluted in acetone and water to mimic concentrations in the field in a final volume of 15 ml. Acetone was used because the pesticides readily dissolve in it and it evaporates quickly. We first calculated the weight of an average milkweed leaf disc (n=18 discs), which was 0.065691 g, and adjusted accordingly through a process of serial dilutions of pure compounds in acetone as a solvent, which were applied in 20 µl (Table 3.1). The 20 µl were applied, using an Eppendorf repeater plus pipette, on the lower surface, where trichomes helped retain the solution on the disc surface. A mix of all 6 pesticides was also created to test for possible synergistic effects of individual compounds. Two controls, leaf discs treated with the solvent control (acetone) and untreated discs were used to compare with the pesticide treatments. Discs were left in the fume hood for 30 min until totally dry to ensure that the acetone had fully evaporated. Treated discs were then collected and placed into individually labeled plastic cups (3.5 oz) for each caterpillar. Discs were handled with forceps specific to each pesticide to avoid cross-contamination.

To confirm that our treated leaf discs contained the targeted pesticide concentrations, we weighed 1 g of untreated leaf discs and then treated them with the pesticide solutions at the concentrations

in Table 3.1. We had two replicates of each of the 14 treatments (mean and maximum field concentrations of 6 pesticides, and the combination of all). Treated leaf discs were allowed to dry in a fume hood and then homogenized. We followed the QuEChERS and LC/MS protocol that was used to extract, quantify and identify pesticides in leaf tissue from the field and measured the 6 pesticides that were being tested.

3.2.3 Monarch Development

The two trials were performed with 5 replicates for each of the 9 treatments (i.e., 6 individual pesticides, 1 mix, 2 controls) and for the 2 concentrations (maximum and mean) for a total of 90 individuals per trial and 180 individuals across both trials. Larvae were randomly assigned to a treatment (2^{nd} instars in the first trial, 1^{st} instars in the second trial), and were weighed using a semi-micro balance (Mettler Toledo NewClassic MF MS205DU) before being placed in an individually labeled 5.5 oz plastic deli container along with a damp strip of paper towel. The towel was moistened with DD water to ensure that larvae had sufficient humidity and 1 inch incisions were added to lids so that some airflow was possible.

Individual containers were arranged in a randomized complete block design, with one caterpillar of each of the 9 treatments in each block. Blocks 1-5 used pesticides at mean field concentrations while blocks 6-10 used maximum field concentrations. Within blocks, caterpillar were randomly assigned treatments. The spatial arrangement of blocks in the greenhouse was also randomized and rotated several times throughout the experiment to reduce any effects of minor environmental differences due to positional effects. Caterpillar containers were kept within a PVC frame (130 cm L x 110 cm H x 80 cm D) covered with shading cloth to reduce direct sunlight and a fan operating at low speed to cool the area even further. Each day, frass was removed and containers were cleaned with DD water and paper towel strips were replaced. Caterpillars were fed with freshly treated leaf discs and larval survival was recorded daily (Table 3.2).

Once larvae reached the pupal stage, each pupa was weighed on an analytical balance (Mettler Toledo XS64) and moved to mesh cages (95.25 cm L x 57.15 cm W x 59.69 cm D) separated by treatment. Cardboard platforms were taped to the top of the cage to provide shade and a location to attach pupae. Cages were arranged randomly on a greenhouse bench and mesh was draped over

a frame to reduce direct sunlight. Pupae attached to the 5.5 oz container lids were taped to the cardboard at the top of the cage, labelled with the specific treatment and concentration. Pupae that had fallen from the lids of their containers were reattached with synthetic silk and tape. This allowed the adult to emerge and extend their wings. Pupal survival and development time (i.e., time until adult emergence) were recorded (Table 3.2).

When adults emerged, their date of emergence and sex were recorded. After their wings dried, but before they started flying, a small amount of paint from Painters' opaque paint markers (Elmer's Products, Inc.) was applied to the hindwing with a fine paintbrush to track butterflies by date of emergence and register the day of death with precision. A different paint color was used each day to differentiate between emergence dates. Individuals that emerged the same day were marked with the same color. Butterflies were fed with banana and tangerine slices, fresh DD water, and Gatorade, which were replaced every other day. In general temperature varied between 23 and 29°C, but some days temperature reached as high as 32°C. To avoid temperature-induced stress, cages were misted with cold water and fans were placed on nearby benches to increase airflow. Cages were checked daily and adult longevity was calculated as time from butterfly emergence from their pupae until death (Table 3.2).

After a butterfly died, date of death was recorded and its wing span was measured by folding the butterfly's wings together, so it was facing right and measuring from the farthest tip of the wing to the first white dot on the thorax (Van Hook et al., 2012, Table 3.2). Butterflies were then photographed on white paper, and examined under a microscope for physical malformations (Table 3.2). Dead butterflies were stored in 3.5 x 6 inch coin envelopes at -20°C.

3.2.4 Quantifying Pesticide Consumption

We quantified the amount of each pesticide consumed per larva during the entire experiment, since oral exposure is a function of concentration applied and the amount of leaf tissue eaten by each individual. To do so, any leaf disc fragments that had not been eaten were taken out and photographed underneath a clear 4 x 4 mm grid. Then, we used these data to measure herbivory per day per caterpillar. The concentration consumed was calculated following the formula:

$$CC\ (ng) = ldc\ (\#) * ldw\ (g) * pc\ (\frac{ng}{g}) \quad (3.1)$$

Where cc = concentration consumed (ng), # ldc = number of leaf discs consumed, ldw (g) = average leaf disc weight, pc = pesticide concentration. The total number of leaf discs consumed was calculated based on the total % of herbivory. For example:

$$\frac{1\ leaf\ disc}{100\%} = \frac{x\ leaf\ discs}{5353.28\ \%} \quad (3.2)$$

$$\text{x leaf discs} = \frac{5353.28\ \%}{100\ \%} \cong 54\ \text{leaf discs} \quad (3.3)$$

3.2.5 Statistical Analysis

We used R software 3.5.1 and the packages car, lme4, multcomp, ggplot2, Matrix, lmerTest.

3.2.5.1 Immature Development, Pupal Weight, Adult Longevity and Wing Span

A generalized linear model with random effects (i.e. model = lmer(response variable ~ Trial + concentration consumed (ng) + treatment*trial + sex + treatment*concentration, data = data) was used to test effect of individual variables and interactions. For the interaction treatment*concentration we used categorical descriptors (mean and max), but we also tested concentration consumed (ng) as a continuous variable within the model. Interactions between trial*treatment or trial*concentration were observed, which is why we analyzed the data separately for each of the two trials. Models were performed using acetone or control as a reference and a pairwise mean contrast Dunnett's test was used to compare controls vs treatments.

We expected that the concentration consumed was related to the leaf area consumed (LAC); thus, we tested for collinearity using the variance inflation factor and the two were highly correlated (vif = 5.47). As a result, LAC was removed from the models. Residuals and qqplot graphs were used to see if the model was appropriate for our data, given statistical assumptions.

We tested if female adult longevity was related to wing span with a simple linear regression, because wing span can be associated with lifespan and fecundity.

3.2.5.2 Developmental Deformation

A generalized linear model with binary data was used to test the effects of pesticides on the proportion of adults with deformed wings. The model used was glm(Deformed_wings ~ treatment *concentration+ CC_ng + Trial*treatment + Sex_ratio, data = data). Dunnett's test was used to compare treatments versus controls. We used acetone as a baseline and then the control.

3.3 Results

Statistical results using acetone or control as a baseline to compare only differed in the results for wing span and developmental deformations where concentration interacts with the treatments.

3.3.1 Larval Development

Larval development was not affected by the concentration consumed per pesticides ($p = 0.133$) or by the treatments ($p>0.05$). Differences between trials (Figure 3.1) in time of development was attributed to the instar at which each trial was started (Trial 1 = 2nd instars, Trial 2 = 1st instars). Pairwise comparisons did not show significant differences in larval development between pesticide treatments and controls; every comparison had a p value > 0.05.

Larval development time in trial 1 was 8-11 days, while in trial 2 it was 15-17 days (Table 3.2). Sex had a significant effect on larval development in trial 1 with males spending longer in their larval stage (females = 10 days; males = 11 days; $p < 0.001$; trial 2, $p= 0.18$). The highest mortality (60 – 80%) at this stage was observed for acetone, clothianidin, azoxystrobin, trifloxystrobin and the mix treatments (Table 3.2). None of the larvae fed with untreated leaf discs died at this stage. All instars had at least one dead individual; however, more individuals died during the second (8) and fifth (9) instars and in J form (9).

3.3.2 Pupal Development

Clothianidin had a significant effect on pupal development in trial 1 ($p < 0.01$). Larvae treated with clothianidin, had longer pupal development than the acetone treatment ($p< 0.011$), (Figure 3.2). No differences were observed when clothianidin was compared to the second control (untreated leaf discs) ($p= 0.328$). All other pesticide treatments did not have an effect on pupal development

within each trial ($p>0.05$). The average development time in trial 1 was 8-9 days and in trial 2, 11-12 days. Females and males did not differ in development time (trial 1, $p = 0.06$; trial 2, $p= 0.18$). At this stage, at least one individual died in the treatments containing clothianidin, atrazine, azoxystrobin, pyraclotrobin, mix and the control (Table 3.2).

3.3.3 Pupal Weight

Pesticide treatments did not affect pupal weight within trials, or when compared with the controls ($p>0.05$). Differences between trials were observed ($P<0.001$), and pupal weight was significantly lower in trial 2 (Figure 3.3). The average weight in the second trial was 56% lower than in the first trial (1.66g in trial 1 versus 0.93g in trial 2; Tables 3.3 and 3.6). Differences in pupal weight between females and males was consistent within trials and males had higher pupal weights ($p < 0.001$) (females = 1.28g; males = 1.37g).

3.3.4 Adult Longevity

Adult longevity was not affected by pesticide treatments in both trials and the controls did not show significant differences compared with the treatments (Figure 3.4). A marginally significant effect was observed for clothianidin ($p = 0.08$). A total of 138 individuals out of 180 tested survived to the adult stage; from these, 77 were alive for less than 2 weeks across all treatments (see Table 3.2 for mean longevity per trial and treatment). Sex did not affect adult longevity ($p = 0.3161$).

3.3.5 Wing Span

Significant differences in wing span between control and acetone were found in Trial 1 ($p = 0.01$); individuals reared in the untreated control had longer wings (Figure 3.5). Results for trial 2 were inconsistent due to an interaction between treatment and concentration. Results were obtained using a linear model per treatment and these analyses showed that in clothianidin the individuals exposed to mean concentrations had longer wings ($p = 0.009$; Figure 3.6A). The opposite was observed with azoxystrobin where wings were longer under exposure to maximum concentrations ($p = 0.002$; Figure 3.6B). Other treatments did not affect wing span ($p > 0.05$). Males had higher forewing length in trial 2 ($p < 0.05$). Wing span was also unrelated to female lifespan ($F_{(1,60)} = 0.5521$, $p = 0.46$, $R^2 = 0.009$).

3.3.6 Developmental Deformation

A total of 20 individuals had deformed wings; some of them were crumpled, some were abnormally asymmetric (Figure 3.7). The fungicides pyraclostrobin and trifloxystrobin and the pesticide mix caused more deformed wing than other treatments (Table 3.4 and 3.7). This result was mainly observed in individuals exposed to mean pesticide concentrations (Table 3.4 and 3.7). In addition, males had more deformed wings than females. This difference was significant for trial 1 (10 males vs 3 females, $p < 0.05$), but not trial 2 (4 males vs 3 females).

3.4 Discussion

The pesticides tested in this study are widely used to protect corn and soybean in the Midwest (https://water.usgs.gov/nawqa/pnsp/usage/maps/compound_listing.php) where monarch larvae consume milkweed tissue during the breeding season (Malcolm 2018; Pleasants 2017). Herbicides can harm butterflies through a reduction of host-plants (e.g., glyphosate reduces milkweed density in agricultural areas; Hartzler 2010), but herbicides can also increase insecticide toxicity as demonstrated for fruit flies, mosquito larvae and houseflies when atrazine is combined with parathion (Lichtenstein et al., 1973). Reduced number of larvae reaching the pupal and adult stages (Stark et al., 2012), smaller sizes, lower pupal weights, shorter development time and lower survival (Russell and Schultz 2009) are some of the negative effects that herbicides cause to butterflies. Fungicides, like herbicides, create synergy and increase susceptibility to insecticides, pathogens and viruses in bees (Tadei et al., 2019; Pettis et al., 2013) and cause anti-feeding and mortality in Lepidoptera (Woods and Gent 2014; Nicodemo et al., 2018).

Many studies use field rate application of the pesticide active ingredients or use doses above real pesticide residues found in the field to test for possible negative effects on non-target organisms. Basley and Goulson (2018) tested the effect of clothianidin field realistic residues in *Lotus corniculatus* and *Trifolium hybridum* (Fabaceae) on the blue butterfly (*Polyommatus Icarus*) larval development. Clothianidin caused high larval mortality, but it was not associated with the dose, and they did not observe negative effects on larval or pupal development time, adult weight or pupal weight. They did find, however, a reduction in larval size at the early stage of development. Monarch caterpillar neonates exposed to clothianidin showed body length and weight reduction

(Pecenka and Ludgren 2015). In our experiment, monarch larvae consumed 49.9 and 58.9 ng of clothianidin at mean concentrations and 83 and 371.1 ng at maximum concentrations, for trials 1 and 2, respectively, during their fifth instar. Like Basley and Goulson (2018), we did not observed differences in larval development, pupal weight and adult longevity. A longer pupal development time was observed, but in only one of the two trials. Although we did not measure larval length or weight, presumably those variables are highly correlated with traits such as pupal/adult size, which we did measure.

Field realistic concentrations of clothianidin, atrazine, metolachlor, azoxystrobin, pyraclostrobin, trifloxystrobin and their combination showed no apparent negative effects on monarch butterfly development through their life cycle. The development times, pupal weight and adult longevity were within values reported in other studies (Glass and Pan 1983; Singh and Clare 1988). A possible explanation for the lack of lethal effects could be related to an increase in the level of detoxification enzymes, such as the cytochrome P450 monooxygenases, carboxylesterases and glutathione transferases, which are specialized in dealing with toxic plant compounds to larvae (Després et al., 2007). While herbivores use enzymes to detoxify compounds (Kao et al., 1995; Miota et al., 2000), this comes with a fitness cost; for example, insecticide resistant individuals of the codling moth have smaller adults than susceptible ones. Small size can reduce adult longevity, mating success and reproduction. In addition, they have longer development time, resulting in a longer exposure window to natural enemies (Konopka et al., 2012), which can decrease survival and population density.

In monarchs, female size is associated with longer lifespan and higher fecundity; however, factors such as abiotic stress can counteract this relationship (Oberhauser 1997). Bigger females are chosen over smaller ones by males to mate, and bigger males can more easily overcome the resistance of some females to mate (Frey et al., 1998). Reduced wing size can thus be considered as a sublethal effect of pesticides to monarchs because a deformation of the wings has negative consequences for migration. Further, the transformation of the landscape limits milkweeds to distant patches, which requires robust wings for effective host-plant finding. Individuals consuming <100 ng of clothianidin reached a wing span of 4.6 and 5.0 cm, while those consuming

>350 ng had wing spans <4.6 cm (Fig 3.6A). The opposite was observed in individuals treated with azoxystrobin (Figure 3.6B), suggesting that pesticides vary in their effects.

In bees, clothianidin and imidacloprid affect immunity and increase the proliferation of the virus causing deformed wings, even at field realistic concentrations (0.1-10 ppb) (Di Prisco et al., 2013). In monarchs, the protozoan parasite *Ophryocystis elektroscirrha* causes deformed wings and reduce lifespan (Leong et al., 1997). In our study, this was not the case, even though our eggs came from a colony where this parasite is studied. To ensure that individuals with deformed wings were not infested with *O. elektroscirrha* we sampled adults by applying clear tape to their abdomen, transferring it to paper and searching for parasite spores; we did not find any across all individuals tested. In general, insecticides produce deformed wings in bees as showed in a study by Atkins (1986) who tested 31 pesticides and found that six of them produced deformed wings in bees.

3.5 Conclusion

Although we did not find obvious negative effects of pesticide treatments on monarch butterfly development, there was some evidence for negative effects on survival; however, this varied widely among pesticides and trials showing that individuals respond in different ways to these stressors. Due to the large number of pesticide treatments included, at both concentration levels, our study was somewhat limited in terms of number of experimental replicates, which limited our statistical power for teasing apart treatment effects. Alternatively, specialist insects like monarchs may be predisposed to detoxifying pesticides due to their well-developed detoxification abilities because of long-term interactions with toxic milkweed host-plants and the cardenolides they produce (see, for instance, Hardy et al., 2018). This hypothesis remains to be tested.

3.6 References

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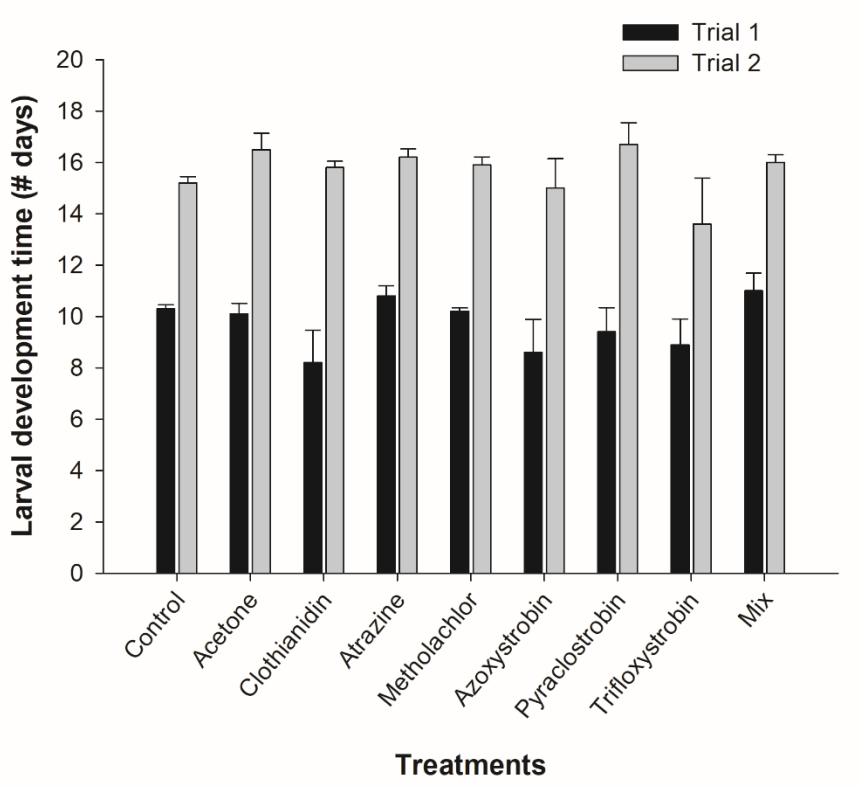

Figure 3.1 Development time (mean + se) in *D. plexippus* larvae exposed to six different pesticides: clothianidin (insecticide), atrazine and metolachlor (herbicides), azoxystrobin, pyraclostrobin and trifloxystrobin (fungicides) and their mix. Trial 1 larvae were 2nd instar; trial 2 larvae were 1st instar.

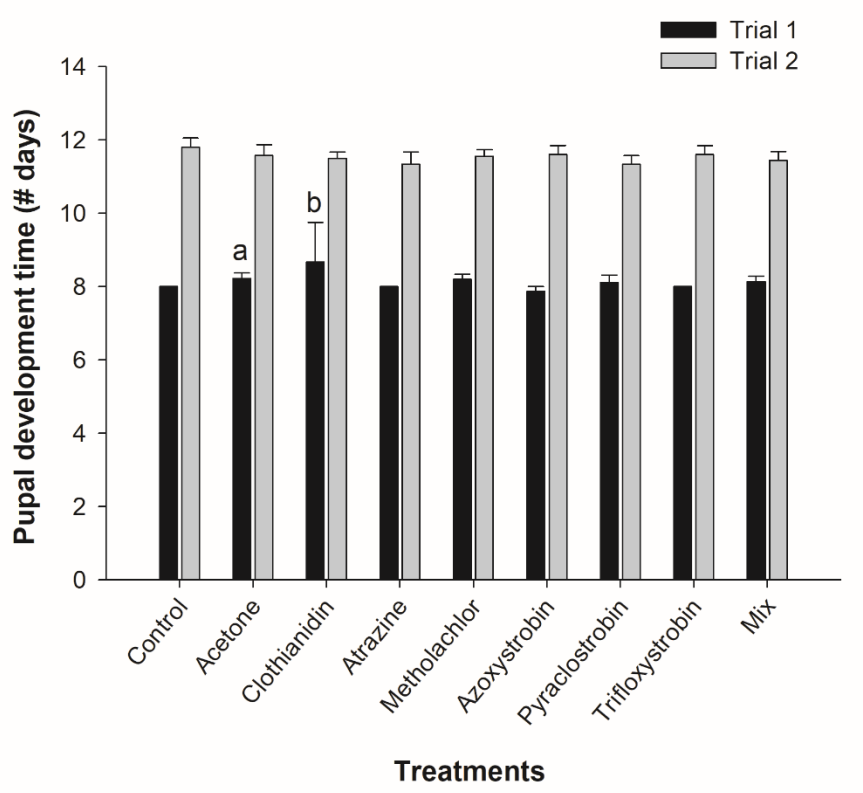

Figure 3.2 Pupal development time (mean + se) in *D. plexippus* after larvae were exposed to six different pesticides: clothianidin (insecticide), atrazine and metolachlor (herbicides), azoxystrobin, pyraclostrobin and trifloxystrobin (fungicides) and their mix. Different lowercase letters represent significant differences at $p<0.05$.

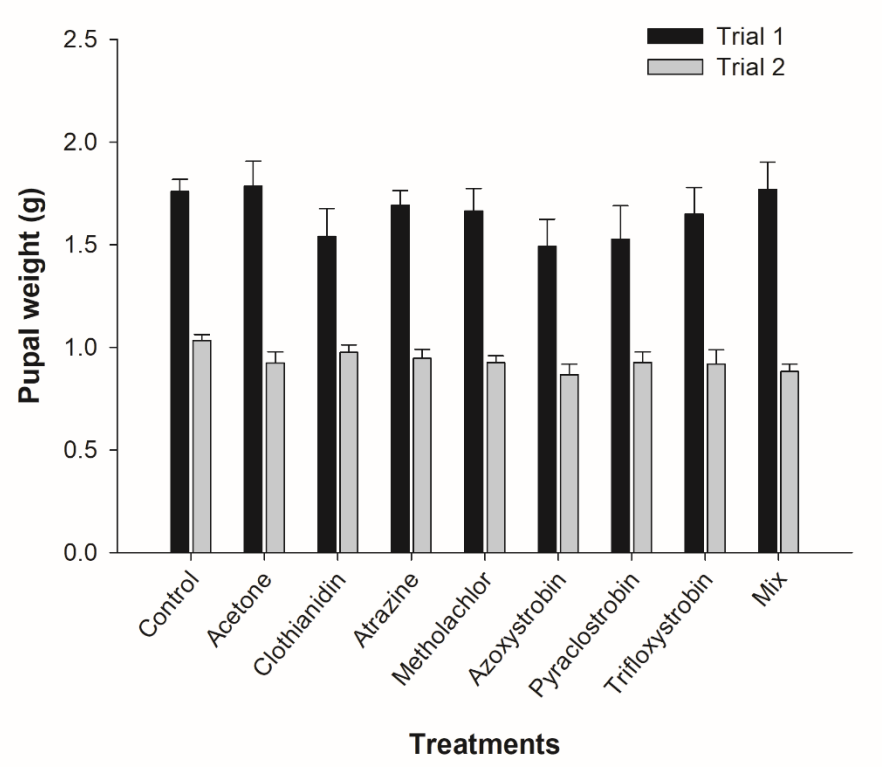

Figure 3.3 Pupal weight (mean + se) after *D. plexippus* larvae exposure to six different pesticides: clothianidin (insecticide), atrazine and metolachlor (herbicides), azoxystrobin, pyraclostrobin and trifloxystrobin (fungicides) and their mix.

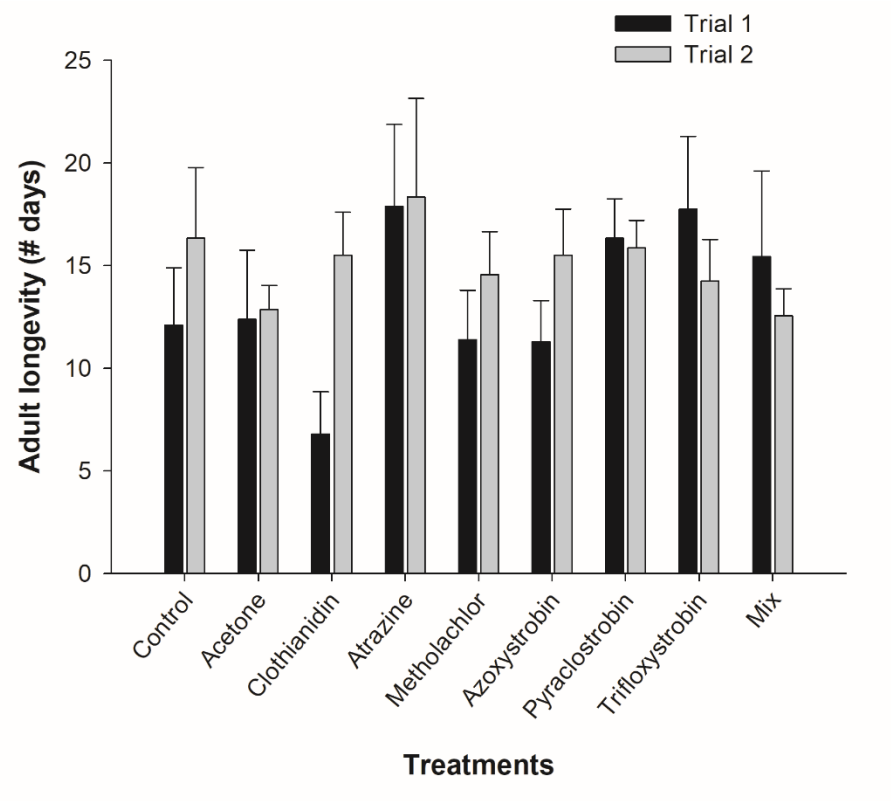

Figure 3.4 Adult longevity (mean + se) after larval exposure to six different pesticides: clothianidin (insecticide), atrazine and metolachlor (herbicides), azoxystrobin, pyraclostrobin and trifloxystrobin (fungicides) and their mix. Clothianidin showed a marginally significant effect (p=0.08).

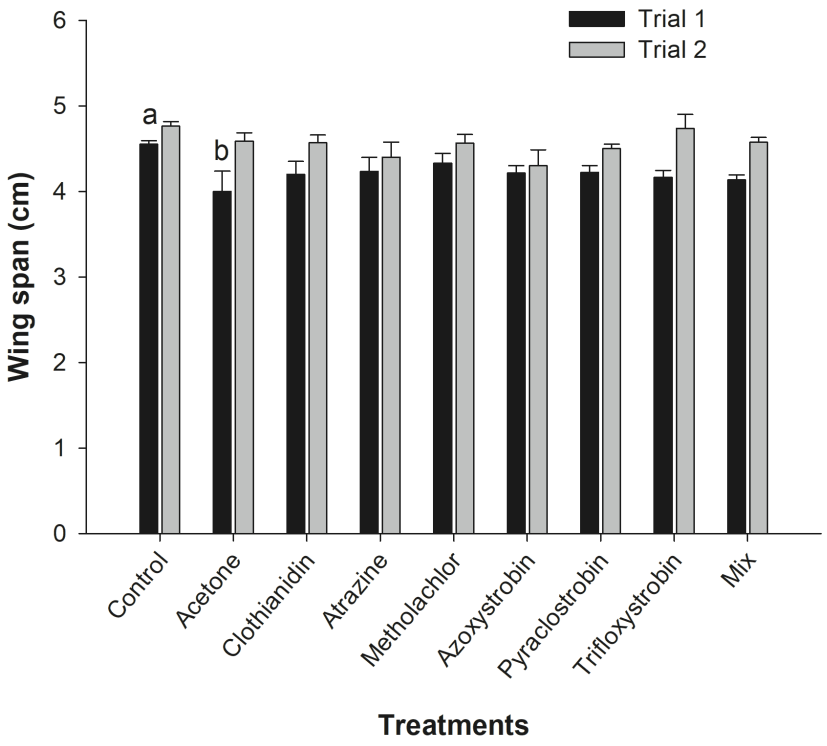

Figure 3.5 Wing span (mean + se) in *D. plexippus* adults after larvae were exposed to six different pesticides: clothianidin (insecticide), atrazine and metolachlor (herbicides), azoxystrobin, pyraclostrobin and trifloxystrobin (fungicides) and their mix. Different lowercase letters represent significance at $p<0.05$.

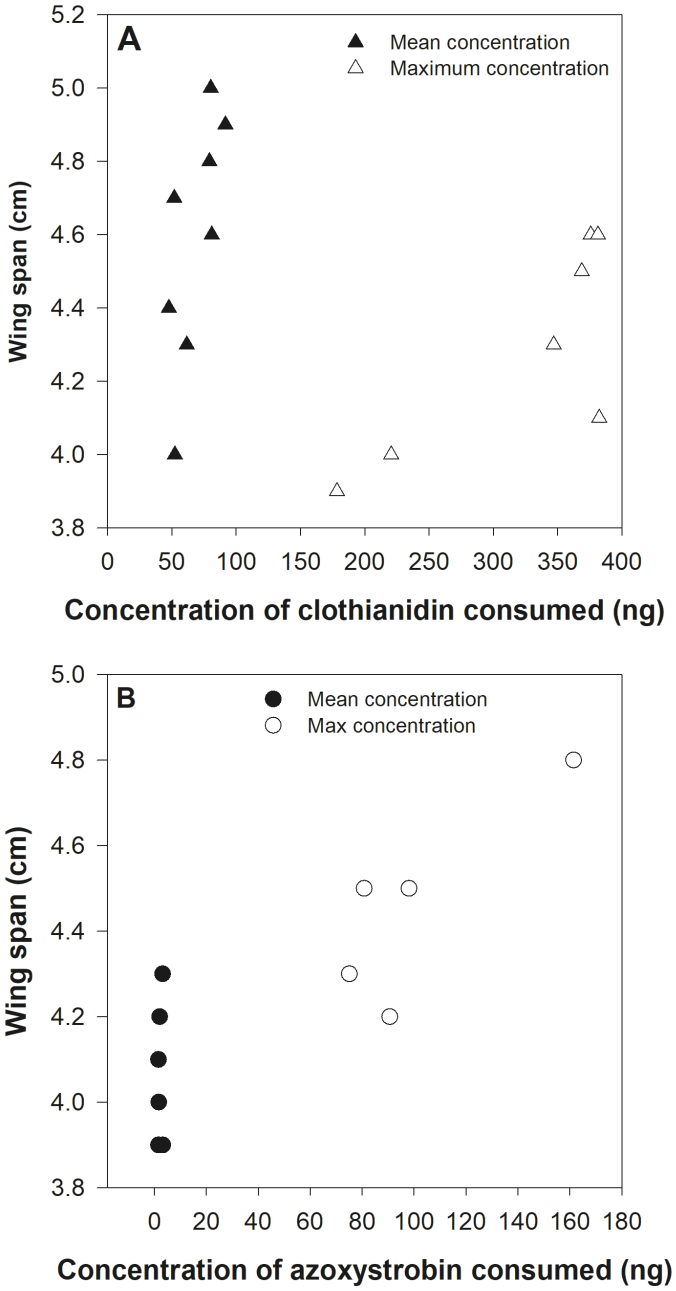

Figure 3.6 Effect of clothianidin (A; p = 0.009) and azoxystrobin (B; p = 0.002) on *D. plexippus* wing span (cm).

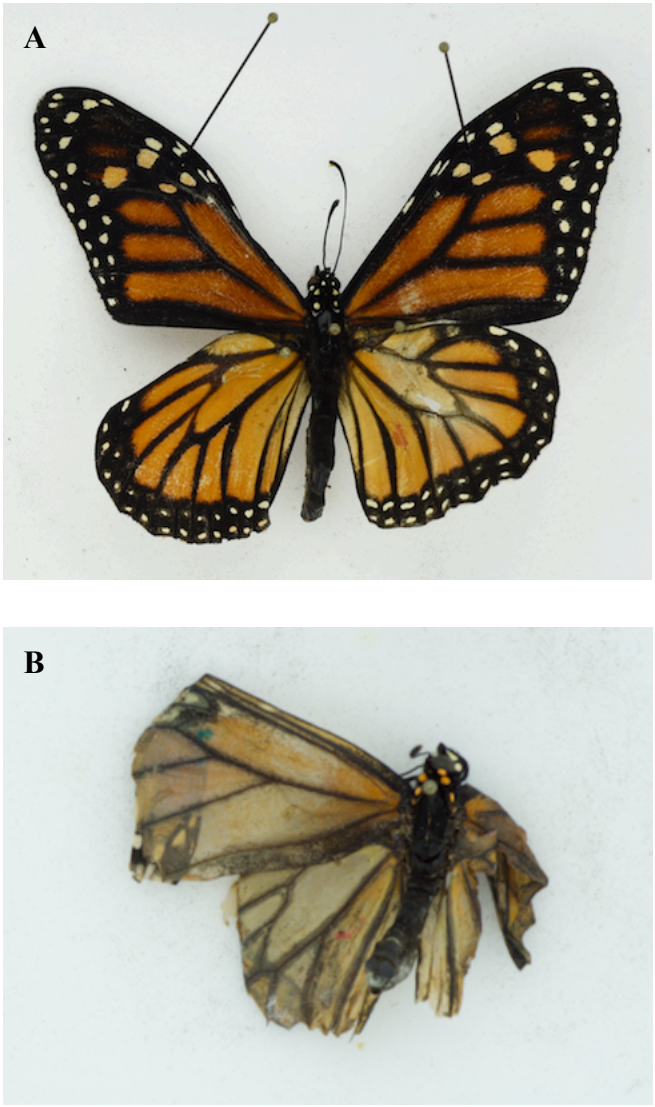

Figure 3.7. Deformed wings in individuals exposed to mean (A) and maximum (B) concentrations of the herbicide atrazine.

Table 3.1 Concentrations of six pesticides commonly found in milkweeds around corn and soybean fields. The concentration applied to each leaf discs used to feed *Danaus plexippus* during its larvae stage and the corroboration that we target in the laboratory the concentrations found in 1 g on milkweed leaf tissue.

Pesticide type	**Active ingredient**	**Concentration in the field (ng/g)**		**Concentration applied in leaf discs (ng/0.065691g), with dilutions prepared in the lab**		**Concentration in 1 g of leaf discs from HPLC analysis (ng/g)**	
		Mean	**Maximum**	**Mean**	**Maximum**	**Mean**	**Maximum**
Insecticide	Clothianidin	15.28	56.55	2.91	11.31	14.36	54.47
Herbicides	Atrazine	8.59	238.7	1.64	47.74	9.7	329.5
	S-metolachlor	1.23	15.31	0.23	3.06	1.36	15.6
Fungicides	Azoxystrobin	0.67	31.06	0.13	6.21	0.48	23.2
	Pyraclostrobin	8.51	211.75	1.62	42.35	11.7	236.6
	Trifloxystrobin	4.48	164.25	0.85	32.85	6.17	157.9

Table 3.2 Trial 1, larval development and pupal development time in *D. plexippus* exposed to six pesticides and two controls. Abbreviations were used to save space: pesticides (Cl = clothianidin, At = atrazine, M = metolachlor, Azo= azoxystrobin, P= pyraclostrbin, Tri= trifloxystrobin and Mix = all six pesticides combined), controls (Ace = acetone, which was the pesticide solvent and Control = untreated leaf discs).

Trial	Treatments	Average concentrations consumed (ng)	Larval development (# days) & survival					Pupal development (# days) & survival				
			n = larval	mean	se	range	alive (total)	n = pupae	mean	se	range	alive (total)
1	Ace	0	5	9.4	0.60	7-10	4 (5)	4	8.0	0.00	8-8	4 (4)
1	Control	0	5	10.2	0.20	10-11	5 (5)	5	8.0	0.00	8-8	5 (5)
1	Cl	49.9	5	10.4	0.40	10-12	4 (5)	4	9.3	1.43	7-14	3 (4)
1	At	36.2	5	11.2	0.73	10-14	5 (5)	5	8.0	0.00	8-8	5 (5)
1	M	5.1	5	10.2	0.20	10-11	5 (5)	5	8.2	0.20	8-9	5 (5)
1	Azo	1.4	5	8.6	1.94	1-12	4 (5)	4	7.8	0.22	7-8	3 (4)
1	P	34.0	5	8.4	1.86	1-11	4 (5)	4	8.0	0.00	8-8	4 (4)
1	Tri	21.3	5	10.0	0.00	10-10	2 (5)	2	8.0	0.00	8-8	2 (2)
1	Mix	105.6	5	12.0	1.26	10-17	2 (5)	2	8.5	0.32	8-9	1 (2)
1	Ace	0	5	10.8	0.37	10-12	5 (5)	5	8.4	0.24	8-9	4 (5)
1	Control	0	5	10.4	0.24	10-11	5 (5)	5	8.0	0.00	8-8	5 (5)
1	Cl	83.0	5	6.0	2.14	1-11	2 (5)	2	7.5	0.32	7-8	2 (2)
1	At	1166.3	5	10.4	0.24	10-11	5 (5)	5	8.0	0.00	10-11	4 (5)
1	M	58.6	5	10.2	0.20	10-11	5 (5)	5	8.2	0.20	8-9	5 (5)
1	Azo	69.0	5	8.6	1.91	1-11	4 (5)	4	8.0	0.00	8-8	4 (5)
1	P	874.1	5	10.4	0.24	10-11	5 (5)	5	8.2	0.37	7-9	5 (5)
1	Tri	390.0	5	7.8	1.98	2-11	3 (5)	3	8.0	0.00	8-8	3 (5)
1	Mix	2613.9	5	10.0	0.00	10-10	5 (5)	5	8.0	0.00	8-8	5 (5)

Table 3.3 Trial 1, pupal weight (g), adult longevity and wing span in *D. plexippus* exposed to six pesticides and two controls. Abbreviations were used to save space: pesticides (Cl = clothianidin, At = atrazine, M = metolachlor, Azo= azoxystrobin, P= pyraclostrbin, Tri= trifloxystrobin and Mix = all six pesticides combined), controls (Ace = acetone, which was the pesticide solvent and Control = untreated leaf discs).

Trial	Treatments	Average concentrations consumed (ng)	Pupal weight (g)			Adult longevity (# days)			Wing span (cm)		
			mean	se	range	mean	se	range	mean	se	range
1	Ace	0	1.59	0.06	1.48-1.77	8.5	3.04	2-18	4.0	0.30	3.0-4.5
1	Control	0	1.73	0.10	1.53-2.11	9.0	1.79	5-15	4.5	0.07	4.3-4.7
1	Cl	49.9	1.42	0.08	1.23-1.64	4.7	0.52	4-6	4.4	0.16	4.0-4.7
1	At	36.2	1.73	0.10	1.46-1.97	21.4	5.81	4-34	4.4	0.07	4.2-4.6
1	M	5.1	1.47	0.14	0.93-1.75	12.4	3.88	6-27	4.3	0.22	3.4-4.6
1	Azo	1.4	1.43	0.06	1.29-1.52	13.3	3.14	6-20	4.0	0.04	3.9-4.1
1	P	34.0	1.17	0.23	0.62-1.82	18.8	2.17	14-25	4.1	0.02	4.1-4.2
1	Tri	21.3	1.70	0.14	1.34-2.12	21.6	4.98	9-33	4.1	0.12	3.8-4.5
1	Mix	105.6	1.62	0.29	1.16-2.08	12.0	5.69	3-21	3.9		3.9
1	Ace	0	1.94	0.19	1.51-2.59	16.3	5.02	3-29	4.0	0.31	3.2-4.4
1	Control	0	1.78	0.07	1.51-1.91	15.2	4.48	9-33	4.6	0.06	4.5-4.8
1	Cl	83.0	1.77	0.25	1.37-2.17	10.0	4.43	3-17	4.0	0.03	3.9-4.0
1	At	1166.3	1.65	0.11	1.25-1.86	13.5	3.68	6-24	4.1	0.34	3.1-4.9
1	M	58.6	1.86	0.13	1.67-2.33	10.4	2.25	6-19	4.4	0.09	4.1-4.6
1	Azo	69.0	1.54	0.21	0.90-2.05	9.8	2.20	5-14	4.4	0.07	4.2-4.5
1	P	874.1	1.81	0.11	1.62-2.15	14.4	2.48	5-19	4.3	0.14	3.8-4.6
1	Tri	390.0	1.57	0.23	0.99-1.91	11.3	0.68	10-13	4.3	0.04	4.2-4.4
1	Mix	2613.9	1.83	0.11	1.57-2.11	16.8	5.68	4-32	4.2	0.05	4.0-4.3

Table 3.4 Trial 1, sex ratio and number of individuals with deformed wings in *D. plexippus* exposed to six pesticides and two controls. Abbreviations were used to save space: pesticides (Cl = clothianidin, At = atrazine, M = metolachlor, Azo= azoxystrobin, P= pyraclostrbin, Tri= trifloxystrobin and Mix = all six pesticides combined), controls (Ace = acetone, which was the pesticide solvent and Control = untreated leaf discs).

Trial	Treatments	Average concentrations consumed (ng)	Sex ratio		Deformed wings
			Females	Males	
1	Ace	0	2	2	1
1	Control	0	3	2	0
1	Cl	49.9	2	1	0
1	At	36.2	3	2	0
1	M	5.1	3	2	1
1	Azo	1.4	3	.	0
1	P	34.0	1	3	2
1	Tri	21.3	2	3	2
1	Mix	105.6	1	.	1
1	Ace	0	2	2	2
1	Control	0	1	4	1
1	Cl	83.0	1	1	1
1	At	1166.3	1	3	1
1	M	58.6	3	2	0
1	Azo	69.0	3	1	1
1	P	874.1	3	2	0
1	Tri	390.0	0	3	0
1	Mix	2613.9	4	1	1

Table 3.5 Trial 2, larval development and pupal development time in *D. plexippus* exposed to six pesticides and two controls. Abbreviations were used to save space: pesticides (Cl = clothianidin, At = atrazine, M = metolachlor, Azo= azoxystrobin, P= pyraclostrbin, Tri= trifloxystrobin and Mix = all six pesticides combined), controls (Ace = acetone, which was the pesticide solvent and Control = untreated leaf discs).

Trial	Treatments	Average concentrations consumed (ng)	Larval development (# days) & survival					Pupal development (# days) & survival				
			n = larval	mean	se	range	alive (total)	n = pupae	mean	se	range	alive (total)
2	Ace	0	5	17.0	1.26	15-22	5 (5)	5	11.4	0.40	10-12	5 (5)
2	Control	0	5	14.8	0.37	14-16	5 (5)	5	12.0	0.00	12-12	4 (5)
2	Cl	58.9	5	15.6	0.40	14-16	5 (5)	5	11.6	0.24	11-12	5 (5)
2	At	78.8	5	15.8	0.20	15-16	3 (5)	3	11.7	0.26	11-12	3 (3)
2	M	9.0	5	15.8	0.20	15-16	5 (5)	5	11.4	0.24	11-12	5 (5)
2	Azo	2.8	5	16.0	0.32	15-17	4 (5)	4	11.8	0.22	11-12	3 (4)
2	P	73.2	5	17.6	1.60	16-24	4 (5)	4	11.0	0.00	11-11	4 (4)
2	Tri	32.1	5	13.8	2.46	4-17	5 (5)	5	11.5	0.32	11-12	4 (5)
2	Mix	172.8	5	15.6	0.40	14-16	5 (5)	5	11.4	0.40	10-12	5 (5)
2	Ace	0	5	16.0	0.32	15-17	2 (5)	2	12.0	0.00	12-12	2 (2)
2	Control	0	5	15.6	0.24	15-16	5 (5)	5	11.6	0.51	10-13	5 (5)
2	Cl	371.1	5	16.0	0.32	15-17	5 (5)	5	11.4	0.24	11-12	5 (5)
2	At	1922.2	5	16.6	0.60	16-19	3 (5)	3	11.0	0.45	16-19	3 (5)
2	M	90.8	5	16.0	0.63	14-18	4 (5)	4	11.8	0.22	11-12	4 (5)
2	Azo	84.4	5	14.0	2.30	5-18	1 (5)	1	11.0		11	1 (1)
2	P	1575.4	5	15.8	0.49	14-17	5 (5)	5	11.6	0.40	11-13	3 (5)
2	Tri	660.7	5	13.4	2.87	2-17	3 (5)	3	11.7	0.26	11-12	3 (3)
2	Mix	4283.3	5	16.4	0.40	16-18	4 (5)	4	11.5	0.26	11-12	4 (4)

Table 3.6 Trial 2, pupal weight (g), adult longevity and wing span in *D. plexippus* exposed to six pesticides and two controls. Abbreviations were used to save space: pesticides (Cl = clothianidin, At = atrazine, M = metolachlor, Azo= azoxystrobin, P= pyraclostrbin, Tri= trifloxystrobin and Mix = all six pesticides combined), controls (Ace = acetone, which was the pesticide solvent and Control = untreated leaf discs).

Trial	Treatments	Average concentrations consumed (ng)	Pupal weight (g)			Adult longevity (# days)			Wing spam (cm)		
			mean	se	range	mean	se	range	mean	se	range
2	Ace	0	0.85	0.05	0.81-0.96	12.2	1.66	6-16	4.5	0.10	4.1-4.7
2	Control	0	1.02	0.04	0.91-1.14	19.3	6.53	10-41	4.9	0.05	4.8-5.0
2	Cl	58.9	0.97	0.08	0.73-1.18	14.0	0.63	13-16	4.7	0.12	4.3-5.0
2	At	78.8	0.98	0.04	0.92-1.09	10.0	1.95	7-15	4.6	0.20	4.1-5
2	M	9.0	0.92	0.04	0.81-1.05	12.6	0.75	11-15	4.6	0.08	4.5-4.9
2	Azo	2.8	0.82	0.03	0.76-0.90	16.7	3.24	12-25	4.1	0.09	3.9-4.3
2	P	73.2	0.99	0.05	0.88-1.17	15.8	2.29	11-23	4.4	0.07	4.3-4.6
2	Tri	32.1	0.78	0.02	0.74-0.81	22.0		22			.
2	Mix	172.8	0.87	0.06	0.75-1.08	12.6	2.25	8-19	4.6	0.10	4.3-4.8
2	Ace	0	1.10	0.01	1.09-1.11	14.5	0.95	13-16	4.9	0.03	4.8-4.9
2	Control	0	1.04	0.04	0.95-1.19	14.0	1.70	8-18	4.7	0.06	4.5-4.8
2	Cl	371.1	0.98	0.02	0.94-1.03	17.0	3.78	12-32	4.4	0.10	4.1-4.6
2	At	1922.2	0.91	0.06	0.77-1.04	26.7	6.88	9-37	4.2	0.03	4.1-4.2
2	M	90.8	0.94	0.05	0.81-1.05	17.0	3.90	12-30	4.5	0.19	4.0-4.9
2	Azo	84.4	1.04		1.04	12.0		12	4.8		4.8
2	P	1575.4	0.87	0.07	0.64-1.02	16.0	0.89	14-18	4.6	0.03	4.5-4.6
2	Tri	660.7	1.01	0.05	0.88-1.08	11.7	1.37	9-15	4.7	0.13	4.4-4.9
2	Mix	4283.3	0.90	0.02	0.85-0.95	12.5	0.77	10-14	4.6	0.04	4.5-4.7

Table 3.7 Trial 2, sex ratio and number of individuals with deformed wings in *D. plexippus* exposed to six pesticides and two controls. Abbreviations were used to save space: pesticides (Cl = clothianidin, At = atrazine, M = metolachlor, Azo= azoxystrobin, P= pyraclostrbin, Tri= trifloxystrobin and Mix = all six pesticides combined), controls (Ace = acetone, which was the pesticide solvent and Control = untreated leaf discs).

Trial	Treatments	Average concentrations consumed (ng)	Sex ratio		Deformed wings
			Females	Males	
2	Ace	0	3	2	0
2	Control	0	1	3	1
2	Cl	58.9	2	3	0
2	At	78.8	2	1	0
2	M	9.0	3	2	0
2	Azo	2.8	1	2	1
2	P	73.2	1	3	1
2	Tri	32.1	.	1	1
2	Mix	172.8	3	2	1
2	Ace	0	1	1	0
2	Control	0	1	4	0
2	Cl	371.1	2	3	1
2	At	1922.2	2	1	1
2	M	90.8	1	3	0
2	Azo	84.4	1	1	0
2	P	1575.4	2	1	0
2	Tri	660.7	1	2	0
2	Mix	4283.3	2	1	0

VITA

Paola Andrea Olaya Arenas was born in Cali, Colombia. She received her bachelor's degree in biology with a concentration in botany from Universidad del Valle, Colombia, and earned a master's degree in ecology from University of Puerto Rico. Before coming to Purdue, she gained experience developing research in plant taxonomy and systematics, forest restoration and plant response to climate change. She came to Purdue University supported by the Colombian government (Colciencias), to pursue a Ph.D. degree in entomology. Her doctoral research was advised by Prof. Ian Kaplan and it focuses on the effects of land use on the frequency and concentrations of pesticides in milkweeds (monarch butterfly host-plants) in natural habitats around corn and soybean fields and the risk for monarchs when exposed to those pesticides and their combination. This topic has been well-studied in bees, but not in non-target butterfly species.