

**Characterization of honey bee (*Apis mellifera*) and monarch butterfly (*Danaus plexippus*)
exposure to pesticides: Risks and benefits of establishing pollinator habitat within Iowa
agricultural landscapes**

by

Maura Jacqueline Hall

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Program of Study Committee:
Steven Bradbury, Co-major Professor
Joel Coats, Co-major Professor
David Borts
Keri Carstens
Lisa Schulte Moore
Michelle Soupir

The student author, whose presentation of the scholarship herein was approved by the program of study committee, is solely responsible for the content of this dissertation. The Graduate College will ensure this dissertation is globally accessible and will not permit alterations after a degree is conferred.

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DEDICATION

To my parents, Christine and Timothy Hall, who have given me a lifetime of unconditional love, made me laugh, and helped me leap when I needed it. Without you, this degree would not have been possible. My partner, Rebecca Morgan Kropp, has been an unwavering light along this journey. Thank you for keeping me strong and believing in me when I didn't believe in myself. I admire your strength daily, I love you.

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ABSTRACT

North American insect pollinators have experienced significant population declines in the last decade. Multiple factors are associated with these declines, including pesticide use and loss of foraging habitat. Declines in wild bees, managed honey bees, and other flower-visiting insects, including monarch butterflies (*Danaus plexippus*) in the North Central United States (U.S.), has triggered research on effective conservation methods within agroecosystems dominated by maize and soybean. Pesticide exposure is likely, given the spatial-temporal overlap of pesticide use and utilization of habitat by pollinators. There is, however, limited understanding of the potential risk of pesticides to pollinators populating restored habitat located within and adjacent to conventional agriculture fields. Characterizing pesticide risk to pollinators within these habitats requires an understanding of the chemicals and species-specific exposure pathways. Quantification of species-specific exposure levels is needed to characterize risks based available toxicity data. This dissertation reports research undertaken to assess bee and monarch pesticide exposure within established pollinator habitat (prairie strips), including milkweed (*Asclepias* spp.) and a diversity of other blooming forbs, and the broader Iowa agricultural landscape. These data were subsequently used to advance refined pesticide risk characterization for these species of conservation concern.

To undertake the research described in this dissertation, new and innovative analytical methodologies, liquid chromatography tandem mass spectrometry (LC-MS/MS), gas chromatography with an electron capture detector (GC-ECD), enzyme-linked immunosorbent assays (ELISA) and QuantiGene, were developed and evaluated for quantification of both chemical pesticides and dsRNA.

Exposure data for five insecticides (chlorpyrifos, clothianidin, imidacloprid, lambda cyhalothrin and thiamethoxam) and two fungicides (azoxystrobin and pyraclostrobin) were characterized for pollen and nectar collected within prairie strips and pollen and nurse bees collected from honey bee colonies located within prairie strips and at margins of maize and soybean fields. Neonicotinoids (clothianidin, imidacloprid, thiamethoxam), commonly used as seed treatments, were prevalent in soil and milkweed plant tissue samples, but were detected less frequently in pollen and nectar collected from prairie strips. Comparison of these measured concentrations to available species-specific toxicity data indicates that neonicotinoid exposure poses little or no dietary risk to monarch larvae or honey bees foraging in this habitat. Azoxystrobin (a strobilurin fungicide) and chlorpyrifos (an organophosphate insecticide) were the most commonly detected pesticides in pollen collected from honey bee colonies. Honey bee colonies experienced more frequent acute exposure events from foliar applied pesticides as compared to the neonicotinoids, which are primarily used as seed treatments. The temporal trends of periodic foliar pesticide exposure were consistent with applications for pests in maize and soybean. Based on pollen concentrations, these exposures are unlikely to cause adverse effects to honey bees; however, a complete risk characterization is not possible due to the lack of information on pesticide concentrations in nectar. Using an extensive toxicity data base for monarch butterfly larvae, chronic exposure to neonicotinoids on milkweed is unlikely to cause adverse effects on survival and development. However, acute exposure to pyrethroid and organophosphate insecticides following foliar applications are likely to cause high mortality rates downwind of treated fields. After a comprehensive review of the literature, including a comprehensive evaluation of exposure data, there is limited empirical data available to assess risks of foliar-applied insecticides to other lepidopteran species of conservation concern.

This dissertation also evaluates the toxicity of double-stranded RNA (dsRNA) to the monarch butterfly. dsRNA technology has the potential to provide more selective insecticides as compared to conventional, chemical insecticides. Results from this investigation suggest the monarch may be recalcitrant to dsRNA-based insecticides.

Pesticide risk assessments, supported by high-quality exposure characterizations, can help determine the conservation risks and benefits of establishing pollinator habitat in close proximity to crop fields. The development of new analytical methods provided the means to quantify conventional and biological insecticides in a diverse set of environmental matrices. These analytical techniques will help support future monitoring studies of contaminants within the environment. Future studies with increased sampling frequency and with more diverse sets of matrixes (e.g., plant tissue, nectar, bee wax, and honey) will improve estimates of foliar-applied pesticide exposure to honey bee hives placed within or adjacent to crop fields and support more refined conservation risk-benefit analyses.

Keywords: Pollinators, Honey bee (*Apis mellifera*), Wild bee (Hymenoptera: Apoidea), Monarch butterfly (*Danaus plexippus*), Lepidoptera, Prairie strips, Pesticides, Neonicotinoid, Organophosphate, Pyrethroid, Strobilurin, Exposure assessment, Conservation, Double-stranded RNA, ELISA, LC-MS/MS

CHAPTER 1. GENERAL INTRODUCTION

Background

In North America several pollinators and flower visiting insects (anthophiles) have experienced population declines, including monarch butterflies (*Danaus plexippus*), honey bees (*Apis mellifera*) and certain native bee species (Hymenoptera: Apoidea) (Potts *et al.*, 2010; Thogmartin *et al.*, 2017). Potential causes for these declines include habitat loss (e.g. loss of food sources); pesticide, predominately insecticide, use; parasites and diseases (Goulson *et al.*, 2015; Stenoien *et al.*, 2018; Forister *et al.*, 2019; U.S. Fish and Wildlife Service, 2020). In the north central states, including Iowa, these declines have triggered the development of conservation practices that can be integrated into intensive agricultural landscapes (Koh *et al.*, 2016). Conservation practices such as prairie strips (CP-43) have been shown to support wild bee communities, increase plant biodiversity and support managed honey bee colony productivity (Schulte *et al.*, 2017; Kordbacheh *et al.*, 2020; Zhang, 2020; Murray, 2021). There is, however, concern that establishment of pollinator habitat in close proximity to intensive row cropping systems could result in increased exposure to insecticides, herbicides, and fungicides, and create ecological sinks (Mullin *et al.* 2010; Botias *et al.* 2015; Botias *et al.* 2016; Topping *et al.*, 2015; Uhl and Brühl, 2019; U.S Fish and Wildlife Service, 2020; Topping *et al.*, 2020). Improved risk characterization of pesticide exposure to pollinator habitat in agricultural settings is necessary to understand the conservation costs and benefits of establishing new habitat within row-crop dominated landscapes.

Characterization of pesticide risks requires toxicological data for species of concern and exposure data in relevant matrices for the north central cropping system. We have focused on generating a robust exposure data set for commonly used insecticides and fungicides that have

been shown to cause direct and indirect effects in pollinators. More specifically, we quantified exposure pathways for five insecticides to monarchs and bees, encompassing three modes of action, and two fungicides, encompassing one mode of action, in soil, plant tissue, pollen, nectar, and bee bodies. These compounds were chosen because they are used to manage a wide variety of pests and pathogens in maize and soybean, which are the major crops grown in the north central U.S. during periods when both monarch larvae and bees would be present within the landscape (e.g. monarch larvae feeding on contaminated milkweed; bees foraging on contaminated nectar and/or pollen) (Mullin *et al.*, 2010; Botias *et al.*, 2015; Krishnan *et al.*, 2020). The insecticides that were analyzed included lambda-cyhalothrin (a pyrethroid) and chlorpyrifos (an organophosphate), which are commonly deployed as foliar insecticides in maize and soybean (Hodgson *et al.*, 2012; Dunbar *et al.*, 2016). Samples were also analyzed for clothianidin, imidacloprid, and thiamethoxam (neonicotinoids), which are registered for both foliar and seed treatment use in maize and soybean (Hodgson *et al.*, 2012, Douglas and Tooker, 2015; Tooker *et al.*, 2017). Strobilurin fungicides, specifically azoxystrobin and pyraclostrobin, are commonly applied to soybean and maize (US Geological Survey, 2019a; US Geological Survey, 2019b). Recent research has suggested some fungicides may enhance the toxicity of co-applied insecticides to honey bees (Tsvetkov *et al.*, 2017).

Without robust monitoring data, pesticide exposure characterization in risk assessments relies on spray drift models (AgDRIFT; US Environmental Protection Agency, 2011), which often use conservative estimates in screening level risk assessments. In the case of seed treatments, there are no models available to predict neonicotinoid concentrations in plants next to crop fields. The research reported in this dissertation advances refined pesticide exposure characterizations to support pollinator risk assessments.

Outlines and Objectives of the Dissertation Chapters

This dissertation reports research undertaken to characterize pesticide exposure to monarch larvae, wild bees and honey bees to help inform habitat conservation efforts in the north central U.S. This research effort included the development of innovative analytical methods to quantify a diversity of conventional and biological pesticides in water; soil; plant foliage, nectar and pollen; and bees. Using these analytical methods, studies were completed to quantify pesticide exposure in field and laboratory settings. In the course of this research, I evaluated and integrated results from these environmental monitoring studies, as well as previously published monitoring and toxicity studies reported in peer-reviewed literature, to develop screening-level risk characterizations for bees and lepidopteran species of conservation concern. Finally, I co-led a monarch larval risk analysis for a new insecticide mode of action.

Chapter 2: Optimization of LC-MS/MS Method for Simultaneous Quantification of Neonicotinoid Residues in Plant Matrices

Application of robust analytical chemistry methods are required to characterize neonicotinoid exposure concentrations in complex environmental samples. Accurate quantification of neonicotinoid insecticide exposures in pollen and milkweed leaf tissue supports robust characterizations of risks to bees and monarch larvae, respectively. In this chapter, I describe a single extraction and quantitation method developed for a suite of neonicotinoids (clothianidin, imidacloprid, and thiamethoxam) and two imidacloprid metabolites (5-OH imidacloprid and imidacloprid olefin). A single method to quantify these compounds increases sample throughput time while also increasing sensitivity to detect these compounds more efficiently and accurately than currently published protocols. The method was modified as needed to support the quantification of neonicotinoids and additional pesticides that were the

subject of research presented in Chapters 3, 4, and 5. This Chapter was published in the journal *Molecules*.

Chapter 3: Quantification of Commonly Used Seed Treatment Insecticides in Milkweed and Other Non-Crop Plants Sampled from Restored Prairie Implemented in Soybean and Maize

Decline in the abundance and diversity of pollinators and flower-visiting insects is associated with a loss of habitat and exposure to pesticides. Efforts to reverse these declines include restoring native habitat in agricultural landscapes. By embedding habitat within working lands, there is a risk that insecticide exposure could limit the means of achieving conservation goals. Neonicotinoids applied to maize and soybean seeds can be transported from crop fields to adjacent habitat through dust drift during planting and/or through overland runoff or subsurface flow following planting. Bees (e.g. Apidae) and monarch larvae could be exposed to neonicotinoids through ingestion of contaminated pollen or milkweed leaves, respectively. Neonicotinoids have previously been detected in pollinator-attractive habitats near crop fields; however, the magnitude and seasonal variation of the concentrations of these insecticides has not been reported. In chapter 3, we quantified concentrations of clothianidin, imidacloprid, and thiamethoxam in soil and forb leaves, including milkweed (*Asclepias spp.*), collected from prairie strips throughout the growing season. We then compared the concentrations detected in milkweed leaf tissue to available chronic dietary toxicity data for monarch larvae to characterize risks associated with feeding on milkweed plants within prairie strips. Chapter 3 has been accepted for publication in the journal *Agriculture, Ecosystems and the Environment*.

Chapter 4: Assessing Honey Bee Hive Exposure to Pesticide Mixtures in Iowa's Landscape

Establishment of pollinator-attractive habitat within agricultural ecosystems can support increased biodiversity and honey bee colony productivity. There is, however, concern that pollinator habitat established close to conventional crop fields may be exposed to pesticides and

create an ecological trap for honey bees and other pollinators. Insecticide exposure to bees can cause lethal and sublethal effects. Depending on the fungicide class, exposure to bees can impact their gut microbiome and synergize the toxicity of some insecticides. Chapter 4 reports the results of two pesticide monitoring surveys. The first survey assessed native forbs in prairie strips exposure to systemic neonicotinoids by quantifying concentrations of clothianidin, imidacloprid, and thiamethoxam in nectar and pollen. In the second survey, honey bee hive exposure was assessed by quantifying concentrations of neonicotinoid, organophosphate, and pyrethroid insecticides and strobilurin fungicides in pollen and honey bees collected from hives located within prairie strips or roadside ditches adjacent to conventional maize or soybean fields. These data were used to characterize pesticide risks to honey bees using pollinator habitat in close proximity to in crop fields. Chapter 4 is being prepared for submission as a manuscript to *Agricultural Science and Technology*.

Chapter 5: Investigation of an Enzyme-Linked Immunosorbent Assay (ELISA)-Based Method to Quantify Neonicotinoid Insecticides in Water and Plant Tissues

Neonicotinoids are one of the most widely deployed insecticides globally due to their use as seed treatments in agricultural production. These compounds have been detected in both terrestrial and aquatic habitats. Extensive monitoring studies are needed to further understand their fate and transport in the environment. In chapter 5, the use of an enzyme-linked immunosorbent assay (ELISA) to quantify neonicotinoids in water and plant tissue is evaluated as an alternative to liquid chromatography tandem mass spectrometry (LC-MS/MS). LC-MS/MS, although very effective in quantifying neonicotinoids, is an expensive approach and requires highly trained personnel. The monetary and personnel costs associated with this method can constrain the number of samples that can be processed, which in turn can place limitations on monitoring study designs and the means to accurately assess neonicotinoid concentrations

across relevant spatial and temporal scales. In this chapter we compare ELISA methods to LC-MS/MS with regard to sensitivity and specificity. Chapter 5 is currently being prepared for submission as a manuscript to *Environmental Toxicology and Chemistry*.

Chapter 6: Assessing Screening Level Risk of Insecticide Exposure to Lepidopteran Species of Conservation Concern in North Central U.S.

Chapter 6 presents a screening-level risk characterization for lepidopteran species of conservation concern found within the north central U.S. Chapters 3 and 4 illustrate an approach to characterize risk associated with pesticide exposure to bee pollinators and the monarch within north central U.S. agroecosystems. Toxicity testing and exposure monitoring data is not, however, generally available for other insect species, especially butterflies of conservation concern in the north central states. Risk estimates for these species is based on data reported in Chapters 3 and 4, publications led by colleagues at Iowa State University, and an evaluation of data reported in environmental monitoring and toxicity studies published in the peer-reviewed literature. Chapter 6 addresses the utility of existing insecticide residue data to estimate species-specific larval host plant exposure. Based on available lepidopteran toxicity data, we developed Species Sensitivity Distribution (SSD) models for topical exposures to pyrethroid and organophosphate insecticides; inadequate data sets were available for other classes of insecticides and dietary exposures. Using the generated SSD models with the available exposure data, we explored potential insecticide risks associated with establishing non-target lepidopteran habitat in agricultural landscapes. We also discuss the types of exposure and toxicity data needed to generate additional SSD models and reduce uncertainties in model predictions. This research has been published as a peer-reviewed book chapter in ‘*Crop Protection Products for Sustainable Agriculture*’ as part of the *American Chemical Society Symposium Series* Vol. 1390.

Chapter 7: Assessing Toxicity of an Emerging Insecticide Technology to the Monarch Butterfly

As discussed in chapters 1 through 6, the main classes of insecticides currently used in agriculture for plant protection include neonicotinoids, organophosphates and synthetic pyrethroids, which are all considered to have a broad spectrum of activity across target and non-target insect species. In recent years, the scientific community has begun addressing the use of RNA interference (RNAi) and CRISPR/Cas9 as potential insect control technologies. In chapter 7, we evaluate the toxicity of an RNAi-based insecticide designed to manage the varroa mite (*Varroa destructor*) to monarch butterfly larvae. The dsRNA was hypothesized to cause adverse effects to the monarch larvae due to the base pair overlap between the mite and monarch nucleotide sequences. The concentration of varroa mite double-stranded RNA (dsRNA) applied to milkweed leaves consumed by monarch larvae was quantified using a specialized analytical method. Monarch larvae showed a lack of mortality and sublethal effects following dietary exposure to the varroa dsRNA, suggesting monarchs may be refractory to silencing by dsRNA. As more dsRNA products are developed and enter the marketplace, there will be an increased need for research looking at their potential adverse effects to beneficial insects and their quantification in various matrices. Chapter 7 gives insights into how the potential risks of this newly emerging technology can be addressed in monarch larvae and other non-target butterflies. This chapter has been published in *PLOS One*.

Chapter 8: General Conclusions

The final chapter of this dissertation synthesizes research findings presented in chapters two through seven and outlines application of these findings to habitat conservation practices and suggests future research to address remaining uncertainties.

Contributions of Dissertation

The research reported in this dissertation has improved understanding of the potential risks of pesticide exposure to pollinator habitat in agricultural settings. Research results indicate that systemic uptake of neonicotinoid insecticides by non-target plants within prairie strips is a potential exposure pathway for monarch larvae and foraging bees. However, the concentrations detected are well below the acute and chronic thresholds for honey bees and monarch larvae, suggesting little or no risk anticipated from these formulations. The dissertation describes results from survey studies that quantify foliar pesticide exposures, which support more refined risk characterizations than previously possible. Foliar applied insecticide applications are likely to result in pulsatile exposure to pollinator habitats. While acute and chronic risks of concern to honey bees from these exposures is unlikely, additional research is needed to understand impacts on wild bee communities and on honey bee colonies when exposed to multiple applications of pesticide mixtures.

These pesticide risk assessments, supported by high quality exposure characterizations, can help determine the conservation risks and benefits of establishing habitat in close proximity of crop fields. The development of new analytical methods provided methods for quantifying conventional and biological insecticides in a diverse set of environmental matrices. These analytical techniques will help support future monitoring studies of contaminants in the environment. Future studies with increased frequency of sampling with more diverse sets of matrixes (e.g. plant tissue, nectar, bee wax and honey) would improve understanding of exposure of honey bee colonies placed within or adjacent to crop fields to foliar applied pesticides and support more refined conservation risk-benefit analyses.

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CHAPTER 2. OPTIMIZATION OF QuEChERS METHOD FOR SIMULTANEOUS DETERMINATION OF NEONICOTINOID RESIDUES IN POLLINATOR FORAGE

Maura J. Hall¹, Viet Dang², Steven P. Bradbury^{1,3} and Joel R. Coats¹

¹ Department of Entomology, Iowa State University, 2007 ATRB, Iowa State University, Ames, IA 50011, USA; mjhall@iastate.edu (M.J.H); spbrad@iastate.edu (S.P.B.)

² Department of Veterinary Diagnostic and Production Animal Medicine, Veterinary Diagnostic Laboratory, Analytical Chemistry Services Section, 1850 Christensen Drive, Iowa State University, Ames, IA 50011; vdang.duc@gmail.com

³ Department Natural Resource Ecology and Management, 339 Science II, Iowa State University, Ames, IA 50011, USA

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Abstract

Consistent with the large-scale use of pesticide seed treatments in U.S. field crop production, there has been an increased use of neonicotinoid-treated corn and soybean seed over the past decade. Neonicotinoids can move downwind to adjacent off-field pollinator habitats in dust from planting and/or move downslope to habitats in surface water. The extent of potential neonicotinoid exposure to pollinators from neonicotinoid movement into these adjacent pollinator habitats is unclear. Pollen and leaf tissue extractions were completed using a quick, easy, cheap, effective, rugged, and safe (QuEChERS) extraction procedure. Samples were subjected to a clean-up step using dispersive solid-phase extraction (dSPE) techniques prior to analysis. The compounds in the extracts were separated on a reversed-phase column with gradient elution and confirmed with tandem mass spectrometry. The extraction method showed acceptable recoveries of analytes ranging from 78.4% to 93.6% and 89.4% to 101% for leaf tissue and pollen, respectively. The method's detection limits ranged from 0.04 to 0.3 ng/g in milkweed leaf tissue and 0.04 to 1.0 ng/g in pollen. The method is currently being employed in

ongoing studies surveying pollen from a diversity of forbs and milkweed leaves obtained from habitat patches established within fields with a history of using neonicotinoid-treated seeds.

Introduction

Since neonicotinoids entered the market in the 1990s, they have become the fastest-growing class of insecticide worldwide [1,2]. Due to their wide-scale use as seed treatments, as well as foliar applications, neonicotinoids are now the most widely used class of insecticide in the world [3]. Their effectiveness against a broad spectrum of sucking and chewing pests and their unique mechanism of action have made them a commonly used group of insecticides in modern crop protection [4]. Neonicotinoids are synthetic compounds designed to act as agonists in the nicotinic acetylcholine receptors in the insects' central nervous system, causing paralysis and death [5,6]. Imidacloprid, clothianidin, and thiamethoxam act systemically due to their relatively high water solubility (0.61, 0.34, and 0.41 g/L, respectively) [7]. Any insecticide that has widespread use can potentially have nontarget impacts on mammals, birds, and other vertebrates, as well as on nontarget insects and other invertebrates. The United State Environmental Protection Agency (U.S. EPA) does not consider there to be risks of concern for human health via dietary (i.e., food and drinking water consumption), residential, or bystander exposure to imidacloprid [8], clothianidin [9], or thiamethoxam [9]. Ecological risk assessments have been crucial for informing the registration of neonicotinoid insecticides. Potential toxicity to nontarget vertebrates has been summarized by Gibbons et al. [10] and Hladik et al. [6]. Neonicotinoids in surface water can also have impacts on invertebrates [6]. When formulated in seed treatments, these insecticides can be taken up by the roots of a plant and translocated throughout the stem, leaves, flowers, and pollen [5,6]. Studies have documented the presence of neonicotinoids in pollinator habitats; however, the extent to which exposures are within the range that produces detrimental effects in monarch butterfly larvae, honey bees, and native bees is

unclear [11–16]. Efficient, multi-analyte residue analyses are needed to develop an accurate understanding of neonicotinoid exposure levels in order to achieve a better understanding of the potential effect of these insecticides on pollinators [5,6].

The objective of this project was to develop a fast and precise single extraction and analysis method for the three commonly used neonicotinoids (clothianidin, imidacloprid, and thiamethoxam) and two metabolites (5-OH-imidacloprid and imidacloprid olefin) in a pollinator-relevant matrix (see <https://www.mzcloud.org> for structures and fragmentation schemes). The goal of developing this method was allow for more effective exposure-monitoring studies to take place in pollinator habitats where potential exposure could occur.

Results

2.1. UHPLC-MS/MS Method Optimization

To optimize multiple reaction monitoring (MRM) transitions for the individual compounds, standard solutions at 500 µg/L combined with 50:50 mobile phases A and B (1:1 v/v) were infused into the mass spectrometer at 10 µL/min. Each compound was examined under two different ionization techniques, ES⁺ and ES[−], to achieve optimal sensitivity and selectivity. The best results were obtained using ES⁺ mode for parent compounds and two metabolites. Two MRM transitions were chosen for each analyte: one for quantitation and a second transition for confirmation.

The two MRM transitions used for each analyte with the optimized mass spectrometry (MS) parameters are presented in Table 1. Several experiments were performed to evaluate chromatographic conditions, and better results were obtained with gradient elution settings with a flow rate of 300 µL/min. Neonicotinoids and metabolites were separated using the Accucore aQ column, with Retention Time (RT) ranging from 3.5 to 5.5 min. The typical MRM chromatograms of five compounds in spiked blank plant tissue are depicted in Figure 1.

2.2. Optimization of Sample Preparation

Clean-up of environmental samples is essential to minimize impairment of the analytical equipment and to eliminate matrix interference in the mass analyzer. Reliable clean-up is challenging with plant tissue and pollen samples due to the presence of pigments and lipids. Two major advancements for sample clean-up are the use of a quick, easy, cheap, effective, rugged, and safe (QuEChERS) procedure and dispersive solid phase extraction (dSPE). QuEChERS and dSPE are simple and robust techniques for the extraction and clean-up of analytes in different matrices, including biological tissues, food products, and environmental matrices [13,14,17].

2.2.1. Plant tissue matrix

To date, extraction of neonicotinoids from plant leaf tissues, specifically milkweed leaf tissue, is labor-intensive and involves complicated clean-up steps [16,18–21]. These methods use various components, such as Celite, C₁₈ cartridges, concentration steps, sodium chloride, anhydrous magnesium sulfate, filtration, and solvent exchange. By optimizing QuEChERS extraction and dSPE we have developed a method that reduces labor costs, variability, and the use of solvents [13,15,17,22].

To assess this approach with leaf tissue, we compared two commonly used QuEChERS methods. One method involved adding 5 g of milkweed powder into a 50 mL QuEChERS extraction tube containing 4 g MgSO₄, 1 g NaCl, 1 g trisodium citrate dehydrate, and 0.5 g sodium citrate. The other method used 50 mL QuEChERS extraction tubes containing 4 g MgSO₄ and 1 g NaCl. Samples, including blank and calibration standards, were spiked with internal standard mixture solution, and eight control samples were also spiked with an analyte mixture solution to make calibration standards. The samples were then extracted and cleaned up using dispersive solid-phase extraction (dSPE) containing 150 mg MgSO₄, 25 mg primary secondary amine (PSA), and 7.5 mg graphitized carbon black (GCB) (data not shown). The

extracts from each method were analyzed by liquid chromatography tandem mass spectrometry (LC-MS/MS). There was a clear correlation between a decrease in response for all analytes of interest when the 1 g trisodium citrate dehydrate and 0.5 g sodium citrate were not present. The 1 g trisodium citrate dehydrate and 0.5 g sodium citrate maintained the pH during the extraction, which improved the recovery. We thus moved forward using the QuEChERS extraction method of 4 g MgSO₄, 1 g NaCl, 1 g trisodium citrate dehydrate, and 0.5 g sodium citrate followed by dSPE containing 150 mg MgSO₄, 25 mg PSA, and 7.5 mg GCB.

2.2.2. Pollen matrix

Common practices for analyzing contaminants in pollen include the use of QuEChERS followed by clean-up using dSPE [14,17]. Most techniques require 1.0 g of pollen or more [17,22,23]. When working on field-level studies, it is often difficult to obtain 1.0 g of pollen. Hence, we adapted methods to quantify neonicotinoids in samples of 0.2 g or less. Given the low mass of our samples, we chose to use dSPE to remove complex compounds found within the pollen. Spiked pollen extract (1 mL) was transferred into a 1.5 mL micro-centrifuge tube containing 150 mg MgSO₄, 25 mg PSA, and 7.5 mg GCB. The mixture was thoroughly vortexed for 1 min and centrifuged at 6500 rpm for 5 min. The same procedure was also performed with a 1.5 mL micro-centrifuge tube containing 150 mg MgSO₄, 50 mg PSA, and 50 mg C₁₈, as well as with a 1.5 mL micro-centrifuge tube containing 150 mg MgSO₄, 50 mg PSA, 50 mg GCB, and 50 mg C₁₈. In both dSPEs containing GCB, there was a substantial decrease in recovery for all analytes. However, the most significant decrease in recovery was for imidacloprid 5-hydroxy and imidacloprid olefin (up to 60%). We thus moved forward with the dSPE containing 150 mg MgSO₄, 50 mg PSA, 50 mg GCB, and 50 mg C₁₈, injecting 2 µL of the crude extract into the LC-MS/MS to minimize interfering compounds that can hamper method sensitivity. In addition, we

used isotopically labeled internal standards for each of the analytes to correct for recovery throughout the extraction and analysis processes.

2.3. Method Validation

Identification of the five analytes of interest was accomplished by comparing the retention time, peak shape, and ion ratio between solvent standards and sample spikes [24]. A total run time of 8 min was used for the separation of analytes (Figure 1). The performance of the LC-MS/MS method was validated using standard solutions spiked into control samples, sample blanks, and Quality Control (QC) samples. Linearity, matrix effects, method detection limit, precision, and recovery were examined.

2.3.1. Evaluation of linearity

Linearity for the five compounds was examined by analyzing eight calibration standards. Calibration curves were constructed by plotting the corresponding peak area ratios of analytes/internal standards against the concentration ratios of analytes/internal standards. The matrix-matched calibration curves obtained using simplified QuEChERS procedures were linear over the concentration range for the five analytes in pollen and plant tissue. Linearity, tested using the least-square regression method, gave a correlation coefficient (r^2) greater than 0.980 in all the linear ranges.

2.3.2. Evaluation of the method detection limit

Method detection limit (MDL) was estimated using the lowest concentration for which percentage relative standard deviation (%RSD) was less than or equal to 15%. Once that level had been determined, MDL was calculated using the formula $S \times 3.143$, where S is the standard deviation of the calculated concentration among 7 replicates and 3.143 is the value for Student's t-test for 6 degrees of freedom. The MDLs for the five analytes of interest ranged from 0.04 to 0.3 ng/g in milkweed leaf tissue (Table 2) and from 0.04 to 1.0 ng/g in pollen (Table 3).

2.3.3. Evaluation of recovery

The recovery (extraction efficiency) was calculated by dividing the peak area of an analyte from a pre-extraction spiked sample by the peak area of an analyte from a post-extraction spiked sample. The extraction recoveries ranged from 85.4% to 93.6% for milkweed leaf tissue (Table 2) and from 89.4% to 101% for pollen (Table 3).

2.3.4. Evaluation of trueness and precision

Intra-assay trueness [25] and precision were determined by analyzing three replicates of QC samples in a single LC-MS/MS run, while inter-assay trueness and precision were determined by analyzing four replicates of QC samples on two or more different days. The concentrations of QC samples were determined using calibration standards prepared on the same day. The assay trueness, presented as percentage, was calculated using the following equation: $\text{trueness} = \text{mean of calculated concentration/actual concentration} \times 100$. The assay precision was determined by the relative standard deviation (%RSD) of the measured concentrations. The intra- and inter-day precision (%RSD) for the five analytes were within 20% of the reference values. The trueness of the method for milkweed leaf tissue ranged from 90.0% to 109% for the low QC level and from 78.4% to 103% for the high QC level (Table 2). The trueness of the method for pollen ranged from 93.6% to 111% for the low QC level and from 92.9% to 108% for the high QC level (Table 3).

Discussion

Our LC-MS/MS method is a sensitive, standardized, and labor-effective technique for analyzing pollen and milkweed leaf tissue, which are dietary sources for bees and monarch butterfly larvae, respectively. The proposed method allows for the quantification of the compounds in a single run at sub-ng/g concentrations using a faster and/or more sensitive method than those found in the literature [12–14,17,22,23,26–28]. The method can quantify

specific analytes in a mixture of neonicotinoids at trace levels in small quantities of milkweed leaf and pollen to facilitate exposure assessment for honey bees, native bees, and monarch butterflies [12,18,19,29].

Pollinators are high-profile non-target organisms that may be exposed to neonicotinoids at levels of concern. The three active ingredients, clothianidin, imidacloprid, and thiamethoxam, are classified as highly toxic to bees, while little is known about their toxicity when monarch larvae are exposed to them [6,29]. Milkweed plant tissue and pollen taken from plants and bees located in close proximity to crop fields that are known to have had neonicotinoid seed treatment are key matrices to evaluate pollinator exposure levels. To accurately, precisely, and efficiently measure these levels, a robust multi-analyte method with a low MDL is needed. The method reported here is being used to analyze 500 plant tissue samples and 600 pollen samples. The adaption of these methods into other laboratories can help support the standardization of analytical techniques used to consistently evaluate neonicotinoid exposure for non-target organisms across research efforts.

Materials and Methods

4.1. Standards, Reagents, and Solvents

A neat standard of imidacloprid (CAS 138261-41-3, 98.8% pure), thiamethoxam (CAS 153719-23-4, 95.2% pure), clothianidin (CAS 210880-92-5, 99.6% pure), imidacloprid-olefin (CAS 115086-54-9, 97.9% pure), and 5-OH-imidacloprid (CAS 380912-09-4, 96.7% pure) were received as a gift from Bayer CropScience (Research Triangle Park, NC, USA). Deuterated internal standards clothianidin-d₃ and thiamethoxam-d₃ were obtained from Sigma-Aldrich (St. Louis, MO, USA). Imidacloprid olefin-¹³C₃, ¹⁵N and imidacloprid-pyr-d₄-methyl-d₂, ¹³C were received from Bayer CropScience, and 5-OH-imidacloprid-¹³C, ¹⁵N was received from Clearsynth (Mississauga, Ontario, Canada). Organic solvents (Optima LC-MS grade methanol,

water, and acetonitrile), ammonium formate (99% pure), and 99% pure formic acid were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Fifty-milliliter QuEChERS tubes (part number 60105-216) and dispersive solid-phase extraction (dSPE) equipment (part number 60105-202; 60105-222; 60105-223) were obtained from ThermoFisher Scientific.

4.2. Spiking Solution Preparation

Stock solutions (0.5 mg/mL) of individual standards and internal standards were prepared by dissolving 5 mg (corrected for salt and purity) in 10 mL solvent (e.g., acetonitrile, dimethylformamide, methanol, or dimethyl sulfoxide). Dilutions of the stock solutions were prepared in acetonitrile for spiking pollen (0.005 to 0.05 ng/ μ L) and leaf tissue (0.2 ng/ μ L). Internal standard solutions were prepared in acetonitrile at a concentration of 0.4 ng/ μ L. Working solutions of analytes and internal standards were stored at $-200\text{ }^{\circ}\text{C}$ and were freshly prepared monthly.

4.3. Leaf Sample Preparation

Unexposed “control” common milkweed (*Asclepias syriaca*) leaves were obtained from Iowa State University (Ames, IA, USA) greenhouses with no history of neonicotinoid use (as noted in the method validation, levels were below the method detection limit). Leaf samples were stored at $-80\text{ }^{\circ}\text{C}$ prior to extraction. On the day of extraction, samples were pulverized using a blender with a small amount of dry ice. The leaf powder was then placed in a fume hood to sublimate the remaining dry ice. The leaf powder was extracted following a generic QuEChERS method with some modifications [13]. In brief, approximately 5 g of powder was weighed into a 50 mL QuEChERS extraction tube (Thermo Fisher, catalog number 60105-216) containing 4 g MgSO_4 , 1 g NaCl, 1 g trisodium citrate dehydrate, and 0.5 g sodium citrate. Samples, including blank, QC levels, and calibration standards, were spiked with 100 μ L of an internal standard mixture solution (8 ng/g sample). Eight control samples were also spiked with

an analyte mixture solution (0.2 ng/ μ L) to make calibration standards of 0.2, 0.5, 1, 2, 5, 10, 20, and 40 ng/g samples. Two QC levels (low = 1 ng/g and high = 20 ng/g) in triplicates were also included. Samples were solvent extracted with 10 mL of LC-MS-grade acetonitrile, followed by vortexing for 30 s and shaking on a multi-tube shaker for 10 min at 2500 rpm. The samples were then centrifuged for 6 min at 3700 rpm. After centrifugation, 1 mL of supernatant was transferred into a 2 mL dSPE tube (Thermo Fisher, catalog number 60105-222) containing 150 mg MgSO₄, 25 mg primary secondary amine (PSA), and 7.5 mg graphitized carbon black (GCB), after which it was vortexed for 1 min. The sample tubes were subsequently centrifuged for 5 min at 5000 rpm. The supernatants (~700 μ L) were filtered using a 0.45 μ m filter and transferred into amber autosampler vials prior to LC-MS analysis.

4.4. Pollen Sample Preparation

Pesticide-free pollen (Buzzy Bee; purchased from Amazon) was analyzed for background levels of neonicotinoids before the spiking tests, and it was found that levels were below the method detection limits. The milkweed leaf sample extraction method was modified for low-mass pollen samples collected during monitoring studies. In brief, approximately 0.2 g of pollen was weighed into a 2 mL prefilled tube kit containing high impact zirconium beads of 1.5 mm diameter (Benchmark, catalog number D1032-15).

Samples were extracted with 0.3 mL of water and then shaken on a multi-tube shaker for 5 min at 2500 rpm. Acetonitrile (1.2 mL) was then added to all samples, followed by shaking on a multi-tube shaker for 5 min at 2500 rpm. The samples were then centrifuged for 5 min at 6000 rpm. After centrifugation, 1 mL of supernatant was transferred into a 2 mL dSPE tube (Fisher Scientific, catalog number 03150625) containing 150 mg MgSO₄, 50 mg PSA, and 50 mg C₁₈ and then shaken on a multi-tube shaker for 2 min at 2500 rpm. The sample tubes were

centrifuged for 5 min at 6000 rpm. The supernatant (300 μ L) was transferred into an amber autosampler vial with an insert prior to LC-MS analysis.

4.5. LC-MS Conditions

The LC-MS/MS consisted of a Vanquish Flex UHPLC system, including a binary pump, autosampler, and column heater compartment, and a TSQ Altis triple quadrupole mass spectrometer equipped with heated electrospray source (Thermo Fisher Scientific, San Jose, CA).

Chromatographic separation was carried out on an Accucore aQ column (100 \times 2.1 mm, 2.6 μ m; Thermo Fisher Scientific). The column was maintained at 30 $^{\circ}$ C. The mobile phase consisted of water:methanol (95:5 v/v) containing 0.1% formic acid and 5 mM ammonium formate (A) and methanol:water (95:5 v/v) containing 0.1% formic acid and 5 mM ammonium formate (B). The elution gradient was held at 0% B for the first 0.5 min, increased from 0% to 80% B from 0.5 to 6 min, held at 80% B from 6 to 8 min, decreased from 80% to 0% B from 8 to 9 min, and held at 0% B for 1 min. The flow rate was 0.3 mL/min for the duration of the run. Injection volume was 2 μ L. The needle wash was a mixture of water:methanol (80:20 v/v).

The MS ionization source conditions were optimized via direct infusion of standard solutions into the mass spectrometer. The mass spectrometer was operated in positive ion heated electrospray ionization mode. The electrospray voltage was set at 3700 V for positive mode. Nitrogen was used as a sheath gas (30 arb), auxiliary gas (6 arb), and sweep gas (1 arb). Argon was used as a collision gas. Ion transfer tube and vaporizer temperatures were set at 325 and 350 $^{\circ}$ C, respectively. Acquisition was performed in selected reaction monitoring (SRM) mode, and two or three main transitions were monitored for each compound (supporting information can be found in Table 1). Data analysis was performed on TraceFinder 4.1 software (Thermo Fisher Scientific,).

Conclusions

We were able to develop a single extraction and quantitation method for a suite of neonicotinoids that are commonly used as seed treatments for corn and soybean (clothianidin, imidacloprid, and thiamethoxam) and two imidacloprid metabolites (5-OH-imidacloprid and imidacloprid olefin) in a pollinator-relevant matrix. Analysis of leaf tissue and pollen is essential to allow for reasonable estimates of exposure for monarch larvae and bees. Since collection of these samples is resource intensive, it is critical to develop efficient and accurate extraction and quantification methods. Currently, sample preparation in combination with LC-MS/MS for neonicotinoid quantification in plant tissue and pollen has been limited to more intensive extraction methods with high-mass samples and longer LC-MS/MS runs [14,16–19,22,23,30]. To address these limitations, we developed a single extraction and analytical method for multiple neonicotinoids from milkweed leaf tissue and pollen. Our method's performance is comparable to, and in some cases superior to, existing methods. The method will support more cost-effective monitoring studies that improve understanding of the spatiotemporal variation of these compounds within agro-ecosystems. The method we report could be evaluated and adapted as needed to support the quantification of multiple neonicotinoid concentrations in animal tissues and other environmental matrices relevant to human health, aquatic life, and wildlife risk assessments.

Author Contributions

Conceptualization, V.D., J. R. C., S. P. B., and M. J. H.; methodology, M. J. H. and V.D.; software, M. J. H. and V. D.; validation, M. J. H. and V. D.; formal analysis, M. J. H. and V.D.; investigation, M. J. H. and V. D.; resources, J. R. C., S. P. B., and M. J. H.; data curation, M. J. H. and V. D.; writing—original draft preparation, M. J. H.; writing—review and editing, V. D., J. R. C., S. P. B., and M. J. H.; visualization, M. J. H. and V. D.; supervision, V. D., J. R. C., and

S. P. B.; project administration, J. R. C. and S. P. B.; funding acquisition, J. R. C. and S. P. B..

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Conflicts of Interest

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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Tables and Figures

Table 1. Select reaction monitoring (SRM) table of three neonicotinoids and their metabolites.

Compound	Retention Time (min)	Precursor (m/z)	Product (m/z)	Collision Energy (V)	RF Lens (V)
Imidacloprid olefin	4.08	254	171	17.2	46
Imidacloprid olefin	4.08	254	205	13.8	46
Imidacloprid	4.41	256	175	19.5	41
Imidacloprid	4.41	256	209	15.4	41
Imidacloprid	4.41	256	212	10.2	41
Clothianidin	4.42	250	113	26.2	34
Clothianidin	4.42	250	169	14.3	34
Thiamethoxam	3.88	292	132	21.9	34
Thiamethoxam	3.88	292	181	22.3	34
Thiamethoxam	3.88	292	211	10.2	34
5-OH-imidacloprid	3.96	272	134	41.2	53
5-OH-imidacloprid	3.96	272	191	19.1	53
5-OH-imidacloprid	3.96	272	225	15.0	53
Imidacloprid olefin- 13C ₃ ,15N	4.08	259	176	17.2	43
Imidacloprid olefin- 13C ₃ ,15N	4.08	259	211	10.2	43
Imidacloprid olefin- 13C ₃ ,15N	4.08	259	241	10.2	43
Imidacloprid-pyr-d4- CH ₃ -d ₂ , ¹³ C	4.39	260	179	18.6	47
Imidacloprid-pyr-d4- CH ₃ -d ₂ , ¹³ C	4.39	260	213	16.1	47
Imidacloprid-pyr-d4- CH ₃ -d ₂ , ¹³ C	4.39	260	214	10.5	47
Clothianidin-d ₃	4.41	253	132	16.6	44
Clothianidin-d ₃	4.41	253	172	12.7	44
Thiamethoxam-d ₃	3.88	295	184	22.4	45
Thiamethoxam-d ₃	3.88	295	214	11.7	45
5-OH-imidacloprid-d ₄	3.95	276	195	19.7	51
5-OH-imidacloprid-d ₄	3.95	276	229	13.3	51

Table 2. Method detection limit (MDL), recovery, trueness, and matrix effect for five target analytes in a common milkweed leaf matrix.

Compound ID	MDL (ng/g)	Recovery (%)	Trueness	
			low QC	high QC
Thiamethoxam	0.04	93.6	90.1	100
5-OH-imidacloprid	0.1	78.4	96.6	78.4
Imidacloprid olefin	0.1	90.2	109	98.1
Imidacloprid	0.2	86.7	90.0	103
Clothianidin	0.3	85.4	90.0	100

Table 3. Method detection limit (MDL), recovery, trueness, and matrix effect for five target analytes in the pollen matrix.

Compound ID	MDL (ng/g)	Recovery (%)	Trueness	
			low QC	high QC
Thiamethoxam	0.04	101	99.7	95.5
5-OH-imidacloprid	0.3	89.4	95.8	100
Imidacloprid olefin	1.0	94.5	111	108
Imidacloprid	0.06	99.7	93.6	95.0
Clothianidin	0.06	95.8	96.0	92.9

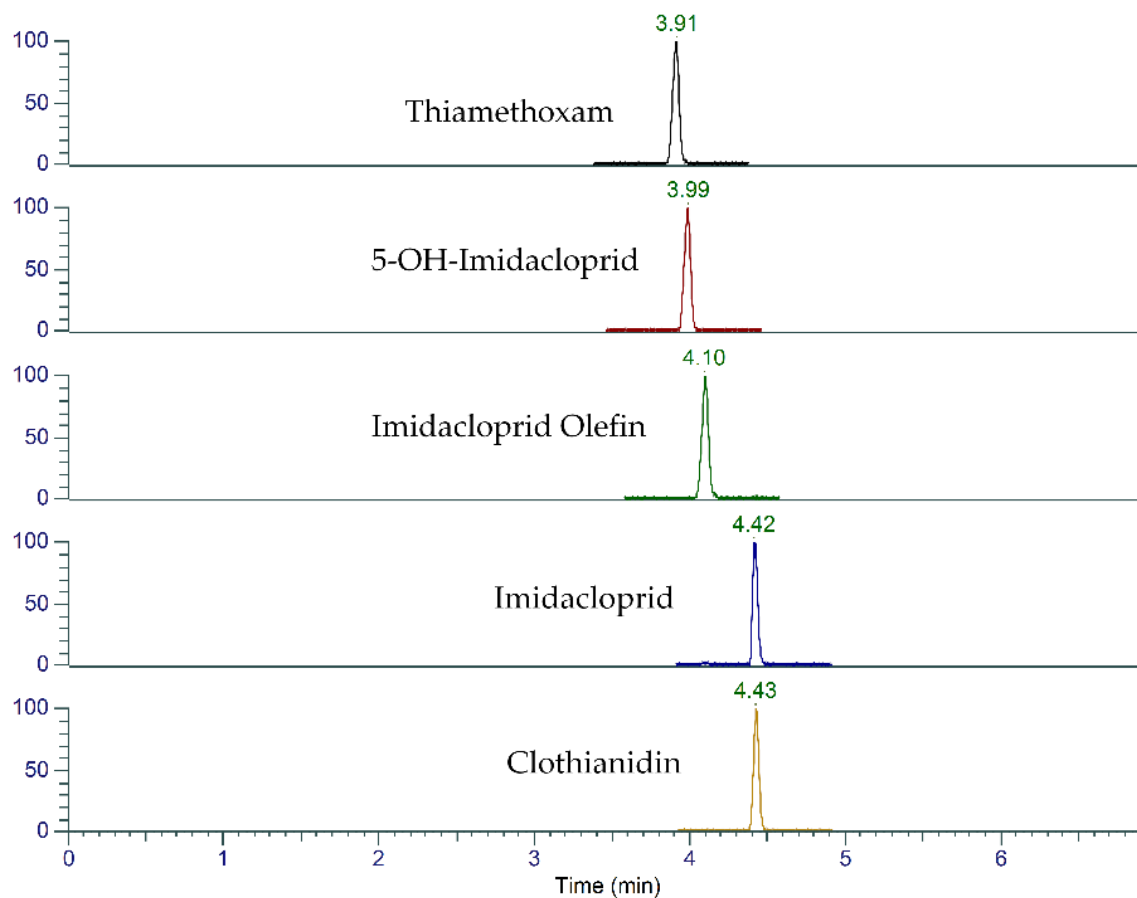


Figure 1. The liquid chromatography tandem mass spectrometry (LC-MS/MS) chromatogram of five neonicotinoids spiked into milkweed leaf tissue extract at 40 ng/g. Across the chromatographic peak, 10 to 12 data points were obtained.

CHAPTER 3. QUANTIFYING NEONICOTINOID INSECTICIDE RESIDUES IN MILKWEED AND OTHER FORBS SAMPLED FROM PRAIRIE STRIPS ESTABLISHED IN MAIZE AND SOYBEAN FIELDS

Maura J. Hall^a, Ge Zhang^{a1}, Matthew O’Neal^a, Steven P. Bradbury^{a,b}, Joel R. Coats^a

^a Iowa State University, Department of Entomology, Ames, IA 50011, and ^b Natural Resource Ecology and Management, Ames, IA 50011

¹ ‘Present address’ Washington State University, Department of Entomology, Pullman, WA 99163

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Abstract

Adding habitat within agricultural ecosystems is necessary to reverse declines in pollinator abundance and diversity. Understanding pesticide exposure to pollinator habitat near crop fields is necessary to support risk assessments. Neonicotinoids applied to maize and soybean seeds can be transported from crop fields to adjacent habitat through dust drift during planting and/or through overland runoff or subsurface flow following planting. Pollinators, especially bees (e.g. *Apidae*) and monarch butterflies (*Danaus plexippus*), could be exposed to neonicotinoids through ingestion of contaminated plant material (e.g. milkweed leaves). Neonicotinoids have been detected in pollinator-attractive habitats near crop fields; however, the magnitude and seasonal variation of the concentration of these insecticides has not been determined. We quantified concentrations of clothianidin, thiamethoxam, and imidacloprid in soil and forb leaf tissue, including milkweed (*Asclepias spp.*) located within reconstructed prairies (3-4 years post-establishment) within or adjacent to maize or soybean fields. Samples taken from April through August in 2017 and 2018 were analyzed with liquid chromatography-tandem mass spectrometry; 100% of soil, 80% of vegetation from blooming forbs, and 80% of milkweed leaf tissue samples had at least one neonicotinoid present above the method detection

limit (0.1 to 0.9 ng g⁻¹). The maximum concentrations detected in 2017 or 2018 of clothianidin, thiamethoxam, and imidacloprid in milkweed leaf tissue samples were 6.6, 12.9, and 2.8 ng g⁻¹, respectively. These values are 10 to 130-fold lower than the chronic LC₁₀ values for monarch larvae, indicating it is unlikely that this route of neonicotinoid exposure will cause adverse effects to monarch larvae.

Introduction

Decline in the abundance and diversity of pollinators is associated with a loss of habitat, floral resources, and exposure to pesticides (Goulson et al., 2015; Stenoien et al., 2018; Forister et al., 2019). Efforts to reverse these trends include restoring native habitat that has been converted to agriculture (Isaacs et al., 2009). By embedding habitat for pollinators within working lands, there is a risk that insecticide exposure could limit the means of achieving this conservation goal. This challenge is most noticeable in the North Central United States (U.S.A.), which has experienced a historic shift from tallgrass prairie and mixed farming into high-yielding monocultures (Plourde et al., 2013). For example, 85% of Iowa is committed to agriculture, and 64% of that area is used for annual crop production (Brown and Schulte, 2019; USDA NRCS, 2019). Conversion of grasslands to monocultures is associated with a decrease in biodiversity, including declines in beneficial insects, including pollinators (Koh et al., 2016; Sánchez-Bayo and Wyckhuys, 2019).

The North Central U.S.A. has been identified as a critical region for declines in wild bees and a high priority for monarch butterfly (*Danaus plexippus*) conservation efforts (Flockhart et al., 2017; Koh et al., 2016; USFWS, 2020). To help maintain a resilient eastern North American monarch population, an estimated 1.3 to 1.6 billion additional milkweed stems (*Asclepias spp.*) need to be added to the North Central U.S.A. landscape (Thogmartin et al., 2017). To meet this and other conservation goals for the North Central U.S.A., habitat for

pollinators and other flower visiting insects needs to be added into agricultural landscapes. These efforts will need to encompass the conversion of marginal cropland, roadsides, and grassy areas bordering maize and soybean fields (Thogmartin et al., 2017).

Insecticides are an important tool for farmers as part of an integrated pest management program for growing maize and soybean. The U.S.A. Fish and Wildlife Service (2020) has identified insecticide exposure as a threat to the recovery of the North American monarch population. Insecticides applied to seeds and foliage of crop fields may contribute to pollinator decline and/or potentially undermine conservation efforts by contaminating the forage used by bees and adult monarchs, which often feed on wildflowers within the landscape (Botías et al., 2016, 2015; Thogmartin et al., 2017). This exposure can affect many pollinators, including generalists like honey bees (*Apis mellifera*), that feed on a wide variety of flowering plants throughout the growing season. More specialized insects, like monarchs, could be exposed in multiple ways, including exposure to the adults who, like bees, feed on flowers. These insects could be exposed to spray drift following the application of an insecticide applied to crop foliage. Monarch larvae can also be exposed to insecticides through the consumption of milkweed leaves contaminated by the uptake of insecticides, like the neonicotinoids that are applied to maize and soybean seeds (Halsch et al., 2020; Krishnan et al., 2020; Olaya-Arenas and Kaplan, 2019).

Neonicotinoid insecticides, specifically thiamethoxam, imidacloprid, and clothianidin, are used extensively in the North Central U.S.A. (Douglas et al., 2020; Douglas and Tooker, 2015; Hladik et al., 2014; USGS, 2014). Neonicotinoids act as agonists at nicotinic acetylcholine receptors and are active against a broad spectrum of insects (Hladik et al., 2018). Neonicotinoids are systemic insecticides and are frequently applied to seeds of a variety of crops, including maize and soybeans (Douglas and Tooker, 2015; Krupke et al., 2017). Neonicotinoids applied to

seeds can be transported from maize and soybean fields to adjacent habitat by the dust produced at planting and/or through surface/subsurface flow following precipitation events (Berens et al., 2020; Botías et al., 2016; Goulson and Kleijn, 2013; Hladik et al., 2017; Schaafsma et al., 2018; Stewart et al., 2014). Neonicotinoids applied to crops have been found in pollinator-attractive habitats planted adjacent to crop fields (Botías et al., 2016, 2015; Halsch et al., 2020; Main et al., 2020; Olaya-Arenas and Kaplan, 2019; Pecenka and Lundgren, 2015) and in pollen collected by honey bees (Botías et al., 2015; Long and Krupke, 2016). Furthermore, imidacloprid, clothianidin, and thiamethoxam have been detected in Iowa streams, presumably, due to overland or subsurface flow (Frame et al., 2021; Hladik et al., 2017, 2014; Thompson et al., 2021), raising concern that non-target plants down-slope of crop fields could systemically take up neonicotinoids. If conservation programs are going to successfully restore pollinator abundance and diversity within agricultural landscapes using neonicotinoids, a more thorough understanding of the spatial and temporal patterns of neonicotinoid uptake by non-target plants is needed.

The addition of prairie strips into agricultural land is a newly adopted practice by the conservation reserve program (CP-43) that increases the abundance and diversity of beneficial insects (Cox et al., 2014; Schulte et al., 2017), especially that of native pollinators (Kordbach et al., 2020). However, due to the close proximity to crop fields prairie strips may expose pollinators to neonicotinoids (Botías et al., 2016, 2015; Hladik et al., 2017; Main et al., 2020). This practice is based on strategically replacing a portion of a crop field (~10%) with a diverse mixture of native, flowering, perennial vegetation (i.e. prairie). Replacing 10% of a cropped field with a prairie strip along a contour or as a buffer before a body of water can yield disproportionate benefits for improving soil and water quality (Schulte et al., 2017; Zhou et al.,

2010). When applied to fields committed to maize and soybean production, prairie strips reduced runoff by 37% and retained 4.3 times more phosphorus and 3.3 times more nitrogen than fields without prairie strips (Schulte et al., 2017; Zhou et al., 2010). Offsite transport of neonicotinoids through groundwater and surface water was reduced with prairie strips (Hladik et al., 2017). Furthermore, prairie strips increased biodiversity, including native plants, birds, and beneficial insects, including native bees (Kordbach et al., 2020; Schulte et al., 2017). Additionally, when these prairie strips include milkweed species, they can serve as breeding habitat for monarch butterflies.

As noted above, there is concern that habitat used by pollinators located within agricultural fields, like prairie strips, could expose pollinators to neonicotinoids. Plants growing in contaminated soil could systemically take up residual neonicotinoids resulting in exposure to bees and adult monarchs foraging on blooming flowers and monarch larvae feeding on milkweed plants down-slope of the agricultural field (Botías et al., 2016, 2015; Halsch et al., 2020; Hladik et al., 2017; Krishnan et al., 2020; Olaya-Arenas and Kaplan, 2019). To better quantify the potential neonicotinoid exposure to pollinators in North Central U.S.A. agroecosystems, we designed a study on commercial farms with prairie strips that included sampling soil, plant tissue, pollen, nectar, and bees foraging on flowering plants from early spring through the summer. The analyses of pollen and nectar collected by bees (*A. mellifera* and *Bombus* spp.) within these prairie strips will be the subject of a future manuscript. This paper summarizes the analysis of soil and plant foliage, including milkweed, which are used to explore the potential for adverse effects to monarch larvae.

Materials and Methods

2.1. Sampling Locations

We selected established prairie strips with flourishing forage for pollinators and adult monarchs. Sites were selected in consultation with the Science-based Trials of Rowcrops Integrated with Prairie Strips (STRIPS; <https://www.nrem.iastate.edu/research/STRIPS/>) project that has helped over 200 farmers establish prairie strips consistent with the CP-43 practice. A subset of farms was selected based on the year of prairie strip establishment (Table 1), conventional production practices, including the use of neonicotinoid-treated seeds, and proximity to the Iowa State University campus (maximum distance 150 km). The sampled prairie strips average widths and lengths were ~10 m and ~330 m, respectively. Seven sites were selected in 2017 (see Fig. 1 for site location); four planted with maize; three were planted with soybeans (both crops sown between May 8th and May 22nd). Nine sites were sampled in 2018; four were planted with maize and five with soybean (crops sown between April 29th and May 24th). Five of the farms were the same in 2017 and 2018.

Farmers followed conventional practices typically used in this region, including a rotation of maize following soybeans, use of herbicide-tolerant crop varieties, pesticide-treated seeds, and application of pesticides during the growing season. A survey describing the use of neonicotinoids-treated seeds by farmers participating in this study is provided in Table 1. The sampled prairie strips were established between two to three years prior to the first sampling to ensure ample forage for pollinators (Table 1). Prairies were sown with a variety of forbs and grasses (Table A1). The sites had a high percentage of grass species to decrease soil erosion and slow water flow (Hirsh et al., 2013). Hectares of prairie strips within the crop field for each site are provided in Table 1.

2.2. Sample Collection

Soil and plant samples were collected six times during the 2017 growing season and four times during the 2018 growing season. Samples were collected from April 11th through August 15th. These time periods were selected to assess prairie strip exposure to neonicotinoids prior to, during, and after planting.

When possible, samples were collected after a regional rain event to capture the potential movement of neonicotinoids from the crop to a down-slope prairie strip. All samples were labeled with contents (soil, plant species) and the date of sampling. Samples were stored in a -20°C freezer immediately upon return to the laboratory.

2.2.1. Soil samples

We collected 85 soil samples over the two years (2017, 2018). Soil cores (15-cm depth, 5-cm diameter) were collected 30 cm into the down-slope edge of a prairie strip adjacent to the crop field (Fig. A1). We collected five samples per location per sampling period. Samples were pooled prior to analysis. The five samples were 2 to 5 m apart along a 10 to 25 m transect. Soil samples were taken from the same transect at each sampling period. On-site, the five samples were pooled in a plastic bag and labeled with farm site and date of sampling. Sample weights were recorded in the lab (measured with a calibrated, tared balance). Representative soil samples from each field site were analyzed by Agvise Laboratories (Northwood, ND) for % sand, % silt, % clay, USDA textural class (hydrometer method), bulk density (disturbed), cation exchange capacity, % moisture at 1/3 bar, % moisture at 15 bar, % organic matter (Walkey-Black), pH (water), buffer pH (Adams-Evans), % total nitrogen, and Olsen phosphorus (Table A2 and A3).

2.2.2. Leaf tissue samples

Leaf tissue samples were collected 0 to 30 cm into prairie strips down-slope from the crop field (Fig. A1). When plants were available, samples were taken approximately every 5 – 15

m along the length of the strip. If there were multiple prairie strips at a site, the same strip was sampled at each time point. Leaves were taken from the bottom, middle, and top of a plant to ensure a representative sample. Throughout the study, we focused on collecting samples from forbs in bloom (native and non-native), as well as common milkweed (*A. syriaca*) and butterfly milkweed (*A. tuberosa*), regardless of their flowering status. Foliage was collected from non-milkweed species to assess differences in residues across species. Plants in bloom were sampled. Milkweed samples were collected regardless of flowering status because monarch larvae feed directly on the foliage. When analyzing these results, we separated plant data into two categories; milkweed species and other plant species labeled “forbs”.

Eighteen plant species were collected (Table 2). Black-eyed Susan (*Rudbeckia hirta*) and golden alexanders (*Zizia aurea*) were the most commonly collected forbs comprising 26.7% and 19% of the total forbs sampled (n=105), respectively.

2.3. Neonicotinoid Analysis

2.3.1. Soil

Pooled soil samples were homogenized in the laboratory. An aliquot of soil samples (5 ± 0.1 g) was spiked with 4 ng of the deuterated pesticide standards (clothianidin-d₃, thiamethoxam-d₃, imidacloprid olefin-¹³C₃, ¹⁵N and imidacloprid-pyr-d₄-methyl-d₂, ¹³C, and 5-OH imidacloprid- ¹³C, ¹⁵N) and extracted. Samples were extracted by adding 12 mL of acetonitrile and vortexing for 30 sec, followed by shaking for 10 min at 2500 rpm. Samples were then centrifuged at 3700 rpm for 5 minutes. A 1.5-mL aliquot of the supernatant was transferred to a dSPE tube (Thermo Fisher, Catalog# 60105-222) containing 150 mg MgSO₄, 25 mg primary secondary amine (PSA), and 7.5 mg graphitized carbon blackened (GCB) and vortexed for 1 minute followed by centrifugation for 5 minutes at 6000 rpm. The supernatants (~700 µL) were

transferred into amber autosampler vials prior to LC-MS analysis. An aliquot of 1.5 g of each wet soil sample was dried for 24 h at 105 °C to determine the water content.

2.3.2. Leaves

Leaf tissue extraction followed a previously published method (Hall et al., 2020). Samples were homogenized using a blender with a small amount of dry ice. Approximately 4-5 g of plant tissue were placed into a 50-ml QuEChERS extraction tube (Thermo Fisher, catalog# 60105-216) containing 4 g MgSO₄, 1 g NaCl, 1 g trisodium citrate dehydrate, and 0.5 g sodium citrate. Samples were spiked with an internal standard mixture solution. Samples were extracted with 10 ml of LC-MS grade acetonitrile, followed by vortexing for 30 sec and shaking on a multi-tube shaker for 10 min at 2500 rpm. The samples were then centrifuged for 6 min at 3700 rpm. After centrifugation, a portion of supernatant was transferred into a 2-ml dSPE tube (Thermo Fisher, catalog# 60105-222) containing 150 mg MgSO₄, 25 mg PSA, and 7.5 mg GCB. The tubes were then vortexed for 5 min at 5000 rpm. The supernatants were filtered using a 0.45- μ m filter and transferred into amber autosampler vials prior to LC-MS analysis.

2.3.3. LC-MS/MS analysis

Extracts were analyzed by a Vanquish Flex ultra-high-performance liquid chromatography system (UHPLC) coupled to a TSQ Altis triple quadrupole mass spectrometer (MS-MS) equipped with a heated electrospray source (ThermoFisher Scientific, San Jose, CA). The instrument settings are as described by Hall et al. (2020). Five compounds: imidacloprid, clothianidin, thiamethoxam, 5-OH imidacloprid, and imidacloprid olefin were measured. The method detection limit (MDL; USEPA, 1997) of the compounds ranged from 0.07 ng g⁻¹ to 0.9 ng g⁻¹ for soil; 0.1 ng g⁻¹ to 0.3 ng g⁻¹ for plant tissue.

2.3.4. QA/QC

Quality control samples were used to validate concentrations of neonicotinoids, including method blanks, matrix blanks, standard replicates, quality control replicates, and soil and milkweed leaf recovery samples. The calibration curve and quality control replicates were completed in blank matrix matches. No compounds were detected in any of the blanks. Standards (7 samples) had relative percentage differences (RPDs) between replicate samples of <20%. Standards were analyzed at least twice during each LC-MS/MS sequence, once prior to sample analysis and once following sample analysis. Quality control samples at three concentrations were analyzed in each sequence; RPDs between the nominal and measured concentrations were <20%. Matrix spike recoveries ranged from 70 to 94%. Soil and leaf data presented here were not recovery-corrected.

2.4. Data Analysis

The median, min, max, and % detection of each neonicotinoid in soil and plant leaf tissue samples were reported. Non-detects were set at one-half of the method detection limit (MDL), hence the calculated median concentrations are conservative. We compared the yearly and monthly medians calculated for each insecticide from soil data using ANOVA. We used a repeated measures linear mixed model to compare concentrations of compounds in the soil across differing sample dates and locations. The model included sampling date and specific neonicotinoid-treated seeds planted in the sampling year and an interaction between these two terms. Site was considered a random effect, and a separate model was constructed for each year and compound. Residual plots for all soil data showed an increase in variance with concentration; consequently, data were log transformed to stabilize the variance. For the data summarizing the concentration of thiamethoxam in soil collected during 2017, the repeated measures linear mixed model resulted in a singular fit when using site as the random effect. To

correct for this, we used a Bayesian model to estimate the median concentrations for thiamethoxam from the 2017 soil data. Pairwise comparisons were made over site and month to evaluate difference in median concentrations of compounds at sites where it was applied versus where it was not applied. The Shapiro-Wilks Normality Test for all forb leaf tissue and milkweed leaf tissue data showed departures from normality. To determine if forb leaf tissue residues were different across sampling months within a year, the non-parametric Kruskal-Wallis Test was conducted. The same statistical analysis was conducted with the milkweed leaf tissue residue data. Data were analyzed with RStudio 1.1.383 (Ver 3.5.2) and SigmaPlot 14.0.

A dose addition model was used to calculate risk quotients (RQs) for plant samples with multiple neonicotinoids (Eq 1).

$$RQ = CCLO/LC10CLO + CIMI/LC10IMI + CTMX/LC10TMX \quad (1)$$

Where C is the concentration of the specified compound (clothianidin (CLO), imidacloprid (IMI), and thiamethoxam (TMX)) within the milkweed leaf tissue sample, and LC10 is the chronic lethal dose causing 10% mortality, as reported by Krishnan et al. (2021).

Results and Discussion

3.1. Soil

At least one neonicotinoid was detected in all soil samples collected in both years. Clothianidin was the most frequently detected neonicotinoid, found in 100% of soil samples analyzed (n=85). Imidacloprid and thiamethoxam were detected in > 90% of the soil samples. Thiamethoxam is a commonly used seed treatment, and its major breakdown product is clothianidin, which helps explain the frequency of clothianidin detected in our samples. These results are similar to those reported by Berens et al. (2020), who detected clothianidin in 100% of water samples obtained from streams and rivers in agricultural watersheds in Minnesota, U.S.A. (median clothianidin concentration 15 ng/L). The median concentration (ng g⁻¹) of clothianidin,

imidacloprid, and thiamethoxam in soil samples across all sample locations were 3.63, 1.03, and 0.29 in 2017 and 2.74, 1.21, and 0.24 in 2018, respectively (Table 3).

In 2017 imidacloprid was not used as a seed treatment at any of the sites sampled; however, it was detected at least once at every site. Detection of imidacloprid at sites where it was not used as a seed treatment could be due to spray drift from foliar applications in surrounding farms. Alternatively, we may have detected legacy imidacloprid residues from use prior to the start of this study. Botías et al. (2015) and Hladik et al. (2017) detected imidacloprid and clothianidin in soil taken from sites where treated seeds had not been planted for at least two years prior to sampling. These trends are possible due to the widespread use of neonicotinoids in the North Central U.S.A. and their persistence (Bonmatin et al., 2015; Douglas and Tooker, 2015; Goulson and Kleijn, 2013; Hladik et al., 2018). We expected the median residue concentrations of a given neonicotinoid to be higher at sites where the adjacent field was planted with seeds treated with the same compound compared to sites that were planted with a different neonicotinoid seed treatment. When such comparisons could be made, we saw no significant difference in median concentrations (Fig. 2), which may indicate accumulation of neonicotinoids in the crop fields over multiple growing seasons, consistent with reported soil degradation half-lives of 7 to >1000 days (AERU, 2020). In part, detection of neonicotinoids in soil samples are likely a result of a legacy of neonicotinoid use on the adjacent field and before the crop was converted to prairie (Hladik et al., 2017; Thompson et al., 2021).

There were no discernable temporal patterns in neonicotinoid concentrations. There was no significant difference between the median clothianidin, thiamethoxam, and imidacloprid soil concentrations across months (Fig. 3). Additionally, the variations in median concentrations are within the method variability.

The highest concentrations of clothianidin, imidacloprid, and thiamethoxam in 2017 were detected in May (11.6 ng g⁻¹), August (5.2 ng g⁻¹), and April (40.7 ng g⁻¹), respectively. In 2018 the highest concentrations were detected in May (18.1 ng g⁻¹), June (73.4 ng g⁻¹), and June (0.72 ng g⁻¹), respectively. Berens et al. (2020) attributed elevated early season concentrations of neonicotinoids in the water surrounding agricultural areas to runoff from spring storms that mobilized neonicotinoids from seeds planted in the current season, as well as neonicotinoid residues remaining in the soil from previous years. Frame et al. (2021) reported concentrations of thiamethoxam and clothianidin in surface flow pathways were greatest in May during the first two storm events after planting. However, thiamethoxam concentrations in subsurface waters also peaked in May, while clothianidin subsurface concentrations peaked in early July. Hladik et al. (2014) reported spikes in the concentrations of clothianidin, imidacloprid, and thiamethoxam in surface water during spring months following snowmelt prior to planting. These spikes were hypothesized to be a result of repeated use over multiple growing seasons.

3.2. Forbs

Forbs, excluding milkweed, contained at least one neonicotinoid in 81% of the samples taken in 2017 (n=33) and 80% taken in 2018 (n=72). Consistent with the soil samples, clothianidin was the most frequently detected compound in the foliage (Table 3). Detection of neonicotinoids in forbs through systemic uptake was expected based on their water solubility (340 – 4,100 mg/L at 20°C and pH 7) and minimal adsorption to soils (log K_{oc} 1.4 – 2.3) (AERU, 2020; Bonmatin et al., 2015; Goulson and Kleijn, 2013; Hladik et al., 2018).

The maximum concentrations detected for all three compounds occurred in the leaf tissue of golden alexander (1.8 ng g⁻¹ – 7.1 ng g⁻¹) (Table A4 and A5). Concentrations of neonicotinoids detected in flowering forbs ranged from <MDL ng g⁻¹ to 7.1 ng g⁻¹, with median concentrations from <MDL to 0.23 ng g⁻¹ (Table 3). Approximately 90% of the neonicotinoid detections were <

1 ng g⁻¹. Detection of neonicotinoids in forbs within prairie strips adjacent to crop fields that did not plant treated seed may reflect the persistence of these compounds within the soil, as noted previously. Detection of neonicotinoids in foliage indicates likely systemic uptake of neonicotinoids into non-target blooming plants; however, leaf residue levels should not be assumed representative of potential concentrations in nectar or pollen (USEPA/HCPMRA/CDPR, 2014).

Neonicotinoid leaf concentrations we report are within the same range as those reported by Botias et al. (2016) and Main et al. (2020). Botías et al. (2016) collected foliage samples from herbaceous and woody plants located 1-2 m from the field edge of oilseed rape (*Brassica napus*) planted with thiamethoxam–treated seeds. Botias et al. (2016) reported higher maximum concentration of clothianidin, thiamethoxam, and imidacloprid (11.45 ng g⁻¹ to 106.2 ng g⁻¹) in the leaves of wild herbaceous and woody plants than what was detected in our study; however, the medians were within the same order of magnitude (≤ 0.20 ng g⁻¹ to ≤ 0.20 ng g⁻¹). Main et al. (2020) sampled non-target vegetation, including wildflowers and flowering shrubs, along margins of row cropped fields in Missouri, U.S.A. They reported less frequent detections of neonicotinoids (<7% overall), with a maximum concentration of 9.8 ng clothianidin/g. Hladik et al. (2017) did not detect neonicotinoids (level of detection of 1 ng g⁻¹) in vegetative tissue from blooming forbs sampled in prairie strips located in crop fields that had not been planted with neonicotinoid-treated seeds for 2 years. Several factors can contribute to the different results across these studies, including variation in neonicotinoid seed concentration, seeding densities when crops were planted, soil types, frequencies and intensities of precipitation events, sampling time points, different non-target plants, and differences in detection limits.

3.3. Milkweed

Neonicotinoids were detected in 80% of the milkweed leaves collected from the prairie strips (n=358). Clothianidin was the most frequently detected neonicotinoid in 2017 (58%), while imidacloprid was the most frequently detected in 2018 (67%). In 2017 the highest median clothianidin and thiamethoxam concentrations per month across all sample locations were detected in June, 0.315 ng g⁻¹ and 0.12 ng g⁻¹, respectively (Fig. 5). Imidacloprid median concentrations were < 0.1 ng/g for all months sampled. In 2018 the highest median clothianidin, thiamethoxam, and imidacloprid concentrations per month across all sample locations were detected in July (0.285 ng g⁻¹), May (0.20 ng g⁻¹), and May (0.24 ng g⁻¹), respectively (Fig. 5). The maximum concentrations detected for clothianidin, thiamethoxam, and imidacloprid in milkweed leaf tissue samples (n= 360) in 2017 and 2018 were in June (6.64 ng g⁻¹), May (12.93 ng g⁻¹), and June (2.8 ng g⁻¹), respectively.

In 2017 and 2018, we observed a similar range of concentrations within leaves of milkweed (<MDL to 12.9 ng g⁻¹) and leaves of other forbs (<MDL to 7.1 ng g⁻¹). Median concentrations of neonicotinoids in leaves of milkweed and forbs also showed minimal difference (Table 3). Differences were seen in how frequently imidacloprid, thiamethoxam, and clothianidin were detected in milkweed leaf tissue as compared to other forbs. In 2017, the detection frequency of imidacloprid, thiamethoxam, and clothianidin in milkweed and forbs were within ≤ 10% of each other (Table 3). In 2018, the percentage detection of clothianidin, thiamethoxam, and imidacloprid in milkweed was > 20% of what was observed for other forbs for all three compounds (Table 3). We were unable to discern any statistical differences due to the relatively low number of non-milkweed plant samples and the small difference between the milkweed and non-milkweed median concentrations. While not statistically significant, variation in residue levels across plant species could be due to variation in neonicotinoid absorption.

Differences in plant physiology and morphology could affect uptake capacity, metabolism, or bioaccumulation of neonicotinoids (Botías et al., 2016; Castle et al., 2005).

Compared to other studies, our data typically shows more frequent detection of neonicotinoids in milkweed leaf tissue samples. Olaya et al. (2019) collected common milkweed in northwestern Indiana from a variety of sites across various distances from agricultural fields (0 – 2,398 m). They reported less frequent detections of clothianidin and imidacloprid ($\leq 8.1\%$ and $\leq 0.2\%$) and less frequent detections of thiamethoxam in 2015 (4.6%) with more frequent detections in 2016 (75.4%). Reported median concentrations of clothianidin ($< 1.060 \text{ ng g}^{-1}$), thiamethoxam ($\leq 1.44 \text{ ng g}^{-1}$), and imidacloprid ($< 0.640 \text{ ng g}^{-1}$) were within the same range as our results. The maximum concentrations of clothianidin, thiamethoxam, and imidacloprid detected (56.5 ng g^{-1} , 151.3 ng g^{-1} , and 3.7 ng g^{-1}) were, however, higher than what we report. Our sampling took place 0 to 30 cm from the edge of field, which could account for our high frequency of detection. The majority of our farmers confirmed no foliar application of neonicotinoids, which may explain the lower maximum detected concentration. Pecenka and Lundgren (2015) analyzed common milkweed leaves collected $1.47 \pm 0.39 \text{ m}$ from maize fields in Brookings County, SD shortly after planting. They reported a maximum concentration of 4 ng g^{-1} of clothianidin with a mean of $1.14 \pm 0.10 \text{ ng g}^{-1}$ per contaminated plant. These authors used ELISA for quantification of clothianidin. Kits used to quantify clothianidin (Product #500800, Abraxis, Warminster, PA) were also used to quantify imidacloprid, and the product manual notes cross-reactivity of the antigens. Therefore, it is possible that the clothianidin concentrations reported by Pecenka and Lungren (2015) reflect detection of clothianidin and imidacloprid.

Our results confirm that plants, including milkweed, growing in prairie strips down-slope of agricultural fields can contain neonicotinoids. The likely source of these neonicotinoid

residues is from seed treatment applications. Clothianidin, thiamethoxam, and imidacloprid comprise the majority of total neonicotinoids sales (~85%; Bass et al. 2015) and are commonly applied to seeds of maize and soybeans (Tooker, 2017). Although we cannot rule out other sources of exposure to the prairie strips, they are unlikely. Potential exposure could be due to dust drift at/shortly after planting; however, our data does not demonstrate early season/post plant spikes in neonicotinoid leaf concentrations. Alternatively, neighboring producers could have applied foliar neonicotinoid formulations in July or August to manage soybean aphid (*Aphis glycines*), and resultant spray drift could have contaminated milkweed sampled in the strips. This exposure route seems unlikely in Iowa, where pyrethroids and organophosphates are predominately used (Krishnan et al., 2021; USDA, 2019). Although registered for foliar applications, neonicotinoids are not commonly employed (Hodgson et al., 2012).

While our data provides a reasonable approximation of a high-end systemic exposure consistent with other papers (Botías et al., 2016; Halsch et al., 2020; Main et al., 2020; Olaya-Arenas and Kaplan, 2019; Stewart et al., 2014), likely due to surface/subsurface flow (Frame et al., 2021; Hladik et al., 2017, 2014; Thompson et al., 2021), future studies with increased sampling within and along strips from additional farms with different topography/hydrology/soil properties would improve understanding of spatial variability of neonicotinoid concentration in milkweed and other forbs. More intensive sampling of soil, soil runoff/subsurface water, and plant tissues prior to, during, and after rainfall events at different times post-planting would also improve understanding of temporal variation in milkweed and other forbs in prairie strips.

3.4. Potential Effects of Neonicotinoids on Monarch Larvae

Potential risk of neonicotinoids used as a seed treatment to pollinators is a function of both toxicity and exposure. For monarch larvae, the neonicotinoid concentrations within milkweed leaves serve as the basis for characterizing dietary exposure. Concentrations in

milkweed leaf tissue will be related to the plant's proximity to the crop field planted with neonicotinoid-treated seeds, the neonicotinoid concentration in the seed treatment, seed planting density, and environmental conditions. One pathway to milkweed exposure could be dust drift at planting time; a second could be subsurface water flow down-slope of fields planted with treated seeds (Frame et al., 2021; Hladik et al., 2017, 2014; Thompson et al., 2021). These pathways could have an acute or chronic effect on monarch larvae consuming contaminated milkweed tissue. To estimate the risk to monarch larvae residing within prairie strips, we compared the reported concentrations of neonicotinoids in milkweed to toxic effects thresholds obtained from published toxicity studies with monarch larvae.

Our data indicate that monarch larvae feeding on milkweed in a prairie strip are exposed to neonicotinoids at a range of concentrations during the summer. Monarch larvae that feed on the milkweed in these habitats could be exposed to milkweed leaf tissues with neonicotinoid residues as high as 8.0 ng g⁻¹ and 12.9 ng g⁻¹ in late May, which is prior to the predicted apex of larval abundance in Iowa (Krishnan et al., 2020). However, these maximum concentrations are well below acute oral LC₁₀ (lethal milkweed leaf concentration to 10% of a tested population) values for the most sensitive instars ranging from 270 to 2,400 ng g⁻¹ (Krishnan et al., 2020). It is, however, likely that larvae could be chronically exposed to variable concentrations of neonicotinoids over their lifetime. To more rigorously characterize potential risks it is necessary to compare the milkweed leaf concentrations to toxicity data reported in chronic dietary studies.

Within the peer-reviewed literature, Krishnan et al. (2021) is the only study that reports chronic toxicity data for clothianidin, thiamethoxam, and imidacloprid. Additional chronic dietary studies are reported for clothianidin (Bargar et al., 2020; Olaya-Arenas et al., 2020). Bargar et al. (2020) reported chronic LC_{50s} (lethal concentration to 50% of a tested population)

of 47 to 210 ng g⁻¹ swamp milkweed (*A. incarnata*) leaf, while Krishnan et al. (2021) reported a LC₅₀ of 74 ng g⁻¹ tropical milkweed (*A. curassavica*) leaf. Olaya-Arenas et al. (2020) reported 30% mortality at a clothianidin concentration of 57 ng g⁻¹ common milkweed leaf; Krishnan et al. (2021) reported 23% mortality at 57 ng g⁻¹ tropical milkweed leaf. Although these studies used different monarch colonies and milkweed species, they reported similar chronic toxicity data (Krishnan et al., 2021). Due to the similar sensitivity reported for clothianidin across all three studies and the inclusion of thiamethoxam and imidacloprid in the Krishnan et al. (2021) data sets, we used their chronic toxicity values to characterize risks from consumption of neonicotinoids in milkweed leaves at concentrations reported from this study

Krishnan et al. (2021) reported chronic dietary LC₁₀ values of 46 ng g⁻¹, 36 ng g⁻¹, and 420 ng g⁻¹ for clothianidin, imidacloprid, and thiamethoxam, respectively. The highest concentrations of neonicotinoids we detected in milkweed leaf tissue are 10 to 130-fold below these chronic LC₁₀ values (see Figs. 6 and 7).

Since the neonicotinoids typically co-occurred in milkweed leaf tissue samples, we characterized the risk of these mixtures assuming dose-addition, consistent with these compounds having the same mechanism of action (Casida and Durkin, 2013). The highest mixture RQ from this data set was 0.14, indicating that for those samples with multiple neonicotinoid residues, the combined concentrations were at least 7-fold lower than a dose expected to elicit 10% mortality in an exposed monarch population (Krishnan et al., 2021). The results indicate that while dietary neonicotinoid exposure to monarch larvae is likely from consumption of milkweed down-slope of fields planted with treated seeds, the level of exposure is not likely to affect monarch larvae adversely.

Conclusion

Neonicotinoids are highly water-soluble, and research has shown that the majority of their transport is expected to occur through subsurface and surface runoff (Berens et al., 2020; Bonmatin et al., 2015; Botías et al., 2016; Frame et al., 2021; Hladik et al., 2017, 2014; Thompson et al., 2021). This high water solubility makes them available for plant uptake (Hladik et al., 2017), and plants located down-slope of fields planted with maize, or soybean-treated seeds are a worst-case scenario for estimating systemic neonicotinoid exposure. Our results indicate that neonicotinoids from treated seeds planted in adjacent maize and soybean fields can move down-slope into habitat and are systemically taken up by non-target plants.

The median neonicotinoid levels in milkweed were ≥ 200 -fold lower than the chronic, dietary LC₁₀ values reported for monarch larvae, while the highest concentrations detected in the foliage of milkweed are 10 to 130-fold lower. Our data suggest systemic uptake of neonicotinoids in milkweed down-slope of fields planted with treated seeds is not expected to cause observable adverse effects to monarch larvae. Prairie strips have been shown to increase pollinator diversity and abundance (Kordbacheh et al., 2020; Schulte et al., 2017). When these prairies incorporate in milkweed, they can be a viable habitat for monarch larvae (Grant et al., 2021). These data indicate that even with continued use of neonicotinoid seed treatment as part of an integrated pest management program for the adjacent and surrounding crop field exposure to monarch larvae is below the threshold of concern.

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Data Availability

Upon publication, all data will be made available.

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Tables and Figures

Table 1. Farm codes, prairie strip history and attributes, soil characterization, cropping and neonicotinoid seed treatment history and years soil, forbs and milkweed were sampled at nine farms in central Iowa, USA.

Farm Code	Year Prairie Planted	Farm Hectares	Prairie Hectares	Soil Class ^a	% Organic Matter	Crop 2017	Crop 2018	A.I. 2017	A.I. 2018	Year(s) Sampled
GES	2015	48	1.2	Clay Loam	3.6	Maize	Soybean	TMX	None	2017 & 2018
MCN	2014	29	2	Clay	4.6	Maize	Soybean	TMX	IMI	2017 & 2018
DMW-N	2014	29	0.4	Silty Clay Loam	3.9	Soybean	Maize	TMX	CLO	2017 & 2018
DMW-S	2014	7	0.2	Silty Clay Loam	3.1	Soybean	Maize	TMX	CLO	2017 & 2018
SME	2014	24	0.8	Sandy Clay Loam	4.3	Maize	Soybean	CLO	None	2017 & 2018
GUT	2014	26	2.1	Sandy Loam	2.2	Maize	Soybean	None	TMX	2017 & 2018
WOR	2015	12	0.9	Sandy Clay Loam	2.9	Soybean	Maize	None	TMX	2017 & 2018
SMI	2015	8	1.6	Clay Loam	3.4	Maize	Soybean	N/A	IMI	2018
STO	2016	15	2.2	Silty Clay Loam	4.5	Soybean	Maize	N/A	TMX	2018

^a – Soil classification bases on USDA taxonomy

A.I. – active ingredient

N/A – Non-applicable indicating that site was not sampled that year.

None – Indicating seed formulation did not include a neonicotinoid insecticide

Table 2. Distribution of plant species collected at nine prairie strips from late April through August 2017 and 2018.

Sampling period	Plants common name (species name)
Pre-planting (Late April)	Dandelion (<i>T. officinale</i>), Milkweed
Planting (May)	Golden Alexander, Dandelion, White Clover (<i>T. repens</i>), Red Clover (<i>T. pratense</i>), Milkweed
Post-planting (June)	Beebalm (<i>Monarda fistulosa</i>), Black-eyed Susan, Golden Alexander, Red Clover, Purple Clover (<i>T. pratense</i>), Oxeye Sunflower (<i>H. helianthoides</i>), Thistle (<i>Cirsium discolor</i>), Vervain (<i>Prairie Verbena</i>), Coneflower (<i>Echinacea</i>), Milkweed
Post-planting (July)	Beebalm, Black-eyed Susan, Golden Alexander, Grey-Headed Cornflower (<i>Ratibida pinnata</i>), Oxeye Sunflower, Purple-Headed Coneflower (<i>Echinacea purpurea</i>), White Clover, Red Clover, Sweet Clover (<i>Melilotus officinalis</i>), Purple Clover, Oxeye Daisy (<i>Leucanthemum vulgare</i>), Queens Anne's Lace (<i>Daucus carota</i>), Oxeye Sunflower, Thistle, Milkweed
Post planting (August)	Grey-Headed Coneflower, Milkweed

*Throughout the sampling period we collected leaf samples from milkweed when available, regardless of flowering status.

Table 3. Summary of neonicotinoid residues in soil and plant tissues (forbs and milkweed) sampled in prairie strips at nine central Iowa, USA conventional farms in 2017 and 2018.

Matrix	Year	N		CLO	TMX	IMI	Olefin
Soil	2017	49	MDL (ng g ⁻¹)	0.1	0.07	0.2	0.9
			Detections (%)	100	91.8	91.8	0
			Median (ng g ⁻¹)	3.63	0.29	1.03	NA
			Range (ng g ⁻¹)	1.15 – 11.6	< MDL – 40.8	<MDL – 5.3	NA
	2018	36	Detections (%)	100	97.2	97.2	0
			Median (ng g ⁻¹)	2.74	0.24	1.21	<MDL
			Range (ng g ⁻¹)	0.78 – 18.1	< MDL – 0.7	<MDL – 73.4	<MDL
Plant Tissue			MDL (ng g ⁻¹)	0.1	0.1	0.1	0.3
Forbs	2017	33	Detections (%)	66.7	33.3	12.1	10.34
			Median (ng g ⁻¹)	0.23	<MDL	<MDL	<MDL
			Range (ng g ⁻¹)	<MDL – 1.2	<MDL – 3.8	<MDL – 0.27	<MDL – 5.19
	2018	72	Detections (%)	68.1	15.3	43.1	0
			Median (ng g ⁻¹)	0.2	<MDL	<MDL	NA
			Range (ng g ⁻¹)	<MDL – 4.5	<MDL – 7.1	<MDL – 1.8	NA
Milkweed	2017	26	Detections (%)	57.7	42.3	23.1	0
			Median (ng g ⁻¹)	<MDL	<MDL	<MDL	NA
			Range (ng g ⁻¹)	<MDL – 1.9	<MDL – 12.9	<MDL – 0.27	NA
	2018	332	Detections (%)	46.9	35	67	0
			Median (ng g ⁻¹)	<MDL	<MDL	<MDL	NA
			Range (ng g ⁻¹)	<MDL – 6.6	<MDL – 8.0	<MDL – 2.8	NA

N- Number of samples

CLO – clothianidin; TMX – thiamethoxam; IMI – imidacloprid; Olefin – imidacloprid-olefin

MDL – Method Detection Limit

Note: 5-OH-Imidacloprid was not detected in any samples

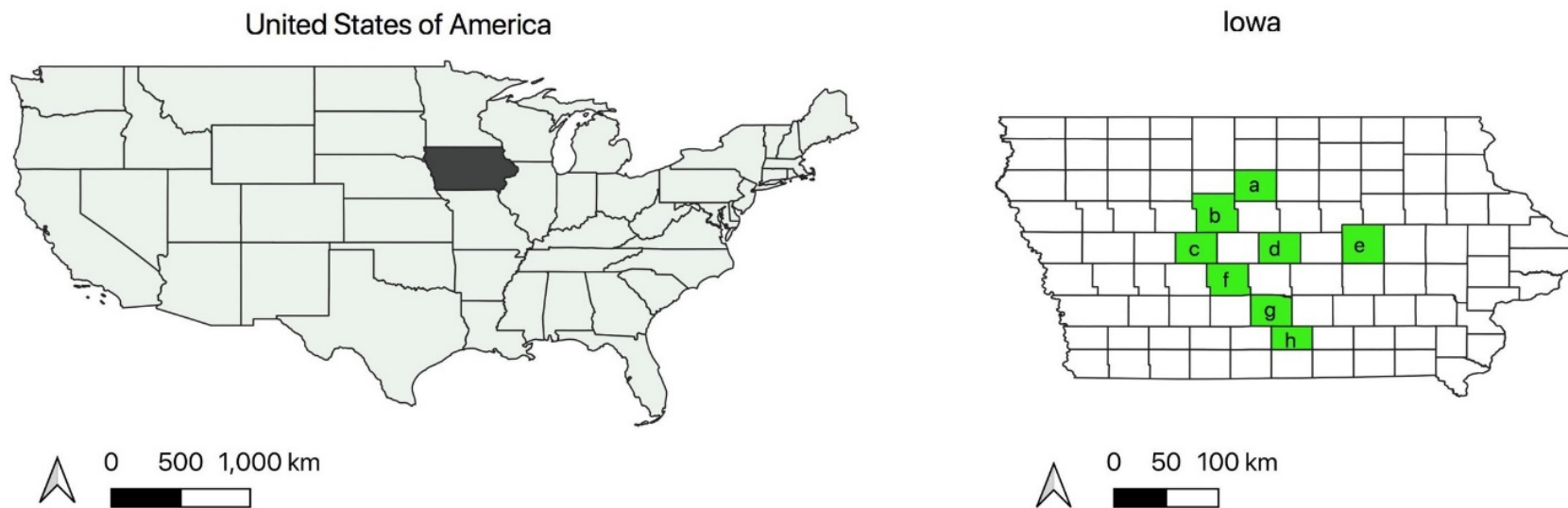


Fig. 1. Map of the United States highlighting the location of Iowa and a map of Iowa highlighting county locations of the study farm sites where prairie strips had been established 2 to 3 years prior to sampling soil and forb, including milkweed, leaves. County d contained two sampling sites. All other highlighted counties contained one (a-c, e-h).

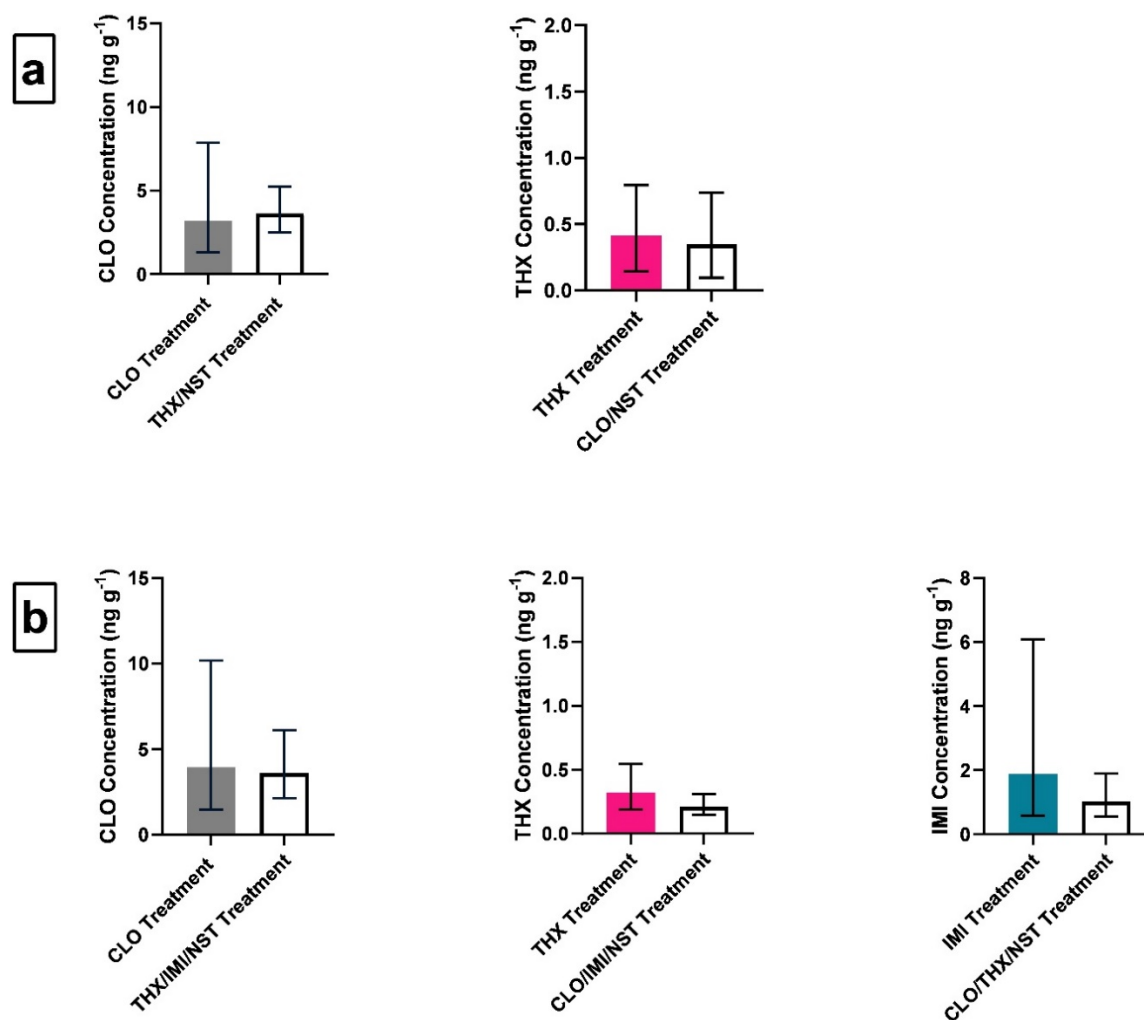


Fig. 2. Median concentration ($\pm 95\%$ confidence interval) of neonicotinoid detected in soil at sites organized into two categories: sites where treated seeds with the neonicotinoid quantified were planted versus sites where a different neonicotinoid seed treatment was planted, or no neonicotinoid-treated seeds were planted, in 2017 (a) and 2018 (b). Based on the analysis of variance, there was no statistical difference between these two categories at a $p = 0.05$ level of significance. IMI = imidacloprid, CLO = clothianidin, THX = thiamethoxam, NST = No Seed Treatment.

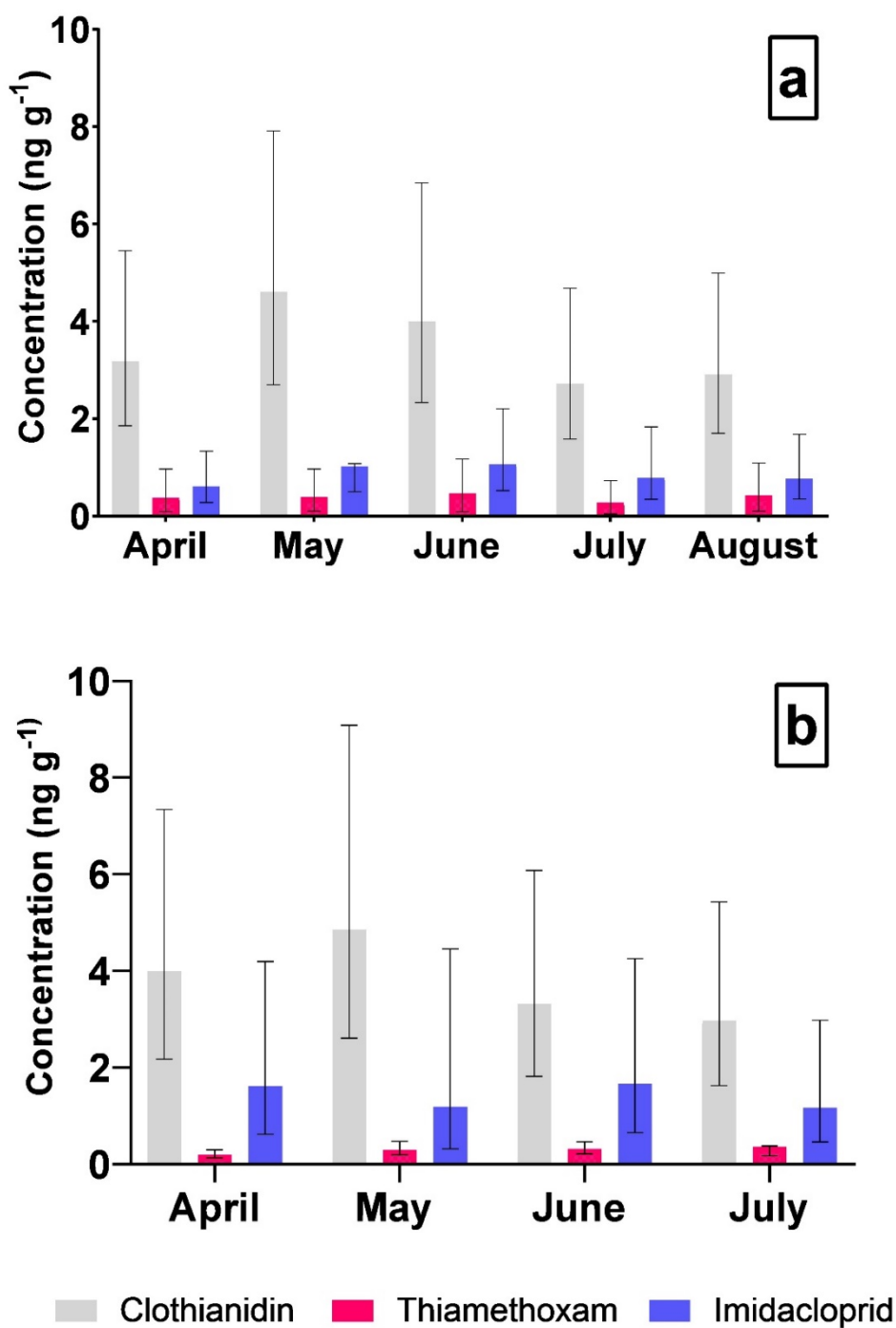


Fig. 3. Median soil concentration ($\pm 95\%$ confidence interval) of neonicotinoids per prairie strip for months sampled in 2017 (a) and 2018 (b). Based on an analysis of variance, there were no statistical differences among the individual compounds in any month at a $p = 0.05$ level of significance. The following neonicotinoids were analyzed; IMI = imidacloprid, CLO = clothianidin, THX = thiamethoxam.

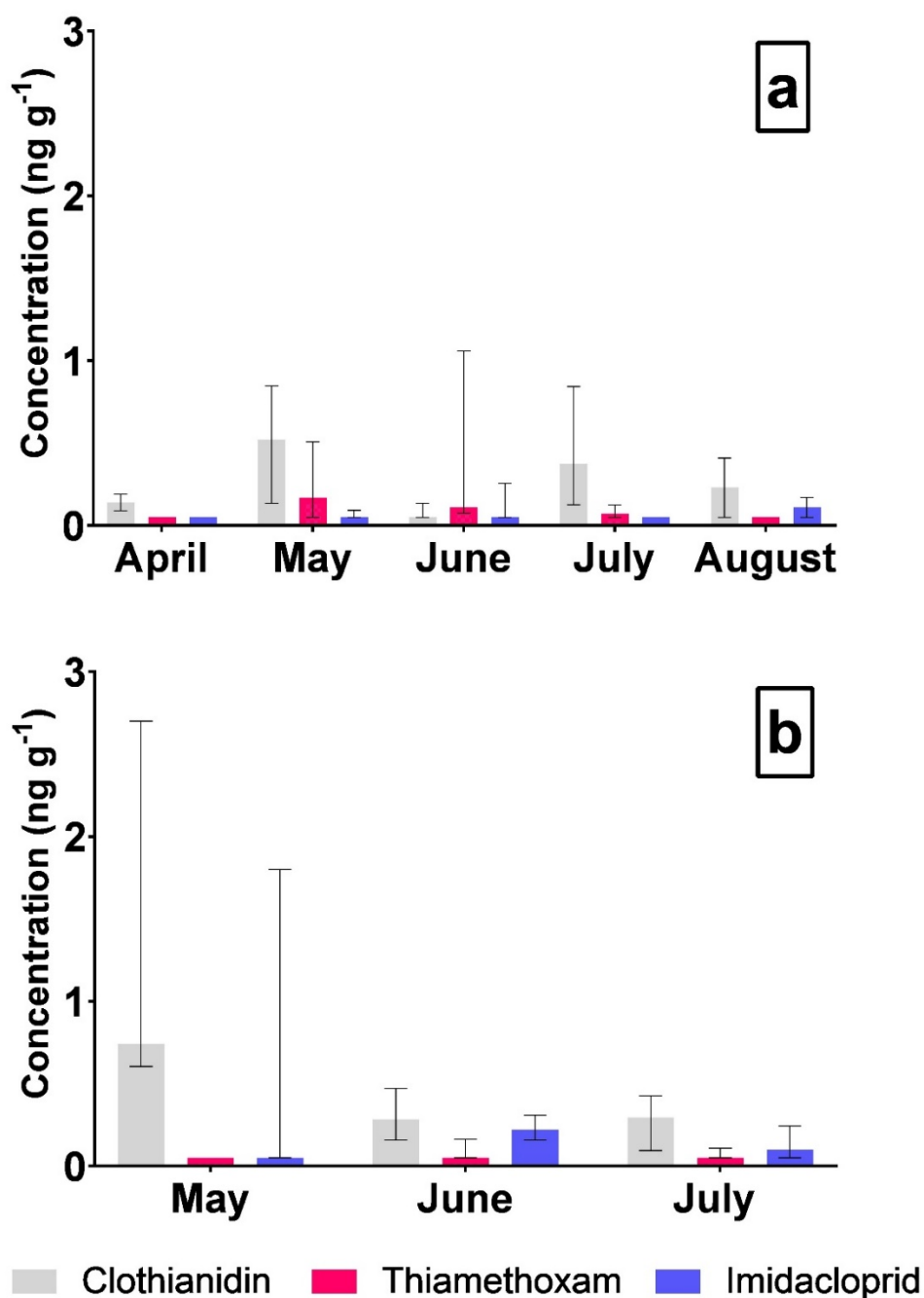


Fig. 4. Median (bar plots) and 25% (lower error bar) and 75% percentiles (upper error bar) of neonicotinoid concentrations per sampling month in foliage of flowering forbs collected from prairie strips in 2017 (a) and 2018 (b). Kruskal-Wallis H tests revealed no statistical difference in the median concentration of each compound between the different sampling months at a $p = 0.01$ level of significance. (a) Clothianidin: chi-squared = 8.79, $df = 4$, p -value = 0.066. Thiamethoxam: chi-squared = 4.8, $df = 4$, p -value = 0.31. Imidacloprid: chi-squared = 3.2, $df = 4$, p -value = 0.53. (b) Clothianidin: chi-squared = 5.4 $df = 2$, p -value = 0.068. Thiamethoxam: chi-squared = 1.3, $df = 2$, p -value = 0.51. Imidacloprid: chi-squared = 2.2, $df = 2$, p -value = 0.33.

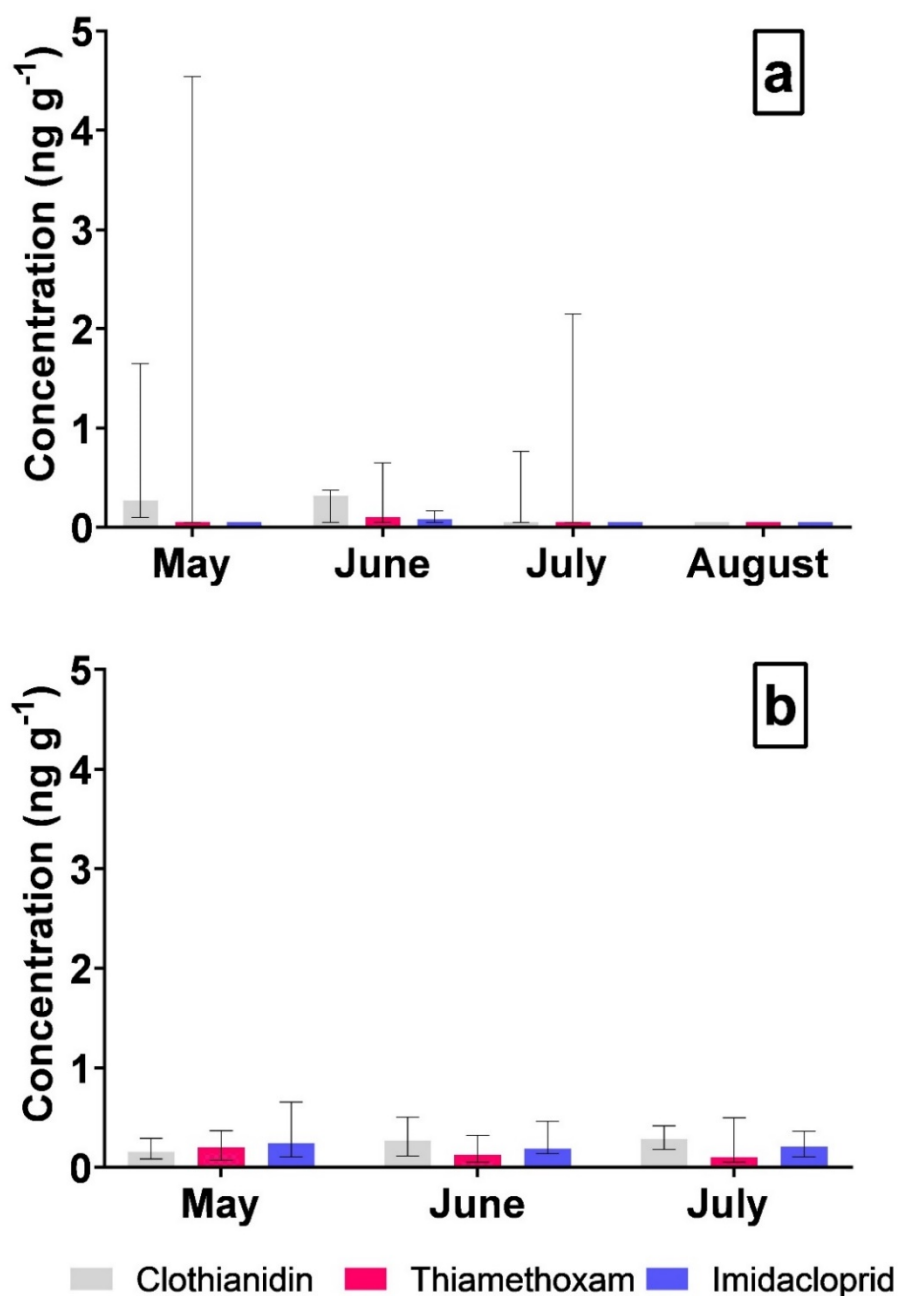


Fig. 5. Median (bar plots) and 25% (lower error bar) and 75% percentiles (upper error bar) concentration per sampling month of neonicotinoid compounds detected in the foliage of milkweed plants collected from prairie strips in 2017 (a) and 2018 (b). Kruskal-Wallis H tests revealed no statistical difference in the median concentration of each compound between the different sampling months ($p > 0.01$). (a) Clothianidin: chi-squared = 3.7, df = 3, p-value = 0.29. Thiamethoxam: chi-squared = 1.6, df = 3, p-value = 0.66. Imidacloprid: chi-squared = 7.5, df = 3, p-value = 0.058. (b) Clothianidin: chi-squared = 2.1, df = 2, p-value = 0.36. Thiamethoxam: chi-squared = 0.2, df = 2, p-value = 0.91. Imidacloprid: chi-squared = 0.07, df = 2, p-value = 0.97.

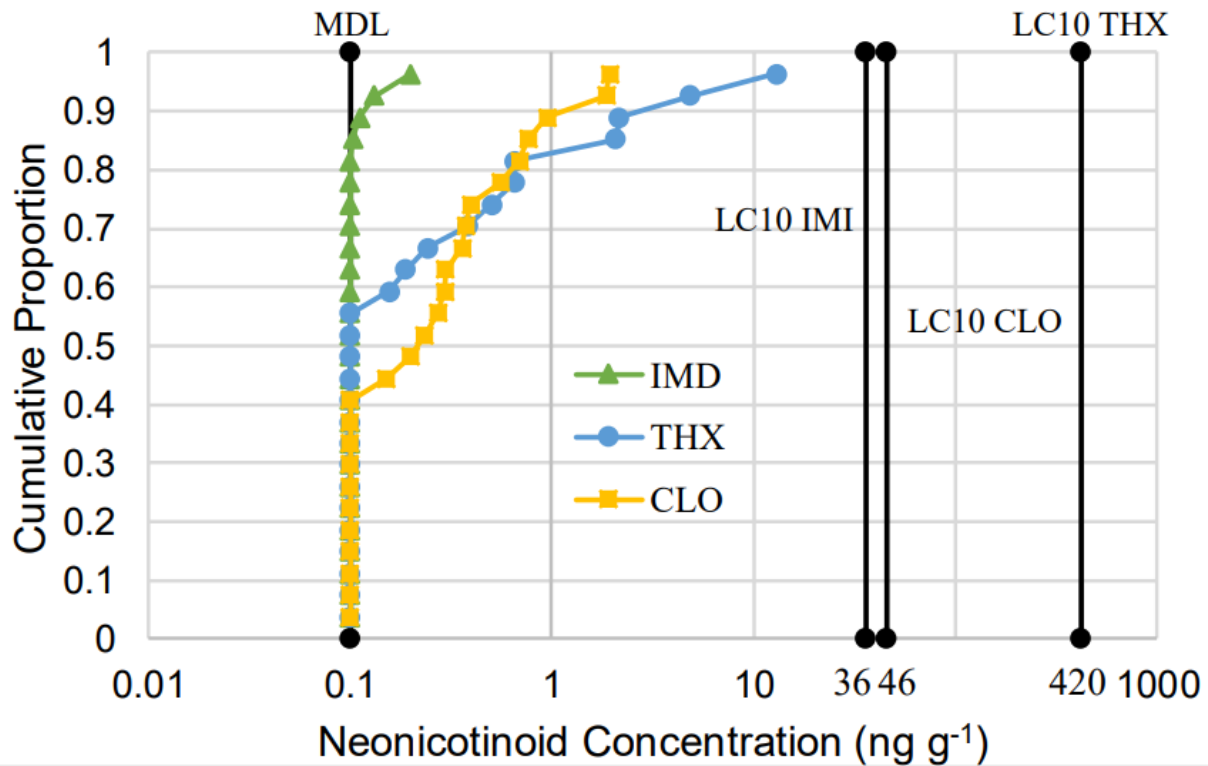


Fig. 6. Proportional distribution graph of concentrations of clothianidin (CLO), imidacloprid (IMI), and thiamethoxam (THX) detected in 2017 milkweed samples compared to chronic, dietary LC₁₀ values for monarch larvae (Krishnan et al. (2021); IMI = 36 ng g⁻¹; CLO = 46 ng g⁻¹; THX = 420 ng g⁻¹). The colored lines represent the distribution of detected concentrations in milkweed leaf samples collected in 2017 (n=26). The symbols on each line represent individual milkweed samples analyzed. Method detection limit (MDL) for clothianidin, imidacloprid, and thiamethoxam is 0.1 ng g⁻¹.

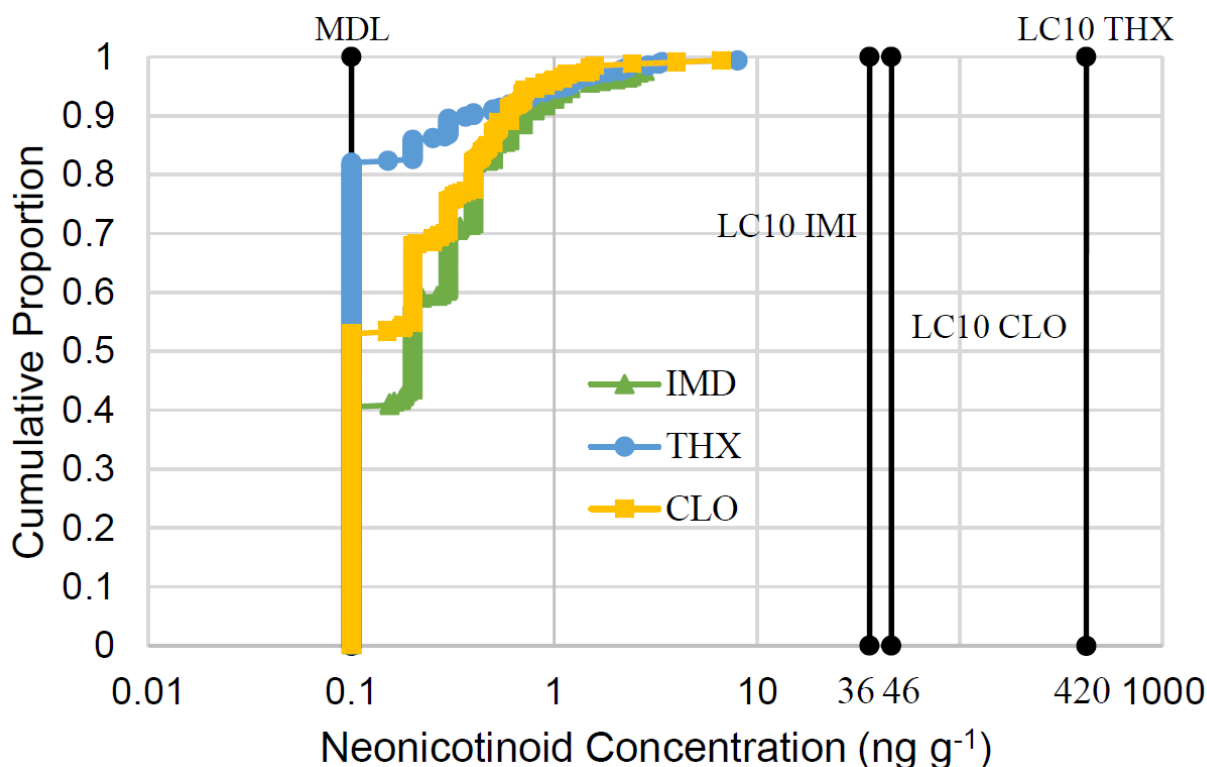


Fig. 7. Proportional distribution graph of concentrations of clothianidin (CLO), imidacloprid (IMI), and thiamethoxam (THX) detected in 2018 milkweed samples compared to chronic, dietary LC₁₀ values for monarch larvae (Krishnan et al. (2021); IMI = 36 ng g⁻¹; CLO = 46 ng g⁻¹; THX = 420 ng g⁻¹). The colored lines represent the distribution of detected concentrations in milkweed leaf samples collected in 2018 (n=332). The symbols on each line represent individual milkweed samples analyzed. Method detection limit (MDL) for clothianidin, imidacloprid, and thiamethoxam is 0.1 ng g⁻¹.

Appendix. Supplemental Tables and Figures

Table A1. Description of seed mixes used to establish prairie within conventional farms.

Site Location	Date Seeded	Seed Mix	Notes/Additions
GES	3/31/2015	Blue bluestem, Indian grass, Virginia wild rye, Canada wild rye, switchgrass, prairie dropseed, fox sedge, black-eyed Susan, gray headed coneflower, prairie mimosa, purple prairie clover, partridge pea, round headed bush clover, white prairie clover, smooth blue aster, showy tick trefoil, tall thimbleweed, butterfly milkweed, sky-blue aster, white wild indigo, pale coneflower, rattlesnake master, wild bergamot, common evening primrose, compass plant, white heath aster, stiff goldenrod, ox-eye sunflower, culver's root, common mountain mint, false dragonhead, flowering spurge, red columbine, wild petunia, prairie cinquefoil, lead plant, golden alexander, Canadian milkvetch	N/A
MCN	6/18/2014	Grasses: Big Bluestem, Canada Wild rye, Indian grass, Little Bluestem, Switchgrass, Prairie Junegrass, Sideoats grama, Composite Dropseed, Slender Wheatgrass, Virginia Wild rye, Western Wheatgrass, Fox Sedge Forbs: Seedbox, Great Lobelia, Rattlesnake Master, White Sagebrush, Foxglove Penstemon, Common Spiderwort, Western Yarrow, Golden Alexanders, Hoary Vervain, Black Eyed Susan, Partridge Pea, Canada Milk Vetch, Prairie Mimosa, Gray-headed Coneflower, Common Evening Primrose, White Prairie Clover, Stiff Goldenrod, Ox-eye Sunflower, Purple Prairie Clover, Prairie Blazing Star, Long-headed Coneflower, Purple Coneflower, Prairie Cinquefoil, Blue Vervain, New England Aster, Sneezeweed, Ironweed, Brown-eye Susan, Lead Plant, Culvers Root, Field Goldenrod, Hairy White Oldfield Aster, Wild Bergamot	N/A
DMW-N & S	4/9/2014	Big bluestem, Sideoats grama, brown fox sedge, Canada wild rye, Virginia wild rye, prairie Junegrass, switchgrass, little bluestem, Indian grass, rough dropseed, prairie dropseed, sky blue aster, Canada milk vetch, partridge pea, purple prairie clover, Illinois bundle flower, pale purple coneflower, rattlesnake master, sweet joe pye weed, wild bergamot, prairie cinquefoil, yellow coneflower, black-eyed Susan, gray goldenrod, hoary Vervain, culver's root, golden alexanders	N/A
SME	12/5/2014	Pheasants Forever Pollinator Mix (https://www.pfhabitatstore.com/store/14351/RM/Midwest-Pollinator-Plus)	N/A

Table A1. Continued

GUT	11/20/2014	Big bluestem, bottle gentian, Canada goldenrod, Canada wild-rye, compass plant, culver's root, cup-plant, cut-leaved water-horehound, false boneset, false white indigo, frost aster, giant sunflower, golden alexanders, gray-headed coneflower, heath aster, Indian grass, little bluestem, meadow rue, milk vetch, mountain mint, ox-eye sunflower, pale purple coneflower, pale-spiked lobelia, prairie dropseed, prairie loosestrife, purple prairie clover, rattlesnake mater, rigid goldenrod, rosinweed, rough blazing-star, round-headed bush clover, saw-tooth sunflower, showy tick-trefoil, smooth aster, sneezeweed, switch grass, tall blazing-star, tall cinquefoil, tall dropseed, tall thistle, white gentian, wing-angled loosestrife, woolly Vervain	N/A
WOR	4/1/2015	Statewide Mesic 10-30, Iowa Pollinator Mix: .5lb Little bluestem, 15lb Big bluestem, .04lb Prairie Junegrass, .04lb Prairie dropseed, .09lb Fox sedge, .1lb Sideoats grama Forbs: Black-eyed Susan .08lb, Gray-headed coneflower .2lb, Prairie Mimosa .15lb, Purple prairie clover .5lb, Partridge Pea .5lb, Round-headed Bush .03lb, White prairie clover .12lb, Compass plant .1lb, Smooth blue aster .09lb, Showy tick trefoil .1lb, Tall thimbleweed .01lb, Butterfly milkweed .1lb, Sky blue aster .05lb, Wild white indigo .1lb, Pale coneflower .1lb, Rattlesnake master .1lb, Wild bergamot .08lb, Common evening primrose .05lb, White heath aster .02lb, Stiff goldenrod .14lb, Golden Alexander's .06lb, Culver's root .01lb, Alumroot .01lb, Tall blazingstar.03lb, Ox-eye .1lb, Prairie cinquefoil .02lb, Red Columbine .04lb, Flowering Spurge .01lb, Wild Petunia .1lb.	<p>Additions:</p> <p>Butterfly milkweed 1pls oz, Whorled milkweed 1pls oz, Swamp milkweed 3pls oz, Common milkweed 1pls oz, Canada wild rye .095 pls lb, Indian grass .190 pls lb</p>
SMI	3/31/2015	1.2lb Little bluestem, .9lb Side oats gramma, .01lb Prairie dropseed, .9lb Big bluestem, .8lb Indian grass, .2lb Canada wildrye, .3lb Virginia wildrye, .1lb Switchgrass, .15lb Composite dropseed, .045lb Fox Sedge Forbs: Black-eyed Susan .08lb, Gray-headed coneflower .2lb, Prairie Mimosa .15lb, Purple prairie clover .5lb, Partridge Pea .5lb, Round-headed Bush .03lb, White prairie clover .12lb, Compass plant .1lb, Smooth blue aster .09lb, Showy tick trefoil .1lb, Tall thimbleweed .01lb, Butterfly milkweed .1lb, Sky blue aster .05lb, Wild white indigo .1lb, Pale coneflower .1lb, Rattlesnake master .1lb, Wild bergamot .08lb, Common evening primrose .05lb, White heath aster .02lb, Stiff goldenrod .14lb, Golden Alexander's .06lb, Culver's root .01lb, Alumroot .01lb, Tall blazingstar.03lb, Ox-eye .1lb, Prairie cinquefoil .02lb, Red Columbine .04lb, Flowering Spurge .01lb, Wild Petunia .1lb.	N/A

Table A1. Continued

STO	1/27/2016	Big Bluestem, Indiangrass, Stiff Goldenrod, Rattlesnake Master, Little Bluestem, Purple Prairie Clover, Golden Alexanders, White False Indigo, Canada Wild Rye, Round-Headed bush clover, Rough Blazing Star, Cup Plant, Switchgrass, Prairie Dropseed, Partridge Pea, Pale Purple Coneflower, Giant Sunflower, Sawtooth Sunflower, Compass Plant, Oxeye Sunflower, Showy Tick-Trefoil, Rough Dropseed, Canada Milkvetch, Rosinweed, Sedge	N/A
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PLS = pure live seed

Table A2. Soil properties as determined from a single composite sample taken prairie strips located in conventional farms sites. Other than SMI and STO all samples were taken in 2017. Samples analyzed by Agvise Laboratories.

Site ID	Percent (%) Make-up			USDA Textural Class [Hydrometer Method]	Bulk Density (gm/cc) [Disturbed]	Cation Exchange Capacity (meq/100 g)	Percent (%) Moisture		Percent (%) Organic Matter [Walkley-Black]	pH [Water]	Buffer pH [Adams-Evans]	Percent (%) Total Nitrogen [Analyzer]	Olsen Phosphorus (ppm)	Soluble Salts 1:1 (mmhos/cm)
	Sand	Silt	Clay				at 1/3 Bar	at 15 Bar						
GES	43	28	29	Clay Loam	1.11	20.6	21.8	13.7	3.6	6.5	7.6	0.186	23	0.44
DMW-N	17	52	31	Silty Clay Loam	1.03	24.1	28.5	13.7	3.9	7.8	7.7	0.172	14	0.22
SME	58	18	23	Sandy Clay Loam	1.17	19.3	18.1	12.9	4.3	8.2	7.8	0.248	16	0.36
MCN	19	38	43	Clay	1.06	29.7	32	22.9	4.6	7.1	7.6	0.247	74	0.99
DMW-S	13	50	37	Silty Clay Loam	1.02	21.4	31.7	22.5	3.1	6.1	7.4	0.168	7	0.22
WOR	51	24	25	Sandy Clay Loam	1.11	16.1	17.4	10.8	2.9	5.2	7.3	0.128	17	0.12
GUT	61	20	19	Sandy Loam	1.13	20.9	14.7	8.3	2.2	8	7.8	0.139	35	0.17
SMI	43	24	33	Clay Loam	1.03	19.6	22.3	16.2	3.4	6.3	7.6	0.179	6	0.19
STO	13	52	35	Silty Clay Loam	0.98	23	31.7	20.2	4.5	7	7.6	0.271	13	0.29

Table A3. Soil base saturation data from soil composites taken from prairie strips located in conventional farm sites analyzed by Agvise laboratories.

Site ID	Cation									
	Potassium		Calcium		Magnesium		Sodium		Hydrogen	
	Percent	ppm	Percent	ppm	Percent	ppm	Percent	ppm	Percent	ppm
GES	2.2	180	62.9	2597	19.4	481	0.3	14	15.1	31
DMW-N	2.4	230	75.9	3654	12.2	351	0.2	11	9.3	22
SME	2	148	71.8	2767	16.8	388	0.4	17	9.1	18
MCN	2	230	72.8	4331	13.6	487	0.3	19	11.3	34
DMW-S	2	163	56.2	2404	21	538	0.3	17	20.6	44
WOR	2.7	170	49.8	1604	13.5	261	0.3	11	33.7	54
GUT	1.2	100	77.2	3227	13.3	334	0.2	11	8	17
GES	4.1	294	54.6	2021	16.3	362	0.3	13	24.7	46
SMI	1.7	128	63.2	2478	16.4	385	0.4	16	18.4	36
STO	1.5	134	67.4	3103	15.6	430	0.3	14	15.3	35

Table A4. Specified neonicotinoid concentrations found in individual plant species, excluding milkweed, collected from prairie strips located in conventional farms in 2017. Sampling site is location that the plant samples was taken from, sampling date was date the sample was collected, CLO = clothianidin, THX = thiamethoxam, and IMI = imidacloprid.

Sampling Site	Sampling Date	Plant Species	CLO (ng g ⁻¹)	THX (ng g ⁻¹)	IMI (ng g ⁻¹)
DMW-N	4/19/2017	Dandelion	0.15	<MDL	<MDL
DMW-N	4/19/2017	Dandelion	0.23	<MDL	<MDL
DMW-N	6/19/2017	Mixed Clover	<MDL	<MDL	0.32
DMW-S	5/25/2017	Golden Alexander	0.60	1.46	<MDL
DMW-S	5/25/2017	Dandelion	0.44	0.23	0.22
DMW-S	6/13/2017	Red Clover	0.39	3.79	<MDL
DMW-S	6/19/2017	White Clover	<MDL	0.11	<MDL
DMW-S	7/7/2017	White Clover	<MDL	<MDL	<MDL
DMW-S	7/17/2017	Black Eyed Susan	0.43	<MDL	<MDL
DMW-S	7/17/2017	Yellow Coneflower	<MDL	<MDL	<MDL
GES	5/22/2017	Mixed Clover	0.15	<MDL	<MDL
GUT	5/23/2017	Golden Alexander	0.56	0.17	<MDL
GUT	6/15/2017	Mixed Clover	<MDL	0.10	0.19
MCN	5/25/2017	Golden Alexander	1.15	<MDL	<MDL
MCN	5/25/2017	Dandelion	1.12	0.29	<MDL
MCN	6/13/2017	Red Clover	<MDL	0.17	<MDL
MCN	7/10/2017	Grey Coneflower	0.61	<MDL	<MDL
MCN	7/10/2017	Oxeye Sunflower	1.16	0.31	<MDL
MCN	7/10/2017	Black Eyed Susan	0.44	<MDL	<MDL
MCN	7/18/2017	Purple-Headed Coneflower	1.11	<MDL	<MDL
SME	4/10/2017	Dandelion	0.09	<MDL	<MDL
SME	5/22/2017	Mixed Clover	0.12	<MDL	<MDL
SME	6/9/2017	Mixed Clover	<MDL	<MDL	<MDL
SME	6/12/2017	White Clover	<MDL	<MDL	<MDL
SME	6/16/2017	Red Clover	<MDL	0.23	<MDL
SME	7/17/2017	Sunflower	0.19	<MDL	<MDL
SME	7/17/2017	Mixed Clover	<MDL	<MDL	<MDL
SME	8/9/2017	Grey-Headed Coneflower	<MDL	<MDL	<MDL
WOR	7/13/2017	Black Eyed Susan	0.88	<MDL	<MDL
WOR	7/13/2017	Oxeye Sunflower	0.77	<MDL	<MDL
WOR	7/13/2017	Bergamot	0.31	0.39	<MDL
WOR	7/13/2017	Grey Coneflower	0.46	<MDL	<MDL
WOR	8/8/2017	Grey-Headed Coneflower	0.41	<MDL	0.17

(<MDL) Indicates value below the method detection limit for our methodology

Table A5. Specified neonicotinoid concentrations found in individual plant species, excluding milkweed, collected from prairie strips located in conventional farms in 2018. Sampling site is location that the plant samples was taken from, sampling date was date the sample was collected, CLO = clothianidin, THX = thiamethoxam, and IMI = imidacloprid.

Sampling Site	Sampling Date	Plant Species	CLO (ng g ⁻¹)	THX (ng g ⁻¹)	IMI (ng g ⁻¹)
DMW-N	6/26/2018	Black-Eyed Susan	<MDL	<MDL	0.60
DMW-N	6/26/2018	Black-Eyed Susan	0.20	<MDL	0.20
DMW-N	6/26/2018	Thistle	<MDL	<MDL	<MDL
DMW-N	7/19/2018	Bergamot	0.20	<MDL	<MDL
DMW-N	7/19/2018	Black-Eyed Susan	<MDL	<MDL	<MDL
DMW-N	7/19/2018	Black-Eyed Susan	<MDL	<MDL	0.20
DMW-S	6/26/2018	Thistle	0.40	<MDL	0.80
DMW-S	7/19/2018	Thistle	0.20	<MDL	<MDL
DMW-S	7/19/2018	Queen Anne's Lace	<MDL	<MDL	<MDL
GES	6/5/2018	Golden Alexander	0.60	0.30	0.50
GES	6/27/2018	Bergamot	<MDL	<MDL	0.30
GES	6/27/2018	Thistle	<MDL	<MDL	<MDL
GES	6/27/2018	Black-Eyed Susan	<MDL	<MDL	<MDL
GES	7/16/2018	Bergamot	0.20	<MDL	<MDL
GES	7/16/2018	Oxeye Daisy	0.40	<MDL	<MDL
GES	7/16/2018	Black-Eyed Susan	<MDL	<MDL	<MDL
GUT	6/28/2018	Black-Eyed Susan	<MDL	<MDL	<MDL
GUT	6/28/2018	Black-Eyed Susan	0.20	<MDL	<MDL
GUT	7/17/2018	Sunflower	0.60	0.20	<MDL
GUT	7/17/2018	Bergamot	0.30	<MDL	<MDL
GUT	7/17/2018	Black-Eyed Susan	0.20	0.20	<MDL
GUT	7/17/2018	Black-Eyed Susan	0.20	<MDL	<MDL
MCN	5/29/2018	Golden Alexander	2.70	<MDL	1.80
MCN	6/26/2018	Black-Eyed Susan	0.40	<MDL	<MDL
MCN	6/26/2018	Purple Prairie Clover	0.20	<MDL	0.20
MCN	6/26/2018	Sunflower	0.70	<MDL	0.40
MCN	6/26/2018	Coneflower	1.20	<MDL	0.40
MCN	6/26/2018	Coneflower	0.20	<MDL	<MDL
MCN	7/19/2018	Coneflower	1.30	<MDL	0.30
MCN	7/19/2018	Purple Prairie Clover	<MDL	<MDL	<MDL
MCN	7/19/2018	Coneflower	0.30	<MDL	<MDL
MCN	7/19/2018	Black-Eyed Susan	0.40	<MDL	0.30
MCN	7/19/2018	Black-Eyed Susan	0.50	<MDL	0.80
MCN	7/19/2018	Black-Eyed Susan	0.20	<MDL	<MDL
SME	6/5/2018	Golden Alexander	0.30	<MDL	0.30

Table A5. Continued

SME	6/27/2018	Black-Eyed Susan	<MDL	<MDL	0.20
SME	6/27/2018	Black-Eyed Susan	0.20	<MDL	<MDL
SME	6/27/2018	Golden Alexander	<MDL	<MDL	0.20
SME	6/27/2018	Golden Alexander	0.20	<MDL	<MDL
SME	7/16/2018	Golden Alexander	<MDL	<MDL	0.30
SME	7/16/2018	Golden Alexander	0.20	<MDL	<MDL
SME	7/16/2018	Black-Eyed Susan	<MDL	<MDL	0.50
SME	7/16/2018	Black-Eyed Susan	<MDL	<MDL	<MDL
SMI	6/5/2018	Golden Alexander	0.55	0.20	0.30
SMI	6/5/2018	Red Clover	<MDL	0.10	0.30
SMI	7/16/2018	Sunflower	<MDL	0.10	0.10
SMI	7/16/2018	Clover	0.24	0.10	0.10
SMI	7/16/2018	Coneflower	0.55	0.10	0.30
SMI	7/16/2018	Coneflower	0.37	0.10	0.10
STO	5/31/2018	Dandelion	0.60	<MDL	<MDL
STO	5/31/2018	Dandelion	0.40	<MDL	<MDL
STO	5/31/2018	Red Clover	<MDL	<MDL	<MDL
STO	5/31/2018	Golden Alexander	1.40	<MDL	<MDL
STO	6/27/2018	Black-Eyed Susan	0.20	<MDL	<MDL
STO	6/27/2018	Black-Eyed Susan	0.40	<MDL	<MDL
STO	6/27/2018	Bergamot	<MDL	<MDL	0.40
STO	6/27/2018	Golden Alexander	0.50	<MDL	0.30
STO	6/27/2018	Golden Alexander	4.50	7.10	<MDL
STO	6/27/2018	Golden Alexander	0.50	<MDL	0.30
STO	6/27/2018	Golden Alexander	0.40	<MDL	<MDL
STO	6/27/2018	Vervain	<MDL	<MDL	<MDL
STO	7/17/2018	Black-Eyed Susan	0.30	<MDL	<MDL
STO	7/17/2018	Black-Eyed Susan	0.60	<MDL	<MDL
STO	7/17/2018	Oxeye Sunflower	0.50	<MDL	<MDL
STO	7/17/2018	Coneflower	0.20	<MDL	<MDL
WOR	5/31/2018	Golden Alexander	0.74	<MDL	<MDL
WOR	6/26/2018	Golden Alexander	0.21	<MDL	0.16
WOR	7/19/2018	Bergamot	0.39	<MDL	0.29
WOR	7/19/2018	Bergamot	0.27	<MDL	<MDL
WOR	7/19/2018	Black-Eyed Susan	<MDL	<MDL	1.03
WOR	7/19/2018	Black-Eyed Susan	<MDL	<MDL	0.66
WOR	7/19/2018	Black-Eyed Susan	3.37	0.40	<MDL
DMW-N	6/26/2018	Black-Eyed Susan	<MDL	<MDL	0.60
DMW-N	6/26/2018	Black-Eyed Susan	0.20	<MDL	0.20
DMW-N	6/26/2018	Thistle	<MDL	<MDL	<MDL

Table A5. Continued

DMW-N	7/19/2018	Bergamot	0.20	<MDL	<MDL
DMW-N	7/19/2018	Black-Eyed Susan	<MDL	<MDL	<MDL
DMW-N	7/19/2018	Black-Eyed Susan	<MDL	<MDL	0.20
DMW-S	6/26/2018	Thistle	0.40	<MDL	0.80
DMW-S	7/19/2018	Thistle	0.20	<MDL	<MDL
DMW-S	7/19/2018	Queen Anne's Lace	<MDL	<MDL	<MDL
GES	6/5/2018	Golden Alexander	0.60	0.30	0.50
GES	6/27/2018	Bergamot	<MDL	<MDL	0.30
GES	6/27/2018	Thistle	<MDL	<MDL	<MDL
GES	6/27/2018	Black-Eyed Susan	<MDL	<MDL	<MDL
GES	7/16/2018	Bergamot	0.20	<MDL	<MDL
GES	7/16/2018	Oxeye Daisy	0.40	<MDL	<MDL
GES	7/16/2018	Black-Eyed Susan	<MDL	<MDL	<MDL
GUT	6/28/2018	Black-Eyed Susan	<MDL	<MDL	<MDL
GUT	6/28/2018	Black-Eyed Susan	0.20	<MDL	<MDL
GUT	7/17/2018	Sunflower	0.60	0.20	<MDL
GUT	7/17/2018	Bergamot	0.30	<MDL	<MDL
GUT	7/17/2018	Black-Eyed Susan	0.20	0.20	<MDL
GUT	7/17/2018	Black-Eyed Susan	0.20	<MDL	<MDL
MCN	5/29/2018	Golden Alexander	2.70	<MDL	1.80
MCN	6/26/2018	Black-Eyed Susan	0.40	<MDL	<MDL
MCN	6/26/2018	Purple Prairie Clover	0.20	<MDL	0.20
MCN	6/26/2018	Sunflower	0.70	<MDL	0.40
MCN	6/26/2018	Coneflower	1.20	<MDL	0.40
MCN	6/26/2018	Coneflower	0.20	<MDL	<MDL
MCN	7/19/2018	Coneflower	1.30	<MDL	0.30
MCN	7/19/2018	Purple Prairie Clover	<MDL	<MDL	<MDL
MCN	7/19/2018	Coneflower	0.30	<MDL	<MDL

(<MDL) Indicates value below the method detection limit for our methodology



Fig. A1. Representative map of land cover surrounding prairie strips within conventionally-managed maize or soybean fields. The photo is an example of a prairie strip located within a soybean field. Red arrows indicate the anticipated downslope movement of neonicotinoids from planted soybean seeds in surface/subsurface water to the prairie strip. The red line is a transect 30 cm into the prairie strip where plants and soil were sampled.

CHAPTER 4. ASSESSING POLLINATOR HABITAT EXPOSURE TO PESTICIDE MIXTURES IN IOWA USA AGROECOSYSTEMS

Authors: Maura J. Hall^{a,b}, Ge Zhang^{a1}, Matthew O'Neal^a, Steven P. Bradbury^{a,b,c}, Joel R. Coats^{a,b*}

^a Iowa State University, Department of Entomology, ^b Toxicology Program, ^c Department of Natural Resource Ecology and Management, Ames, IA 50011

¹ Present address Washington State University, Department of Entomology, Pullman, WA 99163

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Abstract

In the past decade, insect pollinators have experienced significant population declines in the US. Multiple factors are associated with the decline including loss of foraging habitat and pesticide use. Establishing habitat in agricultural landscapes of the north central US is critical; however, there is concern that pollinator habitat established in close proximity to conventional crop fields may increase risks from pesticide use. While previous studies indicate a variety of pesticides can be detected in forbs within pollinator habitat, differences in insecticide and fungicide exposure to honey bees foraging in row crop areas with and without pollinator habitat is uncertain. In this study, concentrations of insecticides (neonicotinoid, pyrethroid, organophosphate) and strobilurin fungicides were quantified in nectar, pollen and bees. Results indicate honey bee hives are experiencing more frequent exposure to foliar applied insecticides and fungicides as compared to neonicotinoids used as seed treatments. When neonicotinoids are detected, the concentrations likely pose *de minimis* risk to honey bee colonies.

Introduction

Pollinators deliver an important ecological service through pollination of food crops; approximately 35% of the global food supply requires some form of pollination¹ with both honey bees (*Apis mellifera*) and wild bees (Hymenoptera: Apoidea) being essential for crop

pollination.¹ Declines in wild bee abundance and reduced honey bee colony survival have been reported across the United States.²⁻⁵ These declines are likely not the result of one factor but the interaction of multiple factors including diseases, pathogens, pests, pesticide exposure, and reduced forage availability.⁶

The loss of forage is due to intensive agriculture^{2, 5}, as exemplified in the north central U.S. For example, in Iowa 85% of the land is used for agricultural, with majority of that land devoted to maize and soybean production (64%).⁷ Average annual honey bee colony losses in Iowa can reach 60%, which is substantially above what commercial beekeepers consider sustainable.^{4, 8, 9} This decline is concurrent with a reduction in wild bees.⁵ Efforts to reverse these declines have included re-introducing native, perennial flowering habitat in agricultural landscapes.¹⁰ Native grasses and forbs used in re-establishments are typically species in native prairies.^{11, 12} Historically, the majority of what is now Iowa was covered in tallgrass prairie (80%), but due land use changes < 0.1% of Iowa's natural tallgrass prairie remains.^{13, 14} Multiple practices within the United States Department of Agriculture's Conservation Reserve Program support establishment of habitat that can support pollinators; e.g. CP-42 (pollinator habitat) and CP-43 (prairie strips).^{15, 16} Prairie strips involve strategic planting of linear arrangements of native prairie grasses and forbs in crop fields.¹⁵ Prairie strips have been shown to reduce nutrient and sediment loss and increase biodiversity,^{17, 18} including an increase in the abundance and diversity of native bees and improved honey bee colony productivity.¹⁹⁻²¹ There is, however, concern that pollinator habitat established in close proximity to conventional crop fields may be exposed to pesticides and create an ecological trap for honey bees and other pollinators.²²⁻²⁶

Bees can be exposed to pesticides used as seed treatments and/or those that are sprayed. Neonicotinoid insecticides, specifically clothianidin, thiamethoxam and imidacloprid are

extensively used as seed treatments on maize and soybean in the north central United States (U.S.) to manage early season pests.^{27, 28} Approximately 100% of maize and 50% of soybean are planted with neonicotinoid-treated seeds nationally.²⁸ These compounds can move off-field in dust produced at planting^{23, 29-34} and via overland runoff and subsurface flow post planting. Off field neonicotinoids can be taken up by the roots of non-target plants and translocated to their pollen and nectar.^{23, 30, 35-38} These insecticides have been shown to cause adverse effects to both native bees and honey bee colonies including mortality, reduction in population densities, foraging impairment and increased susceptibility to disease and parasites.³⁹⁻⁴³ The extent to which neonicotinoid concentrations in nectar and pollen brought back to the hive by bees foraging in contaminated pollinator habitat is not clear.

Approximately, 5-20% of maize and 2-29% of soybean acres across the north central U.S. are also treated with foliar and soil-applied insecticides.^{44, 45} Foliar applied insecticides are used for the conventional production of soybean and maize to manage pests throughout the growing season. Insecticides are commonly used on soybeans to manage soybean aphids⁴⁶, Japanese beetles (*Popillia japonica* Newman)⁴⁷, and spider mites (*Tetranychus urticae*).⁴⁸ Foliar applied insecticides used in maize production to manage Western corn rootworm (*Diabrotica virgifera virgifera*)⁴⁹ and European corn borer (*Ostrinia nubilalis* Hübner).⁵⁰ Regardless of the crop or pest, pyrethroids and organophosphates are the most commonly used foliar applied insecticides.⁵¹ Within the US, insecticide use in maize production has declined over the last 20 years, but increased in soybeans due to the invasion and establishment of the soybean aphid.⁵² There has also been an increase in foliar fungicide use in soybean⁵³ and maize⁵⁴; fungicides and insecticides are typically applied as a tank mix. During foliar application, pesticides can drift off field and deposit directly on bees resulting in direct exposure or on flowers resulting in indirect

exposure to bees who are foraging within these areas. Fungicides are not considered acutely toxic to honey bees⁵⁵; however, depending on the fungicide class, exposure to bees could potentially affect their gut microbiota communities.^{56, 57}

The objectives of this studies were to (1) determine if levels of seed treatment and foliar pesticide exposures vary between honey bee colonies located at control sites (>1.5 km from pollinator habitat) and colonies established in prairie strips; (2) determine neonicotinoid concentrations in nectar and pollen collected from native plants in prairie strips; and (3) characterize risks of adverse effects to honey bees based on the range of measured pesticide exposures. Improved understanding of pesticide exposure in pollinator habitat can help inform the extent to which honey bee productivity will be impacted if hives are placed in close proximity to treated crop fields. These findings can also help inform the conservation risks and benefits for wild bees if pollinator habitat is established within row-crop dominated landscapes.

Methods

2.1. Prairie Strip and Control Sites: Comparison of Pollen and Nurse Bee Pesticide-Concentrations as Indicators of Honey Bee Colony Pesticide Exposure

We hypothesized (H_0 = null hypothesis) colony exposure between control (μ_{control}) and prairie strip sites (μ_{prairie}) would not be significantly different (1). The alternative hypothesis (H_a) is that apiary exposure at control and prairie sites is significantly different (2).

$$H_0: \mu_{\text{prairie}} = \mu_{\text{control}} \quad (1)$$

$$H_a: \mu_{\text{prairie}} \neq \mu_{\text{control}} \quad (2)$$

If the exposure concentrations between prairie strips and control sites is significantly different, we predicated exposure concentrations would be higher for prairie strip colonies compared to control site colonies. This predication is based on results from a multi-year study of prairie strips as a source of forage for bees especially honey bees. These studies showed that

there are more blooming flowers in prairie strips than control sites,²¹ more pollen brought back to honey bee colonies at sites with prairie strips,²⁰ and that, although honey bees can forage from across the landscape, bees bring back pollen that can be found in prairie strips.²⁰ Additionally, there is evidence that neonicotinoids are present in prairie strips.^{31, 58} To test if honey bee colony pesticide exposure varied between sites with and without prairie strips we quantified a suite of commonly used pesticides (chlorpyrifos, lambda-cyhalothrin, imidacloprid, clothianidin, thiamethoxam, azoxystrobin, and pyraclostrobin) in pollen collected from pollen traps and nurse bees collected from hives as indicators of colony exposure.

2.1.1. Site selection

Commercial farms producing maize and soybeans formed our experimental unit. Fields with prairie strips (referred to as prairie strips sites) were selected based on proximity to Iowa State University in Ames, Iowa. Selected prairie strip sites were established at least three years prior to sampling, to ensure ample forage for bees. Four prairie strips sites were selected in 2018, and an additional site was selected in 2019 (5 total) (Table B1). The number of prairie strips within a field varied between prairie strip sites.²¹ A control site for each prairie strips site (i.e., fields without prairie strips) was assigned by locating a maize or soybean field within the same county that was at least 3.2 km from the prairie strip site. This distance was chosen to minimize the potential of honey bees foraging in both types of sites.^{20, 59, 60}

2.1.2. Apiaries

An apiary of four honey bee colonies were placed at both prairie strips and control sites. One strip of prairie was randomly selected for an apiary at each prairie strip site. The apiary was placed 3 m into the selected prairie from the edge of a soybean or maize field. At each control site, an apiary was placed at the margin of the corn or soybean field. Colonies were generated from “nucleus” colonies containing a honey comb, queen, workers, and brood of Italian honey

bees (*Apis mellifera ligustica*) purchased in Iowa.^{20, 21} To control for variation in colony size between sites, colonies were assigned to apiaries based on weight.²⁰ Each colony contained approximately 7,000 adult honey bees.²⁰ Hive boxes were added throughout the season as needed to respond to colony growth. Data was collected on the productivity and survival of honey bee colonies and is reported by Zhang et al.²⁰

2.1.3. Collection of honey bee-collected pollen

Pollen traps (Brushy Mountain Bee Supply, Wilsonville, USA) were attached to the entrances of colonies and activated for 24-hour periods. Traps, when activated, removed pollen from foraging honey bees as they reentered the colony. At each apiary, traps were placed on two of the four colonies. Pollen traps were used on the same two colonies at each apiary at each sampling point. For pesticide analysis, one to two pollen samples were collected from each pollen trap every month from June to September in 2018-2019 (Table B1). Each pollen trap sample collected was weighed and stored at - 20 °C for subsequent pesticide analysis.

2.1.4. Collection of nurse bees from honey bee colony

Nurse bees were collected from hives one to three times per month from June through October in 2018 and 2019 (Table B2). Nurse bees are adults <21 days old that specialize in brood feeding and consume a large portion of the pollen stores.²⁰ To ensure that nurse bees were collected, they were identified on a frame with larvae in each colony.²⁰ Nurse bees were collected in a 15 mL plastic falcon tube in the field and held on ice and upon return to the laboratory stored at -80°C for subsequent pesticide analysis.

2.2. Prairie Strip Sites: Nectar and Pollen Neonicotinoid Concentrations

Neonicotinoids specifically clothianidin, imidacloprid and thiamethoxam have been detected in both soil and the leaf tissue of flowering plants sampled from prairie strips.⁵⁸ These data indicate that neonicotinoid contamination of prairie sites is occurring and there is potential

for these compounds to be present within the pollen and nectar of prairie plants that bees are visiting. To confirm that that prairie strips are a potential source of neonicotinoids exposure, we surveyed the pollen and nectar of plants being visited by bees (Apidae) for concentrations of imidacloprid, clothianidin and thiamethoxam. In this way, we attempt to confirm a route of exposure not only for honey bees but all foraging adult bees that could use prairie strips as forage.

2.2.1. Site selection

Site selection for nectar and pollen sampling is described in detail in Hall et al (2021).⁵⁸ In short, we selected commercial crop fields with prairie strips identified through consultation with the Science-based Trials of Row crops Integrated with Prairie Strips (STRIPS; <https://www.nrem.iastate.edu/research/STRIPS/>) project. Farms were selected based on year of prairie strip establishment (>3 years), proximity to Iowa State University (≤ 150 km) and conventional production practices, including the use of neonicotinoid seed treatments. Samples were collected at seven sites in 2017, nine sites in 2018, and five sites in 2019 (Table B1).

2.2.2. Nectar and pollen collection

Wild bees and honey bees were collected while foraging on flowers located within prairie strips using a Heavy Duty Hand-Held Vacuum (BioQuip, Rancho Dominguez, CA). Bees were collected into a 5-inch insect collecting chamber. Once in the chamber an end cap was secured onto the tube and it was placed on ice. Once in the lab the bees were transferred to a 50-mL plastic falcon tube and stored at -20°C until dissection (typically 1 to 30 days). On the day of dissection, bees were removed from the freezer and warmed to room temperature. Vials for pollen and nectar were labeled and weighed before the dissection. Pollen from individual bees was removed from their pollen basket (corbiculae) and placed into an amber vial labeled with contents, date of sampling, date of processing and weight of sample (calibrated and tared

balance). Pollen from bees collected from the same site on the same sampling day were pooled. Nectar from bees was removed from their honey-stomachs and amber vials. Nectar samples from bees collected from the same site on the same day were pooled. Samples were stored at -20°C prior to neonicotinoid analysis. Collected bees were identified to species (Table B3).

2.3. Residue Analysis

Pollen collected from pollen traps and nurse bees sampled from hives located in prairie strips and control sites were analyzed for clothianidin, imidacloprid, thiamethoxam, azoxystrobin, pyraclostrobin, chlorpyrifos and lambda-cyhalothrin. Pollen and nectar collected from bees foraging in prairie strips were analyzed for imidacloprid, thiamethoxam, clothianidin, imidacloprid olefin and 5-OH imidacloprid to estimate the extent to which these compounds are absorbed systemically by blooming forbs in the prairie strips and translocated to pollen and nectar. Sample preparations for LC-MS/MS analysis of pollen collected from pollen traps or by bees are the same. Analytical methods are summarized below.

2.3.1. Chemicals and reagents

Neat standards of imidacloprid (CAS 138261-41-3, 98.8% purity), thiamethoxam (CAS 153719-23-4, 95.2% pure), clothianidin (CAS 210880-92-5, 99.6% pure), imidacloprid-olefin (CAS 115086-54-9, 97.9% pure), and 5-OH-imidacloprid (CAS 380912-09-4, 96.7% pure) were received as a gift from Bayer CropScience (Research Triangle Park, NC, USA). Neat standards of chlorpyrifos (Lot BCBR6591V, >95% pure), lambda-cyhalothrin (Lot BCBW5903, >95% pure), azoxystrobin (Lot BCBT1118V, >95% pure), and pyraclostrobin (Lot BVBT7756, >95% pure) were purchase from Sigma Aldrich (St. Louis, MO, USA). Aldrin (Lot AC-10, >95%) was purchased from Shell Chemicals (Houston, Texas). Deuterated internal standards, clothianidin-d₃, thiamethoxam-d₃, chlorpyrifos-d₁₀, azoxystrobin-d₄, and pyraclostrobin N-methoxy-d₃, were obtained from Sigma-Aldrich (St. Louis, MO, USA). Imidacloprid olefin-¹³C₃, ¹⁵N and

imidacloprid-pyr-d4-methyl-d2, ^{13}C were provided as a gift from Bayer CropScience, Five-OH-imidacloprid- ^{13}C , ^{15}N was purchased from Clearsynth (Mississauga, Ontario, Canada). Organic solvents (Optima LC-MS grade methanol, water, and acetonitrile), ammonium formate (99% pure), and formic acid (99% purity) were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Pre-filled tubes containing high impact zirconium beads of 1.5 mm diameter were purchased from Benchmark (catalog number D1032-15). Dispersive solid-phase extraction (dSPE) equipment (Catalog number: S2-2-FW-AOAC-KIT) were obtained from Thermo Fisher Scientific.

2.4. LC-MS/MS Analysis

2.4.1. Nurse bee sample preparation

Individual samples of approximately 20 bees were flash frozen and homogenized with a mortar and pestle. The bee homogenate was flash-frozen with liquid nitrogen and ground with a mortar and pestle. A portion (0.2 g) was transferred to a 2-mL plastic flip-top tube, and internal standard was added. Acetonitrile was then added (1.5 mL), and the samples were placed on a multi-tube shaker for 5 minutes at 2500 rpm. Samples were then centrifuged at 6000 rpm for 5 minutes. After centrifugation, a portion of supernatant was transferred into a 2-ml dSPE tube with 150 mg magnesium sulfate (MgSO_4), 50 mg primary secondary amine (PSA), and 50 mg C_{18} . The tubes were then placed on the multi-tube shaker for 5 minutes at 2500 rpm followed by centrifugation for 5 minutes at 6000 rpm. The supernatants ($\sim 700\ \mu\text{L}$) were transferred into autosampler vials prior to LC-MS analysis.

2.4.2. Pollen sample preparation

In brief, approximately 0.2 g of homogenized pollen was weighed into a 2-mL prefilled tube kit containing high 1.5 mm diameter impact zirconium beads. The appropriate internal standard was added and samples were extracted with 0.3 mL of water and then shaken on a

multi-tube shaker for 5 min at 2500 rpm. Acetonitrile (1.2 mL) was then added to all samples, followed by shaking on the multi-tube shaker for 5 min at 2500 rpm. The samples were then centrifuged for 5 min at 6000 rpm. After centrifugation, 1 mL of supernatant was transferred into a 2 mL dSPE tube containing 150 mg MgSO₄, 50 mg PSA, and 50 mg C18 and then shaken on a multi-tube shaker for 2 min at 2500 rpm. The sample tubes were centrifuged for 5 min at 6000 rpm. The supernatant (300 µL) was transferred into an amber autosampler vial with an insert prior to LC-MS analysis.

2.4.3. Nectar sample preparation

An aliquot of nectar (0.2 g) was transferred to a 2.0-mL ultracentrifuge tube and spiked with deuterated pesticide standards and then extracted by adding acetonitrile (1.2 mL) and vortexing for ~30 seconds. Samples were then placed on a multitube shaker for 5 minutes at 20,000 rpm for 5 minutes followed by centrifugation at 6,000 rpm for 5 minutes. A portion of the supernatant was transferred to an autosampler vial for analysis.

2.4.4. UHPLC-MS/MS analysis

Extracts were analyzed by a Vanquish Flex ultra-high-performance liquid chromatography system (UHPLC) coupled to a TSQ Altis triple quadrupole mass spectrometer (MS-MS) equipped with a heated electrospray source (ThermoFisher Scientific, San Jose, CA). The instrument settings as described by Hall et al.⁶¹ was used for analysis of the pollen and nectar for imidacloprid, clothianidin, thiamethoxam, 5-OH imidacloprid, and imidacloprid olefin. That method was modified for quantification of azoxystrobin, and pyraclostrobin. An additional method was developed for the quantification of chlorpyrifos. The parameters of these methods are outlined in the supplemental materials.

2.5. Sample Preparation for GC-ECD Analysis of Lambda-Cyhalothrin

2.5.1. Pollen and nurse bee sample preparation

Approximately 0.2 g of pollen or homogenized nurse bee sample was weighed into a 15-mL tube. Acetonitrile was added to all samples to make a total volume of 2 mL. All samples were then placed on a multi-tube shaker for 10 min at 2500 rpm. All samples were centrifuged for 5 min at 3000 rpm. Approximately 1.5 mL of supernatant was transferred to a 2-mL dSPE tube containing 150 mg MgSO₄, 50 mg PSA, and 50 mg C₁₈. All dSPE tubes were placed on a multi-tube shaker for 2 min at 2000 rpm. Samples were then centrifuged for 5 min at 6000 rpm. Approximately 1 mL of supernatant was transferred into a glass tube and concentrated to dryness. All samples were reconstituted in 100 µL of ethyl acetate containing aldrin (used as an internal standard) at 10 ppb. Approximately 100 µL of sample was transferred to an autosampler vial with an insert prior to GC/ECD analysis.

2.5.2. Gas Chromatography – Electron Capture Detector (GC-ECD) analysis

Extracts were analyzed by GC-ECD. For quantification, an Agilent Technologies 7890B GC equipped with an ECD and an Restek Rtx-5MS w/Integra-Guard 30 m x 0.25 mm i.d x 0.25 µm column (catalog # 12623-124) were used. Column flow was 1 mL/min. The oven temperature program was: 100°C (1 min) ramp 25°C/min to 250°C (1 min) followed by 10°C/min to 300°C(8). Helium was used as the carrier gas at a flow rate of 54.5 mL/min. Injection volume was 1 µL, and sample injection was carried out splitless at 250°C. The electron-capture detector was set to 250°C, and the makeup flow was argon/methane at 60 mL/min.

2.6. Data Analysis

Median, minimum, and maximum concentrations of neonicotinoids in pollen, obtained from the bees' pollen baskets, and nectar samples are reported, as well as the % detection greater

than compound-specific limits of quantification (LOQ). The median, minimum, maximum and % detection of each pesticide >LOQ in bee bodies and pollen obtained from traps are reported for control and prairie strip sites. Non-detects were set at one-half of the LOQ, therefore the calculated concentrations are conservative estimates and likely overestimate the actual field concentration.

To evaluate the effect of prairie strips on honey bee colony pesticide exposure, we treated each apiary deployed in 2018 and 2019 as an experimental unit. Concentration of each pesticide was averaged for each location over the entire year. The distribution of the average concentration of each compound was checked for normality. If necessary, a log transformation was applied to correct for heterogeneity. Residual plots for all compounds, except clothianidin and lambda-cyhalothrin in 2019 pollen and chlorpyrifos in 2019 bee samples, showed an increase in variance with concentration; consequently, data were log transformed to stabilize the variance. Similarly, concentrations of azoxystrobin, pyraclostrobin, chlorpyrifos and lambda-cyhalothrin in 2018 pollen and pyraclostrobin in 2019 bee samples were log transformed to correct for heterogeneity. For each compound in each year, a Welch Two Sample t-test was used to evaluate differences between honey bee colony exposure at control and prairie strips sites based on the hypotheses in equations 1 and 2.

Due to the limited number of detections for some compounds in pollen and bee bodies, a t-test could not be used. In 2018, this was the case for clothianidin and thiamethoxam data sets in both nurse bee and pollen samples collected from the hives. Similarly, in 2019 this was the case for clothianidin and thiamethoxam nurse bee and pollen data sets, as well as lambda-cyhalothrin bee samples. Data were analyzed with RStudio 1.1.383 (Ver 3.5.2) and SigmaPlot 14.0.

Risk quotients (RQs) for individual adult honey bees were calculated using BeeRex Version 1.0⁶¹ based on pesticide toxicity data summarized in Table B8-B12 and pesticide residue data reported in this study. To assess neonicotinoid exposure from pollen and nectar combined, pollen residue levels were converted to total nectar equivalents (equation 3),

$$C_{\text{total-t}} = C_{\text{nectar-t}} + C_{\text{pollen-t}} / 20 \quad (3)$$

where $C_{\text{total-t}}$ is the sum of the concentration in nectar ($C_{\text{nectar-t}}$) and pollen ($C_{\text{pollen-t}}$). The pollen concentration is adjusted by a weighing factor of 20 to account for differences in bee pollen and nectar consumption rates.⁶³

Results and Discussion

3.1. Prairie Strip and Control Sites: Comparison of Pollen and Nurse Bee Pesticide-Concentrations as Indicators of Honey Bee Colony Pesticide Exposure

Establishment of pollinator-attractive habitat within agricultural ecosystems can support increased honey bee colony productivity.²⁰ There is, however, concern that pollinator habitat established in close proximity to conventional crop fields may be exposed to pesticides. Previous studies indicate a variety of pesticides can be detected in non-target flowering plants^{30, 64}; however, differences in insecticide and fungicide exposure for honey bees foraging in row crop areas with and without pollinator habitat is uncertain. In this first objective, we used pollen collected from pollen traps and nurse bees sampled from honey bee colonies as indicators of honey bee colony pesticide exposure at prairie strip sites and control sites.

3.1.1. Honey bee-collected pollen

Pollen traps were used to collect pollen brought back to honey bee colonies located in prairie strip and control sites. Zhang et al.,²⁰ showed that 58 plant taxa were represented in the pollen collected. Although colonies were placed adjacent to soybean fields, soybean pollen was not present in the samples.²⁰ The lack of soybean pollen within the pollens traps is consistent

with previous findings in similar studies conducted in Iowa.^{65, 66} The 58 plant taxa confirm that wildflowers were used as source of forage by the honey bees.

Thiamethoxam was the only neonicotinoid detected in pollen collected from honey bee hives in 2018. In 2019, all three neonicotinoids were detected in pollen from control and prairie strip sites (Table 1). However, neonicotinoids were detected in less than 10% of all the samples collected at both control and prairie strip sites in 2019, with 100% of the detections occurring during the first sampling time period (June 11th). When possible, we compared the average concentration of each neonicotinoid found in prairie strip sites to those found in the control sites. No statistical difference was detected at a level of significance of 0.05 (Table B4 and B5). That indicated that the exposure to honey bee colonies located in prairie strips is no different from that occurring at control sites.

The detection of neonicotinoids during the early season sampling is likely due to dust contamination from planting neonicotinoid-treated seeds. Neonicotinoid seed treatments use is prevalent in maize and soybean and has the potential to move off field in the form of dust during planting resulting in deposition on non-target plants and/or from overland runoff and subsurface flow, which could result in systemic contamination of non-target plants.^{29-31, 36, 67}

In the pollen traps we observed more frequent detections and higher maximum concentration of the two fungicides, as well as chlorpyrifos and lambda-cyhalothrin, as compared to all three neonicotinoids. Azoxystrobin was detected in 57.2% of pollen samples collected in 2019 and 31% of the 2018 pollen samples. Pyraclostrobin was also detected in pollen samples in 2018 and 2019, but less frequently than azoxystrobin (<25%). Lambda-cyhalothrin and chlorpyrifos were detected in 11% and 15% of pollen collected in 2018, respectively, and 15% and 28% of pollen collected in 2019. When comparing differences in exposure between honey

bee colonies located within prairie strips and controls sites, there was no significant difference between the mean lambda-cyhalothrin, chlorpyrifos, pyraclostrobin and azoxystrobin pollen concentrations at 0.05 level of significance (Figure 1; Table B4 and B5).

When tracking concentrations detected over time, we observed pulses of lambda-cyhalothrin, chlorpyrifos, pyraclostrobin and azoxystrobin in pollen sampled in both years. These pulses are likely reflecting foliar applications of these compounds on the surrounding agricultural fields. Although the means of these compounds vary over time at control sites and prairie strip sites, the variations are within the analytical method variability.

The highest detections of chlorpyrifos and lambda-cyhalothrin occurred in late-July and early-August, respectively. Chlorpyrifos pulses also occurred in early and late-August. Lambda-cyhalothrin had additional pulses in late-August and early-September. These compounds are commonly used foliar applied insecticides in Iowa for control of pests in both soybean and maize fields.^{46, 49} Two major maize pests in Iowa often requiring foliar applied insecticides include true armyworms (*Mythimna unipuncta*)⁴⁶ and adult western corn rootworms.⁶⁸ In maize, foliar application for true armyworm typically occurs between mid-May and early-June;⁴⁹ however, we see minimal detections of chlorpyrifos and lambda-cyhalothrin during the early sampling window for pollen. To manage western corn rootworms, applications occur when female egg laying adults are present.⁶⁸ Adult emergence historically occurs between mid-July and mid-August lasting between four to six weeks.^{69, 70} This application window temporally overlaps with maximum detections of lambda-cyhalothrin and chlorpyrifos in pollen. Soybean aphid is the major economic pest requiring foliar applied insecticides in Iowa.⁴⁶ Soybean aphids are present in fields from mid-July to mid-September with the majority of insecticide spraying occurring between late-July to late-August.^{46, 71} These application windows coincide with pulses detected

for chlorpyrifos and lambda-cyhalothrin. Only one site in both years had a confirmed application of a foliar insecticide on the crop next to the apiary location ($< 3\text{m}$). This occurred in 2019 at a prairie strips site where chlorpyrifos was applied in late-July for control of soybean aphids. Sampling following application showed a major chlorpyrifos pulse in pollen (late July 2019; Figure 1A). However, the majority of detections within pollen samples are likely a result of application within the surrounding fields where honey bees are foraging.

Pyraclostrobin and azoxystrobin were commonly detected in pollen with the highest concentrations detected in early-July and additional pulses occurring in late-July and late-August. Fungicides are used on corn and soybean crops to control for fungal diseases such as northern leaf blight, grey leaf spot and soybean rust.⁷²⁻⁷⁴ Application of fungicides for maize have been recorded at vegetative stage 5 and reproduction stage 1 (R1), which depending on time of planting, occur in late-May/early-June and late July/early August, respectively.⁷³ For soybeans, foliar applications of fungicides typically occur during reproductive stages 3 and 4, which occur, depending on planting date, in late-July to mid-August in Iowa.⁷⁵ The expected application windows of these compounds overlap with when they are detected in pollen.

Honey bee hives placed at prairie strips were shown to have greater average colony weight and larger worker bee populations compared to control sites.²⁰ Subsequently, we saw no increase in pesticide exposure to hives placed in prairie strips compared to those placed in control sites. Overall, we see more frequent detections of lambda-cyhalothrin, chlorpyrifos, pyraclostrobin and azoxystrobin, compared to the three neonicotinoids, with substantially higher maximum concentrations ($> 10\text{-fold}$). Concentrations of these compounds within the pollen dissipates over time, likely due abiotic and biotic degradation indicating that honey bees are likely experiencing pulsatile exposure throughout the growing season. These exposures are likely

occurring in non-*Apis* bee species as well. Additionally, these compounds are sometimes premixed (e.g., Cobalt Advanced which has chlorpyrifos and lambda-cyhalothrin)⁷⁶ and in tank mixtures.⁷⁷ Consistent with the use of premixes and tank mixes, multiple pesticides were frequently detected in the same pollen sample, which raises concerns regarding potential pesticide mixture effects on bee communities.

3.1.2. Nurse bees

Nurse bees are young (< 21 days old), in-hive females that specialize in brood feeding.^{20,}
^{78, 79} While nectar is the major source of food for adult bees, nurse bees consume the largest portion of pollen compared to other adult honey bees.^{20, 78, 79} Therefore, if residues in pollen are of concern, nurse bees are the most vulnerable adults.^{72, 73}

Nurse bees contained at least one neonicotinoid in < 1% of the samples taken in 2017 (n=218) and 2019 (n=311). These residues are likely due to the nurse bees consuming contaminated pollen. Chlorpyrifos (7%), azoxystrobin (17%), and pyraclostrobin (11%) were detected more frequently. Consistent with the pollen trap samples, azoxystrobin was the most frequently detected compound in nurse bees and was also observed at the highest concentration (133 ng g⁻¹) (Table 1). The other fungicide, pyraclostrobin, was also detected in nurse bee tissue, but less frequently and at lower concentrations (maximum concentration of 14.3 ng g⁻¹) (Table 2). Chlorpyrifos was detected in both 2018 and 2019 and was the second most frequently detected pesticide, but at concentrations lower than azoxystrobin (maximum 16.6 ng g⁻¹). The maximum concentrations of chlorpyrifos, pyraclostrobin and azoxystrobin occurred in mid-August, mid-July, and early-July, respectively. The concentrations of these compounds follow similar trends to that seen in the pollen samples (Figure 1). The average concentration of compounds in nurse bees sampled from prairie strip sites and controls sites was not significantly different in either year at a level of significance of 0.05 (Figure 1; Tables S6 and S7).

The frequency of pesticides detected in nurse bee samples varied between sampling time points and year. The highest concentrations of azoxystrobin and pyraclostrobin in bees occurred in early-July. The timing of the maximum concentration detected of azoxystrobin and pyraclostrobin in bees coincides with when their highest concentrations are detected in pollen (early-July). For lambda-cyhalothrin, the highest concentration detected in pollen occurred in early-August, and the highest concentration detected in bees occurred in mid-August. The maximum concentration of chlorpyrifos was detected in bees in early-September, while the highest concentration in pollen was detected in early-July. There was also a pulse in late-August. For many of the compounds, detection frequencies were higher in 2018 compared to 2019. This likely reflects changes in pesticide use for a given pest species as well as exposure variability due to maize-soybean rotations, types of pesticides applied, and the timing of nurse bee collection.

Our findings are consistent with previous studies documenting various pesticides in honey bees²² and native bees.⁸⁰ Hladik et al.⁸⁰ analyzed 54 composite native bee samples and detected 18 pesticides including clothianidin, thiamethoxam, imidacloprid, chlorpyrifos, azoxystrobin, and pyraclostrobin. Mullen et al.²² analyzed wax, pollen, and bees sampled from honey bee colonies for 121 different pesticides and metabolites. They reported no detections of clothianidin, imidacloprid, or thiamethoxam in honey bee bodies. Their reported maximum concentrations of pyraclostrobin (8 g ng^{-1}) and chlorpyrifos (10.7 ng g^{-1}) were lower than what was reported in our study (32 ng g^{-1} ; 17 ng g^{-1}) with medians within the same order of magnitude ($<2.2 \text{ ng g}^{-1}$). Similar to our data they reported substantially lower pesticide concentrations being detected in honey bees compared to pollen. This trend may be due to biotransformation and excretion of pesticides by honey bees.²²

3.2. Prairie Strip Sites: Nectar and Pollen Neonicotinoid Concentrations

As noted in the prairie strips – control site study, neonicotinoids were detected in pollen and nurse bees collected from honey bee colonies located in prairie strips. However, the extent to which that exposure was a result of prairie strip contamination is unknown. Previous research reported clothianidin, imidacloprid and thiamethoxam being present in the foliage of flowering plants collected from prairie strip sites.⁵⁸ Detection of neonicotinoids in foliage indicates systemic uptake of neonicotinoids into non-target blooming plants. To confirm that that prairie strips are a potential source of neonicotinoids exposure to bees, we measured imidacloprid, clothianidin and thiamethoxam concentrations in pollen and nectar collected by Apidae from forbs within prairie strips.

3.2.1. Pollen and nectar samples

The number of samples analyzed per site and timing of collection was dependent on the number of bees that were captured per sampling event and the amount of pollen and/or nectar those bees were carrying. Over the three sampling years, 50 and 66 samples had sufficient pollen and nectar (by weight) for neonicotinoid analysis, respectively. During late summer, more bees were at the prairie strip sites, likely a result of more available floral resources during this period of time.¹⁸ Consequently, residue analyses were possible for samples collected from mid-July to mid-August.

At least one neonicotinoid was detected in 20 % of the total pollen samples analyzed. Imidacloprid was the most commonly detected compound, followed by thiamethoxam, with the majority of these detections occurring in July. Clothianidin was not detected in any pollen samples collected from foraging bees in 2017 or 2019. Across all three years, the median pollen concentrations of imidacloprid, clothianidin, and thiamethoxam was <LOQ with maximum detected concentrations of 22.7 ng g⁻¹, 2.41 ng g⁻¹, and 5.12 ng g⁻¹, respectively (Table 2).

Neonicotinoid residues were not always detected in pollen samples taken from prairie strips, even though the compounds were present within the soil and the foliage of flowering plants. Heterogeneity in soil properties (e.g., sand, silt, clay, and organic matter) and differences in plant physiology may influence the persistence and translocation of these compounds into pollen from systemic uptake.^{30, 32, 58} The maximum concentration of imidacloprid was detected in 2017; however, imidacloprid was not used as a seed treatment in crop fields at any of the sites. The detection of imidacloprid in pollen at sites where it was not used as a seed treatment could be due to spray drift from foliar application in surrounding farms or could be a result of previous field use and persistence within the soil, as noted in previous literature.^{31, 58}

Neonicotinoid pollen concentrations we report are within the range reported by Biotas et al.²³; median concentrations of all three compounds from both studies were also within the same order of magnitude. Botías et al.²³ collected nectar and pollens samples from wildflowers located an average distance of 1.5 m from the edge of winter-sown oilseed rape (OSR) or winter-sown wheat (WW) fields planted with neonicotinoid-treated seeds. While ranges of concentrations were similar, Botias et al.²³ did report a higher maximum concentration of thiamethoxam (86.02 ng g⁻¹) in pollen, as well as more frequent detections of thiamethoxam (58.1% of samples) than what was observed in our study (5.12 ng g⁻¹; 4% of samples). For imidacloprid and clothianidin, we reported more frequent detections with higher maximum concentrations.

In the 66 nectar samples we collected, thiamethoxam and imidacloprid were the only compounds detected (Table 2). Thiamethoxam was detected in 6% of the samples and imidacloprid was detected in 3%. The maximum detection of thiamethoxam occurred in early July (4.2 ng g⁻¹), while the maximum detection of imidacloprid occurred in late July (2.0 ng g⁻¹). Botias et al. (2015) reported lower maximum concentration of thiamethoxam and imidacloprid

(1.80 ng g⁻¹ to < 0.17 ng g⁻¹) in the nectar of wild flowers than what was detected in our study; however, the medians were within the same order of magnitude (≤ 0.17 ng g). Botias et al.²³ also reported more frequent detection of thiamethoxam, clothianidin and imidacloprid in nectar collected from wildflowers from OSR margins; however, they reported no detections within wildflowers from WW margins.

Hall et al.⁵⁸ reported frequent detection of thiamethoxam (91.8-97.2%) and imidacloprid (91.8-97.2%) in soil at the same prairie strip sites, as well as detections (12.1% - 43.1%) in foliage collected from flowering plants. It is likely that detections in the nectar and pollen reported here are from systemic uptake from the contaminated soil and translocation throughout the plants. The lower frequency of detection and lower concentrations in pollen and nectar, compared to that in soil and plant foliage, is likely due to metabolism within the plants and growth dilution. The more frequent detection of these compounds in pollen compared to nectar could be due to increased hydrolysis and photolysis of the compounds in nectar.²³ These data confirm that that prairie strips are a potential source of neonicotinoids exposure to foraging bees.

3.3. Characterizing Risks of Pesticides Detected in Pollen and Nectar

Potential risk of these pesticides to bees is a function of both toxicity and exposure. For bees the concentrations in nectar (consumed as honey) and pollen (consumed fresh or stored as bee bread, a combination of pollen and honey) serves as the basis for characterizing dietary exposure.^{76,77} Concentrations in pollen and nectar will be related to the forage plant's proximity to the crop field(s) planted with treated seeds or managed with foliar applications, the concentrations of active ingredient within these formulations, application rates and seed planting density, as well as environmental conditions. For neonicotinoid-treated seeds, dust drift at planting time and/or subsurface water flow down-slope of fields post-planting could result in exposure through pollen and nectar. Alternatively, foliar insecticide and fungicides applications

can result in spray drift deposition on the pollen and/or nectar of flowering plants. These exposure pathways could cause acute or chronic effects on bee species consuming contaminated pollen and/or nectar.

Our data indicate that bees foraging in prairie strips and other pollinator habitat in Iowa's North Central agroecosystems are likely exposed to commonly applied foliar insecticides (chlorpyrifos and lambda-cyhalothrin) and fungicides (azoxystrobin and pyraclostrobin), as well as commonly used neonicotinoid seed treatments, albeit less frequently. Bees feeding on fresh pollen or beebread could be exposed to chlorpyrifos, lambda-cyhalothrin, azoxystrobin and pyraclostrobin residues as high as 505 ng g^{-1} , $> 100 \text{ ng g}^{-1}$, 409 ng g^{-1} , and 90 ng g^{-1} , respectively. For thiamethoxam, clothianidin, and imidacloprid, bees feeding on pollen could be exposed to residues as high as 31.9 ng g^{-1} , 13.8 ng g^{-1} , and 28.1 ng g^{-1} , respectively. If also feeding on nectar they could be exposed to concentrations of thiamethoxam and imidacloprid as high as 4.16 ng g^{-1} and 1.95 ng g^{-1} , respectively.

To estimate the risk associated with neonicotinoid exposure to individual bees within prairie strips and control sites, we used the EPA Bee-Rex model⁶² to calculate acute and chronic risk quotients (RQs) for adult honey bees and the chronic RQ for honey bee larvae. The exposure concentrations used to calculate the RQs were the maximum concentrations of imidacloprid, thiamethoxam and clothianidin detected in pollen, including pollen from pollen traps and pollen collected from individual bees, and nectar, to provide the highest exposure scenario from these data and the most conservative RQ estimates. The toxicity endpoints used in the Bee-Rex model can be found in Table B8, B9 and B10. The RQs calculated for clothianidin and imidacloprid did not exceed the level of concern (LOC) for acute risk ($\text{LOC} \geq 0.4$) or chronic risk ($\text{LOC} \geq 1$). The adult chronic RQ calculated for thiamethoxam (3.38) did exceed the LOC for chronic risk to the

most sensitive adult honey bees, indicating that there is potential for chronic risk for both worker bees foraging for nectar and nurse bees tending to the brood and queen. Bee-Rex is considered a screening tool and employs conservative exposure assumptions (e.g., pesticides do not degrade while in honey and bee bread stored in hives)^{76, 77} as well as the most sensitive toxicity endpoints from laboratory studies of individual bees. We also used the highest concentrations in pollen and nectar to calculate the LOC to give a worst-case scenario. However, the majority of the detections (> 90%) were below the LOQ. A more refined risk characterization for thiamethoxam would likely suggest a low likelihood of adverse effects.

We also compared the maximum concentrations of imidacloprid, thiamethoxam and clothianidin detected in pollen, including pollen from pollen traps and pollen collected from individual bees, and nectar to the No Observed Adverse Effect Concentrations (NOAECs) based on colony feeding studies (6-weeks).^{78, 79} To assess total dietary exposure to honey bee colonies, we combined maximum concentration data for pollen and nectar (Equation 3).⁶³ The maximum estimated exposures for all three neonicotinoids were 7 to 20 – fold lower than the NOAEC for colony feeding studies, indicating a low likelihood of adverse effects for honey bee colonies within these prairie strips.

For lambda-cyhalothrin, the Bee-Rex model was used to calculate acute and chronic risk RQs for adult honey bees and larvae. The exposure concentrations used to calculate the RQs were based on concentrations detected in pollen and the toxicity endpoints provided in Table B11. The calculated RQ did not exceed the LOC for acute risk ($LOC \geq 0.4$) or chronic risk ($LOC \geq 1$) for individual bees. The RQ was calculated based only on pollen exposure; however, research has shown that honey bees consume more nectar than pollen.⁸¹ Therefore, additional research evaluating nectar concentrations of these compounds is necessary to fully evaluate the

acute and chronic risks lambda-cyhalothrin exposure poses to honey bees foraging in prairie strips.

Limited honey bee toxicity data is available for chlorpyrifos. Currently the only data set available is for acute oral exposure of honey bee larvae. An acute oral RQ for honey bee larvae was calculated using the available toxicity data (Table B12) and the maximum concentration of chlorpyrifos detected in pollen (505 ng g^{-1}), which resulted in a $\text{RQ} < 0.4$ indicating low risk to individual honey bee larvae. To gain a more thorough understanding of risk associated with exposure to chlorpyrifos, more robust toxicity studies are needed as well as empirical field data for concentration in nectar.

The majority of research to date is focused on the effects of insecticides on bees.⁸⁰ Limited attention has been given to fungicides and potential adverse effects of pesticides mixtures. There is, however, research indicating that fungicides increase honey bee susceptibility to the pathogen *Nosema*^{82, 83}, as well as increase their sensitivity to insecticides, resulting in lower lethal doses.⁸⁴ Additional research is needed to further understand how these fungicides are affecting bees consuming pollen and nectar contaminated with these compounds, as well as mixtures of other pesticides.

Our results indicate that while dietary neonicotinoid exposure is likely from consumption of pollen and nectar in forbs in pollinator habitat embedded within Iowa agroecosystems, there is low risk for honey bees. We reported concentrations of chlorpyrifos and lambda-cyhalothrin in pollen that are also below levels of concern given the currently available toxicity data. Based on maximum consumption rates of pollen and nectar for foraging honey bees, insecticides in nectar is the most important dietary exposure pathway.^{76, 77} Additional information on levels of lambda-

cyhalothrin and chlorpyrifos in nectar are needed to more accurately characterize the risk to honey bees foraging in these landscapes.

The extent to which risk characterizations for honey bees are appropriate for non-*Apis* bee species is an active area of research. Individual bee toxicity data for honey bees is generally representative or protective of other species of bees.³⁶ However, the extent to which NOAECs for honey bee hives is protective of native bee populations is a complex issue being addressed by ongoing research.

Conclusion

Imidacloprid, thiamethoxam, and clothianidin are extensively used to treat maize and soybean seeds, which are the major crops grown in Iowa.^{28, 85} Although these compounds are present in the nectar and pollen of forbs in pollinator habitat, these seed treatments pose low dietary risk to honey bees.

Pyrethroids and organophosphates are commonly used foliar insecticides.^{7, 51} Aerial applications, particularly of lambda-cyhalothrin and chlorpyrifos, can result in exposure of honey bee hives. However, additional research is needed to further characterize how these exposures may adversely affect honey bees and other non-*Apis* bee species. Fungicide use is increasing in the North Central states.⁷² Research has shown that exposure to fungicides can adversely affect honey bees and non-*Apis* bee species. Fungicides were the most commonly detected pesticide in both pollen and bee bodies, indicating that additional research to understand the long-term impacts they have on pollinators and the services they provide are necessary.

Overall, these data show that the benefits associated with placing honey bee hives in prairie strips or other pollinator habitat in close proximity to crop fields are not likely to negatively impact bees through increased pesticide exposure.²⁰ Additional research on colony-level effects from foliar-applied insecticide and fungicide mixtures would help decrease

uncertainties about the risks associated with these exposure pathways for honey bees and native bees.

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Tables and Figures

Table 1. Pesticide median concentrations, total detections >LOQ, maximum concentrations and total number of pollen traps and nurse bee bodies sampled from hives in prairie strip and control sites embedded in or adjacent to conventional soybean or maize fields.

Matrix	Year		Control							Prairie Strips						
			CLO	TMX	IMI	LCH	CPS	AZY	PYC	CLO	TMX	IMI	LCH	CPS	AZY	PYR
Pollen	2018	LOQ (ng g ⁻¹)	0.5	0.5	0.5	5	1	1	1	0.5	0.5	0.5	5	1	1	1
		Median (ng g ⁻¹)				<LOQ	<LOQ	<LOQ	<LOQ		<LOQ		<LOQ	<LOQ	<LOQ	<LOQ
		% detections	0	0	0	8.2	20.4	32.7	12.2	0	1.8	0	14.3	8.9	30.4	17.9
		Max (ng/g)				37.9	8.4	365.6	90.0		0.7		>100.0	9.1	225.3	28.7
		N	49	49	49	49	49	49	49	56	56	56	56	56	56	56
	2019	Median (ng g ⁻¹)	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	2.2	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	2.0	<LOQ
		% detections	8.6	8.6	7.1	7.2	27.1	61.4	24.3	8.8	1.5	5.9	22.4	29.4	52.9	20.6
		Max (ng/g)	13.8	31.9	28.1	65.5	24.2	238.7	64.6	9.8	1.3	6.0	>100.0	505.3	408.6	19.5
		N	70	70	70	69	70	70	70	68	68	68	67	68	68	68
Bee Bodies	2018	LOQ (ng g ⁻¹)	0.5	0.5	0.5	5	1	1	1	0.5	0.5	0.5	5	1	1	1
		Median (ng g ⁻¹)		<LOQ		<LOQ	<LOQ	<LOQ	<LOQ					<LOQ	<LOQ	<LOQ
		% detections		0.9		0.9	3.7	16.5	7.3				1.8	16.1	28.6	14.3
		Max (ng/g)		0.5		39.6	3.7	6.7	1.8				59	4.2	133.1	31.9
		N		109		109	109	109	109				109	109	109	109
	2019	Median (ng g ⁻¹)		<LOQ			<LOQ	<LOQ	<LOQ		<LOQ			<LOQ	<LOQ	<LOQ
		% detections	0	0	1.9	0	5.8	16.0	1.3	0	0.7	0	0	3.3	9.2	0
		Max (ng/g)			3.2		16.6	3.2	2.4		1.0			3.5	5.8	0.5
		N	159	159	159	159	159	159	159	152	152	152	152	152	152	152

N- Number of samples

CLO – clothianidin; TMX – thiamethoxam; IMI – imidacloprid; LCH – lambda-cyhalothrin; CPS – chlorpyrifos; AZY – azoxystrobin; PYC – pyraclostrobin

LOQ – Limit of Quantification

Table 2. Summary of neonicotinoid residues in pollen and nectar collected from bees^a foraging in prairie strips in 2017, 2018, and 2019.

Matrix	Year	N		CLO	TMX	IMI
Pollen	2017	16	LOQ (ng g ⁻¹)	0.5	0.5	0.5
			Detections (%)	25	0	31.3
			Median (ng g ⁻¹)	<LOQ	n/a	<LOQ
			Max (ng g ⁻¹)	2.41	n/a	22.7
	2018	18	Detections (%)	22.2	11.1	33.3
			Median (ng g ⁻¹)	<LOQ	<LOQ	<LOQ
			Max (ng g ⁻¹)	2.41	5.12	1.27
	2019	24	Detections (%)	4.2	0	8.3
			Median (ng g ⁻¹)	<LOQ	n/a	<LOQ
			Max (ng g ⁻¹)	0.79	n/a	11.0
Nectar	2017	16	LOQ (ng g ⁻¹)	0.2	0.09	0.2
			Detections (%)	0	6.25	0
			Median (ng g ⁻¹)	n/a	<LOQ	n/a
			Max (ng g ⁻¹)	n/a	0.174	n/a
	2018	14	Detections (%)	0	7.17	0
			Median (ng g ⁻¹)	n/a	<LOQ	n/a
			Max (ng g ⁻¹)	n/a	0.176	n/a
	2019	46	Detections (%)	0	4.35	4.35
			Median (ng g ⁻¹)	n/a	<LOQ	<LOQ
			Max (ng g ⁻¹)	n/a	4.16	1.95

^a Various species of bees were sampled (Table B3)

N- Number of samples

CLO – clothianidin; TMX – thiamethoxam; IMI – imidacloprid;

LOQ – Limit of Quantification

n/a – not applicable

Table 3. Summary of acute and chronic risk quotients (RQ) for adult bees based on seed-treatment applications of clothianidin, thiamethoxam and imidacloprid.

Compound	Pollen (ng g ⁻¹)	Nectar (ng g ⁻¹)	Adult Acute RQ	Adult Chronic RQ	Larval Chronic RQ
Clothianidin	13.8	0.2	0.04 ^a	0.45 ^c	0.02 ^d
Thiamethoxam	31.9	4.16	0.33 ^a	3.38 ^c	0.14 ^d
Imidacloprid	28.1	1.95	0.15	0.52	0.19

^a Based on an adult 48-h acute oral LD₅₀ (Lethal dose to 50% of test population) of 0.0037 µg/bee⁷⁷

^b Based on adult 10-day adult chronic NOAEC (No observed adverse effect concentration) of 0.00036 µg/bee⁷⁷

^c Based on adult 21-day larval chronic NOAEC of 0.0043 µg /bee⁷⁷

^d Based on an adult 48-h acute oral LD₅₀ of 0.0039 µg/bee⁷⁶

^e Based on adult 10-day adult chronic NOAEC of 0.0011 µg/bee⁷⁶

^f Based on adult 21-day larval chronic NOAEC of 0.0018 µg /bee⁷⁶

Table 4. Maximum pollen concentration detected, maximum pollen concentration converted to nectar equivalent, maximum nectar concentration detected and no observed adverse effect concentration (NOAEC) for imidacloprid, thiamethoxam and clothianidin.

	Max Concentration Pollen (ng g ⁻¹)	Nectar Equivalent Concentration (ng g ⁻¹) ^a	Max Concentration Nectar (ng g ⁻¹)	Estimated Total Concentration (ng g ⁻¹)	NOAEC (ng g ⁻¹) ^b
Imidacloprid	28.1	1.41	2	3.4	25
Thiamethoxam	31.9	1.60	4.2	5.8	44
Clothianidin	13.8	0.7	0.2	0.9	19

^a Calculated from equation 3.⁶²

^b Based on 6-week honey bee colony feeding study.^{77, 78}

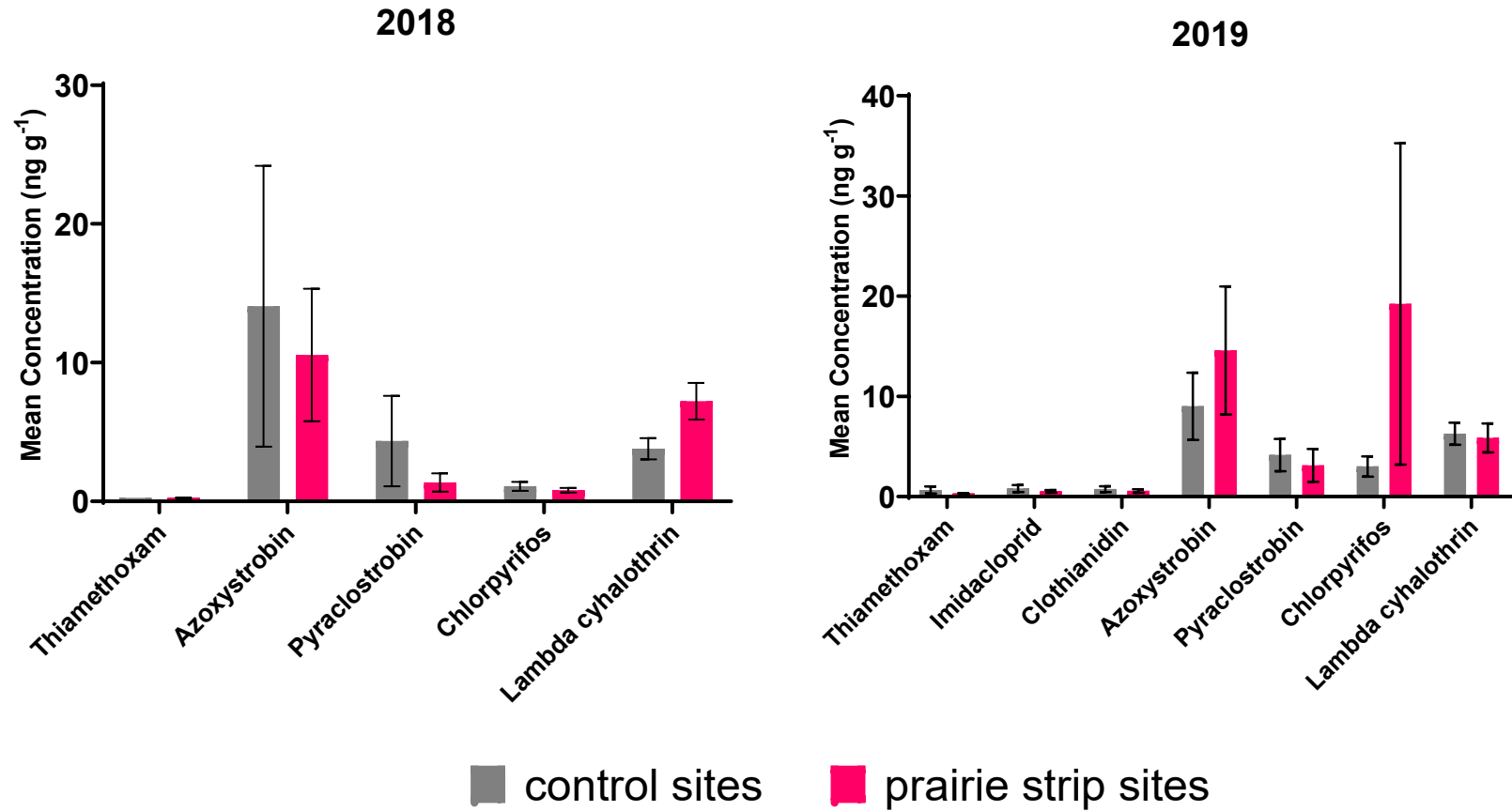


Figure 1. The concentration of each pesticide in pollen was averaged (\pm SD) for each location over yearly sampling (May through September) for prairie strip and control sites in 2018 and 2019. A Welch two sample t-test, indicated no statistical differences among the individual compounds in any year at a $p = 0.05$ level of significance.

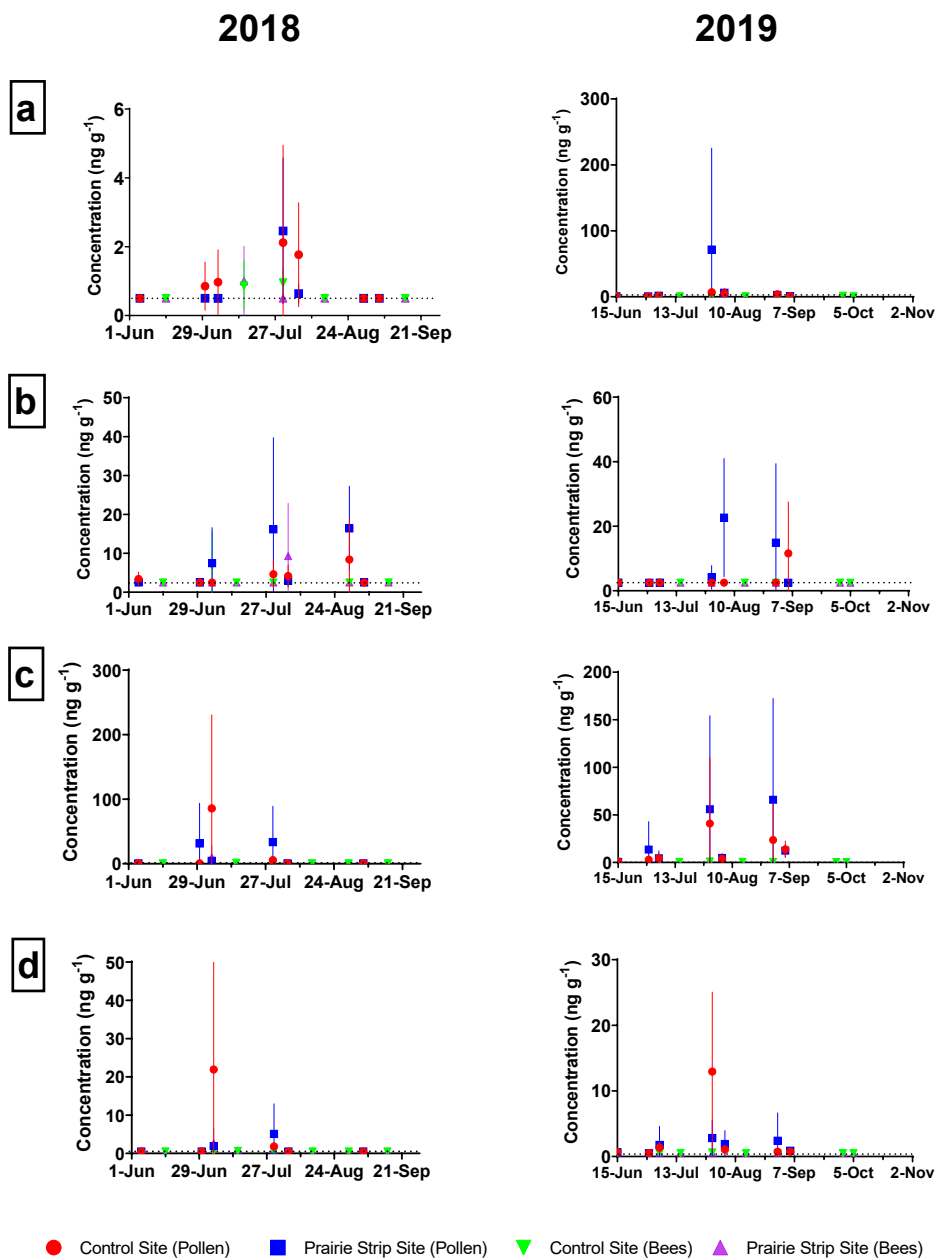


Figure 2. Mean pollen trap and nurse bee concentrations (\pm SD) of (a) chlorpyrifos, (b) lambda-cyhalothrin, (c) azoxystrobin and (d) pyraclostrobin for hives in prairie strip or control sites for sampling time points in 2018 and 2019.

Appendix A. Supplemental Information

LC-MS/MS Analysis of Pollen from Pollen Trap Extracts for Clothianidin, Imidacloprid, Thiamethoxam, Azoxystrobin, Pyraclostrobin

A Vanquish Flex LC pump interfaced with a TSQ Altis mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA) was used for the analysis. The source conditions were as

follows: spray voltage - 3700 V, sheath gas - 30 Arb, auxiliary gas - 6 Arb, sweep gas – 1 Arb, ion transfer tube temperature - 325 °C, and vaporizer temperature – 350 °C. The total run time of the method was 12 minutes. The resolution of Q1 and Q3 was 0.7 FWHM. The CID gas was set to 2 mTorr. The chromatographic peak width was 4 sec and the cycle time was 0.3 sec. Analysis was performed in positive electrospray ionization mode.

The column used for the analysis was Accucore aQ 100 x 2.1mm, 2.6 µm particle size (Thermo Fisher Scientific). Mobile Phase A was water with 2% methanol, 5 mM ammonium formate and 0.1% formic acid. Mobile Phase B was methanol with 2% water, 5 mM ammonium formate and 0.1% formic acid. The column oven was set to 30 °C. The LC gradient profile was the following: linear ramp 5.5 minutes from 0-80% organic, hold at 80% organic for 4 minutes, return to 0% organic in 1.5 minutes, and hold at starting conditions for 0.5 minutes. An injection volume of 2 µL was used for all analyses.

LC-MS/MS Analysis of Bee Extracts for Clothianidin, Imidacloprid, Thiamethoxam, Azoxystrobin, Pyraclostrobin

A PAL Vanquish Flex LC pump interfaced with a TSQ Altis mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA) was used for the analysis. A TriPlus RSH autosampler system (CTC Analytics AG, Zwingen, Switzerland) was used for injection of all samples onto the analytical column. The mass spectrometer source conditions were as follows: spray voltage – 3700 V, sheath gas - 30 Arb, auxiliary gas - 6 Arb, sweep gas - 1 Arb, ion transfer tube temperature - 325 °C, and vaporizer temperature – 350 °C. The total run time of the method was 12 minutes. The resolution of Q1 and Q3 was 0.7 FWHM. The CID gas was set to 2 mTorr. The chromatographic peak width was 4 sec and the cycle time was 0.3 sec. Analysis was performed in positive electrospray ionization mode.

The column used for the analysis was Accucore aQ 100 x 2.1mm, 2.6 μ m particle size (Thermo Fisher Scientific). Mobile Phase A was water with 2% methanol, 5 mM ammonium formate and 0.1% formic acid. Mobile Phase B was methanol with 2% water, 5 mM ammonium formate and 0.1% formic acid. The column oven was set to 30 °C. The LC gradient profile was the following: linear ramp 5.5 minutes from 0-80% organic, hold at 80% organic for 4 minutes, return to 0% organic in 1.5 minutes, and hold at starting conditions for 0.5 minutes. An injection volume of 6 μ L was used for all analyses.

LC-MS/MS Analysis of Bee Extracts and Pollen from Pollen Trap Extracts for Chlorpyrifos

A Vanquish Flex LC pump interfaced with a TSQ Altis mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA) was used for the analysis. The source conditions were as follows: spray voltage - 3700 V, sheath gas - 30 Arb, auxiliary gas - 6 Arb, sweep gas - 1 Arb, ion transfer tube temperature - 325 °C, and vaporizer temperature – 350 °C. The total run time of the method was 10 minutes. The resolution of Q1 and Q3 was 0.7 FWHM. The CID gas was set to 2 mTorr. The chromatographic peak width was 4 sec and the cycle time was 0.3 sec. Analysis was performed in positive electrospray ionization mode.

The column used for the analysis was HypersilGold Aq 100 x 2.1mm, 1.9 μ m particle size (Thermo Fisher Scientific). Mobile Phase A was water with 2% methanol, 5 mM ammonium formate and 0.1% formic acid. Mobile Phase B was methanol with 2% water, 5 mM ammonium formate and 0.1% formic acid. The column oven was set to 30 °C. The LC gradient profile was the following: linear ramp 2 minutes from 0-100% organic, hold at 100% organic for 5.5 minutes, return to 0% organic in 1.5 minutes, and hold at starting conditions for 0.5 minutes. An injection volume of 2 μ L was used for all analyses.

Appendix B. Supplemental Tables and Figures

Table B1. Crops, samples collected and year of collection for research sites.

Site Name	Site Type	Crop 2017	Crop 2018	Crop 2019	Pollen and Nectar Collected	Honey Bee Colony Pollen Traps Collected	Honey Bee Colony Nurse Bees Collected
GES	Strips	Maize	Soybean	N/A	2017 & 2018	N/A	N/A
MCN	Strips	Maize	Soybean	N/A	2017 & 2018	N/A	N/A
DMW-N	Strips	Soybean	Maize	N/A	2017 & 2018	N/A	N/A
DMW-S	Strips	Soybean	Maize	N/A	2017 & 2018	N/A	N/A
SME	Strips	Maize	Soybean	Maize	2017, 2018 & 2019	2018 & 2019	2018 & 2019
GUT	Strips	Maize	Soybean	Maize	2017, 2018 & 2019	2018 & 2019	2018 & 2019
WOR	Strips	Soybean	Maize	Maize	2017, 2018 & 2019	2019	2019
SMI	Strips	Maize	Soybean	Soybean	2018 & 2019	2018 & 2019	2018 & 2019
STO	Strips	Soybean	Maize	Maize	2018 & 2019	2018 & 2019	2018 & 2019
HAR	Control	N/A	Soybean	Maize	N/A	2018 & 2019	2018 & 2019
JER	Control	N/A	Maize	Soybean	N/A	2018 & 2019	2018 & 2019
KOE	Control	N/A	Soybean	Maize	N/A	2018 & 2019	2018 & 2019
HER	Control	N/A	Soybean	Maize	N/A	2018 & 2019	2018 & 2019
DAI	Control	N/A	N/A	Maize	N/A	2019	2019

N/A – Non-applicable indicating that site was not sampled that year.

Table B2. Sampling time for pollen and nurse bees from hives during 2018 and 2019.

Year	Pollen Trap Collection Data	Nurse Bee Collection Data
2018	June 13	June 20
	July 11, 27	July 9, 18, 31
	August 11, 28	August 15, 30
	September 7, 28	September 17-21
2019	June 11, 25	June 20
	July 9, 24	July 2, 18, 30
	August 6, 23	August 14, 28
	September 6	September 26
		October 9

Table B3. Common foraging bee species collected using bee vacuum.

Family	Genus	Species	Scientific Name
Andrenidae	Andrena	rudbeckiae	Andrena rudbeckiae
Apidae	Bombus	griseocollis	Bombus griseocollis
Apidae	Svastra	obliqua	Svastra obliqua
Apidae	Apis	mellifera	Apis mellifera
Apidae	Melissodes	bimaculatus	Melissodes bimaculatus
Megachilidae	Megachile	latimanus	Megachile latimanus
Apidae	Bombus	pennsylvanicus	Bombus pennsylvanicus

Table B4. Welch two sample t-test results for pollen samples 2018. (Degrees of Freedom=3)

Compound	t	p-value
Azoxystrobin	1	0.8045
Pyraclostrobin	0.00080499	0.5003
Chlorpyrifos	-0.78726	0.2337
Thiamethoxam	1	0.8045
Lambda cyhalothrin	0.67591	0.67591

Table B5. Welch two sample t-test results for pollen samples 2019. (Degrees of Freedom=4)

Compound	t	p-value
Azoxystrobin	0.28136	0.6063
Pyraclostrobin	-0.63528	0.2716
Chlorpyrifos	0.8154	0.7773
Imidacloprid	-0.4758	0.3248
Clothianidin	-0.53851	0.3043
Thiamethoxam	-0.90826	0.2048
Lambda cyhalothrin	-0.2323	0.4113

Table B6. Welch two sample t-test results for bee samples 2018. (Degrees of Freedom=3)

Compound	t	p-value
Azoxystrobin	0.79475	0.2422
Pyraclostrobin	1.0219	0.8097
Chlorpyrifos	0.050759	0.5192
Imidacloprid	1	0.8045
Lambda cyhalothrin	0.28702	0.6074

Table B7. Welch two sample t-test results for bee samples 2019. (Degrees of Freedom=4)

Compound	t	p-value
Azoxystrobin	0.021367	0.5082
Pyraclostrobin	-1	0.187
Chlorpyrifos	-0.69375	0.2593
Imidacloprid	1	0.813

Table B8. Clothianidin toxicity endpoints used for BeeRex inputs.

Description	Value (µg a.i./bee) ^a
Adult contact LD50	0.0275
Adult oral LD50	0.0037
Adult oral NOAEL	0.00036
Larval NOAEL	0.0043

^a Values taken from: Final Bee Risk Assessment to Support the Registration Review of Clothianidin and Thiamethoxam; U.S. Environmental Protection Agency, 2020;
<https://www.regulations.gov/document/EPA-HQ-OPP-2011-0865-1164> (Accessed 10/20/201).

Table B9. Thiamethoxam toxicity endpoints used for BeeRex inputs.

Description	Value (µg a.i./bee) ^a
Adult contact LD50	0.021
Adult oral LD50	0.0038
Adult oral NOAEL	0.0025
Larval LD50	0.03
Larval NOAEL	0.0037

^a Values taken from: Final Bee Risk Assessment to Support the Registration Review of Clothianidin and Thiamethoxam; U.S. Environmental Protection Agency, 2020;
<https://www.regulations.gov/document/EPA-HQ-OPP-2011-0865-1164> (Accessed 10/20/201).

Table B10. Imidacloprid toxicity endpoints used for BeeRex inputs.

Description	Value (µg a.i./bee)^a
Adult oral LD50	0.0039
Adult oral NOAEL	0.0011
Larval NOAEL	0.0018

^a Values taken from: Final Bee Risk Assessment to Support the Registration Review of Imidacloprid; U.S. Environmental Protection Agency, 2020; <https://www.regulations.gov/document/EPA-HQ-OPP-2008-0844-1611> (Accessed 10/20/21).

Table B11. Lambda cyhalothrin toxicity endpoints used for BeeRex inputs.

Description	Value (µg a.i./bee)^a
Adult oral LD50	0.0039
Adult oral NOAEL	0.0349
Larval LD50	0.032
Larval NOAEL	0.005

^aLambda-Cyhalothrin: Transmittal of Four Data Evaluation Records for Bee Toxicity Studies and Updated Tier I Bee Risk Assessment. (2020). Retrieved from <https://www.regulations.gov/document/EPA-HQ-OPP-2010-0480-0316> (Accessed 10/20/21).

Table B12. Chlorpyrifos toxicity endpoints used for BeeRex inputs.

Description	Value (µg a.i./bee)^a
Larval LD50	0.0165

^a Chlorpyrifos: Draft Ecological Risk Assessment for Registration Review. (2020). Retrieved from <https://www.regulations.gov/document/EPA-HQ-OPP-2008-0850-0940> (Accessed 10/20/21).

CHAPTER 5. EVALUATION OF ELISA AND LC-MS/MS METHODS OF QUANTIFYING NEONICOTINOID CONCENTRATIONS IN WATER AND PLANT FOLIAGE

Maura J. Hall^{a*}, Dwayne E. Schrunck^b, Laura E. Burns^b, Steven P. Bradbury^{a,c}, and Joel R. Coats^a

^a Toxicology Program and Department of Entomology, Iowa State University, Ames, Iowa, USA

^b Analytical Chemistry Services, Veterinary Diagnostic Laboratory, Iowa State University, Ames, Iowa, USA

^c Department of Natural Resource Ecology and Management, Iowa State University, Ames, Iowa, USA

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Abstract

Neonicotinoids are one of the most widely deployed insecticides globally, largely due to their wide-scale use as seed treatments, in agricultural production. These compounds can move off-field into terrestrial and aquatic habitats where they pose a potential risk to non-target organisms. Extensive monitoring studies are needed to quantify the fate and transport of these compounds to improve exposure characterizations for ecological risk assessments. Quantification of neonicotinoids in water and plant tissue by liquid chromatography tandem mass spectrometry (LC-MS/MS) is generally accepted as the preferred analytical method; however, the equipment and trained personnel are not readily available to many research groups. The per sample cost associated with LC-MS/MS can limit the number of samples and constrain the means to adequately quantify temporal or spatial patterns of neonicotinoid residues. Here we evaluate enzyme linked immunosorbent assay (ELISA) kits as a rapid and cost effective alternative to LC-MS/MS for the three most commonly used neonicotinoids: clothianidin, imidacloprid, and thiamethoxam. Estimates of thiamethoxam and clothianidin concentrations in water and thiamethoxam concentration in leaf tissue were not significantly different between the two

evaluated methods when samples were fortified with only the specified compound of interest. Neonicotinoid concentrations were positively correlated between the ELISA and LC-MS/MS methods, but there was poor agreement, in environmental water and plant tissue samples fortified with mixtures of compounds. In addition to matrix effects, ELISA results tended to overestimate neonicotinoid-specific concentrations due to cross reactivity with other neonicotinoids. Matrix interferences observed were reduced by using matrix-matched calibration curves. Currently available ELISA kits are sufficient to identify the presences/absence of neonicotinoids within water and leaf tissue and could be used to prioritize samples for LC-MS/MS analyses. The presence of cross reactants precludes the means to quantify neonicotinoid-specific concentrations by ELISA. with confidence. Confirmation of ELISA results by LC-MS/MS is suggested to identify and quantify estimate neonicotinoid concentrations in water and plant foliage.

Introduction

Neonicotinoids are widely used insecticides due to their effective management of a broad spectrum of insect pests combined with their low avian and mammalian toxicity (Bass *et al.*, 2015; Thompson *et al.*, 2021). Neonicotinoids are systemic insecticides making them ideal for seed coatings (Simon-Delso *et al.*, 2015; Hladik *et al.*, 2018). Neonicotinoids are also registered as foliar sprays, soil drenches, and granules for crop protection (Simon-Delso *et al.*, 2015; Hladik *et al.*, 2018). In urban and forested areas, they are applied as soil drenches or injections to control pests such as the emerald ash borer (*Agrilus planipennis*) (Cowles, 2009; Hladik *et al.*, 2018). Additionally, imidacloprid has residential uses for insect pests in lawns and gardens, as well as management of fleas on pets (Jeschke *et al.*, 2011; Hladik *et al.*, 2018).

Neonicotinoids are relatively water-soluble with degradation half-lives in soil ranging from 39 to 545 days (AERU, 2021). Consistent with these properties, neonicotinoids are readily

transported from the site of application to non-target terrestrial habitats and water bodies (Goulson and Kleijn, 2013; Bonmatin *et al.*, 2014). In agricultural areas, clothianidin, imidacloprid, and thiamethoxam, which are primarily used as seed coatings, have been detected in soil and non-target plants outside of crop fields, in addition to waterbodies contaminated through overland runoff or subsurface flow (Bonmatin *et al.*, 2014; Hladik *et al.*, 2014; Hladik *et al.*, 2017; Hladik *et al.*, 2018; Frame *et al.*, 2021). In urban areas imidacloprid is typically detected, in conjunction with its registered uses (Hladik and Kolpin, 2016; Berens *et al.*, 2021). Neonicotinoids could pose indirect risks to non-target terrestrial insects that use contaminated non-crop plants as food sources (e.g. monarch larvae feeding on contaminated milkweed; Olaya-Arenas and Kaplan, 2019; Hall *et al.*, 2021) as well as aquatic invertebrates in contaminated waterbodies.

To better understand potential environmental risks, numerous studies have been published to assess the fate and transport of neonicotinoids (Hladik *et al.*, 2014; Botias *et al.*, 2015; Botias *et al.*, 2016; Hladik and Kolpin, 2016; Hladik *et al.*, 2016; Hladik *et al.*, 2017; Thompson *et al.*, 2021; Hall *et al.*, 2021). To quantitate neonicotinoid concentrations in environmental samples, these studies have typically employed liquid chromatography tandem mass spectrometry (LC-MS/MS) methods due to their sensitivity and selectivity in complex matrices. However, LC-MS/MS is an expensive option both in terms of capital and operational expenses. It also requires highly trained personnel to operate the specialized instrumentation and analyze the data. The net result of these costs is reduced sample throughput, which can constrain monitoring study designs and the means to adequately assess neonicotinoid concentrations across spatial and temporal scales required for environmental risk assessments.

Enzyme-linked immunosorbent assay (ELISA) is one technique that could be used as a less expensive and faster alternative to LC-MS/MS to facilitate analysis of a larger volume of samples. ELISA has many benefits as a quantitative method, including cost effectiveness, simplicity, automation, and high sensitivity (Hennion and Barcelo, 1998; Watanabe *et al.*, 2004; Gross *et al.*, 2021). In some applications, ELISA can also provide rapid sample turn-around times (Hennion and Barcelo, 1998; Watanabe *et al.*, 2004). However, like any analytical methodology, it also has its challenges. ELISA antibodies can have cross-reactivity with compounds that are structurally similar to the target compounds (Li and Li, 2000; Lee *et al.*, 2001; Watanabe *et al.*, 2004; Gross *et al.*, 2021). Cross reactivity can result in false positives and/or overestimation of analyte concentrations. Consequently, ELISA kit results may not distinguish the compound of interest from cross reactants, which could include both endogenous compounds in an environmental water or plant sample, as well exogenous compounds, such as other insecticides and/or metabolites (Li and Li, 2000; Lee *et al.*, 2001; Watanabe *et al.*, 2004; Gross *et al.*, 2021).

The objectives of this study were to: (1) evaluate the performances of ELISA and LC-MS/MS methods for the quantification of imidacloprid, clothianidin, and thiamethoxam in water and leaf tissue using accuracy and precision as the criteria; and (2) examine matrix interference with ELISA analysis. Data from this study will provide researchers insights into the benefits and limitations of using ELISA analysis to determine imidacloprid, clothianidin, and thiamethoxam concentrations in environmental matrices.

Methods

Fortified water and common milkweed (*Asclepias syriaca*) samples were extracted and analyzed using ELISA and LC-M/MS protocols to quantify clothianidin, imidacloprid, and

thiamethoxam. Matrix-matched calibration curves were used for both ELISA and LC-MS/MS to limit the interference of matrix components. ELISA kit standards were used to examine matrix effects. Accuracy and precision of the methods were compared using fortified samples.

Chemicals and Materials

Analytical standards (purity > 98%) of imidacloprid, clothianidin, and thiamethoxam were purchased from Sigma Aldrich (Milwaukee, WI, USA). Quality control standards of imidacloprid, clothianidin and thiamethoxam were purchased from Cayman Chemical (Ann Harbor, MI, USA). Internal standards of thiamethoxam-d₃, clothianidin-d₃ were purchased from Sigma -Aldrich. Imidacloprid-pyr-d₄-methyl-d₂ was received as a gift from Bayer CropScience (St. Louis, MO, USA). Imidacloprid ELISA kits were purchased from Eurofins Abraxis (Warminster, PA). Thiamethoxam kits were purchased from Beacon Analytical Systems, INC (Saco, ME).

Standard Solutions

All stock standard solutions were made at 1 mg/mL in acetonitrile. Solutions used to fortify “control” leaf tissue extracts and environmental water samples were prepared as dilutions from the 1-mg/mL stock solutions. Internal standards used for LC-MS/MS analysis were thiamethoxam-d₃, clothianidin-d₃, and imidacloprid-pyr-d₄-methyl-d₂. A 0.1-μg/mL mix of all internal standards was added to all LC-MS/MS samples.

Sample Preparation

Fortified water

Environmental water was collected from a 12.5 hectare lake located in Ames, Iowa USA (42.0857° N, 93.5958° W). All control water samples were analyzed by LC-MS/MS to confirm no analytes of interest were present. Control environmental water was filtered to remove debris and a 10-mL portion was transferred to a 15-mL polypropylene tube prior to fortification. All

environmental water samples were fortified in a total volume of 10 mL. Imidacloprid and clothianidin samples were fortified at 0.5, 1.5, and 3.5 ng/mL. Thiamethoxam samples were fortified at 0.4, 4, 7, 60, 300, and 700 ng/mL. To ensure samples were homogeneous, all tubes were capped and mixed using a roller apparatus (Bellco Biotechnology, Vineland, N.J., USA) for 30 minutes prior to ELISA and LC-MS/MS analysis. Sample preparation for blinded water samples followed the same preparation protocol.

Fortified leaf tissue

Control common milkweed was sampled from a restored prairie in Ames, Iowa, USA (42°02'14.7"N 93°38'38.7"W). Milkweed leaves were flash-frozen using liquid nitrogen and homogenized using a mortar and pestle. A 0.2-g portion of material was weighed into a 15-mL polypropylene tube. Samples were fortified at 5, 15, and 30 ng/g milkweed leaf tissue for clothianidin and imidacloprid. Samples were fortified at 5, 15, 30, and 350 ng/g milkweed leaf tissue for thiamethoxam. Sample preparation for blinded leaf tissue samples followed the same preparation protocol.

Blind samples

A blind method comparison was performed in both water and plant material to compare the ELISA test kits to the LC-MS/MS analysis method. The purpose of the blinded method comparison was to evaluate how the two analytical methods would perform in a routine application. The blinded method comparison was designed to emulate environmentally-relevant neonicotinoid concentrations in water and plants. One co-author (DES) prepared spike solutions, fortified, and randomized the samples. The other authors were blinded to the spike concentrations and randomization scheme. The extractions and ELISA and LC-MS/MS analyses

were undertaken by MJH (extractions and ELISA analyses) and LEB (extractions and LC-MS/MS analyses).

The water samples were fortified in triplicate with imidacloprid, clothianidin, and thiamethoxam at low and high concentrations based on published concentrations of these compounds detected in environmental samples (Hladik *et al.*, 2014; Frame *et al.*, 2021). Additionally, three negative controls along with four challenge samples were included in the method comparison. Challenge samples were fortified at various levels with two or more of the neonicotinoid compounds. Finally, five tile water samples were collected from bioreactors and saturated riparian buffers (Jaynes and Isenhardt, 2019; Feyereisen *et al.*, 2020) and included in the analytical runs.

Plant samples were fortified in triplicate at a low concentration along with three challenge samples, three negative controls, and 12 previously analyzed naturally incurred milkweed samples (Hall *et al.*, 2021). Following fortification all samples were randomized and portioned out for ELISA and LC-MS/MS analysis.

LC-MS/MS

Environmental water extraction

A 980- μ L portion of sample was transferred to a 1.5-mL polypropylene tube, and 20 μ L of internal standard solution was added to the tube. All samples were vortexed for approximately 30 seconds prior to centrifugation at 10,000 rpm for 5 minutes. A 150 μ L portion of sample was transferred to a 1.5-mL microvial with insert for LC-MS/MS analysis. An injection volume of 5 μ L was used in all analyses.

Milkweed plant material extraction

After the addition of 20 μL of internal standard solution each sample was extracted into a total volume of 1.5 mL of acetonitrile. Samples were placed on a multitube shaker (Fisher Scientific, Waltham, MA, USA) at 2500 rpm for 10 minutes prior to centrifugation at 3000 rpm for 5 minutes. The extract was transferred to a 2-mL dispersive solid-phase extraction (dSPE) tube containing 150 mg of magnesium sulfate, 50 mg of primary and secondary amines, and 50 mg of C_{18} . All dSPE tubes were vortexed for approximately 1 minute prior to centrifugation at 10,000 rpm for 5 minutes. A 150- μL portion of sample was transferred to a 1.5-mL microvial with insert for LC-MS/MS analysis. An injection volume of 5 μL was used in all analyses.

Linear ranges

Neonicotinoid insecticides in environmental water were quantified using the linear range of 0.05 to 100 ng/mL. The limit of quantification (LOQ) of neonicotinoids in environmental water was the following: thiamethoxam 0.05 ng/mL, imidacloprid 0.3 ng/mL, and clothianidin 0.3 ng/mL. A calibration curve with a linear range from 1 to 50 ng/g milkweed leaf tissue was used to quantify all milkweed plant samples. The LOQ for all three neonicotinoid insecticides in milkweed plant was 1 ng/g milkweed leaf tissue. All LOQ values were determined based on the lowest calibration standard that met the criteria of a bias and %CV less than 20%. The criteria were evaluated from the average measured value of 5 biological replicates.

Instrument method

A Vanquish Flex LC pump interfaced with a TSQ Altis mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA) was used for the analyses. The mass spectrometer source conditions were as follows: spray voltage 3700 V, sheath gas 30 Arb, auxiliary gas 6 Arb, sweep gas 1 Arb, ion transfer tube temperature 325 $^{\circ}\text{C}$, and vaporizer temperature 350 $^{\circ}\text{C}$. The total run

time of the method was 6 minutes. The resolution of Q1 was 1.2 FWHM, and the resolution of Q3 was 0.7 FWHM. The collision-induced dissociation (CID) gas was set to 2 mTorr. The chromatographic peak width was 4 sec, and the cycle time was 0.3 sec.

Chromatographic separation was performed on a HypersilGold 50 x 3 (1.9 μ m) analytical column (Thermo Fisher Scientific, San Jose, CA, USA). The mobile phases used were (A) water containing 2% methanol, 5 mM ammonium formate, and 0.1% formic acid and (B) methanol containing 2% water, 5 mM ammonium formate, and 0.1% formic acid. The column oven was maintained at 30 °C. The following chromatographic separation gradient was used for all analyses: start with 100% A, linear ramp to 100% B for 5 minutes, hold at 100% B for 0.5 minutes, drop to 100% A in 0.01 minutes, and hold at 100% A for 0.49 minutes.

ELISA

Environmental water preparation

Water samples were prepared following the user guide provided by the manufacturer of the ELISA kit (Eurofins Abraxis, 2021). In brief, samples were filtered through a 0.45- μ m polyethersulfone (PES) filter and diluted with deionized water (1:4). Diluted samples were then vortexed for ~ 30 seconds.

Milkweed extraction

Samples were extracted into a total volume of 1.2 mL of deionized water. Samples were placed on a multitube shaker (Fisher Scientific, Waltham, MA, USA) at 2500 rpm for 60 minutes prior to centrifugation at 3000 rpm for 5 minutes. The supernatant was transferred to a 5 mL polypropylene tube. A portion of the supernatant was diluted using deionized water. Diluted samples were then vortexed for ~ 30 seconds. If analysis was not completed same day samples were stored at -20°C.

Matrix matched calibration standards

Matrix-matched calibration curves were prepared using control environmental water and milkweed. Control samples were prepared following the above outlined extraction methods with final dilutions performed with standard solutions in deionized water. Separate 2.4-ng/mL working solutions of imidacloprid and clothianidin were prepared in deionized water. These working solutions were serially diluted 1:1 to prepare working solutions at 1.2, 0.60, 0.30, and 0.15 ng/mL. Calibrants (0.075-1.2 ng/mL) and a zero calibrant for imidacloprid and clothianidin were prepared by mixing equal portions of the appropriate working solutions with control matrix extracts.

Working solutions of thiamethoxam at 4 and 400 ng/mL were prepared in deionized water for analysis. The 4.0-ng/mL working solutions were serially diluted to prepare working solutions at 2, 1.5, 1.2, 0.6 and 0.1 ng/mL. The 400-ng/mL working solution was diluted to prepare working solutions at 200, 100, 40, and 20 ng/mL. For the thiamethoxam HS plate kit (Cat# 20-0102) thiamethoxam calibrants (0.05-2 ng/mL) and a zero calibrant were prepared by mixing equal portions of the prepared standards and the control matrix diluents. For the thiamethoxam high range plate kit (Cat# 20-0103), thiamethoxam calibrants (10-200 ng/mL) and a zero calibrant were prepared by mixing equal portions of the prepared standards and the control matrix diluents.

Analysis

ELISA quantification was conducted following the assay procedure in the user guides (Eurofins Abraxis, 2021; Beacon Analytical Systems, 2021). The absorbance at 450 nm was quantified using a Gen5™ software with a Synergy HTX plate reader (Biotek®).

Calculation of results

Quantitative interpretation of results required graphing the compound's absorbance as a function of calibrant concentrations. Quantitative analysis for imidacloprid and clothianidin was performed by plotting the normalized absorbance of the analyte (B/B_0) as a function of concentration. This resulted in a non-linear regression. The normalized absorbance of an unknown sample was used to calculate noenicitinoid concentrations in ng/mL based on interpolation of standard curves. Quantitative interpretation for thiamethoxam was accomplished by plotting the absorbance of the analyte as a function of the log of calibrant's concentrations. A linear standard curve was obtained and used to interpolate the log concentration of the sample absorbance. The LOQ in water for imidacloprid, clothianidin and thiamethoxam were 0.3 ng/mL, 0.3 ng/mL and 0.2 ng/mL, respectively. The LOQ in leaf tissue for imidacloprid, clothianidin and thiamethoxam were 2.25 ng/mL, 2.25 ng/mL and 1.5 ng/mL, respectively. The lowest limit of quantification for each kit was given in the kit manual as the lowest calibrant. This value was multiplied by the corresponding dilution factor to calculate LOQ for that specific compound and matrix.

Data Analysis

The relative standard deviation (%RSD), percentage recovery and average recovery (%) were calculated for the fortified plant and water samples for the ELISA and LC-MS/MS methods. The accuracy and precision were calculated for the water and plant samples. The probability of detection (POD) was calculated for the blinded plant and water samples. Probability of detection is a statistical means of evaluating the false-positive and false-negative rate of an analytical method. Positive POD is calculated by dividing the number of samples in which a compound was detected by the total number of samples expected to be positive and

multiplying by 100. Negative POD is calculated by dividing the number of samples where a compound was detected by the total number of samples expected to be negative and multiplying by 100. A high positive POD value is expected for positive controls whereas a low negative POD value is expected for negative controls (Wehling *et al.*, 2011).

The calculated LC-MS/MS and ELISA concentrations of each compound were plotted against the expected concentrations in water and leaf tissue. A two-tailed F test was used to evaluate the null hypothesis that the slopes for the two methods were not different. A second two-tailed F test compared the slopes and intercepts of the lines. The comparison of the intercepts and slopes was undertaken if the p-value from the slope analysis was > 0.05 . The same analysis was done to compare the slopes and intercepts of the thiamethoxam low and high standard curves in different matrices.

The calculated LC-MS/MS and ELISA concentration of each compound were plotted against each for water and plant tissue blinded samples. The 95% confidence intervals for the linear regressions were calculated. If those intervals overlapped with 1 it was concluded the concentrations calculated by the methods were not statistically different.

Results and Discussion

Comparison of ELISA and LC-MS/MS Quantification of Fortified Samples

Environmental water

LC-MS/MS and ELISA methods were used to analyze fortified environmental water samples to determine the mean (\pm SD) measured concentration, relative standard deviation (%RSD), and the average recovery (Table 1). The nominal concentrations were plotted against measured concentrations (Figure 1). The slope of the ELISA linear regression of imidacloprid concentrations in water was statistically different than the slope based on LC-MS/MS at a 0.05

level of significance ($F = 23.98$, $DFn = 1$, $DFd = 2$, $p = 0.0393$) The imidacloprid graph shows that ELISA is likely to overestimate the concentration present compared to LC-MS/MS (Figure 1). The slopes for the clothianidin regressions were also statistically different ($F = 23.37$, $DFn = 1$, $DFd = 2$, $p = 0.0402$); however, in this case ELISA is likely to underestimate the concentration as compared to the LC-MS/MS results. The thiamethoxam low kit and high kit showed no significant difference between the slopes or intercepts for concentrations measured in water by LC-MS/MS and ELISA at a 0.05 level of significance (thiamethoxam low: slope- $F = 3.489$, $DFn = 1$, $DFd = 2$, $p = 0.2029$ and intercept - $F = 2.046$, $DFn = 1$, $DFd = 3$, $p = 0.2480$; thiamethoxam high: slope - $F = 0.3115$, $DFn = 1$, $DFd = 2$, $p = 0.6329$ and intercept - $F = 0.2386$, $DFn = 1$, $DFd = 3$, $p = 0.8871$).

Both methods had acceptable %RSD ($\leq 20\%$); however, the acceptable range of recovery (70 – 120%; SANCO 2012) was not uniformly reached. The RSD for the LC-MS/MS method was $<10\%$ for all the neonicotinoids, while the ELISA method RSD was $<15\%$ for all the compounds and all concentrations, excluding clothianidin at 0.5 ng/mL (RSD = 18.66%). The average recovery for the LC-MS/MS method for all the compounds at all concentrations ranged from 97.4% to 107.4%. The average recovery for the clothianidin and thiamethoxam ELISA methods ranged from 78.2% to 118%. The imidacloprid ELISA method had an average recovery ranging from 137% to 164.5%. These data indicate that for both methods, clothianidin and thiamethoxam were within the acceptable range of recovery, but with imidacloprid the ELISA method was outside the acceptable range. These results indicate if clothianidin or thiamethoxam are the analytes of interest, and there are no other neonicotinoids or neonicotinoid metabolites present in a sample, the two methods are comparable. However, the data also indicates that the

ELISA method employed in this study would overestimate imidacloprid concentrations. For this compound, LC-MS/MS is a more accurate quantification methodology.

Milkweed leaf tissue

LC-MS/MS and ELISA methods were used to measure fortified samples to determine the mean measured concentration, %RSD, and average recovery (Table 1). Expected concentrations versus the measured concentration of the plant matrix for both methods were plotted (Figure 2). The slope of the imidacloprid and clothianidin ELISA concentrations in leaf tissue were statistically different from those determined using LC-MS/MS at a 0.05 level of significance (imidacloprid: slope - $F = 1423$, $DF_n = 1$, $DF_d = 2$, $p = 0.0007$; clothianidin: slope - $F = 39.23$, $DF_n = 1$, $DF_d = 2$, $p = 0.0246$). With the thiamethoxam low concentration kit, no significant difference was detected between the LC-MS/MS method and ELISA method linear regression slopes or intercepts (thiamethoxam low: slope - $F = 6.803$, $DF_n = 1$, $DF_d = 2$, $p = 0.1209$; intercept - $F = 0.4456$, $DF_n = 1$, $DF_d = 3$, $p = 0.5521$).

Both methods had acceptable %RSD (≤ 20) and range of recovery (70 % - 120 %) (Table 1). However, for imidacloprid the data indicates that the ELISA method is likely to underestimate the concentration present at 30 ng/g milkweed leaf tissue, while for clothianidin the data indicates that the ELISA method is likely to underestimate the concentration present at ≤ 15 ng/g milkweed leaf tissue. For these compounds and concentration ranges, the LC-MS/MS method provides a more accurate quantification.

Routine Application of ELISA and LC-MS/MS

Blind environmental sample analysis was used to compare how these two methods would perform with water and plant samples representative of those collected in monitoring studies. Samples were used to characterize the accuracy and precision of both analytical methods in

workflow scenarios in which the analysts did not know if any neonicotinoids were in the sample, and if so, their expected concentrations.

Environmental water

Concentrations of imidacloprid, clothianidin, and thiamethoxam quantified by ELISA and LC-MS/MS were well-correlated (r^2 of 0.88, 0.89, and 99, respectively); however, slopes for imidacloprid and thiamethoxam plots were significantly different from 1 based on 95% confidence intervals indicating overestimation or underestimation by one method in relation to the other (Figure 3). Both methods had acceptable range of recovery for clothianidin and thiamethoxam (70 % - 120 %) when they were the analyte of interest and there were no other neonicotinoids or neonicotinoid metabolites present in the sample. LC-MS/MS had acceptable range of recovery (70 % - 120 %) for imidacloprid and thiamethoxam when it was the analyte of interest and no other neonicotinoids or neonicotinoid metabolites present in the sample. However, ELISA-measured concentrations for the imidacloprid samples were outside an acceptable range of recovery. These data indicate that if clothianidin or thiamethoxam is the analytes of interest, and there are no other neonicotinoids or neonicotinoid metabolites present in a sample, the two methods are comparable. However, these data also indicate that at low concentrations, the ELISA method will overestimate the imidacloprid concentration and at higher concentrations, it will underestimate the imidacloprid concentration.

The positive POD for both methods for all three compounds was 100%. The negative LC-MS/MS method POD was 0%. However, the ELISA method negative POD for imidacloprid, clothianidin, and thiamethoxam were 43%, 33% and 0%, respectively. A negative POD of 43% for imidacloprid indicates that the ELISA kit provides a positive response 43% of the time even if imidacloprid is not in the sample (i.e., it consistently generated false positives in these

samples). The high negative POD with the ELISA imidacloprid kit is not unexpected given the reported 121% cross-reactivity with clothianidin noted in the manual (Eurofins). While this cross-reactivity allows this kit to be used for quantifying clothianidin, the method cannot identify which of the two compounds are present with a positive detection. If both compounds are present in a sample, the imidacloprid ELISA method will overestimate the actual concentration of imidacloprid (or clothianidin), if it is assumed there is one neonicotinoid present (Table 2). Finally, the he ELISA method for quantification of imidacloprid had detects in all samples including samples where no neonicotinoids were present. This was likely caused by an endogenous compound(s) present in the matrix that bound to the imidacloprid antibody, which produced a false positive. Field samples analyzed by the imidacloprid kit can, however, serve as a screen for the presence of imidacloprid and/or clothianidin; however, a more selective analytical method is required to distinguish which compound(s) are present and their concentrations.

Clothianidin and imidacloprid are reported to have cross-reactivity with the antibodies in the commercial thiamethoxam ELISA kit. The reported cross-reactivity for both clothianidin and imidacloprid is reported at <0.1 (Beacon, 2021). When low levels of these two compounds are present, they will not skew the thiamethoxam results. However, when a sample with 90 ng/mL of both imidacloprid and clothianidin was analyzed with the thiamethoxam ELISA kit, elevated concentrations of thiamethoxam were reported (Table 2). The measured concentration reported was 124 ng/mL of thiamethoxam with a % average recovery of 138%. The data shows that when there are mixtures of neonicotinoids at environmentally relevant concentrations the thiamethoxam ELISA kit is likely to overestimate the concentration of thiamethoxam due to cross reactivity. As noted with the imidacloprid kit, a more selective analytical method is

required to confirm the identities and concentrations of neonicotinoid(s) present in a 'positive' thiamethoxam ELISA sample.

Milkweed leaf tissue

Estimated concentrations of thiamethoxam by ELISA and LC-MS/MS were strongly correlated ($r^2=0.97$; slope of 1.02), while correlations for imidacloprid and clothianidin were modest ($r^2= 0.66$ and 0.77 ; slope of 1.636 and 1.561) (Figure 3). The 95% confidence intervals for all three slopes overlapped with one indicating that the ELISA: LC-MS/MS correlation was not significantly different than 1:1 (Figure 3). However, for most correlations, the ELISA method overestimated the neonicotinoid concentrations by up to approximately 1.5 to 2.0-fold as compared to LC-MS/MS. This bias could lead to misinterpretation of the toxicological significance ELISA-based measurements. Blinded samples with only imidacloprid, clothianidin or thiamethoxam present showed % average recovery by the ELISA method of 45.0, 50.5%, and 68.5%, respectively. The LC-MS/MS method showed a percent recovery for compounds of interest between 82% and 104%.

The ELISA and LC-MS/MS positive POD for imidacloprid and thiamethoxam in leaf tissue was 100%. The POD for clothianidin with ELISA and LC-MS/MS was 83% and 100%, respectively. The ELISA negative POD for imidacloprid, clothianidin, and thiamethoxam were 40%, 44%, and 27%, respectively. The LC-MS/MS negative POD were 0%, 0% and 0.09%, respectively. Similar to the water samples, the ELISA imidacloprid kit will have a high negative POD because of its noted cross-reactivity with clothianidin and its inability to distinguish whether both compounds and/or one compound is present in a sample. When both compounds are present in a sample, elevated concentrations are likely to be reported by the imidacloprid kit for the compound being quantified (Table 2). Low concentrations of thiamethoxam were

reported in samples where it was not present, likely a result of an interaction between endogenous compounds in the matrix in the antibody. The imidacloprid and thiamethoxam ELISA kits are less selective than the LC-MS/MS method. However, the ELISA kits could be used as a screen for the presence/absence of neonicotinoids in leaf tissue samples and help prioritize samples for LC-MS/MS analysis to confirm the presence and concentrations of specific neonicotinoids (Hennion and Barcelo, 1998; Gross *et al.*, 2021).

Matrix Interferences

Hall et al (2020) reported matrix effects for milkweed leaf tissue analysis by LC-MS/MS (< 15%). However, this was addressed within the LC-MS/MS leaf tissue method by using matrix-matched calibrants and stable isotope labeled internal standards (SILIS) (Hall *et al.*, 2020). SILIS and matrix-matched calibrants were also used for the environmental water analysis. Matrix effects were determined for the ELISA analysis by comparing ELISA analyses of the kit-provided standard curves to matrix-matched standard curves prepared with environmental water and milkweed leaf tissue from control sites (Figure 4). The IC₅₀ values for the imidacloprid curves were 0.37 ng/mL, 0.42 ng/mL, and 0.46 ng/mL for kit, environmental water, and leaf tissue, respectively. Clothianidin standards were not provided with the test kits; however, the IC₅₀ values for the clothianidin curves were 0.36 ng/mL and 0.49 ng/mL in water and leaf tissue, respectively.

The thiamethoxam standard curves generated linear regressions. To evaluate differences between the kit, environmental water, and leaf tissue standard curves were compared. The low-concentration-range thiamethoxam kits showed significant differences between the slopes of the three curves at a 0.05 level of significance (thiamethoxam low: slope - $F = 24.02$, $DFn = 2$, $DFd = 12$, $p < 0.0001$) (Figure 5). The high-range-thiamethoxam kits show no significant differences

between the slopes of the standard curves (thiamethoxam high: slope - $F = 0.4270$, $DFn = 2$, $DFd = 24$, $p = 0.657$); however, there was evidence of a significant difference between the intercepts at a 0.05 level of significance (thiamethoxam high: intercept - $F = 4.466$, $DFn = 2$, $DFd = 26$, $p = 0.0215$). The deviation of these curves highlights the importance of using matrix-matched curves when dealing with complex samples.

Matrix effects could over estimate actual neonicotinoid concentrations for all three ELISA kits. Using the imidacloprid-kit-provided standard curve to quantify the zero standards for environmental water and resulted in concentrations of 0 ng/mL and 5.13 ng/mL, respectively. Using the thiamethoxam-low-kit standards curve to quantify the zero standards for environmental water and leaf tissue resulted in 0 ng/mL and 4 ng/mL. The thiamethoxam-high-range kit curve resulted in 0 ng/mL for both environmental water and leaf tissue zero standards. The complex matrix within the crude sample extract could artificially elevate imidacloprid, clothianidin, and thiamethoxam concentrations similarly that observed by cross-reactants when the kit-provided curves are used. Therefore, it is necessary to test the matrix interference prior to running ELISA on complex matrices.

Comparison to Relevant Toxicity Benchmarks

Aquatic environment

The U.S. Environmental Protection Agency (USEPA) acute aquatic invertebrate benchmarks for clothianidin, thiamethoxam, and imidacloprid are 0.385, 11 and 17.5 ng/mL, respectively (U.S. Environmental Protection Agency, 2021). The clothianidin ELISA kit reported a mean concentration of 0.48 ± 0.09 at the fortified concentration of 0.5 ng/mL with a bias of -2.08%. The LC-MS/MS method reported a mean concentration of 0.54 ± 0.01 with a bias of 7.44%, indicating that both methods are within acceptable parameters for measuring

concentrations approximating the acute invertebrate benchmarks for clothianidin. Environmental water samples were fortified at imidacloprid concentrations of 4.2 ng/mL and 35 ng/mL. The ELISA method reported measured concentrations of 6.0 ± 0.5 and 22.1 ± 8.42 , and the LC-MS/MS method reported measured concentrations at 3.94 ± 0.16 and 35.4 ± 0.46 . The % accuracy by LC-MS/MS was within the acceptable range of 70-120% while the ELISA method showed % accuracy of 143.0% and 63.5%, respectively. The ELISA method is likely to inaccurately quantify the concentration of imidacloprid in environmental water approximating the acute aquatic invertebrate benchmark. The thiamethoxam ELISA and LC-MS/MS methods showed accuracies of 84.2% and 101% for samples fortified at 6.8 ng/mL with a bias of -28.15% and -1.19% for samples fortified at 7 ng/mL, respectively. Based on these results, the ELISA method is likely to underestimate thiamethoxam concentrations in environmental water approximating the acute the benchmark concentration (17 ng/mL).

The USEPA chronic aquatic invertebrate benchmarks for clothianidin, thiamethoxam, and imidacloprid are 0.01, 0.05, and 0.75 ng/mL, respectively (Agency, 2021). The chronic values for clothianidin are below the LOQ for both the ELISA and LC-MS/MS methods (0.3 ng/mL). The chronic aquatic benchmark for thiamethoxam is below the LOQ for the ELISA method (0.2 ng/mL); therefore, the LC-MS/MS method (LOQ = 0.05 ng/mL) would be a more suitable method for monitoring water bodies for exceedance of the chronic aquatic invertebrate benchmark for thiamethoxam. Environmental water samples were fortified at imidacloprid concentrations of 0.5 ng/mL and 1.5 ng/mL. The mean measured concentration based on the LC-MS/MS method was 0.52 ± 0.01 ng/mL and 1.49 ± 0.09 ng/mL. Measured concentrations based on the ELISA method were 0.823 ± 0.12 and 2.06 ± 0.21 , respectively. The bias for the LC-MS/MS and ELISA methods at 0.5 ng/mL were 3.29% and 64.51%, respectively. The ELISA

imidacloprid kit overestimated concentration between 0.5 ng/mL to 1.5 ng/mL. Consequently, it is likely to provide false positive measurements suggesting an exceedance of the imidacloprid chronic benchmark (0.75 ng/mL); at these low concentrations the LC-MS/MS method is more likely to provide an unbiased estimate.

These three neonicotinoids have been simultaneously detected in water monitoring studies (Hladik *et al.*, 2014; Frame *et al.*, 2021). If both imidacloprid and clothianidin are present, the ELISA kit will overestimate concentrations. If the compounds are present at elevated concentrations, the thiamethoxam kit is also likely to overestimate the actual concentration. Water samples can have different profiles of endogenous components that could also interfere with the ELISA response. While these kits are good indicators of presence/absence and total neonicotinoid concentration, confirmatory methods are needed to provide quantitative data that can be used for estimating risks to aquatic invertebrates (Hennion and Barcelo, 1998; Gross *et al.*, 2021).

Terrestrial environment

Krishnan *et al.* (2020) reported acute LC₁₀ (LC₁₀ = lethal dose to 10% of the test population) values for 2nd instar monarch larvae of 1,000, 4,000, and 40 ng/g milkweed leaf for imidacloprid, clothianidin, and thiamethoxam, respectively. The acute LC₁₀ values for 2nd-instar monarch larvae for clothianidin (4,000 ng/g milkweed leaf tissue) and imidacloprid (1,000 ng/g milkweed leaf tissue) are outside the concentration range tested in this study. However, samples with neonicotinoids at these elevated levels could be diluted to a concentration range appropriate for the ELISA kit (2.25 to 60 ng/g milkweed leaf tissue) and the LC-MS/MS method (1 to 50 ng/g milkweed leaf tissue). In the present study, milkweed leaf tissue was fortified with thiamethoxam at 30 ng/g milkweed leaf tissue. The mean measured concentration

for the LC-MS/MS, and ELISA methods were 28 ± 0.50 and 22.2 ± 3.75 ng/g milkweed leaf tissue, respectively. The ELISA method is likely to underestimate milkweed leaf concentration in field samples approximating the 40 ng/g milkweed leaf tissue acute toxicity threshold.

Krishnan et al. (2021) reported chronic dietary LC_{10} values for imidacloprid, clothianidin, and thiamethoxam to monarch larvae of 36, 46, and 420 ng/g milkweed leaf tissue, respectively. Milkweed leaf tissue was fortified with imidacloprid at 30 ng/g milkweed leaf tissue. The mean measured concentration for the LC-MS/MS, and ELISA methods were 29.75 ± 0.35 and 17.61 ± 1.06 , respectively. Clothianidin-fortified milkweed leaf samples at 30 ng/g milkweed leaf tissue had mean measured concentrations of 22.5 ± 1.10 and 32.5 ± 0.67 by ELISA and LC-MS/MS, respectively. These data indicate that the ELISA method is likely to underestimate the concentration present in milkweed leaf tissue samples at the chronic LC_{10} range of 36 and 46 ng/g milkweed leaf tissue for imidacloprid and clothianidin, respectively.

Imidacloprid, clothianidin, and thiamethoxam have been detected within the same leaf tissue sample (Stewart *et al.*, 2014; Olaya-Arenas and Kaplan, 2019; Halsch *et al.*, 2020; Main *et al.*, 2020; Hall *et al.*, 2021). Consequently, there is potential for cross-reactivity responses by the ELISA kits for leaf tissue samples collected in monitoring studies. As noted previously, ELISA is a useful tool for detecting presence/absence of neonicotinoids within leaf tissues collected in the field; however, confirmation of neonicotinoid presence and quantification requires a more selective and sensitive analytical method.

Conclusions

Accurate detection and quantification of neonicotinoids are necessary to determine their environmental fate and concentrations in biologically relevant matrices. In turn, these data can support risk assessments. Limitation for researchers undertaking environmental monitoring

studies is the availability of appropriate instrumentation and the cost of analyses. ELISA has the potential to be a less expensive and readily available, rapid screening tool for prioritizing neonicotinoid analyses. While neonicotinoid ELISA kits are sensitive, they have limited selectivity due to cross-reactivity with neonicotinoids and endogenous compounds in sample extracts. Monitoring study samples are likely to have a variety of both endogenous and exogenous compounds present limiting ELISA kits quantitative functionality. However, ELISA has the potential to support laboratory experiments, such as bioassays where a single neonicotinoid is being used, if appropriate matrix matched calibrations standards are implemented. Confirmation by LC-MS/MS could be done on a subset of samples. Additionally, ELISA kits could function as indicators of presences/absence of compounds within water and leaf tissue for monitoring studies. ELISA-positive samples would then require confirmation by LC-MS/MS for accurate concentrations and chemical identification. Liquid chromatography-tandem mass spectrometry methods have high selectivity for imidacloprid, thiamethoxam, and clothianidin, which is advantageous when collecting and analyzing environmental samples that contain both endogenous and exogenous compounds. Using ELISA before LC-MS/MS could reduce the costs of monitoring studies by reducing the number of samples that need to be analyzed by LC-MS/MS. Further research into developing ELISA methods that limit cross-reactivity with other neonicotinoids would significantly increase their utility in monitoring studies.

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Tables and Figures

Table 1. Mean (\pm SD) measured concentration, % RSD, and average recovery of imidacloprid, clothianidin and thiamethoxam in fortified water and leaf tissue samples analyzed by ELISA and LC-MS/MS.

Method	Matrix	Compound	Expected Concentration (ng/mL or ng/g) ^a	Measured Concentration Mean (\pm SD) ng/mL	%RSD	Average recovery
ELISA	Water	Imidacloprid	0.5	0.823 \pm 0.12	14.92	164.51
			1.5	2.06 \pm 0.21	10.09	137
			3.5	5.01 \pm 0.37	7.39	143.1
		Clothianidin	0.5	0.48 \pm 0.09	18.66	97.92
			1.5	1.30 \pm 0.17	13.01	86.6
			3.5	3.17 \pm 0.34	10.76	90.55
		Thiamethoxam (low) ^b	0.4	0.31 \pm 0.04	11.29	78.24
			4	3.69 \pm 0.1	5.62	92.21
			7	5.03 \pm 0.09	2.25	71.85
		Thiamethoxam (high) ^b	60	48.17 \pm 5.79	12.03	80.28
			300	353.95 \pm 32.03	9.05	117.98
			700	659.77 \pm 54.82	8.31	94.25
	Plant tissue	Imidacloprid	5	5.38 \pm 0.31	5.79	107.52
			15	10.55 \pm 0.82	7.78	70.33
			30	17.61 \pm 1.06	6.00	58.69
		Clothianidin	5	2.65 \pm 0.40	14.74	53.87
			15	9.61 \pm 0.61	6.31	64.05
			30	22.50 \pm 1.10	4.91	75
		Thiamethoxam (low) ^b	5	5.51 \pm 1.08	19.67	110.16
			15	14.58 \pm 0.80	5.52	97.23
			30	22.21 \pm 3.75	16.89	74.02
		Thiamethoxam (high) ^b	350	340.56 \pm 52.8	15.51	97.30
LC-MS/MS	Water	Imidacloprid	0.5	0.52 \pm 0.01	1.32	103.29
			1.5	1.49 \pm 0.09	5.82	99.53
			3.5	3.71 \pm 0.04	1.06	105.98
		Clothianidin	0.5	0.54 \pm 0.01	1.50	107.44
			1.5	1.56 \pm 0.07	4.57	104.23
			3.5	3.7 \pm 0.042	1.15	106.07
		Thiamethoxam (low) ^b	0.4	0.42 \pm 0.03	7.09	104.35

Table 1. Continued

Leaf tissue	Thiamethoxam (low) ^b	4	3.90±0.04	0.98	97.41
		7	6.92±0.13	1.81	98.81
	Thiamethoxam (high) ^b	60	60.41±1.12	1.90	100.69
		300	0.48±0.09	1.11	102.49
		700	708.52±9.35	1.32	101.22
	Imidacloprid	5	4.90±0.27	5.49	98.07
		15	15.75±0.14	0.95	98.34
		30	29.75±0.35	1.18	99.15
	Clothianidin	5	5.273±0.38	7.26	105.47
		15	15.98±0.49	3.05	106.55
		30	32.51±0.67	2.07	108.36
	Thiamethoxam (low) ^b	5	4.471±0.30	6.78	89.42
		15	13.72±0.08	0.61	91.43
		30	27.97±0.50	1.80	93.24
	Thiamethoxam (high) ^b	350	340.56±14.60	4.14	100.77

^ang/mL units for environmental water samples; ng/g units for plant tissue samples

^b Thiamethoxam (low) indicates that the low range thiamethoxam ELISA kit was used; Thiamethoxam (high) indicated that the high range thiamethoxam kit was used

Table 2. Expected concentrations, measured concentrations by LC-MS/MS and ELISA methods, and average recovery (measured concentration/expected concentration X 100) for blinded environmental water and leaf tissue samples fortified with mixtures of clothianidin, thiamethoxam and imidacloprid.

	LC-MS/MS			ELISA		
	Environmental Water Samples					
	Imidacloprid	Clothianidin	Thiamethoxam	Imidacloprid	Clothianidin	Thiamethoxam
Measured (ng/g)	2.01	1.57	ND	5.76	3.09	ND
Expected (ng/g)	2.2	1.5	None	2.2	1.5	None
Average recovery %	91.4	104.7	N/A	261.9	206.0	N/A
Measured (ng/g)	ND	1.63	5.72	4.07	2.10	4.97
Expected (ng/g)	None	1.34	5.6	None	1.34	5.6
Average recovery %	N/A	108.7	102.1	N/A	156.4	88.7
Measured (ng/g)	4.07	ND	3.78	5.96	3.21	3.88
Expected (ng/g)	4.3	None	3.8	4.3	None	3.8
Average recovery %	94.7	N/A	99.5	138.7	N/A	102.0
Measured (ng/g)	86.5	94.9	80.4	144.73	115.17	128.43
Expected (ng/g)	90	90	90	90.00	90.00	90.00
Average recovery %	96.1	105.4	89.3	160.8	127.97	137.76
Leaf tissue						
	Imidacloprid	Clothianidin	Thiamethoxam	Imidacloprid	Clothianidin	Thiamethoxam
Measured (ng/g)	5.53	9.72	ND	10.81	8.07	ND
Expected (ng/g)	6.5	9.5	None	6.5	9.5	None
Average recovery %	85.1	102.3	N/A	166.3	84.9	N/A
Measured (ng/g)	ND	6.23	6.62	ND	ND	4.22
Expected (ng/g)	None	6	7	None	6	7
Average recovery %	N/A	103.8	94.6	N/A	0.0	60.3
Measured (ng/g)	21.4	20.6	22.9	38.44	28.13	23.15
Expected (ng/g)	25	25	25	25	25	25
Average recovery %	85.6	82.4	91.6	153.8	112.5	92.6

ND = None detected

N/A = Not applicable

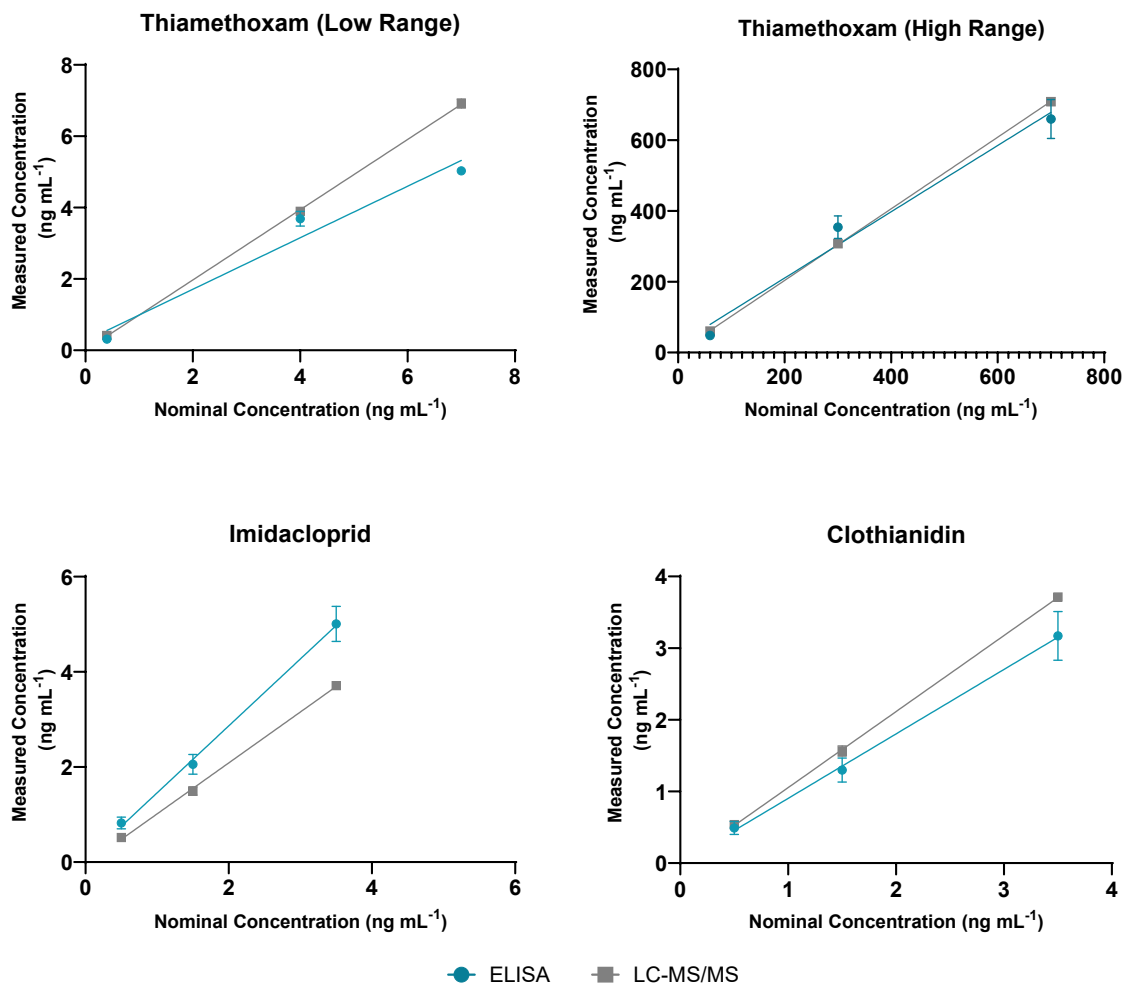


Figure 1. ELISA and LC-MS/MS measured mean (\pm SD) aqueous concentrations plotted against expected concentrations for each neonicotinoid. Results based on a two-tailed F test with the null hypothesis that the slopes are not different. Thiamethoxam (low range) p-value = 0.2029; Thiamethoxam (high range) p-value = 0.6329; Imidacloprid p-value 0.039; Clothianidin p-value = 0.0402. The bars are the standard deviation when not present it indicates that it is smaller than the marker.

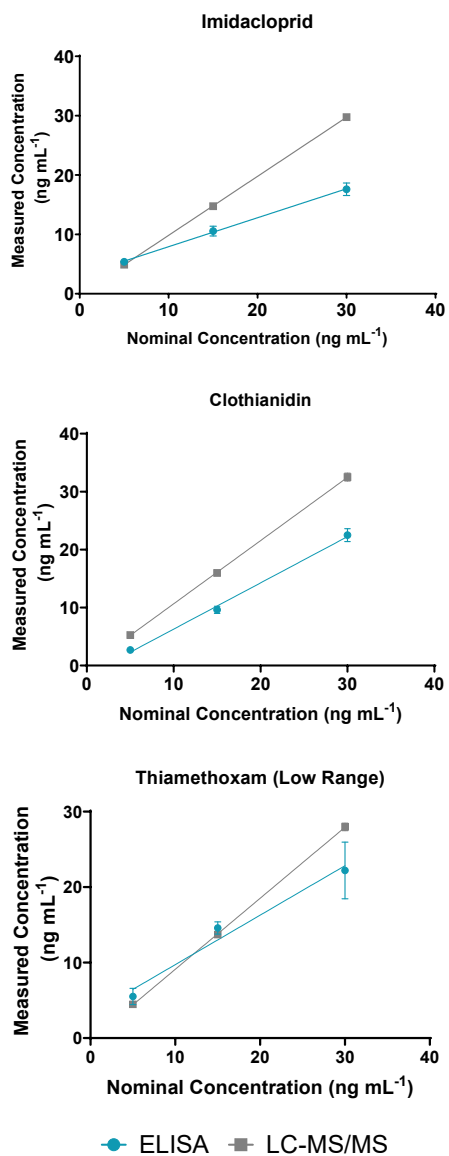


Figure 2. ELISA and LC-MS/MS measured mean (\pm SD) leaf tissue concentrations plotted against expected concentrations for each neonicotinoid in. Results based on a two-tailed F test with the null hypothesis that the slopes are identical. Imidacloprid p-value = 0.0007; Clothianidin p-value = 0.0246; Thiamethoxam (low range) p-value = 1.209. The bars are the standard deviation of the mean when not present it indicates that it is smaller than the marker.

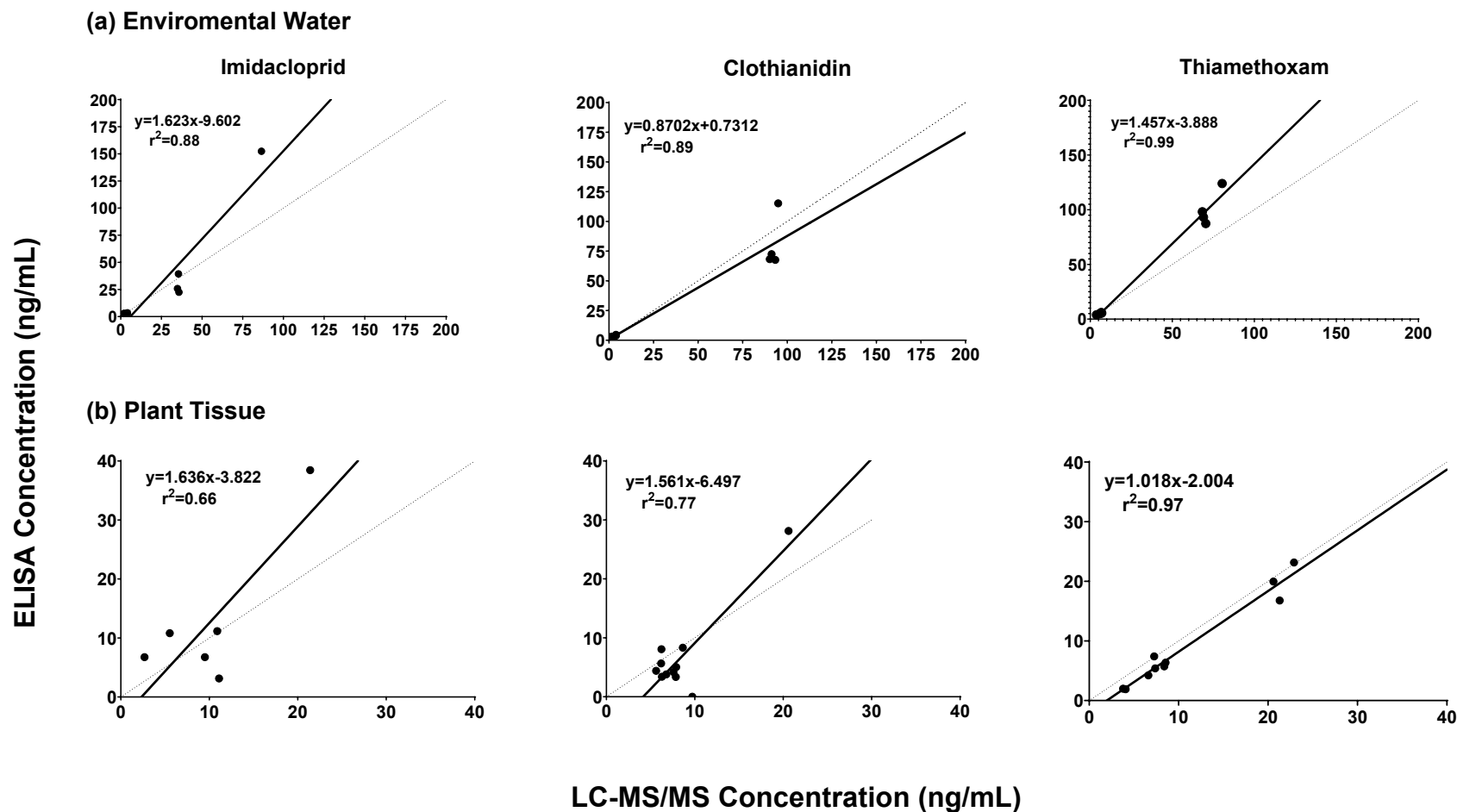


Figure 3. Correlation of measured concentrations obtained from ELISA and LC-MS/MS analyses; row (a) environmental water and row (b) leaf tissue matrices. The dotted lines indicate a 1:1 correlation. . Slope 95% confidence intervals (a) Imidacloprid = 1.134 to 2.113; Clothianidin = 0.6195 to 1.121; Thiamethoxam = 1.309 to 1.605; (b) Imidacloprid = 0.01518 to 3.257; Clothianidin = 0.9259 to 2.197; Thiamethoxam = 0.8637 to 1.173

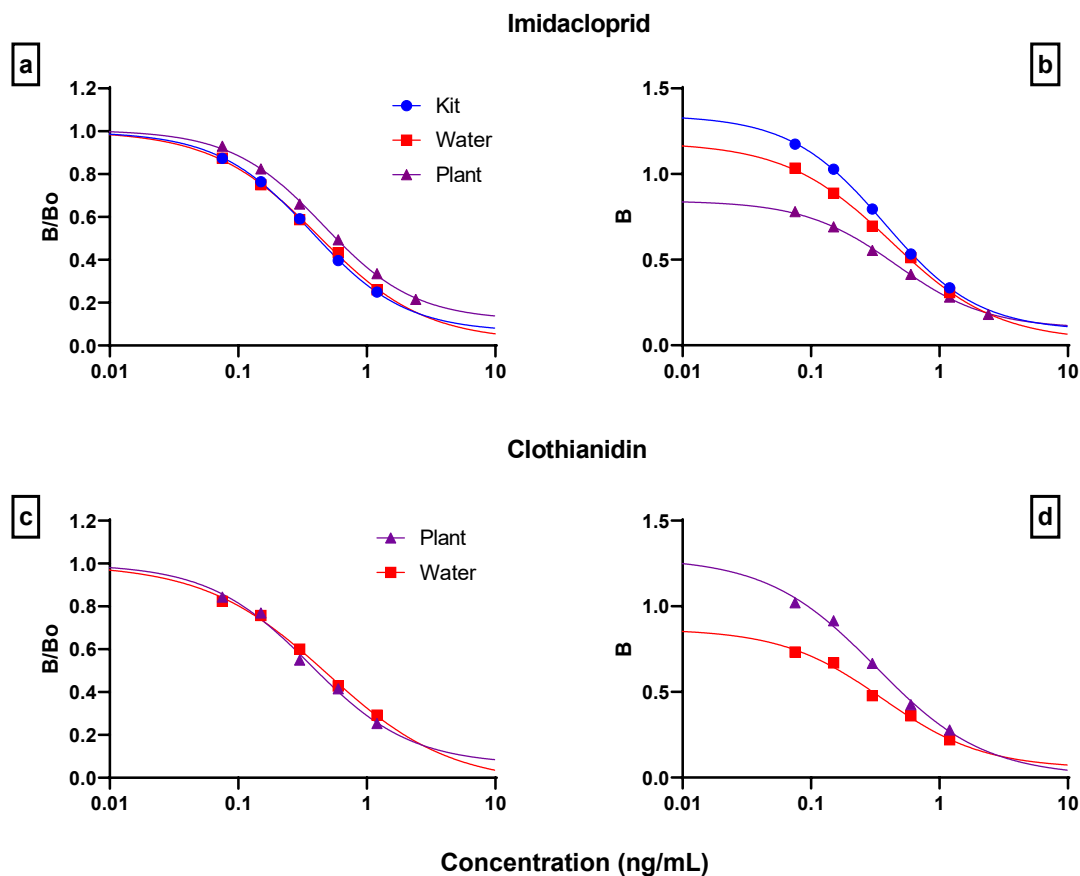


Figure 4. ELISA imidacloprid (a and b) and clothianidin (c and d) calibration curves; y-axis in a and c plots is zero normalized absorbance (B/B_0); y-axis for b and d plots is absorbance at 450 nm (B); and x-axis for all plots is neonicotinoid concentration on a logarithmic scale.

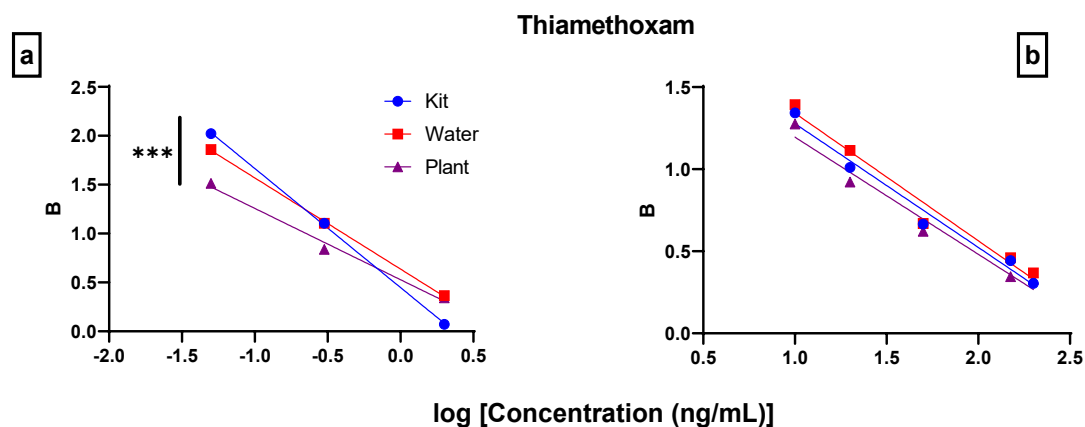


Figure 5. Thiamethoxam calibration curves (a and b) plotted as absorbance at 450 nm (B) plotted against the log concentration. Thiamethoxam low kit standard curve (a) ranging from 0.05 to 2 ng/mL and thiamethoxam high kit standard curves ranging from 10 ng/mL to 200 ng/mL. Results based on a two-tailed F test with the null hypothesis that the slopes are not different. Thiamethoxam low kit (a) p -value < 0.0001 ; Thiamethoxam high kit (b) p -value = 0.6573.

CHAPTER 6. ESTIMATING SCREENING-LEVEL RISKS OF INSECTICIDE EXPOSURE TO LEPIDOPTERAN SPECIES OF CONSERVATION CONCERN IN AGROECOSYSTEMS

Maura J. Hall^{1¶*}, Niranjana Krishnan^{2¶}, Joel R. Coats¹, Steven P. Bradbury^{1,3}

¹ Toxicology Program and Department of Entomology, Iowa State University, Ames, Iowa 50011-0001

² Department of Biological Sciences, Missouri University of Science and Technology, Rolla, Missouri 65409

³ Department of Natural Resource Ecology and Management, Iowa State University, Ames, Iowa 50011-0001

¶ These authors contributed equally to this work.

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Abstract

The U.S. Fish and Wildlife Service defines ‘at risk’ species as those that have either been petitioned for listing, proposed for listing, or assigned a candidate species status under the Endangered Species Act. There are over 30 ‘at risk’ lepidopteran species in the U.S., several of which are found in the north central states. For these species, loss of habitat and exposure to pesticides, particularly insecticides, is often considered a threat to population recovery. Given their range, re-establishment of habitat in agricultural landscapes is typically identified as a primary conservation practice to support species recovery. To evaluate risks associated with habitat established in close proximity to crop fields, estimates of insecticide exposure and toxicity are needed for these species. Based on an evaluation and integration of environmental monitoring and toxicity studies reported in the peer-reviewed literature, we explore an approach to develop screening-level risk analyses for lepidopteran species of conservation concern using data obtained for pyrethroid, organophosphate, neonicotinoid, and diamide insecticides. More

specifically, we interpret the utility of existing insecticide residue data to estimate host plant-mediated exposure for lepidopteran species. Based on available lepidopteran topical and dietary toxicity data, we generate Species Sensitivity Distribution models for topical exposures to pyrethroid and organophosphate insecticides. We compare the toxicity results with modeled or available exposure data to explore the potential insecticide risks associated with establishing non-target lepidopteran habitat in agricultural landscapes. Finally, we identify data gaps and needs for future monitoring and toxicity studies.

Introduction

Worldwide there has been a decline in biodiversity associated with urbanization and agricultural development in natural and semi-natural landscapes (1-3). Agriculture production benefits from increased insect biodiversity, to help suppress insect pest populations by predators and parasitoids, and support crop yields through insect pollination (4). The north central states of the United States (U.S.) have been identified as a critical area for pollinator conservation (5). This region has experienced a decline in wild bee populations and reduced production of other flower-visiting insects, including ‘at risk’ lepidopteran species such as the monarch butterfly (*Danaus plexippus*) (6-9).

The U.S. Fish and Wildlife Service (USFWS) defines ‘at risk’ species as those that have either been petitioned for listing, proposed for listing, or assigned a candidate species status under the Endangered Species Act (ESA) (10). While many insect populations have declined over the last decade, species from Lepidoptera, which include butterflies and moths, are among the most impacted (11). In the lower 48 United States, there are currently 25 lepidopterans (24 butterflies and one moth) listed as endangered and five lepidopterans (four butterflies and one moth) listed as threatened under the ESA. Several more have been designated as a candidate species or are being considered for listing (12). Of these, the Dakota skipper (*Hesperia dacotae*),

Karner blue (*Lycaeides melissa samuelis*), Mitchell's satyr (*Neonympha mitchellii mitchellii*), and Poweshiek skipperling (*Oarisma poweshiek*) are listed species found in the north central states, in addition to the monarch, which was designated as a candidate species in 2020 (13, 14). The regal fritillary (*Speyeria idalia*), which is also found in the north central states, is currently being considered for listing (15).

Dakota skipper, Poweshiek skipperling, regal fritillary, and monarch butterfly rely on native tallgrass prairies for habitat. Larval host plants for the Dakota skipper and Poweshiek skipperling include native grasses [e.g., little bluestem (*Schizachyrium scoparium*), prairie dropseed (*Sporobolus heterolepis*)] and, for the Poweshiek skipperling, also sedges [e.g., spike-rush (*Eleocharis elliptica*)]. Regal fritillary and monarch larvae require violets (*Viola* spp.), and milkweed (*Asclepias* spp.), respectively (13, 16-18). The Karner blue butterfly range in Wisconsin and Minnesota include oak savanna and pine barren ecosystems that promote the growth of wild lupine (*Lupinus perennis*), which is their larval host plant. This butterfly has also been found along utility and road rights-of-way, abandoned agricultural fields, and managed forests (19). Mitchell's satyr butterflies use fens that maintain a large quantity of sedges, which are larval hosts (20). Although all these species were historically found throughout the north central states, they have experienced habitat loss due, in part, to urbanization and agricultural development (1-3). To sustain these species, habitat restoration within cultivated areas is needed, with a focus in the Dakotas, Illinois, Indiana, Iowa, Minnesota, and Wisconsin (13, 17, 20-24).

Unique to these 'at risk' species, the North American monarch is migratory with multi-voltine sub-populations that migrate east and west of the Rocky Mountains. Monarchs that migrate east of the Rocky Mountains overwinter in Mexico. In early spring, the overwintering generation migrates to Texas and, in late spring, subsequent generations migrate to the north

central and northeastern U.S. The second and third generations during the summer are not migratory (25). The fourth (and sometimes a fifth) generation migrates back to Mexico in the fall. Approximately 40-55% of the overwintering monarchs hatch from eggs laid in the north central U.S. (26, 27).

There are several factors that contribute to the decline of Lepidoptera and/or threaten the recovery of these species in the north central states, including habitat loss, climatic changes, susceptibility to diseases, and insecticide exposure; however, habitat loss is typically identified as the primary cause of decline (13, 17, 20-24). Given the range of these ‘at risk’ species, re-establishment of habitat in agricultural landscapes is typically identified as a primary conservation practice to support recovery. For example, recovery of the eastern monarch butterfly population is highly dependent on restoring milkweed in agricultural landscapes (8, 25). The Dakota skipper, Poweshiek skipperling, and regal fritillary are also found, or were previously found, in landscapes that are partly in row crop agriculture and livestock production, with larvae present from April through August depending on the species (17, 18, 20-22). There is concern that establishing new habitat within or near these agricultural landscapes could increase potential insecticide exposure to the butterfly species themselves and/or their host plants and undermine recovery efforts (13, 17, 20-22). In addition, pesticide runoff or drift from adjacent agricultural fields has been listed as a potential threat to Karner blue (23, 28) and Mitchell’s satyr (24).

Potential insecticide exposure is likely given the spatial-temporal overlap of insecticide use patterns with ‘at risk’ species’ utilization of habitat in close proximity of agricultural crops. As summarized by Krishnan et al. (29, 30), 8 to 20% and 6 to 30% of maize and soybean fields in the U.S. north central states are treated with foliar or soil-applied insecticides (typically

organophosphate, pyrethroid, and neonicotinoid insecticides), while nearly 100% of maize and 50% of soybean acres in the U.S. employ neonicotinoid-treated seeds (31). Consistent with these use patterns, insecticide spray drift exposure to downwind habitat up to 300 m from row crop fields is predicted based on modeling (29, 32), while exposure to host plants, typically within 100 m of fields planted with treated seeds, has been reported in monitoring studies (33-36). Additionally, insecticides are mostly applied during the growing season of crops, when lepidopteran species in north central U.S. are often active, which increases likelihood of exposure (e.g., see Krishnan. et al. (29)).

To characterize spatial-temporal risks of insecticide uses to lepidopteran species of conservation concern requires information on the species life history, estimates of environmental exposure, and species-specific dietary and topical toxicity data. Currently, the only species-specific assessment of insecticide risk has been reported for the monarch butterfly (29, 30, 37). In this case, monarch acute and chronic topical and dietary toxicity data were generated for all life stages and were used to characterize risks from estimated environmental exposures based on spray drift modeling and measured insecticide concentrations in milkweed plants.

Characterizing insecticide risks to additional lepidopteran species requires estimates of species-specific exposure levels and toxicity endpoints. While there are a number of monitoring studies reporting neonicotinoid residues in milkweed leaf as a result of seed treatment formulations, the extent to which these data could be used as a surrogate for host plant species of other lepidopterans of conservation concern in the north central states (e.g., sedges, grasses, violets, wild lupine) is unclear. In addition, the extent to which recent monitoring studies provide empirical exposure data due to spray drift, which could be used as another line of evidence with spray drift modeling predictions, is unknown. While a number of chemical insecticide toxicity

studies have been reported for lepidopterans (38), the monarch is the only species of conservation concern for which data are available. Toxicity testing with other ‘at risk’ lepidopteran species would be difficult, if not impossible, due to constraints in collecting ‘at risk’ species, inadequate knowledge on their biology and life history, potential difficulties in rearing and handling under laboratory conditions, and unknown performance in toxicological bioassays. As an alternative to empirical testing, estimates of insecticide toxicity values for ‘at risk’ species could be generated through the development of Species Sensitivity Distribution (SSD) models, which are statistical distributions that describe variations in toxicity among species to a compound (or a group of similar compounds) based on existing data (39).

In this paper we demonstrate an approach to estimate insecticide exposures, adverse effects, and potential risks of insecticide applications to ‘at risk’ lepidopteran species.

Specifically, we:

- a. Conducted literature searches to retrieve pyrethroid, organophosphate, neonicotinoid, and diamide insecticide residue concentrations in terrestrial non-crop plant tissues and toxicity data for lepidopteran larvae.
- b. Summarized relevant residue and toxicity data and generated SSD models,
- c. Compared insecticide-specific toxicity data with insecticide-specific modeled and measured residues on larvae and non-crop plant tissues, respectively, to estimate non-target lepidopteran risks to foliar and seed treatment formulations, and
- d. Identified gaps in available residue and toxicity data, along with uncertainties in risk estimates, and outlined research needs.

Methods

Insecticide Exposure Data

To collect and evaluate the nature and extent of foliar and seed treatment insecticide residues in terrestrial non-crop plant tissue, a literature search was conducted in February and March 2021 for the years of 1900 to 2021 using the following search terms within Google Scholar: “pyrethroid” OR “organophosphate” OR “diamide” OR “neonicotinoid” AND "concentration" AND "wildflowers" OR "milkweed specie" OR "non-crop plant" OR "forbs". Residue data were collected from published papers reporting monitoring studies within agricultural settings; residues reported in greenhouse studies were not used. Neonicotinoid residues based on ELISA analyses were not included due to reported cross reactivity of the kits (Product #500800, Abraxis, Warminster, PA). Although some papers reported residue data for a variety of neonicotinoids, we are only reporting concentrations of clothianidin, thiamethoxam, and imidacloprid, which are widely deployed throughout the north central U.S.

Estimated insecticide concentrations deposited on larvae following foliar applications were obtained using the Tier I Aerial and Ground models for terrestrial assessments within AgDRIFT, Ver 2.1.1 (40) for the following selected formulated products: Warhawk (chlorpyrifos; 34704-857), GOWAN MALATHION 8 (Malathion; 10163-21), Permethrin (Permethrin, 34704-873), Delta Gold® (Deltamethrin; 264-1011-1381), Fastac™ CS (alpha-cypermethrin; 7969-364), and F9114 EC Insecticide (zeta-cypermethrin; 279-3426). For all scenarios, spray drift exposure was modeled at point depositions of 0 to 100 ft (or 0 to 30 m) from the edge of an application area. For the Tier I ground model, the 50th percentile prediction was used as it excludes outlier and high wind speed effects.

Insecticide Toxicity Data

Lepidopteran toxicity data for pyrethroid, organophosphate, neonicotinoid, and diamide insecticides were obtained from published literature. We specifically focused on the larval life stage and two routes of insecticide exposure: topical and dietary. To facilitate comparisons across species and insecticides, we only searched for studies that reported LD50 (lethal dose that kills 50% of the treated population) and LC50 (lethal concentration that kills 50% of the treated population) values. Thus, a Google Scholar search was conducted in February and March 2021 with the following terms: “butterfly” OR "moth" OR "lepidoptera" AND “larva” OR “caterpillar” AND “topical” OR “cuticular” OR “dietary” OR “oral” AND “LD50” OR "LC50" AND “pyrethroid” OR “organophosphate” OR "diamide" OR "neonicotinoid". Over 1600 results were obtained; however, only data from 85 papers were utilized (29, 41-123). Studies were excluded for the following reasons:

- a. Larval dose (topical studies) or diet concentration (dietary studies) units were not provided or could not be deciphered.
- b. No ‘susceptible’ (i.e., non-resistant) lab populations were tested.
- c. Single active ingredient compounds were not tested.
- d. An appropriate solvent control was not utilized.
- e. Insecticide solvent carrier for toxicity bioassays not provided.
- f. Published in a language other than English.

In addition, studies that provided topical larval concentrations (for example, contact toxicity studies where larvae were placed on known concentrations of treated surface areas) or dietary larval doses (for example, the insecticide dose consumed by the larvae was estimated; see (121, 124)) were excluded as the testing methods and/or dose metrics were different from the vast majority of studies, and did not allow for comparisons with exposure concentrations. For a

similar reason, two monarch chronic dietary studies were excluded (30, 125). For topical exposure studies, LD50 values were obtained in the following units: ng/larva and ng/g larva. If one of the two units and weight of the treated larvae were provided, the other dose unit was calculated. For dietary studies, LC50 values were obtained in the following units: ng/g diet, ng/cm² diet, and ng/cm³ diet. Often, the units were calculated from the information provided.

A few laboratories had, over the span of several months to years, retested the same colony using the same method and reported multiple LD/LC 50 values. In such instances, the lowest reported LD/LC 50 value was selected [except in one instance (122), difference across replicates did not exceed four-fold; (51, 62, 97, 99, 115)]. A few papers conducted toxicity bioassays under different temperatures (109) or reported LD/LC 50 values over multiple days following treatment (112); in such instances, optimal temperatures and lowest reported LD/LC 50 values were selected.

The U.S. Environmental Protection Agency's Species Sensitivity Distribution Generator v1 (126), which fits a log-probit distribution to the data, was used to generate the SSDs. Based on skewness of the data, an arithmetic or geometric mean was used to calculate the mean LD/LC50 value for each species.

Results and Discussion

Based on available insecticide residue and toxicity data, we explore the potential risks associated with establishing lepidopteran habitat in agricultural landscapes.

Residue/Exposure Data

Seed treatment and foliar applications: Dietary exposure

For species of conservation concern, a major dietary exposure pathway is from seed treatment insecticides during the larval stages from consumption of contaminated host plants that have systemically absorbed insecticide residues as they move off-field with overland runoff and

subsurface flow (127-131). Larvae could also have dietary exposure to foliar applied insecticides through spray drift deposition on their host plants and, to a lesser extent, from spray drift onto soil and systemic movement into host plants. Common host plants of lepidopteran species found throughout north central U.S. are provided in Table 1. To evaluate potential dietary neonicotinoid exposure to larvae of these species, it is necessary to estimate concentrations in these host plants.

Neonicotinoid seed treatments are commonly used throughout north central U.S. for control of early season pests (31). They are also registered for foliar application, though they are not widely employed (136, 137). Clothianidin, imidacloprid, and thiamethoxam are commonly used in seed treatments and frequently detected in overland runoff, subsurface flow, and groundwater (127-131). There is concern that these compounds could harm pollinators through systemic contamination of downslope plants and cause dietary exposure to non-target insects (138). Most studies thus far have focused on exposure to managed bees, with fewer data available on potential exposures to other non-target insects such as butterflies (138).

As summarized in Table 2, over the last seven years, several studies have analyzed tissues from plants in close proximity to crop fields (33-36, 127, 139-141). Samples collected for analysis were either from individual plants or a composite of multiple species. The majority of studies evaluated foliage as a matrix; the most common species being milkweed. Neonicotinoids, specifically clothianidin, thiamethoxam, and imidacloprid, were the most commonly detected insecticides with means ranging from < Limit of Quantification (LOQ) to 41 ng/g and medians ranging from <LOQ to 1.4 ng/g (Table 2). We assume these residues are primarily due to systemic uptake of residues moving downslope of crop fields planted with treated seeds. However, there is insufficient information available on the surrounding crop fields to rule out

foliar insecticide deposition as a possible route, especially with regard to samples with high residue concentrations. Individual host plant residue data for lepidopteran larvae are provided in Table 3. It is important to note that information on the timing of sampling post-planting and distance from sampling site to crop field edges were not always provided in the reviewed studies. This information is necessary to fully understand spatial and temporal overlaps with plant species of interest.

Anthranilic diamides are a more recent chemistry used for pest control. In the last decade, chlorantraniliprole seed-treatment products have entered the U.S. market and have been registered for use in maize (142, 143). Currently, they are not widely deployed, but they may have increased use in the future. They are also registered for foliar applications on a variety of crops including pome fruit, stone fruit, grapes, leafy vegetables, cucurbit vegetables, fruiting vegetables, potatoes, cotton, rice, oilseeds, and soybean (144). There is insufficient diamide residue data for non-target plant species. To date, there is only one peer-reviewed paper that analyzed milkweed plants to determine concentrations of chlorantraniliprole (Table 2). The mean plant concentration from samples collected in California across a diversity of agricultural sites was 17 ng/g (141). Due to the physiochemical properties of chlorantraniliprole and the location of the sampling (Central Valley of California), it is likely the residues are due to foliar applications (30, 145).

Pyrethroids and organophosphates are commonly used as foliar insecticides in soybean and maize fields to manage a variety of early- and late-season pests (29, 30, 136). However, few field studies have evaluated residues of these compounds in non-target habitat within agroecosystems. Currently, residues have been detected only in milkweed plants; mean organophosphate concentrations are <LOQ (1 ng/g) and mean pyrethroid concentrations range

from LOQ to 3.78 ng/g milkweed leaf tissue (Table 2). Spray drift exposure is a function of habitat proximity to treated field, wind speed and direction at time of foliar application, and the time/rate of insecticide use patterns within and across growing seasons.

While concentrations of insecticides detected across plant species and studies are variable, the data clearly indicate that non-target lepidopterans could be exposed to insecticides through consumption of host plants in agroecosystems. Native grasses, host plants for the Dakota skipper and Poweshiek skipperling, had residues of neonicotinoids ranging from not detected (nd) to 25.2 ng/g, and milkweed species, host plants for monarchs, dogbane tiger moth, and unexpected cynthia larvae, had residues ranging from nd to 151.3 ng/g. Some of the differences in plant residues could be due to the insecticide itself, e.g., certain neonicotinoids can persist longer and accumulate while others may be metabolized more quickly (35). Individual studies also differed in insecticide formulations, application rates, application routes, and soil type, with some studies providing no information. Additionally, field history and seeding density information were often unavailable. Studies also varied in plant species collected, geographical location, sampling time points, and distance of sample collection from crop fields. For example, higher seed treatment residues would be expected if sampling was done downslope of a field and higher foliar insecticide residues would be expected if sampling was done downwind of a field. Differences could also be due to variability in plant physiology, such as plant longevity and growth rate, which would affect the uptake capacity and metabolic pathways of degradation within plant species (35). Consequently, it is difficult to compare studies and characterize with confidence the route of exposure to non-target plants.

Foliar application: Topical exposure

Empirical topical larval exposure data are not available in the literature. Consequently, we used AgDRIFT (40) to estimate spray drift exposure from aerial and ground boom

applications of chlorpyrifos, permethrin, deltamethrin, alpha-cypermethrin and zeta-cypermethrin, for soybean aphid (*Aphis glycines*) management. Exposure was estimated at 0 and 100 feet (30 m) downwind. Malathion applications to manage adult western corn rootworm (*Diabrotica virgifera virgifera*) was also modeled for both aerial and ground boom applications. The AgDRIFT outputs were provided in ng/cm² area. To estimate the concentration of insecticide landing on larval cuticle, it is necessary to estimate the larval surface area. Of the studies we analyzed, only one had estimated the surface area of a lepidopteran larvae, specifically monarch butterfly larvae (26). The authors estimated the surface area for all five instars, which ranged from 0.17 (\pm 0.05) to 7.1 (\pm 1.3) cm². As third instars were most commonly tested in bioassays retrieved from the literature, we compared the AgDRIFT exposure concentrations to the monarch third instar surface area (0.65 cm²) to estimate larval exposure concentrations in ng/larva (ng/cm² x cm²/larva). Due to lack of surface area data for other species and to facilitate risk comparisons between scenarios, we assume these larval exposure concentrations apply to all species.

Toxicity Data

Description of toxicity dataset

We obtained 317 LD/LC50 values from 85 studies published over 51 years (1970 to 2021). These data were obtained following treatment of 34 species, which included 13 butterflies and 21 moths, with 59 active-ingredient insecticides (which included metabolites and isomers). Most data were for pyrethroids (168 LD/LC 50 values), followed by organophosphates (116 LD/LC 50 values). The species with the most data were cotton bollworm (*Helicoverpa armigera*; 87 LD50 values), monarch butterfly (38 LD/LC50 values), and tobacco budworm (*Heliothis virescens*; 24 LD50 values). While cotton bollworms were exposed to 36 insecticides (pyrethroids and organophosphates), the monarch butterflies were exposed to only 7 insecticides

(pyrethroids, organophosphates, neonicotinoids, and diamides). Common cutworms (*Spodoptera litura*) and corn earworms (*Helicoverpa zea*) were exposed to 12 and 9 insecticides (pyrethroids and organophosphates), respectively. Other species were exposed to fewer insecticides.

Toxicity bioassays were conducted on all instars (first to fifth/sixth instar), but nearly half the studies were done on third instars followed by fifth and fourth instar larvae. The observation periods for the bioassays ranged from 24 to 168 hours; however, except for the monarchs, the observation period for all butterfly species was 24 hours. Only 18% of moth bioassays had a 24-hour observation period; approximately 29 and 36% of bioassays had 48- and 72- hour observation periods, respectively. Nearly 84% of moth larvae were provided an artificial diet while the rest of the moth species were provided host plant diet. Excluding one study (41), all butterfly larvae were reared on leaves of their host plants. Only two studies (29, 124) reported analytically verified insecticide concentrations employed in the bioassays.

Topical toxicity data and generation of SSDs

Topical toxicity data were obtained for 33 species (13 butterflies and 20 moths) and 58 insecticides (29 pyrethroids, 24 organophosphates, 3 neonicotinoids, and 2 diamides; see Table 4). Of the 286 LD50 values obtained, 271 were generated for pyrethroids and organophosphates. Currently, monarch butterflies are the only lepidopteran species for which there are topical LD50 values for neonicotinoids. Diamide topical LD50 values are only available for monarchs, fall armyworms (*Spodoptera frugiperda*), and diamondback moths (*Plutella xylostella*). Out of the 81 studies analyzed, 76 had employed acetone as a solvent; the others had employed hexane, a mixture of olive oil and acetone, or a mixture of ethyl methyl ketone and acetone. Nearly 76% of the LD50 values could be expressed as both ng/larva and ng/g larva.

As a minimum of 8 to 10 species are needed to generate reliable SSDs (35), we could only develop models for topical exposures to pyrethroids and organophosphates (see Table 4;

Figures 1, 2 and Figures A1 and A2 in the Appendix). A single pyrethroid study was excluded (88) as it reported a LD50 that was nearly 100-fold greater than the nearest LD50 value (see Figure A3). With both pyrethroids and organophosphates, similar correlations were obtained between insecticide dose and proportion of species affected when SSDs based on LD50 values were generated using ng/g larvae, which accounted for variation in the body weights across lepidopteran larvae, and ng per larvae, which did not account for body weight.

The pyrethroid SSD generated with ng/g LD50 values has a slope of 1.25 and an R^2 of 0.972 (Figure 1). The hazardous concentration for the 5th percentile species (or HC5) is 14.3 ng/g larva or 1.22 ng/larva. The spread in LD50s values is three orders of magnitude (ca. 10 to 10,000 ng/g); the most sensitive species were Atala hairstreak (*Eumaeus atala*) and Zebra longwing (*Heliconius charithonia*), while the least sensitive species were the diamondback moth and European corn borer (*Ostrinia nubilalis*). Monarchs, the only species of conservation concern for which there is empirical toxicity data, was among the most sensitive species. However, the data do not strongly suggest butterflies are generally more susceptible to pyrethroids; white peacock (*Anartia jatrophae*), and large cabbage white (*Pieris brassicae*) butterflies are among the least sensitive species. The SSD generated using LD50 values based on ng/larva (R^2 of 0.971) indicated white peacock, painted ladies (*Vanessa cardui*), and common buckeye (*Junonia coenia*) were the least sensitive species, while the monarch was intermediately sensitive (Figure A1). Interestingly, except for European corn borers, the five least sensitive larval species on a ng/larva basis have hairy cuticles. This may suggest that the presence of cuticular hair reduces topical insecticide uptake.

SSDs were also generated separately for Type I and Type II pyrethroids (see Figures A4 and A5). While some species showed greater sensitivity to one class of pyrethroid (e.g.,

monarchs), most showed similar sensitivity to both classes. Soybean looper (*Chrysodeixis includens*) was highly sensitive to both pyrethroid classes. SSDs were also generated individually for cypermethrin (type II pyrethroid), deltamethrin (type II), fenvalerate (type II), and permethrin (type I pyrethroid) (see Figures A6-A9). Deltamethrin was most toxic to larvae and permethrin was the least toxic. However, as these SSDs had significantly fewer species compared to the combined pyrethroid SSD and had relatively wide toxicity distributions, the correlations were sometimes poor. Often, the SSDs generated using ng/larva LD50 values produced a better fit.

The organophosphate SSD generated using ng/g LD50 values has a slope of 1.29 and an R^2 of 0.978 (Figure 2). The HC5 is 533 ng/g larva or 16 ng/larva. The spread in LD50s is approximately three orders of magnitude (ca. 5000 to 500,000 ng/g). The five most sensitive species are butterflies while three of the five least sensitive species are moths. Monarchs, the only species of conservation concern for which there is empirical toxicity data, is among the least sensitive along with green-veined white (*Pieris napi*) and common blue (*Polymornatus icarus*) butterflies. When SSDs were generated using LD50 values based on ng/larva (R^2 of 0.976), painted ladies, common buckeyes, and monarchs were among the least sensitive species (Figure A2).

SSDs were also generated separately for thioate (R^2 of 0.965 and 0.980 when LD50 values were plotted on a ng and ng/g basis, respectively) and phosphate (R^2 of 0.980 and 0.952) subclasses of organophosphates (see Figures A10 and A11). Twenty-one species were treated with insecticides from the thioate subclass while only eight or nine species were treated with insecticides from the phosphate subclass, making robust comparisons difficult. SSDs were generated individually for chlorpyrifos and malathion (thioate subclasses; see Figures A12 and

A13). Both insecticides produced similar HC5s and distributions. Again, these individual SSDs had relatively poor correlations (R^2 of 0.874 to 0.943).

Generally, the data from both the pyrethroid and organophosphate SSDs showed that, on a ng basis, the most sensitive species was large cabbage white butterfly while the least sensitive species was painted lady butterfly. On a ng/g basis, *Atala* hairstreak and zebra longwing butterflies were among the most sensitive species, while European corn borers and diamondback moths were among the least sensitive. Interestingly, soybean loopers and western spruce budworms (*Choristoneura occidentalis*) were highly sensitive to pyrethroids, but insensitive to organophosphates.

Neonicotinoid topical data were obtained for first, third, and fifth instar monarch butterflies that were exposed to imidacloprid, thiamethoxam, and clothianidin; LD50 values ranged from 0.69 to 30,000 ng/larva or 190 to 35,000 ng/g larva (29). Clothianidin was the most toxic while thiamethoxam was the least toxic. Monarchs exposed to the diamide chlorantraniliprole had LD50s ranging from 0.034 to 150 ng/larva or 12 to 190 ng/g larva (29), while diamondback moths exposed to the same compound had a LD50 of 900 ng/g larva (56). Fall armyworms exposed to chlorantraniliprole and flubendiamide had LD50s of 1 and 3 ng/larva, respectively (52). Given the limited dataset and the variabilities in LD50 values, it is difficult to draw conclusions on topical toxicity of neonicotinoid and diamide insecticides across lepidopteran species.

Dietary toxicity data

Dietary toxicity data were obtained for 5 species (1 butterfly and 4 moths) and 11 insecticides (4 pyrethroids, 1 organophosphate, 4 neonicotinoids, and 2 diamides; see Table 4). Of the 31 LC50 values obtained, 13 were generated for pyrethroids and organophosphates. Currently, apart from monarch butterflies, dietary toxicity data are only available for soybean

loopers, northern armyworms (*Mythimna separata*), codling moths (*Cydia pomonella*), and black cutworms (*Agrotis ipsilon*). Out of the six studies analyzed, four employed acetone as a solvent while two employed a water: surfactant mixture. Monarchs were the only butterfly species studied, and insecticides were applied on milkweed leaf. For the moth species, insecticides were incorporated in artificial diets. For three studies (29, 46, 55), LC50 values were reported or derived in two of three units; ng/g, ng/cm², or ng/cm³ diet. For the remaining bioassays, LC50s were expressed in only one unit.

For monarch butterflies and codling moths, data were available for three classes of insecticides (Table 5). For both species, pyrethroids were the most toxic, while organophosphate chlorpyrifos and neonicotinoid imidacloprid were less toxic. Additionally, codling moths were more susceptible than monarchs to all three insecticide classes. For both monarchs and soybean looper, diamides were more toxic than pyrethroids.

Risk Estimation

Dietary risks from seed treatment insecticides

Potential dietary risk to larvae is a function of insecticide toxicity and concentration of insecticide residues in or on host plant leaves. The majority of available dietary toxicity data is for monarch larvae and neonicotinoid insecticides. Simultaneously, the majority of plant residue data is for neonicotinoids in milkweed species. Risks to monarch larvae through dietary exposure in these scenarios are currently characterized in the literature (29, 30, 33, 36, 37, 125) and indicate that establishment of monarch habitat in close proximity to agricultural fields using treated seeds is not a high-risk scenario. Neonicotinoid residue data are available for additional species that are host plants to other non-target lepidopteran larvae (Tables 2 and 3). There is, however, an insufficient number of neonicotinoid toxicity studies to generate a SSD model to estimate dietary risk associated with neonicotinoid seed treatments for other “at risk” species.

Limited plant residue data are available for pyrethroids, organophosphates, and diamides. Although restricted to the monarch and milkweed species, chlorantraniliprole is the only insecticide for which dietary toxicity values and plant residue data are available. Monarch risks from dietary chlorantraniliprole exposure can be significant; see Halsch et al. (141) and Krishnan et al. (30).

Topical risks from foliar insecticides

Topical risk from foliar insecticides was estimated by comparing the exposure concentrations derived from AgDRIFT with the SSD distributions. Most application scenarios resulted in high levels of estimated mortality for a large proportion of species (see Table 6). Following aerial and ground boom applications of organophosphate insecticides, 50 to 100% of larvae at the downwind edge of a treated field are estimated to be affected (i.e., exposure concentrations are greater than respective LD50 values). At 100 feet (30 m) downwind, ground boom and aerial applications are estimated to affect 0 to 20% and 10 to 75% of the species, respectively. Aerial and ground boom applications of pyrethroid insecticides are estimated to affect 90 to 100% of species at the downwind field edge. At 100 feet downwind, 45 to 95% of species are predicted to be affected following aerial applications; ground booms are predicted to affect 5 to 40% of species.

While topical SSDs were not generated for other insecticide classes, Krishnan et al. (29) reported risks for representative neonicotinoid and diamide foliar insecticide products for monarch butterfly larvae. While neonicotinoid data are lacking for other species, diamide data are available for only two other species, diamondback moths and fall armyworms, both of which are pest species.

Conclusions

Characterizing dietary exposure of foliar and seed treatment insecticides to non-target, ‘at risk’ lepidopteran species within agroecosystems requires detailed residue data for a variety of insecticide classes across a diversity of host plant species. Optimally, sampling frequency and sampling locations of host plants should overlap spatially and temporally with the life history of lepidopteran species of interest. If possible, sampling sites should be georeferenced, historical insecticide use patterns obtained, and cropping history for focal and nearby crop fields noted. This information will help refine sampling designs and elucidate if detected residues were likely due to foliar and/or seed treatment formulations. Ideally, samples should be taken at various distances downwind/downslope from the edge of a treated field. Appropriate chemical-specific analytical techniques, including the method and data used to derive a LOQ, should be employed for chemical quantification.

To date, neonicotinoids are the most represented class of insecticide monitored in field studies, with the most robust data being available for milkweed species. There are, however, a variety of other host plants for non-target lepidopteran species within agroecosystems of the north central states and a variety of other insecticides used on crop fields. Therefore, residue studies focusing on additional plant species and a broader set of insecticide classes would provide more information on the dietary exposure route for non-target lepidopteran species. Additionally, a more thorough understanding of differences in physiology between host plants could help determine if residues found in sampled plant species could reasonably serve as surrogates for non-sampled host plants.

Currently, lepidopteran toxicity bioassays are not standardized, and there are several uncertainties in published data that can influence toxicity estimates. The vast majority of data are from acute studies that have varying observation periods post exposure. A variety of instars are

employed, though information on susceptibility across instars is generally lacking (note: data from Robertson et al. (122), Karoly et al. (91), and Krishnan et al. (29) suggest that younger instars are more susceptible to insecticides on a ng/larva basis; however, see Salvato et al. (76)). The majority of dietary bioassays published to date did not provide dosimetry information that can be used in a risk assessment (e.g., leaf dip studies report the concentration of the insecticide solution used to treat leaves, but not the concentrations of the leaves the larvae were provided). In addition, many studies employed formulated products without utilizing an appropriate control and only two studies had analytically verified the concentrations of the insecticides used in the bioassays. Finally, there is a dearth of data for newer classes of insecticides. All these issues increase uncertainty in ecological risk assessments and risk management decisions for non-target species.

SSDs require that the species employed are from a similar taxon, of a similar stage, and exposed to similar compounds and testing methods, which requires consistently employed methods to generate toxicity endpoint data with supporting dosimetry information. To derive more robust and relevant SSDs for Lepidoptera, it is important to generate data for additional species, particularly butterfly species that are currently underrepresented in existing datasets. Full dose-response toxicity data or LD/LC 50 values for pest (mostly moth) species generated in pesticide discovery efforts could be a useful addition to the knowledge base needed to refine Lepidoptera SSDs; however, this information is typically not published. While SSDs can be useful in predicting toxicity for hard-to-rear species, selecting surrogate species for toxicity testing guidelines, and setting environmental standards (39), there are additional or alternate approaches that can be considered to support risk assessments. For example, allometric scaling, which relates toxicity to species' body mass, are often recommended for interspecies

comparisons (146). Some toxicokinetic and toxicodynamic properties that influence toxicity may, however, not scale with mass (147); of note, in the current study, we obtained slightly better SSDs when body weights were not accounted.

While residue and toxicity data from seed treatment insecticides suggest minimal risk to non-target lepidopterans, we predicted foliar insecticide applications can pose significant risk to a large proportion of lepidopteran species downwind of treated fields, including ‘at risk’ species. However, it is important to consider the bioavailability of an insecticide in the environment, a species’ likelihood of exposure, interactions within ecosystems, combined effects of multiple stressors, etc. to accurately estimate a species’ risk to an insecticide exposure. For a vagile species like the monarch butterfly, a landscape-scale analysis that accounts for wind direction at time of foliar insecticide use, location of milkweed with respect to the crop field, frequency of insecticide application, and behavior of different life stages, indicates that more monarchs would be produced if habitat is established everywhere in agricultural landscape vs. only outside buffer zones of 125 ft (38 m) (37). However, most non-target lepidopteran ‘at risk’ species are patch residents (17, 21, 23, 24) and landscape-scale, metapopulation analyses with different spatial arrangements of habitat patches would be needed to ascertain the extent to which habitat near treated crop fields could serve as population sinks. Ultimately, the strategic generation of more robust exposure and toxicity data will lead to more refined insecticide risk assessments and risk mitigation measures and help inform habitat restoration plans for non-target Lepidoptera, including species of conservation concern.

Author Contributions

MJH analyzed the residue data and drafted the corresponding components of the manuscript. NK analyzed the toxicity data and drafted the corresponding components of the manuscript. All authors were involved in designing the study and editing the manuscript.

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Data Availability

The residue and toxicity datasets that were generated from the literature are publicly available at this GitHub link: <https://github.com/Niranjana296/Insecticide-risk-to-non-target-lepidopterans>.

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Tables and Figures

Table 1. Examples of Lepidopteran Species Found in North Central U.S. and their Larval Host Plants

Lepidopteran Species	Example Larval Host Species ⁺	References
Dakota skipper	Native Grasses (e.g., <i>little bluestem</i>)	(21)
Mitchell's satyr	Native Sedges (e.g., <i>Carex stricta</i>)	(24)
Poweshiek skipperling	Native Grasses/Sedges	(17)
Regal fritillary	Violet Species	(18)
Karner blue butterfly	Wild lupine	(19)
Monarch	Milkweed Species	(13)
Dogbane tiger moth (<i>Cycnia tenera</i>)	Milkweed & Spreading Dogbane (<i>Apocynum androsaemifolium</i>)	(132)
Unexpected cycnia (<i>Cycnia inopinatus</i>)	Milkweed	(133)
Clouded sulphur (<i>Colias philodice</i>)	Clover Species (<i>Trifolium</i> spp.) & Alfalfa (<i>Medicago sativa</i>)	(132)
Eastern tailed blue (<i>Cupido comyntas</i>)	Clover Species, Alfalfa, Wild Pea (<i>Lathyrus</i>)	(134)
Painted lady (<i>Vanessa cardui</i>)	Thistle (<i>Cirsium</i>), Nettle (<i>Urtica dioica</i>), Mallow (<i>Malva</i>)	(132)
Bordered patch (<i>Chlosyne lacinia</i>)	Sunflower species (<i>Helianthus</i> spp.)	(135)

⁺Some lepidopteran species listed have additional host plants that are not included in this table.

Table 2. Summary of Neonicotinoid, Pyrethroid, Diamide, and Organophosphate Residues in Non-crop Plants within Agricultural Landscapes

Species	Average distance from field (m)	Insecticide	Concentration (ng/g)				% Detections	Reference
			Mean	Median	Max	LOQ ^a		
Wildflowers (Table A1)	20	Clothianidin	1.4	nr	53	1	6	(139)
		Thiamethoxam	7.2	nr	256	1	14	
		Imidacloprid	1.1	nr	48	1	6	
nr	~1	Clothianidin	1.2	nr	5.9	nr	nr	(140) ^b
			1		6.5			
Wild plants (Table A2)	1.5	Clothianidin	0.51	≤0.2	11.45	0.6	22	(35)
		Thiamethoxam	8.71	≤0.1	106.2	0.3	35	
		Imidacloprid	1.19	≤0.2	26.1	0.6	29	
Prairie plants (Table A3)	~2	Clothianidin	nd	nd	nd	1	nd	(127)
		Thiamethoxam	nd	nd	nd	1	nd	
		Imidacloprid	nd	nd	nd	1	nd	
Milkweed species	100	Clothianidin	2015=0.71; 2016=0.48	2015 = <LOQ; 2016 = <LOQ	56.5	1.06	2015=4.6; 2016 = 8.1	(33)
		Thiamethoxam	2015=0.19; 2016=1.87	2015 = <LOQ; 2016 = 1.44	151.3	0.23	2015=1.8; 2016 = 75.4	
		Imidacloprid	2015 = <LOQ; 2016 = nd	2015 = <LOQ; 2016 = nd	3.7	0.64	2015=0.2; 2016 = n.d.	
		Deltamethrin	2015 = nd; 2016 = 3.78	2015 = nd; 2016 = 1.91	248.5	0.42	2015=n.d. 2016 = 98.9	
Milkweed species	nr	Clothianidin	41	nr	nr	1	nr	(141) ^{cd}
		Thiamethoxam	4			1		
		Imidacloprid	0.34			1		
		Cyantraniliprole	0.22			1		
		Chlorantraniliprole	17			1		
		Malaoxon	0			1		
		Methamidophos	0			1		
		Trichlorfon	0.01			1		
		Etofenprox	0.14			1		
Wildflowers and flowering shrubs (Table A4)	~2	Clothianidin	0.22	nr	9.8	6	2.2	(34) ^e
		Thiamethoxam	0.07		1	1	6.7	
		Imidacloprid	nd		nd	2	0	

Table 2. Continued

Milkweed species	~0.15	Clothianidin	nr	2017 = <LOQ; 2018 = <LOQ	6.6	0.1	2017= 58; 2018=47	(36)
		Thiamethoxam		2017 = <LOQ; 2018 = <LOQ	12.9	0.1	2017= 42; 2018=35	
		Imidacloprid		2017 = <LOQ; 2018 = <LOQ	2.8	0.1	2017= 23; 2018=67	
Forbs (Table A5)		Clothianidin		2017 = 0.23; 2018 = 0.2	4.5	0.1	2017= 67; 2018=68	
Thiamethoxam		2017 = <LOQ; 2018 = <LOQ		7.1	0.1	2017= 33; 2018=15		
Imidacloprid		2017 = <LOQ; 2018 = <LOQ		1.8	0.1	2017= 12; 2018=43		

^a Various techniques and definitions were used across papers to characterize the lowest concentration that was quantified and reported. LOQ represents the lowest concentration that was determined quantifiable within each paper. We did not standardize estimates of LOQs across papers.

^b Reporting residue data for non-target plants collected next to fields planted with neonicotinoid treated seeds. Overall mean was not reported within paper. Mean values here are for individual sampling times.

^c The average reported for this study are calculated from supplemental data that reports the average concentration of each compound per plant (ng/g) at sites that are characterized as “agricultural”. These average concentrations are per agricultural site.

^d Limit of quantification interpreted to be 1 ng/g.

^e Reporting residue data for non-target plants collected from field margins of treated fields.

LOQ- Limit of Quantification; nr-not reported within the paper; nd-not detected

Table 3. Neonicotinoid Concentrations in Individual Plants or Plant Composites that are Host Plants for Lepidopteran Species in North Central United States

Larval host species	Clothianidin concentration ranges (ng/g)	Thiamethoxam concentration ranges (ng/g)	Imidacloprid concentration ranges (ng/g)
Grasses	nd — <0.2	nd — <0.1	nd — 25.20
Clover	nd — 0.97	nd — 11.47	nd — 0.32
Sunflowers	nd — 1.16	nd — 1.3	nd — 0.4
Milkweed species	nd — 56.5	nd — 151.3	nd — 3.7
Thistle	nd — <0.20	nd — 106.16	nd — 26.06
Nettle	nd — <0.2	nd — 88.5	nd — <0.6

nd-not detected

Table 4. Summary of Lepidopteran Toxicity Data Obtained from Literature ^a

Mode of action	# Species tested	# Insecticides tested	# LD/LC 50 values
Topical exposure			
Pyrethroid	25	29 (11 type I; 17 type II; 1 non-ester)	160
Organophosphate	30	24 (18 thioate; 6 phosphate)	111
Neonicotinoid	1	3 (3 nitroguanidine)	9
Diamide	3	2 (1 anthranilic; 1 phthalic)	6
Total	33 ^b	58	286
Dietary exposure			
Pyrethroid	4	4 (4 type II)	8
Organophosphate	2	1 (1 thioate)	5
Neonicotinoid	2	4 (3 nitroguanidine; 1 cyanoamidine)	12
Diamide	3	2 (1 anthranilic; 1 phthalic)	6
Total	5 ^b	11	31

^a Data were obtained from (29, 41-123).

^b Not cumulative as a single species could have been exposed to multiple modes of action.

#: number of; LD/LC50: lethal dose/concentration that kills 50% of the population.

Table 5. Summary of Lepidopteran Dietary Toxicity Studies

Mode of action	Insecticide	Species	LC50 values			Citation
			ng/g diet	ng/cm ² diet	ng/cm ³ diet	
Pyrethroid	Beta-cyfluthrin	Monarch butterfly ^a	210-940	5-26	NA	(29)
	Lambda-cyhalothrin	Soybean looper	NA	260	NA	(44)
		Northern armyworm	NA	3392	6784	(46)
		Codling moth	NA	NA	9.4	(54)
	Cypermethrin	Codling moth	NA	4.41	157.5	(55)
	Deltamethrin	Codling moth	NA	0.266	9.5	(55)
Organophosphate	Chlorpyrifos	Monarch butterfly ^a	6000-10000	140-250	NA	(29)
		Codling moth	NA	56.7	2025	(55)
		Codling moth	NA	NA	7126	(54)
Neonicotinoid	Imidacloprid	Monarch butterfly ^a	5100-17000	130-410	NA	(29)
		Codling moth	NA	7.84	280	(93)
		Codling moth	NA	NA	262.7	(54)
	Thiamethoxam	Monarch butterfly ^a	350-33000	87-1100	NA	(29)
	Clothianidin	Monarch butterfly ^a	800-7800	22-230	NA	(29)
	Acetamiprid	Codling moth	NA	0.77	27.5	(55)
Diamide	Chlorantraniliprole	Monarch butterfly ^a	8.3-970	0.19-23	NA	(29)
		Soybean looper	NA	50	NA	(44)
		Black cutworm	187	NA	NA	(47)
	Flubendiamide	Soybean looper	NA	130	NA	(44)

^aData for multiple instars.

LC50: lethal concentration that kills 50% of the population; NA: not available.

Table 6. Percentage of Lepidopteran Species with Estimated Pyrethroid (see Species Sensitivity Distribution (SSD) in Figure A1) and Organophosphate (see Figure A2) LD50 Values Exceeded by Predicted Larval Exposure Concentrations at Field Edge and 100 feet (30 m) Downwind Following Foliar Applications (Exposure Concentration Predictions Based on AgDRIFT (40) Outputs and Estimated Larval Surface Areas (see Methods))

Method	Aerial application		Ground boom application	
Distance	0 feet	100 feet	0 feet	100 feet
Malathion				
Exposure (ng/larva)	260	32	585	2.5
% spp. affected (organophosphate SSD)	50	10	70	0
Chlorpyrifos				
Exposure (ng/larva)	3600	390	7300	32
% spp. affected (organophosphate SSD)	95	60	> 95	10
% spp. affected (chlorpyrifos SSD)	> 95	75	100	20
Permethrin				
Exposure (ng/larva)	390	42	720	3.2
% spp. affected (pyrethroid SSD)	> 95	75	> 95	15
% spp. affected (permethrin SSD)	> 95	60	> 95	5
Deltamethrin				
Exposure (ng/larva)	130	12	200	0.90
% spp. affected (pyrethroid SSD)	90	45	> 95	< 5
% spp. affected (deltamethrin SSD)	100	95	100	40
Alpha-cypermethrin				
Exposure (ng/larva)	260	31	520	2.4
% spp. affected (pyrethroid SSD)	> 95	70	> 95	10
% spp. affected (cypermethrin SSD)	100	85	100	10
Zeta-cypermethrin				
Exposure (ng/larva)	520	62	1105	4.8
% spp. affected (pyrethroid SSD)	> 95	80	100	20
% spp. affected (cypermethrin SSD)	100	95	100	30

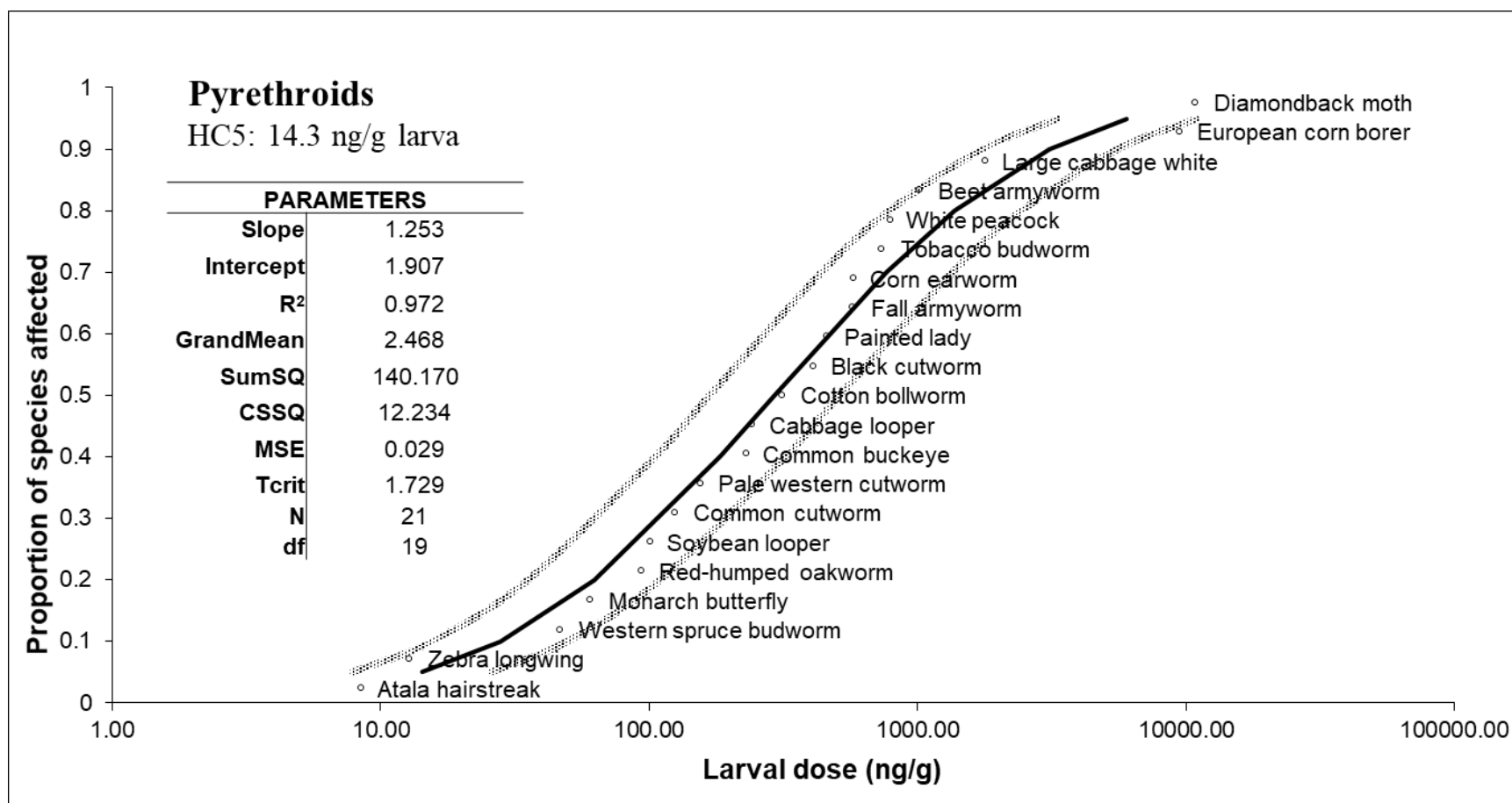


Figure 1. A species sensitivity distribution (with LD50 values in ng/g) for lepidopteran larvae topically exposed to pyrethroid insecticides.

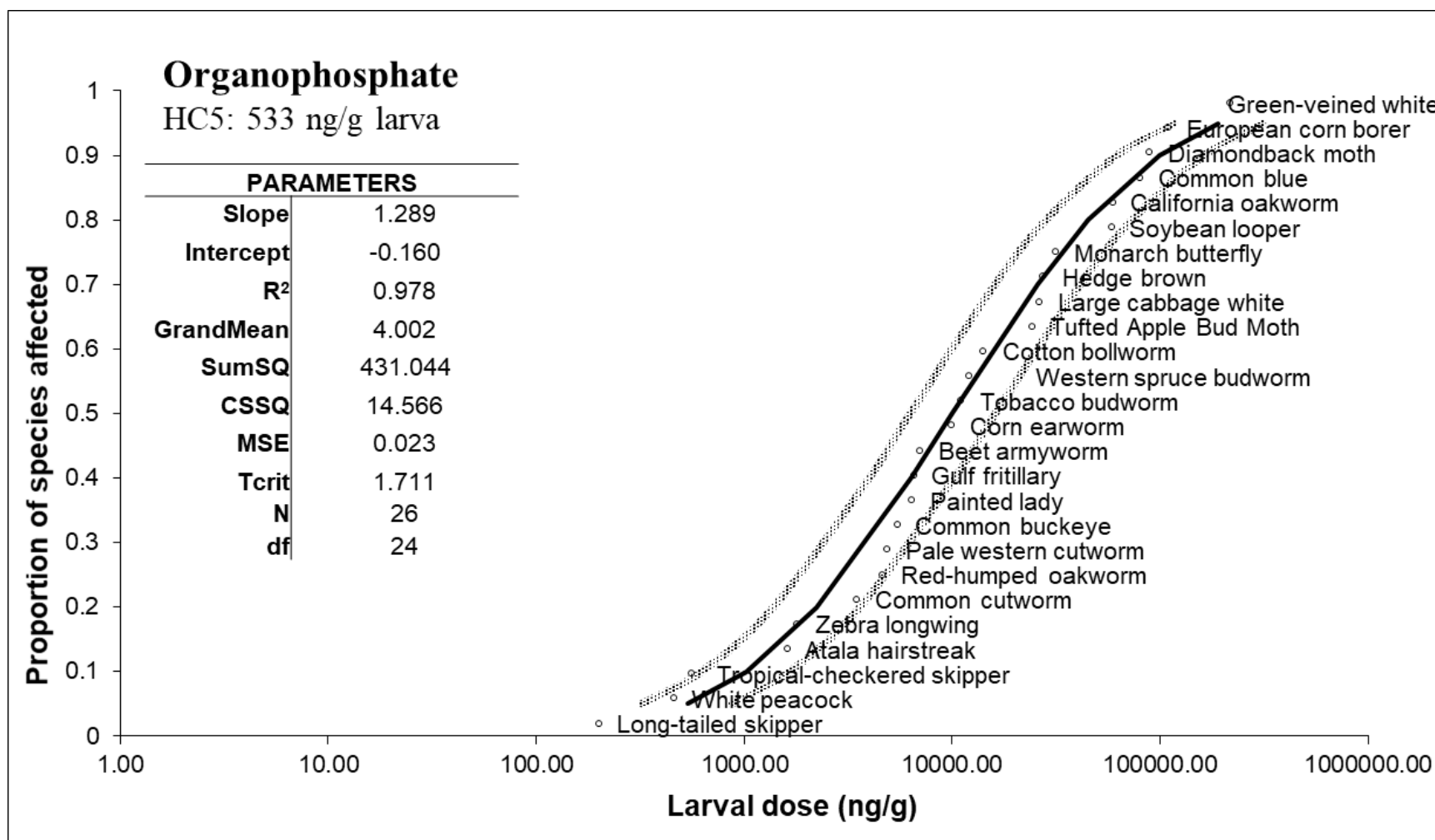


Figure 2. A species sensitivity distribution (with LD50 values in ng/g) for lepidopteran larvae topically exposed to organophosphate insecticides.

Appendix. Supplemental Tables and Figures

Table A1. Commonly Sampled Plant Species Sampled in Stewart et al. (139)

Scientific name	Common name
Rubus spp.	Blackberry & Dewberry
Trifolium	Clovers
Taraxacum officinale Wiggers	Dandelion
Erigeron spp.	Fleabane
Vicia sativa L.	Vetch
Vicia villosa Roth.	Hairy vetch
Rosa multiflora Thunb.	Multiflower rose
Phlox divaricata L.	Wild blue phlox
Tradescantia spp.	Spiderwort
Lonicera spp.	Honeysuckle

Table A2. Plant Species Sampled in Botias et al. (35)

Scientific name	Common name
Lamium purpureum	Purple dead nettle
Glechoma hederacea	Creeping charlie
Lamium album	White dead nettle
Vicia sativa	Common vetch
Trifolium pratense	Red clover
Dactylis glomerata	Orchard grass
Cardamine partensis	Meadow cress
Papaver rhoeas	Common poppy
Trifolium repens	White clover
Ranunculus repens	Creeping buttercup
Galium aparine	Stickywilly
Crataegus monogyna	Common hawthorn
Rubus fruticosus	Bramble blackberry
Viola arvensis	Field pansy
Calystegia sylvatica	Giant bindweed
Malva sylvestris	Common mallow
Matricaria recutita	Chamomile
Sonchus oleraceus	Common sowthistle
Silene latifolia	White campion
Cirsium vulgare	Common thistle
Hieracium agg.	Hawkweed
Sonchus arvensis	Field sowthistle
Silene vulgaris	Bladder campion
Anthriscus sylvestris	Cow parsley
Heracleum sphondylium	Cow parsnip
Stachys sylvatica	Hedge woundwort

Table A2. Continued

<i>Veronica persica</i>	Field speedwell
<i>Senecio jacobaea</i>	Common ragwort
<i>Carduus</i>	Thistle
<i>Fallopia convolvulus</i>	Wild buckwheat
<i>Fumaria officinalis</i>	Common fumitory
<i>Cirsium arvense</i>	Canada thistle
<i>Sherardia arvensis</i>	Blue field-madder
<i>Pimpinella saxifraga</i>	Burnet saxifrage
<i>Avena fatua</i>	Common wild oat
<i>Euphorbia helioscopia</i>	Sun spurge
<i>Polygonum aviculare</i>	Common knotgrass
<i>Convolvulus arvensis</i>	Field bindweed
<i>Solanum dulcamara</i>	Bitter nightshade
<i>Ligustrum vulgare</i>	Common privet
<i>Urtica dioica</i>	Stinging nettle
<i>Sisymbrium vulgare</i>	Oriental mustard
<i>Calystegia sepium</i>	Hedge bindweed
<i>Centaurea nigra</i>	Common knapweed
<i>Hedera helix</i>	Common ivy

Table A3. Plant Species Sampled in Hladik et al. (127)

Scientific name	Common name
<i>Schizachyrium scoparium</i>	Little bluestem
<i>Solidago</i> spp.	Goldenrods
<i>Zizia aurea</i>	Golden alexanders

Table A4. Plant Species Sampled in Main et al. (34)

Scientific name	Common name
<i>Solanum carolinense</i>	Carolina horsenettle
<i>Solidago</i>	Goldenrods
<i>Cichorium intybus</i>	Chicory
<i>Alium</i>	Garlic
<i>Teucrium canadense</i>	American germander
<i>Chamaecrista fasciculata</i>	Partridge pea
<i>Trifolium pratense</i>	Red clover
<i>Gypsophila</i>	Baby's breath
<i>Bidens frondosa</i>	Devil's beggarticks
<i>Tradescantia</i>	Spiderwort
<i>Cirsium</i>	Thistle
<i>Eupatorium</i>	Boneset

Table A4. Continued

<i>Asclepias tuberosa</i>	Butterfly milkweed
<i>Asclepias syriaca</i>	Common milkweed
<i>Erigeron strigosus</i>	Daisy fleabane
<i>Vicia villosa</i>	Hairy vetch
<i>Vernonia missurica</i>	Missouri ironweed
<i>Helianthus</i>	Sunflower
<i>Malus</i>	Apple tree
<i>Monarda</i>	Bergamot
<i>Verbascum</i>	Mullein
<i>Leucanthemum vulgare</i>	Oxeye Daisy
<i>Ipomoea pandurata</i>	Wild potato vine
<i>Campanula americana</i>	American bellflower
<i>Daucus carota</i>	Queen Anne's lace
<i>Rosa setigera</i>	Prairie Rose
<i>Rudbeckia</i>	Coneflower
<i>Asclepias incarnata</i>	Swamp Milkweed
<i>Vitis</i>	Grapevine
<i>Eupatorium altissimum</i>	Tall boneset
<i>Ipomoea</i>	Morning glory
<i>Melilotus albus</i>	White sweetclover
<i>Pastinaca sativa</i>	Parsnip
<i>Achillea</i>	Yarrow
<i>Barbarea vulgaris</i>	Bittercress

Table A5. Plant Species Sampled in Hall et al. (36)

Scientific name	Common name
<i>Taraxacum</i>	Dandelion
<i>Asclepias</i> spp.	Milkweed
<i>Zizia aurea</i>	Golden alexanders
<i>Trifolium repens</i>	White clover
<i>Trifolium pratense</i>	Red clover
<i>Monarda</i>	Beebalm
<i>Rudbeckia hirta</i>	Black-eyed susan
<i>Heliopsis helianthoides</i>	Oxeye sunflower
<i>Trifolium pratense</i>	Purple clover
<i>Cirsium</i>	Thistle
<i>Verbena</i>	Vervain
<i>Echinacea</i>	Coneflower
<i>Ratibida pinnata</i>	Grey-headed coneflower

Table A5. Continued

Echinacea purpurea	Purple-Headed coneflower
Melilotus	Sweet Clover
Leucanthemum vulgare	Oxeye Daisy
Daucus carota	Queens Anne's Lace

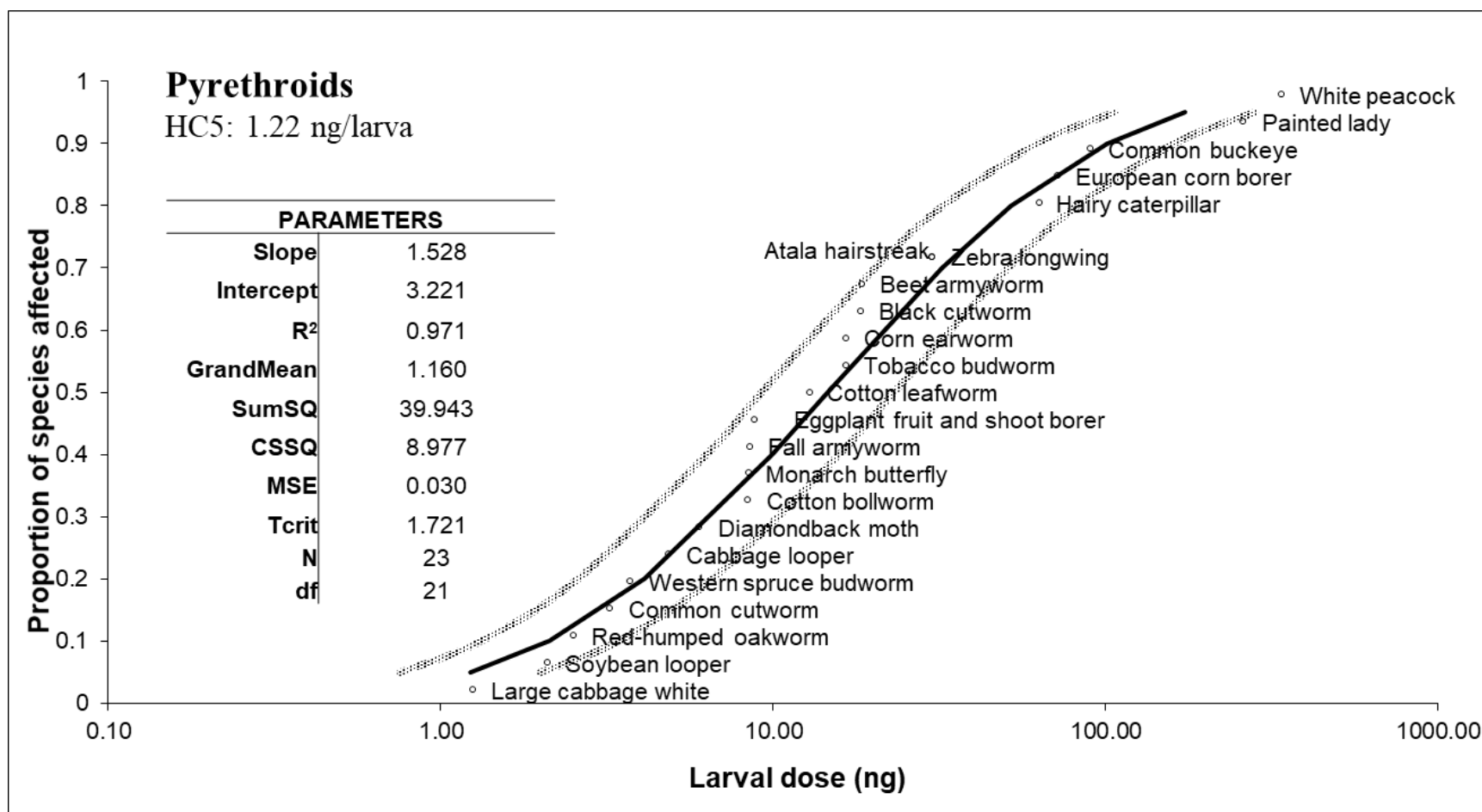


Figure A1. A species sensitivity distribution (with LD50 values in ng) for lepidopteran larvae topically exposed to pyrethroid insecticides.

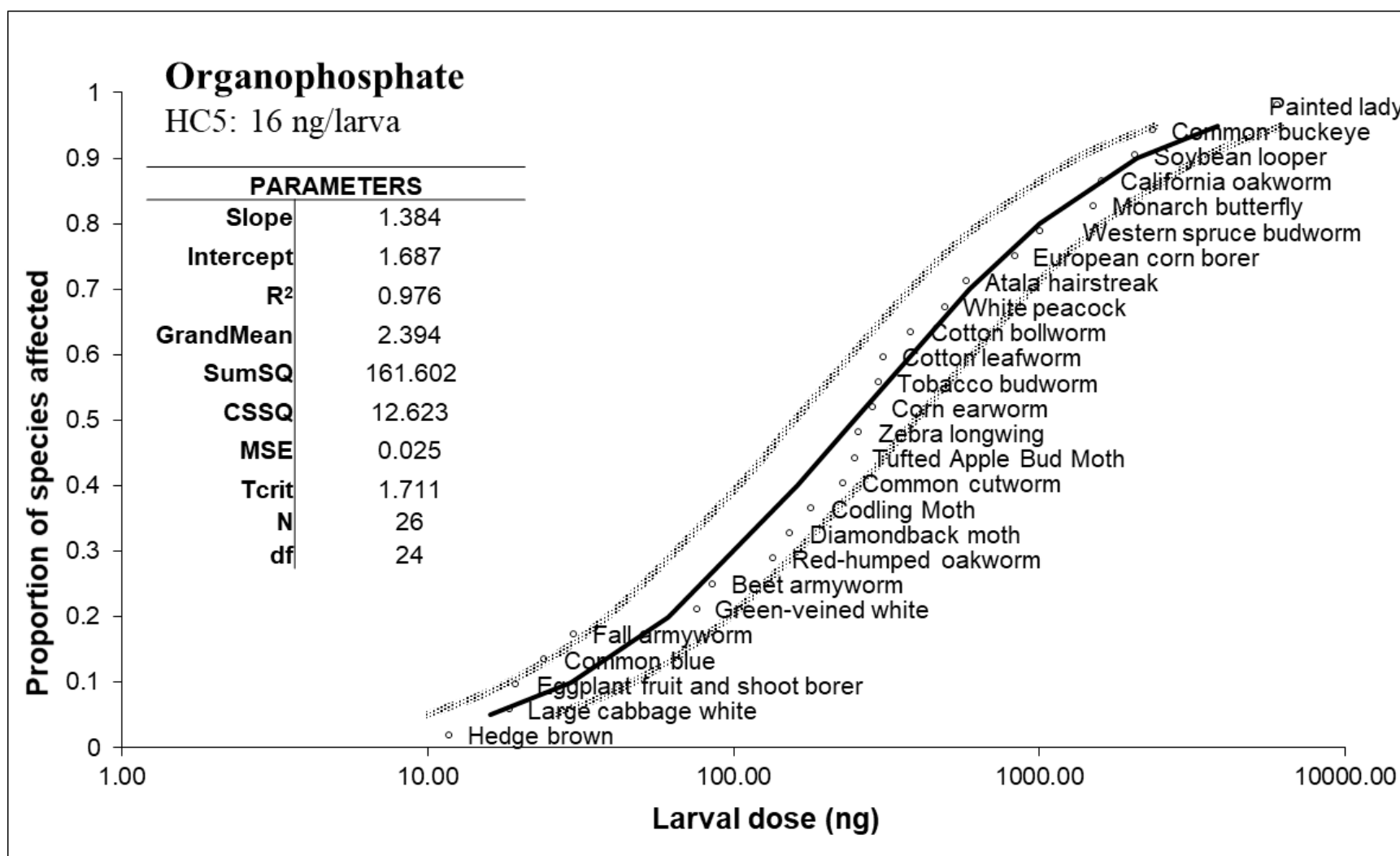


Figure A2. A species sensitivity distribution (with LD50 values in ng) for lepidopteran larvae topically exposed to organophosphate insecticides.

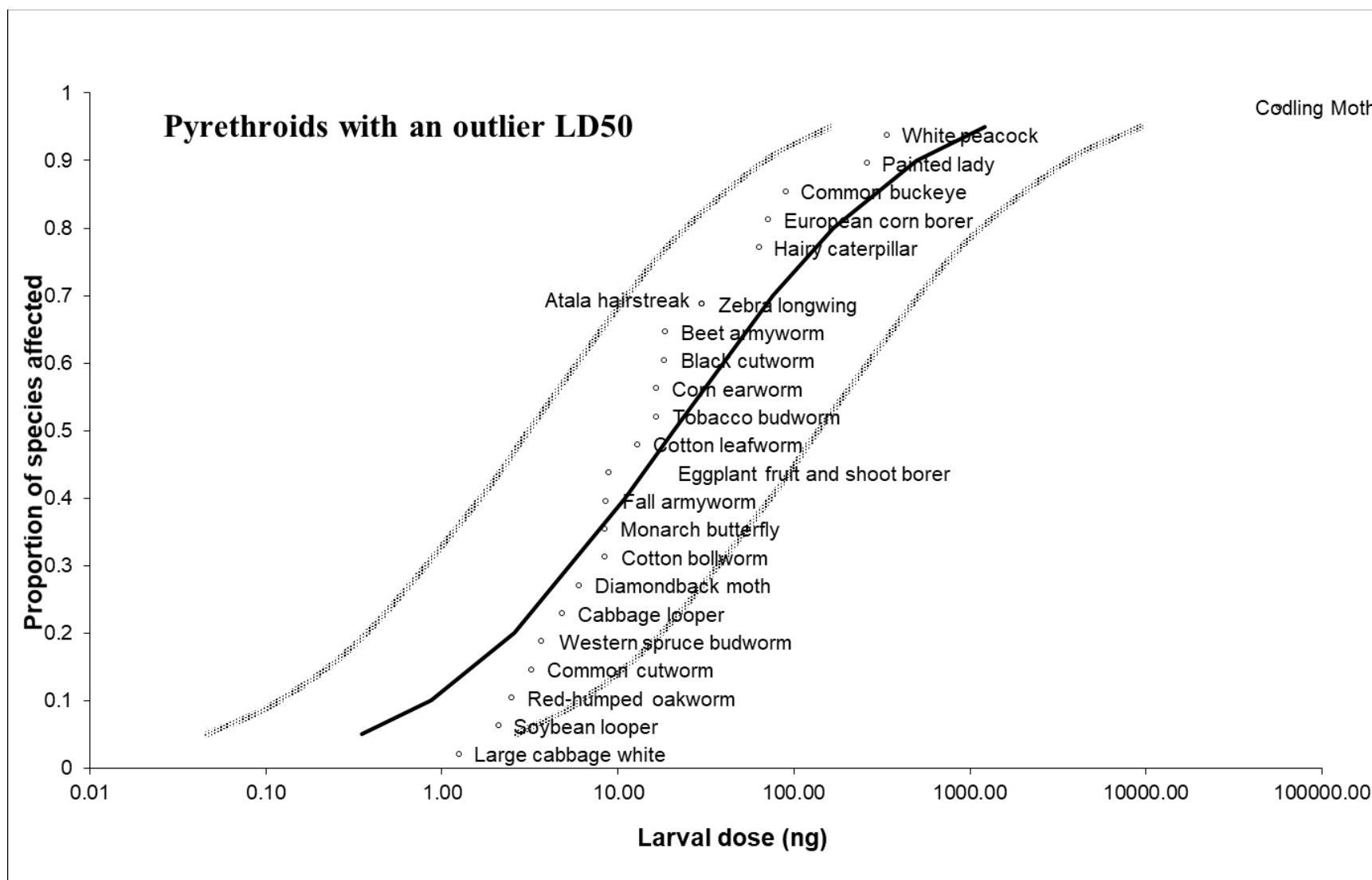


Figure A3. A species sensitivity distribution (with LD50 values in ng) that includes an extreme outlier for lepidopteran larvae topically exposed to pyrethroid insecticides.

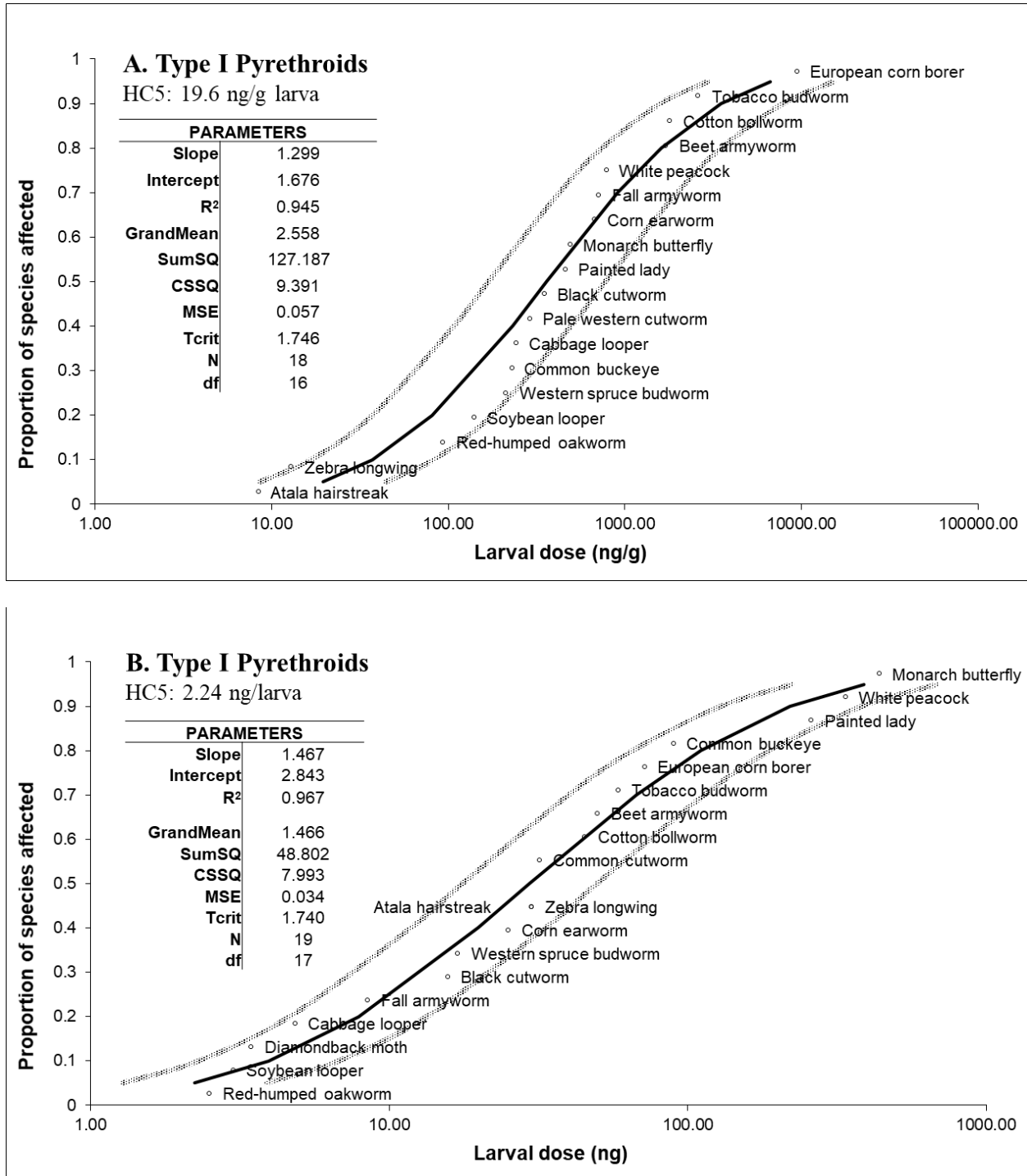


Figure A4. Species sensitivity distributions (A: ng/g larva; B: ng/ larva) for lepidopteran larvae topically exposed to type I pyrethroid insecticides.

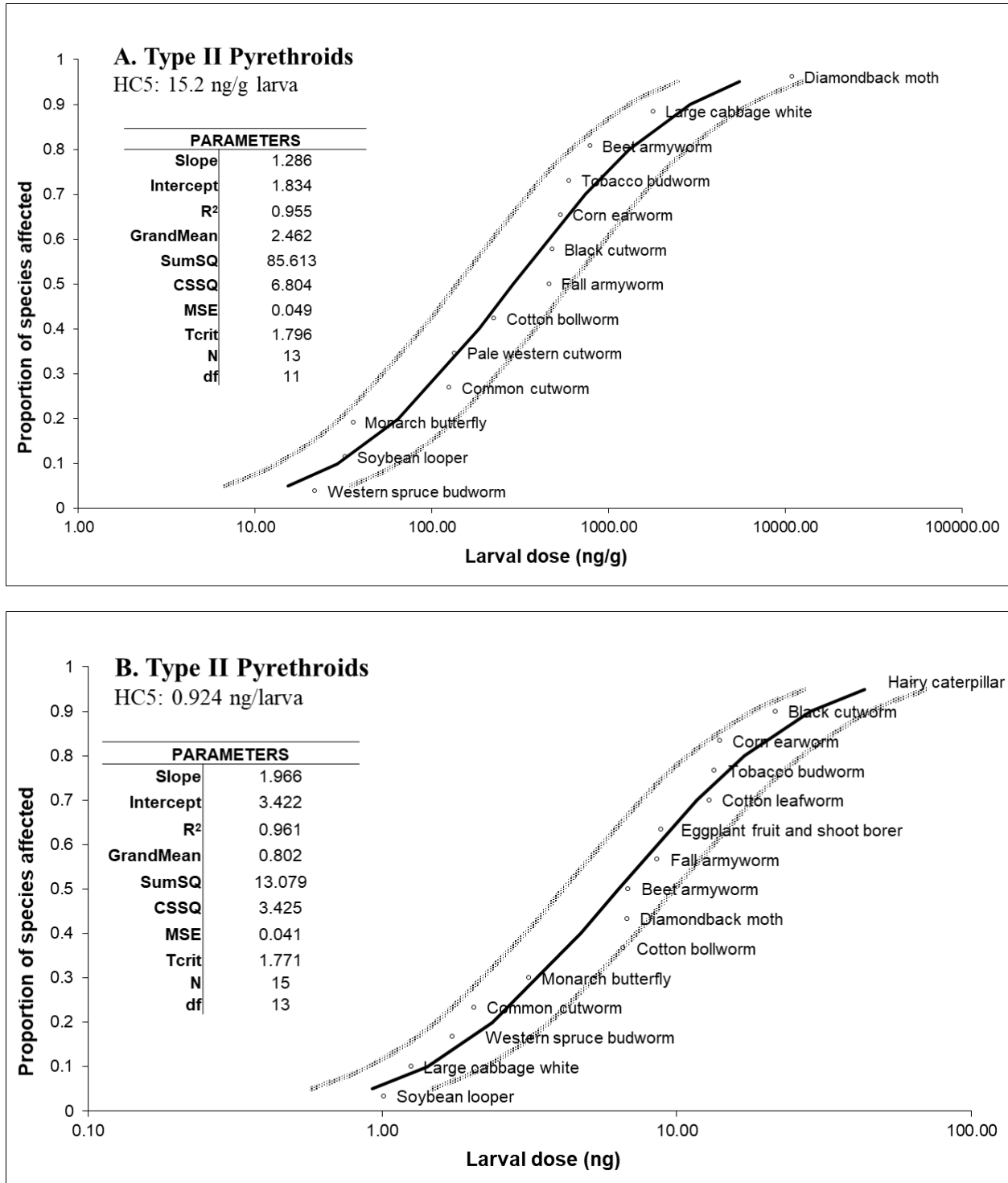


Figure A5. Species sensitivity distributions (A: ng/g larva; B: ng/ larva) for lepidopteran larvae topically exposed to type II pyrethroid insecticides.

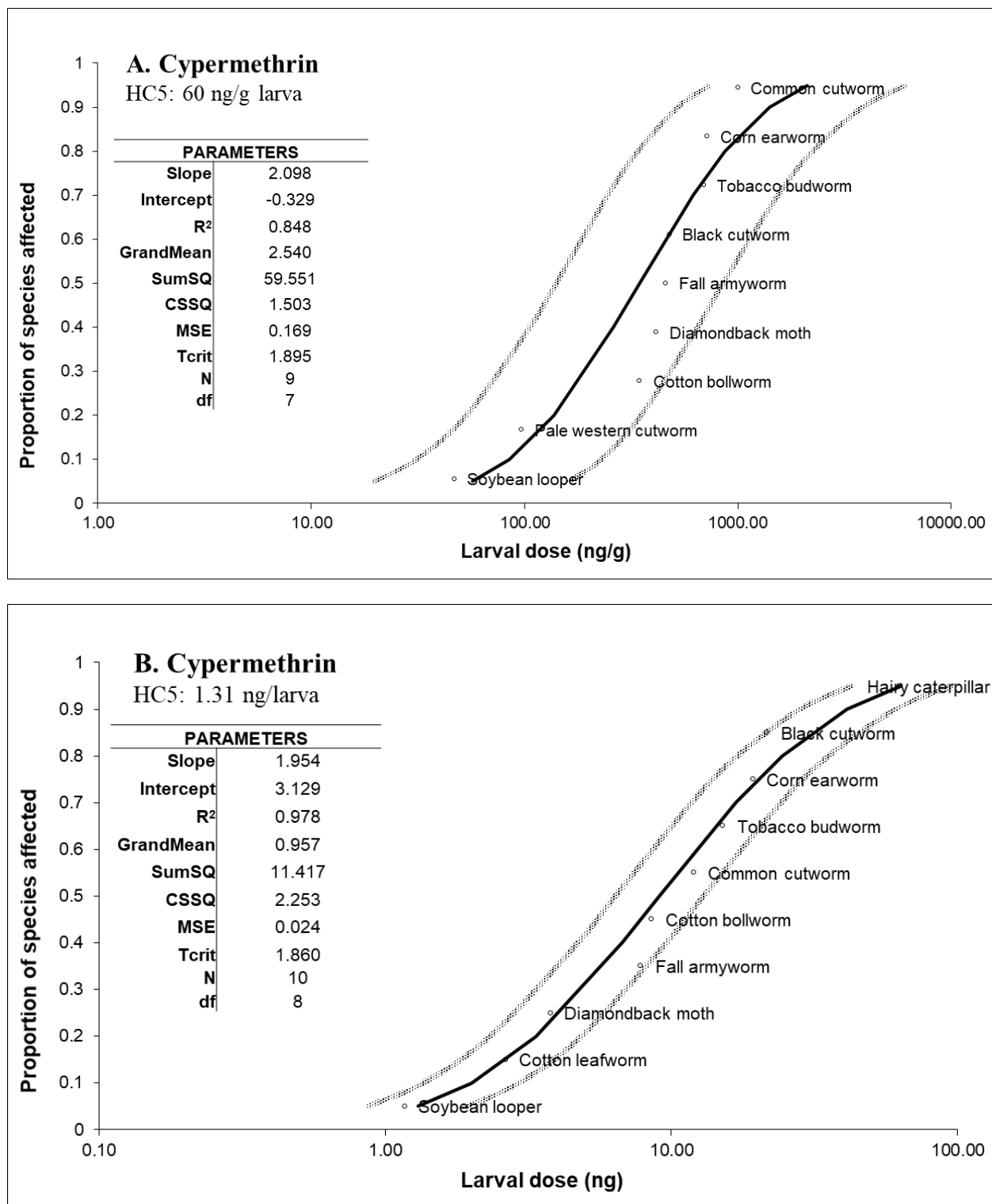


Figure A6. Species sensitivity distributions (A: ng/g larva; B: ng/ larva) for lepidopteran larvae topically exposed to cypermethrin (type II pyrethroid).

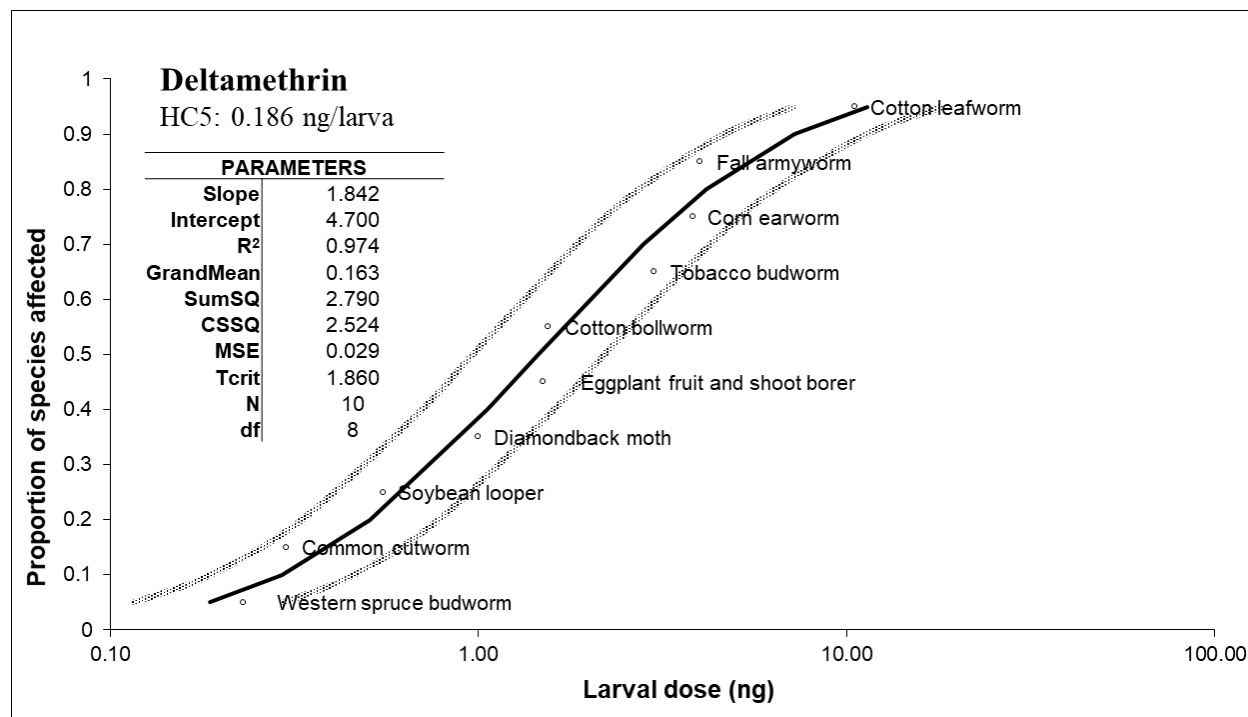


Figure A7. Species sensitivity distributions for lepidopteran larvae topically exposed to deltamethrin (type II pyrethroid). Data for a minimum of eight species were not available when LD50 values were analyzed as ng/g larva.

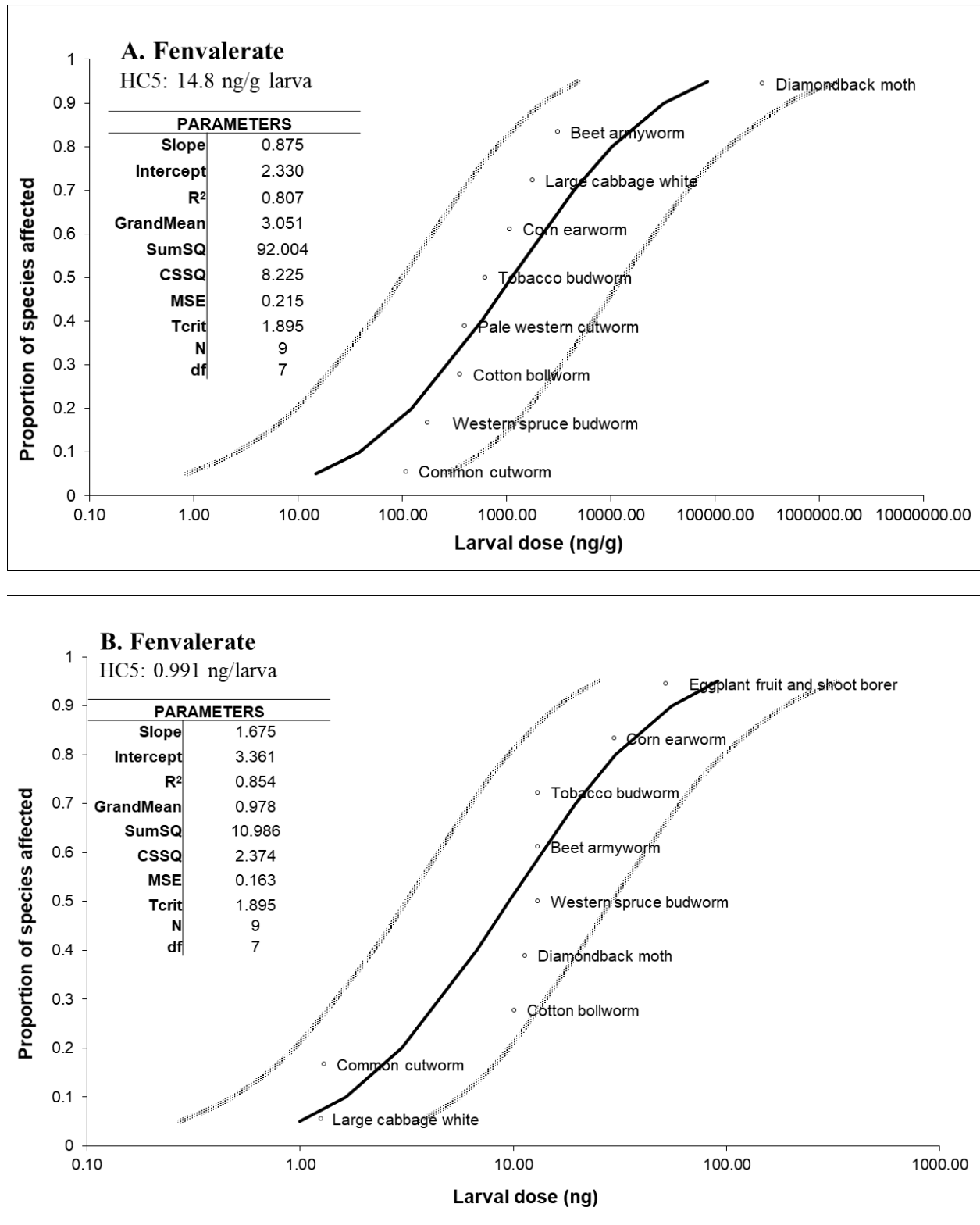


Figure A8. Species sensitivity distributions (A: ng/g larva; B: ng/ larva) for lepidopteran larvae topically exposed to fenvalerate (type II pyrethroid).

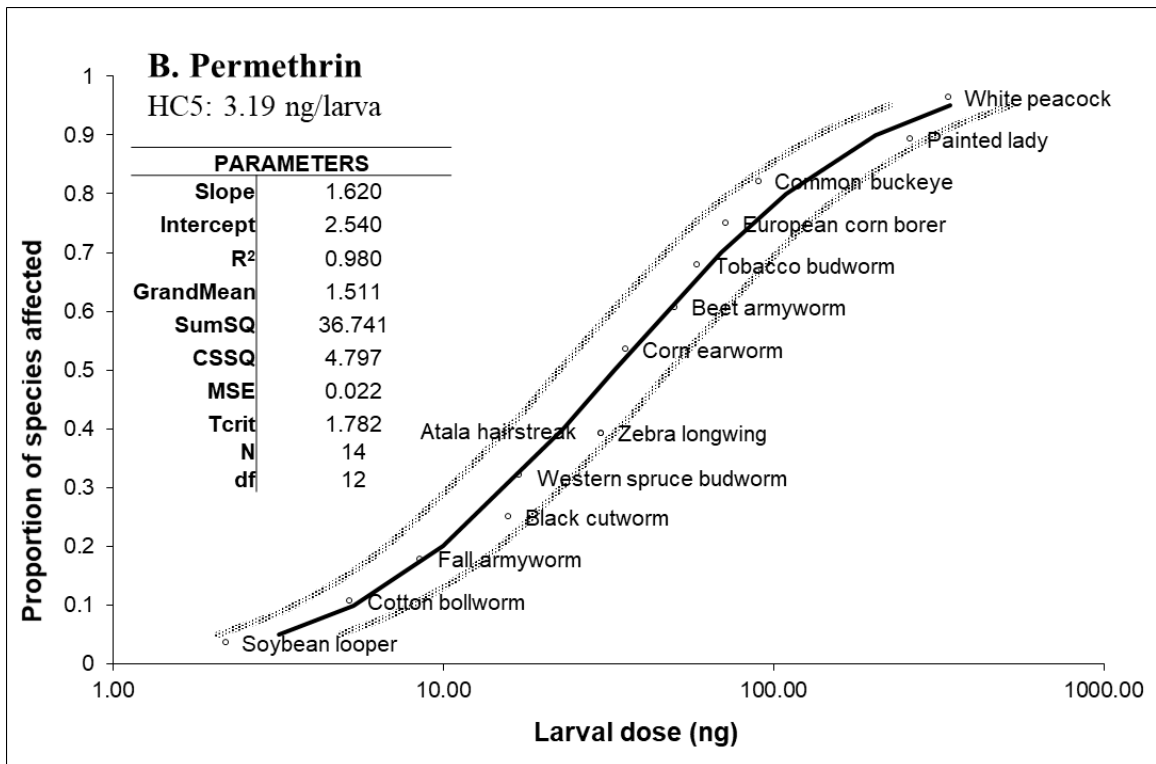
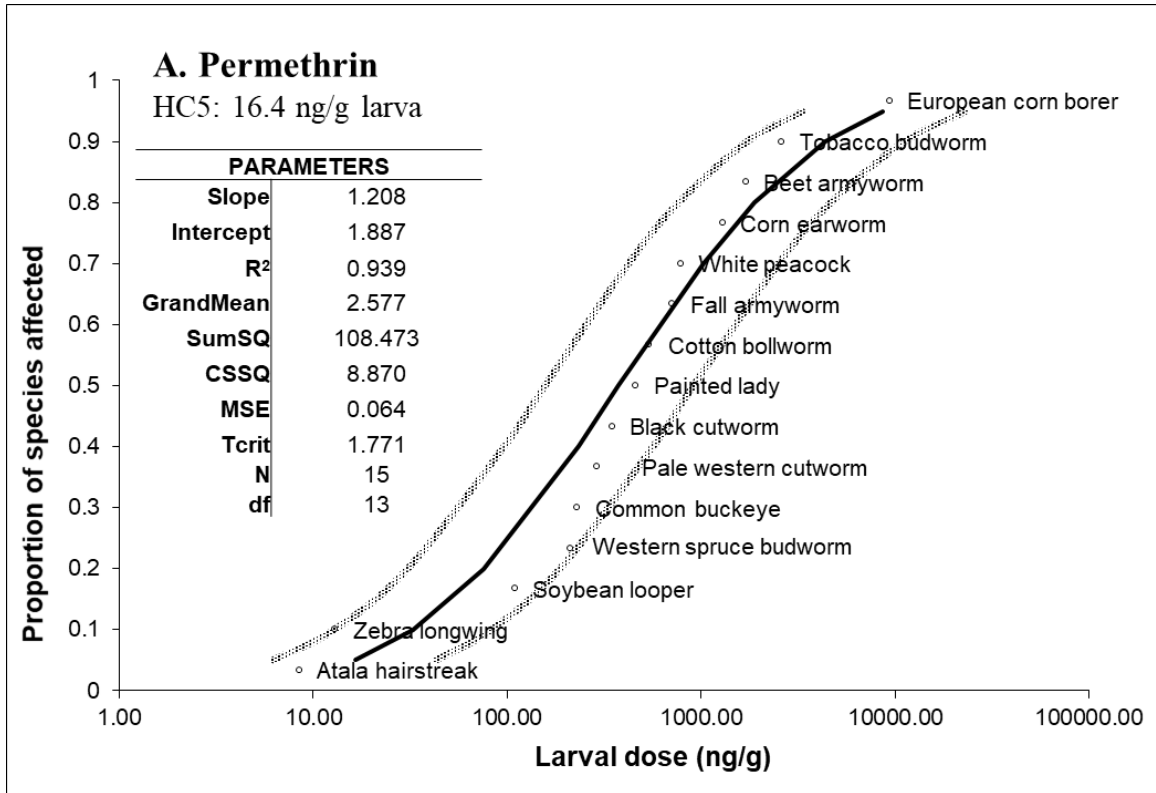


Figure A9. Species sensitivity distributions (A: ng/g larva; B: ng/larva) for lepidopteran larvae topically exposed to permethrin (type I pyrethroid).

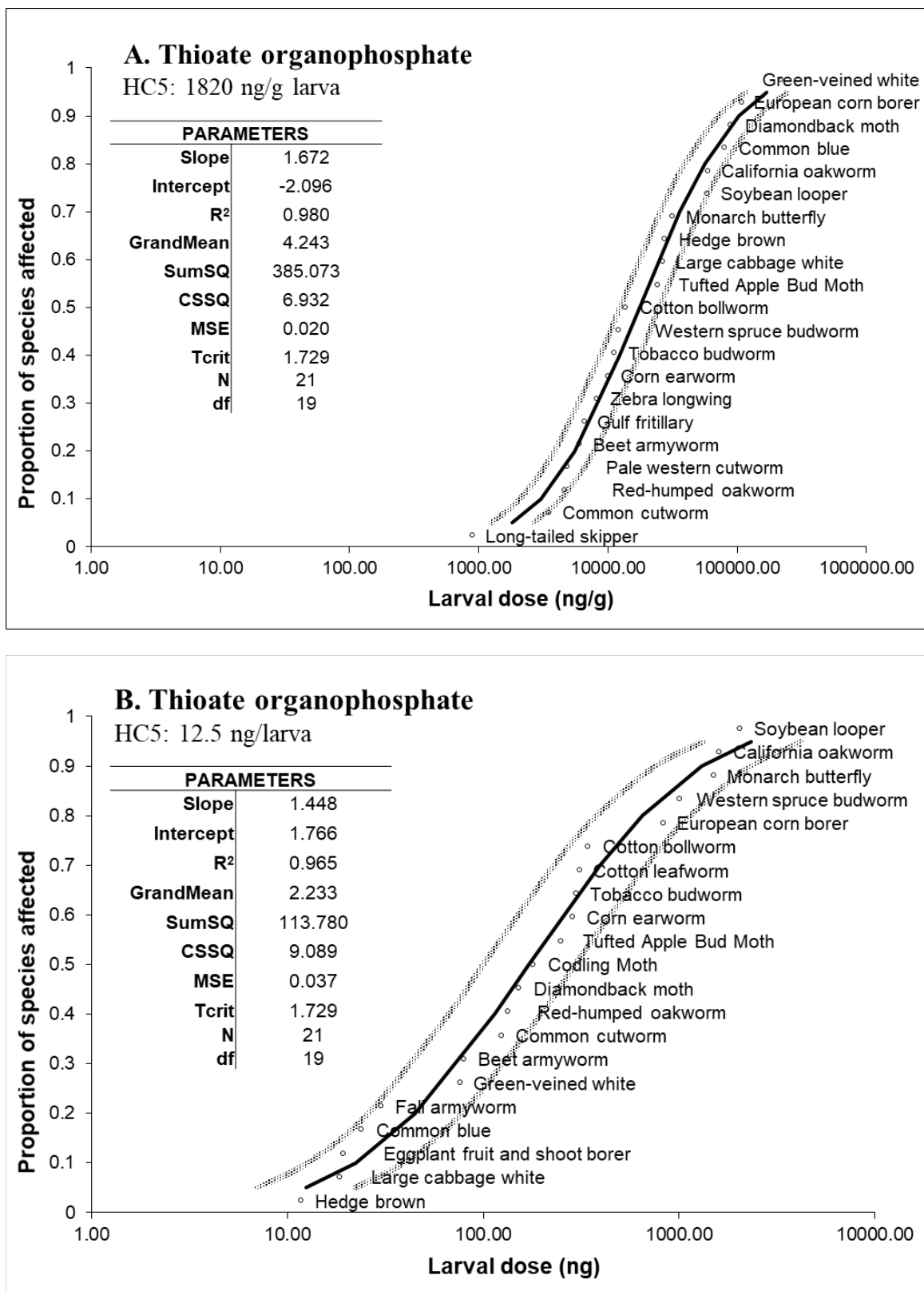


Figure A10. Species sensitivity distributions (A: ng/g larva; B: ng/ larva) for lepidopteran larvae topically exposed to type thioate subclass of organophosphate insecticides.

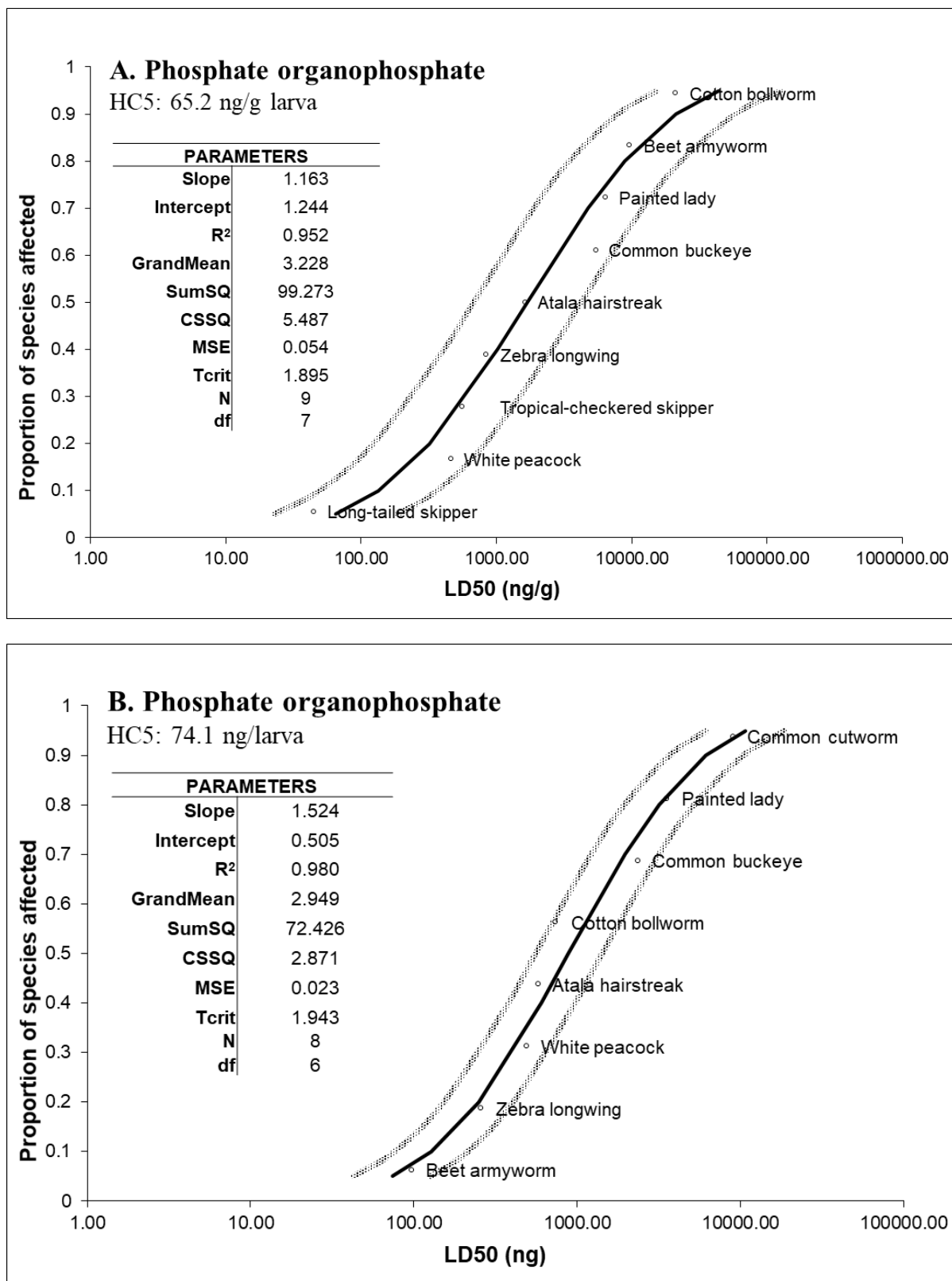


Figure A11. Species sensitivity distributions (A: ng/g larva; B: ng/ larva) for lepidopteran larvae topically exposed to type phosphate subclass of organophosphate insecticides.

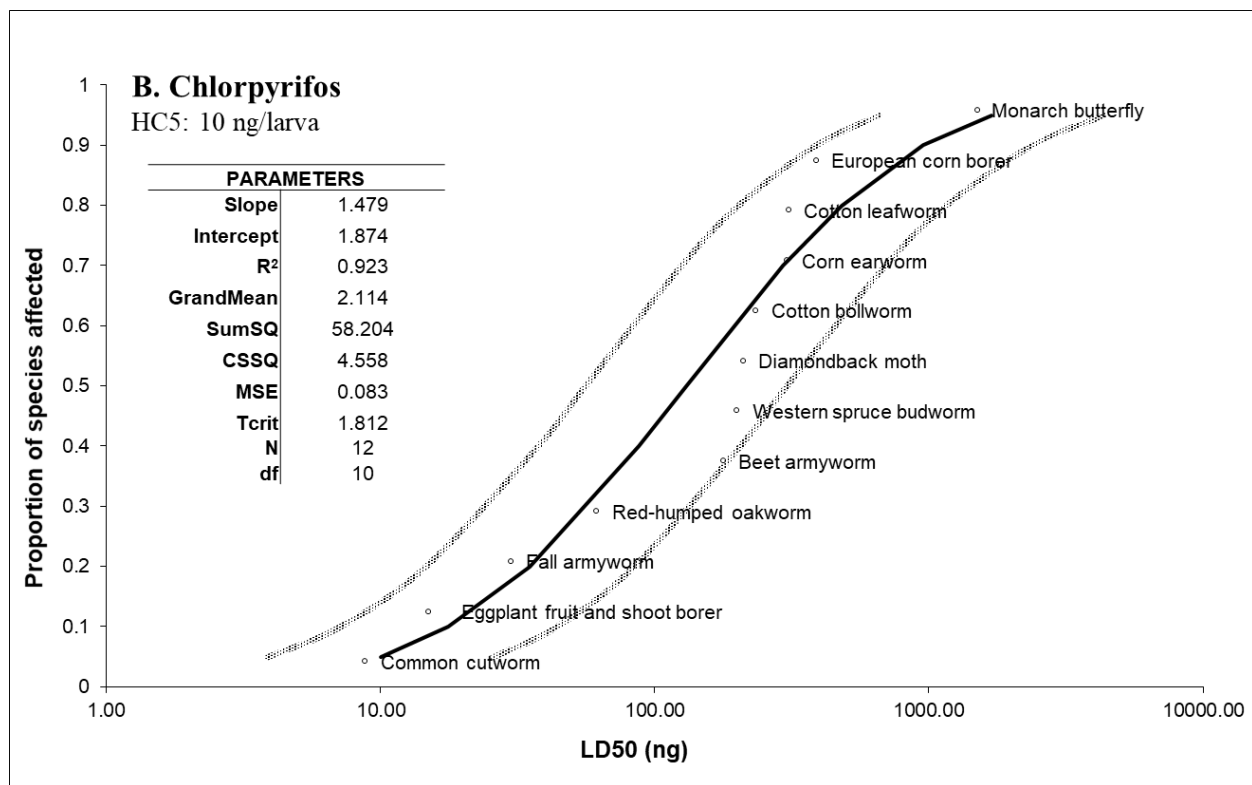
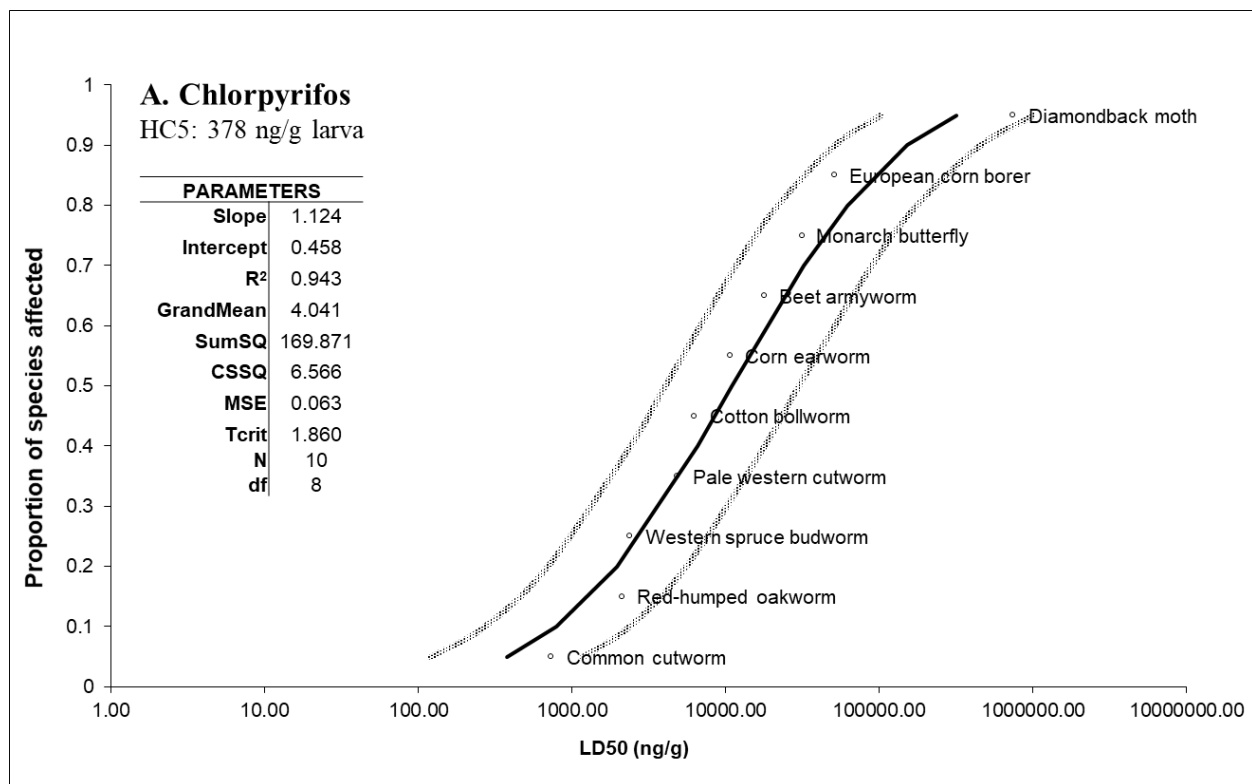


Figure A12. Species sensitivity distributions (A: ng/g larva; B: ng/ larva) for lepidopteran larvae topically exposed to chlorpyrifos (thioate subclass of organophosphates).

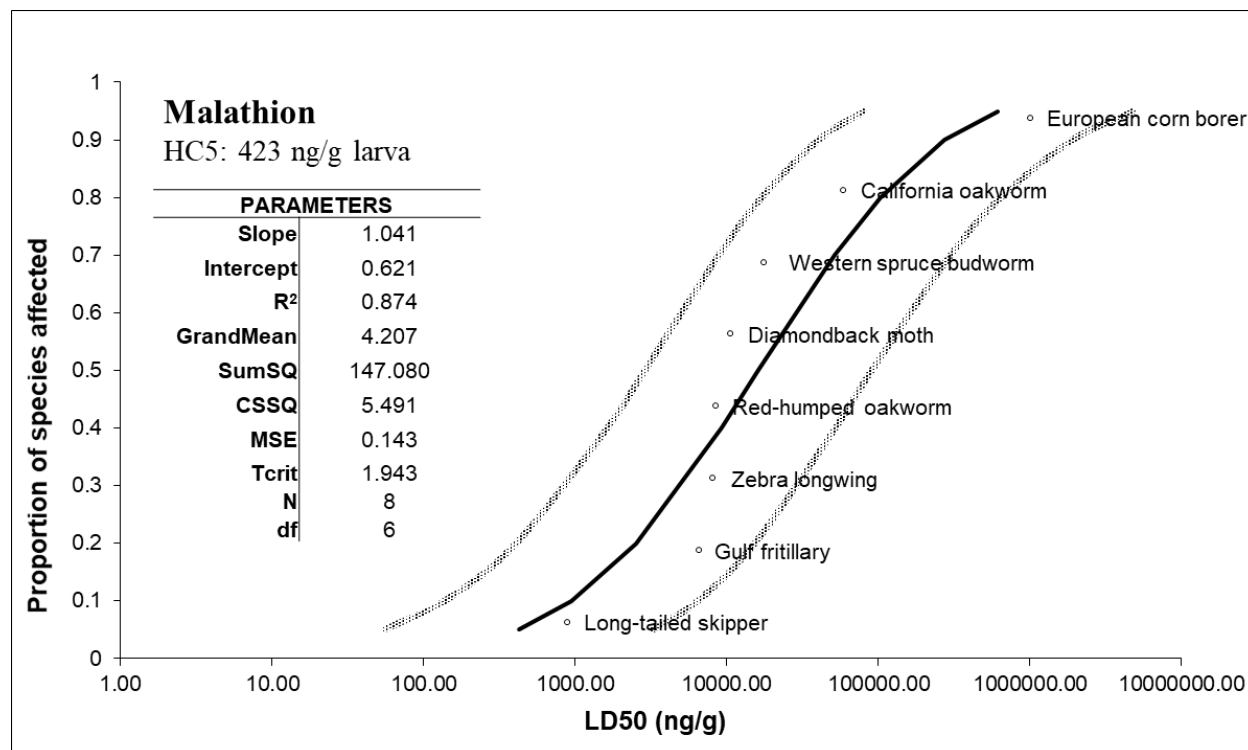


Figure A13. Species sensitivity distributions for lepidopteran larvae topically exposed to malathion (thioate subclass of organophosphates). Data for a minimum of eight species were not available when LD50 values were analyzed as ng/larva.

CHAPTER 7. EVALUATING TOXICITY OF VARROA MITE (*Varroa destructor*)-ACTIVE dsRNA TO MONARCH BUTTERFLY (*Danaus plexippus*) LARVAE

Niranjana Krishnan^{a, +}, Maura J. Hall^{a, +}, Richard L. Hellmich^b, Joel R. Coats^a, Steven P. Bradbury^{a, c}

^a Toxicology Program and Department of Entomology, Iowa State University, Ames, Iowa, USA

^b United States Department of Agriculture, Corn Insects and Crop Genetics Research Unit, Ames, Iowa, USA

^c Department of Natural Resource Ecology and Management, Iowa State University, Ames, Iowa, USA

⁺ Both authors contributed equally.

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Abstract

Varroa mites (*Varroa destructor*) are parasitic mites that, combined with other factors, are contributing to high levels of honey bee (*Apis mellifera*) colony losses. A Varroa-active dsRNA was recently developed to control Varroa mites within honey bee brood cells. This dsRNA has 372 base pairs that are homologous to a sequence region within the Varroa mite calmodulin gene (*cam*). The Varroa-active dsRNA also shares a 21-base pair match with monarch butterfly (*Danaus plexippus*) calmodulin mRNA, raising the possibility of non-target effects if there is environmental exposure. We chronically exposed the entire monarch larval stage to common (*Asclepias syriaca*) and tropical (*Asclepias curassavica*) milkweed leaves treated with concentrations of Varroa-active dsRNA that are one- and ten-fold higher than those used to treat honey bee hives. This corresponded to concentrations of 0.025-0.041 and 0.211-0.282 mg/g leaf, respectively. Potassium arsenate and a previously designed monarch-active dsRNA with a 100% base pair match to the monarch *v-ATPase A* mRNA (leaf concentration was 0.020-0.034 mg/g) were used as positive controls. The Varroa mite and monarch-active dsRNA's

did not cause significant differences in larval mortality, larval or pupal development, pupal weights, or adult eclosion rates when compared to negative controls. Irrespective of control or dsRNA treatment, larvae that consumed approximately 7500 to 10,500-mg milkweed leaf within 10 to 12 days had the highest pupal weights. The lack of mortality and sublethal effects following dietary exposure to dsRNA with 21-base pair and 100% base pair match to mRNAs that correspond to regulatory genes suggest monarch mRNA may be refractory to silencing by dsRNA or monarch dsRNase may degrade dsRNA to a concentration that is insufficient to silence mRNA signaling.

Introduction

Varroa mites are thought to be a significant stressor causing honey bee decline [1]. The mites attach to bees, transmit viruses, and consume the honey bees' fat bodies and, to a lesser extent, hemolymph [2]. The fat body is integral for immune function, pesticide detoxification, hormone regulation, and enhanced overwintering survival [3]. Impairment of fat body function in a sufficient percentage of honey bees can contribute to colony declines [2]. Several control methods are used to reduce Varroa mite populations. Currently, the most effective and economical method is to employ chemical miticides [4]. In the U.S., there are currently 15 miticides approved for controlling Varroa mites in beehives [5]. Due to the heavy reliance on these products, Varroa mites have developed resistance to several compounds [6-9], primarily due to enhanced metabolism and/or target site insensitivity [10]. Three of the miticides for which there are no reported Varroa mite resistance — formic acid, oxalic acid, and thymol — could harm bees by inducing toxicity [11-14], causing stress [15], and affecting brood development [16-18]. Hence, there is a need to develop new miticides that specifically target Varroa mites without negatively affecting honey bees.

The development of double-stranded (dsRNA) insecticides creates the means to selectively target insect pest species. DsRNA insecticides employ RNA interference (RNAi) technology. RNAi is a mechanism whereby specific messenger RNA (mRNA) transcripts are targeted by small interfering RNAs (siRNAs) and silenced via nuclease activity or translational repression [19, 20]. RNAi can be used to design insecticides that specifically target pest species by identifying regions on the pest mRNA that have little or no overlap with mRNA of non-target species [21]. For example, [22, 23] silenced critical genes in several pest insect species without causing adverse effects in a range of taxonomically dissimilar non-target species. It has been hypothesized that a dsRNA could be efficacious only if it shares a minimum sequence of 19-21 nucleotides with the target insect mRNA [22-24].

Bayer Crop Science has developed a dsRNA to control Varroa mites (*Varroa destructor*) within honey bee (*Aphis mellifera*) brood cells [25] (see Fig S1). This dsRNA has 372 base pairs that are homologous to a sequence region within the Varroa mite calmodulin gene (*cam*) [Fig S2]. This gene encodes calmodulin (CaM), which is an essential calcium-binding protein that regulates multiple protein targets. The prototype product is formulated as an 80% sucrose solution that is placed in the hive. Nurse bees consume the dsRNA sucrose solution and deliver it to the brood cells. The mites could be exposed to the dsRNA through contact with the sucrose solution deposited by adult honey bees, brood food made with the 80% sucrose solution, and/or through consumption of larval or adult hemolymph [26].

The Varroa dsRNA has a 99% nucleotide match to the Varroa mite calmodulin mRNA (Fig S2) and a 74% nucleotide match, which includes a contiguous sequence of 14 nucleotides, to the honey bee calmodulin mRNA. There are no contiguous 21-nucleotide overlaps between the Varroa-active dsRNA and the honey bee genome (Fig S3). Previous studies have shown that

honey bees are mostly insensitive to orally delivered dsRNA [27], including dsRNA molecules that have a 100% sequence match to their mRNA [28].

Exposure of the Varroa-active dsRNA product to non-target insects outside the hive, including monarch butterfly (*Danaus plexippus*) larvae, is highly unlikely and supports a low environmental risk determination. However, the Varroa-active dsRNA has a 21-base pair match to monarch calmodulin mRNA (Fig S4). Since dsRNA orthologs could be efficacious against insect mRNA if they share a sequence length of at least 19 to 21 nucleotides [22-24], the potential hazard to monarch larvae, if they are exposed to the Varroa-active dsRNA, cannot be precluded.

To assess risks of dsRNA insecticides to non-target arthropod species, the United States Environmental Protection Agency (USEPA) uses a four-tiered testing scheme based on the microbial pesticide data requirements published under 40 CFR 158.2150 and the associated OCSPP Harmonized Guidelines 885 and 850 series [29, 30]. Tier I studies are designed to estimate hazards to several non-target arthropod taxa under exposure concentrations several times higher than the highest concentrations ($\geq 10X$ when possible) expected to occur under realistic field exposure scenarios. A lack of adverse responses under these exposure conditions, presumably, provide sufficient certainty that there would not be unreasonable effects to the environment if the product were registered, i.e., complex, higher Tier testing with realistic exposure levels is not required.

Previous research by [31] explored the extent to which neonate monarch larvae are sensitive to monarch and western corn rootworm specific dsRNAs that target the *v-ATPase A* mRNA following a two-day dietary exposure (5 mg/mL of respective dsRNAs solutions applied to 0.5 cm diameter honeyvine milkweed [*Cynanchum laeve*] leaf discs). V-ATPase A is a proton

pump that maintains pH equilibrium at the cellular and organismal level and plays an important role in cellular function by interacting with a variety of proteins [32]. Given V-ATPase A's essential physiological function, it was expected monarch *v-ATPase A* mRNA would be silenced by the monarch-active dsRNA, and potentially, the western corn rootworm dsRNA as it shares a high sequence similarity with the monarch mRNA. In turn, silencing monarch *v-ATPase A* mRNA should result in reduced growth leading to a high level of larval mortality [33]. [31], however, reported no adverse effects for either dsRNA. The lack of adverse effects to the rootworm- and monarch-active dsRNA could be due to a short dietary exposure period that may have resulted in an insufficient internal dose and/or a peak internal dose that did not overlap with key development events (i.e., larval molts, pupal formation, and/or adult eclosion).

In the present paper, we expand our understanding of non-target effects of dsRNA insecticides by undertaking chronic dietary studies with the Varroa calmodulin dsRNA, which has a 21-nucleotide overlap with the monarch calmodulin mRNA, and monarch *v-ATPase A* dsRNA, which is assumed to have a 100% nucleotide match with the monarch *v-ATPase* mRNA [31]. We assessed chronic toxicity of Varroa-active dsRNA to monarch larvae by exposing them for approximately two weeks to concentrations 10-fold greater than would be expected if the formulated product were inadvertently applied to milkweed. Given the shared nucleotide sequence, we hypothesized that continuous dietary exposure of the Varroa and monarch-active dsRNA through the entire larval stage could adversely affect larval survival and growth; instar and pupal development; and/or eclosion of adult monarch butterflies.

Materials and Methods

Rearing Monarch Butterflies and Milkweed

Monarch butterfly eggs for four of the six bioassay runs were obtained from the 2016 colony maintained by the U.S. Department of Agriculture (USDA), Corn Insects and Crop Genetics Research Unit in Ames, Iowa (see [34]). The fifth and sixth bioassay runs were conducted using eggs obtained from a colony maintained by the University of Kansas (Dr. Orley Taylor, Director of Monarch Watch). The first three bioassays were undertaken on common milkweed (*Asclepias syriaca*), a native species found in U.S. Midwestern states, using the Iowa monarchs. To see if a different milkweed species and/or a source of monarchs influenced sensitivity to dsRNA, the last three bioassays (one with Iowa monarchs and two with Kansas monarchs) were conducted on tropical milkweed (*Asclepias curassavica*).

Young, non-senescent common milkweed leaves were collected from a restored prairie in Ames, Iowa, in September and October of 2018. Tropical milkweed leaves were reared in Iowa State University greenhouses as described by [34]. All milkweed leaves were washed with 10% bleach solution and rinsed three times with water before use. Leaves were dried using a salad colander and WypAll wiper tissues (Kimberly-Clark Professional) prior to use in the bioassays.

Chemicals Employed and Preparation of Treatment Solutions

A 64 mg/mL aqueous solution of Varroa-active dsRNA (lot number: STG4-0038) was provided by Bayer Crop Science. The prototype dsRNA formulation contains 2.1 mg/mL Varroa-active dsRNA in an 80% sucrose solution (J. Fischer, personal communication). In a preliminary assay, we provided fifth-instar monarchs common milkweed leaves coated with an 80% sucrose aqueous solution (a formulation blank). The larvae did not consume the treated leaves. Consequently, we prepared 2.1 mg/mL (1X environmental concentration) and 21 mg/mL

(10X concentration) Varroa-active dsRNA solutions for bioassays by diluting the 64 mg/mL stock solution in deionized water, rather than a sucrose solution.

Bayer also synthesized and provided a 25.4-mg/mL aqueous solution of monarch butterfly dsRNA (batch number: M1166) with a 100% base pair match to the monarch *v-ATPase A* mRNA. This monarch-active dsRNA was synthesized from forward and reverse primers designed by [31]. The monarch V-ATPase A dsRNA was selected as a putative positive dsRNA control. We prepared a 5-mg/mL monarch-active dsRNA solution in deionized water, which is the same concentration used by [31] in their monarch bioassays with neonates.

Potassium arsenate (CAS number: 7784-41-0; Lot number: SLBN3865V), purchased from Sigma Aldrich, also was used as a positive control. We used an aqueous concentration of 1 mg/mL in the bioassays, which corresponded to the LC₁₀₀ based on a preliminary assay in which larvae were fed treated tropical milkweed leaves.

Toxicity Bioassays

Toxicity bioassay studies were conducted at 24 to 27 °C and 45 to 65% relative humidity, with a 16:8 light: dark cycle. Both common and tropical milkweed bioassays employed six treatments: untreated leaves, deionized water-treated leaves, potassium arsenate-treated leaves, monarch-active dsRNA-treated leaves, and Varroa-active dsRNA-treated leaves at two nominal concentrations of 2.1 and 21 mg/mL. Fifteen and 10 larvae were used per treatment group in the common and tropical milkweed bioassays, respectively. Both milkweed bioassays were conducted three times, with each run employing a different larval generation. Thus, 45 and 30 larvae were employed per treatment group ($n = 6$) in the common and tropical milkweed bioassays, respectively. Water, monarch-active dsRNA, and the Varroa-active dsRNA solutions were applied using a 59-mL fingertip sprayer bottle (Equate brand). Both sides of the leaves

were sprayed with the solutions (multiple sprays were carried out for bigger leaves) and manually spread across the leaf surface using clean nitrile gloves (VWR International), as needed, to ensure complete coating. The leaves were then hung on a wire and clamped with paper clips until dry (10 to 20 minutes). The potassium arsenate solution was applied on one side of the leaf using a micropipette (20 to 30 μ L was spread over a 250 mg leaf). These leaves were placed on a tray with absorbent bench paper and allowed to dry.

Monarch larvae were reared according to methods described in [34]. Neonates were plated on a treated or untreated leaf (220 to 280 mg) in individual petri plates (60 mm x 15 mm containing a thin layer of 2% agar: water) using a paintbrush. Freshly treated (1 or 10X Varroa-active dsRNA, monarch-active dsRNA, or deionized water) or untreated leaves were provided every two days for the first six to eight days of a bioassay, and daily thereafter. Increasing leaf mass (up to 2700 to 3300 mg per day) was provided as the larvae developed. Every 24 hours, larval mortality, abnormal behavior, and leaf consumption (i.e., minimal consumption vs. consumption of most or entire leaf mass provided) were recorded. Instar was recorded every 96 hours. Days to pupation, pupal weights, and adult eclosion (i.e., adult emergence) were recorded for the surviving larvae. Results were analyzed from individual bioassays where both the negative controls (larvae fed untreated and water-treated leaves) produced less than 35% mortality from neonate to pupation. This upper bound control mortality was based on a maximum control mortality of 30% in 96-hour monarch larval dietary bioassays (see [34]).

Three times during each bioassay, three additional leaf samples (mass range: 221 to 2192 mg) were randomly treated with water or one of the three dsRNA solutions. These leaves were allowed to dry, then were wrapped in aluminum foil and stored in Ziploc[®] bags at -20 °C for QuantiGene analysis.

Sample Extraction and Processing

Prior to RNA extraction from treated leaves, the laboratory bench was wiped with RNaseZap to ensure an RNAase-free environment. Each frozen leaf sample was weighed and placed in a mortar with a small amount of liquid nitrogen. Each sample was ground, and the resultant powder was transferred to a pre-chilled phase lock gel tube (Qiagen, Catalog# 129065 & 129073). One mL of TRIzol (Ambion Life Technologies) was added per 0.1 g of leaf tissue. Samples were vortexed for three minutes and then incubated at room temperature (RT) for one hour. Chloroform (Fisher Scientific) was then added to the samples (0.3 mL for every mL of TRIzol). Samples were vortexed again for one minute and incubated at RT for 10 minutes. Samples were then centrifuged at 9000 Relative Centrifugal Force (RCF) at 2 to 6°C. The upper aqueous phase was transferred to a 15-mL falcon tube. The RNA was precipitated by adding 0.5 mL of isopropyl alcohol (Fisher Scientific) per ml of supernatant. The solutions were then mixed by inverting the tubes multiple times. Samples were stored in either a -20°C or -80°C freezer for 0.5 to 24 hours, and then centrifuged at 9000 RCF for 15 to 20 minutes at 2 to 6°C. The supernatant was discarded, and the RNA pellet was washed with ~5 ml of 70% ethanol prepared in nuclease-free Ultrapure Distilled Water (Invitrogen Lot#2063810). The pellets were then centrifuged at ~9000 RCF for 10 minutes at 2 to 6°C, and the supernatant was discarded. Another centrifugation at ~9000 RCF for one minute at 2 to 6°C was conducted, and the residual liquid was removed with a pipette. The RNA pellets were briefly air dried (≤ 10 minutes) and dissolved in an appropriate volume of nuclease-free Ultrapure Distilled Water (100 to 250 μ L per gram of starting tissue). The RNA was stored in a -20°C or -80°C freezer until quantification. Prior to QuantiGene analysis, each milkweed leaf extract was normalized with sample diluent to fall within the standard curve.

QuantiGene Analysis

Total extracted RNA was quantified using a QuantiGene® (QG) 2.0 Singleplex assay kit (Invitrogen Ref#13216). To begin, 1.2 mL of a custom QuantiGene probe set was combined with 90 µL of the appropriate sample (water background control, reference standards, or the test samples) in a disposable PCR plate. The custom probes were designed by the manufacturer to hybridize to the specific dsRNA sequences used in this study. Separate probes were used for Varroa-active dsRNA and monarch butterfly dsRNA samples. After the addition of all standards and samples, the denaturing plate was sealed with plate foil (ThermoFisher Ref#AB0626) and heated at 98°C (±5°C) for 5 minutes and subsequently held at 55°C (±5°C) for 30 minutes.

A premixed QG 2.0 working solution was prepared by adding nuclease-free water, lysis mixture, and blocking reagent. Eighty µL of QG 2.0 working solution was added to each well of the assay plate. For each well containing 80 µL of denatured standard/sample in the denaturing plate, 20 µL was plated into the wells of the assay plate in triplicate. This resulted in 80 µL QG 2.0 working solution and 20 µL denatured standard/sample per assay plate well. The plate was sealed with foil and incubated at 55°C (±5°C) for 16 to 24 hours.

After overnight hybridization, the wells of each plate were washed three times with 300 µL of QG 2.0 Wash Buffer. The plates were then inverted and tapped to dry. One hundred µL of preamplifier solution was added to each well; plates were then sealed with a plate foil and incubated at 55°C (±5°C) for 55 to 65 minutes. The previous step was repeated for the amplifier solution and the label probe solution. QuantiGene solutions were prepared following the manufacturer's recommendations and are outlined in Table A1. Following incubation with the label probe solution, the plates were washed three times with 300 µL/well of QG 2.0 Wash Buffer and allowed to dry for no more than five minutes.

After the last washes, 100 μ L of QG 2.0 Substrate was added to each well and the plate was sealed with foil and incubated for 5 to 15 minutes at room temperature. The median luminescence of each well was captured by a Synergy-HTX Multi-mode Microplate Reader (Biotek). The concentrations of Varroa-active dsRNA and monarch-active dsRNA were calculated from a standard curve fit with a 4-parameter logistic regression model (Fig S5). Each sample was run in triplicate, and the mean concentrations were calculated.

Statistical Methods

All statistical analyses were done in RStudio 1.1.383 (R version 3.5.2). Common and tropical milkweed bioassay results were analyzed independently. In both milkweed species, potassium arsenate treatments (positive control) caused 100% larval mortality within five days (Fig 1) and were excluded from analyses. Generalized linear models (glm) accounted for both run (three bioassay runs each for common and tropical milkweed) and treatment effects. There was no run-by-treatment interaction ($p > 0.05$); consequently, the following equation was used: $\text{response} \sim \text{run} + \text{treatment}$.

To analyze larval mortality (larvae alive/larvae dead) and adult eclosion (adults emerged/adults not emerged), we fit a binomial or a quasibinomial (to account for overdispersion) glm model and used type 3 ANOVA (obtained from the “car” package) to look for differences between treatments. A quasipoisson (to account for underdispersion) glm model and type 3 ANOVA were used to evaluate days from neonate to pupation. Following the removal of a single outlier in the common milkweed water treatment (this pupa’s weight was one-third the weight of an average pupa in the same treatment group), the residual plots for the pupal weights showed the data were normally distributed and had homogenous variances. Consequently, a gaussian glm model and type 3 ANOVA were used to evaluate differences in

pupal weights between treatments. If significant treatment or run effects were identified ($p < 0.05$), Dunnett's test for multiple comparisons (emmeans package) was used to compare the control response to the insecticide treatment responses.

Results

Sample Extraction and QuantiGene Analysis

In the common milkweed bioassays, a subset of two leaves from each treatment group (5 mg/mL monarch-active dsRNA and 2.1 and 21 mg/mL Varroa-active dsRNA) and bioassay run were analyzed. Measured concentrations for 2.1 (1X) and 21 (10X) mg/mL Varroa-active dsRNA ranged from 0.013 to 0.032 and 0.144 to 0.389 mg/g, respectively. The measured concentration of monarch-active dsRNA ranged from 0.020 to 0.021 mg/g (Table 1).

In the tropical milkweed bioassays, a subset of two to three leaves for each treatment group and run were analyzed. Measured concentrations for 2.1 and 21 mg/mL Varroa-active dsRNA ranged from 0.020 to 0.065 and 0.143 and 0.316 mg/g, respectively. The measured concentration of monarch-active dsRNA ranged from 0.030 to 0.037 mg/g (Table 1). The 21 mg/mL treatment was 2- to 16-fold higher and 5- to 30-fold higher than the 2.1 mg/mL treatment in the tropical and common milkweed bioassays, respectively.

Toxicity Bioassays

In the tropical milkweed bioassays, larvae provided untreated, water-treated, 5 mg/mL monarch-active dsRNA-treated, and 2.1 and 21 mg/mL Varroa-active dsRNA-treated tropical milkweed leaves had 20 (± 10), 23 (± 6), 33 (± 21), 17 (± 21), and 13 (± 6) mean (\pm SD) percent mortality, respectively; no noticeable difference in toxicity was seen between Iowa and Kansas colony larvae. In the common milkweed bioassays, the same treatments caused 18 (± 10), 27 (± 10), 33 (± 7), 40 (± 20), and 39 (± 12) mean percent mortality, respectively from neonate to

pupation (Table 2). When Abbott's formula was used to account for mortality in the untreated control group, the average larval percent mortality rates in the water, monarch-active, 1X Varroa-active, and 10X Varroa-active treatment groups ranged from 4-11%, 16-18%, 0-27%, and 0-26%, respectively, when considering both common and tropical milkweed bioassays. While mortality occurred over multiple days for all treatments (excluding potassium arsenate, which killed all treated larvae within five days), there were some temporal trends in mortality. In the common milkweed bioassays, a greater proportion of larval mortality in the negative controls and dsRNA groups occurred in the first eight days; the opposite was true in the tropical milkweed bioassays (Fig 1).

In general, across all assays, the rates of mortality in dsRNA groups were similar to those observed in the two negative control groups. In both the tropical and common milkweed bioassays, there were no significant differences in larval mortality between treatment groups ($\chi^2 = 4.18$; $df = 4$; $p = 0.382$ and $\chi^2 = 6.89$; $df = 4$; $p = 0.142$, respectively). Combined mortality data from both milkweed species also was not different ($\chi^2 = 4.97$; $df = 4$; $p = 0.290$).

With both milkweed species, the monarch and Varroa-active dsRNA treatments did not delay larval development from first through fifth instar and fifth instar to pupae (Table 3). The mean (\pm SD) developmental time from neonate to pupae ranged from 11.2 (\pm 0.95) to 11.6 (\pm 1.1) days with common milkweed, with no differences between treatment groups ($\chi^2 = 1.44$; $df = 4$; $p = 0.838$). For tropical milkweed, developmental times ranged from 11.2 (\pm 0.67) to 11.5 (\pm 1.2) days ($\chi^2 = 4.96$; $df = 4$; $p = 0.292$). Larvae took 10 to 15 days to pupate, with a median of 11 days in all instances. Mean (\pm SD) monarch pupal weights between treatments in the common and tropical milkweed bioassays ranged from 1140 (\pm 168) to 1218 (\pm 145) mg and 936 (\pm 162) to 1006 (\pm 208) mg, respectively (Fig 2). There were no differences in pupal weights between

groups for both milkweed species ($F = 1.36$; $df = 4$; $p = 0.250$ and $F = 0.521$; $df = 4$; $p = 0.721$ for common and tropical milkweed, respectively). The inclusion of a single outlier in the common milkweed water treatment did not change the results ($F = 1.75$; $df = 4$; $p = 0.142$).

Larvae that pupated within 10-11 days in the common milkweed bioassays and within 11-12 days in the tropical milkweed bioassays generally consumed between 7500 to 10,500 mg fresh leaves after reaching the third instar. These larvae generally had higher pupal weights (Fig 3). In one of the tropical milkweed bioassays, fewer than 7 g of milkweed leaf tissue were provided to larvae that had pupated on the tenth day — these pupae were smaller (Fig 3b). Larvae that did not pupate within 12 and 13 days in the common and tropical milkweed bioassays, respectively, did not consume most of the provided leaves. Thus, even though these larvae were provided a greater mass of leaves (freshly treated leaves were provided daily starting on or about Day 9), their pupal weights were often similar or lower than the pupal weights of larvae that pupated earlier.

There was, however, a significant difference in pupal development time and pupal weights between bioassay runs ($p = 5.4 \times 10^{-10}$ and 1.3×10^{-3} , respectively, for common milkweed and $p = 7.2 \times 10^{-4}$ and 6.3×10^{-4} , respectively, for tropical milkweed). In the common milkweed bioassays, the third bioassay run differed from the first two. The milkweed leaves in the third run had started to senesce, and the larvae took longer to feed on the poorer quality leaves and pupate (12.2 days vs. 11.3 days for each of the first two runs). The quality of the leaves also could have resulted in the significantly lower pupal weights (1111 mg vs. 1215 and 1213 mg in the first two runs), even though individual larvae in each run were provided a minimum of 7500 mg of leaf and the average leaf mass provided across runs was similar (range was 10,100 to 11,000 mg). In the tropical milkweed bioassays, individual larvae in the first run

were provided fewer leaves on average (~7000 mg milkweed vs. ~9000 mg milkweed in the other two runs). The lack of sufficient leaf mass might have triggered pupation at a slightly earlier time (average was 11 days vs. 11.8 and 11.5 days for the last two runs) and also resulted in lower average pupal weights (897 mg vs. 942 and 1068 mg in the second and third bioassay run, respectively). Though larvae in the second and third bioassay runs were provided similar leaf mass, pupae from the second run were also significantly smaller ($p = 0.015$). These analyses show that, under the environmental conditions tested, monarch larvae need at least 7500 mg of fresh milkweed leaf in the first 10-11 days to reach a healthy pupal weight.

In the first two common milkweed bioassays and the first tropical milkweed bioassay, there were low levels of bacterial infection in the pupae that suppressed adult eclosion rates (the overall infection rate in any of the treatment groups did not exceed 15%). These pupae were excluded from eclosion analyses but were included in the other analyses as the infection had no effect on the other measured endpoints. The mean (\pm SE) eclosion rate of uninfected pupae ranged from 0.85 (\pm 0.07) to 0.97 (\pm 0.03) and 0.95 (\pm 0.05) to 1.0 (\pm 0.0) in common and tropical milkweed bioassays, respectively (Fig 4). Again, there were no treatment differences in either milkweed species ($\chi^2 = 7.07$; $df = 4$; $p = 0.132$ and $\chi^2 = 3.57$; $df = 4$; $p = 0.467$ for common and tropical milkweed, respectively).

Discussion

It has been hypothesized that a dsRNA that shares a minimum sequence of 19-21 nucleotides with an insect mRNA could cause mortality or adverse sublethal effects [22-24]. Hence, we expected chronic larval exposure to Varroa-active dsRNA and monarch-active dsRNA would cause high rates of mortality and sublethal effects; however, we observed no significant adverse effects. These results suggest bioinformatic analyses (e.g., 21 base pair

matches) alone cannot predict potential dsRNA sensitivity to target species (and insensitivity to non-target species). Other factors including refractory genes, presence of high levels of dsRNase, and exposure to low environmental concentrations may prevent RNAi-mediated effects [23, 35, 36].

In the present study, monarch larvae were chronically exposed to nominal environmental concentrations of a Varroa-active dsRNA one to ten times greater than what would be applied in honey bee hives to control Varroa mites. Quantification of dsRNA concentrations on treated common and tropical milkweed leaves indicated mean leaf concentrations of 0.025 to 0.041 (1X treatment) and 0.211 to 0.282 mg/g leaf (10X treatment). In the common milkweed bioassays, overall larval mortality was higher in the Varroa-active dsRNA treatments (ca. 40%) compared to untreated (ca. 20%) and water-treated controls (ca. 30%), but the differences in toxicity were not statistically significant. The higher mortality in water and Varroa-active dsRNA treatments could have been caused by water retention in common milkweed. Common milkweed leaves are thick and even if their surfaces are air-dried following treatment, water within the leaves may not completely evaporate. Increased internal water content could reduce the nutritional value of the leaves and lead to slightly increased, but statistically insignificant, larval mortality. In the tropical milkweed bioassays, higher larval mortality was seen in the negative controls (ca. 20% for untreated and water-treated leaves) than in the 2.1 and 21 mg/mL Varroa-active dsRNA solutions (ca. 15%), however, this too was statistically insignificant. These mortality rates are also consistent with the historical control mortality rate of the Iowa State University monarch butterfly colony, which is 20 to 25% from neonate to pupa.

There were also no significant differences when mortality was averaged across both milkweed species. While monarch-active dsRNA-treated leaves had the highest combined

mortality (33% vs 30% for Varroa treatments and 22% for control treatments), its effect on mortality was also not significant. The average larval mortality, when combined across milkweed species and control and dsRNA treatments was 27%. Given the historical mortality rate and comparisons of mortality rates between control and dsRNA-treated leaves, the Varroa-active dsRNA at a dietary concentration 10X higher than would be expected in the environment is essentially non-toxic. Finally, larvae feeding on tropical and common milkweed had similar responses to dsRNA treatment, suggesting that different levels of cardenolides in common and tropical milkweed [37] seemingly do not alter the toxicity of dsRNA molecules through differential metabolic capability of the larvae.

Findings with Varroa-active dsRNA could indicate more than 21 base pair matches are required to elicit adverse effects. The monarch-active dsRNA, having a 100% match with monarch mRNA, was expected to serve as positive control; however, we observed only a marginal, non-significant, increase in mortality. To ascertain if individual cohorts of larvae were uniquely resistant to stomach poisons, we employed potassium arsenate as a positive control with each dsRNA bioassay. A 1 mg/mL solution consistently killed all larvae within 5 days.

There was no correlation between measured leaf concentration and average mortality rate for any of the treatments ($p \geq 0.19$; Fig S6). Across common and tropical milkweed bioassays, we observed up to a 3.3-fold difference in measured dsRNA concentrations for replicates across dsRNA treatments. Across both milkweed species, the average dsRNA leaf concentrations for the 5 mg/mL monarch-active dsRNA and the 2.1 and 21 mg/mL Varroa-active dsRNA treatments were 0.027, 0.033, and 0.246 mg/g leaf, respectively. Assuming a monarch larva consumed approximately 7500 mg of milkweed leaf tissue, we estimate internal doses of 0.20

mg of monarch-active dsRNA and 0.25 and 1.8 mg of Varroa-active dsRNA, respectively, for the 1X and 10X Varroa-active dsRNA treatments.

In four other lepidopteran species, diamondback moth (*Plutella xylostella*), legume pod borer (*Maruca vitrata*), spotted stalk borer (*Chilo partellus*), and tobacco cutworm (*Spodoptera litura*), larvae feeding on fresh plant tissue and provided either 1.2×10^{-4} mg $\beta 1$ integrin dsRNA or 3×10^{-3} mg chitin synthase dsRNA (both dsRNA molecules targeted the individual species' mRNA) had 50 to 100% mortality [38, 39]. These results suggest that monarch larvae are less sensitive to dsRNA molecules and/or the *v-ATPase* mRNA could be recalcitrant to silencing. Lower levels (ca. 10%) of mortality via V-ATPase silencing were also seen in cotton bollworm (*Helicoverpa armigera*) larvae that were provided 0.01 mg/cm² treated leaves (dose not provided) for 10 days [40]. More data across species and genes are needed to make more conclusive comparisons.

In both tropical and common milkweed bioassays, the majority (55 to 70%) of monarchs that successfully pupated were third-instar larvae on the fourth day of observation; of the remaining monarchs, 95% were fourth instars and 5% were second instars. On Day 8, 67 to 92% of monarchs were fifth instars, and the rest were fourth instars. On Day 12, 83 to 100% of monarchs were pupae, and the rest were fifth instars. There were no differences in larval or pupal developmental time between treatments; the mean number of days it took larvae to pupate ranged from 11.2 to 11.6 days. Previous studies reported a mean neonate to pupal developmental time of about 12 and 13 days for monarch larvae reared at 27 and 25 °C, respectively [41, 42]. There were also no differences in pupal weights across treatments in both common and tropical milkweed bioassays. The average pupal weight in the common milkweed bioassays was greater (1176 vs. 970 mg) likely because the larvae were, on average, provided more milkweed leaves

than larvae in the tropical milkweed bioassays (Fig 3). Finally, there was no effect of *Varroa* or monarch-active dsRNA on the eclosion rate across treatments or runs. The average eclosion rates in the common and tropical milkweed runs were 0.93 and 0.97, respectively.

Our results provide evidence that chronic monarch larval exposure to monarch V-ATPase dsRNA has no biologically significant effect on monarch survival, growth, development, or eclosion rates. The results are consistent with [31] who fed first-instar monarchs dsRNA derived from monarch *v-ATPase A* mRNA for two days and then provided the larvae untreated honeyvine milkweed leaves (the first-instar stage lasted 4 to 5 days in this experiment). These researchers observed no effects on survival and overall development time; significant differences in development times for some instars between treatments may have been an artifact of using honeyvine milkweed leaves, which in some cases, can delay larval development [43, 44]. The lack of significant effects observed by [31] could have been due to the abbreviated length of dsRNA exposure, which may have resulted in an internal dose that was insufficient to elicit a toxic response and/or the peak dsRNA internal dose did not correspond to a critical developmental window (e.g., pupation and metamorphosis to the adult). In the present study, we chronically exposed monarch larvae to 0.020 to 0.034 mg/g monarch-active dsRNA milkweed leaf concentration and did not detect an adverse impact on survival, development, growth, or eclosion, as compared to larvae reared on untreated milkweed leaves. These findings are broadly consistent with the conclusions of [35], who reviewed more than 150 RNAi experiments in the insect order Lepidoptera. The authors reported that the technology seemed particularly efficacious at targeting immune genes in the family Saturniidae (species in the family Nymphalidae, to which monarchs belong, were not studied at the time of review). However, genes from the protein binding group, e.g., V-APTase and calmodulin, were refractory to

silencing. [45] also found that while Lepidopteran cell lines absorbed V-ATPase dsRNA, they did not process it to siRNA, which is necessary for gene silencing.

We are aware of only three chronic studies with Lepidopteran larvae that employed dietary dsRNA exposure methods without a bacterial or polymer vehicle. These studies used dsRNA molecules with a 100% base pair match to the mRNA of the target insect. [46] fed dsRNA encoding the pheromone biosynthesis activating neuropeptide (PBAN) gene to corn earworm (*Helicoverpa zea*) and tobacco budworm (*Heliothis virescens*); treated larvae experienced delayed growth, failed pupal development, and increased mortality. Cotton bollworm larvae that were fed artificially synthesized siRNA that targeted their acetylcholine esterase enzyme had higher mortality, diminished growth, smaller pupal weights, and reduced fecundity compared to control larvae [47]. [22] found that tobacco hornworm (*Manduca sexta*) larvae that were fed dsRNA targeting their V-ATPase transcripts had a LC_{50} of 0.011 mg/g diet. These three studies employed dsRNA-treated artificial diets rather than treated-host plant leaves. Of note, [36] showed that tobacco cutworm larvae that fed on cabbage leaves had greater dsRNA-degrading activity than larvae that were reared on an artificial diet. These authors suggest that artificial diet could potentially influence dsRNase expression, dsRNA stability, and RNAi efficiency. As our study employed fresh host plant leaves, a comparison of our results with chronic studies that employed an artificial diet may not be appropriate.

The recalcitrant response of monarch larvae also could be due to high gut pH and/or the presence of dsRNases in the gut. For example, RNA is most stable at a pH of 4.0 to 5.0 and lepidopterans have a gut pH greater than 8.0, which suggests dsRNA molecules may be unstable in this environment [48]. In addition, multiple dsRNases have been found in the gut or hemolymph of several lepidopteran larvae, including tobacco cutworm, fall armyworm

(*Spodoptera frugiperda*), silkworm (*Bombyx mori*), and tobacco hornworm [36, 48, 49]. If the monarch gut contains ribonucleases, it could further reduce the internal dsRNA dose below a level needed to silence mRNA signaling. Low dietary dsRNA concentrations, combined with high gut pH and dsRNase activity, could be another potential factor responsible for the lack of dsRNA effects in Lepidoptera. For example, [35] observed that dietary dsRNA insecticides silenced genes at only high concentrations. We used a 5 mg/mL monarch-active dsRNA suspension in the present study, which represents a practical upper limit of exposure given the solubility of the material. Given these factors, it is not surprising that Lepidopterans demonstrate low sensitivity to dsRNA products, with LC₅₀s often exceeding 1.0 mg/g [48, 49].

While our results show that monarch larvae exposed to dsRNA through their diet are unlikely to show adverse effects, application of foliar dsRNA insecticides could result in cuticular exposure. Penetration and absorption of dsRNA through the cuticle could bypass gut nucleases and alkalinity [49]. For example, [50] found that Lepidoptera Asian corn borer (*Ostrinia furnacalis*) had 100% mortality five days after the larvae and their diet were topically sprayed with dsRNA encoding the chymotrypsin-like serine protease C3 gene. Although there are no currently registered foliar dsRNA products, the technology has shown promise and could be further developed in the near future [51]. For example, [52] applied a dsRNA derived from Colorado potato beetle (CPB) to leaves of potato plants. CPB larvae feeding on the treated plants had high mortality. They also found that dsRNA was stable for at least 28 days under greenhouse conditions, which indicates long-term exposure to the insecticide is possible. Future commercial production and application of foliar dsRNA insecticides could result in spray drift exposure to non-target organisms near agricultural fields [48], including monarch larvae.

Monarch butterfly populations have declined in the last two decades [53, 54], and the U.S. Fish and Wildlife Services recently listed it as a candidate species under the Endangered Species Act [55]. Other non-target Lepidopteran populations are also declining [56-58]. Effective conservation practices involve understanding risks of pesticides, including new technologies such as dsRNA insecticides. In this regard, our study adds to the growing evidence that some Lepidopteran species may not be adversely impacted by dsRNA products, particularly by those that target protein binding groups.

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Tables and Figures

Table 1. The mean concentration measured for each treatment group and the overall mean

Milkweed species	Treatment	Concentration dsRNA (mg/g)			
		Run 1 ^a	Run 2 ^a	Run 3 ^a	Overall ^b
Common milkweed	Monarch	0.020 (± 0.005)	0.020 (± 0.015)	0.021 (± 0.015)	0.020 (± 0.0004)
	1X Varroa	0.013 (± 0.003)	0.030 (± 0.018)	0.032 (± 0.014)	0.025 (± 0.009)
	10X Varroa	0.389 (± 0.32)	0.144 (± 0.138)	0.312 (± 0.274)	0.282 (± 0.102)
Tropical milkweed	Monarch	0.036 (± 0.005)	0.037 (± 0.014)	0.030 (± 0.016)	0.034 (± 0.003)
	1X Varroa	0.020 (± 0.013)	0.065 (± 0.049)	0.036 (± 0.021)	0.041 (± 0.019)
	10X Varroa	0.316 (± 0.062)	0.143 (± 0.036)	0.173 (± 0.091)	0.211 (± 0.075)

^a The mean dsRNA concentration and standard deviation (SD) per designated bioassay run.

^b The mean dsRNA concentration and standard deviation (SD) over all bioassay runs.

Monarch-active dsRNA = 5 mg/mL monarch-active dsRNA solution concentration; 1X and 10X Varroa-active dsRNA = 2.1 and 21 mg/mL Varroa-active dsRNA solution concentrations, respectively.

Table 2. Monarch larval percent mortality following treatment with Varroa-active dsRNA and two positive and two negative controls ^a

Milkweed species (# of larvae treated)	Treatment	Larval percent mortality ^b				Mean corrected mortality ^d
		Run 1	Run 2	Run 3	Mean (\pm SD) ^c	
Common milkweed	Untreated	20	7	27	18 (\pm 10)	0
	Water	33	15	33	27 (\pm 10)	11
	Monarch-active dsRNA	27	40	33	33 (\pm 7)	18
	1X Varroa-active dsRNA	40	60	20	40 (\pm 20)	27
	10X Varroa-active dsRNA	27	50	40	39 (\pm 12)	26
	Potassium arsenate	100	100	100	100 (\pm 0)	100
Tropical milkweed	Untreated	10	20	30	20 (\pm 10)	0
	Water	30	20	20	23 (\pm 6)	4
	Monarch-active dsRNA	10	40	50	33 (\pm 21)	16
	1X Varroa-active dsRNA	10	40	0	17 (\pm 21)	0
	10X Varroa-active dsRNA	10	20	10	13 (\pm 6)	0
	Potassium arsenate	100	100	100	100 (\pm 0)	100

^a Monarch larvae were fed untreated leaves and leaves treated with deionized water, 5 mg/mL monarch-active dsRNA solution, 2.1 (1X) and 21 (10X) mg/mL Varroa-active dsRNA solutions, and 1 mg/mL potassium arsenate solution. All solutions were made in deionized water.

^b The percentage of larvae that died from neonate to pupation in each bioassay run. Six missing larvae (including one accidental death) over all treatments were excluded from analyses.

^c The mean larval percent mortality and standard deviation (SD) over all bioassay runs.

^d Abbott's formula was employed to correct for untreated control mortality. Corrected percent mortality = [1- (number of larvae surviving in treatment group \div number of larvae surviving in untreated control group)] x 100

Table 3. Monarch larval development following treatment with Varroa-active dsRNA and one positive and two negative controls ^a

Milkweed species (# of larvae treated)	Treatment	% of monarch instar/stage observed over all bioassay runs ^b			Mean (\pm SD) days to pupae ^c
		Day 4: Third instar	Day 8: Fifth instar	Day 12: Pupae	
Common milkweed	UN	57	86	86	11.2 (\pm 1.0)
	WT	68	68	87	11.6 (\pm 1.1)
	MB	57	87	93	11.2 (\pm 0.95)
	VL	70	67	93	11.3 (\pm 0.88)
	VH	63	78	93	11.3 (\pm 0.96)
Tropical milkweed	UN	63	92	92	11.5 (\pm 0.88)
	WT	70	87	83	11.5 (\pm 1.2)
	MB	55	90	100	11.2 (\pm 0.67)
	VL	64	84	84	11.4 (\pm 1.1)
	VH	65	92	85	11.4 (\pm 1.0)

^a Monarch larvae were fed untreated leaves (UN) and leaves treated with deionized water (WT), 5 mg/mL monarch-active dsRNA solution (MB), and 2.1 (VL) and 21 (VH) mg/mL Varroa-active dsRNA solutions. All solutions were made in deionized water. Only data from larvae that successfully pupated were analyzed. Data were combined over all bioassay runs.

^b The percentage of surviving monarchs in a treatment that belonged to the third instar (Day 4), fifth instar (Day 8) and pupa (Day 12). Larvae that were molting to a new instar were considered to have molted on the same day.

^c The mean [and corresponding standard deviation (SD)] number of days it took surviving larvae in each treatment to form pupae. Larvae that were in “J” form were considered to have pupated on the same day.

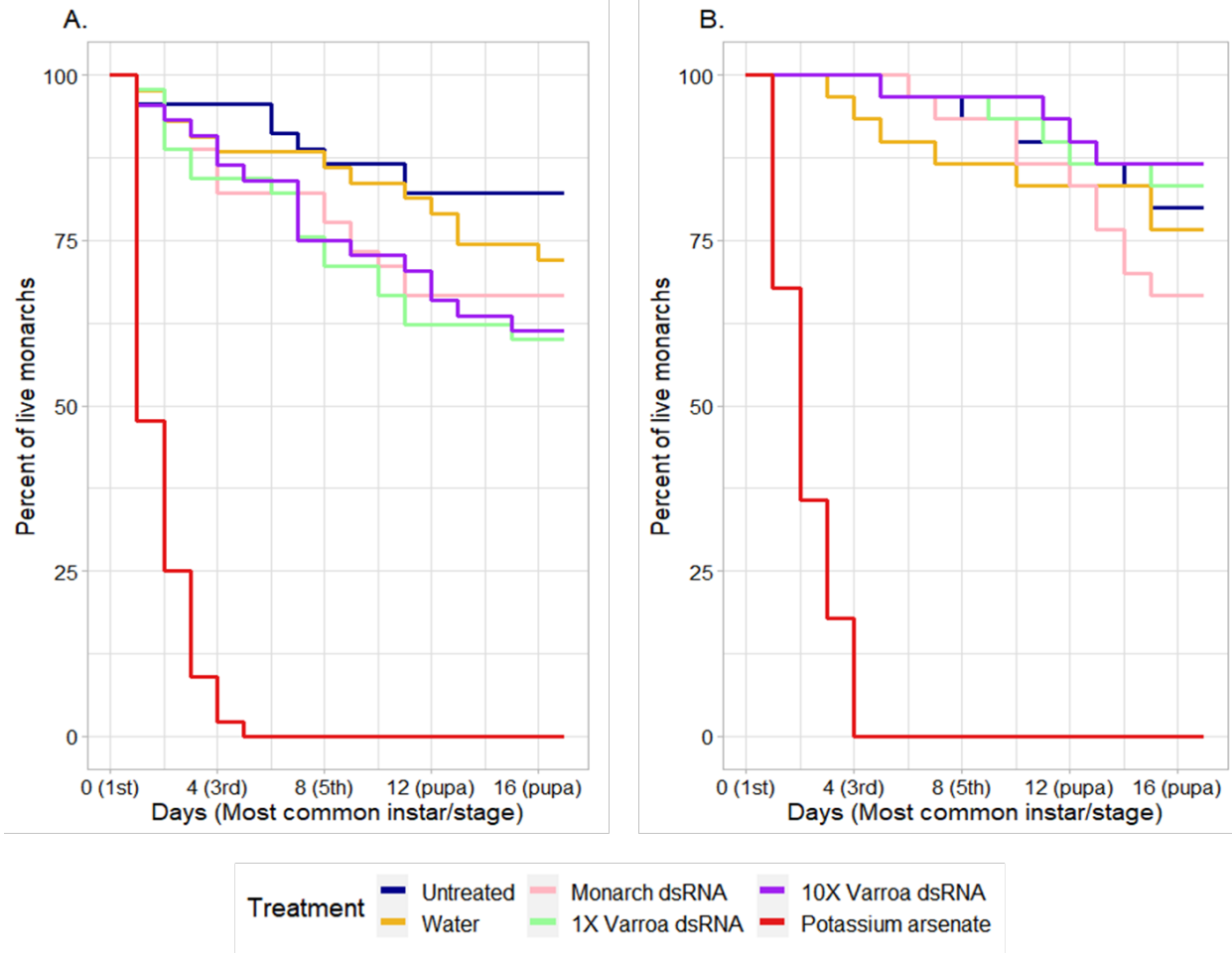


Figure 1. Monarch mean percent mortality over time, from neonate larvae to pupae, with data combined over all bioassay runs. Larvae were fed common (A) or tropical (B) milkweed leaves that were untreated (UN), treated with deionized water (WT), 5 mg/mL monarch dsRNA solution (MB), 2.1 (VL) and 21 (VH) mg/mL Varroa dsRNA solutions, or 1 mg/mL potassium arsenate solution (KA). Missing larvae (including 1 larva that was accidentally killed and five that went missing) were excluded from analysis.

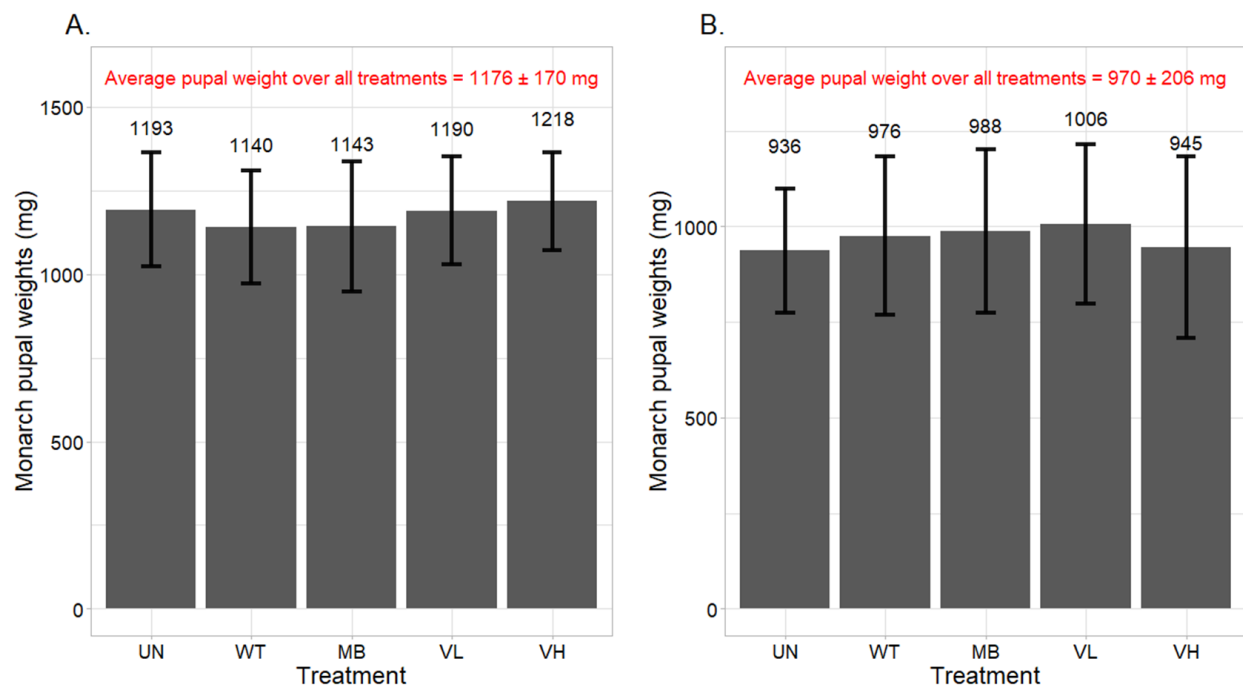


Figure 2. Average monarch pupal weight (in mg) in each treatment (data combined over all bioassay runs). Larvae were fed common (A) or tropical (B) milkweed leaves that were untreated (UN), treated with deionized water (WT), 5 mg/mL monarch dsRNA solution (MB), or 2.1 (VL) and 21 (VH) mg/mL Varroa dsRNA solutions. Bars represent the mean \pm one standard deviation. A single pupa in the common milkweed water treatment was excluded from analyses.

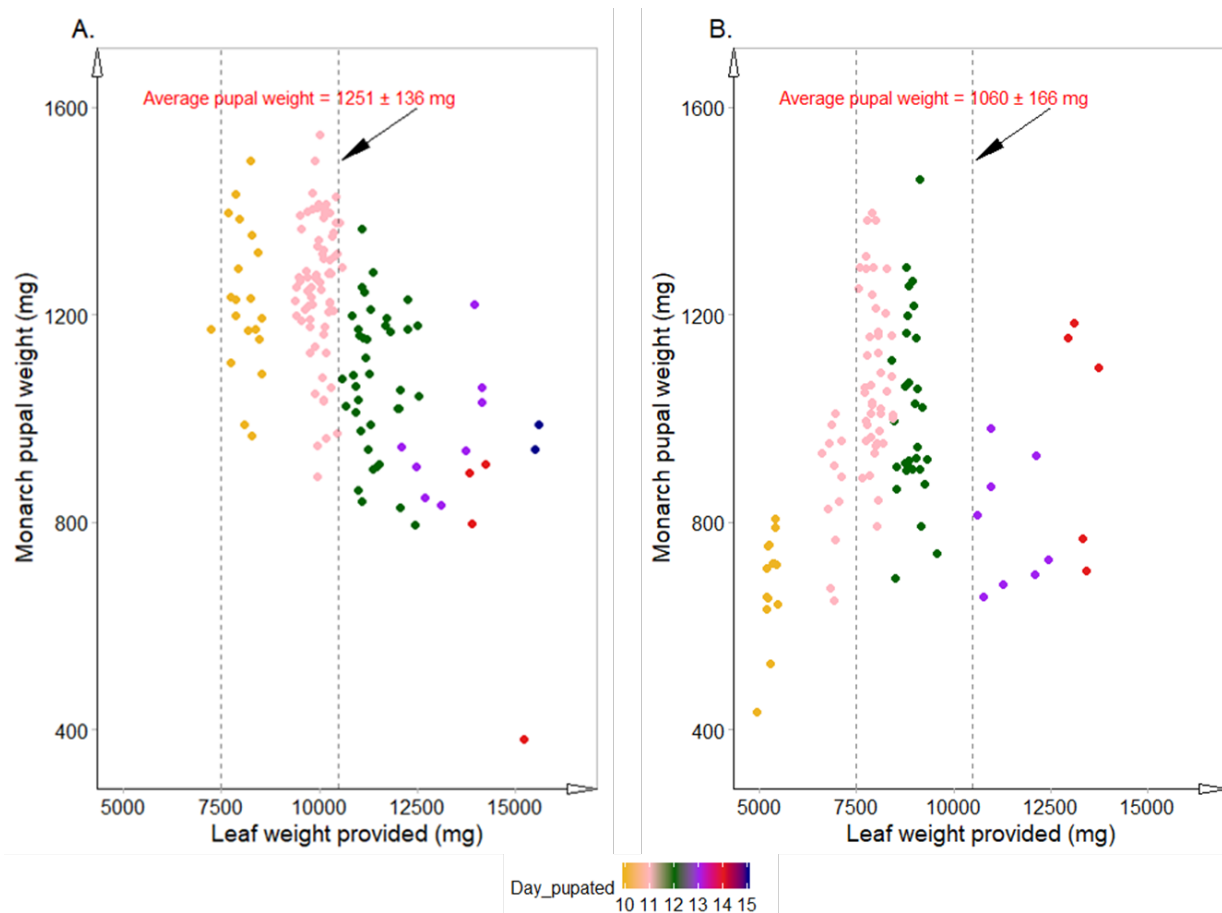


Figure 3. Individual monarch pupal weights (mg) plotted against individual weights (mg) of common (A) and tropical (B) milkweed leaf provided to each larva. Data were combined over all treatments and bioassay runs. The different colored dots represent the range of days it took the monarchs to pupate (see legend). The vertical dotted lines bound monarch pupae that were provided 7500 and 10,500 mg of milkweed leaf. The average weights of these pupae are provided.

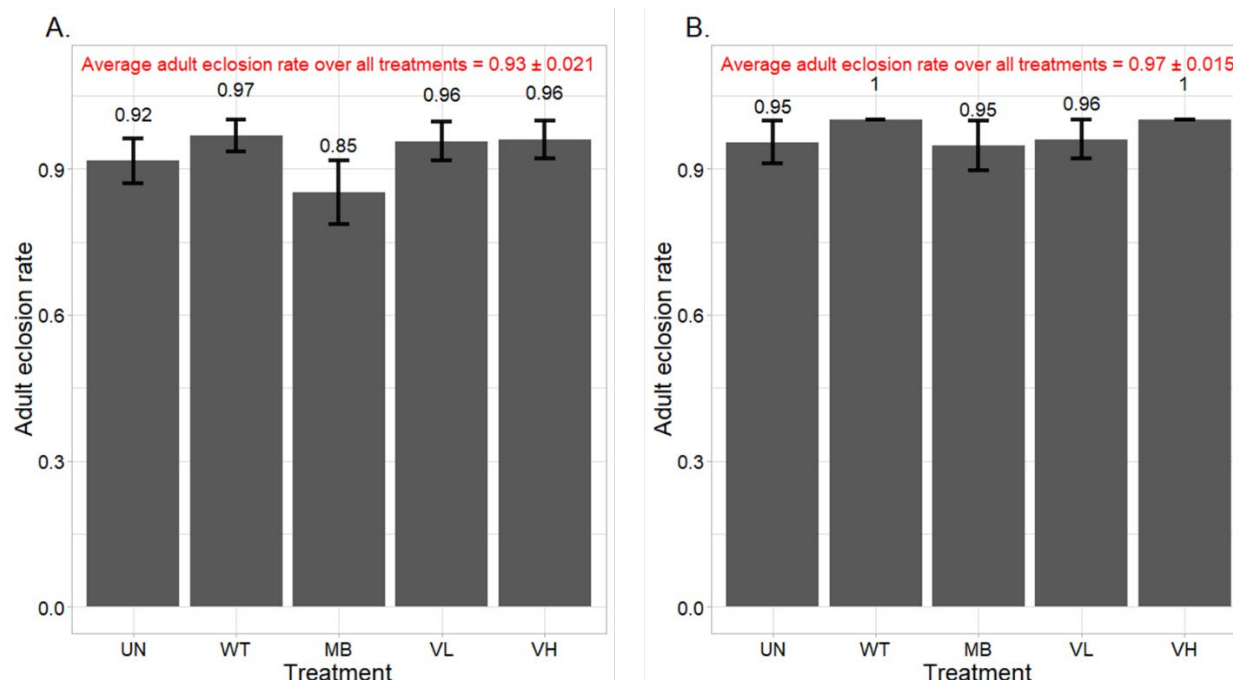


Figure 4: Average monarch adult eclosion rates of uninfected pupae in each treatment (data combined over all bioassay runs). Larvae were fed common (A) or tropical (B) milkweed leaves that were untreated (UN), treated with deionized water (WT), 5 mg/mL monarch dsRNA solution (MB), or 2.1 (VL) and 21 (VH) mg/mL Varroa dsRNA solutions. Bars represent the mean \pm standard error.

Appendix. Supporting Information

Table A1. Preparation of solutions used in the QuantiGene® Singleplex Assay Kit.

Solution	Brand/Company	Components	Notes
Sample diluent (actually used)	Baker's Yeast RNA [Lot #:SLBV7182]: <i>Sigma-Aldrich, St. Louis, MO, USA</i>	1:1000 dilution of Baker's Yeast Solution made with UltraPure™ water [e.g. 100 μ L of Baker's Yeast stock solution and 100 mL of UltraPure™ water]	Keep both stock solution and diluted solution refrigerated
	UltraPure™ water [Ref #:10977-015]: <i>Invitrogen by Life Technologies, Grand Island, NY, USA</i>	(baker's yeast stock solution: 10 mg of baker's yeast RNA and 1 mL of UltraPure™ water)	
Working solution	UltraPure™ water [Ref #:10977-015]: <i>Invitrogen by Life Technologies, Grand Island, NY, USA</i>		
	Lysis mixture [Ref #:10093]: <i>Invitrogen by Thermo Fisher Scientific, Affymetrix Inc., Santa Clara, CA, USA</i>	5.3 mL UltraPure™ Water 3.8 mL lysis mixture*	Vortex 10 sec. Make fresh daily. Makes enough for 1 plate.
	Blocking reagent [Ref #:13254]: <i>Invitrogen by Thermo Fisher Scientific, Affymetrix Inc., Santa Clara, CA, USA</i>	115 μ L blocking reagent*	

Table A1. Continued

Wash buffer	Buffer component #1 [Ref #:10842]: <i>Invitrogen by Thermo Fisher Scientific, Affymetrix Inc., Santa Clara, CA, USA</i>		
	Buffer component #2 [Ref #:10845]: <i>Invitrogen by Thermo Fisher Scientific, Affymetrix Inc., Santa Clara, CA, USA</i>	1.05 mL buffer component #1* 1.75 mL buffer component #2* 350 mL nuclease-free water	Mix well. Make fresh daily. Makes enough for 1 plate.
	Nuclease-free water [CAT #:9153-1]: <i>RICCA Chemical Company, Arlington, TX, USA</i>		
Pre-amplifier solution	Amplifier/label probe diluent [Ref #:14539]: <i>Invitrogen by Thermo Fisher Scientific, Affymetrix Inc., Santa Clara, CA, USA</i>	11 mL Amplifier/label probe diluent* 11 µL pre-amplifier reagent*	Vortex 10 sec. Mix prior to use. Makes enough for 1 plate.
	Pre-amplifier reagent [Ref #:15094]: <i>Invitrogen by Thermo Fisher Scientific, Affymetrix Inc., Santa Clara, CA, USA</i>		
Amplifier solution	Amplifier/label probe diluent [Ref #:14539]: <i>Invitrogen by Thermo Fisher Scientific, Affymetrix Inc., Santa Clara, CA, USA</i>	11 mL Amplifier/label probe diluent* 11 µL amplifier reagent*	Vortex 10 sec. Mix prior to use. Makes enough for 1 plate.
	Amplifier reagent [Ref #:15097]: <i>Invitrogen by Thermo Fisher Scientific, Affymetrix Inc., Santa Clara, CA, USA</i>		
Label probe Solution	Amplifier/label probe diluent [Ref #:14539]: <i>Invitrogen by Thermo Fisher Scientific, Affymetrix Inc., Santa Clara, CA, USA</i>	11 mL Amplifier/label probe diluent* 11 µL label probe reagent*	Vortex 10 sec. Mix prior to use. Makes enough for 1 plate.
	Label probe reagent [Ref #:10087]: <i>Invitrogen by Thermo Fisher Scientific, Affymetrix Inc., Santa Clara, CA, USA</i>		

```

<210> SEQ ID NO 91
<211> LENGTH: 372
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: synthetic sequence

<400> SEQUENCE: 91

ggaacagauc gccgaguuca aagaggcguu uagccuguuu gacaaggacg gagauggcac      60
gaucacgaca aaggagcucg guacgguaau gcgaucucuc ggccagaacc cacugaggcu      120
gaacugcagg acaugaucaa cgaggucgac gccgacggcu ccggaacgau agauuucccu      180
gaguuccuca caaugaugca agaaagauga aggacaccga cucggaggag gagaucgaga      240
ggcguuccgc guauucgaca aggaugcaac gguuucuuu cggcgggccga gcucaggcac      300
guuaugacca accuuggcga gaagcuuacg gacgaggagg uagaugagau gauucgggag      360
gcagauauug ac                                                                372

```

Fig S1. Sequence of the Varroa-active dsRNA (Inberg and Mahak 2016).

A: Closest predicted sequence match.

PREDICTED: Varroa destructor calmodulin-like (LOC111247793), mRNA

Sequence ID: [XM_022799184.1](#) Length: 1498 Number of Matches: 1

Range 1: 807 to 1182 [GenBank](#) [Graphics](#)

▼ [Next Match](#) ▲ [Previous Match](#)

Score	Expect	Identities	Gaps	Strand	
669 bits(362)	0.0	372/376(99%)	4/376(1%)	Plus/Plus	
Query 1	GGAACAGATCGCCGAGTTCAAAGAGGCGTTTAGCCTGTTTGACAAGGACGGAGATGGCAC	60			
Sbjct 807	GGAACAGATCGCCGAGTTCAAAGAGGCGTTTAGCCTGTTTGACAAGGACGGAGATGGCAC	866			
Query 61	GATCACGACAAAGGAGCTCGGTACGGTAATGCGATCTCTCGGCCAGAA-CCCACTGAGGC	119			
Sbjct 867	GATCACGACAAAGGAGCTCGGTACGGTAATGCGATCTCTCGGCCAGAACCCCACTGAGGC	926			
Query 120	TGAACTGCAGGACATGATCAACGAGGTCGACGCCGACGGCTCCGGAACGATAGATTTCCC	179			
Sbjct 927	TGAACTGCAGGACATGATCAACGAGGTCGACGCCGACGGCTCCGGAACGATAGATTTCCC	986			
Query 180	TGAGTTCCTCACAATGAT-GCAAGAAAGATGAAGGACACCGACTCGGAGGAGGAGAT-CG	237			
Sbjct 987	TGAGTTCCTCACAATGATGGCAAGAAAGATGAAGGACACCGACTCGGAGGAGGAGATCCG	1046			
Query 238	AGAGGCGTTCGCGTATTCGACAAGGAT-GCAACGGTTTCATTTGGCGGCCGAGCTCAG	296			
Sbjct 1047	AGAGGCGTTCGCGTATTCGACAAGGATGGCAACGGTTTCATTTGGCGGCCGAGCTCAG	1106			
Query 297	GCACGTTATGACCAACCTTGGCGAGAAGCTTACGGACGAGGAGGTAGATGAGATGATTCTG	356			
Sbjct 1107	GCACGTTATGACCAACCTTGGCGAGAAGCTTACGGACGAGGAGGTAGATGAGATGATTCTG	1166			
Query 357	GGAGGCAGATATTGAC	372			
Sbjct 1167	GGAGGCAGATATTGAC	1182			

Fig S2. Varroa-active dsRNA closest predicted sequence match and location in Varroa mite genome.

B: Varroa-active dsRNA (query) overlap in the Varroa mite genome (subject).

Varroa destructor unplaced genomic scaffold, Vdes_3.0 BEIS01000003.1Sequence ID: [NW_019211456.1](#) Length: 58536683 Number of Matches: 1Range 1: 31901306 to 31901681 [GenBank](#) [Graphics](#)[▼ Next Match](#) [▲ Previous Match](#)

Score	Expect	Identities	Gaps	Strand
669 bits(362)	0.0	372/376(99%)	4/376(1%)	Plus/Minus
Features: calmodulin-like				
Query 1	GGAACAGATCGCCGAGTTCAAAGAGGCGTTTAGCTGTTTGACAAGGACGGAGATGGCAC	60		
Sbjct 31901681	GGAACAGATCGCCGAGTTCAAAGAGGCGTTTAGCTGTTTGACAAGGACGGAGATGGCAC	31901622		
Query 61	GATCACGACAAAGGAGCTCGGTACGGTAATGCGATCTCTCGGCCAGAACCC-CTGAGGC	119		
Sbjct 31901621	GATCACGACAAAGGAGCTCGGTACGGTAATGCGATCTCTCGGCCAGAACCCACTGAGGC	31901562		
Query 120	TGAACTGCAGGACATGATCAACGAGGTGCGACGCCGACGGCTCCGGAACGATAGATTTC	179		
Sbjct 31901561	TGAACTGCAGGACATGATCAACGAGGTGCGACGCCGACGGCTCCGGAACGATAGATTTC	31901502		
Query 180	TGAGTTCCTCACAATGATG-CAAGAAAGATGAAGGACACCGACTCGGAGGAGGAGATC-G	237		
Sbjct 31901501	TGAGTTCCTCACAATGATGGCAAGAAAGATGAAGGACACCGACTCGGAGGAGGAGATCCG	31901442		
Query 238	AGAGGCGTTCCGCGTATTCGACAAGGATG-CAACGGTTTCATTTCGGCGGCCGAGCTCAG	296		
Sbjct 31901441	AGAGGCGTTCCGCGTATTCGACAAGGATGGCAACGGTTTCATTTCGGCGGCCGAGCTCAG	31901382		
Query 297	GCACGTTATGACCAACCTTGGCGAGAAGCTTACGACGAGGAGGTAGATGAGATGATTTCG	356		
Sbjct 31901381	GCACGTTATGACCAACCTTGGCGAGAAGCTTACGACGAGGAGGTAGATGAGATGATTTCG	31901322		
Query 357	GGAGGCAGATATTGAC	372		
Sbjct 31901321	GGAGGCAGATATTGAC	31901306		

Fig S2. Continued

SUMMARY: The closest sequence to the Varroa dsRNA is predicted to be the Varroa mite calmodulin mRNA. The same region of sequence overlap is seen when the Varroa dsRNA sequence is compared to the whole Varroa mite genome.

A: Varroa-active dsRNA (query) overlaps in honeybee genome (subject).

Apis mellifera strain DH4 linkage group LG12, Amel_HAv3.1

Sequence ID: [NC_037649.1](#) Length: 11514234 Number of Matches: 1

Range 1: 8579150 to 8579361 [GenBank](#) [Graphics](#)

▼ [Next Match](#) ▲ [Previous Match](#)

Score	Expect	Identities	Gaps	Strand
133 bits(147)	7e-30	159/212(75%)	3/212(1%)	Plus/Plus

Features: calmodulin

Query	163	GGAACGATAGATTTCCTTGAGTTCCTCACAATGATG-CAAGAAAGATGAAGGACACCGAC	221
Sbjct	8579150	GGCACAAATCGATTTCCGGAATTCTTAACATATGATGGCTCGTAAAATGAAAGATACTGAT	8579209
Query	222	TCGGAGGAGGAGATC-GAGAGGCGTTC CGCGTATTCGACAAGGATGCAAC-GGTTTTCA TT	279
Sbjct	8579210	AGTGAGGAAGAAATTAGGGAGGCCCTTCAGAGTATTTGATAAGGATGGA AATGGTTTTCAT A	8579269
Query	280	TCGGCGGCCGAGCTCAGGCACGTTATGACCAACCTTGGCGAGAAGCTTACGGACGAGGAG	339
Sbjct	8579270	TCCGCAGCAGAACTCAGACATGTTATGACAAATCTTGGCGAGAAACTCACTGATGAAGAA	8579329
Query	340	GTAGATGAGATGATTTCGGGAGGCAGATATTGA	371
Sbjct	8579330	GTTGATGAAATGATTTCGGGAGGCTGACATTGA	8579361

Apis mellifera strain DH4 linkage group LG8, Amel_HAv3.1

Sequence ID: [NC_037645.1](#) Length: 12717210 Number of Matches: 1

Range 1: 7596085 to 7596118 [GenBank](#) [Graphics](#)

▼ [Next Ma](#)

Score	Expect	Identities	Gaps	Strand
44.6 bits(48)	0.003	32/35(91%)	3/35(8%)	Plus/Minus

```
Query    253      ATTCGACAAGGATG--CAACGGTTTCATTTTCGGCG      285
          |||||
Sbjct    7596118  ATTCGACAAGGATGGGCAA-GGTTTCATTTTCGGCG      7596085
```

Fig S3. Varroa-active dsRNA comparison to honeybee sequences.

Sequence ID: [XM_006565254.3](#) Length: 1161 Number of Matches: 2

▼ [Next Match](#) ▲ [Previous Match](#)

Score	Expect	Identities	Gaps	Strand
224 bits(247)	3e-62	277/374(74%)	4/374(1%)	Plus/Plus
Query 2		GAACAGATCGCCGAGTTCAAAGAGGCGTTTAGCCTGTTTGACAAGGACGGAGATGGCAGC		61
Sbjct 401		GAACAAATCGCAGAATTTAAGGAAGCATTTCCTACTATTTGATAAAGATGGAGATGGTACC		460
Query 62		ATCACGACAAAGGAGCTCGGTACGGTAATGCGATCTCTCGGCCAGAA-CCCACTGAGGCT		120
Sbjct 461		ATCACAACTAAAGAGTTGGGTACAGTTATGCGATCACTAGGTCAAAATCCCACAGAAGCT		520
Query 121		GAAGTGCAGGACATGATCAACGAGGTCGACGCCGACGGCTCCGGAACGATAGATTTCCCT		180
Sbjct 521		GAGCTTCAGGATATGATTAATGAAGTTGATGCAGATGGTAATGGCACAATCGATTTTCCG		580
Query 181		GAGTTCCTCACAATGATG-CAAGAAAGATGAAGGACACCGACTCGGAGGAGGAGATC-GA		238
Sbjct 581		GAATTCTTAACTATGATGGCTCGTAAATGAAAGATACTGATAGTGAGGAAGAAATTAGG		640
Query 239		GAGGCGTTCGCGTATTCGACAAGGATGCAAC-GGTTTCATTTCGGCGGCCGAGCTCAGG		297
Sbjct 641		GAGGCCCTTCAGAGTATTTGATAAGGATGAAATGGTTTCATATCCGCAGCAGAACTCAGA		700
Query 298		CACGTTATGACCAACCTTGGCGAGAAGCTTACGGACGAGGAGGTAGATGAGATGATTCGG		357
Sbjct 701		CATGTTATGACAAATCTTGGCGAGAACTCACTGATGAAGAAGTTGATGAAATGATTCGG		760
Query 358		GAGGCAGATATTGA 371		
Sbjct 761		GAGGCTGACATTGA 774		

SUMMARY: The Varroa dsRNA has sequence similarity to four regions in the honeybee genome. One of these regions (DH4 linkage group LG12), which contains a shared 14 nucleotide sequence, overlaps with the honeybee calmodulin mRNA. Another region (DH4 linkage group LG8), which contains a shared 15 nucleotide sequence, did not overlap with the honeybee calmodulin mRNA. There are no shared 21 nucleotide sequences.

A: Varroa-active dsRNA (query) overlaps in monarch butterfly genome (subject).

Danaus plexippus plexippus isolate F-2 chromosome 13, Dplex v4, whole genome shotgun sequence

Sequence ID: [NC_045819.1](#) Length: 8907986 Number of Matches: 2

Range 1: 644607 to 644820 [GenBank](#) [Graphics](#)

▼ [Next Match](#) ▲ [Previous Match](#)

Score	Expect	Identities	Gaps	Strand
200 bits(221)	1e-49	175/214(82%)	3/214(1%)	Plus/Minus

Features: [calmodulin isoform X2](#)
[calmodulin isoform X1](#)

Query	162	CGGAACGATAGATTTCCTGAGTTCCTCACAATGATG-CAAGAAAGATGAAGGACACCGA	220
Sbjct	644820	CGGTACGATAGACTTCCCCGAATTCTCTGACGATGATGGCGCGCAAGATGAAGGACACCGA	644761
Query	221	CTCGGAGGAGGAGATC-GAGAGGCGTTCCGCGTATTTCGACAAGGATG-CAACGGTTTCAT	278
Sbjct	644760	CAGCGAGGAGGAGATCCGCGAGGCGTTCCGCGTGTTCGACAAGGACGGCAACGGATTTCAT	644701
Query	279	TTCCGGCGGCGAGCTCAGGCACGTTATGACCAACCTTGGCGAGAAGCTTACGGACGAGGA	338
Sbjct	644700	ATCCGCGCGGAGCTGCGTCACGTGATGACCAACCTCGGAGAGAAGCTCACCGACGAGGA	644641
Query	339	GGTAGATGAGATGATTCCGGAGGCAGATATTGAC	372
Sbjct	644640	GGTGGACGAGATGATCCGCGAGGCCGACATCGAC	644607

Range 2: 652541 to 652699 [GenBank](#) [Graphics](#)

▼ [Next Match](#) ▲ [Previous Match](#) ▲ [First Match](#)

Score	Expect	Identities	Gaps	Strand
149 bits(164)	2e-34	129/159(81%)	1/159(0%)	Plus/Minus

Features: [calmodulin isoform X2](#)
[calmodulin isoform X1](#)

Query	1	GGAACAGATCGCCGAGTTCAAAGAGGCGTTTTAGCCTGTTTGCACAGGACGGAGATGGCAC	60
Sbjct	652699	GGAACAGATCGCCGAGTTCAAAGAGGCGTTTTACTGTTCGATAAAGATGGGGACGGCAC	652640
Query	61	GATCACGCACAAAGGAGCTCGGTACGGTAATGCGATCTCTCGGCCAGAA-CCCCTGAGGC	119
Sbjct	652639	CATCACCACCAAGGAGCTGGGGACCGTGATGAGGTCGCTGGGACAGAAACCCACGGAGGC	652580
Query	120	TGAACTGCAGGACATGATCAACGAGGTCGACGCCGACGG	158
Sbjct	652579	CGAGCTCCAGGACATGATCAACGAGGTTGACGCTGATGG	652541

Danaus plexippus plexippus isolate F-2 chromosome Z, Dplex_v4, whole genome shotgun sequence

Sequence ID: **NC_045836.1** Length: **15616146** Number of Matches: **1**

Range 1: 2678033 to 2678145 [GenBank](#) [Graphics](#)

▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
52.7 bits(57)	4e-05	80/113(71%)	1/113(0%)	Plus/Minus

Features: neo-calmodulin-like

Query	38	TTTGACAAGGACGGAGATGGCACGATCACGACAAAGGAGCTCGGTACGGTAATGCGATCT	97
Sbjct	2678145	TTCGACAAGGACGGCGATGGCACCATCACAAAGGAAGAGCTCGGGCAGTTCATGAGGAGC	2678086
Query	98	CTCGGCCAGAAAC-CCACTGAGGCTGAACTGCAGGACATGATCAACGAGGTCGA	149
Sbjct	2678085	CTCGGCCAGTTCGCGAGGGTTGAGGAGTTGCAGGACATGTTTCAGGAGGTCGA	2678033

Fig S4. Varroa-active dsRNA comparison to monarch butterfly sequences.

B: Varroa-active dsRNA (query) overlap in the monarch butterfly calmodulin mRNA (subject).

PREDICTED: Danaus plexippus plexippus calmodulin (LOC116769735), transcript variant X2, mRNA

Sequence ID: [XM_032660923.1](#) Length: 1415 Number of Matches: 2

Range 1: 304 to 679 [GenBank](#) [Graphics](#)

▼ [Next Match](#) ▲ [Previous Match](#)

Score	Expect	Identities	Gaps	Strand
340 bits(376)	2e-97	304/376(81%)	4/376(1%)	Plus/Plus
Query 1	GGAACAGATCGCCGAGTTCAAAGAGGCGTTTAGCCTGTTTGACAAGGACGGAGATGGCAC	60		
Sbjct 304	GGAACAGATCGCCGAGTTCAAAGAGGCGTTTTCCTGTTTCGATAAAGATGGGGACGGCAC	363		
Query 61	GATCAGCACAAGGAGCTCGGTACGGTAATGCGATCTCTCGGCCAGAACCC-CTGAGGC	119		
Sbjct 364	CATCACCACCAAGGAGCTGGGGACCGTGATGAGGTCGCTGGGACAGAACCCACGGAGGC	423		
Query 120	TGAATGCGAGGACATGATCAACGAGGTCGACGCCGACGGCTCCGGAACGATAGATTCCC	179		
Sbjct 424	CGAGCTCCAGGACATGATCAACGAGGTTGACGCTGATGGTAACGGTACGATAGACTTCCC	483		
Query 180	TGAGTTCCTCACAATGATG-CAAGAAAGATGAAGGACACCGACTCGGAGGAGGAGATC-G	237		
Sbjct 484	CGAATTCCTGACGATGATGGCGCGCAAGATGAAGGACACGGACAGCGAGGAGGAGATCCG	543		
Query 238	AGAGGCGTTCGCGTATTCGACAAGGATG-CAACGGTTTCATTTCGGCGGCCGAGCTCAG	296		
Sbjct 544	CGAGGCGTTCGCGTGTTCGACAAGGACGGCAACGGATTTCATATCCGCCGCGAGCTGCG	603		
Query 297	GCACGTTATGACCAACCTTGGCGAGAAGCTTACGGACGAGGAGGTAGATGAGATGATTCCG	356		
Sbjct 604	TCACGTGATGACCAACCTCGGAGAGAAGCTCACCACGAGGAGGTGGACGAGATGATCCG	663		
Query 357	GGAGGCAGATATTGAC	372		
Sbjct 664	CGAGGCCGACATCGAC	679		

Fig S4. Continued

SUMMARY: The Varroa dsRNA has sequence similarity to two regions in the monarch butterfly genome. One of these regions (F-2 chromosome 13), which contains a shared 21 nucleotide sequence, overlaps with the monarch butterfly calmodulin mRNA.

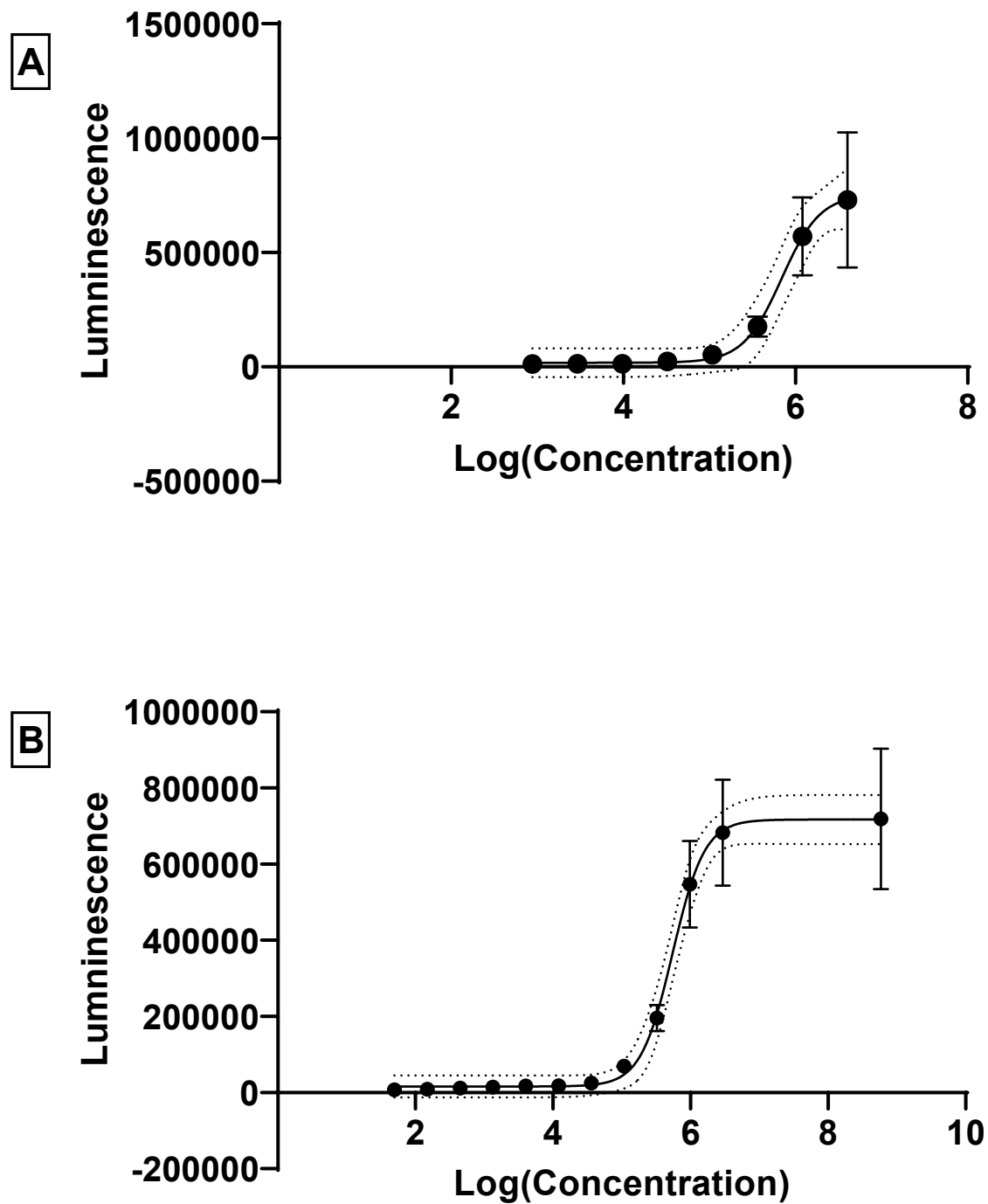


Fig S5. Representative QuantiGene calibration curves for monarch-active dsRNA (A) and Varroa-active dsRNA (B).

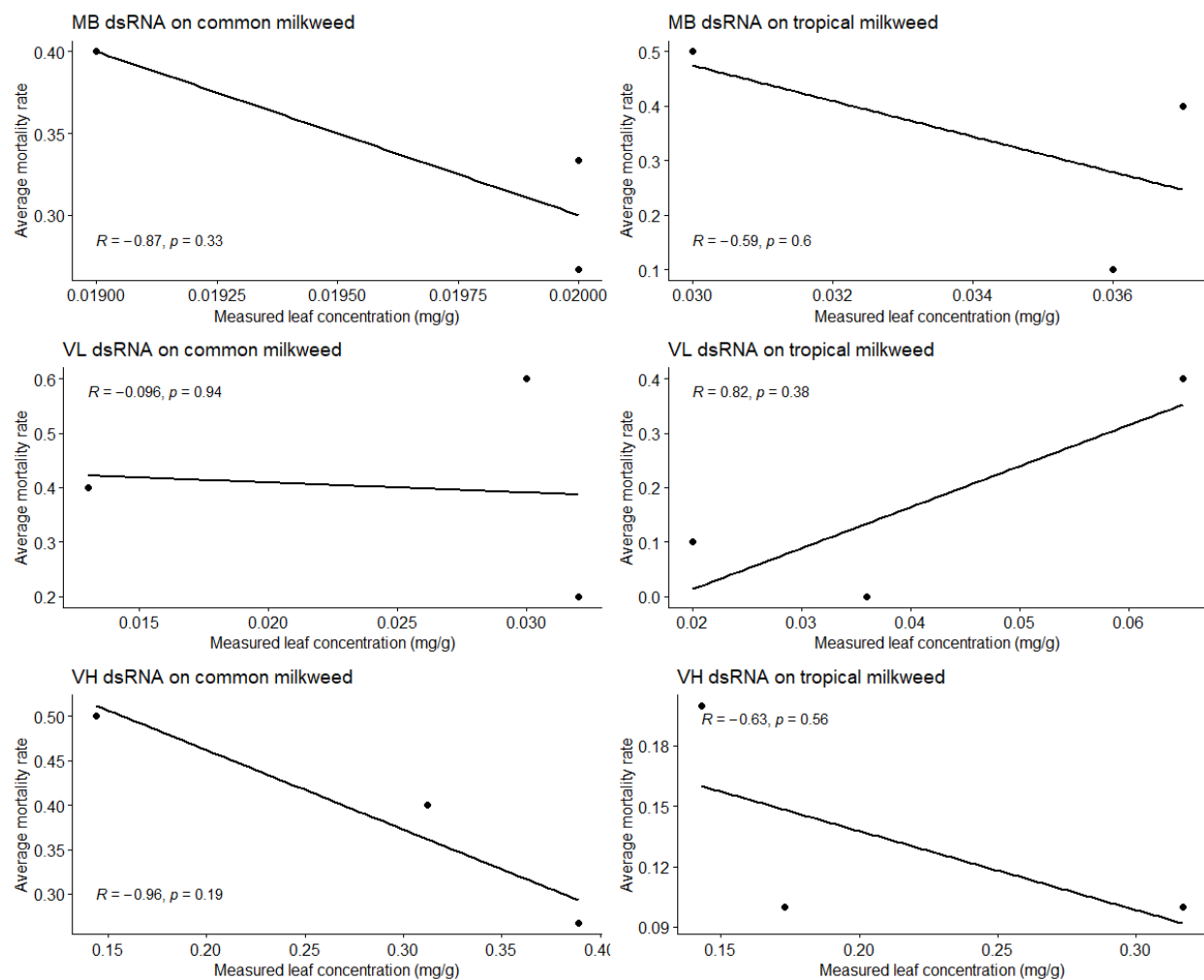


Fig S6. Correlation between measured leaf concentration and mortality for monarch butterfly (MB) dsRNA, 1X Varroa (VL) dsRNA, and 10X Varroa (VH) dsRNA treatments. Data were analyzed separately for common and tropical milkweed. Each point on the graph indicates a bioassay run.

CHAPTER 8. GENERAL CONCLUSION

The research presented in this dissertation successfully addressed gaps in the knowledge of pollinator exposure to pesticides agricultural settings. Wild bees, honey bees, and monarch butterfly populations are at risk due to multiple interacting environmental stressors including pesticides and habitat loss (Goulson *et al.*, 2015; U.S. Fish and Wildlife Service, 2020). Habitat needs to be implemented into agricultural ecosystems in the north central United States to help restore these populations (Koh *et al.*, 2016; Thogmartin *et al.*, 2017). Prairie strips have been shown to increase pollinator diversity and abundance (Kordbacheh *et al.*, 2020; Schulte *et al.*, 2017). When these prairies incorporate milkweed, they can be a viable habitat for monarch larvae (Grant *et al.*, 2021).

Research undertaken throughout this dissertation helps determine the conservation risks and benefits of establishing pollinator habitat (i.e., prairie strips) within or adjacent to conventional maize and soybean fields in which pesticides are used to manage insect pests and fungi. The research included a) development of new analytical methods for accurate and precise quantification of conventional and biological insecticides in a diverse set of environmental matrices; b) generation of species-specific dietary exposure data for comparison with available species-specific acute and chronic toxicity data to characterize risks for monarch butterfly larvae and honey bees; c) evaluation of alternative analytical methodology to support more robust and affordable monitoring studies to characterize fate and transport of pesticides in the environment; and d) development of preliminary screening-level risk analyses for lepidopteran species of conservation concern, based on an evaluation and integration of environmental monitoring and toxicity data generated in my studies and reported in the peer-reviewed literature.

A Brief Overview of Chapter Findings

Application of robust analytical chemistry methods is required to characterize neonicotinoid exposure concentrations in complex environmental samples. In Chapter 2, I developed a single extraction and quantitation method using liquid chromatography tandem mass spectrometry (LC-MS/MS) for a suite of neonicotinoids (clothianidin, imidacloprid, and thiamethoxam) and two imidacloprid metabolites (5-OH imidacloprid and imidacloprid olefin) in pollen and milkweed leaf tissue. The developed method reduced sample preparation time and LC-MS/MS run time, allowing for increased sample throughput. Additionally, the method was able to analyze smaller samples sizes, which allowed for analysis of samples that previously could not be analyzed. The methods performance (accuracy and precision) was comparable and in some cases superior to existing methods. These analytical techniques were modified as needed to support the quantification of neonicotinoids and additional pesticides that were the subject of research presented in Chapters 3, 4, and 5.

Research results presented in Chapters 3 and 4 indicate that systemic uptake of neonicotinoid insecticides by non-target plants within prairie strips is a potential exposure pathway for monarch larvae (e.g., feeding on contaminated milkweed leaves) and foraging bees (e.g., feeding on contaminated pollen and nectar). However, the concentrations detected are well below the acute and chronic thresholds for honey bees and monarch larvae, suggesting little or no risk anticipated from these formulations. These data indicate that continued use of neonicotinoid seed treatment as part of an integrated pest management program for the adjacent and surrounding crop field result in neonicotinoid exposure to monarch larvae and honey bees below thresholds of concern.

Chapter 4 also assessed variation in pesticide exposure between honey bee colonies located in established pollinator habitats (prairie strips) and those located at the edge of maize or soybean fields. Results indicate honeybee colonies experience more frequent acute exposure to foliar-applied pesticides than neonicotinoids that are used as seed treatments. The temporal trends of frequent foliar pesticide exposure were consistent with applications for pests in maize and soybean fields. Overall, these data show that hives placed in prairie strips or other pollinator habitat in close proximity to crop fields are not likely to be negatively impacted by pesticide exposure.

In chapter 6, we developed exploratory Species Sensitivity Distribution models to estimate the toxicity of insecticides to additional lepidopteran species of conservation concern in the north central states (e.g., the Dakota skipper (*Hesperia dacotae*), Karner blue (*Lycaeides melissa samuelis*), Mitchell's satyr (*Neonympha mitchellii mitchellii*), and Poweshiek skipperling (*Oarisma poweshiek*)) for which pesticide toxicity data for is not available. Using these models, preliminary screening-level risk analyses for lepidopteran species of conservation concern were undertaken by using the pesticide monitoring data generated in this dissertation, combined with studies reported in the peer-reviewed literature. These preliminary assessments illustrate how conservation risks and benefits for these other at risk species can be determined. Chapter 6 highlights the need for more intensive monitoring studies to allow for refined exposure characterization for 'at-risk' lepidopteran species.

The per-sample cost associated with LC-MS/MS can limit the number of samples and constrain the means to adequately quantify pesticide residues. In Chapter 5, I compare and contrast the strengths and limitations of using enzyme-linked immunosorbent assay (ELISA) kits as a rapid and cost-effective alternative to LC-MS/MS for the three most commonly used

neonicotinoids: clothianidin, imidacloprid, and thiamethoxam. The data shows that ELISA kits are sufficient to identify the presence/absence of neonicotinoids in water and leaf tissue and could be used to prioritize samples for LC-MS/MS analyses. The presence of cross-reactants precludes the means to quantify neonicotinoid-specific concentrations by ELISA with confidence. Confirmation of ELISA results by LC-MS/MS is suggested to identify and quantify neonicotinoid concentrations in water and plant foliage.

Finally, in Chapter 7 the dietary toxicity of double-stranded RNA (dsRNA) to the monarch butterfly is described. Dietary toxicity bioassays with a dsRNA that targets the v-ATPase mRNA in monarchs (100% base pair match) and a dsRNA designed to kill varroa mites, but with a 21- nucleotide match with monarch RNA produced no adverse effects in monarch larvae. These results suggest that monarch v-ATPase mRNA could potentially be resilient to dsRNA silencing. Additionally, monarch saliva or gut may contain high levels of RNase, which significantly reduce the amount of dsRNA available for binding to the targeted mRNA. These findings indicate this emerging insecticide technology might pose less risks to monarchs in comparison to conventional, chemical insecticides.

Recommendations for Future Work

The generation of species-specific toxicity and exposure data is needed to generate refined risk characterizations for pollinator and flower-visiting insects. Residue studies focusing on a broader set of pesticides classes in various matrices including soil, plant tissue, pollen, nectar and bee wax would provide more information on the dietary exposure route for honey bees and flowering-visiting insects and better support risk benefit analysis for conservation habitat placement.

Monitoring studies are expensive and time-consuming, requiring large research teams and access to appropriate analytical equipment. Additional research evaluating pesticide uptake, distribution and metabolism in representative native forbs could support the development of physiologically based models for extrapolation of pesticide concentrations in pollen and nectar across plant species. Using these physiological based models could decrease the number of plant species and matrices sampled in future monitoring studies.

Currently, limited toxicity data is available for non-*Apis* bee species. Current research has shown the toxicological endpoints in individual honey bees are protective of non-*Apis* bee species; however, little information is available on how endpoints at the honey bee colony level can be extrapolated to wild bee species especially solitary bees. The development of predictive models such as specie sensitive distributions for various classes of pesticides could allow for more robust risk characterization for bee species without available toxicity data. Further research filling current knowledge gaps on pollinator toxicity and exposure could support development of models that will guide conservation efforts in Iowa and throughout the North Central United States.

References

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