Assessing the risk of insecticide exposures on monarch butterflies

by

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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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ABSTRACT

The North American monarch butterfly (Danaus plexippus), an iconic butterfly species, has significantly declined over the last two decades. In December 2020, it was designated as a candidate species under the Endangered Species Act. Conservation of North America's eastern monarch butterfly population requires establishment of 1.3 to 1.6 billion milkweed (Asclepias spp.) stems in the agricultural landscapes of North Central United States (U.S.). As insecticides are commonly used in this landscape, it is important to assess the risk of insecticide exposure on monarch butterflies. Both foliar and seed treatment insecticide use in North Central U.S. could result in topical and/or dietary exposure to different monarch life stages. Topical and dietary acute and chronic toxicity of six insecticides, encompassing four different modes of action, were studied. Chlorantraniliprole (anthranilic diamide) and beta-cyfluthrin (pyrethroid) were the most toxic while thiamethoxam (neonicotinoid) and chlorpyrifos (organophosphate) were the least toxic. Generally, the larvae and eggs were more sensitive than the adults and pupae. Comparison of toxicity data with modelled and measured environmental insecticide concentrations indicated that foliar applications pose significant risks to monarchs downwind of treated maize or soybean fields. Conversely, seed treatment applications pose little or no risk. The field-scale risk estimates were incorporated into a landscape-scale population model to determine the conservation risks and benefits of establishing milkweed in agricultural landscapes. Toxicity data also were generated for double-stranded RNA (dsRNA) molecules, an emerging class of insecticide products. Monarch larvae were recalcitrant to the dsRNA molecules tested. A review of the literature, including development of species sensitivity distribution models, indicated that while monarch and other lepidopteran larvae were similarly susceptible to the chemical modes of action tested, monarchs were comparatively less susceptible to RNA interference. Finally, a

novel mode of action for neonicotinoid insecticides was evaluated. Five of the seven final-instar lepidopteran larvae treated with neonicotinoids failed to expand their pupal appendages and complete pupal ecdysis. Detailed analyses of arrested ecdysis symptomology suggest that neonicotinoids interfere with the function of crustacean cardioactive peptide neurons; adverse outcome pathways for this effect were proposed. Future avenues of research in the field of insect toxicology and risk assessment include the development and use of in vitro and in silico techniques.

Keywords: Monarch butterfly, Lepidoptera, insecticide, toxicology, risk assessment, conservation, pyrethroid, organophosphate, anthranilic diamide, neonicotinoid, double-stranded RNA, adverse outcome pathways, species sensitivity distributions

CHAPTER 1. GENERAL INTRODUCTION

Background

The monarch butterfly (Danaus plexippus) is one of the most recognizable insects in North America. Their aesthetic beauty, remarkable life cycle, dependence on milkweed (Asclepias spp.), and enigmatic migration pattern make them a fascinating species. Monarchs have played, and continue to play, important roles in science education, insect conservation, and development of curiosity towards the natural world (Oberhauser et al. 2015). While monarchs are not the most efficient pollinators, they contribute to the pollination of some wildflowers (National Park Service 2017). They also serve as indicator species, i.e., the health of monarchs is considered to be a good indication of the health of the environment. In addition, habitat that is established for monarchs will likely benefit other butterflies, bees, and birds (Schulte et al. 2017; USFWS 2020a).

In North America, there are two major migratory populations of monarch butterflies, the eastern and western population, that account for approximately 90% of the worldwide population (USFWS 2020b). Over the last two decades, both populations have declined significantly (Semmens et al. 2016; Pelton et al. 2019). This led several organizations to petition the U.S. Fish and Wildlife Service (USFWS) to list the monarchs as a threatened species under the Endangered Species Act (ESA). In December 2020, the USFWS determined the monarchs warranted federal protection but stated they will prioritize other species that are at a greater risk of endangerment before making a listing decision for the monarchs in 2024. In the meantime, they will annually review the status of the species, which will include an assessment of potential threats and

conservation practices (USFWS 2020b). The major potential threats to monarch recovery in the U.S. include limited availability of milkweed and nectar plants and exposure to insecticides (USFWS 2017).

The need to assess the potential risk of insecticides to monarch butterflies is illustrative of the increasingly complex environmental risk assessments needed to support regulatory programs in the United States (National Academy of Sciences 2013). To meet the mounting demands of information required under Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA), Toxic Substances Control Act (TSCA), and ESA, it is necessary to develop efficient risk assessment processes to evaluate the multitude of existing and newly emerging environmental chemicals on hundreds of species in a timely manner. The assessment process involves gathering hazard and exposure information for chemicals through in vivo testing and in vitro and in silico tools. Conceptual models that link information on species-specific toxicity, demographics, life history, spatial-temporal chemical exposures, and habitat quality requirements can provide the means to estimate population-level responses in a defined landscape (Bradbury et al. 2004). In addition, the development of adverse outcome pathways that connect the molecular initiating event to population-level effects can help predict chemical effects on different species and support ecological risk assessments (Ankley et al. 2009).

Outlines and objectives of the dissertation chapters

This dissertation, along with a related companion paper (Grant et al. 2021), employs several steps in the insecticide risk assessment process to inform monarch butterfly conservation. In addition, preliminary adverse outcome pathways and species sensitivity distributions were generated, which can support risk assessments for other lepidopteran species of conservation concern.

Chapters 2 and 3: Assessing risk of chemical insecticides to the monarch butterfly

While successful recovery of eastern and western monarch populations will require significant conservation efforts, the next two chapters in this dissertation focus on the eastern population. Scientists estimate that the eastern population should occupy an average of 6 hectares of overwintering forest canopy in Mexico to increase the probability of maintaining the annual migration (Semmens et al. 2016). Over the last decade, an average of 2.8 hectares of forest canopy have been occupied (Monarch Watch 2020). As nearly half the monarchs in Mexico originate from the North Central states in the U.S., it is important to increase their production in this region (Flockhart et al. 2017; Oberhauser et al. 2017). Studies suggest that monarch population recovery requires establishing 1.3 to 1.6 billion stems of milkweed, which is the only plant that monarch larvae feed on, in 11 North Central states including Iowa (Pleasants et al. 2015; Thogmartin et al. 2017). As 77% of all potential monarch habitat in these states are on agricultural landscapes, utilization of this landcover type is essential to attain a resilient eastern population (Thogmartin et al. 2017).

Due to the deployment of a diversity of insecticides, herbicides, and fungicides on agricultural land, there is a concern that establishing habitat in agroecosystems may undermine insect pollinator conservation. For example, treated crop fields and surrounding areas may be metapopulation sinks that reduce the population growth of a species (Toppings et al. 2020). Given that monarchs are a vagile species (Zalucki and Lammers 2010; Zalucki et al. 2016), the dynamics of sources and sinks may not apply (Grant and Bradbury 2019; Grant et al. 2021). Therefore, it is essential to understand the costs and benefits of utilizing agricultural areas for monarch conservation on a landscape and population level. To undertake such an analysis, it is first important to generate toxicity and exposure data for commonly used pesticides in North

Central crop fields and assess their risk to monarchs at the field-scale. We have focused on generating these data for insecticides as they are likely to pose a direct risk to monarch butterflies.

More specifically, we assessed the risks of six insecticides, encompassing four modes of action, to monarch eggs, larvae, pupae, and adults. These insecticides are used to manage a wide range of insect pests in corn and soybean fields, the dominant crops grown in North Central U.S. Beta-cyfluthrin (pyrethroid) and chlorpyrifos (organophosphate) are used as foliar insecticides, while chlorantraniliprole (anthranilic diamide) and imidacloprid, thiamethoxam, and clothianidin (neonicotinoids) are used as both foliar and seed treatment insecticides. Spatial and temporal overlaps between monarch presence and insecticide applications in North Central U.S. showed that different monarch stages could be exposed to these insecticides near agricultural fields (see Figure 1 in Chapters 2 and 3). Following exposure, monarchs could present different adverse effects including mortality, reduced growth, and impaired or delayed development. Toxicity data, including dose-response curves, for these effects were generated and compared to exposure concentrations that were derived either from computer modeling (for foliar insecticides) or plant residue data (seed treatment insecticides).

The field-scale risk estimates were then incorporated into a spatially explicit agent-based monarch movement and population model (Grant et al. 2018; Grant and Bradbury 2019; Grant et al. 2020) to estimate landscape-scale population responses (Grant et al. 2021). This analysis accounted for factors like pest species and pressure over 10 years, wind direction, monarch stage-specific behavior, demographics, and natural survival rate, and level of milkweed augmentation. Since the Natural Resource Conservation Service had advocated for a 38-m ‘no

milkweed establishment zone' around insecticide-treated crop fields (NRCS 2016), we used this information to answer the question: Would monarch population growth rate be higher if:

Milkweed was established in all available space in agricultural landscapes, but with a high likelihood of insecticide exposure to monarchs in close proximity to crop fields?

OR

Milkweed was established outside a 38-m 'no establishment' zone around crop fields, but with a lower likelihood of insecticide exposure to monarchs?

As described in Chapters 2, 3, and Grant et al. (2021), we were able to evaluate the costs and benefits of utilizing agricultural areas for monarch conservation, including the extent to which insecticide use impacts adult monarch production in habitat established in close proximity of soybean and maize fields in Iowa.

Chapters 2 and 3 address field-scale risks of insecticide use; the second chapter has been published, and the third chapter is under review in *Environmental Toxicology and Chemistry*. The landscape-scale risk assessment paper that incorporates data from these two chapters is under review in *Integrated Environmental Assessment and Management*.

Chapter 4: Assessing toxicity of double-stranded RNA insecticide on monarch butterfly

While chemical insecticides with reduced risk to mammals, amphibians, and other non-insect species have entered the market, most of these insecticides are broad spectrum, which means they affect both insect pests that damage crops and cause diseases and beneficial insects that pollinate plants and sustain ecosystems. Thus, recent efforts have focused on the development of biological insecticides that use technologies like RNA interference (RNAi) and CRISPR/Cas9 to be more 'precise' in their actions. Using nucleotide base pair information, these

insecticides can specifically target pest insects and minimize risk to beneficial insects (de Andrade and Hunter 2016; Courtier-Orgogozo et al. 2017).

The fourth chapter evaluates the toxicity of RNAi-based insecticides on monarch butterfly larvae through the feeding of two double-stranded RNA (dsRNA) sequences. One sequence targets the calmodulin mRNA in Varroa mites (*Varroa destructor*) to which monarchs have an 81% sequence similarity, including a 21 nucleotide overlap in their calmodulin transcriptome. The other sequence targets monarch V-ATPase mRNA, which results in a 100% nucleotide overlap. Both mRNAs code for proteins which are essential for survival and growth. Larvae were chronically exposed to both dsRNA sequences from neonate to pupation: mortality, growth, and development were assessed. Previous studies have shown that dsRNA molecules are effective if they share 21 contiguous nucleotides with an organism's mRNA (Whyard et al. 2009; Bachman et al. 2013). Consequently, we hypothesized both dsRNA sequences would cause an adverse effect in monarchs by silencing the calmodulin and V-ATPase mRNAs.

Presently, the only registered dsRNA products are plant incorporated protectants and monarchs are unlikely to be exposed to them. However, other kinds of dsRNA products are likely to enter the marketplace soon. These include in-hive products like the Varroa dsRNA employed in this study and foliar dsRNA products that could result in exposure to multiple non-target organisms (Romeis and Widmer 2020), including monarchs, within or near crop fields. In this regard, Chapter 4 addresses potential risk to monarch larvae that are environmentally exposed to a newly emerging insecticide mode of action. This chapter is under review in *PLOS One*.

Chapter 5: Assessing a novel mode of action of neonicotinoid insecticides on Lepidoptera

The rationale for this chapter arose from interesting observations made when monarch butterfly final-instar larvae were treated with neonicotinoid insecticides, as noted in Chapters 2 and 3. At certain neonicotinoid doses, larvae died mid-pupation with no symptoms of poisoning. This failure to complete pupation occurred two to three days after a single topical exposure and was manifested only during larval to pupal molts; larval to larval molts were unaffected. This observation suggests that neonicotinoids are disrupting the ecdysis signaling pathway during pupal metamorphosis, an effect not previously reported in the literature. To understand the mode of action through which neonicotinoids cause this arrest in pupal ecdysis (AE), a series of experiments were carried out in seven Lepidoptera species. This included a) treatment of other final-instar lepidopteran larvae with imidacloprid to understand the extent to which this symptomology is preserved across the order, b) treatment of larvae of different ages with imidacloprid to understand how timing of exposure impacted pupal ecdysis and adult eclosion, c) toxicokinetic experiments to understand differences in imidacloprid metabolism and excretion in AE-sensitive and AE-insensitive species, and d) close observations of ecdysis motor process in control and imidacloprid-treated larvae during pupal molt to better inform hypotheses concerning the mechanism(s) through which neonicotinoids cause AE. Based on a review of the literature that describes current understanding of neuroendocrine control of pupal ecdysis and the results from our studies, we propose two adverse outcome pathways that elucidate how neonicotinoids produce this unique symptomology. This chapter is currently being prepared for submission as a manuscript.

Chapter 6: Comparing insecticide sensitivity of monarchs to those of other Lepidoptera and the honey bee (Apis mellifera)

In this chapter, insecticide sensitivity of monarch butterfly is compared to those of other lepidopteran species and the honey bee. This comparison was done for the five insecticide modes of action that were studied in Chapters 2, 3, 4, and 5; toxicity data for other species were obtained from literature. For two insecticide mode of actions with the most butterfly toxicity data, species sensitivity distribution models (SSDs) were developed. Through the generation of toxicity distributions (Posthuma et al. 2004), SSDs can estimate chemical toxicity for difficult-to-rear and at-risk species. As monarchs are the only ESA-listed lepidopteran candidate species with insecticide toxicity data, their role as a surrogate at-risk species was explored. Challenges in rearing monarch butterflies and shortcomings with evaluating interspecies variability and generating sensitivity distributions with currently available toxicity data are discussed. Parts of this chapter are being prepared for submission to a book on 'Sustainability in Agriculture' sponsored by the American Chemical Society.

Overall contributions of the dissertation

1. **Generation of the most extensive monarch butterfly toxicology database.** Toxicity studies were completed with all monarch life stages (including the pupal stage that has not been tested before in butterflies), across all environmentally relevant routes of exposure, and five modes of action, including a new insecticide class. Conceptual models were designed to indicate possible routes, stages, and effects of insecticide exposure. As the monarch butterfly is a species of conservation concern, in-depth information on one of its important stressors, insecticides, is necessary to formulate effective conservation plans. Monarch sensitivity also was compared with other butterflies, moths, and bees;

effective analysis of interspecies variability can help predict chemical toxicity for similar species that are declining and difficult to rear.

2. **Generation of field and landscape-scale risk estimates that can directly aid in the conservation of North America's eastern monarch butterfly population.** The toxicity data were compared with exposure estimates obtained from spray drift models and milkweed residue data reported in the literature. This was used to assess monarch butterfly field-scale risks within and outside North Central U.S. agricultural fields. These field-scale risk estimates were inputs for a spatially explicit, landscape-scale population model that was used to determine how different milkweed establishment options in agroecosystems influence adult monarch production. This information can help support recovery of the eastern population.
3. **Discovery of a novel mode of action for neonicotinoid insecticides and development of new adverse outcome pathways.** Final-instar lepidopteran larvae treated with neonicotinoids, one of the most widely used insecticides in the world, showed a previously unreported failure to pupate due to arrested ecdysis. This observation indicates the final-instar stage is uniquely susceptible to this class of insecticide. Several experiments were conducted to refine the phenotypic description of how neonicotinoids may cause this novel effect. Based on the results of these studies and published information in the literature, adverse outcome pathways were proposed.

References

Ankley GT, Bennett RS, Erickson RJ, Hoff DJ, Hornung MW, Johnson RD, et al. 2009. Adverse Outcome Pathways: A conceptual framework to support ecotoxicology research and risk assessment. Environ Toxicol Chem. 29 (3), 730–741.

Bachman, PM, Bolognesi R, Moar WJ, Mueller GM, Paradise MS, et al. 2013. Characterization of the spectrum of insecticidal activity of a double-stranded RNA with targeted activity against Western Corn Rootworm (Diabrotica Virgifera LeConte). Transgenic Res. 22 (6), 1207–1222.

Bradbury SP, Feijtel TCJ, Leeuwen CJV. 2004. Peer Reviewed: Meeting the scientific needs of ecological risk assessment in a regulatory context. Environ Sci Technol. 38 (23), 463A-470A.

Courtier-Orgogozo V, Morizot B, Boëte C. 2017. Agricultural pest control with CRISPR-based gene drive: Time for public debate: Should we use gene drive for pest control? EMBO Rep 18 (6), 878–880.

de Andrade EC, Hunter WB. 2016. RNA interference – natural gene-based technology for highly specific pest control (HiSPeC). In RNA Interference; Abdurakhmonov IY. Ed.; InTech,

Flockhart DTT, Brower LP, Ramirez MI, Hobson KA, Wassenaar LI, Altizer S, et al. 2017. Regional climate on the breeding grounds predicts variation in the natal origin of monarch butterflies overwintering in Mexico over 38 years. Glob Change Biol. 23 (7), 2565–2576.

Grant TJ, Parry HR, Zalucki MP, Bradbury SP. 2018. Predicting monarch butterfly (Danaus Plexippus) movement and egg-laying with a spatially-explicit agent-based model: The role of monarch perceptual range and spatial memory. Ecological Modelling. 374, 37–50.

Grant TJ, Bradbury SP. 2019. The role of modeling in monarch butterfly research and conservation. Front Ecol Evol. 7, 197.

Grant TJ, Flockhart DTT, Blader TR, Hellmich RL, Pitman GM, Tyner S, et al. 2020. Estimating arthropod survival probability from field counts: a case study with monarch butterflies. Ecosphere. 11 (4).

Grant TJ, Krishnan N, Bradbury SP. 2021. Conservation risks and benefits of establishing monarch butterfly (*Danaus plexippus*) breeding habitat in close proximity to maize and soybean fields in the U.S. Midwest: A landscape-scale analysis of foliar insecticide effect on non-migratory monarch butterfly populations. Integr Environ Assess Manag. *Under Review*.

Monarch Joint Venture. 2020. Eastern monarch butterfly population numbers. Available at: https://monarchjointventure.org/blog/eastern-monarch-population-numbers-decrease-53-from-last-year

National Academy of Sciences. 2013. Assessing risks to endangered and threatened species from pesticides. Available at: https://www.nap.edu/catalog/18344/assessing-risks-to-endangered-and-threatened-species-from-pesticides

Natural Resources Conservation Service. 2016. Monarch butterfly wildlife habitat evaluation guide and decision support tool: Midwest Edition 1.1. Available at: https://www.nrcs.usda.gov/wps/portal/nrcs/detail/national/plantsanimals/pollinate/?cid=nrcseprd402207.

National Park Service. 2017. Pollinators - Monarch Butterfly. Available at: https://www.nps.gov/articles/monarch-butterfly.htm

Oberhauser KS, Nail KR, Altizer S. 2015. Monarchs in a changing world: biology and conservation of an iconic butterfly. Ithaca: Cornell University Press.

Oberhauser K, Wiederholt R, Diffendorfer JE, Semmens D, Ries L, Thogmartin WE, et al. 2017. A trans-national monarch butterfly population model and implications for regional conservation priorities: regional monarch conservation priorities. Ecol Entomol. 42 (1), 51–60.

Pelton EM, Schultz CB, Jepsen SJ, Black SH, Crone EE. 2019. Western monarch population plummets: status, probable causes, and recommended conservation actions. Front Ecol Evol. 7, 258.

Pleasants JM. 2015. Monarch butterflies and agriculture. In Oberhauser KS, Nail KR, Altizer S, eds, Monarchs in a changing world: biology and conservation of an iconic butterfly. Cornell University, Ithaca, NY, USA, pp 169–178.

Posthuma L, Traas TP, Suter GW. 2000. General introduction to species sensitivity distributions. In Species sensitivity distributions in ecotoxicology, Lewis, Boca Raton, FL, USA.

Romeis J, Widmer F. 2020. Assessing the risks of topically applied dsRNA-based products to non-target arthropods. Front. Plant Sci. 11, 679.

Schulte LA, Niemi J, Helmers MJ, Liebman M, Arbuckle JG, James DE, et al. 2017. Prairie strips improve biodiversity and the delivery of multiple ecosystem services from corn–soybean croplands. Proc Natl Acad Sci USA. 114 (42), 11247–11252.

Semmens BX, Semmens DJ, Thogmartin WE, Wiederholt R, López-Hoffman L, Diffendorfer JE, et al. 2016. Quasi-extinction risk and population targets for the eastern, migratory population of monarch butterflies (Danaus Plexippus). Sci Rep. 6 (1), 23265.

Thogmartin WE, López-Hoffman L, Rohweder J, Diffendorfer J, Drum R, Semmens D, et al. 2017. Restoring monarch butterfly habitat in the Midwestern U.S.: 'All hands on deck.' Environ Res Lett. 12 (7), 074005.

Topping CJ, Aldrich A, Berny P. 2020. Overhaul environmental risk assessment for pesticides. Science. 367: 360-363.

U.S. Fish and Wildlife Service. 2017. Monarch Butterfly Species Assessment Update. https://www.fws.gov/savethemonarch/pdfs/Monarch.pdf

U.S. Fish and Wildlife Service. 2020a. Save the monarch butterfly. Available at: https://www.fws.gov/savethemonarch/individual.html

U.S. Fish and Wildlife Service. 2020b. Species Status Assessment Report. Available at: https://www.fws.gov/savethemonarch/pdfs/Monarch-SSA-report.pdf

Whyard S, Singh AD, Wong S. 2009. Ingested double-stranded RNAs can act as species-specific insecticides. Insect Biochem Mol Biol. 39 (11), 824–832.

Zalucki MP, Lammers JH. 2010. Dispersal and egg shortfall in monarch butterflies: what happens when the matrix is cleaned up? Ecol Entomol. 35 (1), 84–91.

Zalucki MP, Parry HR, Zalucki JM. 2016. Movement and egg laying in monarchs: to move or not to move, that is the equation: movement and egg laying. Austral Ecology. 41 (2), 154–167.

CHAPTER 2. ASSESSING FIELD-SCALE RISKS OF FOLIAR INSECTICIDE APPLICATIONS TO MONARCH BUTTERFLY *(DANAUS PLEXIPPUS*) LARVAE

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Abstract

Establishment and maintenance of milkweed plants (*Asclepias* spp.) in agricultural landscapes of the North Central United States is needed to reverse the decline of North America's eastern monarch butterfly (*Danaus plexippus*) population. Due to lack of toxicity data it is unclear how insecticide use may reduce monarch productivity when milkweed habitat is placed near maize and soybean fields. To assess the potential effects of foliar insecticides, acute cuticular and dietary toxicity of five representative active ingredients were determined: beta-cyfluthrin (pyrethroid), chlorantraniliprole (anthranilic diamide), chlorpyrifos (organophosphate), and imidacloprid and thiamethoxam (neonicotinoids). Cuticular LD50 values for first instars ranged from 9.2×10^{-3} to 79 μg/g larvae for beta-cyfluthrin and chlorpyrifos, respectively. Dietary LC50 values for second instars ranged from 8.3×10^{-3} to 8.4 μg/g milkweed leaf for chlorantraniliprole and chlorpyrifos, respectively. To estimate larval mortality rates downwind from treated fields, modeled insecticide exposures to larvae and milkweed leaves were compared to dose-response curves obtained from bioassays with first-, second-, third-, and fifth-instar larvae. For aerial applications to manage soybean aphids, mortality rates at 60 m

downwind were highest for beta-cyfluthrin and chlorantraniliprole following cuticular and dietary exposure, respectively, and lowest for thiamethoxam. To estimate landscape-scale risks, field-scale mortality rates must be considered in context of spatial and temporal patterns of insecticide use.

Keywords: Monarch butterfly; Insecticides; Toxicology; Risk assessment; Conservation

Introduction

In North America, the eastern population of monarch butterflies (*Danaus plexippus*) has declined significantly in the last two decades (Brower et al. 2012; Oberhauser 2017). The historically low overwintering monarch population reported in 2013-14, combined with the two-decade trend, prompted a petition to the United States Fish and Wildlife Service (USFWS) to list the monarch as a threatened species under the Endangered Species Act (USFWS 2016). From 2004 to 2018, the eastern population occupied an average of 3.46 hectares of overwintering forest canopy (Monarch Watch 2019). This level is well below a long-term average of six hectares that is needed to support a resilient population and mitigate the potential loss of the North American migration (Semmens et al. 2016). Approximately 40 to 50% of the monarchs overwintering in Mexico originate in the North Central U.S. (Flockhart et al. 2017) and it is vital to improve summer breeding success in this region (Oberhauser et al. 2017). To maintain a resilient monarch population, an estimated 1.3 to 1.6 billion additional milkweed stems need to be added to the North Central U.S. landscape (Thogmartin et al. 2017). Milkweed species (*Asclepias* spp.), and primarily common milkweed (*Asclepias syriaca*) in the North Central states (Malcolm et al. 1993), are obligate hosts for monarch larvae. The habitat goal for the North Central U.S. can only be met through a significant conservation effort in agricultural landscapes, including rural roadsides, marginal crop land, portions of existing Conservation Reserve

Program (CRP) land, pastures and grassy areas bordering maize and soybean fields (Thogmartin et al. 2017).

In the North Central U.S., monarch larvae are present from mid-May to late September (Prysby and Oberhauser 2004; Pleasants 2015; Nail et al. 2015) and could be exposed to insecticides used to manage early- and late-season pests in conventional maize and soybean production, which are the dominant crops in the region (see Figure 1). Soybean aphid (*Aphis glycines*) is a major late-season pest of soybean (Hodgson et al. 2012) and true armyworm (*Mythimna unipuncta*) is an emerging early-season pest in maize fields containing rye cover crops (Dunbar et al. 2016). These pests are managed with pyrethroid, organophosphate, or neonicotinoid foliar applications (Hodgson et al. 2012; Dunbar et al. 2016). The percentage of maize and soybeans treated with foliar or soil-applied formulations in the North Central states range from 8% in Kansas, Minnesota, and Michigan to 20% in Illinois and from 6% in Michigan to 30% in Minnesota, respectively (USDA 2018). Nationally, at least 79% of maize and 34% of soybeans are planted with neonicotinoid-treated seeds (Douglas and Tooker 2015). Consistent with these use patterns, neonicotinoids have been detected in milkweed growing near maize and soybean fields (Olaya-Arenas et al. 2019). Chlorantraniliprole, an anthranilic diamide, recently entered the market in both foliar and seed treatment formulations (Thrash et al. 2013; Carscallen et al. 2019).

The USFWS has identified insecticide exposure as one of the potential threats to monarch butterfly recovery (USFWS 2017). In 2016 and 2017, the U.S. Department of Agriculture National Resources Conservation Service's (NRCS) *Monarch Butterfly Wildlife Habitat Evaluation Guide* discouraged placement of monarch breeding habitat within 38 m of crop fields treated with herbicides or insecticides (NRCS 2016). Employing a "no habitat buffer" of this size

would significantly reduce the area of land available for establishing breeding habitat and hectares of small habitat patches (e.g. 0.4 to 2.0 hectare) that are crucial for supporting increased monarch egg densities across the landscape (Zalucki et al. 2016; Grant et al. 2018). For example, in Story County, Iowa, a 38-m buffer around conventional maize and soybean fields represents approximately 84% of rural roadside rights-of-ways and 38% of grassland, CRP land, pastures, railroad rights-of-way, riparian corridors, and wetlands.

We are developing a landscape-scale approach (Uhl and Brühl 2019; Grant and Bradbury 2019) to test the hypothesis that conservation benefits of establishing monarch breeding habitat in close proximity to maize and soybean fields will outweigh the risks of increased insecticide exposure. However, the current paucity of insecticide toxicity data precludes the means to assess field-scale and landscape-scale mortality rates. Consequently, we are undertaking a series of acute and chronic toxicity studies that are relevant for foliar and seed treatment insecticide formulations. Here we report larval acute contact and dietary toxicity of five insecticides registered for foliar applications to manage early- and late-season insect pests in maize and soybean fields: beta-cyfluthrin (a pyrethroid), chlorantraniliprole (an anthranilic diamide), chlorpyrifos (an organophosphate), and imidacloprid and thiamethoxam (neonicotinoids). Using data from these toxicity studies and exposure estimates obtained from spray drift modelling, we predict larval mortality rates from the edge of a treated field to 60 m downwind following aerial and ground boom applications.

Materials and methods

Monarch butterfly rearing

Monarch colonies at Iowa State University are maintained by the U.S. Department of Agriculture (USDA), Corn Insects and Crop Genetics Research Unit in Ames, Iowa. Every

spring and summer from 2014 through 2017, monarch butterfly eggs were collected from common milkweed plants in rural roadsides and Iowa State University farms in Boone and Story Counties, Iowa to establish 2014, 2015, 2016, and 2017 colonies. Adult male and female monarchs, obtained from the respective colonies, were housed in aluminum frame cages (~60 cm x 60 cm x 60 cm) with brass screens (14 x 18 mesh). Stems of tropical milkweed (*Asclepias curassavica*) with leaves, and occasionally flowers, were placed in the cages to facilitate egg laying. After 3 to 4 hours, the stems were removed and kept for three days in an I-35VL incubator (Percival Scientific, Perry, Iowa, USA) maintained at 21.1ºC, 65% relative humidity, and a 16:8 light : dark cycle. On Day 4, eggs were moved to another incubator maintained at 26.6ºC (65% relative humidity and 16:8 light: dark cycle) to induce hatching. Newly hatched larvae (0 to 12-h old) were individually plated onto petri plates (60 mm x 15 mm) with a thin layer of 2% agar : water and a freshly picked and surface-sterilized (washed in 10% bleach : water solution, followed by three water rinses) milkweed leaf. The larvae were reared in the 26.6ºC incubator and fed additional tropical milkweed leaves *ad libitum*, except from June through September when larvae were raised on freshly picked and surface-sterilized common milkweed leaves collected from non-agricultural sites in Story and Boone Counties, Iowa. On Day 11, individual larvae were transferred to 8 oz. Comet plastic tumblers (Waddington North America, Covington, Kentucky, USA) inverted over an open petri plate (100 mm x 15 mm) fitted with a 90 mm disk of Whatman No. 1 filter paper (GE Healthcare, Chicago, Illinois, USA). When the larvae initiated pupation (typically Days 15 to 17), they were held at room temperature. After eclosion (typically Days 29 to 32), butterflies were screened for *Ophryocystis elektroscirrha*, using the method described by Altizer et al. (2000); infected individuals were sacrificed. Adult monarchs were provided Gatorade Glacier Cherry Frost Thirst containing sugar

and dextrose (The Gatorade Company, Inc., Pryor, Oklahoma, USA) as a nutritional source. Toxicity bioassay studies were undertaken with the 2014 and 2015 colonies in 2017, 2018, and the first half of 2019. The cumulative survival from egg stage through pupation when bioassays were undertaken ranged from approximately 75 to 80%.

Milkweed production

Tropical milkweed used to support the colonies and bioassay studies were grown from seed (Johnny's Selected Seeds; Winslow, Maine, USA) in Iowa State University greenhouses at 10 to 41ºC with a 16:8 light : dark cycle. Seeds were planted in 128-cell plug trays with potting soil (F1-P potting mix, Sun Gro Horticulture, Agawam, Massachusetts, USA) mixed with a fertilizer (Osmocote Pro 19-5-8 + Minors, Hummert International, Earth City, Missouri, USA; 500 g per 79 liters of soil). After approximately six weeks, one or two plants were transplanted to 8.9-cm square pots or 3.8-liter pots, respectively. Plants were watered twice a day, which included one watering with liquid fertilizer (Peters Professional Peat Lite Special 20-10-20, ICL Specialty Fertilizers, Dublin, Ohio, USA; 100 mg/L nitrogen). To manage oleander aphids (*Aphis nerii*) and western flower thrips (*Frankliniella occidentalis* Pergande), we released parasitic wasps (*Aphidius colemani*), predatory mites (*Neoseiulus californicus* and *Phytoseiulus persimilis*), and rove beetles (*Dalotia coriaria*) on a regular basis.

Insecticides

Toxicity studies were conducted with the following analytical grade insecticides (IUPAC name; CAS number; percentage purity): beta-cyfluthrin ([(*R*)-cyano-(4-fluoro-3-phenoxyphenyl)methyl] (1*S*)-3-(2,2-dichloroethenyl)-2,2-dimethylcyclopropane-1-carboxylate; 1820573-27-0; 99.3%), chlorantraniliprole (5-bromo-*N*-[4-chloro-2-methyl-6-

(methylcarbamoyl)phenyl]-2-(3-chloropyridin-2-yl)pyrazole-3-carboxamide; 500008-45-7; 97.3%), chlorpyrifos (diethoxy-sulfanylidene-(3,5,6-trichloropyridin-2-yl)oxy-λ^5-phosphane; 2921-88-2; 99.3%), imidacloprid (*N*-[1-[(6-chloropyridin-3-yl)methyl]-4,5-dihydroimidazol-2-yl]nitramide; 138261-41-3; 100%), and thiamethoxam (*N*-[3-[(2-chloro-1,3-thiazol-5-yl)methyl]-5-methyl-1,3,5-oxadiazinan-4-ylidene]nitramide; 153719-23-4; 99.3%). Chlorantraniliprole was provided by DuPont Crop Protection (Johnston, Iowa, USA). The remaining compounds were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). To prepare insecticide stock solutions for cuticular and dietary bioassays, certified ACS reagent grade acetone and Silwet L-77 were purchased from Fisher Scientific (Hampton, New Hampshire, USA).

Toxicity studies

Foliar insecticide applications can result in spray drift landing directly on the larvae (cuticular exposure) and/or on the milkweed (dietary exposure). Toxicity studies were undertaken to mimic these two routes of exposure. Cuticular toxicity studies were undertaken using first-, third-, and fifth-instar larvae. Dietary toxicity studies were undertaken with second-, third-, and fifth-instar larvae. First instars were not used in the dietary studies because of their sensitivity to handling required to execute these bioassays. Individual larvae were held in petri plates (first to fourth instars) or plastic tumblers (fourth and fifth instars), as described previously (see *Monarch butterfly rearing*) and maintained at 26.6ºC, 65% relative humidity, on a 16:8 light : dark cycle. For both bioassays, at least five insecticide concentrations and an appropriate control carrier were used. Eleven larvae were used in each concentration and studies were repeated three or four times. Half of the control larvae were weighed prior to treatment; average weights at the time of treatment for cuticular and dietary studies were calculated (Table S1). All bioassays were performed with tropical milkweed. A subset of bioassays was repeated using

common milkweed to determine if milkweed species influenced larval sensitivity. Mortality, growth, reduced feeding, signs of intoxication (e.g. spasms, paralysis, loss of hemolymph), arrested ecdysis, and malformed or discolored pupae were recorded every 24 h. Observations were made up to 96 h for first, second, and third instars; fifth instars were observed to pupation. At the end of 96 h or pupation, weights and developmental stage of the surviving larvae or pupae were noted. Only data obtained from individual bioassays that had less than 30% control mortality were analyzed (94 of 116 initiated bioassays; mean control mortality 10%; range 0% to 27%).

Cuticular toxicity studies: Insecticide stock solutions were prepared in acetone. One µL of an insecticide-acetone solution was placed on the dorsal prothorax using a 50-µL Hamilton syringe (Reno, Nevada, USA). Control larvae were treated with acetone alone. Insecticide stock solution concentrations and subsequent serial dilutions (three-fold or ten-fold) were based on the results of range-finding assays. The measured concentrations of stock solutions were within 75% to 125% of their nominal concentrations. The nominal (measured) stock solution concentrations for beta-cyfluthrin, chlorantraniliprole, imidacloprid, thiamethoxam, and chlorpyrifos were 1 (0.803) µg/µL, 1 (0.810) µg/µL, 10 (9.94) µg/µL, 40 (30.2) µg/µL, and 60 (68.7) µg/µL, respectively. Measured stock solution concentrations were determined by UHPLC-MS/MS or GC-ECD (see Analysis of insecticide stock solutions). Nominal concentrations were used to derive dose-response curves (see Table S2 and Statistical analysis).

Dietary toxicity studies: Larvae were reared on insecticide-treated tropical milkweed leaves for 48 (second and third instars) or 24 h (fifth instars). Second and third instars surviving the exposure period were then fed untreated leaves ad libitum for an additional 48 h. Surviving fifth instars were fed untreated leaves to pupation. See Table S3 for concentrations of insecticide

stock solutions and serial dilutions used in the bioassays. Individual second, third, and fifth instars were provided 0.075 to 0.125, 0.350 to 0.450, and 1.8 to 2.2 g of leaf tissue, respectively. Five, 20 or 100 µL of an insecticide suspension made in 0.1% silwet : water were pipetted on the top surfaces of the leaves (control leaves were treated with 0.1% silwet : water). The insecticide leaf concentrations used to derive concentration-response curves can be found in Table S4. Treated leaves were dried for five minutes and then provided to the larvae. Leaves were photographed prior to treatment and their surface areas were calculated using ImageJ software (National Institute of Health, USA) and task-specific code written in Python using the OpenCV computer vision library (Tripathy 2019).

Analysis of insecticide stock solutions

The insecticide acetone solutions and 0.1% silwet : water suspensions for the neonicotinoids and chlorantraniliprole were analyzed using UHPLC-MS/MS with a Vanquish Flex UHPLC system, including a binary pump, autosampler, and column heater compartment, and a TSQ Altis triple quadrupole mass spectrometer equipped with a heated electrospray source (Thermo Fisher Scientific, San Jose, California, USA). The methods used were as described by Hall et al. (2020), except UHPLC-MS/MS analyses of chlorpyrifos 0.1% silwet : water suspensions employed a Hypersil GOLD Aq column (dimensions 100 x 2.1 mm; particle size 1.9 µm; Thermo Fisher Scientific). The binary mobile phases were water : methanol (98:2, v/v) containing 0.1% formic acid and 5 mM ammonium formate (A) and methanol : water (98:2, v/v) containing 0.1% formic acid and 5 mM ammonium formate (B). Acetone solutions and 0.1% silwet-water suspensions were diluted with acetonitrile prior to injection. The injection volume was 2 µL for the neonicotinoids and chlorantraniliprole and 1 µL for chlorpyrifos. Acetone solutions and 0.1% silwet : water suspensions of beta-cyfluthrin were analyzed by GC-ECD.

Depending on the nominal concentration of a spike solution, a 10 µL or 100 µL aliquot was concentrated to dryness and then brought up to an appropriate volume with ethyl acetate. Concentrations of beta-cyfluthrin were determined using an Agilent 7890B GC equipped with a Ni^{63} micro electron-capture detector (Agilent, Santa Clara, California, USA) and a Restek Rtx®-5MS w/Integra-Guard® (30 m x 0.25 mm ID x 0.25 µm) column. Helium was used as a carrier gas at a flow rate of 1 mL/min and the make-up gas was 5% methane and the remainder were argon at 60 mL/minute. The initial column temperature was 100˚C and held for 1 minute. The temperature was then raised to 250˚C at a rate of 25˚C/minute, held for one minute, and then raised to a final temperature of 300˚C (10˚C/minute), which was held for 10 minutes. Both the inlet and detector temperatures were 250˚C. Beta-cyfluthrin's retention time was 14.4 minutes. Measured concentrations of 0.1% silwet : water insecticide suspensions are provided in Table S3.

Estimated insecticide exposure and field-scale mortality

Estimated insecticide concentrations deposited on larval and milkweed surfaces following foliar applications were obtained using the Tier I Aerial and Ground models within AgDRIFT version 2.1.1 (USEPA 2003) for the following representative formulated products (active ingredients; EPA registration number): Baythroid® XL (beta-cyfluthrin; 264-840), Admire Pro® (imidacloprid; 264-827), Swagger® (imidacloprid and bifenthrin; 34704-1045), Lorsban® (chlorpyrifos; 62719-220), Beseige® (chlorantraniliprole and lambda-cyhalothrin; 100-1402) and Endigo® (thiamethoxam and lambda-cyhalothrin; 100-1276). Assuming a wind speed of 10 mph (maximum wind speed allowed as per label language), concentrations of active ingredients deposited at 0, 15, 30, and 60 m from the edge of the application area were determined using maximum application rates to manage soybean aphids and true armyworms.

Aerial and high ground boom application scenarios were used for soybean aphid applications. For true armyworm, an early-season pest, low and high ground boom scenarios were modeled. Consistent with label instructions, a medium to coarse droplet size was selected for aerial applications and a fine to medium/coarse droplet size was selected for ground applications. Fiftieth percentile model estimates, which exclude outlier and high wind speed effects, were used for ground applications (Table S5).

To estimate larval mortality from cuticular exposure following a spray event, the initial average deposition (µg of insecticide deposited/cm^2 of area) obtained from AgDRIFT was compared to cuticular bioassay dose-response curves, with dose expressed as µg of insecticide/cm^2 larva. Larval surface area was estimated using the cylindrical surface area formula ($2\pi rh + 2\pi r^2$). The radius and height represent the thickness and length of the larvae, respectively.

To estimate larval mortality from dietary exposure to milkweed leaves, the predicted initial average insecticide deposition (µg of insecticide deposited/cm^2 of area) was compared to dietary bioassay concentration-response curves, with concentration expressed as µg of insecticide/cm^2 leaf. Average leaf surface area (and weights) provided to larvae are presented in Table S13.

Statistical analyses

All statistical analyses were done in RStudio 1.1.383 (R version 3.5.2). The “drc” package (version 3.0.1; a nonlinear least square model) was used to generate dose- (or concentration-) response curves and LC/LD values if the data met the assumption of normality. If the data did not meet this assumption, a “mle” maximum likelihood estimate model was used (Dixon et al. 2020). Abbott’s formula was used to account for control mortality. ANOVA was

used to analyze final larval weights and percentage adult eclosion in treatment groups that had less than 70% larval or pupal mortality; when treatment effects were significant post-hoc tests with Dunnett's comparisons were employed.

Results

Cuticular bioassays

Acute cuticular LD10, LD50, and LD90 values for first-, third-, and fifth-instar larvae are provided in Table 1. Based on a comparison of LD50 values and 95% confidence intervals (CIs), beta-cyfluthrin and chlorantraniliprole were the most toxic insecticides (across all instars, LD50 values range from 9.2 x 10-3 µg/g larvae to 4.8 x 10-2 µg/g and 1.2 x 10-2 µg/g to 0.19 µg/g, respectively). Chlorpyrifos was the least toxic to first instars (LD50 of 79 µg/g), and thiamethoxam was the least toxic to fifth instars (35 µg/g; Figure 2). When LD50 values are expressed on a µg/cm2 larva and µg/larva basis, the first instars tend to be the most sensitive (typically 95% CIs do not overlap with CIs of older instars), followed by third and fifth instars (Tables 2 and S6 and Figures S1 and S2). A subset of bioassays were undertaken with common milkweed and results compared to tropical milkweed bioassay toxicity values; LD50 values and associated 95% CIs are provided in Table S10. Responses were similar between the plant species. Except for imidacloprid, ratios of tropical milkweed to common milkweed LD50 values ranged from 0.91 to 1.9, with overlapping 95% CIs. The tropical milkweed imidacloprid LD50 value was 2.3-fold higher (upper bound common milkweed 95% CI = 2.0 µg/larva and lower bound tropical 95% CI= 2.2 µg/larva; this difference is not considered biologically significant).

For all insecticide exposure levels that caused less than 70% larval mortality, there were no differences in final weights between control and surviving insecticide-treated larvae at p = 0.01 level of significance, however at p = 0.05, third instars treated with chlorantraniliprole

weighed less than controls ($p = 0.0092$ for 2.21×10^{-2} μg/g chlorantraniliprole, based on Dunnett's multiple comparison test; Table S7). A slight delay in development was observed when third instars were treated with 2.21×10^{-3} and 2.21×10^{-2} μg/g chlorantraniliprole; at 96 h, the majority of treated larvae (52 to 54%) were third or fourth instars, while the majority of control larvae (60%) were fifth instars. Adult eclosion rates for insecticide-treated and control fifth instars were not significantly different ($p > 0.54$) (Table S8).

Most insecticide-treated first and third instars died within 0 to 48 h after treatment. When fifth instars were treated with beta-cyfluthrin and chlorantraniliprole, mortality generally occurred 0 to 72 h post-exposure and before ecdysis. However, mortality in fifth instars treated with neonicotinoids, and to a lesser extent chlorpyrifos, typically occurred during ecdysis (72 to 96 h after application) and was characterized by a cessation in pupa formation. Larvae died in transition to the pupal stage (suspended in a "J" shape) or after excreting molting fluid. Before onset of pupation, treated larvae rarely showed signs of intoxication. This symptomology was observed with 92%, 87%, and 18% of moribund fifth instars treated with imidacloprid, thiamethoxam, and chlorpyrifos, respectively (Table S9). Dissected fifth instars that exhibited arrested ecdysis had pupal cuticle with adult features; however, the wing buds were not expanded. We also observed melanization in the hemolymph. In subsequent experiments, third instars were treated with the same doses as used in the fifth-instar bioassays and the surviving larvae successfully pupated. Arrested ecdysis also was observed in the imidacloprid fifth-instar bioassays with common milkweed. While arrested ecdysis was observed occasionally in control larvae and in the colony-reared larvae, the rates are much lower than what was observed with the neonicotinoid treatments (Table S9).

At imidacloprid doses of 0.944, 2.98, and 9.44 µg/g larva, all mortality was associated with arrested ecdysis. Prior to ecdysis, most of the treated larvae did not exhibit signs of intoxication and maintained feeding. The 9.44 µg/g dose elicited 91% mortality, all through arrested ecdysis. However, in range-finding assays, all ten fifth instars treated with approximately 100 µg/g larva showed signs of intoxication at 24 h and died prior to ecdysis. These observations indicate that doses that elicited nearly 100% mortality associated with arrested ecdysis are 10 times lower than doses that caused 100% mortality prior to ecdysis, suggesting there may be two modes of action associated with neonicotinoids.

Though clothianidin is not registered for foliar uses in maize and soybean, we undertook range-finding bioassays to compare responses to the other neonicotinoids (Table S11). Clothianidin was more toxic (non-overlapping 95% CIs) than imidacloprid and thiamethoxam, with LD50 values of 0.19, 0.83, and 1.3 µg/g larva for first, third, and fifth instars, respectively (Table S12 and Figure S3). Clothianidin-treated fifth instars also exhibited arrested ecdysis.

Dietary bioassays

Acute dietary LC10, LC50, and LC90 values, and associated 95% CIs for second-, third-, and fifth-instar larvae are provided in Table 3. Chlorantraniliprole was the most toxic insecticide (95% CIs do not overlap with other insecticide CIs) for second (LC50 of 8.3×10^{-3} µg/g leaf) and third instars (LC50 of 4.6×10^{-2} µg/g leaf). Chlorpyrifos, imidacloprid, and thiamethoxam were similarly toxic to second (LC50 values range from 3.5 to 8.4 µg/g leaf) and fifth instars (LC50 values range from 9.4 to 33 µg/g leaf; Table 3 and Figure 3). When toxicity values were reported on a µg/cm^2 leaf basis, 95% CIs also overlapped with these insecticides (Table 4 and Figure S4). Results of select bioassays with common milkweed leaves are provided in Table S16. Leaf

concentrations expected to elicit 50% mortality, based on results of tropical milkweed bioassays, caused 42% to 70% larval mortality. These rates of mortality are within the ranges expected based on the tropical milkweed 95% CIs.

At insecticide concentrations that caused less than 70% larval mortality, the final weights of surviving larvae were significantly lower than larvae fed control leaves in several instances. Reduced weight was typically seen in third instars, where it was often associated with delayed development (Table 5). Adult eclosion rates for treated and control fifth instars were not significantly different ($p > 0.19$) (Table S14). In two of the 15 bioassays, the eclosion rates were suppressed, in part, due to pupal infection observed in both control and treated fifth instars.

With dietary exposure, the rate of arrested ecdysis was less than observed following cuticular exposure. Monarch fifth instars treated with chlorantraniliprole, beta-cyfluthrin, and chlorpyrifos had low rates of arrested ecdysis (10%, 5%, and 2%, respectively). The rate of arrested ecdysis was 16% and 21% with imidacloprid and thiamethoxam treatments, respectively (Table S15). The dietary bioassays, like the cuticular bioassays, were carried out with early fifth instars (approximately 24 h-old). However, when late fifth instars (approximately 72 h-old) were exposed to neonicotinoids through their diet, the rate of arrested ecdysis and corresponding mortality increased. For example, when early fifth instars fed on a concentration of 0.78 µg of imidacloprid/g leaf, 10% died (Table 5). However, when this concentration was provided to late fifth instars, 82% of the larvae died, with 89% of the mortality occurring due to arrested ecdysis.

Results of dietary bioassays with clothianidin were similar to that of imidacloprid and thiamethoxam for second and third instars (overlapping 95% CIs), with LC50 values of 4.2 and 7.8 µg/g leaf, respectively. Clothianidin-treated fifth instars were more sensitive than thiamethoxam-treated fifth instars, producing a LC50 value of 0.80 µg/g leaf (Table S19 and

Figure S5). These values were calculated using measured clothianidin stock solution concentrations and estimated leaf concentrations (Tables S17 and S18). As with the other neonicotinoids, treated larvae showed reduced larval growth and development in a few instances; there was no effect on adult eclosion (Table S20).

Field-scale mortality assessments

Larval cuticular exposure: When aerial applications for beta-cyfluthrin and chlorantraniliprole were modeled for soybean aphid management, predicted monarch larval mortality was between 100% and 32% at all modeled distances (0, 15, 30, and 60 m downwind from the field). Chlorpyrifos, imidacloprid, and thiamethoxam were estimated to cause 99%, 91%, and 67% mortality, respectively to the most sensitive larval instar at the edge of field. There was 0% to 2% mortality predicted for these insecticides at 60 m downwind (Figure 4A). Similar trends were seen with insecticide applications using a high ground boom. However, due to reduced off-site drift, lower mortality was predicted at 15, 30, and 60 m downwind compared to aerial applications, but greater larval mortality was observed at 0 m (Figure 4B). Modeled high and low ground boom applications to manage true armyworm infestations produced similar mortality patterns (Figures 4C and 4D). Ninetieth percentile results for ground applications, to capture worse-case drift scenarios, are provided in Figure S6. Over all the scenarios, the mortality rate was generally highest for the first instars and lowest for fifth instars.

Larval dietary exposure: When beta-cyfluthrin and chlorantraniliprole exposures were modeled for aerial applications to manage soybean aphids, predictions for monarch larval mortality were between 100% and 10% at all modeled distances downwind from the field (0, 15, 30, and 60 m). Chlorpyrifos, imidacloprid, and thiamethoxam were estimated to cause 96%, 80%, and 83% mortality, respectively to the most sensitive larval instar at the edge of field. They caused 64%,

13%, and 3% mortality to the most sensitive larval instar at 60 m downwind (Figure 5A). Similar trends were seen with insecticide applications using a high ground boom; however, due to reduced off-site drift, lower mortality was predicted compared to aerial applications (with the exception of 0 m; Figure 5B). High and low ground boom applications to manage true armyworm infestations produced similar mortality patterns (Figures 5C and 5D). Ninetieth percentile results for ground applications are provided in Figure S7. Over all the scenarios, mortality rates were generally highest for the second instars and lowest for third or fifth instars.

Discussion

Foliar insecticide applications to manage late- and early-season pests can occur when monarch larvae are found in significant numbers in the North Central states (Figure 1). In Iowa, mid- to late-season pests that can require foliar applications include soybean aphids, European corn borers [(*Ostrinia nubilalis*); Hodgson and Rice 2017], adult western and northern corn rootworms [(*Diabrotica virgifera virgifera* and *Diabrotica barberi*); Gassmann and Weber 2016], and corn aphids [(*Rhopalosiphum maidis*); Hodgson 2018)]. The true armyworm is an example of a re-emerging, early-season pest that is associated with the increased use of cover crops (Dunbar et al. 2016). While pyrethroids and organophosphates are the most commonly used foliar insecticides in soybean fields, neonicotinoids and diamides also are being used (Hodgson et al. 2012; Whalen et al. 2016). Potential risk of foliar insecticide applications to monarch larvae is a function of insecticide toxicity and exposure. Exposure is a function of habitat proximity to treated maize or soybean fields, wind speed and direction at time of foliar application, and the nature and extent of insecticide use patterns within and across growing seasons.

Insecticide toxicity

Cuticular and dietary LD/LC50 values for third-instar monarchs found beta-cyfluthrin and chlorantraniliprole to be approximately 10 to 1000-fold more toxic than chlorpyrifos, imidacloprid, and thiamethoxam. Cuticular LD50 values across larval instars for a given insecticide were generally within a factor of 10. For all the insecticides, except chlorantraniliprole, dietary LC50 values across larval instars were within a factor of 10. Fifth instars were approximately 100 times less sensitive to chlorantraniliprole than second instars. Following cuticular exposure to all the insecticides, and dietary exposure to chlorpyrifos, minimal to no adverse effects on growth and development in surviving larvae were observed at doses or concentrations that caused less than 70% larval mortality. Following dietary exposure to the other insecticides, surviving third instar larvae frequently weighed significantly less than controls (1.1- to 2.9-fold lower) and developed slower. There were no adverse effects on adult eclosion for surviving larvae following cuticular or dietary exposures.

Larvae responded similarly when bioassays were conducted with tropical and common milkweed, which suggests, at least with routes of exposures, endpoints, and insecticides examined in this paper, differences in milkweed species did not confound interpretation of results. However, the condition of milkweed used in bioassays, regardless of the species, is an important consideration. Milkweed reared in our greenhouses can be infested with western flower thrips and oleander aphids if cultural and biological pest management practices are not employed. Milkweed reared with significant insect feeding can increase the plant's cardenolide concentrations (Rasmann et al. 2009; Agrawal et al. 2014). Monarchs feeding on stressed milkweed with elevated cardenolide concentrations are smaller than monarchs feeding on unstressed milkweed with lower cardenolide concentrations (Agrawal 2014).

Following cuticular exposure, arrested ecdysis was observed with neonicotinoid- and chlorpyrifos-intoxicated fifth instars. Neonicotinoids also caused arrested ecdysis via the dietary route of exposure, though the rates were lower. The effect seems to be unique to fifth instars. Third instars exposed to imidacloprid at doses that cause arrested ecdysis in fifth instars developed normally. We also observed that the rate of arrested pupal ecdysis depends on the timing of fifth-instar exposure, particularly in dietary bioassays. Based on an experiment in which 72 h-old fifth instars were fed imidacloprid-treated leaves, and results from our preliminary chronic dietary studies with imidacloprid, thiamethoxam, and chlorpyrifos, 24 h-old fifth instars are 10- to 100-fold less sensitive. Higher mortality rates in older fifth instars are associated with arrested ecdysis.

To the best of our knowledge, no previously published studies report neonicotinoids or organophosphates causing arrested pupal ecdysis in insects. Neonicotinoid and organophosphate insecticides increase acetylcholine signaling in the central nervous system (CNS) of insects. Neonicotinoids act as acetylcholine agonists while organophosphates, and their activated oxon metabolites, inhibit acetylcholine esterase (AChE), which increases synaptic concentrations of endogenous acetylcholine. Thany (2011) reported that thiamethoxam may bind to mixed nicotinic/muscarinic receptors in cercal afferent giant interneuron synapses of the American cockroach (*Periplaneta americana*). Aizono et al. (1997) suggested muscarinic, cholinergic transmission may directly regulate prothoracicotropic hormone (PTTH) release from neurosecretory cells in the brain-corpus cardiacum-corpus allatum of the silkworm (*Bombyx mori*). Altered timing or levels of PTTH secretion due to neonicotinoid- or organophosphate-based stimulation of muscarinic receptors could perturb production and release of ecdysone from the prothoracic gland. In turn, the timing of ecdysis triggering hormone (ETH) production and

secretion and/or expression of ETH receptors (ETHRs) in CNS neurons could be disrupted and impact subsequent steps in the signaling cascade that regulates ecdysis behavior, including the production of kinins and diuretic hormones (Kim et al. 2006; Lenaerts et al. 2017). These hormones regulate secretion of fluids in insects (Diao 2016). Premature activation of neurons releasing these hormones could cause fluid loss that interferes with the molting process, consistent with our observation of fluid loss preceding arrested pupal ecdysis.

Notably, we did not observe arrested larval ecdysis. Kim et al. (2006) and Diao et al. (2016) described two ETHRs (ETHR-A and ETHR-B) that are expressed in distinct neurons of *Drosophila* and the hawkmoth, *Manduca*. Diao et al. (2016) showed that ETHR-A expressing neurons are required for ecdysis at all developmental stages, while ETHR-B expressing neurons are only required for pupal and adult ecdysis. The initiation of ecdysis behavior is regulated, in part, by the "disinhibition" of descending inhibitory ETHR-B neurons by segmental interneurons expressing ETHR-A and -B (Zitnan and Adams 2012). Diao et al. (2016) demonstrated that suppression of a subset of cholinergic ETHR-expressing neurons can block ecdysis. Exposure of cholinergic expressing neurons to acetylcholine agonists (e.g. neonicotinoids) or inhibitors of AChE (e.g. organophosphate insecticides) could alter the timing and/or degree of "disinhibition" and disrupt ecdysis. These hypotheses remain to be tested.

While there are no monarch larval cuticular toxicity studies reported in the literature, Pecenka and Lundgren (2015) and Krischik et al. (2015) reported results from dietary bioassays with clothianidin and imidacloprid, respectively. Krischik et al. (2015) exposed early-instar larvae to tropical milkweed plants that were grown in imidacloprid-treated soil. Over a seven-day period, nearly 100% mortality occurred when larvae were reared on tropical milkweed with 10.4 μg imidacloprid/g leaf. In our 2-day dietary exposures, we observed a similar response, with 90%

mortality for second instars feeding on tropical milkweed leaves with 19 µg of imidacloprid/g leaf (Table 3). Pecenka and Lundgren (2015) treated 1-cm-diameter discs of swamp milkweed (*Asclepias incarnata*) with 10 µL of aqueous solutions of clothianidin. A first-instar 36-h LC50 of 15.6 µg clothianidin/L of water was determined. This corresponds to a LC50 value of 2×10^{-4} µg of clothianidin/cm^2 swamp milkweed leaf. Our second-instar 96-h LC50 value is 9.7×10^{-2} µg clothianidin/cm^2 tropical milkweed leaf (Table S19). Differences in these LC50 values may be due to the source of larvae or experimental conditions.

To compare insecticide sensitivity of monarch larvae to other butterfly species, we primarily relied on the review conducted by Braak et al. (2018) and restricted our evaluation to those studies that reported LC or LD values based on mass of insecticide per g larva, per larva, per g diet or per surface area diet. While there is a limited data set of comparable studies, results to date do not suggest a large range of species sensitivity to pyrethroid, organophosphate, and neonicotinoid insecticides. Hoang et al. (2011) estimated fifth-instar 24-h LD50 values of pyrethroid and organophosphate insecticides following cuticular exposure to larvae of five butterfly species: *Anartia jatrophae* (white peacock), *Eumaeus atala* (Atala butterfly), *Heliconius charitonius* (zebra longwing), *Junonia coenia* (common buckeye) and *Vanessa cardui* (painted lady). Permethrin (a pyrethroid) 24-h LD50 values ranged from 8×10^{-2} to 0.79 µg/g larva, while naled and dichlorvos (organophosphates) 24-h LD50 values ranged from 0.19 to 10.82 µg/g larva. Our fifth-instar monarch studies with beta-cyfluthrin and chlorpyrifos produced 96-h LD50 values of 4.8×10^{-2} and 18 µg/g larva, respectively (Table 1). Basley et al. (2018) reported 22% mortality (corrected for control mortality) with seven-day old *Polyommatus icarus* (common blue butterfly) larvae reared on 0.439 µg clothianidin/g white clover leaves until

pupation. Based on our clothianidin 96-h concentration-response curve, 4.9 µg/g milkweed leaf is expected to cause 22% mortality in third instar monarchs (Figure S5).

Results of toxicity studies with the insecticides examined in this paper have also been reported for several pest moth species. Third-instar larvae of cotton bollworm (*Helicoverpa armigera*) topically treated with beta-cyfluthrin produced 72-h LD50s of approximately 4.7×10^{-2} and 9.9×10^{-2} µg/g larva (Tan and McCaffery 2007; Martin et al. 2003). The 96-h LD50 value of third instar monarchs treated with beta-cyfluthrin is 1.8×10^{-2} µg/g. Following cuticular treatment with chlorpyrifos, third-instar common cutworm (*Spodoptera litura*) and cotton bollworm larvae produced 72-h LD50 values of 0.73 and 8.11 µg/g, respectively (Huang et al. 2006; Martin et al. 2003). Chlorpyrifos-treated monarch third instars produced a 96-h LD50 value of 22 µg/g (Table 1), suggesting cotton bollworms and monarch butterflies have similar sensitivities to pyrethroids and organophosphates; however, the common cutworm is approximately 30-fold more sensitive to organophosphates. A dietary clothianidin toxicity study with fourth instar black cutworm (*Agrotis ipsilon*) resulted in a 72-h LC50 of 27.8 µg/g artificial diet (Ding et al. 2018). The 96-h LC50 of third and fifth instar monarchs exposed to clothianidin is 7.8 and 0.80 µg/g leaf, making them approximately four to 35-fold more sensitive than fourth instar black cutworm. He et al. (2019) reported a chlorantraniliprole 72-h LC50 of 0.187 µg/g artificial diet for third instar black cutworms. The 96-h LC50 values of third instar monarchs exposed to chlorantraniliprole is approximately four-fold lower (4.6×10^{-2} µg/g leaf).

A robust lepidopteran species sensitivity distribution could be used to estimate toxicity for insects of conservation concern and minimize, if not avoid, the time, costs and challenges of rearing insects and host plants. Hoang and Rand (2015) carried out a probabilistic risk assessment for three insecticides encompassing two modes of action using toxicity data

generated for five adult butterfly species. Developing an expanded lepidopteran sensitivity distribution with more insecticide modes of action requires clear description of dosimetry information to support a robust compilation of toxicity data. Screening bioassays used to identify candidate insecticides for lepidopteran pest species typically do not incorporate full dose-response curves, late-instar larvae, or extended observation periods, which limits their utility in developing models to support ecological risk assessments. Our observation of arrested pupal ecdysis and increased sensitivity of fifth instar monarchs to neonicotinoid and organophosphate insecticides highlights the need to use standardized bioassay methods to generate well-defined data sets that can be used for species sensitivity modeling.

We also compared the cuticular toxicity values of monarch larvae to adult honey bees and found that monarch larvae are less sensitive to three of the four insecticide modes of action evaluated in this paper. As reviewed by Arena and Sgolastra (2014), cyfluthrin (mixed isomers), imidacloprid, and thiamethoxam, and chlorpyrifos honey bee 24-h LD50 values range from 1 x 10^{-3}, 2.6 x 10^{-3} to 4 x 10^{-2}, 6.1 x 10^{-3}, and 5.9 x 10^{-2} µg/bee, respectively. Assuming an adult honey bee weighs 0.1 g (Thompson 2015), these values correspond to an LD50 range of 1 x 10^{-2} to 0.59 µg/g bee. Based on our first-instar monarch bioassays, beta-cyfluthrin, thiamethoxam, imidacloprid, and chlorpyrifos produced 96-h LD50 values of 9.2 x 10^{-3}, 6.1, 6.7 and 79 µg/g larva, respectively (Table 1), which suggests honey bees have similar sensitivity to cyfluthrin but are significantly more sensitive than monarch larvae to neonicotinoids following cuticular exposure. With the monarch, beta-cyfluthrin is approximately 700 to 9000-fold more potent than the neonicotinoids and chlorpyrifos; however, with the honey bee, cyfluthrin and the neonicotinoids are approximately one to 60-fold more toxic than chlorpyrifos. Wade et al. (2019) and Kadala et al. (2019) topically treated adult honey bees with chlorantraniliprole and reported

48- and 144-h LD50s of 0.706 and 0.250 µg/bee, respectively (or 7.06 and 2.50 µg/g bee, respectively); first-instar monarch larvae are approximately 200 to 600-fold more sensitive (Table 1; chlorantraniliprole 96-h LD50 is 1.2×10^{-2} µg/g larva). Differences in sensitivity to insecticide classes may reflect differences in susceptibility at the molecular sites of action and/or differences in rates of metabolic detoxification and sequestration.

Characterizing mortality risks

We provide estimates of larval mortality at varying distances downwind from treated fields under different application scenarios by integrating exposure estimates to larvae and milkweed with our cuticular and dietary dose (or concentration) response curves, respectively. As there are no studies that measure insecticide residues on monarch larvae or milkweed leaves immediately following foliar applications, we estimated exposure using the AgDRIFT model (USEPA 2003). With this model, insecticide exposure to surfaces up to 300 m downwind of an application are estimated based on droplet size, wind speed, and insecticide-specific application rate, as specified on the label of the formulated product. The formulated products we selected are illustrative of the types of products available to manage early- and late-season pests of maize and soybean in the North Central states. We did not undertake an exhaustive evaluation of all registered products; however, the method we employed could be readily adapted to other foliar formulations.

The cuticular assessment indicated aerial applications of formulated beta-cyfluthrin and chlorantraniliprole products at maximum label rates to manage soybean aphids would be expected to cause 100% to 32% mortality of all larvae at 0 and 60 m downwind from treated fields, respectively. Foliar applications of chlorpyrifos and the neonicotinoids were estimated to cause between 99% to 0% mortality at 0 and 60 m downwind. Due to chlorpyrifos's higher

application rate, there is greater downwind deposition (5.6 to 0.3 µg/cm^2 at 0 and 60 m, respectively, following aerial application of Lorsban) as compared to the other insecticides. Thus, this insecticide causes high mortality near the edges of field despite its comparatively low toxicity. The other insecticides had similarly lower application rates (Table S5). Consequently, beta-cyfluthrin and chlorantraniliprole, the most toxic insecticides, produced the highest downwind mortality rates, while the neonicotinoids produced the lowest mortality rates. Based on results of our toxicity studies, for insecticide exposures estimated to cause less than 70% larval mortality, negligible downwind effects on larval growth or development would be expected. In our analysis we assumed all monarch larvae are exposed to the spray drift plume; however, larvae are most frequently found underneath milkweed leaves (Rawlins and Lederhouse 1981; Fisher 2020). For example, Fisher et al. (2020) reported monarch larvae on the underside of the leaves during approximately 60% of their observations of development from neonate larvae to pupa. Consequently, our estimates of cuticular exposure and field-scale mortality are likely overestimated.

The dietary assessment indicated aerial applications of formulated chlorantraniliprole and chlorpyrifos products at maximum label rates to manage soybean aphids would be expected to cause 100% to 44% mortality of all larvae at 0 and 60 m downwind from treated fields, respectively. Foliar applications of beta-cyfluthrin and the neonicotinoids were estimated to cause between 96% to 1% mortality at 0 and 60 m downwind. Beta-cyfluthrin is expected to cause greater mortality via the cuticular exposure route while chlorpyrifos is expected to cause greater mortality via the dietary route. Downwind effects on monarch larval growth and development could be expected following dietary insecticide exposure.

Two published studies estimated monarch mortality rates from aerial applications of mosquito adulticides. Oberhauser et al. (2006) collected common milkweed leaves following application of permethrin (application rate: 0.109 kg AI/ha). First, second, and third instars that fed on these leaves had over 71% mortality. When larvae were directly exposed to resmethrin (application rate: 0.0039 kg AI/ha), over 60% mortality was seen up to 23 m downwind (Oberhauser et al. 2009). While droplet sizes are much smaller with mosquito adulticide formulations as compared to formulations used for agricultural pests, the level of larval mortality observed in these field studies is qualitatively similar to the larval mortality we estimated with aerial beta-cyfluthrin applications.

Our mortality estimates based on dietary exposure are most relevant for a period of one to two days post-application, however for some of the insecticides, especially chlorantraniliprole, significant mortality may occur for several days post-application. Length of dietary exposure is a function of an insecticide's photolysis, hydrolysis, and oxidation rates. In field and greenhouse studies conducted with growing plants, beta-cyfluthrin was found to have a half-life of 1 to 2 days (Banerjee et al. 2012) while chlorpyrifos, imidacloprid, and thiamethoxam had half-lives of 2 to 6 days (Galietta et al. 2011; Hassanzadeh et al. 2012; Rahman et al. 2015). Chlorantraniliprole has a reported half-life of 16 to 17 days (Szpyrka et al. 2017). Chronic studies to mimic longer-term dietary exposure to foliar insecticides are in progress. Our estimates also do not incorporate additional exposure episodes associated with multiple insecticide applications during the approximately 10 to 14 days of larval development. Label instructions for beta-cyfluthrin and neonicotinoid formulations used in this study require a minimum seven-day interval between the first and second applications; however, the minimum application interval is

five days for Beseige (chlorantraniliprole and lambda-cyhalothrin) and 14 days for Lorsban (chlorpyrifos).

While our risk assessments for individual insecticide applications at the field-scale are conservative in that they employ upper-end exposure estimates, they could underestimate mortality to larvae simultaneously exposed to a mixture of insecticides. For example, with our representative formulated products, Beseige contains chlorantraniliprole and lambda-cyhalothrin; Endigo contains thiamethoxam and lambda-cyhalothrin; and Swagger contains bifenthrin and imidacloprid. Risks for formulated products with multiple active ingredients could be derived by adding the concentrations for insecticides with the same mode of action, or by adding the responses (or mortality rates) for insecticides with different modes of action (National Research Council 2013). This approach would not capture any potential synergistic or antagonistic effects with insecticide-fungicide tank mixes, for example. We also did not assess the combined mortality rates from cuticular and dietary exposures. However, since larvae are typically found under milkweed leaves (Rawlins and Lederhouse 1981; Fisher et al. 2020), cuticular exposure to spray drift is likely low. Therefore, independently assessing mortality risks for the two routes of exposure is a reasonable approach.

Data and field-scale mortality estimates from this paper can augment expert opinion recently used to elucidate the potential impact of insecticide use on recovery of the monarch butterfly (Voorhies et al. 2019). We estimated high monarch larval mortality rates 0 to 15 m downwind of maize and soybean fields treated with foliar insecticide applications; however, these findings are not relevant for all monarch habitat that is in close proximity to crop fields. At the time of application, insecticide spray drift is deposited downwind of a treated field, with less or no insecticide deposition occurring on larvae or milkweed crosswind or upwind. Hence,

similar levels of larval exposure and mortality will likely not occur on all sides of a treated field. In addition, across the North Central states, insect pressure can vary widely within a given year, with some states having pest pressure above economic thresholds and other states with pest levels that do not require insecticide treatment. For example, from 2000 through 2012 soybean aphid pressure varied widely across the North Central states (Bahlai et al. 2015). Variation also occurs within a state in a given year. Schmidt et al. (2008) reported a gradient of soybean aphid pressure that increased from southern to northern Iowa counties in 2005. Similarly, a small percentage of Iowa fields are being treated with foliar insecticides to manage true armyworms. In 2018, about 4% of the maize and soybean hectares had cover crops (Juchems 2019; USDA 2019). In addition, Dunbar et al. (2016) reported in their study only half of the six maize fields with rye cover crops had true armyworm populations exceeding economic thresholds that warranted insecticide use.

Characterizing risks of foliar insecticides to non-migratory monarch populations in agricultural ecosystems requires landscape-scale analyses (Uhl and Brühl 2019). Adult monarchs are vagile (Zalucki et al. 2016), which requires attributes of their movement and reproductive behavior be integrated with spatial and temporal heterogeneity of monarch breeding habitat, agricultural fields, pastures, rural road rights-of-ways, weather conditions, and pest pressure (Grant and Bradbury 2019). Results from the current paper, ongoing acute contact exposures to egg and pupae from foliar insecticides, chronic larval dietary exposures to foliar and seed treatment insecticides, and acute adult oral exposures to seed treatment insecticides are being incorporated into an individual-based model (Grant et al. 2018) to obtain a more complete picture of landscape-scale risks. These analyses will evaluate the conservation risks and benefits of establishing new monarch habitat within agricultural landscapes of the North Central U.S.

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Data availability

Data and metadata pertaining to this manuscript are publicly available at this GitHub repository: https://github.com/Niranjana296/Assessing-risk-of-insecticides-to-monarch-butterfly-larvae

References

Agrawal AA, Hastings AP, Patrick ET, Knight AC. 2014. Specificity of herbivore-Induced hormonal signaling and defensive traits in five closely related milkweeds (*Asclepias* spp.). J Chem Ecol. DOI: 10.1007/s10886-014-0449-6.

Aizono Y, Endo Y, Sattelle DB, Shirai Y. 1997. Prothoracicotropic hormone-producing neurosecretory cells in the silkworm, *Bombyx mori*, express a muscarinic acetylcholine receptor. Brain Research. DOI: 10.1016/S0006-8993(97)00496-4.

Altizer SM, Oberhauser KS, Brower LP. 2000. Associations between host migration and the prevalence of a protozoan parasite in natural populations of adult monarch butterflies. Ecol Entomol. DOI: 10.1046/j.1365-2311.2000.00246.x.

Arena M, Sgolastra F. 2014. A meta-analysis comparing the sensitivity of bees to pesticides. Ecotoxicology. DOI:10.1007/s10646-014-1190-1.

Bahlai CA, Vander Werf W, O'Neal M, Hemerik L, Landis DA. 2015. Shifts in dynamic regime of an invasive lady beetle are linked to the invasion and insecticidal management of its prey. Ecol Appl. DOI: 10.1002/eap.1345.

Banerjee T, Banerjee D, Roy S, Banerjee H, Pal S. 2012. A comparative study on the persistence of imidacloprid and beta-cyfluthrin in vegetables. Bull Environ Contam Toxicol. DOI: 10.1007/s00128-012-0644-5.

Basley K, Goulson D. 2018. Effects of field-relevant concentrations of clothianidin on larval development of the butterfly *Polyommatus icarus* (Lepidoptera, Lycaenidae). Environ Sci Technol. DOI: 10.1021/acs.est.8b00609.

Braak N, Neve R, Jones AK, Gibbs M, Breuker CJ. 2018. The effects of insecticides on butterflies – A review. Environ Pollut. DOI: 10.1016/j.envpol.2018.06.100.

Brower LP, Taylor OR, Williams EH, Slayback DA, Zubieta RR, Ramírez MI. 2012. Decline of monarch butterflies overwintering in Mexico: Is the migratory phenomenon at risk? Insect Conserv Divers. DOI: 10.1111/j.1752-4598.2011.00142.x.

Carscallen GE, Kher S V., Evenden ML. 2019. Efficacy of Chlorantraniliprole Seed Treatments Against Armyworm (*Mythimna unipuncta* [Lepidoptera: Noctuidae]) Larvae on Corn (Zea mays). J Econ Entomol. DOI: 10.1093/jee/toy338.

Diao F, Mena W, Shi J, Park D, Diao Fengqiu, Taghert P, Ewer J, White BH. 2016. The splice isoforms of the *Drosophila* ecdysis triggering hormone receptor have developmentally distinct roles. Genetics. DOI: 10.1534/genetics.115.182121.

Ding J, Zhao Y, Zhang Z, Xu C, Mu W. 2018. Sublethal and Hormesis Effects of Clothianidin on the Black Cutworm (Lepidoptera: Noctuidae). J Econ Entomol. doi:10.1093/jee/toy254.

Dixon PM, Goode KJ, Lay C. 2020. Profile likelihood confidence intervals for ECx. Iowa State University Digital Repository.

Douglas MR, Tooker JF. 2015. Large-scale deployment of seed treatments has driven rapid increase in use of neonicotinoid insecticides and preemptive pest management in U.S. Field crops. Environ Sci Technol. DOI: 10.1021/es506141g.

Dunbar MW, O'Neal ME, Gassmann AJ. 2016. Increased risk of insect injury to corn following rye cover crop. J Econ Entomol. DOI: 10.1093/jee/tow101.

Elmore R. 2012. Iowa State University Extension. Ames (IA, USA). [accessed 2019 October 18]. Available from: https://crops.extension.iastate.edu/cropnews/2012/03/best-corn-planting-dates-iowa

Fisher KE, RL Hellmich, SP Bradbury. 2020. Estimates of common milkweed (*Asclepias syriaca*) utilization by monarch larvae (*Danaus plexippus*) and the significance of larval movement. J of Insect Conserv. DOI: https://doi.org/10.1007/s10841-019-00213-2.

Flockhart DTT, Brower LP, Ramirez MI, Hobson KA, Wassenaar LI, Altizer S, Norris DR. 2017. Regional climate on the breeding grounds predicts variation in the natal origin of monarch butterflies overwintering in Mexico over 38 years. Glob Chang Biol. DOI: 10.1111/gcb.13589.

Galietta G, Egana E, Gemelli F, Maeso D, Casco N, Conde P, Nunez S. 2011. Pesticide dissipation curves in peach, pear and tomato crops in Uruguay. J Environ Sci Health B. DOI: 10.1080/03601234.2010.515504.

Gassman A, Weber P. 2016. Iowa State University Extension. Ames (IA, USA). [accessed 2019 October 18]. Available from: http://www.ent.iastate.edu/dept/faculty/gassmann/files/page/files/2015_Ent_Report.pdf

Grant TJ, Parry HR, Zalucki MP, Bradbury SP. 2018. Predicting monarch butterfly (*Danaus plexippus*) movement and egg-laying with a spatially-explicit agent-based model: The role of monarch perceptual range and spatial memory. Ecol Modell. DOI: 10.1016/j.ecolmodel.2018.02.011.

Grant TJ and Bradbury SP. 2019. The role of modeling in monarch butterfly research and conservation. Front Ecol Evol. DOI: 10.3389/fevo.2019.00197.

Hall MJ, Dang V, Bradbury SP, Coats JR. 2019. Optimization of QuEChERS method for simultaneous determination of neonicotinoid residues in pollinator forage. Rapid Communications in Mass Spectrometry Methods. Submitted.

Hassanzadeh N, Sari AE, Bahramifar N. 2012. Dissipation of imidacloprid in greenhouse cucumbers at single and double dosages spraying. J Agric Sci Technol. 14:557–564.

He F, Sun S, Tan H, Sun X, Qin C, Ji S, Li X, Zhang J, Jiang X. 2019. Chlorantraniliprole against the black cutworm Agrotis ipsilon (Lepidoptera: Noctuidae): From biochemical/physiological to demographic responses. Sci Rep. doi:10.1038/s41598-019-46915-0.

Hoang TC, Pryor RL, Rand GM, Frakes RA. 2011. Use of butterflies as nontarget insect test species and the acute toxicity and hazard of mosquito control insecticides. Environ Toxicol Chem. DOI: 10.1002/etc.462.

Hoang TC, Rand GM. 2015. Acute toxicity and risk assessment of permethrin, naled, and dichlorvos to larval butterflies via ingestion of contaminated foliage. Chemosphere. DOI: 10.1016/j.chemosphere.2014.10.040.

Hodgson EW, McCornack BP, Tilmon K, Knodel JJ. 2012. Management recommendations for soybean aphid (Hemiptera: Aphididae) in the United States. J Integr Pest Manag. DOI: 10.1603/ipm11019.

Hodgson EW, Rice M. 2017. Iowa State University Extension. Ames (IA, USA). [accessed 2019 October 18]. Available from: https://store.extension.iastate.edu/Product/15141

Hodgson EW. 2018. Iowa State University Extension. Ames (IA, USA). [accessed 2019 October 18]. Available from: https://lib.dr.iastate.edu/cropnews/2484/

Huang S, Xu J, Han Z. 2006. Baseline toxicity data of insecticides against the common cutworm Spodoptera litura (Fabricius) and a comparison of resistance monitoring methods. Int J Pest Manag. doi:10.1080/09670870600673962.

Journey North. 2016. Madison (WI, USA). [accessed 2019 October 18]. Available from: https://journeynorth.org/sightings/querylist.html?map=milkweed&year=2016&season=spring

Juchems L. 2019. Iowa State University Extension. Ames (IA, USA) [cited 2019 October 18]. Available from: https://www.extension.iastate.edu/news/cover-crop-acres-increase-rate-growth-declines-2018

Kadala A, Charreton M, Charnet P, Collet C. 2019. Honey bees long-lasting locomotor deficits after exposure to the diamide chlorantraniliprole are accompanied by brain and muscular calcium channels alterations. Sci Rep. doi:10.1038/s41598-019-39193-3.

Kaul RB, Rolfsmeier SB, Esch JJ. 1991. The distribution and reproductive phenology of the milkweeds (Asclepiadaceae : Asclepias and Cynanchum) in Nebraska. Trans Nebraska Acad Sci. 18:127–140.

Kim YJ, Žitňan D, Cho KH, Schooley DA, Misoguchi A, Adams ME. 2006. Central peptidergic ensembles associated with organization of an innate behavior. Proc Natl Acad Sci U S A. DOI: 10.1073/pnas.0603459103.

Krischik V, Rogers M, Gupta G, Varshney A. 2015. Soil-applied imidacloprid translocates to ornamental flowers and reduces survival of adult *Coleomegilla maculata*, *Harmonia axyridis*, and *Hippodamia convergens* lady beetles, and larval *Danaus plexippus* and *Vanessa cardui* butterflies. PLoS One. DOI: 10.1371/journal.pone.0119133.

Lenaerts C, Cools D, Verdonck R, Verbakel L, Vanden Broeck J, Marchal E. 2017. The ecdysis triggering hormone system is essential for successful moulting of a major hemimetabolous pest insect, *Schistocerca gregaria*. Sci Rep. DOI: 10.1038/srep46502.

Malcolm SB, Cockrell BJ, Brower LP. 1993. Spring recolonization of eastern North America by the monarch butterfly: successive brood or single sweep migration? Biol Conserv Monarch butterfly.(January):253–267.

Martin T, Ochou OG, Vaissayre M, Fournier D. 2003. Organophosphorus insecticides synergize pyrethroids in the resistant strain of cotton bollworm, Helicoverpa armigera (Hübner) (Lepidoptera: Noctuidae) from West Africa. J Econ Entomol. doi:10.4103/0972-5229.53114.

Monarch Watch. 2019. Lawrence (KS, USA) [accessed 2019 October 18]. Available from: https://monarchwatch.org/blog/2019/01/30/monarch-population-status-37/

Nail KR, Stenoien C, Oberhauser KS. 2015. Immature monarch survival: Effects of site characteristics, density, and time. Ann Entomol Soc Am. DOI: 10.1093/aesa/sav047.

National Research Council. 2013. Assessing risks to endangered and threatened species from pesticides. The National Academies Press, Washingtom, D.C. (USA), pp 110 -111.

Natural Resources Conservation Service. 2016. Monarch Butterfly Wildlife Habitat Evaluation Guide and Decision Support Tool: Midwest Edition 1.1. https://www.nrcs.usda.gov/wps/portal/nrcs/detail/national/plantsanimals/pollinate/?cid=nrcseprd402207.

Oberhauser KS, Brinda SJ, Weaver S, Moon RD, Read N, Manweiler SA. 2006. Growth and survival of monarch butterflies (Lepidoptera: Danaidae) after exposure to permethrin barrier treatments Pest Manag. 35(6):1626–1634.

Oberhauser KS, Manweiler S a, Lelich R, Blank M, Batalden R V, de Anda A. 2009. Impacts of ultra-low volume resmethrin applications on non-target insects. J Am Mosq Control Assoc. DOI: 10.2987/08-5788.1.

Oberhauser KS, Wiederholt R, Diffendorfer JE, Semmens D, Ries L, Thogmartin WE, Lopez-Hoffman L, Semmens B. 2017. A trans-national monarch butterfly population model and implications for regional conservation priorities. Ecol Entomol. DOI: 10.1111/een.12351.

Olaya-Arenas P, Kaplan I. 2019. Quantifying pesticide exposure risk for monarch caterpillars on milkweeds bordering agricultural land. Front Ecol Evol. DOI: 10.3389/fevo.2019.00223.

Pecenka JR, Lundgren JG. 2015. Non-target effects of clothianidin on monarch butterflies. Sci Nat. DOI: 10.1007/s00114-015-1270-y.

Pedersen P. 2007. Iowa State University Extension. Ames (IA, USA). [accessed 2019 October 18]. Available from: https://crops.extension.iastate.edu/files/article/PlantingDate_000.pdf

Pleasants JM. 2015. Monarch butterflies and agriculture. In Oberhauser KS, Nail KR, Altizer S, *Monarchs in a changing world: biology and conservation of an iconic butterfly*. Cornell University Press, Ithaca, NY, USA, pp 169-178.

Prysby MD, Oberhauser KS. 2004. Temporal and geographic variation in monarch densities: Citizen scientists document monarch population patterns. In K. S. Oberhauser and M. J. Solensky, editors. *The monarch butterfly: biology and conservation.* Cornell University Press, Ithaca, New York, USA, pp 9-20

Rahman MM, Farha W, Abd El-Aty AM, Kabir MH, Im SJ, Jung DI, Choi JH, Kim SW, Son YW, Kwon CH, et al. 2015. Dynamic behaviour and residual pattern of thiamethoxam and its metabolite clothianidin in Swiss chard using liquid chromatography-tandem mass spectrometry. Food Chem. DOI: 10.1016/j.foodchem.2014.11.052.

Rasmann S, Agrawal AA, Cook SC, Erwin AC. 2009. Cardenolides, induced responses, and interactions between above- and below ground herbivores of milkweed (*Asclepias* spp.). Ecology. DOI: 10.1890/08-1895.1.

Rawlins E, Lederhouse C. 2011. Developmental influences of thermal behavior on monarch caterpillars (*Danaus plexippus*): An adaptation for migration (Lepidoptera : Nymphalidae : Danainae). J Kansas Entomol Soc. 54(2):387–408.

Schmidt NP, O'Neal ME, Dixon PM. 2008. Aphidophagous predators in Iowa soybean: A community comparison across multiple years and sampling methods. Ann Entomol Soc Am. 101(2):341–350.

Semmens BX, Semmens DJ, Thogmartin WE, Wiederholt R, López-Hoffman L, Diffendorfer JE, Pleasants JM, Oberhauser KS, Taylor OR. 2016. Quasi-extinction risk and population targets for the Eastern, migratory population of monarch butterflies (*Danaus plexippus*). Sci Rep. DOI: 10.1038/srep23265.

Szpyrka E, Matyaszek A, Słowik-Borowiec M. 2017. Dissipation of chlorantraniliprole, chlorpyrifos-methyl and indoxacarb—insecticides used to control codling moth (*Cydia pomonella* L.) and leafrollers (Tortricidae) in apples for production of baby food. Environ Sci Pollut Res. DOI: 10.1007/s11356-017-8821-z.

Tan J, McCaffery AR. 2007. Efficacy of various pyrethroid structures against a highly metabolically resistant isogenic strain of Helicoverpa armigera (Lepidoptera: Noctuidae) from China. Pest Manag Sci. doi:10.1002/ps.

Thany SH. 2011. Thiamethoxam, a poor agonist of nicotinic acetylcholine receptors expressed on isolated cell bodies, acts as a full agonist at cockroach cercal afferent/giant interneuron synapses. Neuropharmacology. DOI: 10.1016/j.neuropharm.2010.12.008.

Thogmartin WE, López-Hoffman L, Rohweder J, Diffendorfer J, Drum R, Semmens D, Black S, Caldwell I, Cotter D, Drobney P, et al. 2017. Restoring monarch butterfly habitat in the Midwestern US: "All hands on deck." Environ Res Lett. DOI: 10.1088/1748-9326/aa7637.

Thompson H. 2015. Extrapolation of acute toxicity across bee species. Integr Environ Assess Manag. DOI: 10.1002/ieam.1737.

Thrash B, Adamczyk JJ, Lorenz G, Scott AW, Armstrong JS, Pfannenstiel R, Taillon N. 2013. Laboratory evaluations of Lepidopteran-active soybean seed treatments on survivorship of fall armyworm (Lepidoptera: Noctuidae) Larvae. Florida Entomol. DOI: 10.1653/024.096.0304.

Tripathy A. 2019. Calculating leaf surface areas using OpenCV. MINDS@UW-Madison. Available from: http://digital.library.wisc.edu/1793/79417

Uhl P, Brühl CA. 2019. The Impact of Pesticides on Flower-visiting Insects: A Review with Regard to European Risk Assessment. Environ Toxicol Chem. DOI :10.1002/etc.4572.

U.S. Department of Agriculture. 2018. Washington, D.C. (USA) [accessed 2019 October 18]. Available from: https://quickstats.nass.usda.gov/#59CB4138-B1CE-31CD-B29D-A71A0DF9BBE6

U.S. Department of Agriculture. 2019. Iowa Ag News - 2018 Crop Production. Washington, D.C. (USA) [accessed 2019 October 18]. Available from: https://www.nass.usda.gov/Statistics_by_State/Iowa/Publications/Crop_Report/2019/IA-Crop-Production-Annual-01-19.pdf

U.S. Environmental Protection Agency. 2003. AgDRIFT. Washington, D.C. (USA) [accessed 2019 October 18]. Available from: https://www.epa.gov/pesticide-science-and-assessing-pesticide-risks/models-pesticide-risk-assessment#AgDrift

U.S. Fish and Wildlife Service. 2016. Washington, D.C. (USA) [accessed 2019 October 18]. Available from: https://www.fws.gov/savethemonarch/ssa.html

U.S. Fish and Wildlife Service. 2017. Monarch Butterfly Species Assessment Update. https://www.fws.gov/savethemonarch/pdfs/Monarch.pdf

Voorhies KJ, Szymanski J, Nail KR, Fidino M. 2019. A method to project future impacts from threats and conservation on the probability of extinction for North American migratory nonarch (*Danaus plexxipus*) Populations. Front Ecol Evol. DOI: 10.3389/fevo.2019.00384.

Wade A, Lin CH, Kurkul C, Regan ER, Johnson RM. 2019. Combined toxicity of insecticides and fungicides applied to California almond orchards to honey bee larvae and adults. Insects. doi:10.3390/insects10010020.

Whalen RA, Herbert DA, Malone S, Kuhar TP, Brewster CC, Reisig DD. 2016. Effects of diamide insecticides on predators in soybean. J Econ Entomol. DOI: 10.1093/jee/tow173.

Zalucki MP, Parry HR, Zalucki JM. 2016. Movement and egg laying in Monarchs: To move or not to move, that is the equation. Austral Ecol. DOI: 10.1111/aec.12285.

Zitnan D, Adams ME. 2012. Neuroendocrine Regulation of Ecdysis. In Lawrence IG, ed., *Insect Endocrinology*. Academic Press, San Diego, CA, USA, pp. 253-309.

Tables and Figures

Table 1. Cuticular Study: Acute toxicity (expressed as μg insecticide/g larva) of five insecticides to monarch first-, third-, and fifth-instar larvae fed tropical milkweed leaves[a]

Insecticide	Instar	96-h LD values and 95% CIs (μg insecticide/g larva)[b]		
		LD10	LD50	LD90
BCF	First	2.1×10^{-3} (7.4×10^{-5} – 4.2×10^{-3})	9.2×10^{-3} (5.2×10^{-3} – 1.3×10^{-2})	4.0×10^{-2} (1.7×10^{-2} – 6.3×10^{-2})
	Third	2.8×10^{-3} (7.5×10^{-4} – 1.0×10^{-2})[c]	1.8×10^{-2} (9.7×10^{-3} – 3.4×10^{-2})[c]	0.12 (5.7×10^{-2} – 0.32)[c]
	Fifth[d]	1.5×10^{-2} (3.1×10^{-3} – 2.7×10^{-2})	4.8×10^{-2} (2.7×10^{-2} – 6.8×10^{-2})	0.15 (8.7×10^{-2} – 0.22)
CTR	First	1.1×10^{-3} (1.4×10^{-4} – 4.2×10^{-3})[c]	1.2×10^{-2} (5.1×10^{-3} – 2.8×10^{-2})[c]	0.14 (5.4×10^{-2} – 0.60)[c]
	Third	1.3×10^{-2} (4.0×10^{-3} – 3.7×10^{-2})[c]	9.5×10^{-2} (5.2×10^{-2} – 0.17)[c]	0.68 (0.34 – 1.7)[c]
	Fifth[d]	5.8×10^{-2} (1.7×10^{-2} – 0.10)	0.19 (0.12 – 0.26)	0.62 (0.31 – 0.93)
CFS	First	40 (17 – 62)	79 (55 – 100)	150 (100 – 200)
	Third	8.5 (2.7 – 14)	22 (15 – 30)	58 (32 – 84)
	Fifth[d]	8.6 (7.9 – 9.3)	18 (15 – 21)	38 (30 – 45)
IMI	First	2.6 (0.99 – 4.3)	6.7 (4.5 – 8.8)	17 (9.9 – 24)
	Third	1.3 (0.30 – 4.0)[c]	8.4 (4.4 – 16)[c]	56 (30 - 140)[c]
	Fifth[d]	1.0 (0.33 – 1.7)	3.0 (2.0 – 4.0)	9.0 (3.5 – 15)
TMX	First	1.4 (0.27 – 2.5)	6.1 (3.0 – 9.3)	27 (6.6 – 47)
	Third	1.8 (0.58 – 3)	8.8 (5.6 – 12)	43 (19 – 67)
	Fifth[d]	17 (7.2 – 27)	35 (28 – 41)	71 (39 – 100)

[a] Based on combined mortality data from triplicate or quadruplicate toxicity bioassays for each insecticide-instar combination. Larvae were treated with acetone and five insecticide-acetone solutions.

[b] The μg of insecticide per g larva were calculated by dividing the nominal concentrations and volume of insecticide solution applied to each larva using the average weights of control larvae before treatment. Respective control larval weights for each insecticide-instar combination were used (Table S1). Except as noted in the table, LD values were estimated using a nonlinear least square estimate model (see MATERIALS AND METHODS/*Statistical Analyses*). Adjustment for control (acetone) mortality was made using Abbott's formula.

[c] LD values were calculated using a maximum likelihood estimate model (see *Statistical Analyses*).

[d] Observations until pupation (usually 72 or 96 h after treatment).

CIs: confidence intervals; LD10: lethal dose that kills 10% of a treated population; LD50: lethal dose that kills 50% of a treated population; LD90: lethal dose that kills 90% of a treated population

BCF: beta-cyfluthrin; CTR: chlorantraniliprole; CFS: chlorpyrifos; IMI: imidacloprid; TMX: thiamethoxam

Table 2. Cuticular Study: Acute toxicity (expressed as μg insecticide/cm^2 larva) of five insecticides to monarch first-, third-, and fifth-instar larvae fed tropical milkweed leaves[a]

Insecticide	Instar	96-h LD values and 95% CIs (μg insecticide/cm^2 larva)[b]		
		LD10	LD50	LD90
BCF	First	3.4×10^{-5} (1.0×10^{-6} – 6.7×10^{-5})	1.5×10^{-4} (8.4×10^{-5} – 2.1×10^{-4})	6.5×10^{-4} (2.7×10^{-4} – 1.0×10^{-3})
	Third	1.4×10^{-4} (3.6×10^{-5} – 5.0×10^{-4})[c]	8.7×10^{-4} (4.7×10^{-4} – 1.7×10^{-3})[c]	5.6×10^{-3} (2.8×10^{-3} – 1.6×10^{-2})[c]
	Fifth[d]	1.8×10^{-3} (9.8×10^{-4} – 2.9×10^{-3})[c]	6.5×10^{-3} (4.7×10^{-3} – 8.7×10^{-3})[c]	2.3×10^{-2} (1.6×10^{-2} – 3.7×10^{-2})[c]
CTR	First	1.7×10^{-5} (2.3×10^{-6} – 6.9×10^{-5})[c]	2.0×10^{-4} (8.3×10^{-5} – 4.6×10^{-4})[c]	2.3×10^{-3} (8.9×10^{-4} – 9.8×10^{-3})[c]
	Third	9.3×10^{-4} (2.8×10^{-4} – 2.6×10^{-3})[c]	6.6×10^{-3} (3.6×10^{-3} – 1.2×10^{-2})[c]	4.7×10^{-2} (2.4×10^{-2} – 0.12)[c]
	Fifth[d]	6.6×10^{-3} (2.0×10^{-3} – 1.1×10^{-2})	2.2×10^{-2} (1.4×10^{-2} – 2.9×10^{-2})	7.1×10^{-2} (3.5×10^{-2} – 0.11)
CFS	First	0.60 (0.26 – 0.94)	1.2 (0.83 – 1.5)	2.3 (1.6 – 3.1)
	Third	0.60 (0.19 – 1.0)	1.6 (1.1 – 2.1)	4.1 (2.3 – 5.9)
	Fifth[d]	1.1 (1.0 – 1.2)	2.3 (1.9 – 2.7)	4.9 (3.6 – 6.1)
IMI	First	4.3×10^{-2} (1.6×10^{-2} – 7.0×10^{-2})	0.11 (7.5×10^{-2} – 0.15)	0.28 (0.16 – 0.40)
	Third	5.9×10^{-2} (1.4×10^{-2} – 0.19)[c]	0.39 (0.21 – 0.74)[c]	2.6 (1.4 – 6.7)[c]
	Fifth[d]	0.15 (5.0×10^{-2} – 0.25)	0.45 (0.30 – 0.59)	1.3 (0.52 – 2.2)
TMX	First	2.9×10^{-2} (5.5×10^{-3} – 5.2×10^{-2})	0.13 (6.0×10^{-2} – 0.19)	0.55 (0.13 – 0.96)
	Third	0.10 (3.4×10^{-2} – 0.17)	0.51 (0.32 – 0.70)	2.5 (1.1 – 3.9)
	Fifth[d]	2.1 (0.87 – 3.3)	4.2 (3.4 – 5.0)	8.6 (4.7 – 12)

[a] Based on combined mortality data from triplicate or quadruplicate toxicity bioassays for each insecticide-instar combination. Larvae were treated with acetone and five insecticide-acetone solution.

[b] Larvae were assumed to be cylinders. Surface area in cm^2 was estimated by measuring the height (h; or length) and radius (r; or half the thickness) of ten individuals for each larval instar using the following formula: $2\pi rh + 2\pi r^2$. Estimated surface areas of first, third, and fifth instars were 0.17 ± 0.05, 0.65 ± 0.12 and 7.1 ± 1.3 cm^2, respectively. Except as noted in the table, LD values were estimated using nonlinear least square estimate model (see MATERIALS AND METHODS/*Statistical Analyses*). Adjustment for control (acetone) mortality was made using Abbott's formula.

[c] LD values estimated using a maximum likelihood estimate model (see *Statistical Analyses*).

[d] Observations until pupation (usually 72 or 96 h after treatment).

CIs: confidence intervals; LD10: lethal concentration that kills 10% of a treated population; LD50: lethal concentration that kills 50% of a treated population; LD90: lethal concentration that kills 90% of a treated population

BCF: beta-cyfluthrin; CTR: chlorantraniliprole; CFS: chlorpyrifos; IMI: imidacloprid; TMX: thiamethoxam

Table 3. Dietary Study: Acute toxicity (expressed as µg insecticide/g leaf) to monarch second-, third-, and fifth-instar larvae following exposure to tropical milkweed leaves treated with five insecticides[a]

Insecticide	Instar	96-h LC values and 95% CIs (µg insecticide/g leaf)[b]		
		LC10	LC50	LC90
BCF	Second	2.1×10^{-2} (6.1×10^{-3} – 5.5×10^{-2})	0.21 (0.12 – 0.35)	2.1 (1.1 – 5.0)
	Third	0.20 (4.1×10^{-3} – 0.39)[c]	0.94 (0.45 – 1.4)[c]	4.5 (1.2 – 7.8)[c]
	Fifth[d]	3.6×10^{-2} (5.9×10^{-3} – 0.15)	0.62 (0.27 – 1.4)	11 (3.8 – 52)
CTR	Second	4.9×10^{-4} (7.3×10^{-5} – 1.8×10^{-3})	8.3×10^{-3} (3.8×10^{-3} – 1.6×10^{-2})	0.14 (6.0×10^{-2} – 0.49)
	Third	6.0×10^{-4} (6.8×10^{-5} – 2.9×10^{-3})	4.6×10^{-2} (1.8×10^{-2} – 0.11)	3.6 (1.1 – 21)
	Fifth[d]	1.7×10^{-2} (1.8×10^{-3} – 0.10)	0.97 (0.36 – 3.0)	55 (13 – 580)
CFS	Second	0.68 (0.14 – 6.4)	8.4 (4.0 – 19)	100 (24 – 530)
	Third	0.31 (4.4×10^{-2} – 1.6)	6.0 (2.7 – 14)	120 (40 – 630)
	Fifth[d]	0.74 (0.16 – 2.3)	10 (5.0 – 23)	140 (48 – 820)
IMI	Second	1.4 (0.57 – 2.1)[c]	5.1 (3.3 – 6.8)[c]	19 (7.5 – 30)[c]
	Third	3.7 (0.48 – 6.9)[c]	17 (9.4 – 24)[c]	77 (22 – 130)[c]
	Fifth[d]	0.27 (1.4×10^{-2} – 2.3)	9.4 (3.0 – 27)	330 (92 – 3100)
TMX	Second	1.4 (0.36 – 3.6)	3.5 (2.2 – 5.0)	8.8 (NC – 26)
	Third	1.1 (0.48 – 2.1)	5.6 (3.7 – 8.9)	29 (15 – 69)
	Fifth[d]	4.2 (NC – 13)[c]	33 (4.5 – 62)[c]	270 (NC – 550)[c]

[a] Based on combined mortality data from triplicate or quadruplicate bioassays for each insecticide-instar combination. Larvae were fed leaf tissue treated with 0.1% silwet : water and five insecticide suspensions in 0.1% silwet : water.

[b] The µg of insecticide per g leaf tissue were calculated by dividing the concentrations and volume of insecticide solution pipetted on each leaf tissue by the known weights of the leaf tissue. The average weights of leaves provided to larvae in each insecticide, instar, bioassay run, and concentration are available in a supplementary file (*Weights and surface areas of leaves)*. Except as noted in the table, LC values were estimated using maximum likelihood estimate model (see MATERIALS AND METHODS/*Statistical Analyses*). Adjustment for control (0.1% silwet : water) mortality was done using Abbott's formula.

[c] LC values were calculated using nonlinear least square estimate model (see *Statistical Analyses*).

[d] Observations until pupation (usually 72 or 96 h after treatment).

CIs: confidence intervals; LC10: lethal concentration that kills 10% of a treated population; LC50: lethal concentration that kills 50% of a treated population; LC90: lethal concentration that kills 90% of a treated population; NC: not calculable or a negative lower bound CI value

BCF: beta-cyfluthrin; CTR: chlorantraniliprole; CFS: chlorpyrifos; IMI: imidacloprid; TMX: thiamethoxam

Table 4. Dietary Study: Acute toxicity (expressed as μg insecticide/cm^2 leaf) to monarch second-, third-, and fifth-instar larvae following exposure to tropical milkweed leaves treated with five insecticides[a]

Insecticide	Instar	96-h LC values and 95% CIs (μg insecticide/cm^2 leaf)[b]		
		LC10	LC50	LC90
BCF	Second	6.3×10^{-4} (2.1×10^{-4} – 1.6×10^{-3})	5.0×10^{-3} (3.0×10^{-3} – 8.2×10^{-3})	4.0×10^{-2} (2.2×10^{-2} – 9.0×10^{-2})
	Third	5.9×10^{-3} (9.4×10^{-5} – 1.2×10^{-2})[c]	2.6×10^{-2} (1.3×10^{-2} – 4.0×10^{-2})[c]	0.12 (3.0×10^{-2} – 0.21)[c]
	Fifth[d]	8.6×10^{-4} (1.4×10^{-4} – 3.7×10^{-3})	1.7×10^{-2} (7.5×10^{-3} – 4.0×10^{-2})	0.34 (0.11 – 1.8)
CTR	Second	9.8×10^{-6} (1.0×10^{-6} – 4.0×10^{-5})	1.9×10^{-4} (7.4×10^{-5} – 3.8×10^{-4})	3.5×10^{-3} (1.5×10^{-3} – 1.3×10^{-2})
	Third	1.3×10^{-5} (1.2×10^{-6} – 7.5×10^{-5})	1.2×10^{-3} (4.3×10^{-4} – 2.9×10^{-3})	0.11 (3.0×10^{-2} – 0.64)
	Fifth[d]	4.1×10^{-4} (4.5×10^{-5} – 2.4×10^{-3})	2.3×10^{-2} (8.6×10^{-3} – 7.2×10^{-2})	1.3 (0.30 – 14)
CFS	Second	1.5×10^{-2} (3.4×10^{-3} – 0.15)	0.17 (8.6×10^{-2} – 0.39)	2.0 (0.47 – 9.9)
	Third	7.4×10^{-3} (1.0×10^{-3} – 3.9×10^{-2})	0.14 (6.2×10^{-2} – 0.33)	2.7 (0.92 – 15)
	Fifth[d]	1.9×10^{-2} (4.2×10^{-3} – 6.0×10^{-2})	0.25 (0.13 – 0.57)	3.4 (1.2 – 19)
IMI	Second	3.4×10^{-2} (2.2×10^{-2} – 4.6×10^{-2})[c]	0.13 (8.1×10^{-2} – 0.17)[c]	0.48 (0.30 – 0.66)[c]
	Third	8.8×10^{-2} (1.0×10^{-2} – 0.16)[c]	0.41 (0.23 – 0.60)[c]	1.9 (0.53 – 3.4)[c]
	Fifth[d]	7.8×10^{-3} (4.1×10^{-4} – 6.4×10^{-2})	0.25 (7.7×10^{-2} – 0.71)	7.8 (2.2 – 70)
TMX	Second	2.8×10^{-2} (7.8×10^{-3} – 7.1×10^{-2})	8.7×10^{-2} (5.5×10^{-2} – 0.13)	0.27 (0.16 – 0.80)
	Third	2.8×10^{-2} (6.4×10^{-3} – 5.0×10^{-2})[c]	0.17 (9.0×10^{-2} – 0.24)[c]	0.99 (0.22 – 1.8)[c]
	Fifth[d]	0.13 (NC – 0.39)[c]	1.1 (0.14 – 2.0)[c]	8.8 (NC – 18)[c]

[a] Based on combined mortality data from triplicate or quadruplicate bioassays for each insecticide-instar combination. Larvae were fed leaf tissues treated with 0.1% silwet : water and five insecticide suspensions in 0.1% silwet : water.

[b] The cm^2 leaf tissue provided to each larva (see MATERIALS AND METHODS/*Toxicity Studies*/*Dietary toxicity studies*) was used to estimate dietary insecticide concentrations. The average surface areas of leaves given to larvae in each insecticide, instar, bioassay run, and concentration were used (see supplementary file *Weights and surface areas of leaves*). Except as noted in the table, LC values were calculated using maximum likelihood estimate model (see MATERIALS AND METHODS/ *Statistical Analyses)*. Adjustment for control (0.1% silwet: water) mortality was done using Abbott's formula.

[c] LC values were calculated using nonlinear square estimate model (see *Statistical Analyses*).

[d] Observations until pupation (usually 72 or 96 h after treatment).

CIs: confidence intervals; LC10: lethal concentration that kills 10% of a treated population; LC50: lethal concentration that kills 50% of a treated population; LC90: lethal concentration that kills 90% of a treated population

BCF: beta-cyfluthrin; CTR: chlorantraniliprole; CFS: chlorpyrifos; IMI: imidacloprid; TMX: thiamethoxam; NC: not calculable or a negative lower bound CI value

Table 5. Dietary Study: Growth and development of surviving monarch second-, third-, and fifth-instar larvae following exposure to tropical milkweed leaves treated with five insecticides[a]

Insecticide	Instar	Conc[b] (µg insecticide/g leaf)	Larval percent mortality[c]	Number of surviving larvae/pupae (# of replicate bioassays)[d]	Instar/stage at 96 h after application[e]	Mean final Weights[f] (± SD)	Statistical analysis
BCF	Second						$F_{3, 98} = 2.373$; $p = 0.0749$[g]
		0	0%	38 (4)	Fourth	171 (± 85)	
		1.5×10^{-2}	0%	38 (4)	Fourth	165 (± 77)	
		0.13	45%	21 (4)	Fourth	160 (± 55)	
		0.45	68%	9 (2)	Fourth	137 (± 65)	
	Third						$F_{4, 117} = 10.97$; $p = 1.383 \times 10^{-7}$, [h]
		0	0%	30 (3)	Fifth	410 (± 126)	
		1.8×10^{-3}	0%	21 (2)	Fifth	407 (± 136)	df = 87; t-ratio = 0.039; p = 0.9998
		1.4×10^{-2}	0%	32 (3)	Fifth	407 (± 147)	df = 117; t-ratio = 0.028; p = 0.9999
		0.12	7%	28 (3)	Fifth	342 (± 125)	df = 117; t-ratio = 1.907; p = 0.1845
		0.93	35%	13 (2)	Fourth[+]	140 (± 106)***	df = 87; T-ratio = 5.784; p < 0.0001
	Fifth						$F_{3, 85} = 2.615$; $p = 0.05632$[g]
		0	0%	26 (3)	Pupa	1156 (± 137)	
		2.0×10^{-3}	0%	20 (2)	Pupa	1264 (± 131)	
		1.5×10^{-2}	0%	27 (3)	Pupa	1158 (± 160)	
		0.13	14%	23 (3)	Pupa	1129 (± 202)	
CTR	Second						$F_{4, 119} = 6.415$; $p = 1.04 \times 10^{-4}$, [h]
		0	0%	42 (4)	Fourth	207 (± 110)	
		1.9×10^{-6}	5%	20 (2)	Fourth	243 (± 103)	df = 70; t-ratio = 0.139; p = 0.9924
		2.2×10^{-5}	14%	18 (2)	Fourth	148 (± 63)	df = 48; t-ratio = 0.717; p = 0.6911
		2.5×10^{-4}	5%	20 (2)	Fourth	192 (± 126)*	df = 70; t-ratio = 2.982; p = 0.0112
		2.9×10^{-3}	36%	27 (4)	Fourth	162 (± 111)***	df = 119; t-ratio = 4.262; p = 0.0002

Table 5 continued

	Third						$F_{2, 64} = 25.1$; $p = 8.973 \times 10^{-9}$, h
		0	0%	32 (3)	Fifth	377 (± 131)	
		2.6×10^{-4}	1%	21 (2)	Fifth	240 (± 117)**	df = 46; t-ratio = 3.504; p = 0.0020
		3.1×10^{-2}	50%	16 (3)	Fourth+	142 (± 95)***	df = 64; t-ratio = 6.913; p < 0.0001
	Fifth						$F_{3, 84} = 3.722$; $p = 0.01445$g
		0	0%	26 (3)	Pupa	987 (± 237)	
		3.1×10^{-3}	0%	21 (2)	Pupa	1062 (± 158)	df = 61; t-ratio = 0.584; p = 0.8580
		3.2×10^{-2}	8%	24 (3)	Pupa	938 (± 188)	df = 84; t-ratio = 1.397; p = 0.3716
		0.24	21%	20 (3)	Pupa	851 (± 193)*	df = 84; t-ratio = 2.520; p = 0.0374
CFS	Secondi						$F_{4, 97} = 1.705$; $p = 0.1551$g
		0	0%	27 (3)	Fourth	176 (± 133)	
		0.12	0%	27 (3)	Fourth	185 (± 119)	
		0.63	7%	26 (3)	Fourth	155 (± 91)	
		1.1	39%	11 (2)	Fourth	83 (± 38)	
		9.8	45%	13 (3)	Fourth	98 (± 89)	
	Third						$F_{4, 103} = 0.6175$; $p = 0.651$g
		0	0%	31 (3)	Fifth	422 (± 160)	
		3.6×10^{-3}	8%	19 (2)	Fifth	417 (± 220)	
		0.13	3%	20 (2)	Fifth	426 (± 130)	
		0.66	23%	24 (3)	Fifth	434 (± 141)	
		10	48%	16 (3)	Fifth	373 (± 170)	
	Fifth						$F_{4, 117} = 2.149$; $p = 0.07907$g
		0	0%	29 (3)	Pupa	1048 (± 172)	
		3.6×10^{-3}	10%	27 (3)	Pupa	1031 (± 153)	
		0.14	0%	32 (3)	Pupa	987 (± 180)	
		0.69	6%	28 (3)	Pupa	987 (± 127)	
		10	63%	11 (2)	Pupa	947 (± 192)	
IMI	Second						$F_{4, 117} = 12.42$; $p = 1.890 \times 10^{-8}$, h
		0	0%	28 (3)	Fourth	251 (± 70)	
		6.0×10^{-3}	0%	21 (2)	Fourth	258 (± 71)	df = 82; t-ratio = 1.636; p = 0.3038

Table 5 continued

		9.5×10^{-2}	0%	33 (3)	Fourth	260 (± 69)	df = 117; t-ratio = 0.197; p = 0.9924
		0.76	0%	30 (3)	Fourth	247 (± 75)	df = 117; t-ratio = 0.737; p = 0.8341
		7.0	58%	12 (3)	Fourth	145 (± 103)***	df = 117; t-ratio = 5.794; p < 0.0001
	Third						$F_{4, 120} = 16.35$; $p = 1.027 \times 10^{-10}$, h
		0	0%	31 (3)	Fifth	437 (± 121)	
		6.1×10^{-3}	0%	22 (2)	Fifth	418 (± 133)	df = 93; t-ratio = 0.847; p = 0.7761
		9.4×10^{-2}	3%	30 (3)	Fifth	377 (± 126)	df = 120; t-ratio = 2.036; p = 0.1419
		0.75	3%	20 (2)	Fifth	354 (± 69)*	df = 93; t-ratio = 2.741; p = 0.0265
		7.2	23%	24 (3)	Fourth+	199 (± 102)***	df = 120; t-ratio = 7.604; p < 0.0001
	Fifth						$F_{3, 76} = 1.685$; $p = 0.1773$g
		0	0%	26 (3)	Pupa	1010 (± 109)	
		9.7×10^{-2}	14%	23 (3)	Pupa	959 (± 110)	
		0.78	10%	24 (3)	Pupa	959 (± 174)	
		7.3	44%	15 (3)	Pupa	990 (± 137)	
TMX	Second						$F_{4, 97} = 1.944$; $p = 0.1092$g
		0	0%	29 (3)	Fourth	160 (± 81)	
		4.2×10^{-2}	14%	25 (3)	Fourth	144 (± 77)	
		0.52	14%	25 (3)	Fourth	143 (± 90)	
		1.6	17%	16 (2)	Fourth	143 (± 91)	
		4.7	69%	9 (2)	Fourth	120 (± 70)	
	Third						$F_{4, 110} = 9.216$; $p = 1.848 \times 10^{-6}$, h
		0	0%	30 (3)	Fifth	439 (± 161)	
		4.7×10^{-3}	0%	19 (2)	Fifth	411 (± 181)	df = 82; t-ratio = 0.594; p = 0.8978
		4.5×10^{-2}	0%	22 (2)	Fifth	467 (± 109)	df = 82; t-ratio = 0.635; p = 0.8809
		0.54	0%	32 (3)	Fifth	430 (± 134)	df = 110; t-ratio = 0.170; p = 0.9947

Table 5 continued

	4.8	53%	14 (3)	Fourth[+]	175 (± 143)***	df = 110; t-ratio = 5.328; p < 0.0001
Fifth						$F_{4, 101} = 5.779$; $p = 3.142 \times 10^{-4}$, [h]
	0	0%	28 (3)	Pupa	1146 (± 163)	
	4.5×10^{-2}	0%	28 (3)	Pupa	1133 (± 212)	df = 101; t-ratio = 1.031; p = 0.6658
	0.55	0%	29 (3)	Pupa	1138 (± 185)	df = 101; t-ratio = 0.590; p = 0.8994
	4.9	18%	23 (3)	Pupa	1015 (± 173)**	df = 101; t-ratio = 3.464; p = 0.0030
	62	54%	13 (3)	Pupa	957 (± 244)**	df = 101; t-ratio = 3.724; p = 0.0012

[a] Based on combined mortality data from triplicate or quadruplicate bioassays for each insecticide-instar combination. Larvae were fed leaf tissues treated with 0.1% silwet : water and five insecticide suspensions in 0.1% silwet : water.

[b] Concentrations (averaged over runs) that caused equal to or fewer than 70% larval or pupal mortality (i.e., ≥ 30% survival) after adjusting for control (0.1% silwet : water) mortality using Abbott's formula. Only data with concentrations that were used at least twice are provided in the table and analyzed for difference in final weights and development with respect to controls of the same bioassay run.

[c] Larval mortality calculated after setting control mortality to zero and adjusting for it in other concentrations (Abbott's formula).

[d] 11 larvae were treated per concentration per run.

[e] Most common larval instar/stage observed at 96 h after application.

[f] Final weights of larvae were recorded 96 h after application. Final weights of pupae were recorded prior to adult eclosion.

[g] No significant concentration effect on larval weights based on ANOVA.

[h] Significant concentration effect on larval weights based on ANOVA. Post hoc analyses were conducted using Dunnett's test for multiple comparison with control larval weights from the same bioassay runs.

[i] One of the four runs excluded due to hormesis effect (i.e., larval weight gain with increasing concentration).

SD: standard deviation; BCF: beta-cyfluthrin; CTR: chlorantraniliprole; CFS: chlorpyrifos; IMI: imidacloprid; TMX: thiamethoxam

*Treated larvae had significantly lower weights than control larvae at $p < 0.05$.

** Treated larvae had significantly lower weights than control larvae at $p < 0.01$.

*** Treated larvae had significantly lower weights than control larvae at $p < 0.001$.

[+] 79 to 92% of treated larvae were third or fourth instars. 66 to 90% of control larvae were fifth instars.

Table S1. Mean weights and standard deviations (SD) of monarch control first-, second-, third-, and fifth-instar larvae used in the cuticular and dietary toxicity bioassays conducted with tropical milkweed[a]

Insecticide	Cuticular bioassays		Dietary bioassays	
	Instar	Mean larval weight ± SD (g)	Instar	Mean larval weight ± SD (g)
BCF	First	0.0028 ± 0.001 (n = 24)	Second	0.0091 ± 0.004 (n = 24)
	Third	0.0314 ± 0.013 (n = 18)	Third	0.0291 ± 0.012 (n = 18)
	Fifth	0.8118 ± 0.293 (n = 18)	Fifth	0.9247 ± 0.370 (n = 18)
CTR	First	0.0028 ± 0.001 (n = 18)	Second	0.0084 ± 0.003 (n = 24)
	Third	0.0453 ± 0.024 (n = 18)	Third	0.0293 ± 0.015 (n = 18)
	Fifth	0.8061 ± 0.261 (n = 18)	Fifth	0.8237 ± 0.237 (n = 18)
CFS	First	0.0026 ± 0.001 (n = 18)	Second	0.0081 ± 0.005 (n = 24)
	Third	0.0459 ± 0.020 (n = 18)	Third	0.0404 ± 0.026 (n = 18)
	Fifth	0.9188 ± 0.203 (n = 18)	Fifth	0.7567 ± 0.231 (n = 18)
IMI	First	0.0028 ± 0.001 (n = 18)	Second	0.0100 ± 0.006 (n = 18)
	Third	0.0304 ± 0.010 (n = 18)	Third	0.0387 ± 0.014 (n = 18)
	Fifth	1.0591 ± 0.252 (n = 18)	Fifth	0.7454 ± 0.245 (n = 18)
TMX	First	0.0035 ± 0.001 (n = 18)	Second	0.0089 ± 0.003 (n = 18)
	Third	0.0376 ± 0.018 (n = 24)	Third	0.0353 ± 0.022 (n = 18)
	Fifth	0.8572 ± 0.366 (n = 18)	Fifth	0.8971 ± 0.341 (n = 18)

[a] Mean weight of half the control larvae from triplicate or quadruplicate toxicity bioassays for each insecticide-instar combination. Weights of control larvae were measured prior to application of acetone (cuticular bioassays) or feeding with 0.1% silwet : water treated leaves (dietary bioassays).

BCF: beta-cyfluthrin; CTR: chlorantraniliprole; CFS: chlorpyrifos; IMI: imidacloprid; TMX: thiamethoxam

Table S2. Cuticular Study: Nominal insecticide doses used in toxicity bioassays

Insecticide	Instar	Dose units	Doses						
BCF	First	µg/g larva[a]	3.64×10^{-5}	3.64×10^{-4}	3.64×10^{-3}	1.15×10^{-2}	3.64×10^{-2}	0.115	
		µg/cm^2 larva[b]	5.88×10^{-7}	5.88×10^{-6}	5.88×10^{-5}	1.86×10^{-4}	5.88×10^{-4}	1.86×10^{-3}	
		µg/larva[c]	1.00×10^{-7}	1.00×10^{-6}	1.00×10^{-5}	3.16×10^{-5}	1.00×10^{-4}	3.16×10^{-4}	
	Third	µg/g larva[a]	3.19×10^{-4}	3.19×10^{-3}	1.01×10^{-2}	3.19×10^{-2}	0.319	3.19	
		µg/cm^2 larva[b]	1.54×10^{-5}	1.54×10^{-4}	4.86×10^{-4}	1.54×10^{-3}	1.54×10^{-2}	0.154	
		µg/larva[c]	1.00×10^{-5}	1.00×10^{-4}	3.16×10^{-4}	1.00×10^{-3}	1.00×10^{-2}	0.100	
	Fifth	µg/g larva[a]	1.23×10^{-2}	3.89×10^{-2}	0.123	0.265	0.572	1.23	
		µg/cm^2 larva[b]	1.41×10^{-3}	4.45×10^{-3}	1.41×10^{-2}	3.03×10^{-2}	6.54×10^{-2}	0.141	
		µg/larva[c]	1.00×10^{-2}	3.16×10^{-2}	0.100	0.215	0.464	1.00	
CTR	First	µg/g larva[a]	3.60×10^{-5}	3.60×10^{-4}	3.60×10^{-3}	1.14×10^{-2}	3.60×10^{-2}	0.360	3.60
		µg/cm^2 larva[b]	5.88×10^{-7}	5.88×10^{-6}	5.88×10^{-5}	1.86×10^{-4}	5.88×10^{-4}	5.88×10^{-3}	5.88×10^{-2}
		µg/larva[c]	1.00×10^{-7}	1.00×10^{-6}	1.00×10^{-5}	3.16×10^{-5}	1.00×10^{-4}	1.00×10^{-3}	1.00×10^{-2}
	Third	µg/g larva[a]	2.21×10^{-4}	2.21×10^{-3}	2.21×10^{-2}	0.221	0.697	2.21	22.1
		µg/cm^2 larva[b]	1.54×10^{-5}	1.54×10^{-4}	1.54×10^{-3}	1.54×10^{-2}	4.86×10^{-2}	0.154	1.54
		µg/larva[c]	1.00×10^{-5}	1.00×10^{-4}	1.00×10^{-3}	1.00×10^{-2}	3.16×10^{-2}	0.100	1.00
	Fifth	µg/g larva[a]	1.24×10^{-2}	3.92×10^{-2}	0.124	0.392	1.24		
		µg/cm^2 larva[b]	1.41×10^{-3}	4.45×10^{-3}	1.41×10^{-2}	4.45×10^{-2}	0.141		
		µg/larva[c]	1.00×10^{-2}	3.16×10^{-2}	0.100	0.316	1.00		
CFS	First	µg/g larva[a]	3.89	12.3	38.9	123	389		
		µg/cm^2 larva[b]	5.88×10^{-2}	0.186	0.588	1.86	5.88		
		µg/larva[c]	1.00×10^{-2}	3.16×10^{-2}	0.100	0.316	1.00		
	Third	µg/g larva[a]	2.18	6.89	21.8	68.9	218		
		µg/cm^2 larva[b]	0.154	0.486	1.54	4.86	15.4		
		µg/larva[c]	0.100	0.316	1.00	3.16	10.0		
	Fifth	µg/g larva[a]	3.44	10.9	19.4	34.4	61.2		
		µg/cm^2 larva[b]	0.445	1.41	2.50	4.45	7.92		
		µg/larva[c]	3.16	10	17.8	31.6	56.2		
IMI	First	µg/g larva[a]	0.356	1.12	3.56	11.2	35.6		
		µg/cm^2 larva[b]	5.88×10^{-3}	1.86×10^{-2}	5.88×10^{-2}	0.186	0.588		
		µg/larva[c]	1.00×10^{-3}	3.16×10^{-3}	1.00×10^{-2}	3.16×10^{-2}	0.100		
	Third	µg/g larva[a]	1.04	3.29	10.4	32.9	104	329	

Table S2 continued

		µg/cm^2 larva[b]	4.86 x 10^{-2}	0.154	0.486	1.54	4.86	15.4	
		µg/larva[c]	3.16 x 10^{-2}	0.100	0.316	1.00	3.16	10.0	
	Fifth	µg/g larva[a]	9.44 x 10^{-3}	2.98 x 10^{-2}	9.44 x 10^{-2}	0.298	0.944	2.98	9.44
		µg/cm^2 larva[b]	1.41 x 10^{-3}	4.45 x 10^{-3}	1.41 x 10^{-2}	4.45 x 10^{-2}	0.141	0.445	1.41
		µg/larva[c]	1.00 x 10^{-2}	3.16 x 10^{-2}	0.100	0.316	1.00	3.16	10.0
TMX	First	µg/g larva[a]	2.89 x 10^{-2}	0.289	2.89	28.9	289		
		µg/cm^2 larva[b]	5.88 x 10^{-4}	5.88 x 10^{-3}	5.88 x 10^{-2}	0.588	5.88		
		µg/larva[c]	1.00 x 10^{-4}	1.00 x 10^{-3}	1.00 x 10^{-2}	0.100	1.00		
	Third	µg/g larva[a]	2.66 x 10^{-2}	0.266	2.66	8.41	26.6	84.1	266
		µg/cm^2 larva[b]	1.54 x 10^{-3}	1.54 x 10^{-2}	0.154	0.486	1.54	4.86	15.4
		µg/larva[c]	1.00 x 10^{-3}	1.00 x 10^{-2}	0.100	0.316	1.00	3.16	10.0
	Fifth	µg/g larva[a]	1.17	3.69	11.7	23.3	36.9	46.7	
		µg/cm^2 larva[b]	0.141	0.445	1.41	2.82	4.45	5.63	
		µg/larva[c]	1.00	3.16	10.0	20.0	31.6	40.0	

[a] Calculated by multiplying the concentration of the insecticide-acetone solution with the volume of solution applied to each larva (1 µL for all instars) and divided by the average weights of larvae in each insecticide-instar combination (see Table S1).

[b] Calculated by multiplying the concentration of the insecticide-acetone solution with the volume of solution applied to each larva (1 µL for all instars) and divided by the average surface areas of larvae in each instar. Larvae were assumed to be cylinders. Surface area in cm^2 was estimated by measuring the height (h; or length) and radius (r; or half the thickness) of ten individuals for each larval instar using the following formula: $2\pi rh + 2\pi r^2$. Estimated surface areas of first, third, and fifth instars were 0.17 ± 0.05, 0.65 ± 0.12 and 7.1 ± 1.3 cm^2, respectively.

[c] Calculated by multiplying the concentration of the insecticide-acetone solution with the volume of solution applied to each larva (1 µL for all instars).

BCF: beta-cyfluthrin; CTR: chlorantraniliprole; CFS: chlorpyrifos; IMI: imidacloprid; TMX: thiamethoxam

Table S3. Dietary Study: Measured concentrations of 0.1% silwet : water insecticide suspensions used in toxicity bioassays

Nominal concentrations (µg/µL)	Measured concentrations (µg/µL)				
	BCF	CTR	CFS	IMI	TMX
1.00×10^{-7}		5.01×10^{-8} [a]			
1.00×10^{-6}		5.01×10^{-7} [a]			
1.00×10^{-5}		1.34×10^{-6}			
1.00×10^{-4}	4.40×10^{-5}	6.21×10^{-5}	6.97×10^{-6}	1.21×10^{-4}	9.36×10^{-5}
1.00×10^{-3}	3.47×10^{-4}	6.24×10^{-4}	7.18×10^{-5}	1.89×10^{-3}	8.98×10^{-4}
1.00×10^{-2}	2.71×10^{-3}	4.85×10^{-3}	2.72×10^{-3}	1.53×10^{-2}	1.10×10^{-2}
3.16×10^{-2}	9.03×10^{-3} [b]			4.44×10^{-2}	3.43×10^{-2}
0.100	2.10×10^{-2}	4.40×10^{-2}	1.34×10^{-2}	0.145	9.77×10^{-2}
0.316	9.03×10^{-2}		2.26×10^{-2}	0.363	0.340
1.00	0.210	0.700	0.200	1.33	1.21
3.16			0.542	4.74	
10.0	20.6	10.8	13.2	10.0[c]	5.46

[a] Nominal concentration below limit of quantification (1.00×10^{-6} µg/µL); estimated based on average UHPLC/MS-MS recovery (50%) for 0.100 through 1.00×10^{-5} µg/µL (nominal) suspensions.

[b] Not quantified by GC-ECD; estimated concentration based on serial dilution of the 0.316 µg/µL suspension.

[c] Nominal concentration; suspension not quantified by UHPLC/MS-MS.

BCF: beta-cyfluthrin; CTR: chlorantraniliprole; CFS: chlorpyrifos; IMI: imidacloprid; TMX: thiamethoxam

Table S4. Dietary Study: Estimated tropical milkweed insecticide concentrations used in toxicity bioassays

Insecticide	Instar	Conc. units	Tropical milkweed leaf concentrations						
BCF	Second	µg/g leaf[a]	1.9×10^{-3}	1.5×10^{-2}	0.13	0.45	1.0	10	
		µg/cm^2 leaf[b]	5.4×10^{-5}	3.9×10^{-4}	3.0×10^{-3}	1.1×10^{-2}	2.4×10^{-2}	0.24	
	Third	µg/g leaf[a]	1.8×10^{-3}	1.4×10^{-2}	0.12	0.44	0.93	4.7	9.9
		µg/cm^2 leaf[b]	5.7×10^{-5}	4.5×10^{-4}	3.2×10^{-3}	1.5×10^{-2}	2.4×10^{-2}	0.15	0.27
	Fifth	µg/g leaf[a]	2.0×10^{-3}	1.5×10^{-2}	0.13	1.0	11	1000	
		µg/cm^2 leaf[b]	5.8×10^{-5}	4.6×10^{-4}	3.6×10^{-3}	2.9×10^{-2}	0.31	28	
CTR	Second	µg/g leaf[a]	1.8×10^{-6}	2.2×10^{-5}	2.5×10^{-4}	2.9×10^{-3}	3.0×10^{-2}	0.24	2.1
		µg/cm^2 leaf[b]	6.9×10^{-8}	5.3×10^{-7}	1.9×10^{-6}	6.9×10^{-5}	7.4×10^{-4}	5.0×10^{-3}	4.7×10^{-2}
	Third	µg/g leaf[a]	2.3×10^{-5}	2.6×10^{-4}	3.1×10^{-3}	3.1×10^{-2}	0.24	2.2	35
		µg/cm^2 leaf[b]	6.3×10^{-7}	2.0×10^{-6}	7.3×10^{-5}	8.8×10^{-4}	6.9×10^{-3}	5.6×10^{-2}	1.0
	Fifth	µg/g leaf[a]	2.2×10^{-5}	3.1×10^{-3}	3.2×10^{-2}	0.24	2.2	36	570
		µg/cm^2 leaf[b]	7.2×10^{-7}	8.4×10^{-5}	7.7×10^{-4}	5.8×10^{-3}	5.6×10^{-2}	0.85	12
CFS	Second	µg/g leaf[a]	3.6×10^{-4}	3.3×10^{-3}	0.12	0.63	1.1	9.7	620
		µg/cm^2 leaf[b]	8.9×10^{-6}	8.2×10^{-5}	2.9×10^{-3}	1.5×10^{-2}	2.4×10^{-2}	0.22	14
	Third	µg/g leaf[a]	3.6×10^{-4}	3.6×10^{-3}	0.13	0.66	10	27	650
		µg/cm^2 leaf[b]	9.3×10^{-6}	8.8×10^{-5}	3.2×10^{-3}	1.5×10^{-2}	0.23	0.68	16
	Fifth	µg/g leaf[a]	3.6×10^{-3}	0.14	0.69	10	28	670	
		µg/cm^2 leaf[b]	9.0×10^{-5}	3.5×10^{-3}	1.7×10^{-2}	0.26	0.67	16	
IMI	Second	µg/g leaf[a]	6.0×10^{-3}	9.5×10^{-2}	0.76	2.3	7.0	66	
		µg/cm^2 leaf[b]	1.7×10^{-4}	2.5×10^{-3}	1.9×10^{-2}	5.3×10^{-2}	0.20	1.9	
	Third	µg/g leaf[a]	6.1×10^{-3}	9.4×10^{-2}	0.75	7.2	18	66	240
		µg/cm^2 leaf[b]	1.5×10^{-4}	2.3×10^{-3}	1.9×10^{-2}	0.17	0.45	1.7	5.6
	Fifth	µg/g leaf[a]	6.1×10^{-3}	9.7×10^{-2}	0.78	7.3	68	240	540
		µg/cm^2 leaf[b]	1.8×10^{-4}	2.5×10^{-3}	1.9×10^{-2}	0.18	1.7	6.7	12
TMX	Second	µg/g leaf[a]	4.4×10^{-3}	4.2×10^{-2}	0.52	1.6	4.7	57	
		µg/cm^2 leaf[b]	1.3×10^{-4}	1.3×10^{-3}	1.5×10^{-2}	4.1×10^{-2}	0.12	1.6	
	Third	µg/g leaf[a]	4.7×10^{-3}	4.5×10^{-2}	0.54	1.6	4.8	16	60
		µg/cm^2 leaf[b]	1.3×10^{-4}	1.2×10^{-3}	1.5×10^{-2}	5.7×10^{-2}	0.13	0.57	1.7
	Fifth	µg/g leaf[a]	4.8×10^{-3}	4.5×10^{-2}	0.55	4.9	62	280	
		µg/cm^2 leaf[b]	1.5×10^{-4}	1.4×10^{-3}	1.7×10^{-2}	0.15	2.0	8.9	

[a] Calculated by multiplying the measured concentration of 0.1% silwet : water insecticide suspension (see Table S3) with the volume

of suspension applied on each leaf tissue (5, 20, and 100 µL for second-, third-, and fifth-instar bioassays, respectively) and divided by the average weights of leaves used in each insecticide-instar-concentration combination (see supplementary file *Weights and surface areas of leaves*).

[b] Calculated by multiplying the measured concentration of 0.1% silwet : water insecticide suspension (see Table S3) with the volume of suspension applied on each leaf tissue (5, 20, and 100 µL for second-, third-, and fifth-instar bioassays, respectively) and divided by the average surface areas of leaves used in each insecticide-instar-concentration combination (see supplementary file *Weights and surface areas of leaves*).

BCF: beta-cyfluthrin; CTR: chlorantraniliprole; CFS: chlorpyrifos; IMI: imidacloprid; TMX: thiamethoxam; Conc.: concentration

Table S5. Estimated environmental exposure concentrations of five active ingredient insecticides based on representative formulated products registered for foliar application to manage true armyworms (ta) and soybean aphids (sa)[a]

Formulated product (active ingredient) and its application rate (g AI/ha) for sa and ta	Application method[b] (pest)	Point deposition (µg/cm^2) at 0, 15, 30, and 60 m downwind from edge of treated crop field			
		0	15	30	60
Baythroid® XL	Aerial (sa)	0.1	2.8×10^{-2}	1.4×10^{-2}	6.2×10^{-3}
[beta-cyfluthrin: 24.7 (sa, ta)]	High Boom (sa, ta)	0.2	2.1×10^{-3}	1.1×10^{-3}	5.8×10^{-4}
	Low Boom (ta)	0.21	1.1×10^{-3}	6.0×10^{-4}	3.4×10^{-4}
Beseige®	Aerial (sa)	0.3	6.5×10^{-2}	3.4×10^{-2}	1.5×10^{-2}
[chlorantraniliprole; 58.5 (sa) and 73.1 (ta)]	High Boom (sa)	0.6	4.9×10^{-3}	2.6×10^{-3}	1.4×10^{-3}
	High Boom (ta)	0.7	6.2×10^{-3}	3.3×10^{-3}	1.7×10^{-3}
	Low Boom (ta)	0.7	3.2×10^{-3}	1.8×10^{-3}	1.0×10^{-3}
Lorsban®	Aerial (sa)	5.6	1.3	0.7	0.3
[chlorpyrifos; 1121 (sa, ta)]	High Boom (sa, ta)	11	9.5×10^{-2}	5.0×10^{-2}	2.7×10^{-2}
	Low Boom (ta)	11	4.9×10^{-2}	2.7×10^{-2}	1.5×10^{-2}
Admire Pro®	Aerial (sa)	0.3	5.8×10^{-2}	3.0×10^{-2}	1.3×10^{-2}
[imidacloprid; 52.3 (sa)]	High Boom (sa)	0.5	4.4×10^{-3}	2.4×10^{-3}	1.2×10^{-3}
Swagger®	High Boom (ta)	1.1	9.5×10^{-3}	5.0×10^{-3}	2.7×10^{-3}
[imidacloprid; 112 (ta)]	Low Boom (ta)	1.1	4.9×10^{-3}	2.7×10^{-3}	1.5×10^{-3}
Endigo®	Aerial (sa)	0.2	4.6×10^{-2}	2.4×10^{-2}	1.0×10^{-2}
[thiamethoxam; 41.4 (sa)][c]	High Boom (sa)	0.4	3.5×10^{-3}	1.9×10^{-3}	9.8×10^{-4}

[a] Environmental insecticide concentrations deposited on downwind surfaces following an aerial, high or a low ground boom application to manage soybean aphids and true armyworms was estimated using AgDRIFT model version 2.1.1 (USEPA 2003).

[b] For high and low ground boom, 50th-percentile results were used.
[c] Thiamethoxam is not registered for use on true armyworms in maize fields.

Table S6. Cuticular Study: Acute toxicity (expressed as μg insecticide/larva) of five insecticides to monarch first-, third-, and fifth-instar larvae fed tropical milkweed leaves[a]

Insecticide	Instar	96-h LD values with 95% CI (μg insecticide/larva)[b]		
		LD10	LD50	LD90
BCF	First	6.0×10^{-6} ($0 – 1.1 \times 10^{-5}$)	2.5×10^{-5} ($1.4 \times 10^{-5} – 3.6 \times 10^{-5}$)	1.1×10^{-4} ($4.6 \times 10^{-5} – 1.7 \times 10^{-4}$)
	Third	8.8×10^{-5} ($2.4 \times 10^{-5} – 3.3 \times 10^{-4}$)[c]	5.7×10^{-4} ($3.0 \times 10^{-4} – 1.1 \times 10^{-3}$)[c]	3.7×10^{-3} ($1.8 \times 10^{-3} – 1.0 \times 10^{-2}$)[c]
	Fifth[d]	1.3×10^{-2} ($7.0 \times 10^{-3} – 2.0 \times 10^{-2}$)[c]	4.6×10^{-2} ($3.3 \times 10^{-2} – 6.2 \times 10^{-2}$)[c]	0.17 (0.11 – 0.26)[c]
CTR	First	3.0×10^{-6} ($3.9 \times 10^{-7} – 1.2 \times 10^{-5}$)[c]	3.4×10^{-5} ($1.4 \times 10^{-5} – 7.8 \times 10^{-5}$)[c]	4.0×10^{-4} ($1.5 \times 10^{-4} – 1.7 \times 10^{-3}$)[c]
	Third	6.0×10^{-4} ($1.8 \times 10^{-4} – 1.7 \times 10^{-3}$)[c]	4.3×10^{-3} ($2.4 \times 10^{-3} – 7.7 \times 10^{-3}$)[c]	3.1×10^{-2} ($1.6 \times 10^{-2} – 7.6 \times 10^{-2}$)[c]
	Fifth[d]	4.7×10^{-2} ($1.4 \times 10^{-2} – 8.0 \times 10^{-2}$)	0.15 (0.10 – 0.21)	0.50 (0.25 – 0.75)
CFS	First	0.10 ($4.4 \times 10^{-2} – 0.16$)	0.20 (0.14 – 0.26)	0.40 (0.27 – 0.53)
	Third	0.40 (0.13 – 0.67)	1.0 (0.69 – 1.4)	2.7 (1.5 – 3.8)
	Fifth[d]	7.9 (7.3 – 8.6)	17 (14 – 19)	35 (28 – 41)
IMI	First	7.4×10^{-3} ($2.8 \times 10^{-3} – 1.2 \times 10^{-2}$)	1.9×10^{-2} ($1.3 \times 10^{-2} – 2.5 \times 10^{-2}$)	4.8×10^{-2} ($2.8 \times 10^{-2} – 6.8 \times 10^{-2}$)
	Third	3.8×10^{-2} ($9.0 \times 10^{-3} – 0.12$)[c]	0.26 (0.13 – 0.48)[c]	1.7 (0.90 – 4.3)[c]
	Fifth[d]	1.1 (0.35 – 1.8)	3.2 (2.2 – 4.2)	9.5 (3.7 – 15)
TMX	First	4.9×10^{-3} ($9.3 \times 10^{-4} – 8.8 \times 10^{-3}$)	2.1×10^{-2} ($1.0 \times 10^{-2} – 3.2 \times 10^{-2}$)	9.3×10^{-2} ($2.3 \times 10^{-2} – 0.16$)
	Third	6.8×10^{-2} ($2.2 \times 10^{-2} – 0.11$)	0.33 (0.21 – 0.45)	1.6 (0.73 – 2.5)
	Fifth[d]	15 (6.2 – 23)	30 (24 – 35)	61 (33 – 89)

[a] Based on combined mortality data from triplicate or quadruplicate toxicity bioassays for each insecticide-instar combination. Larvae were treated with acetone and five insecticide-acetone solutions.
[b] Except as noted in the table, LD values were estimated using nonlinear least square estimate model (see MATERIALS AND METHODS/*Statistical Analyses*). Adjustment for control (acetone) mortality was done using Abbott's formula.
[c] LD values estimated using a maximum likelihood estimate model (see *Statistical Analyses*).
[d] Observations until pupation (usually 72 or 96 h after treatment).
CIs: confidence intervals; LD10: lethal dose that kills 10% of a treated population; LD50: lethal dose that kills 50% of a treated population; LD90: lethal dose that kills 90% of a treated population
BCF: beta-cyfluthrin; CTR: chlorantraniliprole; CFS: chlorpyrifos; IMI: imidacloprid; TMX: thiamethoxam

Table S7. Cuticular Study: Growth and development of surviving monarch first-, third-, and fifth-instar larvae fed tropical milkweed leaves following application of five insecticides[a]

Insecticide	Instar	Dose[b] (μg insecticide/g larva)	Larval percent mortality[c]	# of surviving larvae/pupae (# of replicate bioassays)[d]	Instar/stage 96 h after application[e]	Mean final weight[f] (± SD)	Statistical analysis
BCF	First						$F_{4, 131} = 1.303$; $p = 0.2723$[g]
		0	0	39 (4)	Fourth	138 (± 74)	
		3.64×10^{-5}	0	30 (3)	Fourth	133 (± 60)	
		3.64×10^{-4}	13	26 (3)	Fourth	129 (± 64)	
		3.64×10^{-3}	30	28 (4)	Fourth	112 (± 66)	
		1.15×10^{-2}	47	16 (3)	Fourth	84 (± 44)	
	Third						$F_{3, 90} = 2.085$; $p = 0.1077$[g]
		0	0	28 (3)	Fourth	290 (± 123)	
		3.19×10^{-4}	0	30 (3)	Fourth	276 (±124)	
		3.19×10^{-3}	11	25 (3)	Fourth	267 (± 93)	
		3.19×10^{-2}	54	13 (2)	Fourth	197 (± 89)	
	Fifth						$F_{2, 71} = 0.2595$; $p = 0.7722$[g]
		0	0	33 (3)	Pupa	1030 (± 164)	
		1.23×10^{-2}	6	30 (3)	Pupa	1025 (± 161)	
		3.89×10^{-2}	41	13 (2)	Pupa	990 (± 144)	
CTR	First						$F_{4, 98} = 0.4057$; $p = 0.8042$[g]
		0	0	28 (3)	Fourth	205 (± 112)	
		3.60×10^{-5}	7	26 (3)	Fourth	180 (± 102)	
		3.60×10^{-4}	4	27 (3)	Fourth	195 (± 96)	
		3.60×10^{-3}	14	16 (2)	Fourth	169 (± 84)	
		3.60×10^{-2}	57	8 (2)	Fourth	165 (± 98)	
	Third						$F_{2, 82} = 4.214$; $p = 0.018$[h]
		0	0	30 (3)	Fifth	342 (± 119)	
		2.21×10^{-3}	0	31 (3)	Fourth[+]	309 (± 172)	df = 82; t-ratio = 1.400; p = 0.2855
		2.21×10^{-2}	13	26 (3)	Fourth[+]	276 (± 143)**	df = 82; t-ratio = 2.903; p = 0.0092

Table S7 continued

	Fifth					$F_{3, 103} = 0.8845$; $p = 0.4518$[g]
		0	0	31 (3)	Pupa	961 (± 150)
		1.24×10^{-2}	6	29 (3)	Pupa	973 (± 136)
		3.92×10^{-2}	7	28 (3)	Pupa	1015 (± 123)
		0.124	32	21 (3)	Pupa	963 (± 131)
CFS	First					$F_{3, 111} = 0.6321$; $p = 0.5958$[g]
		0	0	30 (3)	Fourth	210 (± 81)
		3.89	0	31 (3)	Fourth	218 (± 76)
		12.3	3	29 (3)	Fourth	225 (± 100)
		38.9	10	27 (3)	Fourth	236 (± 73)
	Third					$F_{3, 97} = 1.9295$; $p = 0.1299$[g]
		0	0	30 (3)	Fifth	426 (± 188)
		2.18	0	30 (3)	Fifth	445 (± 171)
		6.89	10	27 (3)	Fifth	441 (± 190)
		21.8	47	16 (3)	Fifth	332 (± 133)
	Fifth					$F_{3, 103} = 2.1687$; $p = 0.09622$[g]
		0	0	33 (3)	Pupa	902 (± 146)
		3.44	0	33 (3)	Pupa	925 (± 145)
		10.9	18	27 (3)	Pupa	861 (± 152)
		19.4	52	16 (3)	Pupa	798 (± 126)
IMI	First[i]					$F_{3, 69} = 3.6132$; $p = 0.0174$[h]
		0	0	20 (2)	Fourth	115 (± 43)
		0.356	0	20 (2)	Fourth	138 (± 46)	df = 69; t-ratio = 1.855; p = 0.1698
		1.12	5	19 (2)	Fourth	116 (± 35)	df = 69; t-ratio = 0.082; p = 0.9975
		3.56	20	15 (2)	Fourth	93 (± 32)	df = 69; t-ratio = 1.545; p = 0.2959
	Third					$F_{3, 73} = 2.3355$; $p = 0.08082$[g]
		0	0	27 (3)	Fourth	209 (± 82)
		1.04	15	23 (3)	Fourth	189 (± 94)
		3.29	19	22 (3)	Fourth	163 (± 53)

Table S7 continued

		10.4	56	8 (2)	Fourth	157 (± 58)	
	Fifth						$F_{4, 119} = 1.0539$; $p = 0.3825$[g]
		0	0	31 (3)	Pupa	1057 (± 177)	
		9.44×10^{-3}	0	22 (2)	Pupa	1032 (± 199)	
		9.44×10^{-2}	6	30 (3)	Pupa	1093 (± 197)	
		0.944	12	28 (3)	Pupa	1117 (± 180)	
		2.98	53	15 (3)	Pupa	1047 (± 127)	
TMX	First						$F_{3, 104} = 0.4029$; $p = 0.7512$[g]
		0	0	27 (3)	Fourth	175 (± 90)	
		2.89×10^{-2}	0	29 (3)	Fourth	189 (± 88)	
		0.289	0	31 (3)	Fourth	162 (± 74)	
		2.89	21	23 (3)	Fourth	186 (± 68)	
	Third						$F_{3, 100} = 0.5515$; $p = 0.6483$[g]
		0	0	33 (3)	Fourth	229 (± 110)	
		2.66×10^{-2}	0	21 (2)	Fourth	202 (± 108)	
		0.266	2	20 (2)	Fourth	190 (± 73)	
		2.66	5	32 (3)	Fourth	219 (± 103)	
	Fifth						$F_{4, 107} = 0.3716$; $p = 0.8284$[g]
		0	0	29 (3)	Pupa	852 (± 144)	
		3.69	0	29 (3)	Pupa	856 (± 132)	
		11.7	3	28 (3)	Pupa	888 (± 131)	
		23.3	22	15 (2)	Pupa	902 (± 113)	
		36.9	52	14 (3)	Pupa	863 (± 103)	

[a] Based on combined mortality data from triplicate or quadruplicate toxicity bioassays for each insecticide-instar combination. Larvae were treated with acetone and five insecticide-acetone solutions.

[b] Doses that caused equal to or fewer than 70% larval or pupal mortality (i.e., ≥ 30% survival) after adjusting for control (acetone) mortality using Abbott's formula. Only data with doses that were used at least twice are provided and analyzed for difference in final weights and development with respect to controls of the same bioassay run.

[c] Larval mortality calculated after setting control mortality to zero and adjusting for it in other concentrations (Abbott's formula).

[d] 11 larvae were treated per concentration per run.

[e] Most common larval instar/stage observed 96 h after application.

[f] Final weights of larvae were recorded 96 h after application, except in four out of 46 instances when weights were recorded at 72 or

120 h (done for beta-cyfluthrin and chlorpyrifos). Final weights of pupae were recorded prior to adult eclosion.
[g] No significant dose effect on larval weights based on ANOVA.
[h] Significant dose effect on larval weights based on ANOVA. Post hoc analyses were conducted using Dunnett's test for multiple comparison with control larval weights from the same bioassay runs.
[i] One of the three runs excluded due to hormesis effect (i.e., larval weight gain with increasing concentration).
SD: standard deviation; BCF: beta-cyfluthrin; CTR: chlorantraniliprole; CFS: chlorpyrifos; IMI: imidacloprid; TMX: thiamethoxam
*Treated larvae had significantly lower weights than control larvae at $p < 0.05$.
** Treated larvae had significantly lower weights than control larvae at $p < 0.01$.
[+] 52 to 54% of treated larvae were third or fourth instars. 60% of control larvae were fifth instars.
SD: standard deviation; BCF: beta-cyfluthrin; CTR: chlorantraniliprole; CFS: chlorpyrifos; IMI: imidacloprid; TMX: thiamethoxam

Table S8. Cuticular Study: Mean adult eclosion rates of fully formed pupae following application of five insecticides to monarch fifth-instar larvae fed tropical milkweed leaves[a]

Insecticide	Dose[b] (μg insecticide/g larva)	Number of surviving pupae (# of replicate bioassays)[c]	Mean adult eclosion rate (Range)	Statistical analyses
BCF				$F_{2, 3} = 0.4620$; $p = 0.6685$[d]
	0	33 (3)	0.85 (0.73 - 1.00)	
	1.23×10^{-2}	30 (3)	0.93 (0.88 - 1.00)	
	3.89×10^{-2}	13 (2)	0.92 (0.83 - 1.00)	
CTR				$F_{3, 6} = 0.7191$; $p = 0.5760$[d]
	0	31 (3)	0.68 (0.45 - 0.82)	
	1.24×10^{-2}	29 (3)	0.55 (0.44 - 0.70)	
	3.92×10^{-2}	28 (3)	0.75 (0.40 - 1.00)	
	0.124	21 (3)	0.62 (0.33 - 0.88)	
CFS				$F_{3, 6} = 0.7885$; $p = 0.5429$[d]
	0	33 (3)	0.82 (0.73 - 0.91)	
	3.44	33 (3)	0.91 (0.82 - 1.00)	
	10.9	27 (3)	0.89 (0.73 - 1.00)	
	19.4	16 (3)	0.75 (0.60 – 1.00)	
IMI				$F_{4, 7} = 0.5275$; $p = 0.7200$[d]
	0	31 (3)	0.90 (0.67 - 1.00)	
	9.44×10^{-3}	22 (2)	0.95 (0.91 - 1.00)	

Table S8 continued

	9.44×10^{-2}	30 (3)	0.93 (0.88 - 1.00)	
	0.944	28 (3)	0.89 (0.78 - 1.00)	
	2.98	15 (3)	1.00 (1.00 – 1.00)	
TMX				$F_{4, 7} = 0.5110$; $p = 0.7306$[d]
	0	29 (3)	0.97 (0.91 - 1.00)	
	3.69	29 (3)	1.00 (1.00 - 1.00)	
	11.7	28 (3)	0.93 (0.83 - 1.00)	
	23.3	15 (2)	0.87 (0.60 - 1.00)	
	36.9	14 (3)	0.93 (0.67 – 1.00)	

[a] Based on combined mortality data from triplicate toxicity bioassays for each insecticide-instar combination. Larvae were treated with acetone and five insecticide-acetone solutions.

[b] Doses that caused equal to or fewer than 70% larval or pupal mortality (i.e., ≥ 30% survival), after adjusting for control (acetone) mortality using Abbott's formula. Only data with doses that were used at least twice are provided and analyzed for difference in eclosion with respect to controls of the same bioassay run.

[c] 11 fifth instars were treated per concentration per run.

[d] No significant dose effect on eclosion rate based on ANOVA.

BCF: beta-cyfluthrin; CTR: chlorantraniliprole; CFS: chlorpyrifos; IMI: imidacloprid; TMX: thiamethoxam

Table S9. Cuticular Study: Rates of arrested ecdysis in monarch fifth-instar larvae fed tropical milkweed leaves following application of five insecticides[a]

Insecticide	Total mortality[b]	Total mortality from arrested ecdysis[c]	Mean mortality due to arrested ecdysis (Range over runs)[d]
Control[e]	4%	1%	29% (0% – 100%)
BCF	67%	2%	3% (0% – 5%)
CTR	48%	1%	3% (0% – 8%)
CFS	52%	9%	18% (13% – 32%)
IMI	31%	28%	92% (78% – 100%)
TMX	37%	32%	87% (69% – 97%)

[a] Based on combined mortality data from triplicate toxicity bioassays for each insecticide-fifth instar combination. Larvae were treated with acetone and five insecticide-acetone solutions. In each bioassay run, 11 larvae were treated with acetone and 55 larvae were

treated with an insecticide (at five different concentrations).
[b] Total mortality across all insecticide concentrations and runs. This includes mortality prior to J formation and mortality due to arrested ecdysis.
[c] Total mortality caused by arrested ecdysis across all insecticide concentrations and runs (total mortality minus mortality prior to J formation).
[d] Percentage of total mortality that occurred due to arrested ecdysis [i.e., (mortality due to arrested ecdysis/total mortality) x 100)].
[e] Control mortality and arrested ecdysis data obtained from 15 fifth-instar cuticular bioassays (control larvae were used in every insecticide-bioassay run combination).
Clothianidin treatments produced an overall mortality of 51%, of which the mean mortality due to arrested ecdysis was 42% (range over runs was 39% to 45%).
BCF: beta-cyfluthrin; CTR: chlorantraniliprole; CFS: chlorpyrifos; IMI: imidacloprid; TMX: thiamethoxam

Table S10. Cuticular Study: Comparison of acute toxicity of five insecticides to monarch first-, third-, and fifth-instar larvae fed common or tropical milkweed leaves

Insecticide	Instar	Common milkweed 96-h LD50 with 95% CI (µg/larva)[a] (A)	Tropical milkweed 96-h LD50 with 95% CI (µg/larva)[b] (B)	96-h LD50 ratio (B/A)
BCF	Third	5.1×10^{-4} (1.6×10^{-4} – 1.9×10^{-3})	5.7×10^{-4} (3.0×10^{-4} – 1.1×10^{-3})	1.1
CTR	First	3.3×10^{-5} (4.7×10^{-6} – 3.1×10^{-4})	3.4×10^{-5} (1.4×10^{-5} – 7.8×10^{-5})	1.0
CFS	First	0.22 (NC – 14)	0.20 (0.14 – 0.26)	0.91
IMI[c]	Fifth[d]	1.4 (0.69 – 2.0)[e]	3.2 (2.2 – 4.2)	2.3
TMX	First	1.1×10^{-2} (NC – 2.9×10^{-2})[e]	2.1×10^{-2} (1.0×10^{-2} – 3.2×10^{-2})	1.9

[a] Based on larval mortality data from one common milkweed bioassay. Larvae were treated with acetone and five doses of each insecticide solutions in acetone (11 larvae treated per dose). Except as noted in the table, LD values were estimated using maximum likelihood estimate model (see MATERIALS AND METHODS/*Statistical Analyses*). Adjustment for control (acetone) mortality was done using Abbott's formula.
[b] Values from Table S6.
[c] Based on larval mortality data from two common milkweed bioassays. The imidacloprid treatments produced an overall mortality of 42%, of which the mean mortality due to arrested ecdysis was 96% (range over runs was 92% to 100%).
[d] Observations until pupation (usually 72 or 96 h after treatment).
[e] LD50 values calculated using nonlinear least square estimate model (see *Statistical Analyses*).
LD50: lethal dose that kills 50% of a treated population; NC: lower bound CI either not calculable or a negative value
BCF: beta-cyfluthrin; CTR: chlorantraniliprole; CFS: chlorpyrifos; IMI: imidacloprid; TMX: thiamethoxam

Table S11. Cuticular Study: Clothianidin doses used in toxicity bioassays

Instar	Dose units	Estimated doses						
First	µg/g larva[a]	2.78×10^{-4}	2.78×10^{-3}	2.78×10^{-2}	0.278	2.78	27.8	
	µg/cm^2 larva[b]	5.88×10^{-6}	5.88×10^{-5}	5.88×10^{-4}	5.88×10^{-3}	5.88×10^{-2}	0.588	
	µg/larva[c]	1.00×10^{-6}	1.00×10^{-5}	1.00×10^{-4}	1.00×10^{-3}	1.00×10^{-2}	0.100	
Third	µg/g larva[a]	4.83×10^{-3}	4.83×10^{-2}	0.483	4.83	48.3		
	µg/cm^2 larva[b]	1.54×10^{-4}	1.54×10^{-3}	1.54×10^{-2}	0.154	1.54		
	µg/larva[c]	1.00×10^{-4}	1.00×10^{-3}	1.00×10^{-2}	0.100	1.00		
Fifth	µg/g larva[a]	9.70×10^{-4}	9.70×10^{-3}	9.70×10^{-2}	0.307	0.970	3.07	9.70
	µg/cm^2 larva[b]	1.41×10^{-4}	1.41×10^{-3}	1.41×10^{-2}	4.45×10^{-2}	0.141	0.445	1.41
	µg/larva[c]	1.00×10^{-3}	1.00×10^{-2}	0.100	0.316	1.00	3.16	10.0

[a] Calculated by multiplying the concentration of the clothianidin-acetone solution with the volume of solution applied to each larva (1 µL for all instars) and divided by the average weights of larvae in each clothianidin-instar combination. The average weights of first-, third-, and fifth-instar control larvae at time of treatment were 0.0036 ± 0.001g, 0.0207 ± 0.004g, and 1.0308 ± 0.371g, respectively (n = 6 each).

[b] Calculated by multiplying the concentration of the clothianidin-acetone solution with the volume of solution applied to each larva (1 µL for all instars) and divided by the average surface areas of larvae in each instar. Estimated surface areas of first, third, and fifth instars were 0.17 ± 0.05, 0.65 ± 0.12 and 7.1 ± 1.3 cm^2, respectively (n= 10 each).

[c] Calculated by multiplying the concentration of the clothianidin-acetone solution with the volume of solution applied to each larva (1 µL for all instars).

Table S12. Cuticular Study: Acute toxicity of clothianidin to monarch first-, third-, and fifth-instar larvae fed tropical milkweed leaves[a]

Instar	Units	96-h LD values with 95% CIs[b]		
		LD10	LD50	LD90
First	µg/g larva[c]	3.8×10^{-2} (3.0×10^{-2} – 4.5×10^{-2})	0.19 (0.15 – 0.23)	0.95 (0.76 – 1.1)
	µg/larva	1.4×10^{-4} (1.1×10^{-4} – 1.6×10^{-4})	6.9×10^{-4} (5.4×10^{-4} – 8.2×10^{-4})	3.4×10^{-3} (2.7×10^{-3} – 4.1×10^{-3})
	µg/cm^2 larva[d]	8.3×10^{-4} (6.5×10^{-4} – 9.6×10^{-4})	4.1×10^{-3} (3.8×10^{-3} – 4.2×10^{-3})	2.0×10^{-2} (1.4×10^{-2} – 2.6×10^{-2})
Third	µg/g larva[c]	0.11 (9.2×10^{-2} – 0.12)	0.83 (0.82 – 0.84)	6.5 (5.5 – 7.5)
	µg/larva	2.2×10^{-3} (1.9×10^{-3} – 2.5×10^{-3})	1.7×10^{-2} (1.6×10^{-2} – 1.8×10^{-2})	0.13 (0.10 – 0.17)
	µg/cm^2 larva[d]	3.4×10^{-3} (2.9×10^{-3} – 3.8×10^{-3})	2.7×10^{-2} (2.6×10^{-2} – 2.7×10^{-2})	0.21 (0.17 – 0.25)
Fifth[e]	µg/g larva[c]	0.44 (0.13 – 0.75)	1.3 (0.84 – 1.7)	3.6 (1.6 – 5.5)
	µg/larva	0.45 (0.13 – 0.77)	1.3 (0.86 – 1.7)	3.7 (1.6 – 5.7)
	µg/cm^2 larva[d]	6.3×10^{-2} (1.9×10^{-2} – 0.11)	0.18 (0.12 – 0.24)	0.52 (0.23 – 0.80)

[a] Based on combined mortality data from two to five bioassays. Larvae were treated with acetone and five clothianidin doses dissolved in acetone (5-11 larvae treated per dose).

[b] LD values were estimated using nonlinear least square estimate model (see MATERIALS AND METHODS/*Statistical Analyses*). Adjustment for control (acetone) mortality was done using Abbott's formula.

[c] The average weights of first-, third-, and fifth-instar control larvae at time of treatment were 0.0036 ± 0.001g, 0.0207 ± 0.004g, and 1.0308 ± 0.371g, respectively (n = 6 each). The µg of insecticide per g larva were calculated by dividing the concentrations and volume of insecticide solution applied to each larva using the average weights of control larvae before treatment.

[d] Estimated surface areas of first, third, and fifth instars were 0.17 ± 0.05, 0.65 ± 0.12 and 7.1 ± 1.3 cm^2, respectively (n= 10 each).

[e] Observations until pupation (usually 72 or 96 h after treatment).

CIs: confidence intervals; LD50: lethal dose that kills 50% of a treated population; LD10: lethal dose that kills 10% of a treated population; LD90: lethal dose that kills 90% of a treated population

Table S13. Dietary Study: Mean weight and surface area of tropical milkweed leaves used in toxicity bioassays conducted with monarch second-, third-, and fifth-instar larvae[a]

Insecticide	Instar	Mean leaf weight ± SD (g)	Mean leaf surface area ± SD (cm^2)
BCF	Second[b]	0.102 ± 0.013 (n = 264)	4.4 ± 1.1 (n = 264)
	Third[b]	0.437 ± 0.056[c] (n = 197)	16 ± 3.5[c] (n = 197)
	Fifth[d]	2.003 ± 0.111 (n = 197)	73 ± 11 (n = 193)
CTR	Second[b]	0.104 ± 0.012 (n = 264)	4.4 ±1.2 (n = 264)
	Third[b]	0.404 ± 0.03 (n = 198)	15 ± 2.3 (n = 198)
	Fifth[d]	1.983 ± 0.121 (n = 198)	81 ± 11 (n = 194)
CFS	Second[b]	0.107 ± 0.013 (n = 264)	4.7 ± 1.2 (n = 264)
	Third[b]	0.405 ± 0.026 (n = 198)	17 ± 3.3 (n = 198)
	Fifth[d]	1.962 ± 0.119 (n = 196)	78 ± 8.5 (n = 179)
IMI	Second[b]	0.102 ± 0.014 (n = 197)	3.9 ± 1.0 (n = 197)
	Third[b]	0.402 ± 0.027 (n = 198)	16 ± 2.4 (n = 198)
	Fifth[d]	1.964 ± 0.124 (n = 197)	78 ± 11 (n = 196)
TMX	Second[b]	0.105 ± 0.013 (n = 198)	3.8 ± 0.8 (n = 198)
	Third[b]	0.405 ± 0.027 (n = 197)	14 ± 3.4 (n = 197)
	Fifth[d]	1.988 ± 0.114 (n = 198)	64 ± 6.4 (n = 198)

[a] Mean weight and surface area of leaves treated with 0.1% silwet : water and five insecticide suspensions in 0.1% silwet : water. Leaves were provided to individual larvae in triplicate or quadruplicate bioassays for each insecticide-instar combination. LC values and concentration-response curves were derived using average weights and surface areas of leaves from each concentration and bioassay run for each insecticide-instar combination. See supplementary file *Weights and surface areas of leaves*. Weights and surface areas of leaves fed to larvae that went missing during a bioassay are excluded from the table.

[b] Second and third instars were fed treated leaves for 48 h. The range of leaf weights selected for second- and third-instar bioassays were 0.075 to 0.125 g and 0.350 to 0.450 g, respectively.

[c] One of the three bioassays was conducted using leaf weights in the range of 0.450 to 0.550 g (instead of 0.350 to 0.450 g).

[d] Fifth instars were fed treated leaves for 24 h. The range of leaf weights selected for fifth-instar bioassays was 1.8 to 2.2 g.

BCF: beta-cyfluthrin; CTR: chlorantraniliprole; CFS: chlorpyrifos; IMI: imidacloprid; TMX: thiamethoxam; SD: standard deviation

Table S14. Dietary study: Adult eclosion rates of fully formed pupae following exposure to tropical milkweed leaves treated with five insecticides[a]

Insecticide	Concentration[b] (μg insecticide/g leaf)	Number of surviving larvae/pupae (# of replicate bioassays)[c]	Mean adult eclosion rate (Range)	Statistical analysis
BCF[d]				$F_{4, 7} = 2.037$; $p = 0.1934$[e]
	0	26 (3)	0.73 (0.44 – 0.89)	
	2.0×10^{-3}	20 (2)	0.70 (0.33 – 1.00)	
	1.5×10^{-2}	27 (3)	0.89 (0.88 – 0.90)	
	0.13	23 (3)	0.61 (0.00 – 0.89)	
CTR				$F_{3, 5} = 0.9617$; $p = 0.4789$[e]
	0	26 (3)	0.96 (0.89 – 1.00)	
	3.1×10^{-3}	21 (2)	0.90 (0.90 – 0.91)	
	3.2×10^{-2}	24 (3)	0.92 (0.78 – 1.00)	
	0.24	20 (3)	0.80 (0.57 – 1.00)	
CFS[d]				$F_{4, 7} = 0.7194$; $p = 0.6051$[e]
	0	29 (3)	0.93 (0.75 – 1.00)	
	3.6×10^{-3}	27 (3)	0.89 (0.50 – 1.00)	
	0.14	32 (3)	0.84 (0.60 – 1.00)	
	0.69	28 (3)	0.86 (0.67 – 1.00)	
	10	11 (2)	0.91 (0.86 – 1.00)	
IMI				$F_{3, 6} = 0.2958$; $p = 0.8275$[e]
	0	26 (3)	0.88 (0.63 – 1.00)	
	9.7×10^{-2}	23 (3)	0.74 (0.50 – 1.00)	
	0.78	24 (3)	0.83 (0.67 – 1.00)	
	7.3	15 (3)	0.93 (0.75 – 1.00)	
TMX				$F_{4, 8} = 1.558$; $p = 0.2747$[e]
	0	28 (3)	0.96 (0.91 – 1.00)	
	4.5×10^{-2}	28 (3)	0.75 (0.50 – 0.90)	
	0.55	29 (3)	0.76 (0.70 – 0.82)	
	4.9	23 (3)	0.83 (0.78 – 0.86)	
	62	13 (3)	0.77 (0.60 – 1.00)	

[a] Based on combined mortality data from triplicate bioassays for each insecticide-instar combination. Larvae were fed leaf tissues treated with 0.1% silwet : water and five insecticide suspensions in 0.1% silwet : water.

[b] Concentrations that caused up to 70% larval or pupal mortality (i.e., ≥ 30% survival), after adjusting for control (0.1% silwet : water) mortality using Abbott's formula. Only data with concentrations that were used at least twice are provided in the table and analyzed for difference in eclosion with respect to controls of the same bioassay run.
[c] 11 fifth instars were treated per concentration per run.
[d] One (of three) bioassay run had suppressed adult eclosion rates due to pupal infection.
[e] No significant concentration effect on eclosion rates based on ANOVA analysis.
BCF: beta-cyfluthrin; CTR: chlorantraniliprole; CFS: chlorpyrifos; IMI: imidacloprid; TMX: thiamethoxam

Table S15. Dietary Study: Percent arrested ecdysis in monarch fifth-instar larvae following exposure to tropical milkweed leaves treated with five insecticides[a]

Insecticide	Total mortality[b]	Total mortality from arrested ecdysis[c]	Mean mortality due to arrested ecdysis (Range over runs)[d]
Control[e]	17%	2%	15% (0% – 50%)
BCF	50%	2%	5% (0% – 6%)
CTR	47%	5%	10% (0% – 26%)
CFS	37%	0.6%	2% (0% – 3%)
IMI	52%	8%	16% (5% – 26%)
TMX	37%	8%	21% (7% – 40%)

[a] Based on combined mortality data from triplicate toxicity bioassays for each insecticide-fifth instar combination. Larvae were fed leaf tissues treated with 0.1% silwet : water and five insecticide suspensions in 0.1% silwet : water.
[b] Total mortality across all insecticide concentrations and runs. This includes mortality prior to J formation and mortality due to arrested ecdysis.
[c] Total mortality caused by arrested ecdysis across all insecticide concentrations and runs (total mortality minus mortality prior to J formation).
[d] Percentage of total mortality that occurred due to arrested ecdysis [i.e., (mortality due to arrested ecdysis/total mortality) x 100)].
[e] Control mortality and arrested ecdysis data obtained from 15 fifth-instar cuticular bioassays (control larvae were used in every insecticide-bioassay run combination).
Clothianidin treatments produced an overall mortality of 59%, of which the mean mortality due to arrested ecdysis was 5% (range over runs is 0 to 8%). The negligable rate of arrested ecdysis in the clothianidin treatments could be due to use of younger (less than 24-h old) fifth instars (see DISCUSSION/*Insecticide Toxicity*) that weighed an average of 0.400 g (Table S19). The fifth instars in the other insecticide treatments weighed an average of 0.830 g (Table S1).

BCF: beta-cyfluthrin; CTR: chlorantraniliprole; CFS: chlorpyrifos; IMI: imidacloprid; TMX: thiamethoxam

Table S16. Dietary Study: Comparison of acute toxicity of four insecticides to monarch second- and third-instar larvae fed common or tropical milkweed leaves

Insecticide	Instar	Tropical milkweed 96-h LC50 and 95% CI (µg/g leaf)[a]	Concentration (µg/g leaf) on common milkweed[b]	96-h larval percent mortality on common milkweed[c]
CTR	Third	4.6×10^{-2} (1.8×10^{-2} – 0.11)	4.4×10^{-2}	70%
CFS	Third	6.0 (2.7 – 14)	4.4	42%
IMI	Second	5.1 (3.3 – 6.8)	4.9	59%
TMX	Second	3.5 (2.2 – 5.0)	5.0	59%

[a] Tropical milkweed data obtained from Table 3.

[b] Based on combined larval mortality data from two common milkweed bioassays with ten larvae per bioassay run. Larvae were fed leaf tissues treated with insecticide suspensions in 0.1% silwet : water at concentrations that approximated the 96-h LC50 values obtained from the tropical milkweed bioassays. Bioassays with beta-cyfluthrin were not conducted.

[c] Percent mortality was calculated after adjusting for control (larvae exposed to 0.1% silwet : water) mortality using Abbott's formula.

LC50: lethal concentration that kills 50% of a treated population

CTR: chlorantraniliprole; CFS: chlorpyrifos; IMI: imidacloprid; TMX: thiamethoxam

Table S17. Dietary Study: Measured concentrations of clothianidin in 0.1% silwet : water suspensions used in toxicity bioassays[a]

Nominal concentrations (µg/µL)	1.00×10^{-4}	1.00×10^{-3}	1.00×10^{-2}	3.16×10^{-2}	0.100	0.316	1.00	10.0
Measured concentrations (µg/µL)	5.15×10^{-5}	6.58×10^{-4}	7.78×10^{-3}	2.54×10^{-2}	9.76×10^{-2}	0.198	0.449	19.7

[a] See MATERIALS AND METHODS/*Analysis of insecticide stock solutions*

Table S18. Dietary Study: Clothianidin tropical milkweed concentrations used in toxicity bioassays

Instar	Concentration units	Estimated tropical milkweed leaf concentrations						
Second	µg/g leaf[a]	2.4×10^{-3}	3.0×10^{-2}	0.37	1.2	4.5	9.6	21
	µg/cm^2 leaf[b]	7.4×10^{-5}	7.9×10^{-4}	8.8×10^{-3}	2.7×10^{-2}	0.11	0.23	0.55
Third	µg/g leaf[a]	2.6×10^{-3}	3.3×10^{-2}	0.38	4.8	10	22	
	µg/cm^2 leaf[b]	7.6×10^{-5}	9.9×10^{-4}	1.2×10^{-2}	0.15	0.31	0.63	
Fifth	µg/g leaf[a]	2.6×10^{-3}	3.4×10^{-2}	0.40	5.1	23	1100	
	µg/cm^2 leaf[b]	6.7×10^{-5}	8.8×10^{-4}	1.1×10^{-2}	0.14	0.62	28	

[a] Calculated by multiplying the measured concentration of 0.1% silwet : water clothianidin suspension (Table S17) with the volume of suspension applied on each leaf tissue (5, 20, and 100 µL for second-, third-, and fifth-instar bioassays, respectively) and divided by the average weights of leaves used in each instar-concentration combination (see supplementary file *Weights and surface areas of leaves*).

[b] Calculated by multiplying the measured concentration of 0.1% silwet : water clothianidin suspension (Table S17) with the volume of suspension applied on each leaf tissue (5, 20, and 100 µL for second-, third-, and fifth-instar bioassays, respectively) and divided by the average surface areas of leaves used in each instar-concentration combination (see supplementary file *Weights and surface areas of leaves*).

Table S19. Dietary Study: Acute toxicity to monarch second-, third-, and fifth-instar larvae following exposure to tropical milkweed leaves treated with clothianidin[a]

Instar	Units	96-h LC values with 95% CI[b]		
		LC10	LC50	LC90
Second	µg/g leaf[c]	2.4 (1.3 – 3.4)	4.2 (3.4 – 5.0)	7.5 (5.2 – 9.7)
	µg/cm^2 leaf[d]	5.8×10^{-2} (3.5×10^{-2} – 8.0×10^{-2})	9.7×10^{-2} (8.0×10^{-2} – 0.11)	0.16 (0.12 – 0.21)
Third	µg/g leaf[c]	3.5 (1.3 – 5.6)	7.8 (5.2 – 10)	17 (9.8 – 25)
	µg/cm^2 leaf[d]	0.11 (5.1×10^{-2} – 0.17)	0.23 (0.16 – 0.31)	0.49 (0.29 – 0.69)
Fifth[e]	µg/g leaf[c]	2.8×10^{-2} (1.1×10^{-3} – 0.54)[f]	0.80 (0.21 – 3.0)[f]	23 (7.3 – 150)[f]
	µg/cm^2 leaf[d]	7.9×10^{-4} (3.0×10^{-5} – 1.4×10^{-2})[f]	2.2×10^{-2} (5.5×10^{-3} – 7.9×10^{-2})[f]	0.59 (0.19 – 3.9)[f]

[a] Based on combined mortality data from triplicate or quadruplicate bioassays for each clothianidin-instar combination. Larvae were fed leaf tissues treated with 0.1% silwet : water and five clothianidin suspensions in 0.1% silwet : water.

[b] Except as noted in the table, LC values were calculated using nonlinear least square estimate model (see MATERIALS AND METHODS/*Statistical Analyses*). Adjustment for control mortality was done using Abbott's formula.

[c] The overall mean weights of leaves for second, third, and fifth instars were 0.106 ± 0.011 g (n = 263), 0.403 ± 0.027 g (n = 197), and 1.936 ± 0.108 g (n = 197), respectively. The weights of leaves given to larvae in each insecticide, instar, bioassay run, and concentration are provided in a supplementary file (*Weights and surface areas of leaves*).
[d] The cm^2 leaf tissue provided to each larvae (see MATERIALS AND METHODS/*Toxicity Studies/Dietary toxicity studies*) was used to estimate dietary insecticide concentrations. The overall average surface areas of leaves provided to second, third, and fifth instars were 4.3 ± 1.0 cm^2 (n = 263), 13 ± 2.4 cm^2 (n = 197) and 75 ± 9.5 cm^2 (n = 195), respectively. The surface area of leaf tissue given to larvae in each clothianidin, instar, bioassay run, and concentration are provided in a supplementary file (*Weights and surface areas of leaves*). Mean weights of second, third, and fifth instars at time of treatment were 0.0093 ± 0.004 g (n= 24), 0.0360 ± 0.015 g (n= 18), and 0.4000 ± 0.116 g, respectively (n= 18).
[e] Observations until pupation (usually 96 h after treatment).
[f] LC values calculated using maximum likelihood estimate model (see *Statistical Analyses*).
CIs: confidence intervals; LC10: lethal concentration that kills 10% of a treated population; LC50: lethal concentration that kills 50% of a treated population; LC90: lethal concentration that kills 90% of a treated population

Table S20. Dietary Study: Growth, development, and eclosion of monarch second-, third-, and fifth-instar larvae following exposure to tropical milkweed leaves treated with clothianidin[a]

Instar	Conc[b] (µg insecticide/g leaf)	Larval percent mortality[c]	Number of surviving larvae/pupae (# of replicate bioassays)[d]	Instar/stage at 96 h after application[e]	Mean final weight[f] (± SD)	Statistical analysis	Mean adult eclosion rate (Range)
Second						$F_{4, 134} = 2.623$; p = 0.03758[g]	
	0	0	40 (4)	Fourth	186 (± 115)		
	2.99×10^{-2}	0	30 (3)	Fourth	258 (± 87)	df = 101; t-ratio = 1.091; p = 0.6279	
	0.37	10	36 (4)	Fourth	184 (± 111)	df = 134; t-ratio = 0.213; p = 0.9909	
	1.20	0	21 (2)	Fourth	169 (± 150)	df = 79; t-ratio = 0.618; p = 0.8882	
	4.53	59	16 (4)	Fourth	144 (± 144)*	df = 134; t-ratio = 2.493; p = 0.0488	

Table S20 continued

Third					$F_{4, 116} = 22.07$; $p = 1.458 \times 10^{-13}$, [g]	
	0	0	28 (3)	Fifth	474 (± 123)	
	2.55×10^{-3}	0	20 (2)	Fifth	431 (± 92)	df = 80; T-ratio = 0.540; p = 0.9179
	3.30×10^{-2}	0	31 (3)	Fifth	453 (± 141)	df = 116; t-ratio = 0.573; p = 0.9060
	0.382	12	24 (3)	Fifth	303 (± 175)***	df = 116; t-ratio = 4.719; p < 0.0001
	4.76	29	20 (3)	Fourth[+]	166 (± 102)***	df = 116; t-ratio = 8.097; p < 0.0001
Fifth					$F_{3, 79} = 1.561$; $p = 0.2054$[h]	
	0	0	29 (3)	Pupa	909 (± 168)	0.86 (0.50 – 1.00)[i]
	2.63×10^{-3}	7	18 (2)	Pupa	865 (± 157)	0.78 (0.67 – 0.89)[i]
	3.41×10^{-2}	28	21 (3)	Pupa	817 (± 125)	0.90 (0.71 – 1.00)[i]
	0.395	31	20 (3)	Pupa	852 (± 179)	0.80 (0.50 – 1.00)[i]

[a] Based on combined mortality data from triplicate or quadruplicate bioassays for each insecticide-instar combination. Larvae were treated with control (0.1% silwet : water) and clothianidin suspensions in 0.1% silwet : water.

[b] Concentrations (averaged over runs) that caused equal to or fewer than 70% larval or pupal mortality (i.e., ≥ 30% survival) after adjusting for control (0.1% silwet : water) mortality using Abbott's formula. Only data with concentrations that were used at least twice are provided in table and analyzed for difference in final weights and development with respect to controls of the same bioassay run.

[c] Larval mortality calculated after setting control mortality to zero and adjusting for it in other concentrations (Abbott's formula).

[d] 11 larvae were treated per concentration per run.

[e] Most common larval instar/stage observed at 96 h after application.

[f] Final weights of larvae recorded 96 h after application. Final weights of pupae were recorded prior to adult eclosion.

[g] Significant concentration effect on larval weight based on ANOVA. Post hoc analyses were conducted using Dunnett's test for multiple comparison with control larval weights from the same bioassay runs.

[h] No significant concentration effect on larval weights based on ANOVA.

[i] No significant concentration effect following ANOVA analysis on adult eclosion. $F_{3, 5} = 0.9332$; $p = 0.4898$.

*Weight of treated larvae less than control larvae at $p < 0.05$.

*** Weight of treated larvae less than control larvae at $p < 0.001$.

[+] 100% of treated larvae were third or fourth instars. 82% of control larvae were fifth instars.

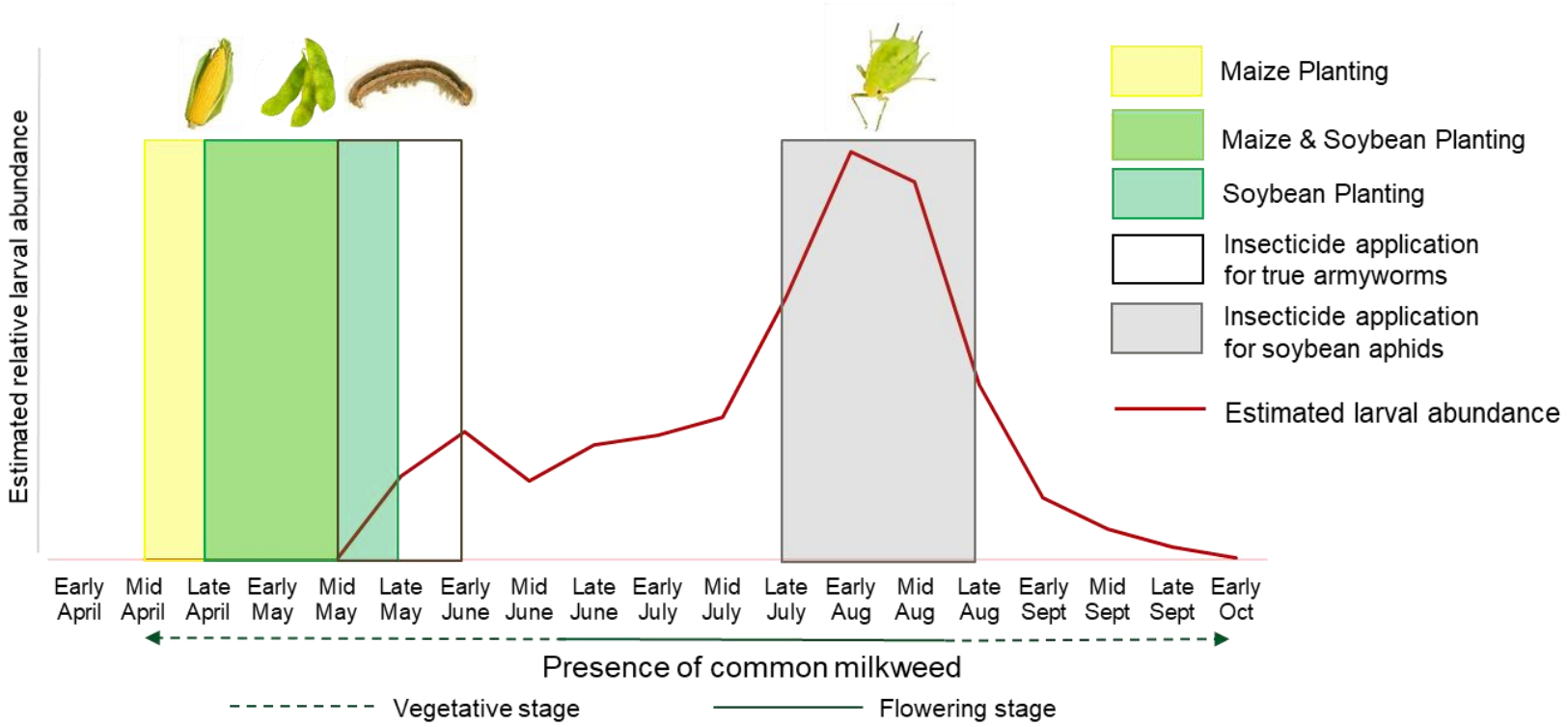

Figure 1. Conceptual model depicting maize and soybean planting dates, periods of economically significant true armyworm and soybean aphid populations, monarch larval abundance, and common milkweed phenology in Iowa. Monarch larval abundance (red line) for the North Central U.S. was estimated for the years 1997 to 2014 (Prysby and Oberhauser 2004; Pleasants 2015; Nail et al. 2015). A supplementary file *(Monarch abundance calculations)* contains data used to derive these estimates. Approximate dates for maize and soybean planting (yellow and green bars, respectively) were obtained from Iowa State University Extension reports (Pedersen 2007; Elmore 2012). Approximate insecticide application dates for managing true armyworm (white bar) and soybean aphid (light grey bar) populations exceeding economic thresholds in Iowa were based on Dunbar et al. (2016) and Hodgson et al. (2012),

respectively. Presence and stage of common milkweed (solid and dotted green line) from April to September in the North Central States was obtained from Kaul et al. (1991) and Journey North (2016).

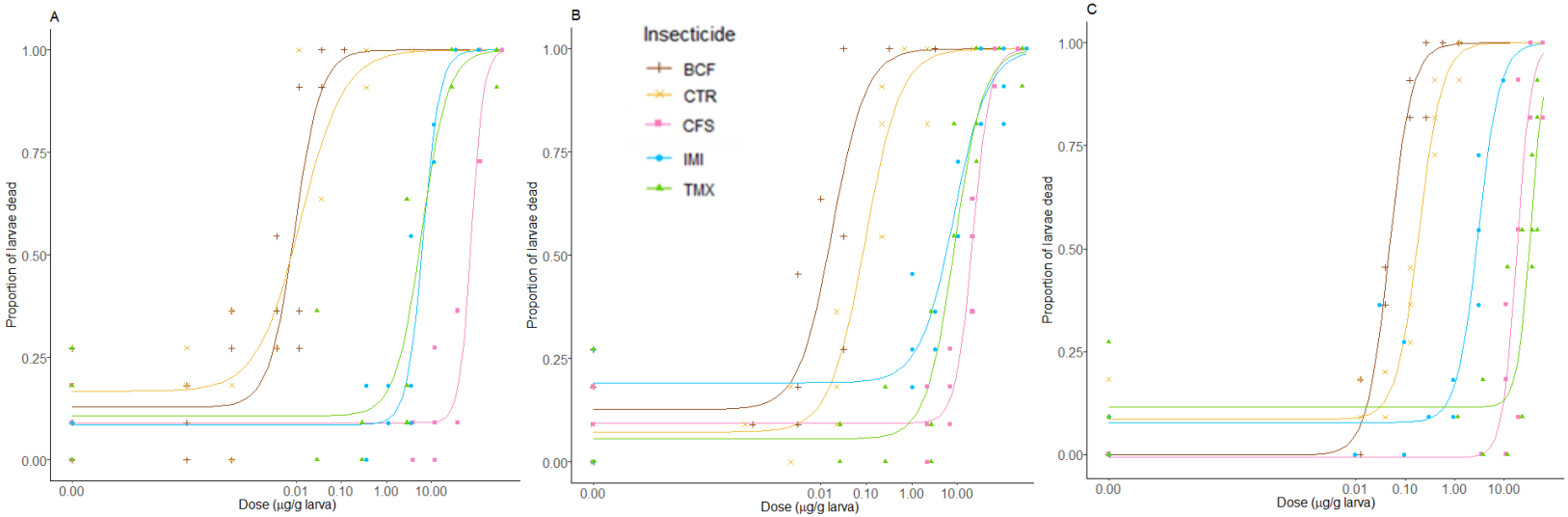

Figure 2. Mortality dose-response curves (µg insecticide/g larva) for first- (A), third- (B), and fifth-instar (C) monarch butterfly larvae following cuticular application of five insecticide solutions in acetone. For the first and third instars, observations were made daily through 96-h post-application. For the fifth instars, observations were made through pupation (usually 72 or 96 h after treatment). BCF: beta-cyfluthrin; CTR: chlorantraniliprole; CFS: chlorpyrifos; IMI: imidacloprid; TMX: thiamethoxam

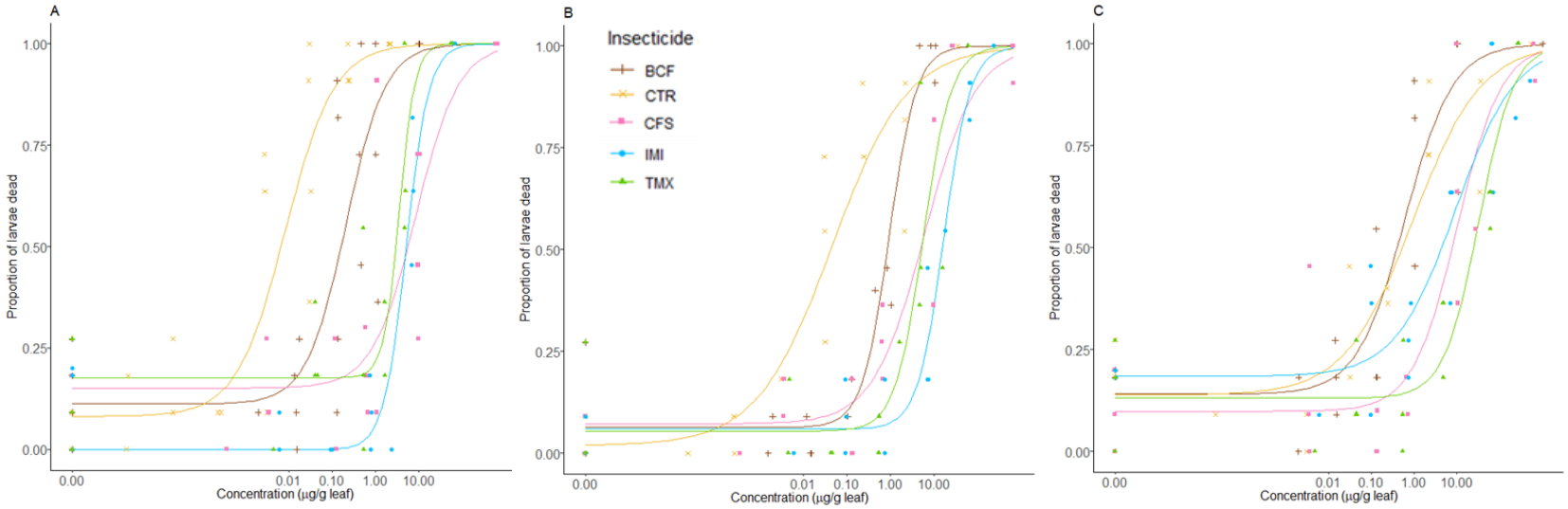

Figure 3. Mortality concentration-response curves (µg insecticide/g leaf) for second- (A), third- (B), and fifth-instar (C) monarch butterfly larvae following dietary exposure to tropical milkweed leaves treated with five insecticide suspensions in 0.1% silwet : water. For the second and third instars, observations were made daily through 96-h post-application. For the fifth instars, observations were made through pupation (usually 72 or 96 h after treatment). BCF: beta-cyfluthrin; CTR: chlorantraniliprole; CFS: chlorpyrifos; IMI: imidacloprid; TMX: thiamethoxam

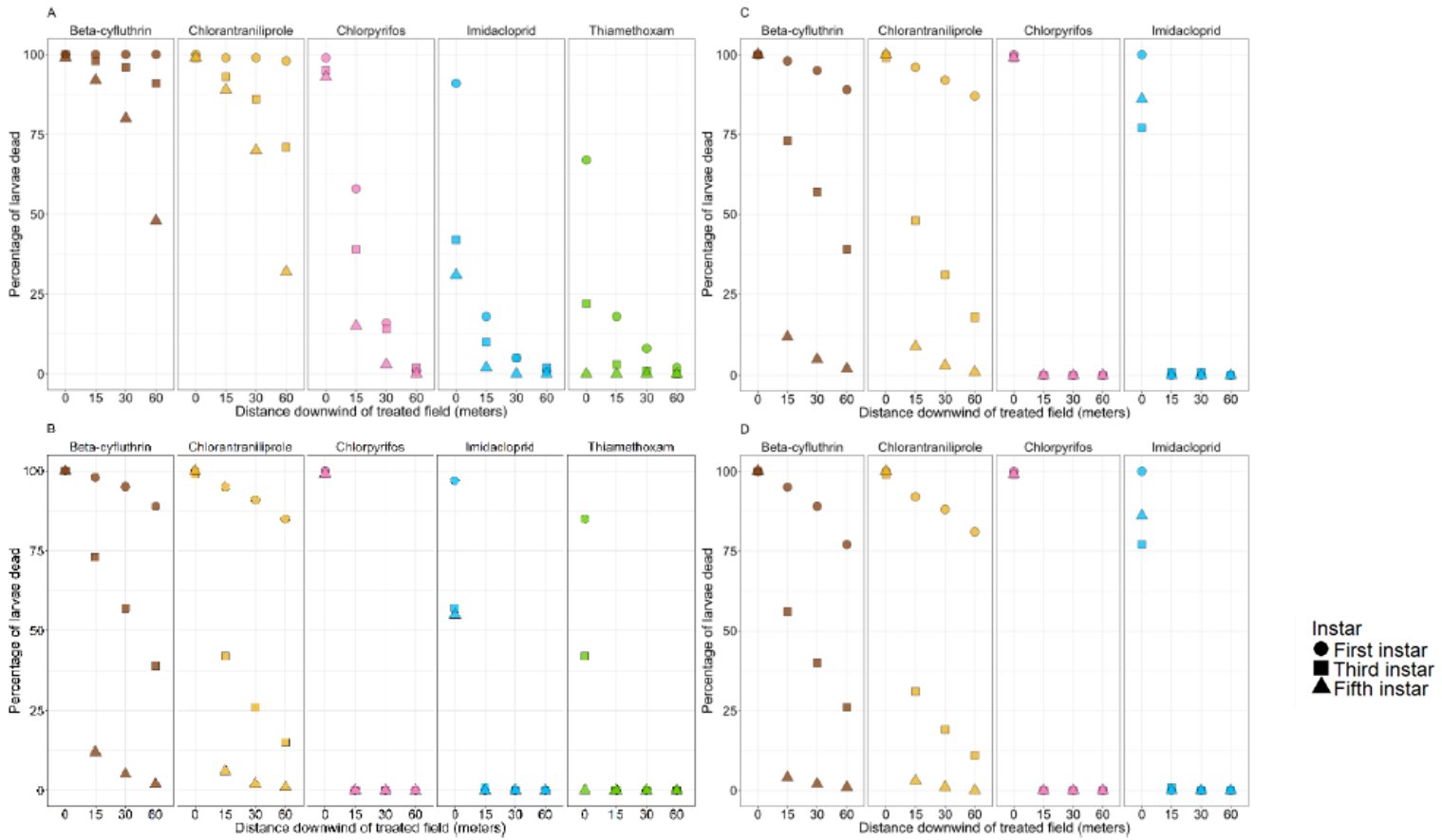

Figure 4. Estimated monarch larval mortality from cuticular exposure due to insecticide spray drift at increasing distances downwind from a treated crop field. Modeled spray drift scenarios using AgDRIFT (USEPA 2003) include: (A) aerial applications to manage soybean aphids; (B) high ground boom applications to manage soybean aphids; (C) high ground boom applications to manage true armyworms; and (D) low ground boom applications to manage true armyworms. Mortality rates were estimated using active ingredient (a.i.)-specific larval dose-response curves (Figure S1) and estimated 50th percentile, a.i.-specific exposures using the AgDRIFT model for ground boom applications (Table S5). Representative formulated products used to derive a.i.-specific exposures can also be found in Table S5. Thiamethoxam is not registered for use on true armyworms in maize or soybean fields. Note the x-axes are not proportionally spaced.

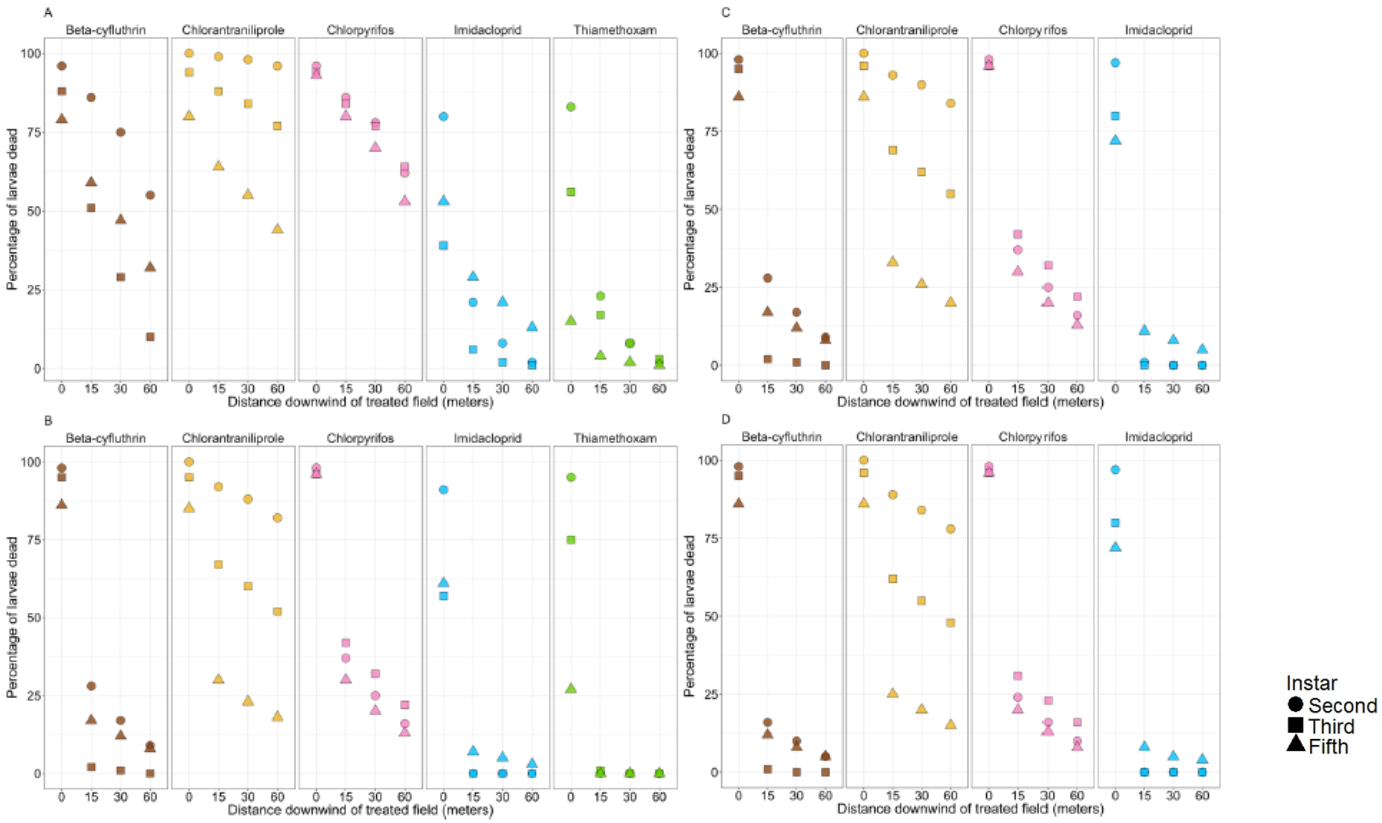

Figure 5. Estimated monarch larval mortality from dietary exposure due to insecticide spray drift at increasing distances downwind from a treated crop field. Modeled spray drift scenarios using AgDRIFT (USEPA 2003) include: aerial applications to manage soybean aphids (A); high ground boom applications to manage soybean aphids (B); high ground boom applications to manage true armyworms (C); and low ground boom applications to manage true armyworms (D). Mortality rates were estimated using a.i.-specific larval concentration-response curves (Figure S4) and estimated 50th percentile, a.i.-specific exposures using the AgDRIFT model for ground boom applications (Table S5). Representative formulated products used to derive a.i.-specific exposures can also be found in Table S5. Thiamethoxam is not registered for use on true armyworms in maize or soybean fields. Note the x-axes are not proportionally spaced.

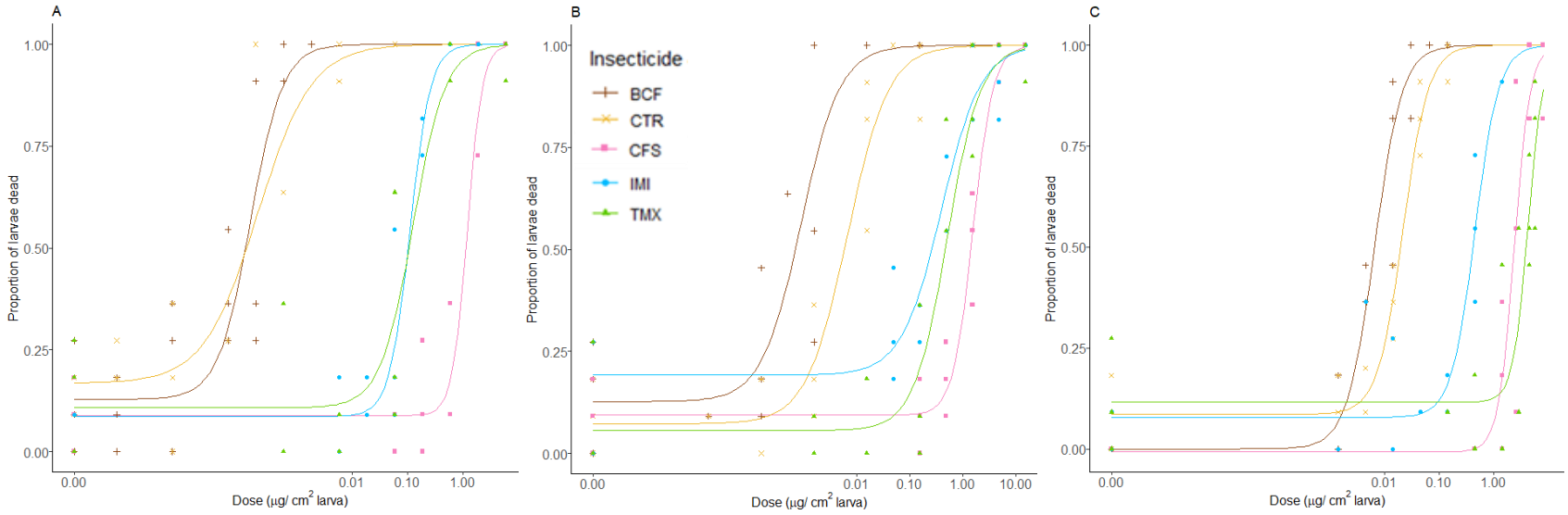

Figure S1. Mortality dose-response curves (µg insecticide/cm^2 larva) for first- (A), third- (B), and fifth-instar (C) monarch butterfly larvae feeding on tropical milkweed leaves following cuticular application of acetone and five insecticide solutions in acetone. For the first and third instars, observations were made daily through 96-h post-application. For the fifth instars, observations were made through pupation (usually 72 or 96 h after treatment). BCF: beta-cyfluthrin; CTR: chlorantraniliprole; CFS: chlorpyrifos; IMI: imidacloprid; TMX: thiamethoxam

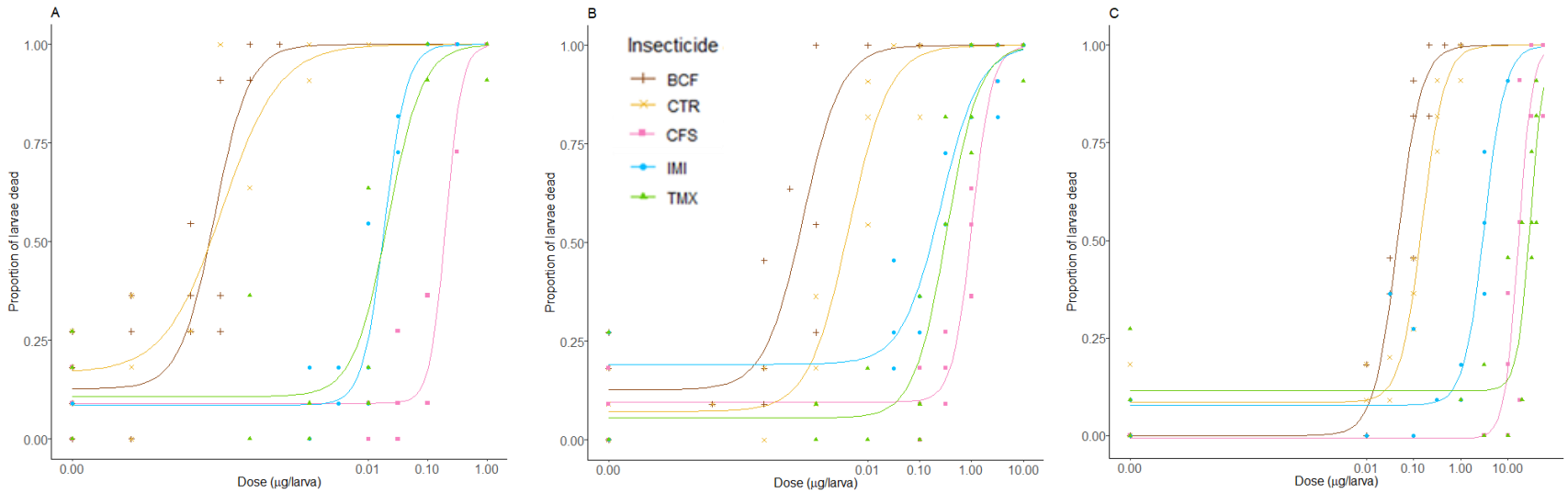

Figure S2. Mortality dose-response curves (µg insecticide/larva) for first- (A), third- (B), and fifth-instar (C) monarch butterfly larvae feeding on tropical milkweed leaves following cuticular application of acetone and five insecticide solutions in acetone. For the first and third instars, observations were made daily through 96-h post-application. For the fifth instars, observations were made through pupation (usually 72 or 96 h after treatment). BCF: beta-cyfluthrin; CTR: chlorantraniliprole; CFS: chlorpyrifos; IMI: imidacloprid; TMX: thiamethoxam

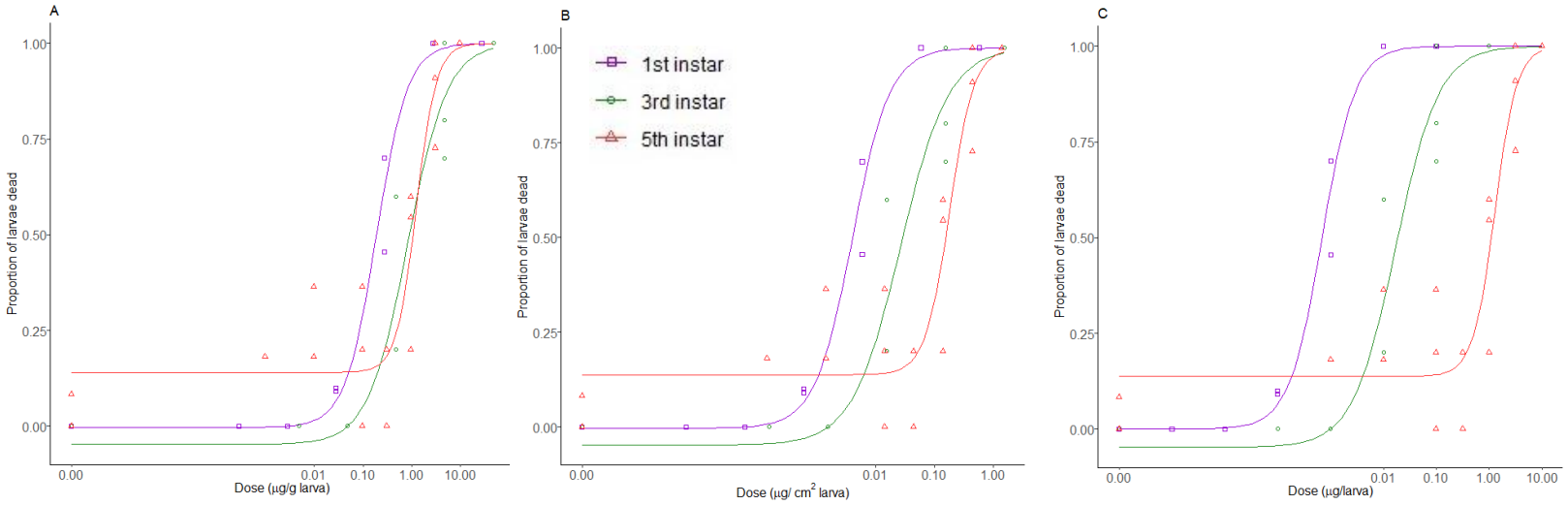

Figure S3. Mortality dose-response curves for first-, third-, and fifth-instar larvae of monarch butterfly larvae feeding on tropical milkweed leaves following cuticular application of clothianidin solutions in acetone. Dose units are µg insecticide/g larva (A), µg insecticide/cm^2 larva (B) and µg insecticide/larva (C). For the first and third instars, observations were made daily through 96-h post-application. For the fifth instars, observations were made through pupation (usually 72 or 96 h after treatment).

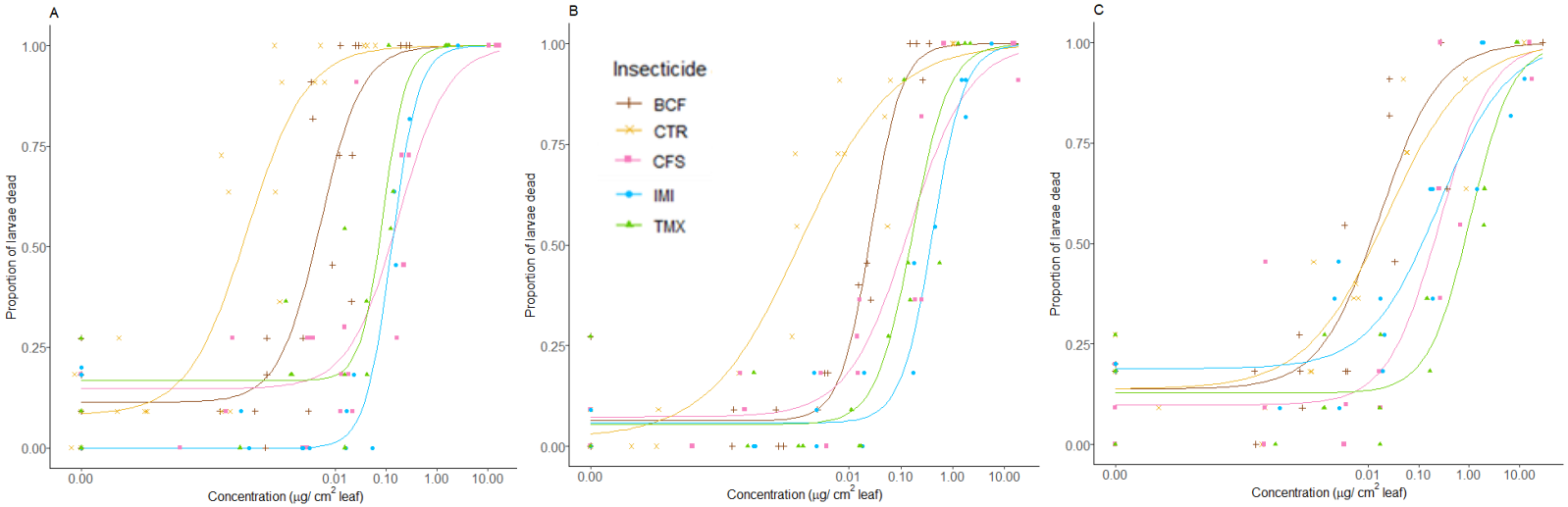

Figure S4. Mortality concentration-response curves (µg insecticide/cm^2 leaf) for second- (A), third- (B), and fifth-instar (C) monarch butterfly larvae following dietary exposure to tropical milkweed leaves treated with 0.1% silwet : water and five insecticide suspensions in 0.1% silwet : water. For the second and third instars, observations were made daily through 96-h post-application. For the fifth instars, observations were made through pupation (usually 72 or 96 h after treatment). BCF: beta-cyfluthrin; CTR: chlorantraniliprole; CFS: chlorpyrifos; IMI: imidacloprid; TMX: thiamethoxam

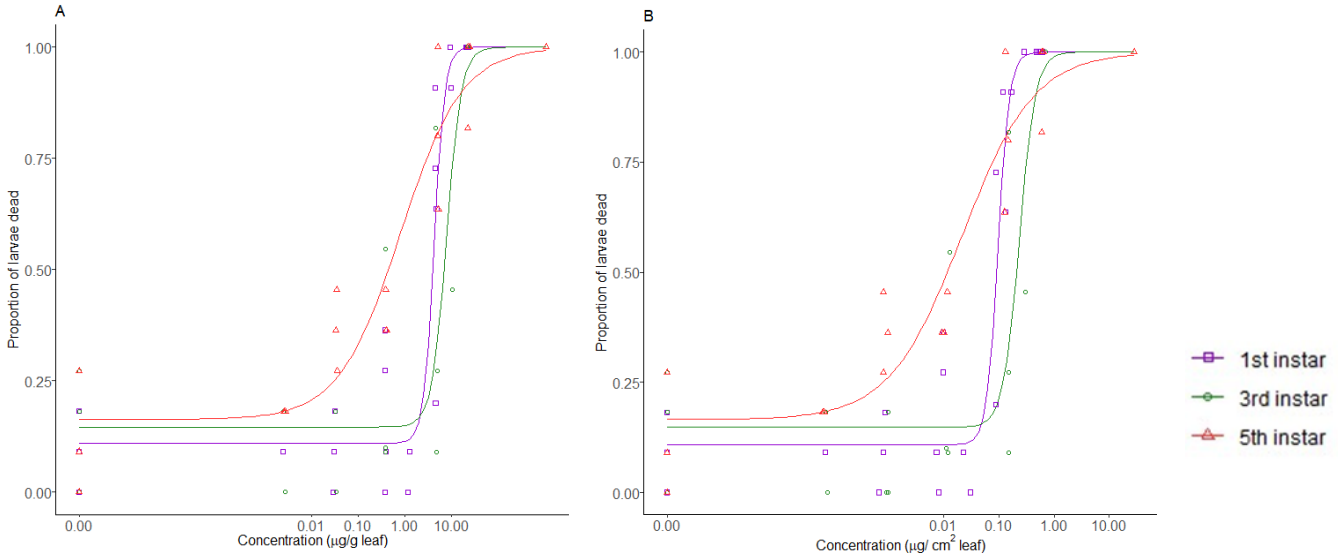

Figure S5. Mortality concentration-response curves for second-, third-, and fifth-instar monarch butterfly larvae following dietary exposure to tropical milkweed leaves treated with clothianidin suspensions in 0.1% silwet : water. Concentration units are µg insecticide/g leaf (A) and µg insecticide/cm^2 leaf (B). For the second and third instars, observations were made daily through 96-h post-application. For the fifth instars, observations were made through pupation (usually 96 h after treatment).

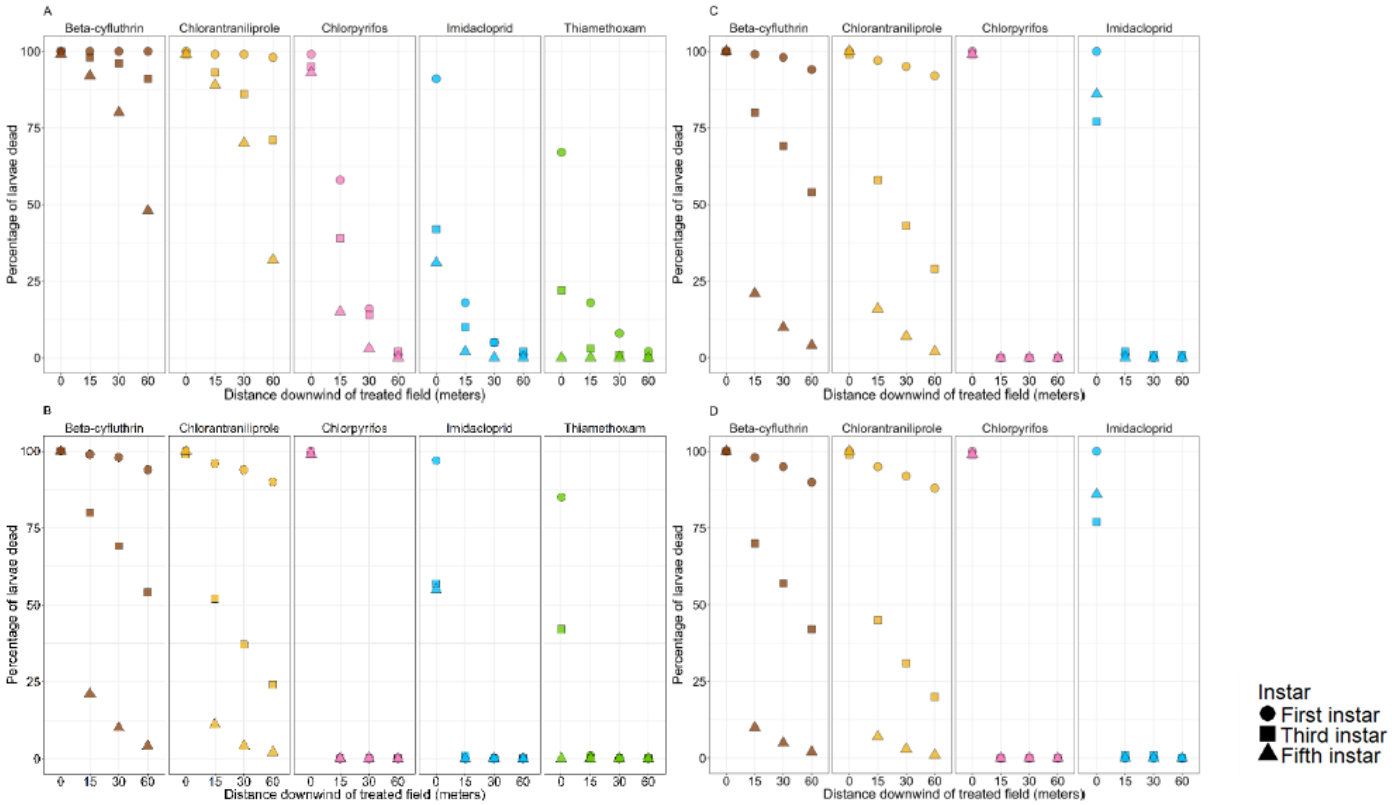

Figure S6. Estimated monarch larval mortality from cuticular exposure due to insecticide spray drift at increasing distances downwind from a treated crop field. Modeled spray drift scenarios using the AgDRIFT model (USEPA 2003) include: (A) aerial applications to manage soybean aphids; (B) high ground boom applications to manage soybean aphids; (C) high ground boom applications to manage true armyworms; and (D) low ground boom applications to manage true armyworms. Mortality rates were estimated using active ingredient (a.i.)-specific larval dose-response curves from cuticular exposure (Figure S1) and 90th percentile, a.i.-specific exposures using the AgDRIFT model for ground boom applications (estimates not shown). Representative formulated products used to derive a.i.-specific exposures can be found in Table S5. Thiamethoxam is not registered for use on true armyworms in maize or soybean fields. The x-axis is not proportionally spaced.

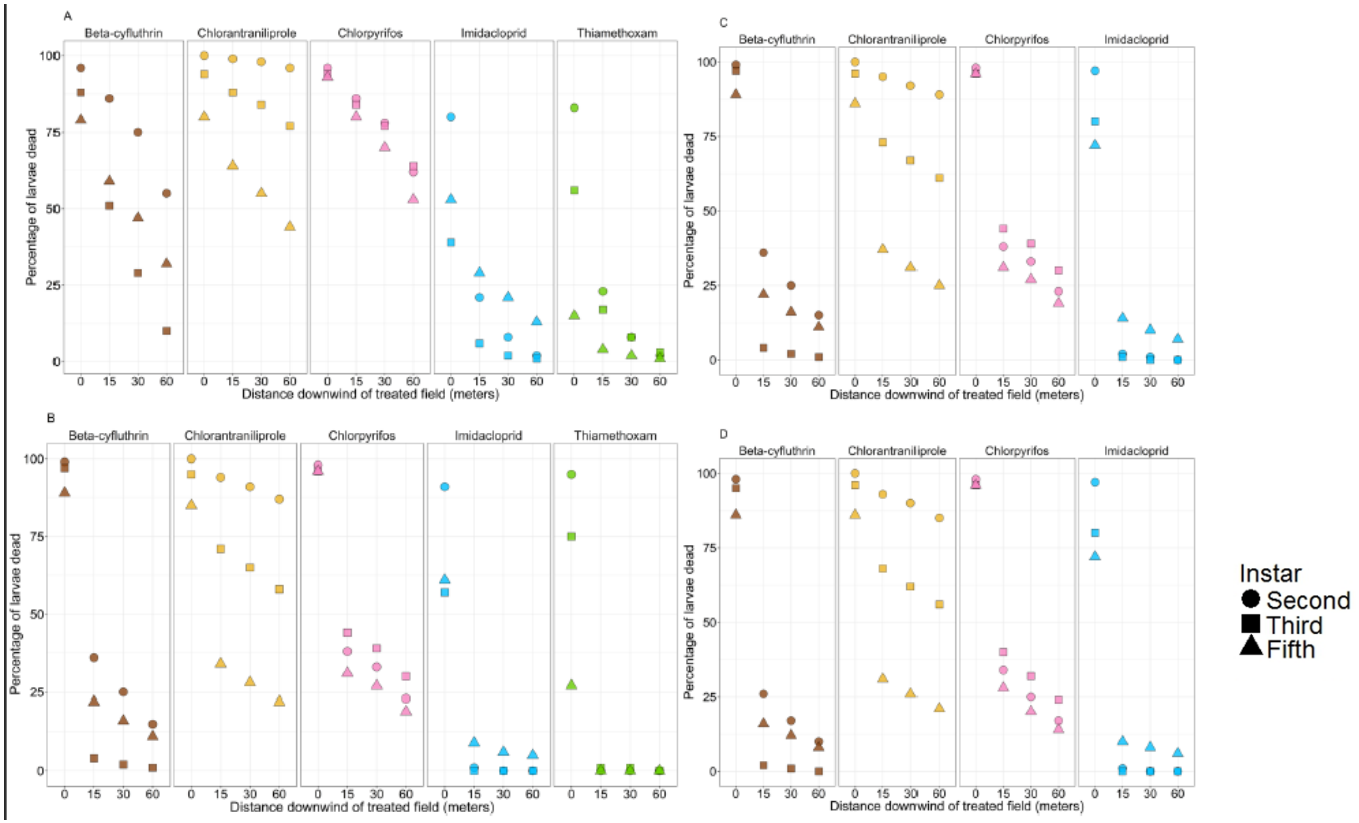

Figure S7. Estimated monarch larval mortality from dietary exposure due to insecticide spray drift at increasing distances downwind from a treated crop field. Modeled spray drift scenarios using AgDRIFT (USEPA 2003) include: aerial applications to manage soybean aphids (A); high ground boom applications to manage soybean aphids (B); high ground boom applications to manage true armyworms (C); and low ground boom applications to manage true armyworms (D). Mortality rates were estimated using a.i.-specific larval concentration-response curves from dietary exposure (Figure S4) and 90th percentile, a.i.-specific exposures using the AgDRIFT model for ground boom applications (estimates not shown). Representative formulated products used to derive a.i.-specific exposures can be found in Table S5. Thiamethoxam is not registered for use on true armyworms in maize or soybean fields. The x-axis is not proportionally spaced.

CHAPTER 3. MONARCH BUTTERFLY (*DANAUS PLEXIPPUS*) LIFE STAGE RISKS FROM FOLIAR AND SEED-TREATMENT INSECTICIDES

Modified from a manuscript under review in Environmental Toxicology and Chemistry

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Abstract

Conservation of North America's eastern monarch butterfly (*Danaus plexippus*) population would require establishment of additional milkweed (*Asclepias* spp.) and nectar plants in the agricultural landscapes of North Central United States (U.S.). A variety of seed-treatment and foliar insecticides are used to manage early- and late-season pests in these landscapes. To inform habitat conservation practices, there is a need to assess risks of these insecticides to monarch butterfly life stages. Chronic and acute dietary toxicity studies were undertaken with larvae and adults, and acute topical bioassays were conducted with eggs, pupae, and adults using six representative insecticides: beta-cyfluthrin (pyrethroid), chlorantraniliprole (anthranilic diamide), chlorpyrifos (organophosphate), and imidacloprid, clothianidin, and thiamethoxam (neonicotinoids). Chronic dietary LC_{50} values for monarch larvae ranged from 1.6 x 10^{-3} (chlorantraniliprole) to 5.3 (chlorpyrifos) µg/g milkweed leaf, with the neonicotinoids

producing high rates of arrested pupal ecdysis. Chlorantraniliprole and beta-cyfluthrin were generally the most toxic insecticides to all life stages, and thiamethoxam and chlorpyrifos were generally the least toxic. The toxicity results were compared to insecticide exposure estimates derived from a spray drift model and/or milkweed residue data reported in the literature. Aerial applications of foliar insecticides are expected to cause high downwind mortality in larvae and eggs, with lower mortality predicted for adults and pupae. Neonicotinoid seed treatments are expected to cause little to no downslope mortality and/or sublethal effects in larvae and adults. Given the vagile behavior of non-migratory monarchs, considering these results within a landscape-scale context suggests adult recruitment will not be negatively impacted if new habitat is established in close proximity of maize and soybean fields in the agricultural landscapes of North Central U.S.

Keywords: Lepidoptera; Conservation; Pesticide; Toxicity; Risk assessment; Agroecosystems

Introduction

Decline of North America's monarch butterfly (Danaus plexippus) populations has spurred collaborative conservation efforts that link federal and state agencies with a diversity of non-governmental organizations and the public (e.g., Monarch Joint Venture 2010; U.S. Fish and Wildlife Service 2015; Natural Resources Conservation Services 2016; Keystone Policy Center 2017). Recovery of the eastern population will require preservation of the overwintering grounds in Mexico, establishment of milkweed (Asclepias spp.) in the spring and summer breeding grounds of northern Mexico, the United States, and southern Canada, and establishment of flowering forbs along the butterflies' 4000-kilometer migratory path (Oberhauser et al. 2017). The U.S. North Central is critical summer breeding ground for the monarchs. An estimated 1.3 to 1.6 billion milkweed stems need to be established over the next 20 years to help support a

sustainable population (Thogmartin et al. 2017). This goal can be reached only with substantial conservation in agricultural landscapes, which represent approximately 75% of the land cover available for establishing new habitat in the North Central states (Thogmartin et al. 2017). Maize and soybean fields account for 75% of this agricultural land cover (U.S. Department of Agriculture 2019).

The percentage of maize and soybeans that are treated with foliar or soil-applied chemical insecticides range from 8 to 20% and 6 to 30% in the North Central states, respectively (U.S. Department of Agriculture 2018). Nearly 100% of maize and 50% of soybean acres in the United States employ neonicotinoid-treated seeds (Tooker et al. 2017). Not surprisingly, insecticide exposure to monarch habitat in close proximity to row crop fields in the North Central states has been reported in modeling (Krishnan et al. 2020) and monitoring studies (Olaya-Arenas and Kaplan 2019; Hall et al. 2020). Figure 1 depicts a conceptual model that outlines environmental transport pathways of foliar and seed treatment insecticide formulations, routes of monarch exposure, and potential adverse effects to different life stages. Uncertainty in the potential risks of these exposures has led the U.S. Fish and Wildlife Service to identify insecticide use as a potential threat to the species' recovery (U.S. Fish and Wildlife Service 2017).

Neonicotinoids and chlorantraniliprole used in maize and soybean seed treatments can move downslope in subsurface runoff, reach monarch habitat, and be taken up systemically by milkweed and flowering forbs (Figure 1). Hall et al. (2020) sampled downslope milkweed in pollinator habitat within neonicotinoid seed-treated maize and soybean fields from May through August and found imidacloprid, clothianidin, and thiamethoxam leaf residues above the method detection limit (MDL; 0.04 to 0.1 ng/g) in approximately 70% of 360 sampled milkweeds.

Approximately 90% of the plants that had detectable neonicotinoid concentrations were sampled in early June through late July (mean concentrations were between 0.21 to 1.6 ng/g; range was <MDL to 13 ng/g). These data suggest larvae could be chronically exposed through consumption of milkweed leaves. Botias et al. (2015) analyzed nectar in flowering plants near seed-treated oil rape fields and detected neonicotinoid residues several months after planting; frequency of detects for imidacloprid, clothianidin, and thiamethoxam were 0 to 21% (range was ≤ 0.10 to 1.8 ng/g). Since adult monarchs, with a life span of two to eight weeks (Oberhauser 1989), are vagile and move across the landscape (Zalucki et al. 2016; Grant et al. 2018), they are unlikely to be chronically exposed to neonicotinoids in nectar. However, acute or subchronic dietary exposures cannot be precluded.

Spray drift from foliar insecticide applications could directly expose monarch eggs, larvae, pupae, and adults, as well as milkweed and other forbs that are downwind to treated fields. Krishnan et al. (2020) estimated field-scale acute topical and dietary risks to different larval instars following single foliar applications of beta-cyfluthrin, chlorantraniliprole, chlorpyrifos, imidacloprid, and thiamethoxam (acute foliar risks for clothianidin presented in Table S1). The half-lives of these insecticides on growing plants range from 1 to 17 days (Mukherjee et al. 2000; Galietta et al. 2011; Banerjee et al. 2012; Chowdhury et al. 2012; Kar et al. 2013; Szpyrka et al. 2017; Lee et al. 2019). Consequently, larvae that survive the initial exposure from a spray drift event, as well as larvae that hatch from eggs laid after a spray drift event, could be exposed to insecticide residues through a significant portion of their life stage, which ranges from 12 to 13 days (Rawlins and Lederhouse 1981; Zalucki 1982).

In the present paper, we provide data to more rigorously test the hypothesis that conservation benefits of establishing milkweed habitat close to maize and soybean fields

outweigh the risk of insecticide exposure due to foliar and seed treatment applications (Krishnan et al. 2020). We evaluated six representative insecticides used in maize and soybean production, beta-cyfluthrin (pyrethroid; foliar), chlorantraniliprole (anthranilic diamide; foliar/seed treatment), chlorpyrifos (organophosphate; foliar), imidacloprid (neonicotinoid; foliar/seed treatment), thiamethoxam (neonicotinoid; foliar/seed treatment), and clothianidin (neonicotinoid; foliar/seed treatment), and undertook the following studies:

1. Chronic dietary toxicity studies with monarch larvae to assess their potential risk to consuming milkweed that are downwind of maize and soybean fields that had foliar applications or downslope of maize and soybean fields planted with treated seeds. We estimate field-scale mortality and sublethal effects based on insecticide exposure estimated from a spray drift model (AgDRIFT; U.S. Environmental Protection Agency 2011a) and milkweed residue data reported in the literature.
2. Acute topical toxicity studies with monarch eggs, pupae, and adults to assess their potential risks to spray drift exposure. We estimate field-scale mortality and sublethal effects based on modeled exposure levels using AgDRIFT.
3. Acute dietary toxicity studies with monarch adults to assess their potential risks to consuming nectar from forbs that are downslope of fields planted with treated seeds. We compare the mortality results to nectar residue data reported in the literature.

These analyses, when combined with previous field- and landscape-scale risk estimates obtained from acute topical and dietary exposure to monarch larvae (Krishnan et al. 2020; Grant et al. 2020a), provide a more complete assessment of the risks and benefits of establishing monarch habitat in different spatial patterns within agricultural landscapes.

MATERIALS AND METHODS

Rearing monarchs and milkweed

Monarch eggs for the egg and pupa topical bioassays and the adult dietary bioassays were obtained from the 2014 and 2015 colonies maintained by the U.S. Department of Agriculture (USDA) Corn Insects and Crop Genetics Research Unit in Ames, Iowa (see Krishnan et al. 2020 for monarch rearing methods). Eggs for the larval dietary and adult topical bioassays were obtained from the University of Kansas. Acute larval dietary toxicity studies with the Kansas colony provided LC_{50} values within 2- to 5-fold of those previously reported using the Iowa colony (Krishnan et al 2020), suggesting similar larval sensitivity across the colonies (see Table S2 and associated summary). Leaves from tropical milkweed (*Asclepias curassavica*) were used to feed larvae in all the bioassays as per Krishnan et al. (2020).

Insecticides

The following analytical grade insecticides were used (IUPAC name; CAS number; percentage purity): beta-cyfluthrin ([(*R*)-cyano-(4-fluoro-3-phenoxyphenyl)methyl] (1*S*)-3-(2,2-dichloroethenyl)-2,2-dimethylcyclopropane-1-carboxylate; 1820573-27-0; 99.3%), chlorantraniliprole (5-bromo-*N*-[4-chloro-2-methyl-6-(methylcarbamoyl)phenyl]-2-(3-chloropyridin-2-yl)pyrazole-3-carboxamide; 500008-45-7; 97.3%), chlorpyrifos (diethoxy-sulfanylidene-(3,5,6-trichloropyridin-2-yl)oxy-λ^5-phosphane; 2921-88-2; 99.3%), imidacloprid (*N*-[1-[(6-chloropyridin-3-yl)methyl]-4,5-dihydroimidazol-2-yl]nitramide; 138261-41-3; 100%), thiamethoxam (*N*-[3-[(2-chloro-1,3-thiazol-5-yl)methyl]-5-methyl-1,3,5-oxadiazinan-4-ylidene]nitramide; 153719-23-4; 99.3%), and clothianidin (1-(2-chloro-1,3-thiazol-5-ylmethyl)-3-methyl-2-nitroguanidine; 210880-92-5; 99%). Chlorantraniliprole was provided by DuPont Crop Protection. The remaining compounds were purchased from Sigma-Aldrich. To prepare

insecticide stock solutions for topical and dietary bioassays, certified ACS reagent grade acetone, certified ACS reagent grade dimethylformamide (DMF), and Silwet L-77 were purchased from Fisher Scientific.

Toxicity bioassays

All toxicity bioassays were conducted between June 2019 and July 2020 in two laboratory rooms that were maintained between 21 to 29 ºC, 20 to 50% relative humidity, and a 14:10 light : dark cycle. Prior to treatment, monarchs were randomly assigned to different insecticides and concentrations.

Chronic dietary toxicity studies with monarch larvae: Bioassays were conducted with chlorpyrifos, chlorantraniliprole, imidacloprid, thiamethoxam, and clothianidin. For each insecticide, four to six concentrations (including a control) were used with 20 to 40 larvae exposed per concentration. A nominal 1 mg/mL insecticide stock solution was made in DMF; dilutions were made using 0.1% silwet : water to ensure an even coating on the leaf surfaces. Leaves were treated with an insecticide or control suspension (0.1% silwet : water suspension containing 10% DMF) using a pipette. The range of leaf mass provided to a larva over the course of a bioassay and the volume of insecticide suspension applied on each leaf is summarized in Table S3. The volume of insecticide suspension to mass ratio was kept constant to ensure instars were exposed to a consistent concentration of insecticide throughout the larval stage. Three extra leaves were treated at each insecticide concentration and collected at 0 and 48-hours following treatment. Leaves were wrapped in aluminum foil and stored in Ziploc bags at -20 ºC for residue analyses (see supplementary section *Residue analyses)*. Based on these analyses, nominal leaf concentrations at time 0 were used in the concentration-response analyses as they were within ±

25% of the measured leaf concentrations (the chlorpyrifos 5 x 10^{-2} µg/g concentration was an exception; see Table S4).

Neonate larvae were individually plated onto petri plates (60 mm x 15 mm) containing a thin layer of 2% agar : water and a milkweed leaf. At the second instar, freshly treated, surface-dried milkweed leaves were provided once every two days for the first six days and daily thereafter (see Krishnan et al. 2020 for methodological details). The average control mortality over all insecticide bioassays was 18% (range was 13 to 28%). Observations of mortality, feeding, signs of intoxication (e.g., spasms, paralysis, loss of hemolymph), arrested ecdysis (see Krishnan et al. 2020), pupation, and eclosion were recorded every 24 h. The larval instar was recorded on the fourth and eighth day following the start of bioassay. Following eclosion, adults were weighed, sexed, and forewing length (thorax to wingtip) was measured unless the wings were crumpled.

Acute topical toxicity studies with monarch eggs, pupae, and adults: Bioassays were conducted with beta-cyfluthrin, chlorpyrifos, chlorantraniliprole, clothianidin, imidacloprid, and thiamethoxam. All insecticide stock solutions were made in acetone, with the exception of a nominal 6 mg/mL chlorantraniliprole: DMF stock solution that was employed in the adult topical bioassays [chlorantraniliprole solubility in acetone is 3.4 mg/mL at 20 ºC (U.S. Environmental Protection Agency 2008)]. Acetone solution was used to treat control eggs and pupae, and acetone or DMF was used to treat control adults. Average control mortality across stages ranged from 0 (pupae) to 21% (eggs). The stock solutions were analyzed to confirm insecticide concentrations (see supplementary section *Residue analyses* and Table S5), and measured concentrations, along with estimated dilution concentrations, were used to conduct statistical analyses. Dose-response curves were derived for the egg bioassays. Pupae and adults were first

treated with doses approaching the highest possible estimated field exposure doses (see *Estimated insecticide exposure and field-scale risks* and Table S6); if adverse effects were observed, lower doses were tested.

To collect individuals for the egg bioassays, sprigs of tropical milkweed, put in a 125 mL flask with water, were placed in adult monarch cages (cages described in Krishnan et al. 2020). Following three to four hours of egg laying, the sprigs were collected and individual eggs with surrounding leaf tissue (separated using an exacto-knife) were placed in a petri plate containing a thin layer of 2% agar : water. After 24 hours, the individual eggs were treated using a 10-µL Hamilton syringe; 0.2 µL of an insecticide-acetone solution (or acetone alone) was placed on the egg surface. Four concentrations were used per insecticide and 20 eggs were treated per concentration. Daily observations for larval emergence were taken for up to 96 hours. Unhatched eggs were observed for two additional days; however, no emergence was observed after the initial 96-h observation period.

For the pupal bioassays, larvae were reared using USDA colony protocols (see Krishnan et al. 2020). Either one- or two-days following pupation, healthy and properly formed pupae were carefully removed from their 8 oz. plastic cups through the pupal stem and weighed. In preliminary pupal bioassays, we applied 1.0 µL of an insecticide-acetone stock solution (concentrations provided in Table S5) using a 50-µL Hamilton syringe to non-spiracle regions of the pupal cuticle; none of the six insecticides suppressed adult eclosion. The same concentrations (including acetone control) and volume were then spread over the four upper pupal spiracles (see Figure 2a) to enhance insecticide uptake. Within five days following treatment, the pupae were affixed to the inner top of their plastic cups using toothpicks and superglue to ensure proper adult emergence. Daily observations were taken up to 15 days following treatment; day of adult

emergence and coloration were recorded. One to two days following adult emergence, the adults were weighed and sexed. Twenty pupae were treated per concentration; if reduced emergence was observed, lower concentrations (n = 10 pupae per concentration) were employed.

For the adult topical bioassays, control adults from the larval dietary toxicity studies as well as adults reared according to USDA colony procedures were used. Within two days following adult emergence, adults were weighed, and females and males were introduced into separate mesh pop-up laundry baskets (57×37×55 cm; Honey-Can-Do HMP-03891 Mesh Hamper with Handles) with "no-see-em" netting (Arrowhead Fabric Outlet). The baskets contained a small petri plate that was refilled every two days with fresh Gatorade Glacier Cherry Frost Thirst (The Gatorade Company, Inc.) that included sugar and dextrose as a nutritional source. The adults were treated within five days of emergence with 1.0 μL of the insecticide solution that was applied to the center of each of the four wings on the dorsal side with a 50-μL Hamilton syringe. They were then placed into the baskets following segregation by sex and treatment. At least two concentrations were tested for all insecticides except thiamethoxam and clothianidin, which caused no effects at the highest tested concentration (Table S5). At least 20 adults (approximately 50:50 female : male) were treated per insecticide concentration. Daily observations were taken up to 96 h following treatment. Mortality and behavioral effects (paralysis, lethargy, abnormal morphological development) were noted.

Acute dietary toxicity studies with monarch adults: Bioassays were conducted with imidacloprid, thiamethoxam, and clothianidin. For each insecticide we used a single concentration that was at least one hundred-fold higher than the highest concentration measured in nectar of wildflowers adjoining seed-treated fields (Botias et al. 2015). The treatment solution

consisted of an insecticide-acetone solution (or acetone control) dissolved in Gatorade in a 1:4 ratio. Results of the bioassays are based on measured insecticide concentrations (Table S5).

Either one or two days following adult emergence (larvae were reared according to USDA methods; see Krishnan et al. 2020), butterflies were weighed, sexed, screened for *Ophryocystis elektroscirrha* (using methods described in Altizer et al. 2000), and randomly assigned an insecticide treatment. Females and males were introduced into separate laundry baskets and provided sponges soaked in Gatorade up until one day prior to treatment. Diet was withheld one day prior to a bioassay to ensure butterflies readily consumed the insecticide solution the following day. The age of butterflies at the time of treatment did not exceed nine days, and at least 20 butterflies were employed in each treatment.

On the day of treatment, butterflies were taken from their baskets and held in a corral that was fashioned from wood, clothespins, and cardboard (see Figure 2b). Fifty µL of a solution was deposited in plastic caps from 5.0 ml microcentrifuge tubes; 78 of the 80 butterflies consumed the entire solution, either voluntarily or through the forced extension of their proboscis with an uncurled metal paper clip. Daily observations were taken up to 96 h following treatment. Mortality and behavioral effects (paralysis, lethargy, abnormal morphological development) were noted.

Estimated insecticide exposure and field-scale risks

To estimate insecticide spray drift exposure to different monarch stages, we selected a representative formulated product for each active ingredient. We identified the maximum label rates to manage true armyworm and/or soybean aphid, which are early and late season pests in maize and soybean fields, respectively (see Krishnan et al. 2020; information for the clothianidin product provided in Table S6). AgDRIFT (U.S. Environmental Protection Agency 2011a) was

used to estimate the µg of insecticide deposited per cm2 up to 60 m downwind from the edge of a field treated by an aircraft or high or low ground boom applications. We compared the estimated insecticide concentrations on a µg/cm2 basis to the µg/cm2 larval concentration-response curves and µg/cm2 egg dose-response curves to estimate dietary and topical mortality rates to larvae and eggs, respectively, at different distances downwind from a treated field. For monarch pupae and adults, we compared the estimated insecticide exposure landing on their surfaces to the limit doses used in the toxicity bioassays to obtain predicted mortality rates.

To estimate the surface area of eggs, we measured the thickness (or diameter) and length (or height) of 10 eggs with a vernier caliper and assumed eggs were cylinders (cylindrical formula for surface area, $2\pi rh + 2\pi r2$, was used). For pupae, we taped the discarded pupal cuticle of five adults to a sheet and measured their surface area using the ImageJ software (National Institutes of Health). For adults, we ventrally spread out nine dead adults (four females and five males) and measured their surface areas through ImageJ. To estimate the amount of insecticide applied per cm2 of milkweed leaf, we relied on our previous study (Krishnan et al. 2020) in which we estimated the weights and surface areas of approximately 3800 leaves that were fed to second, third, and fifth instar monarchs (Table S7). A weight (g) to surface area (cm2) ratio of 0.026 was obtained. We thus converted the µg of insecticide applied per g leaf to 0.026 x µg of insecticide per cm2 leaf.

To estimate larval and adult risks from dietary exposure to the systemic insecticides, we compared the µg/g concentrations of insecticide residues found within milkweed leaves (Olaya-Arenas and Kaplan 2019; Hall et al. 2020) and wildflower nectar (Botias et al. 2015) from plants sampled near agricultural fields to the respective larval and adult toxicity data sets.

Statistical analyses

All statistical analyses were done in RStudio 1.1.383 (R version 3.5.2). All insecticides and monarch stages were analyzed independently. The "drc" package (version 3.0.1) was used to generate mortality concentration- and dose-response curves and LC/LD values for monarch larvae and eggs. Based on AIC estimates, a three-parameter log-logistic model with a fixed upper limit at 1 was chosen to generate the curves. The "predict" function, followed by corrections using Abbott's formula to account for control mortality, was used to estimate percentage mortality to larvae and eggs from the dose-/concentration-response curves based on AgDRIFT outputs.

For analyzing sublethal effects, we excluded insecticide concentrations that had fewer than three surviving monarchs. Bioassay run was accounted for in the models whenever present. A binomial generalized linear model with type 3 ANOVA (obtained from the "car" package) was used to analyze eclosion rate, sex ratio, and rate of crumpled wings in newly emerged adults. A quasi-poisson generalized linear model (to account for underdispersion) with type 3 ANOVA was used to analyze days to egg emergence, days to pupation, and days to adult eclosion. As data residuals for adult wingspan length and adult weights appeared normally distributed and appropriately dispersed, we used a gaussian glm model with type 3 ANOVA to analyze these endpoints. Whenever treatment effects were significant at the $p = 0.05$ level, emmeans (i.e., Dunnett's test) was used to compare the control response to the insecticide treatment responses.

Results

Toxicity bioassays

Chronic dietary toxicity studies with monarch larvae: Chronic dietary LC_{10}, LC_{50}, and LC_{90} values, and associated 95% confidence intervals (CIs), for monarch larvae are provided in Table

1. Chlorantraniliprole was the most toxic insecticide (95% CIs do not overlap with other insecticide CIs) with a LC_{50} of 1.6 x 10^{-3} µg/g leaf. Imidacloprid and clothianidin were similarly toxic (LC_{50} values were 0.13 and 7.4 x 10^{-2} µg/g leaf, respectively, with overlapping CIs) followed by thiamethoxam (LC_{50} of 0.94 µg/g leaf). Chlorpyrifos was the least toxic insecticide (LC_{50} of 5.3 µg/g leaf). Concentration-response curves expressed as µg/g leaf and µg/cm^2 leaf generally had steep slopes that ranged from -1.5 (chlorantraniliprole) to -6.2 (chlorpyrifos; Figures 3 and S1).

The highest leaf concentration used for each insecticide caused between 88% to 100% larval mortality (percentage mortality rates for all insecticide concentrations are provided in Table S8). The highest chlorpyrifos (25 µg/g) and chlorantraniliprole (5 x 10^{-3} µg/g) concentrations caused 100 and 52% of cumulative larval mortality by Day 8, respectively, with mortality typically observed each day (Figure 4). The highest imidacloprid and clothianidin concentration (0.5 µg/g) killed 82 and 60% of larvae, respectively, at the time of pupation (10-12 days after a bioassay was initiated) through arrested ecdysis. The 0.5 and 2.5 µg/g thiamethoxam concentrations killed 44 and 46% of the fifth instars also through arrested ecdysis (Table S8).

Eighty to 100% of all larvae that successfully pupated, irrespective of insecticide or insecticide concentration, were in the fourth instar on day 4 and the fifth instar on day 8 (data not shown). All surviving larvae took an average of 10-11 days to pupate and 11-13 days to eclose (Figure S2), with no differences observed between concentrations ($p > 0.19$ and $p > 0.18$, respectively; see Table S9). Larvae that pupated successfully had a 71 to 100% eclosion success rate, again with no differences between concentrations ($p > 0.055$; see Table S10). Appearance and behavior of butterflies in insecticide treatment groups were similar to controls. Across control and treatment groups, the incidence of crumpled wings ranged from 4 to 25% and 0 to

43%, respectively, with no significant effects noted except in the 0.5 µg/g chlorpyrifos treatment group ($p = 0.045$; Table S10).

The mean wingspan length of butterflies with normal wings in each treatment ranged from 3.9 to 4.5 cm and did not differ between treatments and controls for the neonicotinoids and chlorantraniliprole (Figure 5). Butterflies in the 5 µg/g chlorpyrifos had 8% smaller wings ($p = 0.0007$; Table S9). All chlorpyrifos-treated butterflies ($p < 0.036$ for all concentrations) and the 5 x 10^{-4} µg/g clothianidin-treated butterflies ($p = 0.044$) had reduced adult weights compared to control butterflies; no effects on weights were observed with other insecticide treatments (Figure S3 and Table S9). The sex ratio, defined as number of females divided by number of males, of newly emerged butterflies ranged from 0.62 to 1.5 for the neonicotinoids and chlorantraniliprole; for chlorpyrifos it ranged from 0.5 (control) to 4.0 (5 µg/g). Again, no significant differences were found ($p > 0.097$; see Table S10).

Acute topical toxicity studies with monarch eggs, pupae, and adults: Acute topical LD_{10}, LD_{50}, and LD_{90} values, and associated 95% confidence intervals (CIs), for monarch eggs are provided in Table 2. Beta-cyfluthrin and chlorantraniliprole were the most toxic insecticides (overlapping 95% CIs) with LD_{50} values of 7.3 x 10^{-3} and 1.8 x 10^{-2} µg/g egg, respectively. The neonicotinoids had LD_{50} values of 1.2 (clothianidin), 2.9 (imidacloprid), and 87 (thiamethoxam) µg/g egg. Chlorpyrifos was the least toxic insecticide, with a LD_{50} value of 3600 µg/g egg. Egg percentage mortality rates for all insecticide concentrations are provided in Table S11. Dose-response curves in µg/g egg and µg/cm^2 egg had slopes ranging from -0.040 (beta-cyfluthrin) to -6.4 (chlorpyrifos; Figures S4 and S5). The vast majority of eggs hatched on the third day following treatment (Table S11). No differences in days to hatch were observed, except for eggs treated with 4.3 x 10^{-2} µg/g beta-cyfluthrin (Table S12), which on average hatched on day 4.

Pupae treated on the spiracles with chlorpyrifos and neonicotinoids had 100% eclosion (Table 3) with no effects seen on pupal duration ($p > 0.068$; Table S12); adults that emerged appeared healthy. When pupal spiracles were treated with beta-cyfluthrin and chlorantraniliprole, no adults eclosed (Table 3) even though the pupae had normally developed adult coloration. Serial dilutions of the stock solutions were then tested; the 7.8×10^{-4} µg/g beta-cyfluthrin and the 4.0×10^{-4} µg/g chlorantraniliprole did not suppress or alter the time to adult eclosion (Tables 3). At 7.8×10^{-3} µg/g and 7.8×10^{-2} µg/g beta-cyfluthrin doses, 100 and 40% of the adults emerged, respectively. Of these, 10 and 100% of emerged butterflies, respectively, were weak and died within two days. No adults emerged when pupae were treated with 4.0×10^{-2} µg/g chlorantraniliprole; a ten-fold lower dose had 30% emergence and a shorter pupal duration ($p = 0.034$; see Tables 3 and S12). The butterflies otherwise appeared healthy, and the sex ratios in all treatments were in the expected range.

Adults treated with neonicotinoids at concentrations that were within ± 20% of the highest possible spray drift exposure dose had a control-corrected mortality of 58% with imidacloprid (two- and twenty-fold lower doses caused 26 and 0% mortality, respectively) and 0% with thiamethoxam and clothianidin (Table 4). Both the 86 µg/g chlorpyrifos dose and the 8×10^{-2} µg/g beta-cyfluthrin dose killed 100% of butterflies in four days. Doses that were ten-fold lower caused little to no mortality. The highest chlorantraniliprole dose killed approximately 60% of treated monarchs, while a dose approximately 10-fold lower caused no mortality. Of note, female butterflies were nearly twice as susceptible to the 52 and 104 µg/g imidacloprid doses and three times as susceptible to the 21 µg/g chlorantraniliprole dose, compared to males.

Acute dietary toxicity studies with monarch adults: Butterflies in both the treatment and control groups typically consumed the 50 µL insecticide-treated or untreated Gatorade solution

in two to three minutes. The control butterflies had the highest mortality (18%) followed by imidacloprid (5%). Thiamethoxam and clothianidin caused no mortality (Table 5). No other observable adverse effect occurred within the 96-hr observation period.

Estimated insecticide exposure and field-scale risks

Chronic dietary larval exposure to spray drift from foliar applications: When aerial applications for foliar formulations of chlorpyrifos, chlorantraniliprole, imidacloprid, and clothianidin were modeled for soybean aphid management, predicted monarch larval mortality was between 100% and 93% at all modeled distances downwind from the field (0, 15, 30, and 60 m). Thiamethoxam was estimated to cause between 100 to 24% larval mortality from the field edge to 60 m downwind (Figure 6). High ground boom applications for soybean aphid are expected to cause 100% mortality for all insecticides at the field edge; however, due to reduced off-site drift, lower mortality was predicted for chlorpyrifos and thiamethoxam at 15 (17 to 27% mortality), 30 and 60 m (17 to 19% mortality) downwind. Imidacloprid is expected to cause between 70 to 32% larval mortality at the same distances. Chlorantraniliprole and clothianidin kill nearly 100% of the larvae at all distances downwind. Similar mortality patterns for insecticides were seen for modeled high and low ground boom applications to manage true armyworm outbreaks (Figure S6). While exposure concentrations were based on the fiftieth percentile results for ground applications, ninetieth percentile results to capture worse-case drift scenarios are expected to produce similar results (see Krishnan et al. 2020).

Acute topical egg, pupa, and adult exposure to spray drift from foliar insecticides: When aerial applications for foliar formulations of beta-cyfluthrin, chlorantraniliprole, imidacloprid, and clothianidin were modeled for soybean aphid management, predicted monarch egg mortality was between 100% and 83% at all modeled distances (0, 15, 30, and 60 m downwind from the

field). Chlorpyrifos and thiamethoxam were estimated to cause between 98 to 19% egg mortality from edge of field to 60 m downwind (Figure 6). High ground boom applications for soybean aphid is expected to cause at least 95% mortality for all insecticides at edge of field. However, due to reduced off-site drift, lower mortality was predicted for the neonicotinoids at 15 (27 to 76% mortality), 30 (24 to 72% mortality), and 60 m (23 to 68% mortality) downwind. Chlorpyrifos is predicted to kill a similar percentage of eggs as with aerial application at all distances. Beta-cyfluthrin and chlorantraniliprole are expected to cause between 89 to 93% egg mortality even 60 m downwind. Similar mortality patterns were seen for modeled high and low ground boom applications to manage true armyworm (Figure S6).

Aerial and high ground boom applications for managing soybean aphids and high or low ground boom applications for true armyworm management, are not expected to cause mortality to monarch pupae if spray drift lands on non-spiracular regions of the cuticle. However, if beta-cyfluthrin or chlorantraniliprole exposures contact pupal spiracles, 100% mortality to pupae (and/or butterflies that successfully eclose) is estimated at nearly all distances downwind (0, 15, 30, and 60 m) following aerial applications to manage soybean aphids. When ground boom applications are modeled to manage soybean aphid or true armyworm populations, beta-cyfluthrin is predicted to cause 100% pupal mortality at the edge of field, with little to no mortality occurring further downwind. Chlorantraniliprole boom applications are expected to cause between 70 to 100% pupal mortality at all modeled distances.

No mortality is expected for adult monarchs due to wing exposure from thiamethoxam or clothianidin spray drift. Aerial and ground boom applications of imidacloprid and chlorantraniliprole are predicted to kill up to 60% of butterflies at the edge of field, with no mortality anticipated at 15, 30, and 60 m downwind. Chlorpyrifos and beta-cyfluthrin

applications are estimated to kill nearly all butterflies up to 30 and 60 m downwind following aerial applications, respectively. With ground boom applications, these insecticides are expected to cause 100% mortality at the edge of field with little to no mortality downwind.

Downslope chronic larval dietary and acute adult dietary adult exposure to neonicotinoid residues from seed treatments: No mortality is expected for monarch larvae consuming milkweed containing mean concentrations of neonicotinoids derived from seed treatment uses (Table 6). No mortality is also expected when larvae consume milkweed containing the highest imidacloprid and thiamethoxam concentrations reported; the highest clothianidin concentration (80-fold higher than the corresponding mean reported in Olaya-Arenas and Kaplan 2019) is expected to kill 23% of downslope larvae. No acute monarch mortality is expected for adults consuming the mean or highest reported neonicotinoid concentrations in wildflower nectar (Table 6).

Discussion

Insecticide exposure to monarchs and their habitat is considered a potential threat to recovery of the North American eastern population (U.S. Fish and Wildlife Service 2017). Monarchs in the U.S. North Central agricultural landscapes are likely to be exposed to foliar and seed-treatment insecticides from mid-May to late August, which coincides with peak levels of non-migratory monarchs in the region. In maize and soybean fields, insecticide-treated seeds are routinely used to manage early-season pests (Tooker et al. 2017), while foliar insecticides are used to varying degrees to manage early- and late-season pests (see Krishnan et al. 2020 Figure 1 and references therein). Understanding the potential impact of insecticide use on monarch productivity requires quantitative information on the nature and extent of field-scale insecticide

exposure and the toxicity of the compounds to monarch life stages through different exposure pathways.

Insecticide toxicity

Chlorantraniliprole is approximately 50 to 500 times more toxic to monarch larvae than the neonicotinoids and 3000 times more toxic than chlorpyrifos. Chronic LC_{50}s were 1.1 (chlorpyrifos), 3.7 (thiamethoxam), 5.2 (chlorantraniliprole), 11 (clothianidin), and 39 (imidacloprid) times lower than acute LC_{50}s for the most sensitive instars (Krishnan et al. 2020). Larval and pupal duration, adult eclosion, and sex ratio did not vary between controls and insecticide treatments for the chronic studies. However, chlorpyrifos-treated larvae produced adults that had smaller wingspan and weight; in some instances, it also elicited deformation of the wings. There were no adverse sublethal effects observed with the other insecticides. Overall, 53, 56, and 68% of fifth-instar mortality in the thiamethoxam, clothianidin, and imidacloprid treatments, respectively, occurred due to arrested pupal ecdysis, with no symptoms observed prior to death. Some neonicotinoid concentrations caused over 80% of the fifth instars to die through this phenomenon. Approximately 10 to 20% of fifth-instar mortality following exposure to chlorpyrifos, chlorantraniliprole, and the control solvent was through arrested ecdysis.

To date, clothianidin has the most extensive monarch toxicity data available in the peer-reviewed literature. Bargar et al. (2020) conducted a series of chronic dietary studies and reported LC_{50}s of 4.7×10^{-2} to 0.21 µg/g swamp milkweed (*Asclepias incarnata*) leaf. Olaya-Arenas et al. (2020) observed 30% larval mortality following a chronic clothianidin dietary exposure to 5.7×10^{-2} µg/g common milkweed (*Asclepias syriaca*) leaf. We determined a chronic LC_{50} value of 7.4×10^{-2} µg/g tropical milkweed leaf and observed 23% mortality at 5.7×10^{-2} µg/g. Pecenka and Lundgren (2015) treated 1-cm diameter swamp milkweed leaf discs with 10

µL of clothianidin solutions; however, toxicity was not expressed on µg/g basis. Assuming these swamp milkweed leaf discs weighed 16 mg (based on our independent measurements), their reported acute LC_{50} would be approximately 9.8 x 10^{-3} µg/g swamp milkweed leaf. Previously, we reported acute LC_{50}s ranging from 0.80 to 7.8 µg/g tropical milkweed leaf (Krishnan et al. 2020). We also obtained a similar acute dietary LC_{50} with an artificial diet (see Tables S13 and S14 and *Artificial diet* summary in supplementary). The 100 to 1000-fold greater sensitivity reported by Pecenka and Lundgren (2015) as compared to the results reported in the present study as well as Krishnan et al. (2020), Bargar et al. (2020), and Olaya-Arenas et al. (2020), which used three different sources of monarchs and three different milkweed species, is unclear.

Peterson et al. (2019) chronically fed painted lady (*Vanessa cardui*) larvae an artificial diet spiked with a range of clothianidin concentrations; after correcting for control mortality, approximately 50% of the butterflies pupated at the 5 µg/g concentration. This suggests monarch larvae are about 70-fold more sensitive to clothianidin. To the best of our knowledge, there are no other chronic larval dietary toxicity studies for other butterfly species that report effect values based on mass of insecticide per mass, surface area, or volume of leaf or diet.

Acute topical LD_{50} values for eggs indicated that beta-cyfluthrin and chlorantraniliprole were the most toxic insecticides. Their lipophilicity (log K_{ow} of 6 and 3, respectively; Tomlin 1994 and MacBean 2012) may facilitate greater diffusion into the egg, resulting in a higher delivered dose. Thiamethoxam and chlorpyrifos, both of which are metabolically activated, were 30 and 1600 times less sensitive, respectively, than imidacloprid (2.9 µg/g) and clothianidin (1.2 µg/g). A beta-cyfluthrin concentration that reduced the hatch rate by 75% also delayed larval emergence; this effect was not observed in the other insecticides. Comparisons of our results

with prior insecticide toxicity studies with butterfly eggs (Braak et al. 2018) was not possible because effect concentrations or doses were not provided.

One- to two-day-old monarch pupae were unaffected when the highest modeled exposure concentration for each insecticide was applied to non-spiracular regions of the pupal surface, presumably due to no or low diffusion across the cuticle. When the highest tested beta-cyfluthrin and chlorantraniliprole concentrations were applied to pupal spiracles, no adults emerged even though the treated pupae developed adult coloration. Adults emerged at lower doses, either sooner than controls or in a compromised condition. No effects on pupal duration or adult emergence was observed when pupal spiracles were treated with chlorpyrifos or the neonicotinoid insecticides. As noted above, the higher lipophilicity of beta-cyfluthrin and chlorantraniliprole may facilitate higher uptake into the developing adult body, thereby causing muscle paralysis that hindered emergence. To the best of our knowledge, we are the first to report pupal toxicity studies with a butterfly species.

No mortality was observed with monarch adults topically exposed to clothianidin and thiamethoxam at concentrations that corresponded to the highest predicted spray drift exposure. Imidacloprid and chlorantraniliprole are expected to kill up to 60% of butterflies at the highest expected environmental concentrations; concentrations that are a magnitude lower had no effect. The highest expected environmental beta-cyfluthrin and chlorantraniliprole concentrations, and concentrations that are ten-fold lower, caused 100% mortality. A 10-fold lower concentration caused no mortality. Interestingly, most of the dead chlorpyrifos-treated butterflies had bulging or burst thoraxes due to fluid retention. We also observed sex differences in mortality rates in imidacloprid and chlorantraniliprole treatments. A mechanistic explanation for these symptoms is not readily apparent.

Hoang et al. (2011) treated wings of the white peacock (*Anartia jatrophae*), Atala hairstreak (*Eumaeus atala*), zebra longwing (*Heliconius charitonius*), common buckeye (*Junonia coenia*), and painted lady (*Vanessa cardui*), with permethrin (a pyrethroid) and obtained 24-h LD_{50}s ranging from 0.66 to 8.69 µg/g. Exposures to naled and dichlorvos (organophosphates) resulted in LD_{50}s between 1.31 to 13.6 µg/g. The authors also noted differences in sensitivity based on insecticide application site; the pyrethroid was more toxic when applied to the thorax while the organophosphates were more toxic when applied to the wings. While we only applied insecticides on the wings, our results suggest that monarchs, in general, are slightly more sensitive to pyrethroid and slightly less sensitive to organophosphate [beta-cyfluthrin LD_{50} is between 8×10^{-2} (1% mortality) to 1.2 µg/g (100% mortality), and the chlorpyrifos LD_{50} is between 7.7 (6% mortality) to 86 µg/g (100% mortality)] compared to the other species. When compared to adult honeybees, adult monarchs are less sensitive to all classes of insecticides tested (Arena and Sgolastra 2014; Thompson 2015; Wade et al. 2019; Kadala et al. 2019).

Monarch adults had no acute adverse effects when they consumed an artificial nectar source spiked with 7.0×10^{-3} µg clothianidin (140 µg/L clothianidin), 1.3×10^{-2} µg imidacloprid (250 µg/L imidacloprid), or 1.7×10^{-2} µg thiamethoxam (330 µg/L thiamethoxam; See Table S5). Krischik et al. (2015) reported no increased mortality when monarchs were exposed to 15 and 30 µg/L imidacloprid for 29 days. James (2019) reported that a 22-day exposure of monarch adults to cotton wool treated with a residential formulated imidacloprid product (i.e., a mixture of imidacloprid and ‘inert’ ingredients) diluted with distilled water (23.5 µg/L) caused 74% mortality as compared to adults exposed to distilled water. Because this experimental design likely resulted in topical and oral exposure from the cotton wool, and a control treatment based

on the formulation's inert ingredients was not employed, a meaningful comparison to our results and those of Krischik et al. (2015) is not possible.

Toxicity to mixtures of insecticides and/or other pesticides in foliar tank mixes or seed treatment formulations can be assessed through the use of concentration- or response-addition models (National Research Council 2013). Synergistic effects that might considerably increase toxicity would not be captured by these models, but they are relatively rare (Cedergreen 2014; Belden and Brain 2017). Olaya-Arenas et al. (2020) did not find any synergistic effects on survival when they chronically exposed larvae to milkweed leaves that were treated with a mixture of clothianidin, two herbicides, and three fungicides.

Comparing sensitivity across insecticides, life stages, and exposure routes

To compare sensitivity across different life stages, exposure routes, and lengths of exposure, we expressed toxicity results obtained in the present study and in Krishnan et al. (2020) on a µg of insecticide/g mass basis. The methods used to obtain the doses and the results are described in the supplementary materials (Table S15).

Insecticide comparisons: Beta-cyfluthrin (a pyrethroid) and chlorantraniliprole (a diamide) were the most toxic insecticides, followed by the neonicotinoids. Typically, clothianidin is the most toxic neonicotinoid, while thiamethoxam the least. The organophosphate chlorpyrifos is the least toxic insecticide tested. Since thiamethoxam and chlorpyrifos are pro-insecticides, it is possible that monarchs do not metabolically-activate the parent compounds efficiently to clothianidin and chlorpyrifos-oxon, respectively. A similar pattern of organophosphate toxicity has been observed with other butterfly species. Malathion and fenthion, which require activation to their respective oxons, were approximately 5 to 500 times less toxic than naled and

dichlorvos, which are phosphates and do not require activation (Eliazar and Emmel 1991; Salvato 2001; Hoang et al. 2011).

Life stage comparisons: Following topical exposures to all life stages, we found monarch eggs and larvae (see also Krishnan et al. 2020) to be the most susceptible stages on a µg/g basis. While full dose-response curves with monarch pupae and adult would provide a more extensive life stage comparison, our findings indicate these later life stages are less sensitive. However, as eggs and pupae are undergoing development within their cuticles, it is possible that exposure to insecticides at different times within a stage may alter their susceptibility. While no comparable toxicity studies have been conducted on other butterfly eggs and pupae, topical exposure studies suggest, in general, that butterfly larval stages are more sensitive than their adult stages (Hoang et al. 2011), consistent with our findings.

Exposure route comparisons: On a µg/g basis, beta-cyfluthrin is more toxic to monarch larvae via topical exposure. With the other insecticides, the topical and dietary doses that cause between 20 and 100% larval mortality were generally within the same order of magnitude (see Table S15 and Krishnan et al. 2020). Our data suggest that the dietary bioassays also resulted in topical uptake of insecticide; Olaya-Arenas et al. (2020) had also noted the possibility of combined exposures in their dietary studies. Hoang et al. (2011, 2015) observed differential toxicity with the two exposure routes in Atala hairstreak and common buckeye butterfly larvae; permethrin was 3 to 9 times more toxic via the topical route while naled and dichlorvos were 8 to 23 times more toxic via the dietary route. However, in white peacock larvae, the three insecticides exhibited similar toxicity via both exposure routes. In our adult toxicity studies, acute exposures to 2×10^{-2} to 4×10^{-2} µg/g neonicotinoids caused no effects via both the dietary and topical routes.

Characterizing mortality risks from insecticide seed treatments

To estimate seed treatment risks, we relied on Hall et al. (2020) and Botias et al. (2015) who sampled milkweed leaves and wildflower nectar within and at the edge of fields planted with treated seeds, respectively. No mortality to larvae and adults is predicted at their highest reported neonicotinoid residue concentrations. The lack of monitoring studies for chlorantraniliprole seed treatment applications preclude the means to estimate its likelihood of risk to larvae and adults.

Characterizing mortality risks from foliar applications

In Krishnan et al. (2020), we estimated acute dietary mortality to monarch larvae immediately following a spray drift event. However, larvae that survive the initial 24- or 48-h exposure period or larvae that hatch from eggs laid after a spray drift event could be exposed to insecticide residues on leaves. Assuming there is no insecticide degradation over the entire larval life stage, aerial and ground boom applications of chlorantraniliprole and clothianidin are estimated to kill nearly all exposed larvae up to 60 m downwind. Aerial applications of chlorpyrifos and imidacloprid are also expected to cause nearly 100% larval mortality up to 60 m downwind; however, with ground boom, mortality is ~ 30%. Thiamethoxam was expected to cause the least mortality via both foliar application methods (100 to 17% at 0 and 60 m downwind). A more realistic estimate of mortality could take into account the insecticide half-lives [chlorpyrifos (4-6 days; Galietta et al. 2011; Szpyrka et al. 2017), chlorantraniliprole (3-17 days; Lee et al. 2019; Szpyrka et al. 2017), imidacloprid (2-5 days; Mukherjee et al. 2000; Banerjee et al. 2012), thiamethoxam (4-6 days; Rahman et al. 2015), and clothianidin (4 days; Chowdhury et al. 2012)], which are shorter than the length of the entire larval stage [12 to 13 day at 27 and 25 ºC, respectively (Rawlins and Lederhouse 1981; Zalucki 1982)]. Assuming an

insecticide half-life of 4 days, estimated exposure would drop approximately 2.4-fold for neonates that hatch on the day of application, and 4.8-fold for neonates that hatch four days later. This results in a significant reduction in larval mortality at 60 m downwind for chlorpyrifos, imidacloprid, and thiamethoxam applications. Due to their inherent toxicity, aerial applications of clothianidin and both aerial and ground boom applications of chlorantraniliprole are predicted to cause high downwind mortality even with reduced exposure (see Table S16 and *Foliar insecticide degradation* in the supplementary).

We also compared our toxicity data to field measured insecticide residues reported by Halsch et al. (2020) who quantified pesticide concentrations in four species of milkweed plants sampled from the Central Valley of California. The combined mean concentration of chlorantraniliprole in milkweed plants in nine agricultural sites was 1.6×10^{-2} µg/g, and the lowest and highest mean plant concentrations observed within sites were 6.6×10^{-4} and 6.6×10^{-2} µg/g, respectively. These milkweed residue concentrations are likely due to foliar applications on tree nut crops (California Department of Pesticide Regulation 2019; U.S. Geological Survey 2020). The combined, lowest, and highest mean concentrations are predicted to kill 97, 21, and 100% of larvae consuming milkweed downwind of an application, respectively (Figure 3).

Aerial and ground boom applications of formulated beta-cyfluthrin and chlorantraniliprole products are expected to kill nearly all exposed eggs up to 60 m downwind. Aerial applications of clothianidin and imidacloprid are expected to cause over 80% egg mortality up to 60 m downwind, however, with ground boom, mortality falls down to ~ 50%. Thiamethoxam and chlorpyrifos are expected to cause the least mortality (ca. 100 to 20% at 0 and 60 m downwind). Risk to monarch pupae is expected to be minimal following foliar application of neonicotinoids and chlorpyrifos, while aerial applications of beta-cyfluthrin and

chlorantraniliprole that land on spiracles are expected to kill nearly all pupae (or emergent adults) up to 60 m downwind. Ground boom applications cause lower mortality (100 to 0% for beta-cyfluthrin and 100 to 70% for chlorantraniliprole). Aerial applications of neonicotinoids and chlorantraniliprole are predicted to cause no acute mortality with adult butterflies, while ground boom applications of imidacloprid and chlorantraniliprole are expected to kill 60 to 0% of exposed butterflies at 0 and 60 m downwind. Chlorpyrifos and beta-cyfluthrin applications are estimated to cause nearly 100% adult mortality in all downwind distances following aerial applications; 100 to 0% mortality is expected with ground boom applications.

While these field-scale risk estimates are informative, it is important to consider the behavior of the different monarch stages to accurately assess their risk to insecticides. Monarch eggs and pupae are typically found underneath leaves (Monarch Joint Venture 2020) and are therefore less likely to be exposed to foliar insecticide drift. For the pupae, insecticides have to land on the spiracle to cause any effects. Monarch larvae and adults are likely to have the greatest risk as they could have simultaneous topical and dietary exposure to insecticides. While we did not assess the combined risk of topical and dietary exposures to foliar and seed-treatment insecticides, it is possible to sum the insecticide doses across different exposure routes and uses to obtain an aggregate dose within exposed larvae or adults.

Conclusions

Imidacloprid, thiamethoxam, and clothianidin constitute nearly 85% of total neonicotinoid sales (Bass et al. 2015) and are extensively used to treat maize and soybean seeds (Tooker et al. 2017). We conclude these seed treatment uses pose little risk to monarch larvae and adults, consistent with findings of Krischik et al. (2015), Bargar et al. (2020), and Olaya-Arenas et al. (2020). In the last decade, several chlorantraniliprole seed treatment products have

been registered in maize (U.S. Environmental Protection Agency 2011b and 2020) and their use may increase. Currently, the lack of chlorantraniliprole seed treatment residue data in milkweed leaves or wildflower nectar makes it difficult to assess their risk to monarchs.

Pyrethroids and organophosphates are the most commonly used foliar insecticides; over 190,000 kilograms were applied in Iowa in 2018 (U.S. Department of Agriculture 2019). Neonicotinoids and diamides are also registered for foliar applications, though they are not widely employed to manage soybean aphids (Hodgson et al. 2012; Whalen et al. 2016). Less than a third of maize and soybeans in North Central U.S. are annually treated with foliar insecticides (U.S. Department of Agriculture 2018); however, aerial applications, particularly of chlorantraniliprole, beta-cyfluthrin, and chlorpyrifos, can result in high rates of downwind mortality. Lower mortality is anticipated with ground boom applications.

Our field-scale mortality estimates directly inform a landscape-scale risk analysis to evaluate conservation risks and benefits of establishing monarch habitat in agricultural landscapes (Grant et al. 2020a). This analysis accounts for several factors including adult monarch vagile behavior (Zalucki et al. 2016) and population demographics (Grant et al. 2020b); levels of milkweed augmentation; pest type, levels of pest pressure, and use of Integrated Pest Management; wind direction at the time of insecticide application and predicted field-scale mortality. Even under the assumption that foliar insecticide applications result in 100% downwind mortality, this analysis indicates more adult monarchs will be produced when new milkweed is established in all available space, including within close proximity of treated fields in the agricultural landscapes of the North Central U.S (Grant et al. 2021).

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Data availability

Data and metadata pertaining to this manuscript will be made publicly available at this GitHub repository following acceptance of manuscript:

https://github.com/Niranjana296/Monarch-butterfly-life-stage-risks-to-insecticides

References

Altizer SM, Oberhauser KS, Brower LP. 2000. Associations between host migration and the prevalence of a protozoan parasite in natural populations of adult monarch butterflies. Ecol Entomol 25:125–139.

Arena M, Sgolastra F. 2014. A meta-analysis comparing the sensitivity of bees to pesticides. Ecotoxicology 23:324–334.

Banerjee T, Banerjee D, Roy S, Banerjee H, Pal S. 2012. A comparative study on the persistence of imidacloprid and beta-cyfluthrin in vegetables. Bull Environ Contam Toxicol 89:193–196.

Bargar, TA, Hladik ML, Daniels JC. 2020. Uptake and toxicity of clothianidin to monarch butterflies from milkweed consumption. PeerJ 8: e8669.

Bass C, Denholm I, Williamson MS, Nauen R. 2015. The global status of insect resistance to neonicotinoid insecticides. Pestic Biochem Physiol 121: 78–87.

Belden JB, Brain RA. 2018. Incorporating the joint toxicity of co-applied pesticides into the ecological risk assessment process. Integr Environ Assess Manag 14: 79-91.

Botías C, David A, Horwood J, Abdul-Sada A, Nicholls E, Hill E, Goulson D. 2015. Neonicotinoid residues in wildflowers, a potential route of chronic exposure for bees. Environ Sci Technol 49 (21): 12731–40.

Braak N, Neve R, Jones AK, Gibbs M, Breuker CJ. 2018. The effects of insecticides on butterflies—A review. Environ Pollut 242:507–518.

California Department of Pesticide Regulation. 2019. Summary of Pesticide Use Report Data - 2017 [cited 2020 October 17]. Available from: https://www.cdpr.ca.gov/docs/pur/pur17rep/17sum.htm

Cedergreen N. 2014. Quantifying Synergy: A systematic review of mixture toxicity studies within environmental toxicology. PLoS One 9 (5): e96580.

Chowdhury S, Mukhopadhyay S, Bhattacharyya A. 2012. Degradation dynamics of the insecticide: clothianidin (dantop 50 % wdg) in a tea field ecosystem. Bull Environ Contam Toxicol 89 (2): 340–43.

Douglas MR, Tooker JF. 2015. Large-scale deployment of seed treatments has driven rapid increase in use of neonicotinoid insecticides and preemptive pest management in U.S. field crops. Environ Sci Technol 49 (8): 5088–97.

Dunbar MW, O'Neal ME, Gassmann AJ. 2016. Increased risk of insect injury to corn following rye cover crop. J Econ Entomol 109:1691–1697.

Eliazar PJ, Emmel TC. 1991. Adverse impacts to non-target insects. Mosquito control pesticides: Ecological impacts and management alternatives. Scientific Publishers Inc., Gainesville, Florida, 17-19.

Galietta G, Egana E, Gemelli F, Maeso D, Casco N, Conde P, Nunez S. 2011. Pesticide dissipation curves in peach, pear and tomato crops in Uruguay. J Environ Sci Health B 46:35–40.

Grant TJ, Parry HR, Zalucki MP, Bradbury SP. 2018. Predicting monarch butterfly (Danaus plexippus) movement and egg-laying with a spatially explicit agent-based model: The role of monarch perceptual range and spatial memory. Ecol Modell 374:37–50.

Grant TJ, Krishnan N, Bradbury SP. 2020a. Conservation risks and benefits of establishing monarch butterfly (*Danaus plexippus*) breeding habitat in close proximity to maize and soybean fields in the U.S. Midwest: A landscape-scale analysis of foliar insecticide effect on non-migratory monarch butterfly populations. To Integr Environ Assess Manag. Submitted.

Grant TJ, Flockhart DTT, Blader TR, Hellmich RL, Pitman GM, Tyner S, Norris DR, Bradbury SP. 2020b. Estimating arthropod survival probability from field counts: a case study with monarch butterflies. Ecosphere 11(4).

Hall MJ, O'Neal M, Bradbury SP, and Coats JR 2020. Quantifying neonicotinoid insecticide residues in wildflowers sampled from prairie strips established in corn and soybean fields planted with treated seeds. To Agric Eco Environ. Submitted.

Halsch CA, Code A, Hoyle SM, Fordyce JA, Baert N, Forister ML. 2020. Pesticide contamination of milkweeds across the agricultural, urban, and open spaces of low-elevation northern California. Front Ecol Evol 8: 162.

Hoang TC, Pryor RL, Rand GM, Frakes RA. 2011. Use of butterflies as nontarget insect test species and the acute toxicity and hazard of mosquito control insecticides. Environ Toxicol Chem 30: 997–1005.

Hoang TC, Rand GM. 2015. Acute toxicity and risk assessment of permethrin, naled, and dichlorvos to larval butterflies via ingestion of contaminated foliage. Chemosphere 120:714–721.

Hodgson EW, McCornack BP, Tilmon K, Knodel JJ. 2012. Management recommendations for soybean aphid (Hemiptera: Aphididae) in the United States. J Integr Pest Manag 3:E1–E10.

James DG. 2019. A neonicotinoid insecticide at a rate found in nectar reduces longevity but not oogenesis in monarch butterflies, Danaus Plexippus (L.). (Lepidoptera: Nymphalidae). Insects 10 (9): 276.

Kadala A, Charreton M, Charnet P, Collet C. 2019. Honey bees long-lasting locomotor deficits after exposure to the diamide chlorantraniliprole are accompanied by brain and muscular calcium channels alterations. Sci Rep 9:2153.

Keystone Policy Center. 2017. Monarch Collaborative. [cited 2020 October 17]. Available from: https://www.keystone.org/our-work/agriculture/monarch-collaborative/

Krischik V, Rogers M, Gupta G, Varshney A. 2015. Soil-applied imidacloprid translocates to ornamental flowers and reduces survival of adult Coleomegilla maculata, Harmonia axyridis, and Hippodamia convergens lady beetles, and larval Danaus plexippus and Vanessa cardui butterflies. PLoS One 10:e0119133.

Krishnan N, Yang Z, Bidne KG, Hellmich RL, Coats JR, and Bradbury SP. 2020. Assessing field-scale risks of foliar insecticide applications to monarch butterfly (Danaus Plexippus) larvae. Environ Toxicol Chem 39 (4): 923–41.

Lee J, Byung JK, Eunhye K, Jeong-Han K. 2019. Dissipation kinetics and the pre-harvest residue limits of acetamiprid and chlorantraniliprole in kimchi cabbage using ultra-performance liquid chromatography-tandem mass spectrometry. Molecules 24 (14): 2616.

MacBean C. 2012. The Pesticide Manual: A World Compendium. British Crop Protection Council, The Royal Society of Chemistry, Cambridge.

Malhat F, Hend A, and Islam H. 2012. Dissipation of chlorantraniliprole in tomato fruits and soil. Bull Environ Contam Toxicol 88 (3): 349–51.

Monarch Joint Venture. 2010. [cited 2020 October 17]. Available from: https://monarchjointventure.org/

Mukherjee I, Gopal M. 2000. Environmental behaviour and translocation of imidacloprid in eggplant, cabbage and mustard. Pest Manag Sci 56: 932–36.

National Research Council. 2013. Assessing risks to endangered and threatened species from pesticides. National Academies Press, Washington, DC.

Natural Resources Conservation Service. 2016. [cited 2020 October 17]. Available from: https://www.nrcs.usda.gov/wps/portal/nrcs/detail/national/plantsanimals/pollinate/?cid=nrcseprd402207

Oberhauser KS. 1989. Effects of spermatophores on male and female monarch butterfly reproductive success. Behav Ecol Sociobiol 25 (4): 237–46.

Oberhauser KS, Wiederholt R, Diffendorfer JE, Semmens D, Ries L, Thogmartin WE, Lopez-Hoffman L, Semmens B. 2017. A trans-national monarch butterfly population model and implications for regional conservation priorities. Ecol Entomol 42:51–60.

Olaya-Arenas P, Kaplan I. 2019. Quantifying pesticide exposure risk for monarch caterpillars on milkweeds bordering agricultural land. Front Ecol Evol 7:223.

Olaya-Arenas P, Kayleigh H, Scharf ME, Kaplan I. 2020. Larval pesticide exposure impacts monarch butterfly performance. Sci Rep10 (1): 14490.

Pecenka JR, Lundgren JG. 2015. Non-target effects of clothianidin on monarch butterflies. Naturwissenschaften 102:19.

Peterson EM, Shaw KR, Smith PN. 2019. Toxicity of agrochemicals among larval painted lady butterflies (*Vanessa cardui*). Environ Toxicol Chem, 38: 2629-2636.

Rahman MM, Farha W, Abd El-Aty AM, Kabir MH, Im SJ, Jung DI, Choi JH, Kim SW, Son YW, Kwon CH, Shin HC, Shim JH. 2015. Dynamic behaviour and residual pattern of thiamethoxam and its metabolite clothianidin in Swiss chard using liquid chromatography-tandem mass spectrometry. Food Chem 174:248–255.

Rawlins E, Lederhouse C. 1981. Developmental influences of thermal behavior on monarch caterpillars (Danaus plexippus): An adaptation for migration (Lepidoptera: Nymphalidae: Danainae). J Kans Entomol Soc 54:387–408.

Salvato, M. 2001. Influence of mosquito control chemicals on butterflies (Nymphalidae, Lycaenidae, Hesperiidae) of the lower Florida Keys. J Lepid Soc 55: 8–14.

Szpyrka E, Matyaszek A, Słowik-Borowiec M. 2017. Dissipation of chlorantraniliprole, chlorpyrifos-methyl and indoxacarb—Insecticides used to control codling moth (Cydia pomonella L.) and leafrollers (Tortricidae) in apples for production of baby food. Environ Sci Pollut Res Int 24:12128–12135.

Thogmartin WE, López-Hoffman L, Rohweder J, Diffendorfer J, Drum R, Semmens D, Black S, Caldwell I, Cotter D, Drobney P, Jackson LL, Gale M, Helmers D, Hilburger S, Howard E, Oberhauser K, Pleasants J, Semmens B, Taylor O, Ward P, Weltzin JF, Widerholt R. 2017. Restoring monarch butterfly habitat in the Midwestern US: “All hands on deck.” Environ Res Lett 12:7.

Thompson H. 2015. Extrapolation of acute toxicity across bee species. Integr Environ Assess Manag 12:622–626.

Tomlin CDS. 1994. The Pesticide Manual: Incorporating the Agrochemicals Handbook. British Crop Protection Council, The Royal Society of Chemistry, Cambridge.

Tooker JF, Douglas MR, Krupke CH. 2017. Neonicotinoid seed treatments: limitations and compatibility with integrated pest management. Agric Environ Lett 2 (1): ael2017.08.0026.

US Department of Agriculture. 2018. Quick stats. Washington, DC: National Agricultural Statistics Service. [cited 2020 October 18]. Available from: https://quickstats.nass.usda.gov/#59CB4138-B1CE-31CD-B29D-A71A0DF9BBE6

US Department of Agriculture. 2019. Iowa ag news—2018 crop production. Washington, DC: National Agricultural Statistics Service. Washington, DC. [cited 2020 October 18]. Available from: https://www.google.com/url?sa=t&rct=j&q=&esrc=s&source=web&cd=&ved=2ahUKEwigqJ3eoP7sAhUDVs0KHeQBA8MQFjABegQIAhAC&url=https%3A%2F%2Fwww.nass.usda.gov%2FStatistics_by_State%2FIowa%2FPublications%2FCrop_Report%2F2019%2FIA-Crop-Production-Annual-01-19.pdf&usg=AOvVaw2FOCVLX6r1Db_dNZhDqQki

US Environmental Protection Agency. 2003. AgDRIFT User Manual. Washington, DC. [cited 2020 October 18]. Available from: https://www.epa.gov/pesticide-science-and-assessing-pesticide-risks/models-pesticide-risk-assessment#AgDrift

US Environmental Protection Agency. 2008. Pesticides - Fact Sheet for Chlorantraniliprole. Washington, DC. [cited 2020 October 18]. Available from: https://www3.epa.gov/pesticides/chem_search/reg_actions/registration/fs_PC-090100_01-Apr-08.pdf

US Environmental Protection Agency. 2011a. AgDRIFT model. Washington, DC. [cited 2020 October 18]. Available from: https://www.epa.gov/pesticide-science-and-assessing-pesticide-risks/models-pesticide-risk-assessment#AgDrift

US Environmental Protection Agency. 2011b. Pesticide Registration - DuPont Dermacor. [cited 2020 October 18]. Available from: https://www3.epa.gov/pesticides/chem_search/ppls/000352-00841-20110921.pdf

US Environmental Protection Agency. 2020. Pesticide Registration - Lumivia. [cited 2020 October 18]. Available from: https://www3.epa.gov/pesticides/chem_search/ppls/000352-00841-20200624.pdf

US Geological Survey. 2020. Estimated Annual Agricultural Pesticide Use Maps - Chlorantraniliprole [cited 2020 October 18]. Available from: https://water.usgs.gov/nawqa/pnsp/usage/maps/show_map.php?year=2017&map=CHLORANTRANILIPROLE&hilo=L&disp=Chlorantraniliprole

US Fish and Wildlife Service. 2015. Save the monarch butterfly. [cited 2020 October 17]. Available from: https://www.fws.gov/savethemonarch/

US Fish and Wildlife Service. 2017. Monarch butterfly species assessment update. [cited 2020 February 10]. Available from: https://www.fws.gov/savethemonarch/pdfs/Monarch.pdf

Wade A, Lin CH, Kurkul C, Regan ER, Johnson RM. 2019. Combined toxicity of insecticides and fungicides applied to California almond orchards to honey bee larvae and adults. Insects 10:E20.

Whalen RA, Herbert DA, Malone S, Kuhar TP, Brewster CC, Reisig DD. 2016. Effects of diamide insecticides on predators in soybean. J Econ Entomol 109:2014–2019.

Zalucki MP. 1982. Temperature and rate of development in Danaus Plexippus L. and D. Chrysippus L. (Lepidoptera:Nymphalidae). Aust J Entomol 21 (4): 241–46.

Zalucki MP, Parry HR, Zalucki JM. 2016. Movement and egg laying in monarchs: To move or not to move, that is the equation. Austral Ecol 41 (2): 154–67.

Tables and Figures

Table 1. Chronic dietary toxicity of five insecticides to monarch larvae following exposure to treated tropical milkweed leaves[a]

Insecticide	Conc. unit	LC values and 95% CIs		
		LC_{10}	LC_{50}	LC_{90}
CFS	µg/g leaf[b]	3.7 (0.76 − 18)	5.3 (3.9 − 7.0)	7.5 (0.93 − 60)
	µg/cm^2 leaf[c]	9.6×10^{-2} (2.0×10^{-2} − 0.47)	0.14 (0.10 − 0.18)	0.19 (2.4×10^{-2} − 1.6)
CTR	µg/g leaf[b]	3.8×10^{-4} (1.2×10^{-4} − 1.2×10^{-3})	1.6×10^{-3} (8.8×10^{-4} − 2.9×10^{-3})	6.8×10^{-3} (3.3×10^{-3} − 1.4×10^{-2})
	µg/cm^2 leaf[c]	9.8×10^{-6} (3.0×10^{-6} − 3.2×10^{-5})	4.2×10^{-5} (2.3×10^{-5} − 7.6×10^{-5})	1.8×10^{-4} (8.5×10^{-5} − 3.7×10^{-4})
IMI	µg/g leaf[b]	3.6×10^{-2} (1.2×10^{-2} − 0.11)	0.13 (6.3×10^{-2} − 0.25)	0.44 (0.20 − 0.98)
	µg/cm^2 leaf[c]	9.4×10^{-4} (3.1×10^{-4} − 2.9×10^{-3})	3.3×10^{-3} (1.6×10^{-3} − 6.6×10^{-3})	1.2×10^{-2} (5.2×10^{-3} − 2.6×10^{-2})
TMX	µg/g leaf[b]	0.42 (0.21 − 0.83)	0.94 (0.61 − 1.5)	2.1 (1.3 − 3.4)
	µg/cm^2 leaf[c]	1.1 x 10^{-2} (5.5×10^{-3} − 2.2×10^{-2})	2.4×10^{-2} (1.6×10^{-2} − 3.8×10^{-2})	5.5×10^{-2} (3.4×10^{-2} − 8.8×10^{-2})
CDN	µg/g leaf[b]	4.6×10^{-2} (2.7×10^{-2} − 7.8×10^{-2})	7.4×10^{-2} (1.9×10^{-2} − 0.29)	0.12 (6.0×10^{-3} − 2.3)
	µg/cm^2 leaf[c]	1.2×10^{-3} (7.0×10^{-4} − 2.0×10^{-3})	1.9×10^{-3} (4.8×10^{-4} − 7.6×10^{-3})	3.1×10^{-3} (1.6×10^{-4} − 6.1×10^{-2})

[a] Based on mortality data obtained from treating 20-40 larvae at each insecticide concentration. Larvae were fed leaf tissue treated with 0.1% silwet: water/DMF suspensions (control) or one of five insecticides in 0.1% silwet : water/DMF suspensions.

[b] The µg of insecticide per g leaf tissue were calculated by dividing the nominal insecticide amount pipetted on each leaf by the approximate average weights of the leaf.

[c] Derived from Table S7.

CIs: confidence intervals; LC_{10}: lethal concentration that kills 10% of a treated population; LC_{50}: lethal concentration that kills 50% of a treated population; LC_{90}: lethal concentration that kills 90% of a treated population.

CTR: chlorantraniliprole; CFS: chlorpyrifos; IMI: imidacloprid; TMX: thiamethoxam; CDN: clothianidin; Conc: concentration

Table 2. Acute toxicity of six insecticides to monarch eggs following topical exposure[a]

Insecticide	Conc. unit	LD values and 95% CIs		
		LD_{10}	LD_{50}	LD_{90}
BCF	µg/egg[b]	1.4×10^{-8} (1.2×10^{-10} - 1.7×10^{-6})	3.2×10^{-6} (2.6×10^{-7} - 4.0×10^{-5})	7.4×10^{-4} (4.1×10^{-5} - 1.4×10^{-2})
	µg/g egg[c]	3.2×10^{-5} (2.6×10^{-7} - 3.8×10^{-3})	7.3×10^{-3} (5.9×10^{-4} - 9.0×10^{-2})	1.7 (9.3×10^{-2} - 31)
	µg/cm^2 egg[d]	2.8×10^{-8} (2.3×10^{-10} - 3.4×10^{-6})	6.4×10^{-6} (5.2×10^{-7} - 8.0×10^{-5})	1.5×10^{-3} (8.2×10^{-5} - 2.7×10^{-2})
CFS	µg/egg[b]	1.1 (2.0×10^{-2} - 63)	1.6 (0.31 - 8.1)	2.2 (1.0 - 5.0)
	µg/g egg[c]	2600 (46 - 140000)	3600 (700 - 19000)	5100 (2300 - 11000)
	µg/cm^2 egg[d]	2.3 (4.0×10^{-2} - 130)	3.2 (0.62 - 16)	4.5 (2.0 - 10)
CTR	µg/egg[b]	1.1×10^{-7} (6.8×10^{-10} - 1.9×10^{-5})	8.0×10^{-6} (7.2×10^{-7} - 8.8×10^{-5})	5.6×10^{-4} (4.2×10^{-5} - 7.5×10^{-3})
	µg/g egg[c]	2.6×10^{-4} (1.5×10^{-6} - 4.3×10^{-2})	1.8×10^{-2} (1.6×10^{-3} - 0.20)	1.3 (9.5×10^{-2} - 17)
	µg/cm^2 egg[d]	2.3×10^{-7} (1.4×10^{-9} - 3.8×10^{-5})	1.6×10^{-5} (1.4×10^{-6} - 1.8×10^{-4})	1.1×10^{-3} (8.3×10^{-5} - 1.5×10^{-2})
IMI	µg/egg[b]	1.5×10^{-4} (3.0×10^{-5} - 7.1×10^{-4})	1.3×10^{-3} (5.2×10^{-4} - 3.0×10^{-3})	1.1×10^{-2} (3.0×10^{-3} - 3.8×10^{-2})
	µg/g egg[c]	0.33 (6.8×10^{-2} - 1.6)	2.9 (1.2 - 6.8)	25 (6.9 - 87)
	µg/cm^2 egg[d]	2.9×10^{-4} (6.0×10^{-5} - 1.4×10^{-3})	2.5×10^{-3} (1.0×10^{-3} - 6.0×10^{-3})	2.2×10^{-2} (6.1×10^{-3} - 7.7×10^{-2})
TMX	µg/egg[b]	2.7×10^{-3} (2.1×10^{-4} - 3.5×10^{-2})	3.8×10^{-2} (1.2×10^{-2} - 0.12)	0.54 (0.12 - 2.4)
	µg/g egg[c]	6.2 (0.48 - 79)	87 (27 - 280)	1200 (280 - 5400)
	µg/cm^2 egg[d]	5.4×10^{-3} (4.3×10^{-4} - 7.0×10^{-2})	7.7×10^{-2} (2.4×10^{-2} - 0.25)	1.1 (0.25 - 4.8)
CDN	µg/egg[b]	1.7×10^{-6} (7.6×10^{-10} - 3.8×10^{-3})	5.4×10^{-4} (2.2×10^{-5} - 1.3×10^{-2})	0.17 (7.1×10^{-3} - 4.0)
	µg/g egg[c]	3.9×10^{-3} (1.7×10^{-6} - 8.6)	1.2 (5.0×10^{-2} - 29)	380 (16 - 9100)
	µg/cm^2 egg[d]	3.4×10^{-6} (1.5×10^{-9} - 7.6×10^{-3})	1.1×10^{-3} (4.4×10^{-5} - 2.6×10^{-2})	0.34 (1.4×10^{-2} - 8.0)

[a] Based on mortality data obtained from treating 20 eggs at each insecticide concentration. Eggs were topically treated with 0.2 µL volume of acetone (controls) and insecticide-acetone solutions.

[b] Calculated by multiplying the measured insecticide concentration with the volume of insecticide solution applied on each egg.

[c] Calculated by dividing the µg/egg with the average weight of an egg, which was 0.44 ± 0.02 mg or 4.4×10^{-4} g (n = 32).

[d] Calculated by dividing the µg/egg with the average surface area of an egg, which was 0.5 ± 0.1 cm^2 (n = 10).

CIs: confidence intervals; LD_{10}: lethal dose that kills 10% of a treated population; LD_{50}: lethal dose that kills 50% of a treated population; LD_{90}: lethal dose that kills 90% of a treated population; Conc: concentration

BCF: beta-cyfluthrin; CTR: chlorantraniliprole; CFS: chlorpyrifos; IMI: imidacloprid; TMX: thiamethoxam; CDN: clothianidin

Table 3. Percent eclosion of monarch pupae following topical exposure to six insecticides[a]

Mean (± SD) pupal weight in g	Insecticide	Dose (µg/pupa)[b]	Dose (µg/g pupa)[c]	Dose (µg/cm^2 pupa)[d]	N[e]	Percent adult eclosion	Mean (± SD) pupal duration in days[f]	Sex ratio (F/M)	Mean (± SD) adult weight in g[g]
1.23 (± 0.14)	Control	0	0	0	42	100	11.3 (± 0.7)	1.6	0.48 (± 0.10)
1.17 (± 0.15)	CFS	56	48	8.9	20	100	11.7 (± 0.7)	1.2	0.50 (± 0.08)
1.18 (± 0.15)	IMI	14	12	2.2	20	100	11.5 (± 0.7)	0.82	0.46 (± 0.09)
1.20 (± 0.21)	TMX	24	20	3.8	20	100	11.1 (± 1.0)	1.2	0.45 (± 0.17)
1.19 (± 0.16)	CDN	7.9	6.6	1.3	20	100	11.5 (± 0.8)	1.2	0.46 (± 0.09)
1.22 (± 0.22)	Control	0	0	0	10	100	12.5 (± 0.7)	4.0	0.48 (± 0.09)
1.16 (± 0.20)	BCF (A)	0.93	0.80	0.15	22	0	NA	NA	NA
1.11 (± 0.15)	BCF (C)	9.3×10^{-2}	8.3×10^{-2}	1.5×10^{-2}	10	40	12.5 (± 0.6)	3.0	0.49 (± 0.07)
1.19 (± 0.15)	BCF (C)	9.3×10^{-3}	7.8×10^{-3}	1.5×10^{-3}	10	100	12.4 (± 0.7)	2.3	0.47 (± 0.11)
1.19 (± 0.09)	BCF (C)	9.3×10^{-4}	7.8×10^{-4}	1.5×10^{-4}	10	100	12.0 (± 0.7)	2.3	0.46 (± 0.04)
1.20 (± 0.17)	CTR (A)	0.47	0.39	7.5×10^{-2}	21	0	NA	NA	NA
1.17 (± 0.20)	CTR (C)	4.7×10^{-2}	4.0×10^{-2}	7.5×10^{-3}	10	0	NA	NA	NA
1.13 (± 0.13)	CTR (C)	4.7×10^{-3}	4.2×10^{-3}	7.5×10^{-4}	10	30	12.7 (± 0.6)	0.5	0.47 (± 0.12)
1.17 (± 0.17)	CTR (C)	4.7×10^{-4}	4.0×10^{-4}	7.5×10^{-5}	10	100	11.8 (± 0.6)	1.0	0.46 (± 0.08)

[a] Pupae were topically treated with 1 µL volume of acetone or insecticide-acetone solutions on the spiracles either at 24- or 48-hours following pupation.

[b] Calculated by multiplying the measured insecticide concentration with the volume of insecticide solution applied on each pupa.

[c] Calculated by dividing the µg/pupa with the corresponding mean weight of the treated pupae (see first column).

[d] Calculated by dividing the µg/pupa with the average surface area of a pupa which was 6.3 ± 0.9 cm^2 (n = 5).

[e] The number of pupae treated at each insecticide concentration.

[f] The mean number of days from pupation to adult emergence.

[g] The mean weights of the adult butterflies that emerged following treatment.

BCF: beta-cyfluthrin; CTR: chlorantraniliprole; CFS: chlorpyrifos; IMI: imidacloprid; TMX: thiamethoxam; CDN: clothianidin
SD: standard deviation; F: female; M: male

Table 4. Percent mortality of monarch adults following topical exposure to six insecticides[a]

Sex ratio[b]	Mean (± SD) weight in g	Insecticide	Dose (µg/adult)[c]	Dose (µg/g adult)[d]	Dose (µg/cm^2 adult)[e]	% mortality	Adjusted overall % mortality[f]
12 : 9	0.50 (± 0.14)	Control-A	0	0	0	19	0
9 : 11	0.45 (± 0.10)	Control-D	0	0	0	5	0
12 : 9	0.53 (± 0.15)	BCF	3.7	6.9	0.11	100	100
12 : 8	0.30 (± 0.07)	BCF	0.37	1.2	1.1×10^{-2}	100	100
11 : 9	0.47 (± 0.09)	BCF	3.7×10^{-2}	8.0×10^{-2}	1.1×10^{-3}	15	1
10 : 10	0.48 (± 0.10)	CTR	21	44	0.64	60[g]	58
10 : 10	0.50 (± 0.09)	CTR	1.9	3.8	5.8×10^{-2}	15	0
9 : 11	0.50 (± 0.17)	CFS	224	452	6.8	100	100
11 : 9	0.37 (± 0.09)	CFS	32	86	0.97	100	100
8 : 12	0.42 (± 0.09)	CFS	3.2	7.7	9.7×10^{-2}	15	6
9 : 11	0.40 (± 0.07)	IMI	42	104	1.3	60[h]	58
9 : 11	0.54 (± 0.15)	IMI	28	52	0.85	40[h]	26
11 : 9	0.40 (± 0.06)	IMI	2.8	7.0	8.5×10^{-2}	0	0
9 : 11	0.50 (± 0.07)	TMX	16	32	0.48	5	0
10 : 10	0.39 (± 0.10)	CDN	32	83	0.97	0	0

[a] Adults were topically treated on their wings with a 4 µL volume of acetone or DMF (controls) and insecticide-acetone or insecticide-DMF solutions one to five days following eclosion.

[b] The ratio of number of females : males treated at each concentration.

[c] Calculated by multiplying the measured insecticide concentration with the volume of insecticide solution applied on each adult wing.

[d] Calculated by dividing the µg/adult with the corresponding mean weight of the treated adults (see second column).

[e] Calculated by dividing the µg/adult with the average surface area of an adult which was 33 ± 5 cm^2 (n = 9).

[f] The adult percentage mortality for each insecticide concentration was adjusted for control mortality from the same bioassay runs using Abbott's formula.

[g] Female butterflies had three times the mortality of male butterflies (90 vs. 30%).

[h] Female butterflies had approximately twice the mortality of male butterflies (78 vs. 45% and 56 vs. 27% for 104 and 52 µg/g dose, respectively).

Control-A: Acetone treatment; Control-D: Dimethylformamide treatment; F: female; M: male; SD: standard deviation

BCF: beta-cyfluthrin; CTR: chlorantraniliprole; CFS: chlorpyrifos; IMI: imidacloprid; TMX: thiamethoxam; CDN: clothianidin

Table 5. Percent mortality of monarch adults following dietary exposure to three neonicotinoid insecticides[a]

Sex ratio[b]	Mean (± SD) weight in mg	Concentration (µg/g solution)[c]	Insecticide	Dose (µg/adult)[d]	Dose (µg/g adult)[e]	Overall % mortality
12 : 10	0.46 (± 0.11)	0	Control	0	0	18
10 : 10	0.46 (± 0.07)	0.25	IMI	1.3×10^{-2}	2.8×10^{-2}	5
9 : 11	0.48 (± 0.09)	0.33	TMX	1.7×10^{-2}	3.6×10^{-2}	0
9 : 11	0.46 (± 0.08)	0.14	CDN	7.0×10^{-3}	1.5×10^{-2}	0

[a] Adults were fed 50 µL of acetone-Gatorade solution (controls) or insecticide acetone-Gatorade solution. The age of the treated adults ranged from four to nine days.
[b] The ratio of females: males treated at each concentration.
[c] The µg/µL neonicotinoid concentration in Table S5 was converted to µg/g concentration.
[d] Calculated by multiplying the measured insecticide concentration with the volume of insecticide solution fed to each adult.
[e] Calculated by dividing the µg/adult with the corresponding mean weight of the treated adults (see second column).
IMI: imidacloprid; TMX: thiamethoxam; CDN: clothianidin
F: female; M: male; SD: standard deviation

Table 6. Estimated risk to monarch larvae and adults following dietary exposure to neonicotinoid residues in milkweed leaves and wildflower nectar, respectively, sampled from plants in close proximity to maize and soybean fields planted with neonicotinoid treated seeds

Monarch stage	Insecticide	Residue data in published literature				
		Tissue sampled	Mean conc (µg/g)	Percent mortality[a]	Highest conc (µg/g)	Percent mortality[a]
Larva	IMI	Milkweed[b]	3.0×10^{-4}	0	2.8×10^{-3}	0
		Milkweed[c]	1.0×10^{-5}	0	3.7×10^{-3}	0
	TMX	Milkweed[b]	1.6×10^{-3}	0	1.3×10^{-2}	0
		Milkweed[c]	1.9×10^{-3}	0	0.15	1
	CDN	Milkweed[b]	4.1×10^{-4}	0	6.6×10^{-3}	0
		Milkweed[c]	7.1×10^{-4}	0	5.7×10^{-2}	23
Adult	IMI	Nectar[d]	NA	NA	1.7×10^{-4}	0
	TMX	Nectar[d]	1.0×10^{-4}	0	1.8×10^{-3}	0
	CDN	Nectar[d]	NA	NA	5.0×10^{-4}	0

[a] Larval percent mortality was derived from the concentration-response curves in µg/g leaf (Figure 3) and the adult percent mortality

was obtained by comparing it with the toxicity concentration in µg/g solution (Table 5).
[b] Milkweed residues obtained from Hall et al. (2020); milkweed plants were sampled downslope in maize and soybean fields planted with treated seeds. The largest mean and highest concentration detected over two years were selected.
[c] Milkweed residue data obtained from Olaya-Arenas and Kaplan (2019); milkweed plants were sampled between 0 to > 2 km (most within a 100 m) from maize and soybean fields. The largest mean and highest concentration detected over two years were selected.
[d] Nectar residue data obtained from Botias et al. (2015); nectar was sampled from wildflowers at the edge of oilseed rape and wheat fields planted with treated seeds. The largest mean and highest concentration were selected.

Table S1. Acute risks to monarch larvae following cuticular and dietary exposure to clothianidin foliar application[a]

Instar	Distance away from field (m)	% mortality from acute cuticular exposure		% mortality from acute dietary exposure	
		Aerial	High ground boom[b]	Aerial	High ground boom[b]
First (cuticular)/ Second (dietary)	0	100	100	100	100
	15	99	76	53	0
	30	98	58	16	0
	60	93	36	1	0
Third	0	97	98	94	99
	15	81	25	7	0
	30	72	14	2	0
	60	51	8	0	0
Fifth	0	93	98	90	93
	15	22	0	73	37
	30	10	0	68	28
	60	2	0	54	20

[a] Risks were estimated by comparing clothianidin acute cuticular and dietary dose and concentration-response curves (see Figures S3 and S5 in Krishnan et al. 2020) with spray drift exposure values obtained from AgDRIFT for the formulated product Belay, which contains clothianidin as an active ingredient (see Table S6). Maximum label rates to manage soybean aphids via aerial and high ground boom applications were used to obtain these exposure values.
[b] 50th-percentile results from AgDRIFT were used.

Table S2. Differences in insecticide susceptibilities between two monarch butterfly colonies following acute dietary exposure to five insecticides

Insecticide	Instar	Measured conc[n] (µg/g leaf)[a]	Kansas colony: 96-h percent mortality[b]	Iowa colony: 96-h LC values with 95% CI (µg/g leaf)[c]	Colony comparison[d]
Control	Second	0	0	NA	NA
Control	Third	0	5	NA	NA
CFS	Third	5.5	5	LC_{10}: 0.31 (4.4×10^{-2} – 1.6)	Iowa colony ~ 5X
		28	50	LC_{50}: 6.0 (2.7 – 14)	more sensitive
		56	100	LC_{90}: 120 (40 – 630)	
CTR	Third	3.6×10^{-3}	0	LC_{10}: 6.0×10^{-4} (6.8×10^{-5} – 2.9×10^{-3})	Kansas colony ~ 3X
		1.8×10^{-2}	50	LC_{50}: 4.6×10^{-2} (1.8×10^{-2} – 0.11)	more sensitive
		3.5×10^{-2}	90	LC_{90}: 3.6 (1.1 – 21)	
IMI	Second	4.4	15	LC_{10}: 1.4 (0.57 – 2.1)	Iowa colony ~ 5X
		24	70	LC_{50}: 5.1 (3.3 – 6.8)	more sensitive
		51	85	LC_{90}: 19 (7.5 – 30)	
TMX	Third	3.1	10	LC_{10}: 1.1 (0.48 – 2.1)	Iowa colony ~ 3X
		15	55	LC_{50}: 5.6 (3.7 – 8.9)	more sensitive
		30	90	LC_{90}: 29 (15 – 69)	
CDN	Second	0.48	10	LC_{10}: 2.4 (1.3 – 3.4)	Kansas colony ~ 2X
		2.3	50	LC_{50}: 4.2 (3.4 – 5.0)	more sensitive
		4.6	70	LC_{90}: 7.5 (5.2 – 9.7)	

[a] Calculated by multiplying the measured insecticide stock solution concentrations (data in raw files on GitHub) with the volume of insecticide applied and divided by the average weight of leaves provided to larvae.

[b] Larval percent mortality obtained from conducting acute dietary toxicity studies (methodology described in Krishnan et al. 2020) with monarch larvae acquired from Kansas. Three concentrations were employed for each insecticide and 20 larvae were treated per concentration. Larvae were provided treated leaves for 48 h followed by untreated leaves for another 48 h.

[c] LC values obtained from conducting acute dietary toxicity studies (results described in Krishnan et al. 2020) with monarch larvae acquired from Iowa.

[d] Comparison made by dividing the LC50 value obtained from the Iowa colony with the concentration causing approximately 50% mortality in larvae obtained from the Kansas colony.

CTR: chlorantraniliprole; CFS: chlorpyrifos; IMI: imidacloprid; TMX: thiamethoxam; CDN: clothianidin

SUMMARY: The Kansas larvae were overall less susceptible as they recovered more effectively from concentrations that caused approximately 50% to 90% mortality in the Iowa colony. However, these larvae were often very small and did not advance to the next

instar in the 96-h study duration. Chronic exposure to these concentrations would have likely resulted in high mortality.

Table S3. Ratio of volume of insecticide suspensions applied to mass of tropical milkweed leaves provided to monarch larvae in chronic dietary bioassays

Average (and range) of leaf mass (g) provided to a larva	Volume of insecticide suspension (µL) applied on leaf surfaces	Volume to mass ratio (µL/g)	Larval instars typically fed	Approx. # of times provided to a larva
0.100 (0.075 – 0.125)	5	50 (67 – 40)	Second	1
0.500 (0.450 – 0.550)	25	50 (56 – 45)	Third	1
1.000 (0.900 – 1.100)	50	50 (56 – 45)	Fourth	1
2.000 (1.800 – 2.200)	100	50 (56 – 45)	Fifth	1
3.000 (2.700 – 3.300)	150	50 (56 – 45)	Fifth	2

RESIDUE ANALYSES

Leaf samples were prepared using a previously published method (Hall et al. 2020; see references at the end of supplementary section). All leaf samples were flash frozen with liquid nitrogen and homogenized using a mortar and pestle. A 0.2 g portion of leaf was extracted with acetonitrile. Approximately 1 mL of the extract was transferred to a dispersive solid phase extraction (dSPE) tube containing 150 mg $MgSO_4$, 50 mg PSA, and 50 mg C18. Extracts were diluted with 50:50 methanol : water and internal standards were added prior to LC-MS analysis. An injection volume of 2 µL was used for all samples. Extracts were analyzed on a Vanquish Flex LC pump interfaced with a TSQ Altis triple quadrupole mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA). Instrument details are provided in Hall et al. (2020). Two calibration curves were prepared with untreated tropical milkweed leaves. Samples containing higher concentrations of insecticides were analyzed with a standard curve that ranged from 0.05 to 10 µg/g (Curve 1). Samples containing lower concentrations of insecticides were analyzed with a curve that ranged from 1 to 50 ng/g (Curve 2). Recovery rates of the leaf extractions were 91.5, 97.5, and 98.5%, for chlorantraniliprole; imidacloprid and clothianidin; and thiamethoxam and chlorpyrifos, respectively. Quality control (QC) samples were prepared in triplicate at either 4 µg/g (with Curve 1) or 30 ng/g (with Curve 2) and analyzed with study samples. All QC samples had a calculated concentration within 20% of the nominal value for all insecticides.

For stock solutions (including Gatorade solutions), the calibration curves and dilutions were made in 50 : 50 methanol : water, except for beta-cyfluthrin solutions for which ethyl acetate was used instead of methanol. All insecticide solutions, except for beta-cyfluthrin solutions, were analyzed similar to leaves. Beta-cyfluthrin was analyzed using GC-ECD. Instrument details and methods are provided in Krishnan et al. (2020). Measured concentrations of leaves and stock solutions can be found in Tables S4 and S5,

respectively. With the leaf samples, the 48-h concentrations often exceeded the 0-h concentrations as the leaves had dried up over time.

Table S4. Measured insecticide concentrations of tropical milkweed leaves provided to larvae in chronic dietary bioassays[a]

Insecticide	Nominal concentration (µg/g)[b]	Mean (± SD) measured leaf concentration (µg/g leaf)[c]		Ratio of concentrations (Day 2/Day 0)
		Day 0	Day 2	
CFS	5×10^{-2}	2.3×10^{-2} ($\pm 9 \times 10^{-3}$)	5.4×10^{-3} ($\pm 7 \times 10^{-4}$)	0.235
	0.5	0.379 (± 0.146)	0.205 ($\pm 7.3 \times 10^{-2}$)	0.541
	5	3.988 (± 0.385)	2.695 (± 1.337)	0.676
	25	21.113 (± 4.753)	28.893 (± 6.957)	1.37
CTR	5×10^{-6}	6.5×10^{-6}, [d]	1.2×10^{-5}, [d]	
	5×10^{-5}	6.5×10^{-5}, [d]	1.2×10^{-4}, [d]	
	5×10^{-4}	6.5×10^{-4}, [d]	1.2×10^{-3} ($\pm 3 \times 10^{-4}$)	1.85
	5×10^{-3}	6.5×10^{-3} ($\pm 2 \times 10^{-4}$)	1.1×10^{-2} ($\pm 3.3 \times 10^{-3}$)	1.69
IMI	5×10^{-3}	6.7×10^{-3} ($\pm 1.2 \times 10^{-3}$)	1.2×10^{-2} ($\pm 3 \times 10^{-3}$)	1.79
	5×10^{-2}	5.7×10^{-2} ($\pm 2.8 \times 10^{-3}$)	0.110 ($\pm 4.5 \times 10^{-2}$)	1.93
	0.5	0.478 (± 0.128)	0.989 (± 0.267)	2.07
TMX	5×10^{-3}	4.3×10^{-3} ($\pm 3 \times 10^{-4}$)	4.2×10^{-3} ($\pm 1.3 \times 10^{-3}$)	0.977
			CDN: 2.7×10^{-3} ($\pm 2 \times 10^{-4}$)	
	5×10^{-2}	5.2×10^{-2} ($\pm 1.4 \times 10^{-3}$)	4.8×10^{-2} ($\pm 1.8 \times 10^{-2}$)	0.923
			CDN: 3.0×10^{-2} ($\pm 6.2 \times 1^{-3}$)[e]	
	0.5	0.462 ($\pm 3.9 \times 10^{-2}$)	0.670 (± 0.183)	1.45
			CDN: 0.103 ($\pm 4.2 \times 10^{-2}$)	
	2.5	2.016 (± 0.490)	4.223 (± 1.360)	2.09
			CDN: 0.444 ($\pm 5.7 \times 10^{-2}$)	
	5	4.985 (± 1.620)	5.916 (± 0.886)	1.19
			CDN: 0.607 (± 0.178)	
CDN	5×10^{-4}	4.4×10^{-4}, [d]	8.6×10^{-4}, [d]	
	5×10^{-3}	4.4×10^{-3} ($\pm 8 \times 10^{-4}$)	8.6×10^{-3} ($\pm 3.3 \times 10^{-3}$)	1.95
	5×10^{-2}	5.1×10^{-2} ($\pm 5 \times 10^{-3}$)	6.6×10^{-2} ($\pm 1.4 \times 10^{-2}$)	1.29
	0.5	0.395 (± 0.156)	0.694 (± 0.267)	1.76

[a] LC-MS/MS was used to quantify all insecticide samples.

[b] Calculated by multiplying the nominal insecticide concentration (µg/µL) with the µL of insecticide solution applied and divided by the approximate weight in g of leaf used.
[c] Three replicates per leaf sample were analyzed.
[d] Below limits of quantification; estimated concentration based on serial dilution of the preceding concentration.
[e] Based on quantifiable concentrations in two leaf samples.
CTR: chlorantraniliprole; CFS: chlorpyrifos; IMI: imidacloprid; TMX: thiamethoxam; CDN: clothianidin

Table S5. Measured concentrations of insecticide stock solutions used in acute topical egg, pupa, and adult toxicity bioassays, and acute adult dietary toxicity bioassays[a]

Insecticide	Nominal concentration (µg/µL)	Solvent used	Monarch stage treated	Exposure route	Measured concentration (µg/µL)
BCF	1.0	Acetone	Egg, Pupa, Adult	Topical	0.93
CTR	1.0	Acetone	Egg, Pupa, Adult	Topical	0.47
CTR	6.0	DMF	Adult	Topical	5.3
CFS	70	Acetone	Egg, Pupa, Adult	Topical	56
IMI	2.0×10^{-4}	1:4 Acetone: Gatorade	Adult	Dietary	2.5×10^{-4}
IMI	20	Acetone	Egg, Pupa, Adult	Topical	14
TMX	2.0×10^{-4}	1:4 Acetone: Gatorade	Adult	Dietary	3.3×10^{-4}
TMX	5.0	Acetone	Egg, Pupa, Adult	Topical	4.1
TMX	40	Acetone	Pupa	Topical	24
CDN	2.0×10^{-4}	1:4 Acetone: Gatorade	Adult	Dietary	1.4×10^{-4}
CDN	10	Acetone	Egg, Pupa, Adult	Topical	7.9

[a] LC-MS/MS was used to quantify all insecticides except BCF. BCF was quantified using GC-ECD.
BCF: beta-cyfluthrin; CTR: chlorantraniliprole; CFS: chlorpyrifos; IMI: imidacloprid; TMX: thiamethoxam; CDN: clothianidin

Table S6. Estimated environmental deposition doses of six active ingredient insecticides on monarch pupae and adults, based on representative foliar formulations registered for managing true armyworms (ta) and soybean aphids (sa)[a]

Formulated product [active ingredient; application rate (g AI/ha)]	Pest[b]	Monarch stage	Downwind insecticide doses on monarch (µg/stage) following aerial application[c]		Downwind insecticide doses on monarch (µg/stage) following high or low ground boom application[d]	
			Highest exposure (0 m)	Lowest exposure (60 m)	Highest exposure (0 m)	Lowest exposure (60 m)
Baythroid® XL	sa, ta	Pupa	0.63	3.9×10^{-2}	1.3	2.1×10^{-3}
		Adult	3.3	0.20	6.9	1.1×10^{-2}
Beseige®	sa	Pupa	1.9	9.2×10^{-2}	3.8	8.7×10^{-3}
(CTR; 58.5)		Adult	9.9	0.48	20	4.6×10^{-2}
Beseige®	ta[e]	Pupa	NA	NA	4.4	6.3×10^{-3}
(CTR; 73.1)		Adult	NA	NA	23	3.3×10^{-2}
Lorsban® (CFS;	sa, ta	Pupa	35	1.9	71	9.6×10^{-2}
1121)		Adult	185	9.9	373	0.50
Admire Pro®	sa	Pupa	1.9	8.3×10^{-2}	3.2	7.8×10^{-3}
(IMI; 52.3)		Adult	9.9	0.43	17	4.1×10^{-2}
Swagger® (IMI;	ta[e]	Pupa	NA	NA	6.9	9.6×10^{-3}
112)		Adult	NA	NA	36	5.0×10^{-2}
Endigo® (TMX;	sa	Pupa	1.3	6.5×10^{-2}	2.5	6.2×10^{-3}
41.4)[f]		Adult	6.6	0.34	13	3.2×10^{-2}
Belay®	sa	Pupa	3.8	0.18	6.9	1.7×10^{-2}
(CDN; 112)[f]		Adult	20	0.92	36	8.7×10^{-2}

[a] Environmental insecticide doses deposited on downwind monarch pupae and adults following an aerial, high, or low ground boom application to manage soybean aphids and true armyworms. Spray drift concentrations were estimated using AgDRIFT model version 2.1.1 (U.S. Environmental Protection Agency 2011a) by choosing representative insecticide labels. Clothianidin (Belay) deposition doses (application rate is 112 g AI/ha) were estimated similar to the other insecticides. For high and low ground boom, 50th-percentile results were used.

[b] Maximum insecticide application label rate to manage soybean aphids and/or true armyworms was chosen to estimate downwind drift concentrations.

[c] Estimated using aerial application deposition doses (µg/cm^2 stage) from Table S5 in Krishnan et al. (2020) and multiplying with the surface area of pupae (6.3 ± 0.9 cm^2) and adults (33 ± 5 cm^2).

[d] Estimated using high and low ground boom deposition doses (µg/ cm^2 stage) from Table S5 in Krishnan et al. (2020) and multiplying with the surface area of pupae (6.3 ± 0.9 cm^2) and adults (33 ± 5 cm^2).
[e] As true armyworm is an early season pest, often only ground boom applications are done to manage the pest.
[f] Thiamethoxam and clothianidin are not registered for use on true armyworms in maize fields.
BCF: beta-cyfluthrin; CTR: chlorantraniliprole; CFS: chlorpyrifos; IMI: imidacloprid; TMX: thiamethoxam; CDN: clothianidin
AI: active ingredient; SD: standard deviation

Table S7. Weights and surface areas of tropical milkweed leaves provided to monarch larvae in acute dietary bioassays[a]

Instar fed	Average leaf weight (g)[b] [A]	Average leaf surface area (cm^2) [B]	Weight by area (g/cm^2) [A/B]
Second	0.104 (n= 1450)	4.3 (n = 1450)	0.024
Third	0.409 (n = 1185)	15 (n = 1185)	0.027
Fifth	1.973 (n = 1183)	75 (n = 1155)	0.026
	(total n = 3818)	(total n = 3790)	Average: 0.026

[a] Data obtained from Krishnan et al. 2020.
[b] Range of leaf mass provided to second, third, and fifth instars were 0.075 to 0.0125, 0.350 to 0.450, and 1.800 to 2.200 g, respectively.

Table S8. Larval percent mortality and pupal arrested ecdysis rates following chronic larval exposure to tropical milkweed leaves treated with five insecticides[a]

Insecticide	Concentration (µg/g)[b]	Estimated concentration ($\mu g/cm^2$)[c]	N[d]	% mortality (n)[e]	Adjusted % mortality[f]	% arrested ecdysis in dead (n)[g]
CFS	0	0	40	13 (5)	0	20 (1)
	5×10^{-2}	1.3×10^{-3}	30	27 (8)	12	0 (0)
	0.5	1.3×10^{-2}	30	20 (6)	4	17 (1)
	5	0.13	30	53 (16)	41	6 (1)
	25	0.65	30	100 (30)	100	0 (0)
CTR	0	0	40	15 (6)	0	0 (0)
	5×10^{-6}	1.3×10^{-7}	30	10 (3)	0	33 (1)
	5×10^{-5}	1.3×10^{-6}	40	23 (9)	9	33 (3)
	5×10^{-4}	1.3×10^{-5}	40	28 (11)	15	18 (2)

Table S8 continued

	5×10^{-3}	1.3×10^{-3}	40	88 (35)	85	0 (0)
IMI	0	0	30	23 (7)	0	14 (1)
	5×10^{-3}	1.3×10^{-4}	30	17 (5)	0	0 (0)
	5×10^{-2}	1.3×10^{-3}	30	33 (10)	13	40 (4)
	0.5	1.3×10^{-2}	30	93 (28)	91	82 (23)
TMX	0	0	40	13 (5)	0	20 (1)
	5×10^{-3}	1.3×10^{-4}	20	15 (3)	3	0 (0)
	5×10^{-2}	1.3×10^{-3}	30	23 (7)	12	14 (1)
	0.5	1.3×10^{-2}	30	30 (9)	19	44 (4)
	2.5	6.5×10^{-2}	30	93 (28)	93	46 (13)
	5	0.13	29	100 (29)	100	28 (8)
CDN	0	0	40	28 (11)	0	18 (2)
	5×10^{-4}	1.3×10^{-5}	30	23 (7)	4	29 (2)
	5×10^{-3}	1.3×10^{-4}	40	38 (15)	14	27 (4)
	5×10^{-2}	1.3×10^{-3}	40	40 (16)	17	25 (4)
	0.5	1.3×10^{-2}	30	100 (30)	100	60 (18)

[a] Based on mortality data obtained from treating 20-40 larvae at each insecticide concentration. Larvae were fed leaf tissue treated with 0.1% silwet: water/DMF suspension (control) or one of five insecticides in 0.1% silwet : water/DMF suspensions.
[b] The µg of insecticide per g leaf tissue were calculated by dividing the nominal insecticide amount pipetted on each leaf by the approximate average weights of the leaf.
[c] Derived from Table S7.
[d] The number of larvae treated at each insecticide concentration (a missing larva in TMX 5 µg/g concentration was excluded from the dataset).
[e] The percent and number of treated larvae that died.
[f] The larval percent mortality for each insecticide concentration was adjusted for control mortality from the same bioassay run(s) using Abbott's formula.
[g] A description of the phenomenon can be found in Krishnan et al. (2020).
Bolded lines indicate concentrations that caused high larval mortality due to arrested ecdysis.
CTR: chlorantraniliprole; CFS: chlorpyrifos; IMI: imidacloprid; TMX: thiamethoxam; CDN: clothianidin

Table S9. Statistical analyses of monarch developmental endpoints following chronic larval exposure to tropical milkweed leaves treated with five insecticides[a]

Insecticide	Conc[n] (µg/g)[b]	Endpoints analyzed[c]			
		Larval duration	Pupal duration	Adult wingspan	Adult weight
CFS		$\chi^2 = 4.62$; df = 3; p = 0.202	$\chi^2 = 4.51$; df = 3; p = 0.212	F = 5.30; df = 3; p = 2.71 x 10^{-3}	F = 9.17; df = 3; p = 3.54 x 10^{-5}
	5 x 10^{-2}			t ratio = -1.47; p = 0.336	t ratio = -3.49; p = 0.0024
	0.5			t ratio = -0.669; p = 0.815	t ratio = -2.55; p = 0.0357
	5			t ratio = -3.91; p = 0.0007	t ratio = -4.80; p = <0.0001
CTR		$\chi^2 = 0.327$; df = 4; p = 0.988	$\chi^2 = 6.19$; df = 4; p = 0.186	F = 2.14; df = 4; p = 0.0808	F = 0.290; df = 4; p = 0.884
	5 x 10^{-6}				
	5 x 10^{-5}				
	5 x 10^{-4}				
	5 x 10^{-3}				
IMI		$\chi^2 = 3.25$; df = 2; p = 0.197	$\chi^2 = 2.12$; df = 2; p = 0.347	F = 2.31; df = 2; p = 0.110	F = 0.775; df = 2; p = 0.465
	5 x 10^{-3}				
	5 x 10^{-2}				
TMX		$\chi^2 = 1.25$; df = 3; p = 0.742	$\chi^2 = 1.34$; df = 3; p = 0.720	F = 1.69; df = 3; p = 0.179	F = 1.35; df = 3; p = 0.265
	5 x 10^{-3}				
	5 x 10^{-2}				
	0.5				
CDN		$\chi^2 = 2.08$; df = 3; p = 0.556	$\chi^2 = 3.97$; df = 3; p = 0.265	F = 1.45; df = 3; p = 0.238	F = 3.73; df = 3; p = 0.0143
	5 x 10^{-4}				t ratio = -2.45; p = 0.0441
	5 x 10^{-3}				t ratio = -0.325; p = 0.957
	5 x 10^{-2}				t ratio = 0.963; p = 0.638

[a] A poisson (larval and pupal duration) or gaussian (adult wingspan and weight) generalized linear models with type 3 ANOVA (obtained from "car" package in R) was used to analyze the data. If $p < 0.05$, emmeans was used to compare the control to other treatments.

[b] Only concentrations that had at least three surviving monarchs were analyzed.

[c] Data plotted in Figures 5, S1, and S2.

Values in bold indicate significant effects at p = 0.05 level.

CTR: chlorantraniliprole; CFS: chlorpyrifos; IMI: imidacloprid; TMX: thiamethoxam; CDN: clothianidin

Table S10. Statistical analyses of other sublethal monarch endpoints following chronic larval exposure to tropical milkweed leaves treated with five insecticides

Insecticide	Conc[n] (µg/g)[a]	Endpoints analyzed					
		% adult eclosion	Adult eclosion analyses[b]	% wing crumpled	Crumpled wing analyses[b]	Sex ratio (F/M)	Sex ratio analyses[b]
CFS			$\chi^2 = 3.22$; df = 3; p = 0.359		$\chi^2 = 16.0$; df = 3; p = 1.12 x 10^{-3}		$\chi^2 = 6.31$; df = 3; p = 0.0975
	0	96		4		0.50	
	5 x 10^{-2}	86		0	OR = 1.3 x 10^7; p = 1.00	1.1	
	0.5	88		38	OR = 0; p = 0.0446	1.0	
	5	71		20	OR = 0; p = 0.421	4.0	
CTR			$\chi^2 = 4.94$; df = 4; p = 0.293		$\chi^2 = 3.28$; df = 4; p = 0.511		$\chi^2 = 3.21$; df = 4; p = 0.524
	0	97		6		0.83	
	5 x 10^{-6}	93		12		1.1	
	5 x 10^{-5}	97		10		1.5	
	5 x 10^{-4}	100		10		0.82	
	5 x 10^{-3}	100		0		0.67	
IMI			$\chi^2 = 5.78$; df = 2; p = 0.0555		$\chi^2 = 1.95$; df = 2; p = 0.377		$\chi^2 = 1.37$; df = 2; p = 0.504
	0	87		25		1.0	
	5 x 10^{-3}	100		12		0.67	
	5 x 10^{-2}	85		12		1.4	
TMX			$\chi^2 = 0.657$; df = 3; p = 0.883		$\chi^2 = 8.37$; df = 3; p = 0.0390		$\chi^2 = 0.782$; df = 3; p = 0.854
	0	86		13		0.67	
	5 x 10^{-3}	94		40	OR = 0.0701; p = 0.0657	1.0	
	5 x 10^{-2}	91		19	OR = 0.186; p = 0.342	0.91	
	0.5	95		30	OR = 0.104; p = 0.121	0.67	
CDN			$\chi^2 = 0.985$; df = 3; p = 0.805		$\chi^2 = 6.12$; df = 3; p = 0.106		$\chi^2 = 4.29$; df = 3; p = 0.232
	0	93		15		1.3	
	5 x 10^{-4}	96		14		1.4	

Table S10 continued

5×10^{-3}	88	32	0.83
5×10^{-2}	88	43	0.62

[a] Only concentrations that had at least three surviving monarchs were analyzed.
[b] A binomial generalized linear model with type 3 ANOVA (obtained from "car" package in R) was used to analyze the data. If $p < 0.05$, emmeans was used to compare the control to other treatments.
Value in bold indicates significant effect at $p = 0.05$ level.
CTR: chlorantraniliprole; CFS: chlorpyrifos; IMI: imidacloprid; TMX: thiamethoxam; CDN: clothianidin
F: female; M: male

Table S11. Percent emergence of eggs following topical exposure to six insecticides[a]

Insecticide	Dose (µg/egg)[b]	Dose (µg/g egg)[c]	Dose (µg/cm^2 egg)[d]	N[e]	Percent egg emergence	Adjusted percent egg emergence[f]	Mean (± SD) days to emergence
Control	0	0	0	80	79	100	3.0 (± 0.2)
BCF	1.9×10^{-8}	4.3×10^{-5}	3.8×10^{-8}	20	75	88	3.0 (± 0.0)
	1.9×10^{-5}	4.3×10^{-2}	3.8×10^{-5}	20	20	24	3.8 (± 0.5)
	1.9×10^{-3}	4.3	3.8×10^{-3}	20	10	14	3.0 (± 0.0)
	0.19	430	0.38	20	0	0	NA
CTR	9.4×10^{-9}	2.1×10^{-5}	1.9×10^{-8}	20	75	88	3.0 (± 0.0)
	9.4×10^{-6}	2.1×10^{-2}	1.9×10^{-5}	20	30	41	3.2 (± 0.4)
	9.4×10^{-4}	2.1	1.9×10^{-3}	20	5	6	3.0 (± NA)
	9.4×10^{-2}	210	0.19	20	0	0	NA
CFS	1.6×10^{-3}	3.6	3.2×10^{-3}	20	85	100	3.1 (± 0.2)
	0.16	360	0.32	20	85	100	3.1 (± 0.3)
	1.6	3600	3.2	20	15	21	3.3 (± 0.6)
	5.6	13000	11.2	20	0	0	NA
IMI	1.4×10^{-4}	0.32	2.8×10^{-4}	20	80	94	3.1 (± 0.3)
	1.4×10^{-3}	3.2	2.8×10^{-3}	20	20	28	3.0 (± 0.0)
	1.4×10^{-2}	320	2.8×10^{-2}	20	10	12	3.0 (± 0.0)
	0.14	3200	0.28	20	0	0	NA
TMX	1.6×10^{-4}	0.36	3.2×10^{-4}	20	80	94	3.2 (± 0.4)
	1.6×10^{-2}	36	3.2×10^{-2}	20	45	62	3.0 (± 0.0)

Table S11 continued

	0.16	360	0.32	20	25	29	3.0 (± 0.0)
	0.82	1900	1.6	20	0	0	NA
CDN	1.6×10^{-6}	3.6×10^{-3}	3.2×10^{-6}	20	65	76	3.0 (± 0.0)
	1.6×10^{-3}	3.6	3.2×10^{-3}	20	35	41	3.0 (± 0.0)
	1.6×10^{-2}	36	3.2×10^{-2}	20	15	21	3.3 (± 0.6)
	0.16	360	0.32	20	5	7	3.0 (± NA)

[a] Eggs were topically treated with 0.2 µL volume of acetone (controls) and insecticide-acetone solutions.
[b] Calculated by multiplying the measured insecticide concentration with the volume of insecticide solution applied on each egg.
[c] Calculated by dividing the µg/egg with the average weight of an egg, which was 0.44 ± 0.02 mg or 4.4×10^{-4} g (n = 32).
[d] Calculated by dividing the µg/egg with the average surface area of an egg, which was 0.5 ± 0.1 cm^2 (n = 10).
[e] The number of eggs treated at each insecticide concentration.
[f] The egg percent emergence for each insecticide concentration was adjusted for control mortality from the same bioassay run using Abbott's formula.
BCF: beta-cyfluthrin; CTR: chlorantraniliprole; CFS: chlorpyrifos; IMI: imidacloprid; TMX: thiamethoxam; CDN: clothianidin
SD: standard deviation; NA: Not available

Table S12. Statistical analyses of monarch developmental endpoints following topical egg and pupal exposures to six insecticides[a]

Insecticide	Statistical analyses of egg and pupal endpoints[b]		
	Egg duration[c]	Pupal duration[d]	Sex ratio of eclosed adults[d]
BCF	$\chi^2 = 56.2$; df = 2; $p = 6.23 \times 10^{-13}$, [e]	$\chi^2 = 3.34$; df = 3; p = 0.342	$\chi^2 = 0.357$; df = 3; p = 0.949
CFS	$\chi^2 = 5.88$; df = 3; p = 0.117	$\chi^2 = 3.31$; df = 1; p = 0.0689	$\chi^2 = 0.102$; df = 1; p = 0.749
CTR	$\chi^2 = 2.76$; df = 2; p = 0.251	$\chi^2 = 7.19$; df = 2; p = 0.0274[f]	$\chi^2 = 3.10$; df = 2; p = 0.212
IMI	$\chi^2 = 0.443$; df = 2; p = 0.801	$\chi^2 = 0.499$; df = 1; p = 0.480	$\chi^2 = 0.906$; df = 1; p = 0.341
TMX	$\chi^2 = 6.27$; df = 3; p = 0.0992	$\chi^2 = 0.201$; df = 1; p = 0.654	$\chi^2 = 0.324$; df = 1; p = 0.569
CDN	$\chi^2 = 7.81$; df = 3; p = 0.0500	$\chi^2 = 0.447$; df = 1; p = 0.504	$\chi^2 = 0.102$; df = 1; p = 0.749

[a] A poisson generalized linear models with type 3 ANOVA (obtained from "car" package in R) was used to analyze all the data. If $p < 0.05$, emmeans was used to compare the control to other treatments.
[b] Only concentrations that had at least three surviving monarchs were analyzed.
[c] Endpoint obtained from topically treating monarch eggs with six insecticides (see Table S11).
[d] Endpoint obtained from topically treating monarch pupae with six insecticides (see Table 3).
[e] The 4.3×10^{-2} µg/g egg BCF dose was significantly different from controls ($p < 0.0001$). The 4.3×10^{-5} µg/g egg BCF dose was not

different from control (p = 0.807).
[f] The 4.0 x 10^{-4} µg/g pupa CTR dose was significantly different from controls (p = 0.0341). The 4.2 x 10^{-3} µg/g pupa CTR dose was not different from control (p = 0.888).
Value in bold indicates significant effect at p = 0.05 level.
BCF: beta-cyfluthrin; CTR: chlorantraniliprole; CFS: chlorpyrifos; IMI: imidacloprid; TMX: thiamethoxam; CDN: clothianidin

ARTIFICIAL DIET

Acute dietary toxicity studies were conducted with an artificial diet as an alternate approach to mimic exposure to seed treatment insecticides (i.e., imidacloprid, clothianidin, thiamethoxam, and chlorantraniliprole) within milkweed leaves. The diet consisted of *Heliothis* Stonefly powder (Ward's Science), lyophilized and finely ground tropical milkweed leaf powder, and distilled water containing the insecticide-acetone solution. The dry portion made up 25% of the diet, with a Stonefly powder to milkweed powder ratio of 3 : 2. The wet portion consisted of 70% water and 5% insecticide-acetone solution (or acetone alone for control). Third-instar monarch larvae obtained from the Iowa colony were exposed to the treated diet for two days followed by a two-day exposure to untreated diet (diet was changed daily). Larval responses in bioassays with treated diet were similar to those obtained in assays with treated leaves. Except for thiamethoxam, the LC50s based on the artificial diet (nominal concentrations) overlapped with the 95% CIs of the LC_{50}s based on treated tropical milkweed leaves reported in Krishnan et al. 2020 (Table S13). To assess the extent to which the insecticides were consistently blended in the diet and the extent to which the insecticides degraded in the diet over a 24 h period, three replicate diet samples for each insecticide were spiked at their ~ LC_{50} concentrations and analyzed by LC-MS/MS on Day 0 and Day 1. Results of these analyses indicated uniform insecticide concentrations in the diet with no degradation over 24 h (Table S14).

Table S13. Acute toxicity of four insecticides to monarch third-instar larvae following dietary exposure to artificial diet treated with four insecticides[a]

Insecticide	Exposure media	96-h LC values and 95% CIs		
		LC_{10}	LC_{50}	LC_{90}
IMI	Artificial diet[b]	3.9 (1.6 – 9.3)	16 (10 – 24)	62 (28 – 140)
	Milkweed leaf[c]	3.7 (0.48 – 6.9)	17 (9.4 – 24)	77 (22 – 130)
TMX	Artificial diet[b]	6.1 (2.5 –15)	32 (19 – 55)	170 (69 – 430)
	Milkweed leaf[c]	1.1 (0.48 – 2.1)	5.6 (3.7 – 8.9)	29 (15 – 69)
CDN	Artificial diet[b]	3.6 (2.1 – 6.4)	8.5 (6.3 – 12)	20 (12 – 35)
	Milkweed leaf[c]	3.5 (1.3 – 5.6)	7.8 (5.2 – 10)	17 (9.8 – 25)
CTR	Artificial diet[b]	1.5 x 10^{-3} (NA – NA)	3.7 x 10^{-2} (3.2 x 10^{-2} – 4.3 x 10^{-2})	0.93 (NA – NA)
	Milkweed leaf[c]	6.0 x 10^{-4} (6.8 x 10^{-5} – 2.9 x 10^{-3})	4.6 x 10^{-2} (1.8 x 10^{-2} – 0.11)	3.6 (1.1 – 21)

[a] Based on mortality data obtained from treating 95 (CTR) to 150 (TMX) third instars. Larvae were exposed to diet containing acetone or insecticide: acetone for two days. At least five concentrations were used for each insecticide.
[b] Based on nominal artificial diet concentrations.
[c] LC values based on tropical milkweed bioassays obtained from Krishnan et al. (2020).
CIs: confidence intervals; LC_{10}: lethal concentration that kills 10% of a treated population; LC_{50}: lethal concentration that kills 50% of a treated population; LC_{90}: lethal concentration that kills 90% of a treated population; NA: not available
CTR: chlorantraniliprole; IMI: imidacloprid; TMX: thiamethoxam; CDN: clothianidin

Table S14. Measured insecticide concentrations in an artificial diet used for monarch larvae acute dietary bioassays[a]

Insecticide	Nominal conc (µg/g)[b]	Mean (± SD) measured diet conc (µg/g diet)[c]		Ratio of conc (Day 1/Day 0)	Percent of nominal conc[d]
		Day 0	Day 1		
CTR	5×10^{-2}	5.7×10^{-2} ($\pm 7.4 \times 10^{-3}$)	6.0×10^{-2} ($\pm 8.7 \times 10^{-3}$)	1.1	110
IMI	15	15 (± 0.7)	16 (± 1.8)	1.1	100
TMX	30	17 (± 2.5)	19 (± 4.2)	1.1	57
CDN	10	7.8 (± 1.5)	8.2 (± 1.4)	1.1	78

[a] LC-MS/MS was used to quantify all insecticide samples; leaf sample analyses methods were used (see *Residue analyses*). Recovery rates of the diet extractions were 97% for clothianidin and 99% for chlorantraniliprole, imidacloprid, and thiamethoxam.
[b] Calculated by multiplying the nominal insecticide concentration (µg/µL) with the µL of insecticide solution applied and divided by the approximate weight in g of diet.
[c] Three replicates per diet sample were analyzed. Half the diet was immediately stored in -80ºC (Day 0) and the other half was kept in the incubator (26.6ºC, 65% relative humidity and 16:8 light: dark cycle) for 1 day and then stored in -80ºC (Day 1).
[d] Obtained by dividing the measured concentration on Day 0 with the nominal concentration and multiplying the result by 100.
CTR: chlorantraniliprole; IMI: imidacloprid; TMX: thiamethoxam; CDN: clothianidin

ACUTE DIETARY TOXICTY DOSES IN MONARCH LARVAE

In Krishnan et al. (2020), we estimated the lethal concentrations of insecticides to monarch larvae in acute dietary studies by calculating the amount of insecticide applied per cm^2 of milkweed leaf. We analyzed the surface areas of leaves prior to larval feeding. We also photographed the leaves after larval feeding (two-day feeding for second and third instars, and one-day feeding for fifth instars), but did not quantify surface areas. In the present paper, we estimated leaf surface areas after larval exposure and subtracted those values from the pre-exposure surface areas to estimate mass of insecticide consumed to derive lethal dietary doses. The surface area was analyzed using ImageJ (National Institute of Health, USA) and task-specific code written in Python using the OpenCV

computer vision library (Tripathy 2020). The doses corresponding to µg of insecticide/larva and µg of insecticide/g larva and associated mortality can be found in Table S15. Though the leaves were dried prior to larval feeding, the data indicate larvae also had cuticular exposure to the insecticide applied on the leaf surface.

Table S15. Dose of insecticide consumed by larvae in acute dietary studies[a]

Insecticide	Instar	Conc[n] (µg/cm^2 leaf)	# of leaf pairs analyzed	Leaf area consumed (%)	Dose (µg/larva)[b]	Dose (µg/g larva)[c]	Corrected larval percent mortality
BCF	Second	0	44	84	0	0	0
		3.9×10^{-4}	44	82	1.4×10^{-3}	0.16	0
		3.0×10^{-3}	44	46	6.2×10^{-3}	0.68	45
		1.1×10^{-2}	33	17	7.6×10^{-3}	0.84	68
		2.4×10^{-2}	44	12	1.3×10^{-2}	1.4	74
		0.24	44	10	0.10	11	100
	Third	0	32	82	0	0	0
		5.7×10^{-5}	22	81	7.2×10^{-4}	2.5×10^{-2}	0
		4.5×10^{-4}	32	73	5.1×10^{-3}	0.17	0
		3.2×10^{-3}	32	63	3.4×10^{-2}	1.2	7
		2.4×10^{-2}	22	20	8.3×10^{-2}	2.8	35
		0.27	32	9	0.38	13	97
	Fifth	0	28	89	0	0	0
		5.8×10^{-5}	21	98	4.3×10^{-3}	4.7×10^{-3}	0
		4.6×10^{-4}	33	88	3.1×10^{-2}	3.3×10^{-2}	0
		3.6×10^{-3}	30	64	0.17	0.19	14
		2.9×10^{-2}	33	16	0.34	0.37	66
		0.31	31	5	1.1	1.2	85
CTR	Second	0	33	95	0	0	0
		6.9×10^{-8}	22	96	2.4×10^{-7}	2.9×10^{-5}	5
		5.3×10^{-7}	11	82	2.1×10^{-6}	2.5×10^{-4}	14
		1.9×10^{-6}	20	57	3.9×10^{-6}	4.6×10^{-4}	5
		6.9×10^{-5}	32	43	1.3×10^{-4}	1.6×10^{-2}	36
		7.4×10^{-4}	31	14	4.5×10^{-4}	5.4×10^{-2}	71
		5.0×10^{-3}	20	5	1.3×10^{-3}	0.16	94

Table S15 continued

		4.7×10^{-2}	21	3	5.7×10^{-3}	0.68	100
	Third	0	33	82	0	0	0
		2.0×10^{-6}	22	59	1.6×10^{-5}	5.4×10^{-4}	1
		8.8×10^{-4}	33	16	2.0×10^{-3}	6.8×10^{-2}	50
		6.9×10^{-3}	33	7	7.1×10^{-3}	0.24	78
		5.6×10^{-2}	33	5	4.3×10^{-2}	1.5	75
		1.0	22	3	0.42	14	100
	Fifth	0	30	82	0	0	0
		8.4×10^{-5}	21	74	4.6×10^{-3}	5.6×10^{-3}	0
		7.7×10^{-4}	27	44	2.8×10^{-2}	3.4×10^{-2}	8
		5.8×10^{-3}	29	30	0.15	0.18	21
		5.6×10^{-2}	31	20	0.87	1.1	73
		0.85	22	9	6.1	7.4	71
CFS	Second	0	41	71	0	0	0
		8.3×10^{-5}	21	86	3.1×10^{-4}	3.8×10^{-2}	0
		2.9×10^{-3}	37	96	1.3×10^{-2}	1.6	0
		1.5×10^{-2}	44	66	4.4×10^{-2}	5.5	0
		2.4×10^{-2}	22	38	4.3×10^{-2}	5.3	39
		0.22	41	40	0.41	50	44
		14	33	11	7.3	900	100
	Third	0	33	80	0	0	0
		8.8×10^{-5}	22	75	1.1×10^{-3}	2.7×10^{-2}	8
		3.2×10^{-3}	21	79	4.3×10^{-2}	1.1	3
		1.5×10^{-2}	33	66	0.18	4.4	23
		0.23	31	56	2.3	56	48
		16	33	10	27	660	97
	Fifth	0	33	93	0	0	0
		9.0×10^{-5}	32	83	5.9×10^{-3}	7.8×10^{-3}	10
		3.5×10^{-3}	26	92	0.25	0.33	0
		1.7×10^{-2}	33	88	1.2	1.6	6
		0.26	30	56	11	15	63
		16	11	19	240	320	95

Table S15 continued

IMI	Second	0	31	91	0	0	0
		1.7×10^{-4}	22	95	5.7×10^{-4}	5.7×10^{-2}	0
		2.5×10^{-3}	33	97	9.1×10^{-3}	0.91	0
		1.9×10^{-2}	33	88	6.7×10^{-2}	6.7	0
		0.20	32	33	0.26	26	58
		1.9	33	2	0.14	14	100
	Third	0	33	81	0	0	0
		1.5×10^{-4}	21	87	2.1×10^{-3}	5.5×10^{-2}	0
		2.3×10^{-3}	32	79	3.0×10^{-2}	0.77	3
		1.9×10^{-2}	20	88	0.27	7.0	3
		0.17	33	24	0.70	18	23
		1.7	30	3	0.78	20	87
	Fifth	0	31	89	0	0	0
		2.5×10^{-3}	27	85	0.16	0.21	14
		1.9×10^{-2}	30	79	1.2	1.6	10
		0.18	31	31	4.5	6.0	44
		1.7	32	13	17	23	85
TMX	Second	0	33	100	0	0	0
		1.3×10^{-3}	33	81	3.6×10^{-3}	0.41	14
		1.5×10^{-2}	33	91	5.0×10^{-2}	5.7	14
		4.1×10^{-2}	22	80	0.14	15	17
		0.12	33	31	0.15	17	69
		1.6	33	7	0.45	50	100
	Third	0	33	84	0	0	0
		1.3×10^{-4}	22	80	1.5×10^{-3}	4.3×10^{-2}	0
		1.2×10^{-3}	22	92	1.6×10^{-2}	0.47	0
		1.5×10^{-2}	33	83	0.18	5.2	0
		0.13	31	16	0.32	9.0	53
		1.7	33	3	0.69	20	100
	Fifth	0	33	89	0	0	0
		1.4×10^{-3}	32	92	8.3×10^{-2}	9.2×10^{-2}	0
		1.7×10^{-2}	32	93	1.0	1.1	0

Table S15 continued

		0.15	32	44	4.3	4.8	18
		2.0	33	13	15	17	54
		8.9	22	15	83	93	100
CDN	Second	0	42	91	0	0	0
		7.9×10^{-4}	33	96	3.2×10^{-3}	0.34	0
		8.8×10^{-3}	43	79	3.1×10^{-2}	3.3	10
		2.7×10^{-2}	44	71	9.0×10^{-2}	9.7	0
		0.11	43	30	0.15	16	59
		0.23	21	2	1.5×10^{-2}	1.6	95
		0.55	22	2	3.5×10^{-2}	3.8	100
	Third	0	33	86	0	0	0
		7.6×10^{-5}	22	83	8.5×10^{-4}	2.4×10^{-2}	0
		9.9×10^{-4}	33	86	1.1×10^{-2}	0.31	0
		1.2×10^{-2}	33	57	8.9×10^{-2}	2.5	12
		0.15	33	27	0.53	15	29
		0.63	33	2	0.20	5.5	100
	Fifth	0	28	76	0	0	0
		6.7×10^{-5}	22	70	3.6×10^{-3}	9.1×10^{-3}	7
		8.8×10^{-4}	28	64	4.2×10^{-2}	0.10	28
		1.1×10^{-2}	30	51	0.40	1.0	31
		0.14	33	14	1.4	3.5	79
		0.62	33	4	2.0	5.0	93

[a] Data obtained from Krishnan et al. (2020) where monarch second, third, and fifth instar larvae were acutely exposed to tropical milkweed leaves treated with six insecticides made in 0.1% silwet: water suspensions. Leaves provided were photographed prior and after larval consumption to estimate percent leaf consumption and larval doses. Only leaves from concentrations that were employed at least twice in the toxicity bioassays were analyzed.

[b] The μg/larva dose or the amount of insecticide consumed by a larva was obtained by measuring the area of leaf consumed following applications of known concentrations and volumes of insecticide on leaf surface (the insecticide solution is assumed to have been evenly spread on the leaf surface).

[c] The μg/g dose was obtained by dividing the μg/larva dose with the average weight of larvae in the bioassay (obtained from Tables S1 and S19 in Krishnan et al. 2020).

BCF: beta-cyfluthrin; CTR: chlorantraniliprole; CFS: chlorpyrifos; IMI: imidacloprid; TMX: thiamethoxam; CDN: clothianidin

FOLIAR INSECTICIDE DEGRADATION

Assuming all insecticides have a half-life of 4 days, and assuming the larval life stage is 12 days, we can use the following equation to obtain the average larval exposure concentration (µg/cm^2 leaf) each day over 12 days. We assume the foliar insecticide application takes place on day 0.

$$\frac{1}{12}\int_{a}^{a+12} C_0 \left(\frac{1}{2}\right)^{x/4} dx$$

C_0 is the concentration of the insecticide at day 0 (derived from AgDRIFT), x/4 is the number of half-lives encountered up till the xth day, and a is the first day of larval exposure. In Table S16, we assume larvae are exposed to the insecticide either from day 0 (neonate larvae hatched from eggs laid on the underside of leaves, i.e., eggs were not exposed to the spray drift) or from day 4 (neonate larvae hatched four days after application).

Table S16. Chronic risk to monarch larvae following dietary exposure to a single application of a foliar formulated product[a]

Insecticide	Type of application (pest)	Distance away from field (m)	Larval % mortality from days 0 to 12 assuming no degradation[b]	Larval % mortality from days 0 to 12 assuming degradation[c]	Larval % mortality from days 4 to 16 assuming degradation[d]
CFS	Aerial (sa)	0	100	100	100
		15	100	100	99
		30	100	99	62
		60	99	38	1
	High boom (sa, ta)	0	100	100	100
		15	27	0	0
		30	19	0	0
		60	19	0	0
	Low boom (ta)	0	100	100	100
		15	19	0	0
		30	19	0	0
		60	19	0	0
CTR	Aerial (sa)	0	100	100	100
		15	100	100	100
		30	100	100	100

Table S16 continued

		60	100	100	100
	High boom (sa)	0	100	100	100
		15	100	100	99
		30	100	99	98
		60	100	98	95
	High boom (ta)	0	100	100	100
		15	100	100	99
		30	100	100	99
		60	100	99	96
	Low boom (ta)	0	100	100	100
		15	100	99	99
		30	100	99	97
		60	100	97	92
IMI	Aerial (sa)	0	100	100	99
		15	99	97	91
		30	98	92	76
		60	93	71	42
	High boom (sa)	0	100	100	100
		15	70	27	10
		30	49	11	3
		60	32	4	1
	High boom (ta)	0	100	100	100
		15	89	58	29
		30	74	32	12
		60	53	13	4
	Low boom (ta)	0	100	100	100
		15	74	31	12
		30	54	14	4
		60	37	5	2
TMX	Aerial (sa)	0	100	97	81
		15	87	35	7
		30	58	8	1

Table S16 continued

		60	24	1	0
	High boom (sa)	0	100	99	97
		15	17	0	0
		30	17	0	0
		60	17	0	0
CDN	Aerial (sa)	0	100	100	100
		15	100	100	100
		30	100	100	100
		60	100	100	99
	High boom (sa)	0	100	100	100
		15	100	97	54
		30	99	61	6
		60	87	8	0

[a] Risk was estimated by comparing the chronic concentration-response curves with spray drift exposure obtained from AgDRIFT values (50^{th}-percentile results were employed for ground boom).
[b] Predicted larval percent mortality assuming no insecticide degradation. Neonate larvae were assumed to have hatched on day of foliar application.
[c] Predicted larval percentage mortality assuming all insecticides have a half-life of 4 days. Neonate larvae were assumed to have hatched on day of foliar application.
[d] Predicted larval percentage mortality assuming all insecticides have a half-life of 4 days. Neonate larvae were assumed to have hatched four days after foliar application.
CTR: chlorantraniliprole; CFS: chlorpyrifos; IMI: imidacloprid; TMX: thiamethoxam; CDN: clothianidin
m: meters; sa: soybean aphid; ta: true armyworm

SUPPLEMENTARY REFERENCES

Hall MJ, Dang V, Bradbury SP, and Coats JR. 2020. Optimization of QuEChERS Method for Simultaneous Determination of Neonicotinoid Residues in Pollinator Forage. Molecules 25 (12): 2732

Krishnan N, Yang Z, Bidne KG, Hellmich RL, Coats JR, and Bradbury SP. 2020. Assessing Field-Scale Risks of Foliar Insecticide Applications to Monarch Butterfly (Danaus Plexippus) Larvae. Environ Toxicol Chem 39 (4): 923–41.

Tripathy A. 2019. Calculating leaf surface areas using OpenCV. Madison (WI): MINDS@UW. [cited 2020 November 10]. Available from: http://digital.library.wisc.edu/1793/79417

US Environmental Protection Agency. 2011a. AgDRIFT model. Washington, DC. [cited 2020 October 18]. Available from: https://www.epa.gov/pesticide-science-and-assessing-pesticide-risks/models-pesticide-risk-assessment#AgDrift

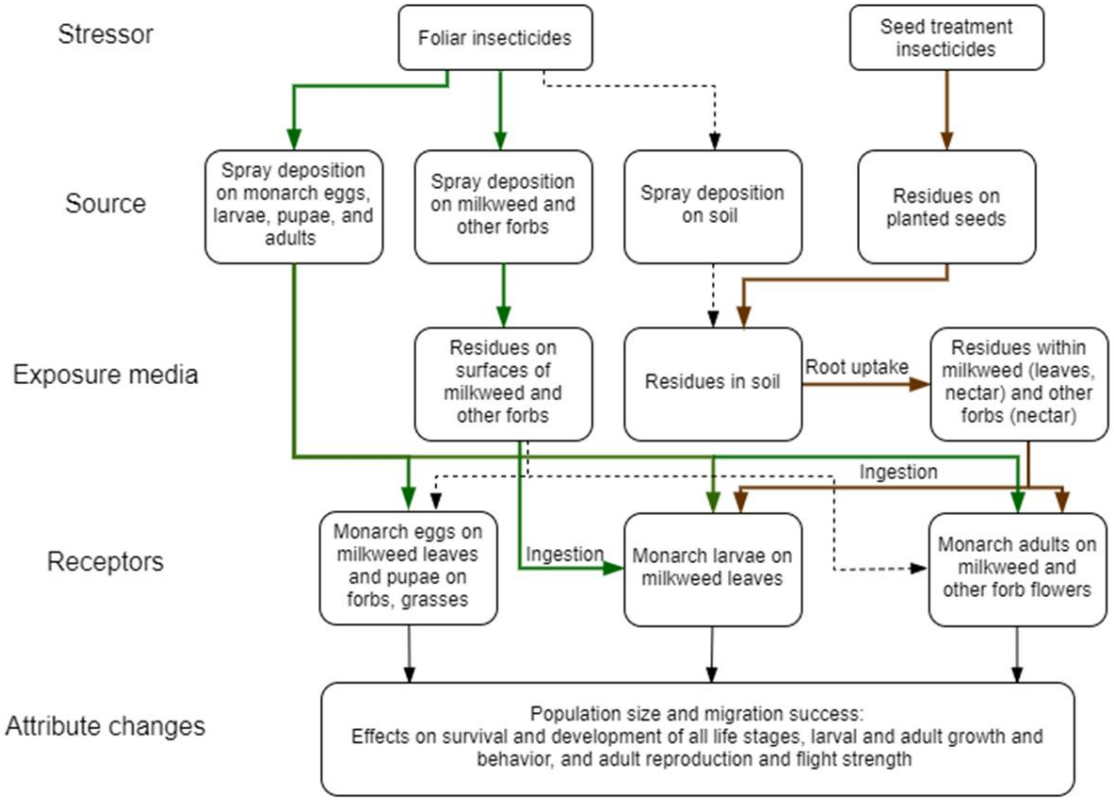

Figure 1. Conceptual model describing how different life stages of monarch butterfly could be exposed to foliar (green arrows) and seed treatment (brown arrows) insecticides and potential adverse effects that could occur from these exposure pathways. Dotted lines are minor exposure pathways.

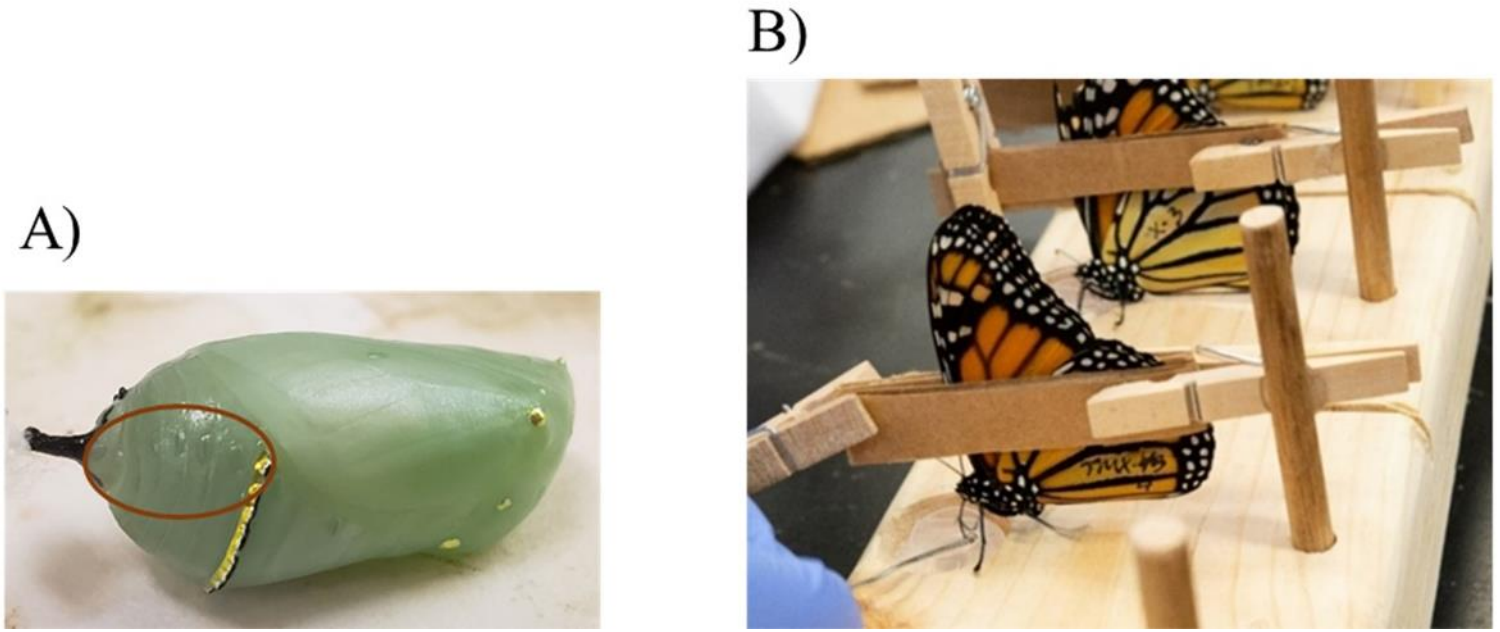

Figure 2. A) A representative monarch pupa treated with an insecticide suspension; suspensions were applied to the four spiracles located within the red circle. B) The experimental apparatus used to restrain monarch adults provided an artificial nectar containing imidacloprid, thiamethoxam, or clothianidin to assess acute dietary toxicity.

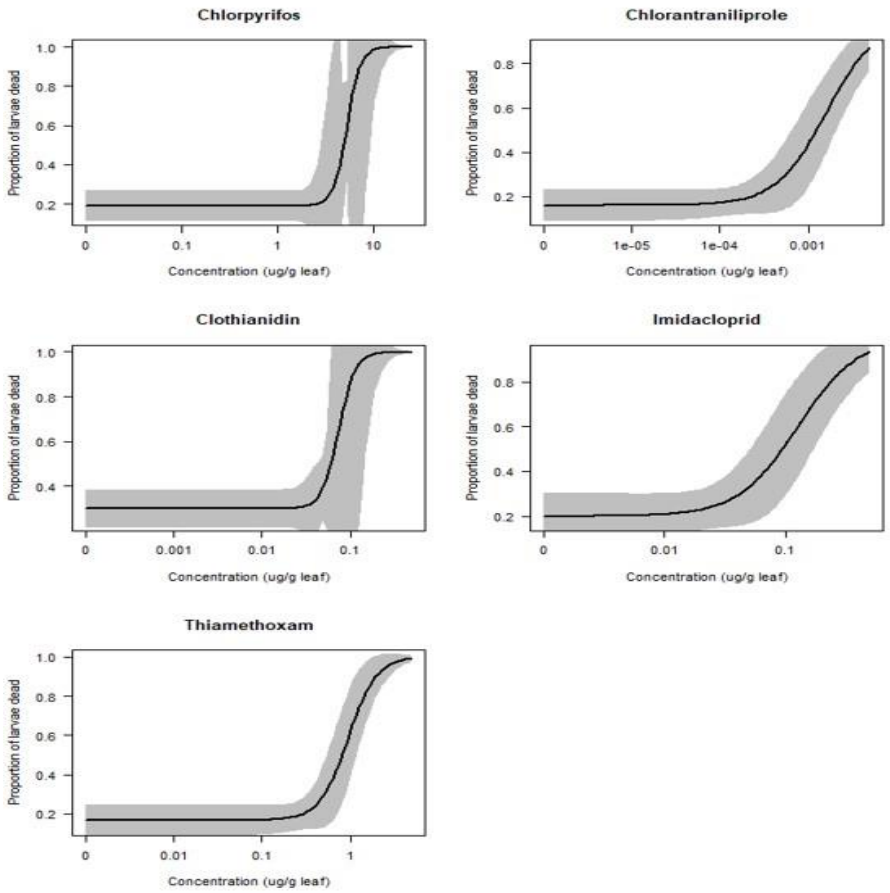

Figure 3. Mortality concentration-response curves (μg insecticide/g leaf) for monarch butterfly larvae following chronic dietary exposure to tropical milkweed leaves treated with five insecticides in 0.1% silwet : water/DMF suspensions. Larvae were exposed from the second instar through pupation.

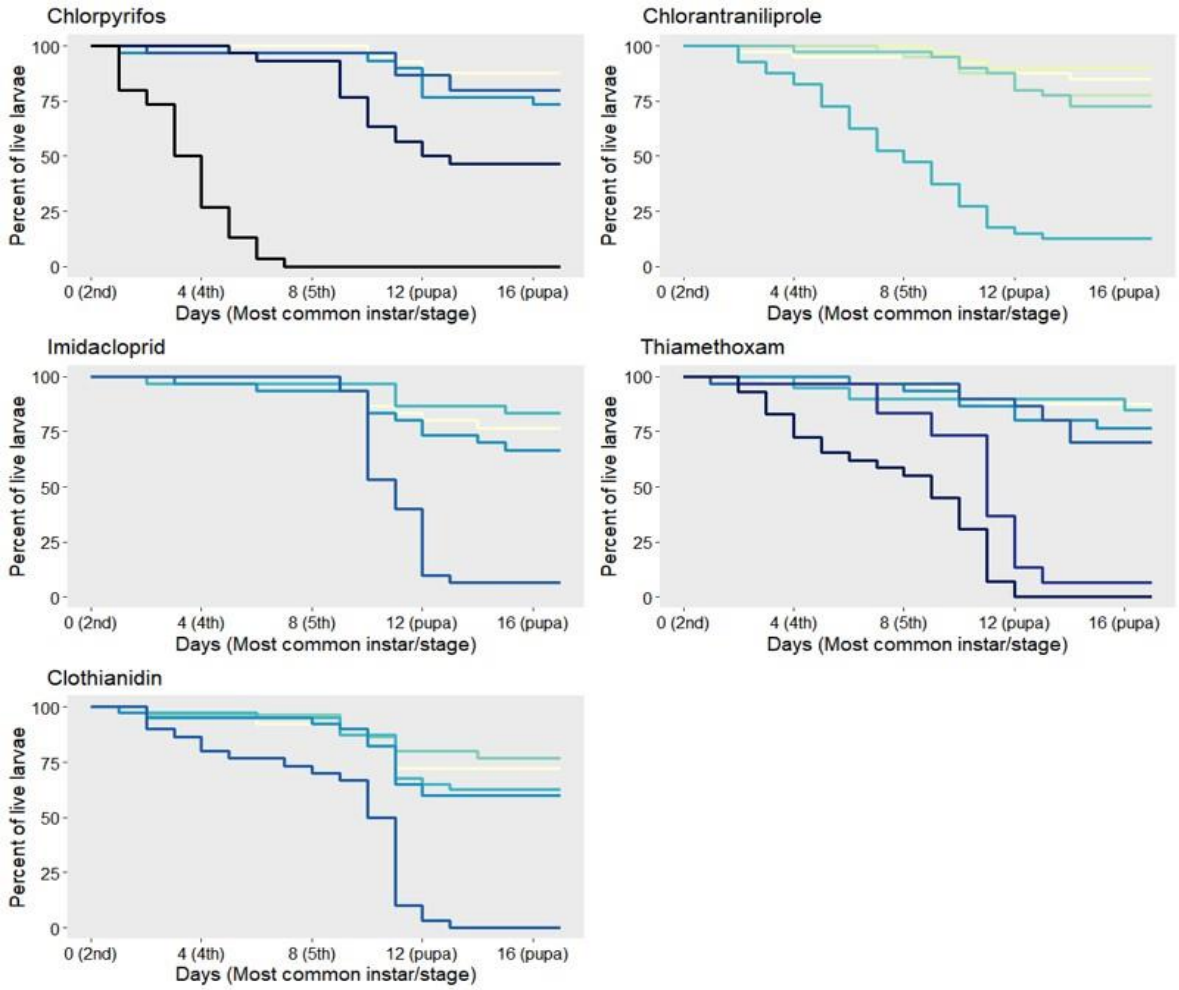

Figure 4. The time to mortality of monarch larvae chronically exposed to tropical milkweed leaves treated with five insecticides. The y-axis is the percentage of larvae living over time for each insecticide concentration. The x-axis is the number of days from initiation of the experiment. The most common instar/life stage observed on days 0, 4, 8, 12, and 16 are noted.

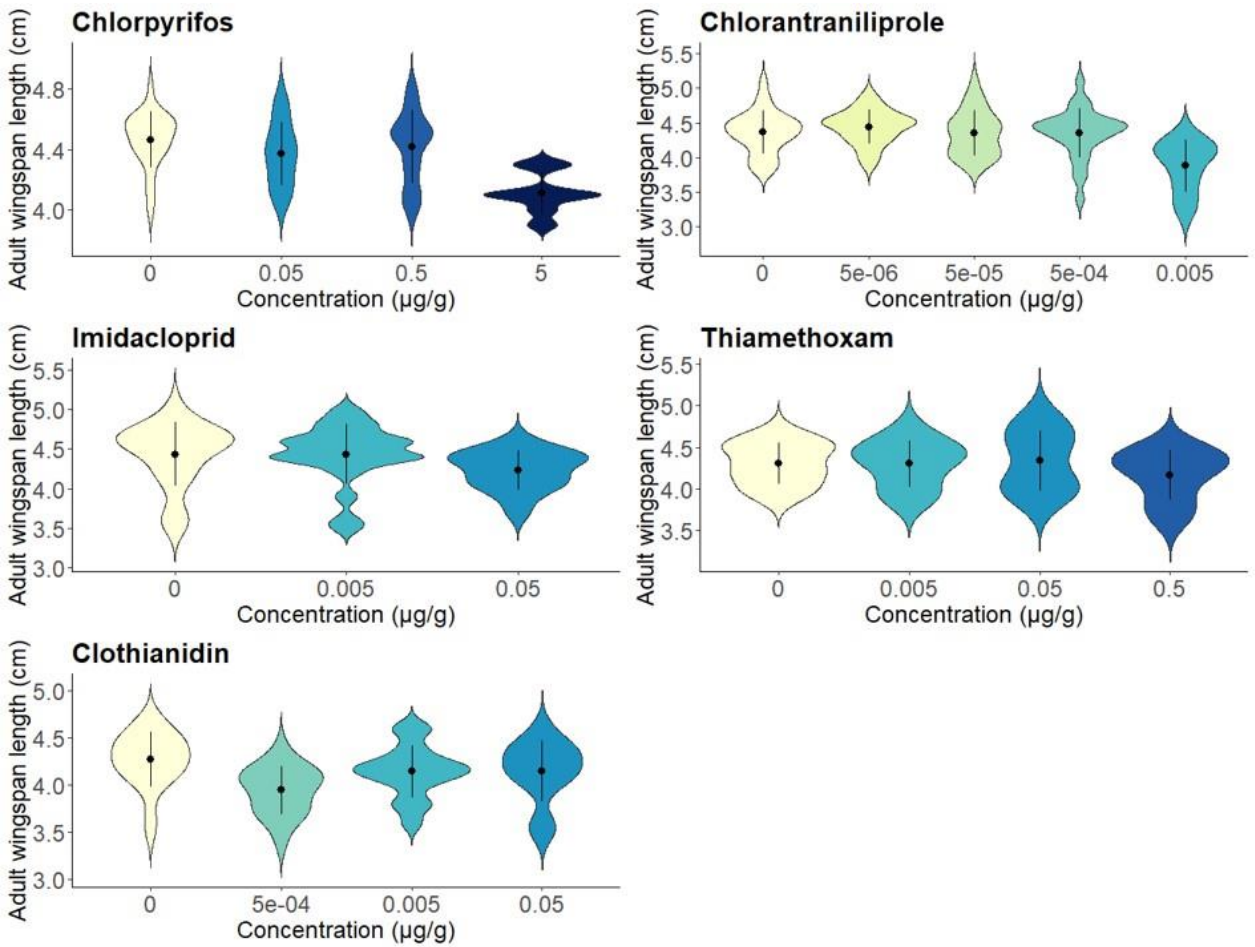

Figure 5. Wingspan length (in cm) of adult monarch butterflies that emerged following chronic exposure in their larval stage to tropical milkweed leaves treated with five insecticides. Insecticide concentrations that had at least three adults emerge are displayed. The violin plots represent the wingspan length associated with each insecticide concentration as the mean ± one standard deviation.

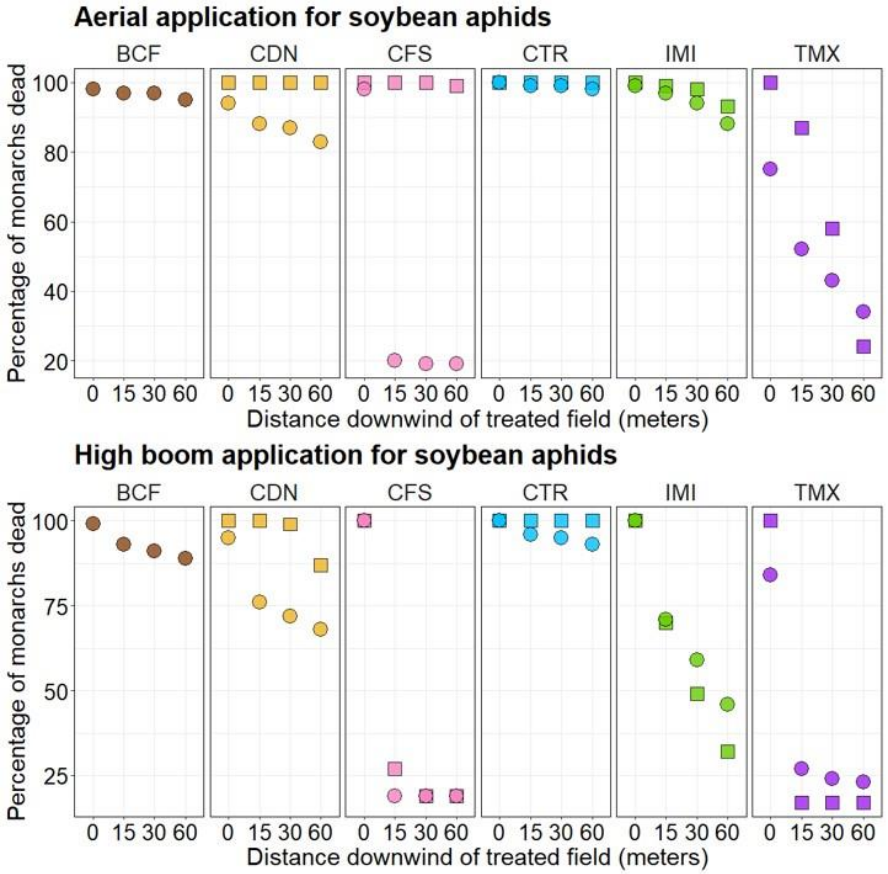

Figure 6. Estimated monarch egg and larval mortality due to insecticide spray drift at increasing distances downwind from a treated soybean field. Squares are predicted larval percent mortality following chronic dietary exposure to five insecticides. The circles are predicted egg mortality following acute topical exposure to six insecticides. Mortality rates were estimated using active ingredient-specific larval and egg concentration- and dose-response curves (Figures S1 and S5), respectively, and estimated 50th percentile, active ingredient-specific exposures using the AgDRIFT model (U.S. Environmental Protection Agency 2011a) for aerial and ground boom applications for representative formulated products (see Table S5 in Krishnan et al. 2020 and Table S3 in current paper). Note the x-axes are not proportionally spaced. BCF: beta-cyfluthrin; CTR: chlorantraniliprole; CFS: chlorpyrifos; IMI: imidacloprid; TMX: thiamethoxam; CDN: clothianidin

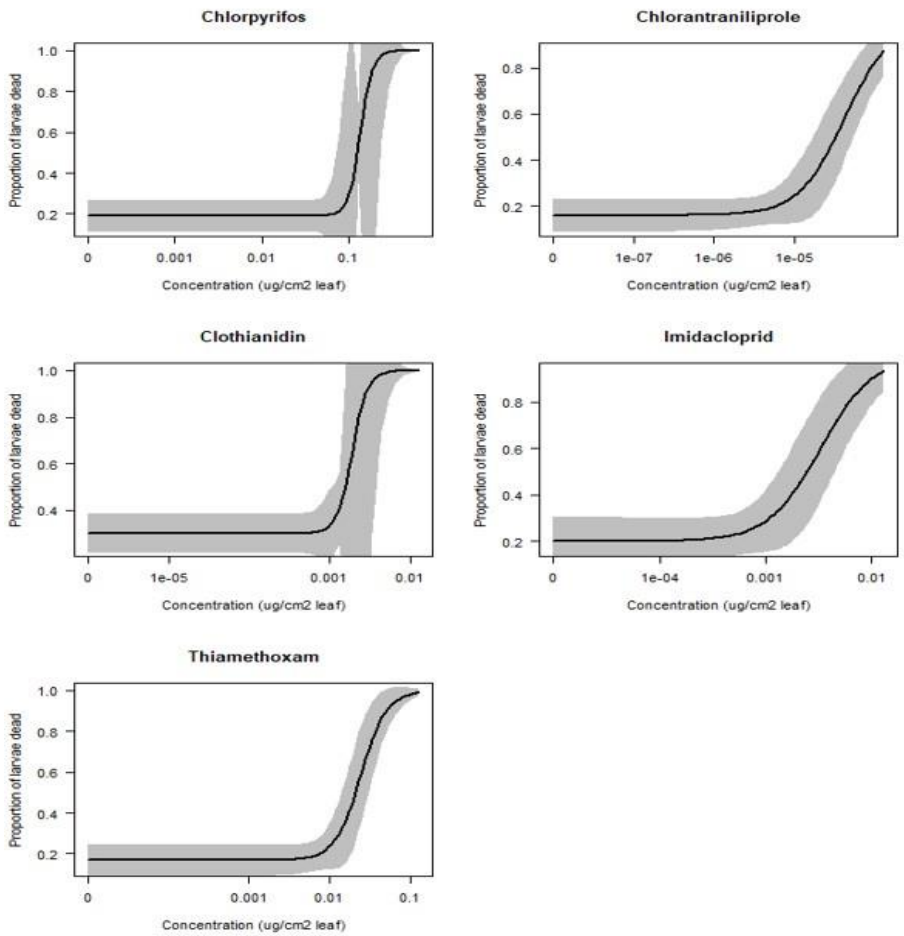

Figure S1. Mortality concentration-response curves (μg insecticide/cm^2 leaf) for monarch butterfly larvae following chronic dietary exposure (second instar to pupation) to tropical milkweed leaves treated with five insecticides in 0.1% silwet : water : DMF suspensions.

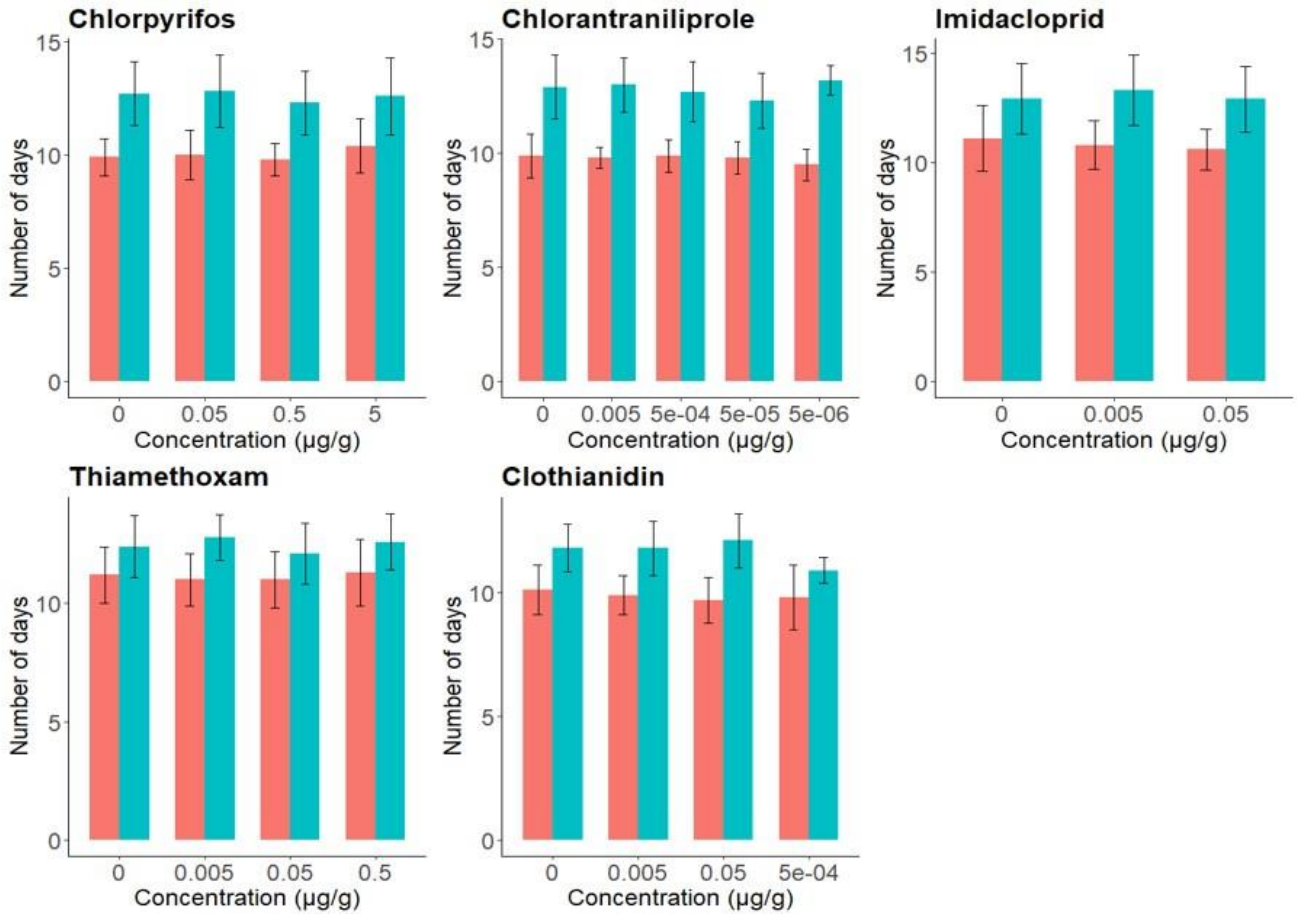

Figure S2. The developmental time (in days) of monarch larvae and pupae following chronic exposure in their larval stage to tropical milkweed leaves treated with five insecticides. Only insecticide concentrations which had at least three larvae pupate are displayed. The bars represent the larval and pupal duration for each insecticide concentration as the mean ± one standard deviation. Pupal duration data were not collected for one imidacloprid and one thiamethoxam bioassay run.

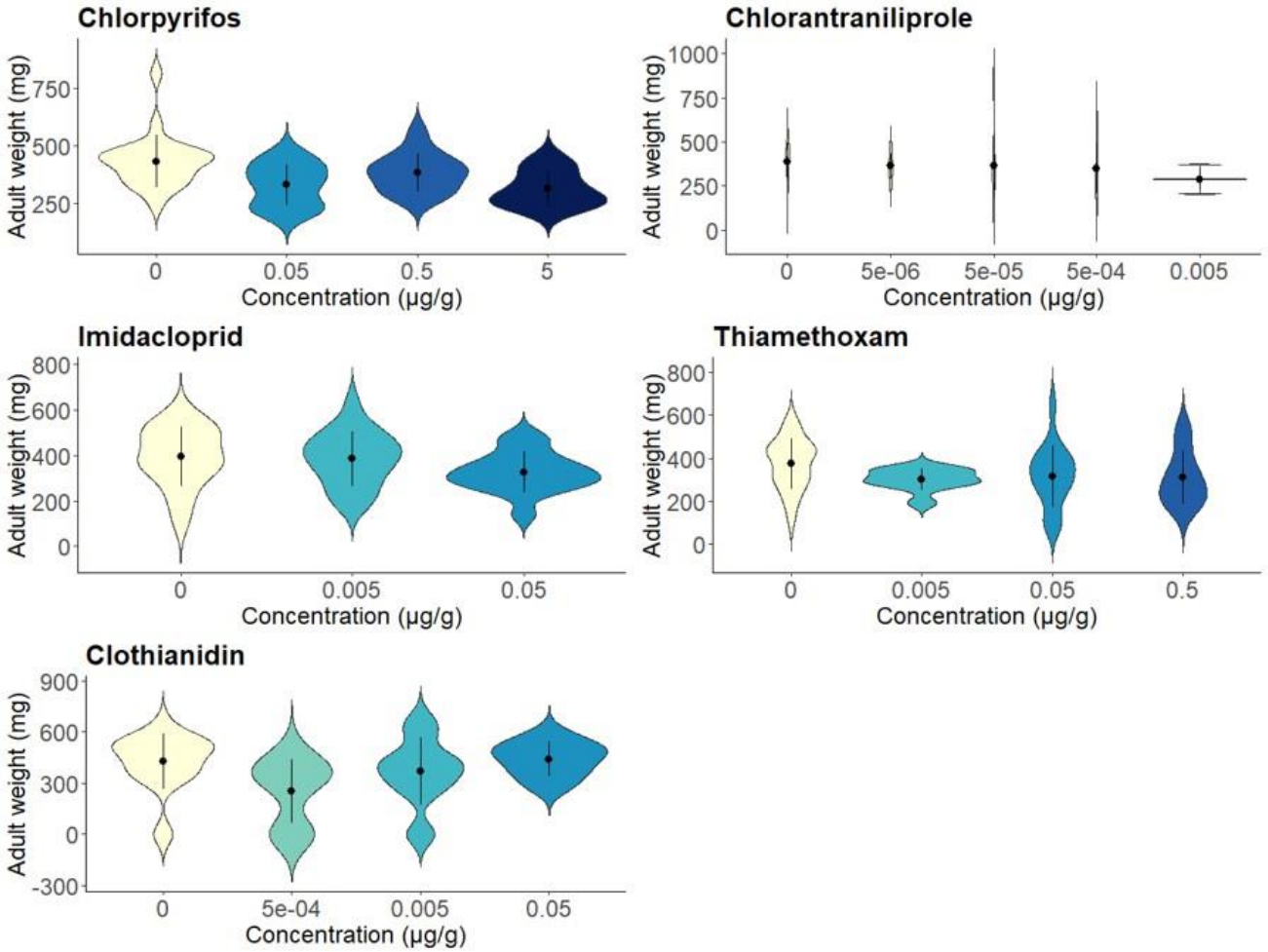

Figure S3. The weight (in mg) of adult monarch butterflies that emerged following chronic exposure in their larval stage to tropical milkweed leaves treated with five insecticides. Only insecticide concentrations which had at least three adults emerge are displayed. The violin plots represent the weight associated with each insecticide concentration as the mean ± one standard deviation.

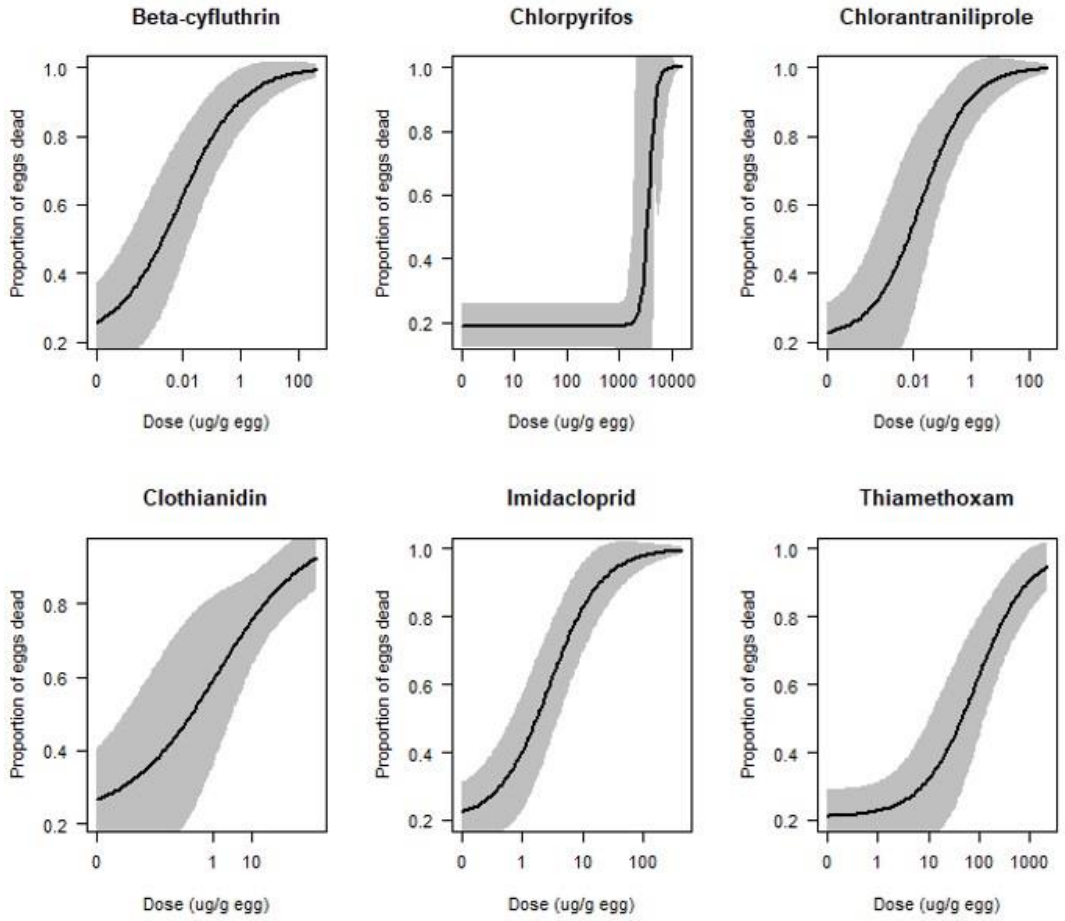

Figure S4. Mortality dose-response curves (μg insecticide/g egg) for monarch butterfly eggs following cuticular application of six insecticides in acetone solution. Observations were made daily through 96-h post-application.

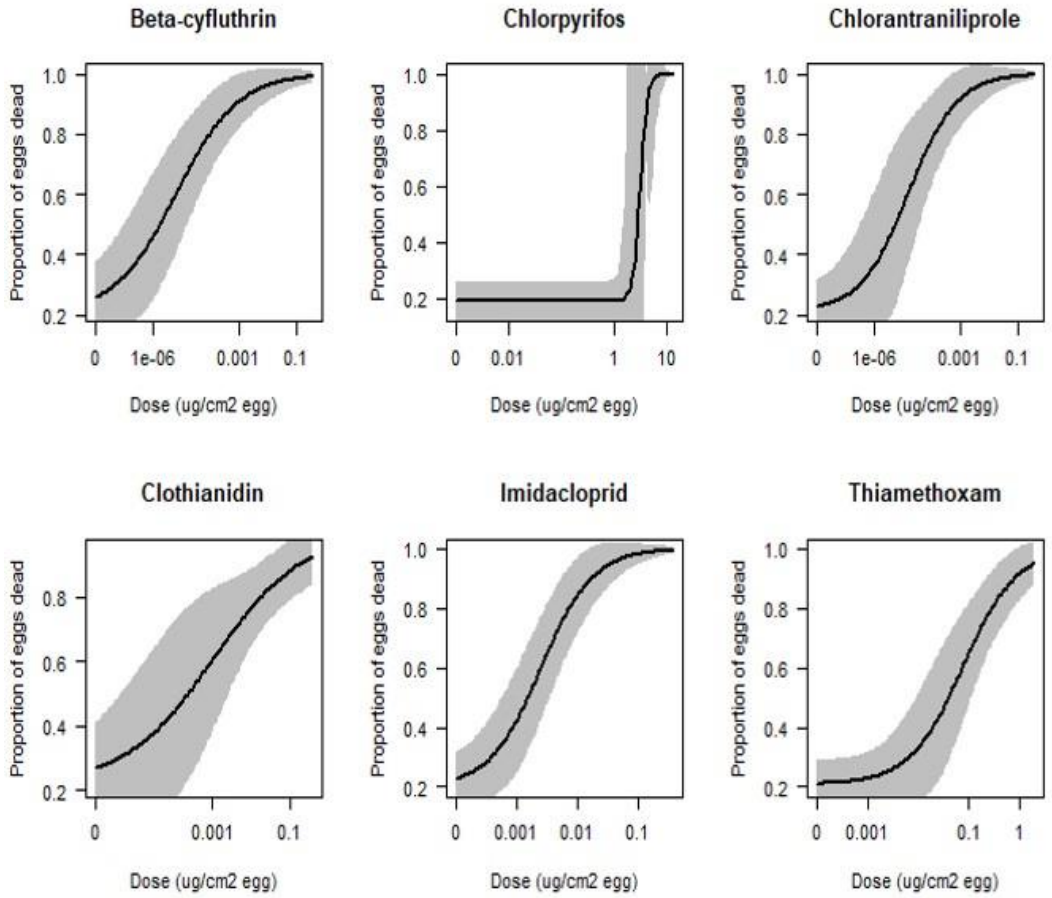

Figure S5. Mortality dose-response curves (μg insecticide/cm^2 egg) for monarch butterfly eggs following cuticular application of six insecticides in acetone solution. Observations were made daily through 96-h post-application.

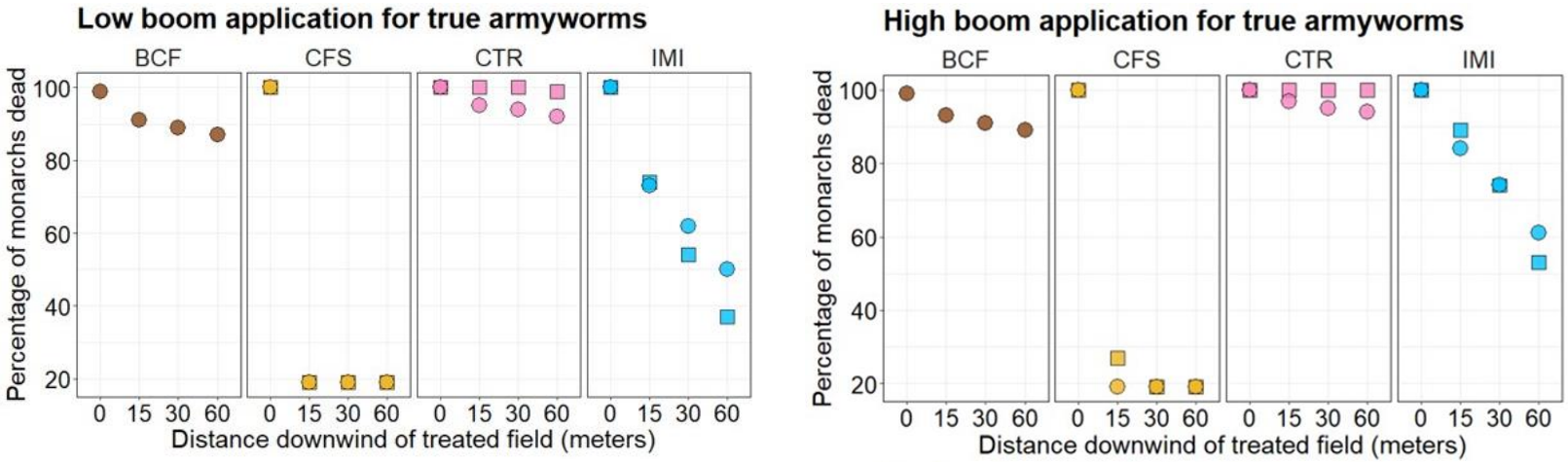

Figure S6. Estimated monarch mortality due to insecticide spray drift at increasing distances downwind from a treated crop field. The squares are predicted larval percentage mortality following chronic dietary exposure to three insecticides. The circles are predicted egg mortality following acute topical exposure to four insecticides. High and low boom spray drift scenarios to manage true armyworms were modeled using AgDRIFT. Mortality rates were estimated using a.i.-specific larval and egg concentration- and dose-response curves (Figures S1 and S5), respectively, and estimated 50th percentile, a.i.-specific exposures using the AgDRIFT model (U.S. Environmental Protection Agency 2011a) for ground boom applications for representative formulated products (see Table S5 in Krishnan et al. 2020 and Table S3 in current paper). Note the x-axes are not proportionally spaced.). Thiamethoxam and clothianidin are not registered for use on true armyworms in maize or soybean fields. BCF: beta-cyfluthrin; CTR: chlorantraniliprole; CFS: chlorpyrifos; IMI: imidacloprid

CHAPTER 4. EVALUATING TOXICITY OF VARROA MITE (*VARROA DESTRUCTOR*)-ACTIVE DSRNA TO MONARCH BUTTERFLY (*DANAUS PLEXIPPUS*) LARVAE

Modified from a manuscript under review in PLOS ONE

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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 (*Aphis 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 milkweed (*Asclepias curassavica*) 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 0.211-0.281 mg/g leaf, respectively. Potassium arsenate and a previously designed monarch 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 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 activity may degrade dsRNA to a concentration that is insufficient to silence mRNA signaling.

Keywords: Lepidoptera, RNA interference, non-target effects, dietary exposure, insecticide, Quantigene assay.

Introduction

RNA interference (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 (Pasquinelli 2002; Agrawal et al. 2003). RNAi technology 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 (Mamta and Rajam 2017). For example, Whyard et al. (2009) and Bachman et al. (2013) silenced critical genes in several pest insect species without causing adverse effects in taxonomically dissimilar non-target species.

Dietary RNAi insecticides silence specific genes through the feeding of double-stranded RNA (dsRNA). DvSnf7 dsRNA, derived from western corn rootworm *(Diabrotica virgifera virgifera*), is a plant-incorporated protectant in maize that was approved for use by the U.S. Environmental Protection Agency (2015). RNAi insecticides also can be formulated for topical uptake by pest species. For example, Bayer Crop Science has developed a dsRNA to control

Varroa mites (*Varroa destructor*) within honey bee (*Aphis mellifera*) brood cells (Inberg and Mahak 2016; see Figure S1). This dsRNA has 372 base pairs that are homologous to a sequence region within the Varroa mite calmodulin gene (*cam*) [Figure 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, which in turn exposes reproductive mites present in the cell.

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 (USEPA 2009, 2012). Tier I studies are designed to estimate hazards to several non-target arthropod taxa under exposure concentrations several times higher ($\geq$ 10X when possible) than the highest concentrations 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.

Exposure of the Varroa 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 dsRNA has a 21-base pair match to monarch calmodulin mRNA (Figure S3). Since dsRNA orthologs could be efficacious against insect mRNA if they share a sequence length of at least 19 to 21 nucleotides (Whyard et al.

2009; Bachman et al. 2013), the potential hazard to monarch larvae, if they are exposed to the Varroa dsRNA, cannot be precluded.

Previous research by Pan et al. (2017) 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 the respective dsRNAs applied to 0.5 cm diameter milkweed 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 (Marshansky et al. 2019). Given V-ATPase A's essential physiological function, it was expected monarch *v-ATPase A* mRNA would be silenced by the monarch dsRNA, and also potentially the western corn rootworm dsRNA as it shared a high sequence similarity. This should result in reduced growth leading to a high level of larval mortality (Bolognesi et al. 2012). Pan et al. (2017), however, reported no adverse effects for either dsRNA. The lack of adverse effects associated with dietary exposure to the western corn rootworm dsRNA may be due to the absence of a 19 to 21-bp-sequence match with the monarch mRNA. Alternatively, the lack of a larval response to the rootworm and monarch dsRNA could be due to a short dietary exposure period that may have resulted in an insufficient internal dose and/or an internal dose window 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 (Pan et al. 2017). We assessed chronic toxicity of Varroa 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 dsRNA through the entire larval stage would adversely affect survival, 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 Krishnan et al. 2020). 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 Krishnan et al. (2020). 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 dsRNA (lot number: STG4-0038) was provided by Bayer Crop Science. The prototype dsRNA formulation contains 2.1 mg/mL Varroa 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 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 dsRNA was synthesized from forward and reverse primers designed by Pan et al. (2017). The monarch V-ATPase A dsRNA was selected as a putative positive dsRNA control. We prepared a 5-mg/mL monarch dsRNA solution in deionized water, which is the same concentration used by Pan et al. (2017) 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 LC_{100} 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 ºC 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 dsRNA-treated leaves, and Varroa 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. Water, monarch dsRNA, and the Varroa dsRNA solutions were applied using a 59-mL fingertip sprayer bottle (Equate brand). Both sides of the leaves were sprayed (multiple sprays were carried out for bigger leaves), with manual spreading using clean nitrile gloves (VWR International) if necessary, to ensure coating of the entire leaf surface. 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 µL 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 Krishnan et al. (2020). 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 dsRNA, monarch 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, growth, 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 h. 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 Krishnan et al. 2020).

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 ºC 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 6ºC, 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 µL 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 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 S1. 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 µL 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 dsRNA and monarch dsRNA were calculated from a standard curve fit with a 4-parameter logistic regression model (Figure S4). 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 (Figure 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: response ~ run + 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 dsRNA and 2.1 and 21 mg/mL Varroa dsRNA) and bioassay run were analyzed. Measured concentrations for 2.1 (1X) and 21 (10X) mg/mL Varroa dsRNA ranged from 0.013 to 0.032 and 0.144 to 0.389 mg/g, respectively. The measured concentration of monarch dsRNA ranged from 0.019 to 0.020 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 dsRNA ranged from 0.020 to 0.065 and 0.143 and 0.317 mg/g, respectively. The measured concentration of monarch 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 dsRNA-treated, and 2.1 and 21 mg/mL Varroa dsRNA-treated tropical milkweed leaves had 20 (± 10), 23 (± 6), 33 (± 21), 17 (± 21), and 13 (± 6) mean (± 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). While mortality was spread across multiple days for all treatments (excluding potassium arsenate, which killed all treated larvae within five days), there were some trends in time to 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 (Figure 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 found no differences ($\chi^2 = 4.97$; df = 4; $p = 0.290$).

With both milkweed species, the monarch and Varroa dsRNA treatments did not delay larval development from first through fifth instar and fifth instar to pupae (Table 3). The mean (± SD) developmental time from neonate to pupae ranged from 11.2 (± 0.95) to 11.6 (± 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 (± 0.67) to 11.5 (± 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 (± SD) monarch pupal weights between treatments in the common

and tropical milkweed bioassays ranged from 1140 (± 168) to 1218 (± 145) mg and 936 (± 162) to 1006 (± 208) mg, respectively (Figure 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 (Figure 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 (Figure 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 (± SE) eclosion rate of uninfected pupae ranged from 0.85 (± 0.07) to 0.97 (± 0.03) and 0.95 (± 0.05) to 1.0 (± 0.0) in common and tropical milkweed bioassays, respectively (Figure 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

Varroa mites are thought to be a significant stressor causing honey bee decline (Rosenkranz et al. 2010). The mites attach to bees, transmit viruses, and consume the bees' fat bodies and, to a lesser extent, hemolymph (Ramsey et al. 2019). The fat body is integral for

immune function, pesticide detoxification, hormone regulation, and enhanced overwintering survival (Arrese and Soulages 2010). Impairment of fat body function in a sufficient percentage of bees in a hive can contribute to colony declines (Ramsey et al. 2019). Several control methods are used to reduce Varroa mite populations. Currently, the most effective and economical method is to employ chemical miticides (Rinkevich et al. 2017). In the U.S., there are currently 15 miticides approved for controlling Varroa mites in beehives (USEPA 2018). Due to the heavy reliance on these products, Varroa mites have developed resistance to several compounds (Milani et al. 1995, 1999; Elzen et al. 2000; Spreafico et al. 2001), primarily due to enhanced metabolism and/or target site insensitivity (Kanga et al. 2016). Three of the insecticides for which there are no reported Varroa mite resistance — formic acid, oxalic acid, and thymol — could harm bees by inducing toxicity (Mattila et al. 2000; Aliano et al. 2006; Martín-Hernández et al. 2007; Giovenazzo et al. 2011), causing stress (Gunes et al. 2017), and affecting brood development (Higes et al. 1999; Ostermann et al. 2004; Boncristiani et al. 2012). Hence, there is a need to develop new miticides that specifically target Varroa mites without negatively affecting honey bees.

The development of dsRNA insecticides creates the means to selectively target insect pest species. It has been hypothesized that a dsRNA could be efficacious if it shares a minimum sequence of 19-21 nucleotides with the target insect mRNA (Whyard et al. 2009; Bachman et al. 2013) Previously published work (Garbian et al. 2012) with a mixture of Varroa dsRNA sequences that targeted housekeeping genes and genes involved in inhibiting apoptosis in Varroa mites had demonstrated efficacy as a dietary miticide. These authors elucidated the exposure pathway by using a dsRNA marker that carried a segment of the green fluorescent protein (GFP). Adult honey bees ingested the dsRNA-GFP added to a sucrose solution and mites attached to the

bees were exposed to the dsRNA when they fed on the adults' hemolymph (Garbian et al. 2012). The Varroa dsRNA examined in this paper is formulated in an 80% sucrose solution and the mites within the brood cells could be exposed 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. The dsRNA has a 99% nucleotide match to the Varroa mite calmodulin mRNA (Figure 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 dsRNA and the honey bee genome (Figure S5). Previous studies have shown that honey bees are mostly insensitive to orally delivered dsRNA (Tan et al. 2016), including dsRNA molecules that have a 100% sequence match to their mRNA (Vélez et al. 2016).

The Varroa dsRNA examined in this study shares a 21-contiguous nucleotide match with the monarch butterfly mRNA, which raises the possibility that the Varroa dsRNA may be toxic to monarchs. While bioinformatic analyses (e.g., base pair matches) can screen for potential dsRNA sensitivity to target species (and insensitivity to non-target species), there may be other barriers including refractory genes, presence of dsRNase, and exposure to low environmental concentrations that may prevent RNAi-mediated effects (Terenius et al. 2011; Bachman et al. 2013; Peng et al. 2019).

In the present study, monarch butterfly larvae were 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 0.281 mg/g leaf (10X treatment). In the common milkweed bioassays,

overall larval mortality was higher in the Varroa 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 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 dsRNA solutions (ca. 15%). The Iowa State University monarch butterfly colony has a historical mortality rate of 20 to 25% from neonate to pupation.

There were also no significant differences when mortality was averaged across both milkweed species. While monarch 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 morality rate and comparisons of mortality rates between control and dsRNA-treated leaves, the Varroa dsRNA at a dietary concentration 10X higher than would be expected in the environment is essentially non-toxic. The monarch 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. Larvae feeding on tropical and common

milkweed had similar responses to dsRNA treatment, suggesting that different levels of cardenolides in common and tropical milkweed (Petschenka and Agrawal 2015) seemingly do not alter the toxicity of dsRNA molecules through differential metabolic capability of the larvae.

There was no correlation between measured leaf concentration and average mortality rate for any of the treatments ($p \geq 0.19$; Figure 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 dsRNA and the 2.1 and 21 mg/mL Varroa 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 dsRNA and 0.25 and 1.8 mg of Varroa dsRNA, respectively, for the 1X and 10X Varroa 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 ß1 integrin dsRNA or 3×10^{-3} mg chitin synthase dsRNA (both dsRNA molecules targeted the individual species' mRNA) had 50 to 100% mortality (Mohamed and Kim 2011; Rana et al. 2020). 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/cm^2 treated leaves (dose not provided) for 10 days (Mao et al. 2014). 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 ºC, respectively (Rawlins and Lederhouse 1981; Zalucki 1982). 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 (Figure 3). Finally, there was no effect of Varroa or monarch 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 Pan et al. (2017) who fed first-instar monarchs dsRNA derived from monarch *v-ATPase A* mRNA for two days and then provided the larvae untreated 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 (*Cynanchum laeve*), which, in some cases, can delay larval

development (Pocius et al. 2017a, 2017b). The lack of significant effects observed by Pan et al. (2017) 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 dsRNA was eliminated prior to critical developmental windows (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 dsRNA 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 Terenius et al. (2011), 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. Shukla et al. (2016) 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 on 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. Choi and Vander Meer (2018) 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 (Kumar et al. 2009). Whyard et al. (2009) 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, Peng et al. (2019) showed that tobacco cutworm larvae that fed on cabbage leaves had greater dsRNA-degrading activity than larvae that were reared on an artificial diet. The 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 gut pH and/or the presence of dsRNases in the gut. RNA is most stable at a pH of 4.0 to 5.0. Lepidopterans have a gut pH greater than 8.0, which suggests dsRNA molecules may be unstable in this environment (Romeis and Widmer 2020). 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 (Baum and Roberts 2014; Peng et al. 2019; Romeis and Widmer 2020). If the monarch gut contains ribonucleases, it could further reduce the internal dsRNA dose below a level needed to silence mRNA signaling. Low dietary concentrations could be another potential factor responsible for observations of non-toxic dsRNA effects in Lepidoptera. For example, Terenius et al. (2011) observed that dietary dsRNA insecticides silenced genes at only high concentrations. We used a 5 mg/mL monarch 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 LC_{50}s (lethal concentration 50) often exceeding 1.0 mg/g (Baum and Roberts 2014; Romeis and Widmer 2020).

While our results show that monarch larvae exposed to dsRNA through their diet are unlikely to show adverse effects, application of foliar dsRNA insecticides also could result in cuticular exposure. Penetration and absorption of dsRNA through the cuticle could bypass gut nucleases and alkalinity (Baum and Roberts 2014). For example, Wang et al. (2011) 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 (Taning 2020). For example, Miguel and Scott (2015) 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. Commercial production and application of foliar dsRNA insecticides could result in spray drift exposure to non-target organisms near agricultural fields (Romeis and Widmer 2020), including monarch larvae.

Monarch butterfly populations have declined in the last two decades (Brower et al. 2012; Semmens et al. 2016), and the U.S. Fish and Wildlife Services recently listed it as a candidate species under the Endangered Species Act (USFWS 2020). Other non-target Lepidopteran populations are also declining (Dirzo et al. 2014; Habel et al., 2016; Sánchez-Bayo and Wyckhuys 2019). 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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Data availability

Data and metadata pertaining to this manuscript will be made publicly available at this GitHub repository following acceptance of manuscript: https://github.com/Niranjana296/Evaluating-Toxicity-of-Varroa-dsRNA-to-Monarch-Larvae

Author contributions

NK conducted the toxicity bioassays, analyzed its results, and wrote the first draft of the paper. MJH quantified concentrations in the leaf samples, analyzed its results, and wrote associated components of the paper. All authors were involved in designing the study and editing the manuscript.

References

Agrawal N, Dasaradhi PVN, Mohmmed A, Malhotra P, Bhatnagar RK, Mukherjee SK. RNA interference: biology, mechanism, and applications. Microbiol Mol Biol Rev. 2003; 12. 67 (4): 657–685.

Aliano NP, Ellis MD, Siegfried SD. Acute contact toxicity of oxalic acid to Varroa Destructor (Acari: Varroidae) and Their Apis Mellifera (Hymenoptera: Apidae) hosts in laboratory bioassays. J Econ Entomol. 2006; 10. 99 (5): 1579–1582.

Arrese EL, Soulages JL. Insect fat body: Energy, metabolism, and regulation. Annu Rev Entomol. 2010; 1. 55: 207–225.

Bachman PM, Bolognesi R, Moar WJ, Mueller GM, Paradise MS, Ramaseshadri P, et al. Characterization of the spectrum of insecticidal activity of a double-stranded RNA with targeted activity against Western Corn Rootworm (Diabrotica Virgifera Virgifera LeConte). Transgenic Res. 2013; 6. 22 (6): 1207–1222.

Baum JA, Roberts JK. Progress towards RNAi-mediated insect pest management. Adv Insect Physiol. 2014. 47, 249–295.

Bolognesi R, Ramaseshadri P, Anderson A, Bachman PM, Clinton W, Flannagan R, et al. Characterizing the mechanism of action of double-stranded RNA activity against Western Corn Rootworm (Diabrotica Virgifera Virgifera LeConte). PLOS ONE. 2012; 10. 7 (10): e47534.

Boncristiani H, Underwood R, Schwarz R, Evans JD, Pettis J, vanEngelsdorp D. Direct effect of acaricides on pathogen loads and gene expression levels in Honey Bees Apis Mellifera. J Insect Physiol. 2011; 12. 58 (5): 613–620.

Brower LP, Taylor OR, Williams EH, Slayback DA, Zubieta RR, Ramírez MI. decline of monarch butterflies overwintering in Mexico: Is the migratory phenomenon at risk? decline of monarch butterflies in Mexico. Insect Conserv Divers. 2011; 3. 5 (2): 95–100.

Choi MY, Vander Meer RK. Phenotypic Effects of PBAN RNAi Using oral delivery of dsRNA to Corn Earworm (Lepidoptera: Noctuidae) and Tobacco Budworm Larvae. *J Econ Entomol*. 2019; 2. 112 (1): 434–439.

Dirzo R, Young HS, Galetti M, Ceballos G, Isaac NJB, Collen B. Defaunation in the Anthropocene. Science. 2014; 7. 345 (6195): 401–406.

Elzen PJ, Baxter JR, Spivak M, Wilson WT. Control of Varroa Jacobsoni Oud. resistant to fluvalinate and amitraz using coumaphos. Apidologie. 2000; 5. 31 (3): 437–441.

Garbian Y, Maori E, Kalev H, Shafir S, Sela I. Bidirectional transfer of RNAi between honey bee and Varroa Destructor: Varroa gene silencing reduces Varroa population. PLOS Pathog. 2012; 12. 8 (12): e1003035.

Giovenazzo P, Dubreuil P. Evaluation of spring organic treatments against Varroa Destructor (Acari: Varroidae) in honey bee Apis Mellifera (Hymenoptera: Apidae) colonies in eastern Canada. Exp Appl Acarol. 2011; 9. 55 (1): 65–76.

Gunes N, Aydın L, Belenli D, Hranitz JM, Mengilig S, Selova S. Stress responses of honey bees to organic acid and essential oil treatments against Varroa mites. J Apic Res. 2017; 3. 56 (2): 175–181.

Habel JC, Segerer A, Ulrich W, Torchyk O, Weisser WW, Schmitt T. Butterfly community shifts over two centuries. Conserv Biol. 2016; 8. 30 (4): 754–762.

Higes M, Meana A, Suárez M, Llorente J. Negative long-term effects on bee colonies treated with oxalic acid against Varroa Jacobsoni Oud. Apidologie. 1999. 30 (4): 289–292.

Inberg A, Mahak K, inventors. Monsanto Technology LLC and Beeologics, Inc. U.S. Patent 62156751. May 3, 2016.

Kanga, LHB, Marshall K, Legaspi JC. Mechanisms of insecticide resistance in field populations of the Varroa mite (Acari: Mesostigmata: Varroidae) in Florida. Fla Entomol. 2016; 6. 99 (2): 324–326.

Krishnan N, Zhang Y, Bidne KG, Hellmich RL, Coats JR, Bradbury SP. Assessing field-scale risks of foliar insecticide applications to monarch butterfly (*Danaus Plexippus*) larvae. Environ Toxicol Chem. 2020; 4. 39 (4): 923–941.

Kumar M, Gupta GP, Rajam MV. Silencing of acetylcholinesterase gene of Helicoverpa armigera by siRNA affects larval growth and its life cycle. J Insect Physiol. 2009; 3. 55 (3); 273-278.

Mamta B, Rajam MV. RNAi Technology: A new platform for crop pest control. Physiol Mol Biol Plants. 2017; 3. 23 (3): 487–501.

Mao J, Zhang P, Liu C, Zeng F. Co-Silence of the coatomer β and V-ATPase A genes by siRNA feeding reduces larval survival rate and weight gain of cotton bollworm, Helicoverpa Armigera. 2015. Pesticide Biochemistry and Physiology. 118, 71–76.

Marshansky V, Hosokawa H, Merkulova M, Bakulina A, Dip PV, Thaker YG, et al. Structural model of A2-bubunit N-terminus and its binding interface for Arf-GEF CTH2: Implication for regulation of V-ATPase, CTH2 function and rational drug design. Curr Top Membr. 2019; 3. 83: 77–106.

Martín-Hernández R, Higes M, Pérez JL, Nozal MJ, Gómez L, Meana A. Short-term negative effect of oxalic acid in Apis Mellifera Iberiensis. Span J Agricul Res. 2007. 5 (4): 474–480.

Mattila HR, Otis GW, Daley J, Schulz T. Trials of Apiguard, a thymol-based miticide. 2. Non-target effects on honey bees. Ame Bee J. 1999; 12. 140(1): 68-70.

Miguel KS, Scott JG. The next generation of insecticides: dsRNA is stable as a foliar-applied insecticide. Pest Manag Sci. 2016; 4. 72 (4): 801–809.

Milani N. The resistance of Varroa Jacobsoni Oud to pyrethroids: A laboratory assay. Apidologie.1995; 11. 26 (5): 415–429.

Milani N. The resistance of Varroa Jacobsoni Oud to acaricides. Apidologie. 1999. 30 (2-3): 229–234.

Mohamed AAM, Kim Y. A target-specific feeding toxicity of β1 integrin dsRNA against diamondback moth, Plutella Xylostella. 2011. Arch Insect Biochem Physiol. 78 (4), 216–230.

Ostermann DJ, Currie RW. Effect of formic acid formulations on honey bee (Hymenoptera: Apidae) colonies and influence of colony and ambient conditions on formic acid concentration in the hive. J Econ Entomol. 2004. 97 (5): 1500–1508.

Pan H, Yang X, Bidne KG, Hellmich RL, Siegfried BD, Zhou X. Dietary risk assessment of V-ATPase A dsRNAs on monarch butterfly larvae. 2017; 2. Front Plant Sci.

Pasquinelli AE. MicroRNAs: Deviants no longer. Trends Genet. 2002; 4. 18 (4): 171–173.

Peng Y, Wang K, Zhu G, Han Q, Chen J, Elzaki MEA, et al. Identification and characterization of multiple dsRNAses from a lepidopteran insect, the tobacco cutworm, Spodoptera Litura (Lepidoptera: Noctuidae). Pestic Biochem Physiol. 2020; 1. 162 (1): 86–95.

Petschenka G, Agrawal AA. Milkweed butterfly resistance to plant toxins is linked to sequestration, not coping with a toxic diet. Proc Royal So B. 2015; 11. 282 (1818): 20151865.

Pocius VM, Debinski DM, Pleasants JM, Bidne KG, Hellmich RL, Brower LP. Milkweed matters: monarch butterfly (Lepidoptera: Nymphalidae) survival and development on nine Midwestern milkweed species. Environ Entomol. 2017a; 10. 46 (5): 1098–1105.

Pocius VM, Debinski DM, Bidne KG, Hellmich RL, Hunter FK. Performance of early instar monarch butterflies (*Danaus Plexippus* L.) on nine milkweed species native to Iowa. J Lepid Soc. 2017b; 9. 71 (3): 153–161.

Ramsey SD, Ochoa, R Bauchan G, Gulbronson C, Mowery JD, Cohen A, et al. Varroa Destructor feeds primarily on honey bee fat body tissue and not hemolymph. Proc Natl Acad Sci. 2019; 1. 116 (5): 1792–1801.

Rana S, Rajurkar AB, Kumar KK, Mohankumar S. Comparative analysis of chitin synthase A dsRNA-mediated RNA interference for management of crop pests of different families of Lepidoptera. 2020. Front Plant Sci. 11, 427.

Rawlins J, Lederhouse R. Developmental influences of thermal behavior on monarch caterpillars (Danaus Plexippus): An adaptation for migration (Lepidoptera: Nymphalidae: Danainae). J Kans Entomol Soc. 1981; 4. 54 (2): 387–408.

Rinkevich FD, Danka RG, Healy KB. Influence of Varroa mite (Varroa Destructor) management practices on insecticide sensitivity in the honey bee (Apis Mellifera). Insects. 2017; 3. 8 (1): 9.

Romeis J, Widmer F. Assessing the risks of topically applied dsRNA-based products to non-target arthropods. Front Plant Sci. 2020; 4. 11: 679.

Rosenkranz P, Aumeier P, Ziegelmann B. Biology and control of Varroa Destructor. J Invertebr Pathol. 2010; 1. 103 Suppl 1 (1): S96-119.

Sánchez-Bayo F, Wyckhuys KAG. Worldwide decline of the entomofauna: A review of its drivers. Biol Conserv. 2019; 4. 232 (4): 8–27.

Semmens BX, Semmens DJ, Thogmartin WE, Wiederholt R, López-Hoffman L, Diffendorfer JE, et al. Quasi-extinction risk and population targets for the eastern, migratory population of monarch butterflies (Danaus Plexippus). Sci Rep. 2016; 3. 6 (1): 23265.

Shukla JN, Kalsi M, Sethi A, Narva KE, Fishilevich E, Singh S, et al. Reduced stability and intracellular transport of dsRNA contribute to Poor RNAi response in lepidopteran insects. RNA Biol.2016; 7. 13 (7): 656–669.

Spreafico M, Francesca RE, Bernardinelli I, Colombo M. First detection of strains of Varroa Destructor resistant to coumaphos. Results of laboratory tests and field trials. Apidologie. 2001; 1. 32 (1): 49–55.

Tan J, Levine SL, Bachman PM, Jensen PD, Mueller GM, Uffman JP, et al. No impact of DvSnf7 RNA on honey bee (*Apis Mellifera* L.) adults and larvae in dietary feeding tests. Environ Toxicol Chem. 2016; 2. 35 (2): 287–294.

Taning CNT, Arpaia S, Christiaens O, Dietz-Pfeilstetter A, Jones H, Mezzetti B, et al. RNA-based biocontrol compounds: current status and perspectives to reach the market. Pest Manag Sci. 2020. 76, 841–845.

Terenius O, Papanicolaou A, Garbutt JS, Eleftherianos I, Huvenne H, Kanginakudru S, et al. RNA interference in Lepidoptera: An overview of successful and unsuccessful studies and implications for experimental design. J Insect Physiol. 2011; 2. 57 (2): 231–245.

U.S. Environmental Protection Agency. Microbial Pesticide Test Guidelines: OPPTS 885.4340 Nontarget Insect Testing, Tier I. 2009 [cited 2020 Nov 19]. Available from: https://www.regulations.gov/document?D=EPA-HQ-OPPT-2009-0159-0036.

U.S. Environmental Protection Agency. OCSPP 850.3000: Background and special considerations - tests with terrestrial beneficial insects, invertebrates and microorganisms. 2012 [cited 2020 Nov 19]. Available from: https://www.regulations.gov/document?D=EPA-HQ-OPPT-2009-0154-0015.

U.S. Environmental Protection Agency. Pesticide Product Label, MON 87411. 2015 [cited 2020 Nov 19]. Available from: https://www.google.com/url?sa=t&rct=j&q=&esrc=s&source=web&cd=&ved=2ahUKEwit3bnGqZDtAhXCPM0KHUSnDA0QFjAAegQIBRAC&url=http%3A%2F%2Fwww3.epa.gov%2Fpesticides%2Fchem_search%2Fppls%2F000524-00618-20151029.pdf&usg=AOvVaw0SyXioRo41--vj0_fE6jUz

U.S. Environmental Protection Agency. EPA-registered pesticide products approved for use against Varroa mites in bee hives. 2018 [cited 2020 Nov 19]. Available from: https://www.epa.gov/pollinator-protection/epa-registered-pesticide-products-approved-use-against-varroa-mites-bee-hives

U.S. Fish and Wildlife Service. Assessing the status of the monarch butterfly. 2020 [cited 2020 Dec 22]. Available from: https://www.fws.gov/savethemonarch/ssa.html

Vélez AM, Jurzenski J, Matz N, Zhou X, Wang H, Ellis M, Siegfried BD. Developing an in vivo toxicity assay for RNAi risk assessment in honey bees, Apis Mellifera L. Chemosphere. 2016; 2. 144 (2): 1083–1090.

Wang Y, Zhang H, Li H, Miao X. Second-generation sequencing supply an effective way to screen RNAi targets in large scale for potential application in pest insect control. PLOS ONE. 2011; 4. 6(4): e18644.

Whyard S, Singh AD, Wong S. Ingested double-stranded RNAs can act as species-specific insecticides. Insect Biochem Mol Biol. 2009; 11. 39 (11): 824–832.

Zalucki MP. Temperature and rate of development in Danaus Plexippus L. and D. Chrysippus L. (Lepidoptera:Nymphalidae). Aust. J Entomol. 1982; 11. 21 (4): 241–246.

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 (± 0.139)	0.312 (± 0.274)	0.281 (± 0.102)
Tropical milkweed	Monarch	0.036 (± 0.006)	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	0.317 (± 0.062)	0.143 (± 0.036)	0.173 (± 0.090)	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 dsRNA = 5 mg/mL monarch dsRNA solution concentration; 1X and 10X Varroa dsRNA = 2.1 and 21 mg/mL Varroa dsRNA solution concentrations, respectively.

Table 2. Monarch larval percent mortality following treatment with Varroa dsRNA and two positive and two negative controls[a]

Milkweed species (# of larvae treated)	Treatment	Larval percent mortality[b]			
		Run 1	Run 2	Run 3	Mean (± SD)[c]
Common milkweed (15 larvae used per treatment for a total of 90 larvae per run)	Untreated	20	7	27	18 (± 10)
	Water	33	15	33	27 (± 10)
	Monarch dsRNA	27	40	33	33 (± 7)
	1X Varroa dsRNA	40	60	20	40 (± 20)
	10X Varroa dsRNA	27	50	40	39 (± 12)
	Potassium arsenate	100	100	100	100 (± 0)
Tropical milkweed (10 larvae used per treatment for a total of 60 larvae per run)	Untreated	10	20	30	20 (± 10)
	Water	30	20	20	23 (± 6)
	Monarch dsRNA	10	40	50	33 (± 21)
	1X Varroa dsRNA	10	40	0	17 (± 21)
	10X Varroa dsRNA	10	20	10	13 (± 6)
	Potassium arsenate	100	100	100	100 (± 0)

[a] Monarch larvae were fed untreated leaves and leaves treated with deionized water, 5 mg/mL monarch dsRNA solution, 2.1 (1X) and 21 (10X) mg/mL Varroa 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.

Table 3. Monarch larval development following treatment with Varroa 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 (± SD) days to pupae[c]
		Day 4: Third instar	Day 8: Fifth instar	Day 12: Pupae	
Common milkweed (45 larvae used per treatment across 3 runs)	UN	57	86	86	11.2 (± 1.0)
	WT	68	68	87	11.6 (± 1.1)
	MB	57	87	93	11.2 (± 0.95)
	VL	70	67	93	11.3 (± 0.88)
	VH	63	78	93	11.3 (± 0.96)
Tropical milkweed (30 larvae used per treatment across 3 runs)	UN	63	92	92	11.5 (± 0.88)
	WT	70	87	83	11.5 (± 1.2)
	MB	55	90	100	11.2 (± 0.67)
	VL	64	84	84	11.4 (± 1.1)
	VH	65	92	85	11.4 (± 1.0)

[a] Monarch larvae were fed untreated leaves (UN) and leaves treated with deionized water (WT), 5 mg/mL monarch dsRNA solution (MB), and 2.1 (VL) and 21 (VH) mg/mL Varroa 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.

Table S1. 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]: Sigma-Aldrich, St. Louis, MO, USA UltraPure™ water [Ref #:10977-015]: Invitrogen by Life Technologies, Grand Island, NY, USA	1:1000 dilution of Baker's Yeast Solution made with UltraPure™ water [e.g. 100 µl of Baker's Yeast stock solution and 100 mL of UltraPure™ water] (Baker's yeast stock solution: 10 mg of Baker's yeast RNA and 1 mL of UltraPure™ water)	Keep both stock solution and diluted solution refrigerated
Working solution	UltraPure™ water [Ref #:10977-015]: Invitrogen by Life Technologies, Grand Island, NY, USA	5.3 mL UltraPure™ Water 3.8 mL lysis mixture* 115 µL blocking reagent*	Vortex 10 sec. Make fresh daily. Makes enough for 1 plate.

Table S1 continued

Wash buffer	Lysis mixture [Ref #:10093]: Invitrogen by Thermo Fisher Scientific, Affymetrix Inc., Santa Clara, CA, USA Blocking reagent [Ref #:13254]: Invitrogen by Thermo Fisher Scientific, Affymetrix Inc., Santa Clara, CA, USA Buffer component #1 [Ref #:10842]: Invitrogen by Thermo Fisher Scientific, Affymetrix Inc., Santa Clara, CA, USA Buffer component #2 [Ref #:10845]: Invitrogen by Thermo Fisher Scientific, Affymetrix Inc., Santa Clara, CA, USA Nuclease-free water [CAT #:9153-1]: RICCA Chemical Company, Arlington, TX, USA	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.
Pre-amplifier solution	Amplifier/label probe diluent [Ref #:14539]: Invitrogen by Thermo Fisher Scientific, Affymetrix Inc., Santa Clara, CA, USA Pre-amplifier reagent [Ref #:15094]: Invitrogen by Thermo Fisher Scientific, Affymetrix Inc., Santa Clara, CA, USA	11 mL Amplifier/label probe diluent* 11 µL pre-amplifier reagent*	Vortex 10 sec. Mix prior to use. Makes enough for 1 plate.
Amplifier solution	Amplifier/label probe diluent [Ref #:14539]: Invitrogen by Thermo Fisher Scientific, Affymetrix Inc., Santa Clara, CA, USA Amplifier reagent [Ref #:15097]: Invitrogen by Thermo Fisher Scientific, Affymetrix Inc., Santa Clara, CA, USA	11 mL Amplifier/label probe diluent* 11 µL amplifier reagent*	Vortex 10 sec. Mix prior to use. Makes enough for 1 plate.

Table S1 continued

Label probe Solution	Amplifier/label probe diluent [Ref #:14539]: Invitrogen by Thermo Fisher Scientific, Affymetrix Inc., Santa Clara, CA, USA Label probe reagent [Ref #:10087]: Invitrogen by Thermo Fisher Scientific, Affymetrix Inc., Santa Clara, CA, USA	11 mL Amplifier/label probe diluent* 11 µL label probe reagent*	Vortex 10 sec. Mix prior to use. Makes enough for 1 plate.

*Provided as part of the QuantiGene® 2.0 Singleplex Assay kit.

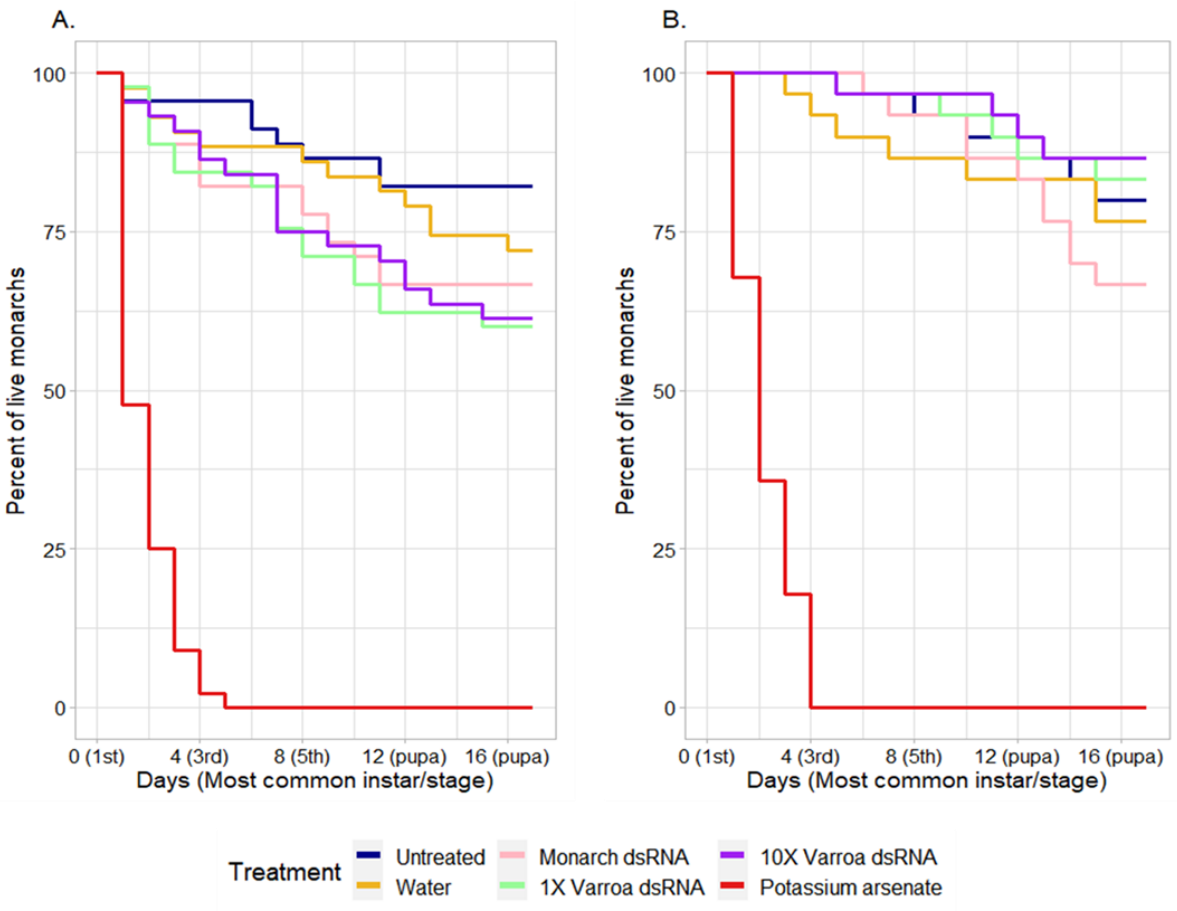

Figure 1: Monarch mean percent mortality over time, from neonate larvae to pupae, with data combined over all bioassay runs. Larvae were fed common (A) or tropical (B) milkweed leaves that were untreated (UN), treated with deionized water (WT), 5 mg/mL monarch dsRNA solution (MB), 2.1 (VL) and 21 (VH) 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.

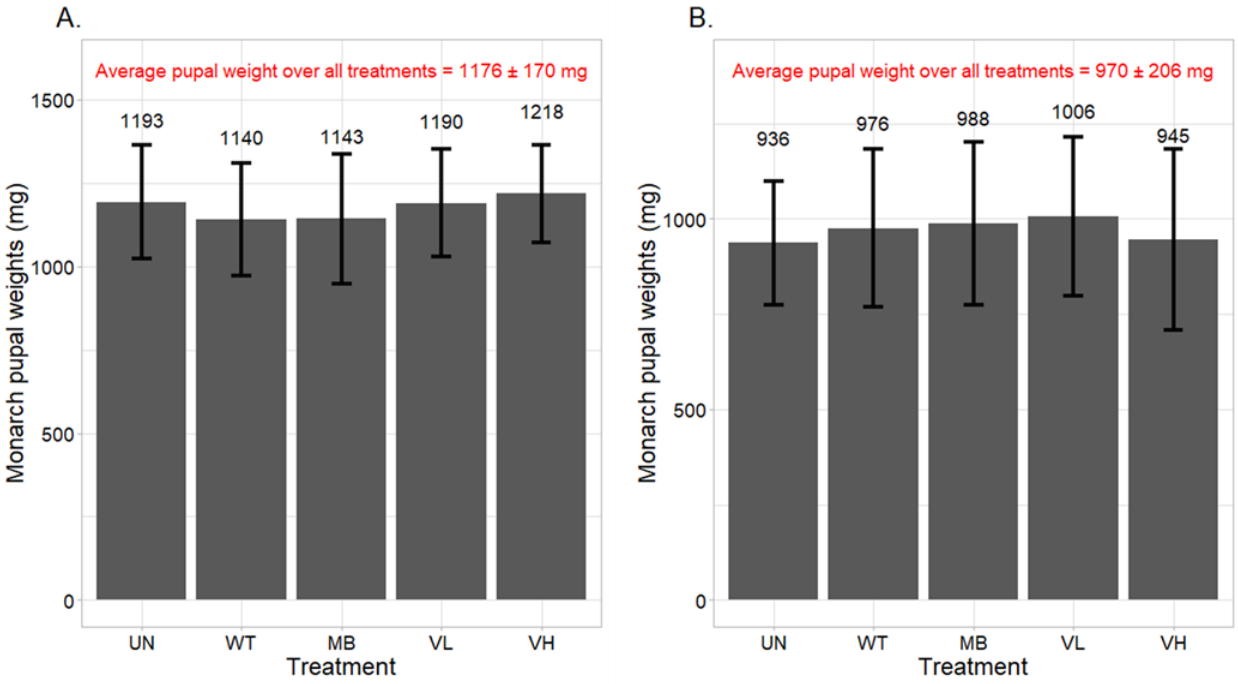

Figure 2: Average monarch pupal weight (in mg) in each treatment (data combined over all bioassay runs). Larvae were fed common (A) or tropical (B) milkweed leaves that were untreated (UN), treated with deionized water (WT), 5 mg/mL monarch dsRNA solution (MB), or 2.1 (VL) and 21 (VH) mg/mL Varroa dsRNA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

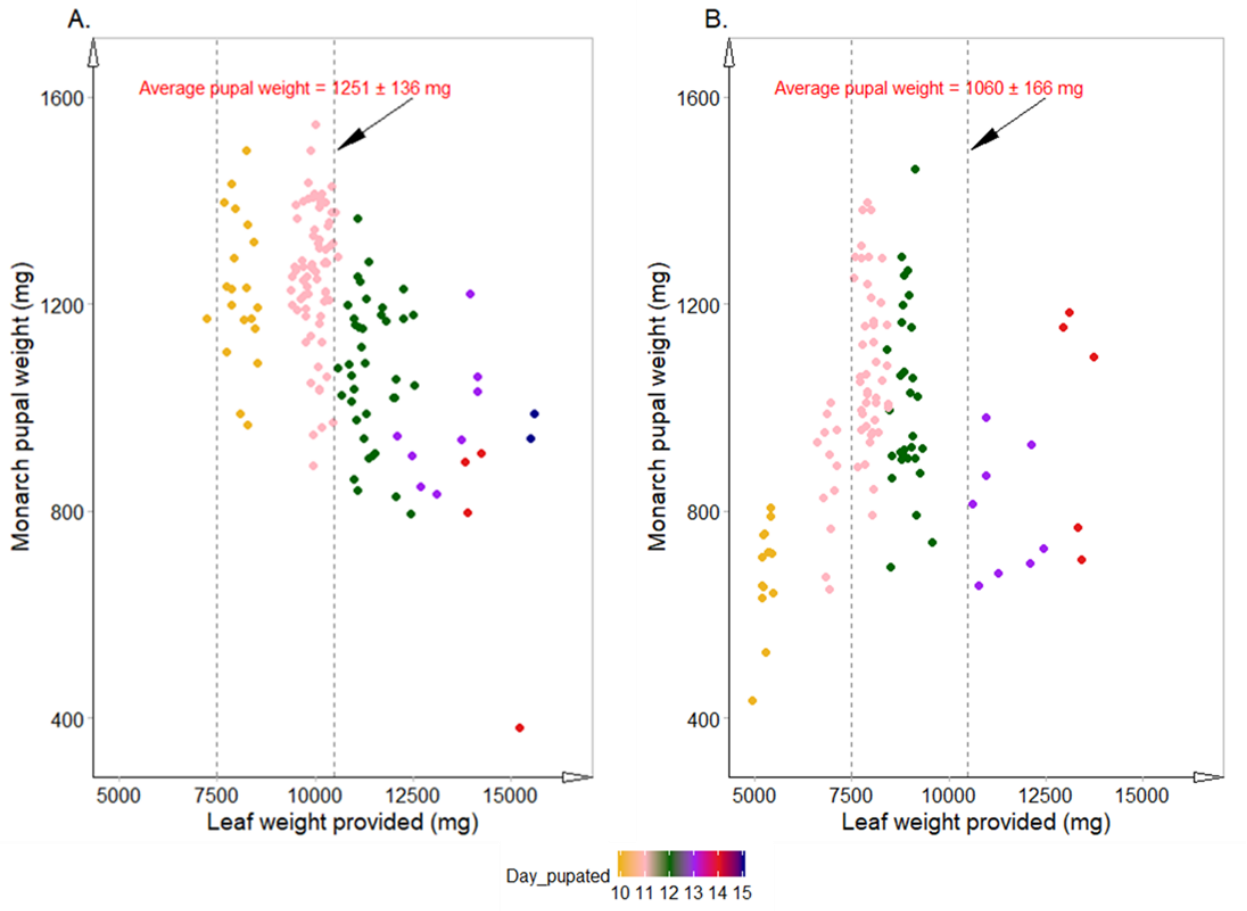

Figure 3: Individual monarch pupal weights (mg) plotted against individual weights (mg) of common (A) and tropical (B) milkweed leaf provided to each larva. Data were combined over all treatments and bioassay runs. The different colored dots represent the range of days it took the monarchs to pupate (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.

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gaacugcagg acaugaucaa cgaggucgac gccgacggcu ccggaacgau agauuucccu 180

gaguuccuca caaugaugca agaaagauga aggacaccga cucggaggag gagaucgaga 240

ggcguuccgc guauucgaca aggaugcaac gguuucauuu cggcggccga gcucaggcac 300

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gcagauauug ac 372

Figure S1. Sequence of the Varroa dsRNA (Inberg and Mahak 2016).

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

Sequence ID: XM_022799184.1 **Length:** 1498 **Number of Matches:** 1

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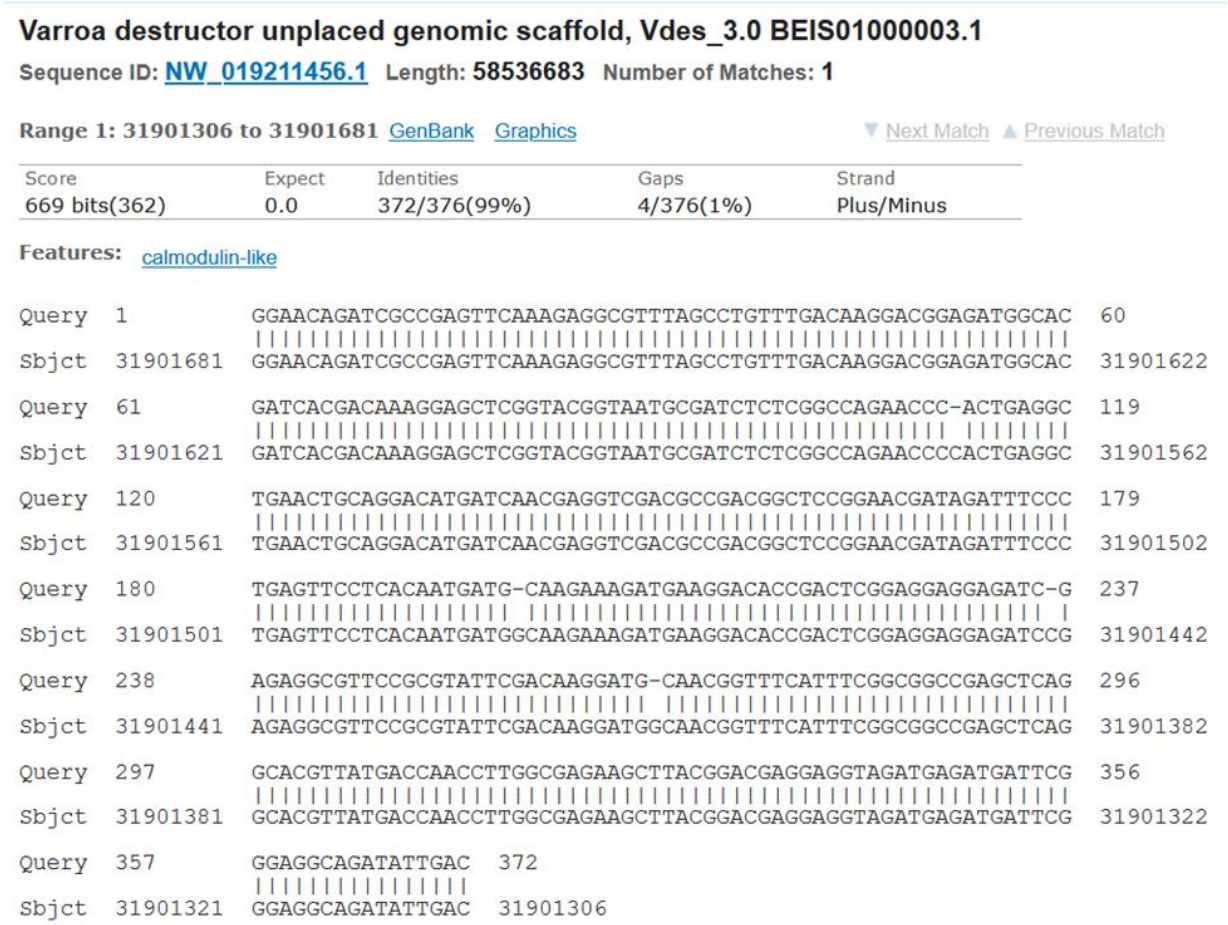
A. Closest predicted sequence match

Figure S2. Varroa dsRNA closest predicted sequence match and location in Varroa mite genome.



Varroa destructor unplaced genomic scaffold, Vdes_3.0 BEIS01000003.1

Sequence ID: NW_019211456.1 **Length:** 58536683 **Number of Matches:** 1

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Features: calmodulin-like

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

Figure 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.

Danaus plexippus plexippus isolate F-2 chromosome 13, Dplex_v4, whole genome shotgun sequence

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calmodulin isoform X1

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

Figure S3. Varroa dsRNA comparison to monarch butterfly sequences.

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

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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  TTCGACAAGGACGGCGATGGCACCATCACAAAGGAAGAGCTCGGGCGAGTCATGAGGAGC  2678086

Query  98       CTCGGCCAGAAC-CCACTGAGGCTGAACTGCAGGACATGATCAACGAGGTCGA  149
                |||||||||  | |||  |  |  ||  |||||||||||  |  | ||||||||
Sbjct  2678085  CTCGGCCAGTTCGCCAGGGTTGAGGAGTTGCAGGACATGTTGCAGGAGGTCGA  2678033
```

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  GGAACAGATCGCCGAGTTCAAGGAGGCGTTTTCACTGTTCGATAAAGATGGGGACGGCAC  363

Query  61   GATCACGACAAAGGAGCTCGGTACGGTAATGCGATCTCTCGGCCAGAACCC-ACTGAGGC  119
             |||||  || |||||||| || || || ||| | || || || || |||||||| || |||||
Sbjct  364  CATCACCACCAAGGAGCTGGGGACCGTGATGAGGTCGCTGGGACAGAACCCCACGGAGGC  423

Query  120  TGAACTGCAGGACATGATCAACGAGGTCGACGCCGACGGCTCCGGAACGATAGATTTCCC  179
             || || ||||||||||||||||||| ||||| || ||    ||| |||||||| |||||
Sbjct  424  CGAGCTCCAGGACATGATCAACGAGGTTGACGCTGATGGTAACGGTACGATAGACTTCCC  483

Query  180  TGAGTTCCTCACAATGATG-CAAGAAAGATGAAGGACACCGACTCGGAGGAGGAGATC-G  237
             || ||||| || |||||| |   | ||||||||||||| |||    |||||||||||  |
Sbjct  484  CGAATTCCTGACGATGATGGCGCGCAAGATGAAGGACACGGACAGCGAGGAGGAGATCCG  543

Query  238  AGAGGCGTTCCGCGTATTCGACAAGGATG-CAACGGTTTCATTTCGGCGGCCGAGCTCAG  296
             |||||||||||||| |||||||||| | |||||| ||||| || || || |||||   |
Sbjct  544  CGAGGCGTTCCGCGTGTTCGACAAGGACGGCAACGGATTCATATCCGCCGCGGAGCTGCG  603

Query  297  GCACGTTATGACCAACCTTGGCGAGAAGCTTACGGACGAGGAGGTAGATGAGATGATTCG  356
             ||||| |||||||||| || |||||||| || |||||||||| || |||||||| ||
Sbjct  604  TCACGTGATGACCAACCTCGGAGAGAAGCTCACCGACGAGGAGGTGGACGAGATGATCCG  663

Query  357  GGAGGCAGATATTGAC  372
             ||||| || || |||
Sbjct  664  CGAGGCCGACATCGAC  679
```

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

Figure S3 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.

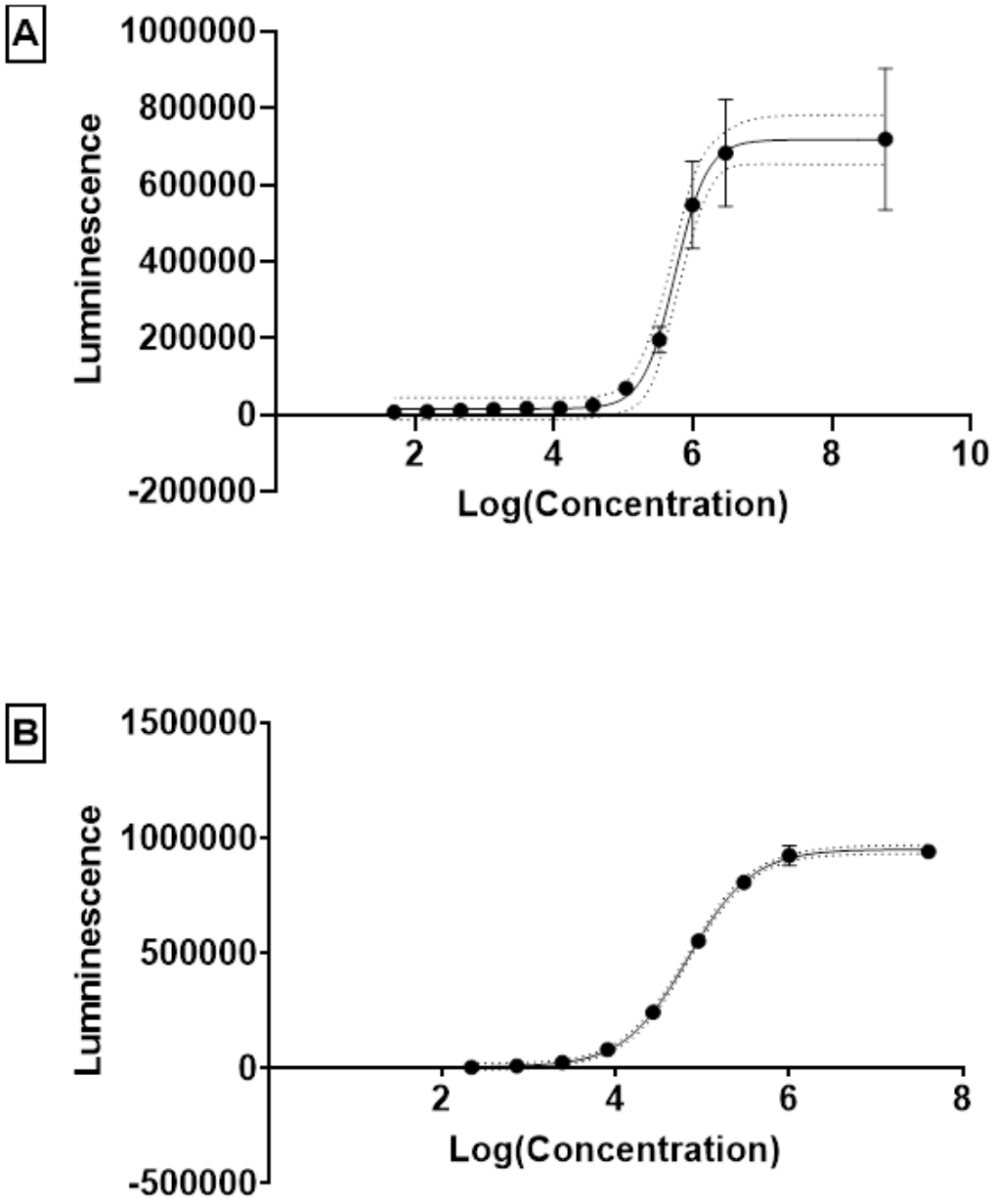


Figure S4. Representative QuantiGene calibration curves for monarch dsRNA (A) and Varroa dsRNA (B).

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      GGAACGATAGATTTCCCTGAGTTCCTCACAATGATG-CAAGAAAGATGAAGGACACCGAC  221
                || || || ||||| || || ||| | || |||||| |  | || ||||| || || ||
Sbjct  8579150  GGCACAATCGATTTTCCGGAATTCTTAACTATGATGGCTCGTAAAATGAAAGATACTGAT  8579209

Query  222      TCGGAGGAGGAGATC-GAGAGGCGTTCCGCGTATTCGACAAGGATGCAAC-GGTTTCATT  279
                   ||||| || ||  | ||||| ||| | ||||| || ||||||| ||  ||||||||
Sbjct  8579210  AGTGAGGAAGAAATTAGGGAGGCCTTCAGAGTATTTGATAAGGATGGAAATGGTTTCATA  8579269

Query  280      TCGGCGGCCGAGCTCAGGCACGTTATGACCAACCTTGGCGAGAAGCTTACGGACGAGGAG  339
                || || || || ||||| || |||||||| || |||||||||| || || || || ||
Sbjct  8579270  TCCGCAGCAGAACTCAGACATGTTATGACAAATCTTGGCGAGAAACTCACTGATGAAGAA  8579329

Query  340      GTAGATGAGATGATTCGGGAGGCAGATATTGA  371
                || ||||| |||||||||||||| || |||||
Sbjct  8579330  GTTGATGAAATGATTCGGGAGGCTGACATTGA  8579361
```

Apis mellifera strain DH4 linkage group LG8, Amel_HAv3.1

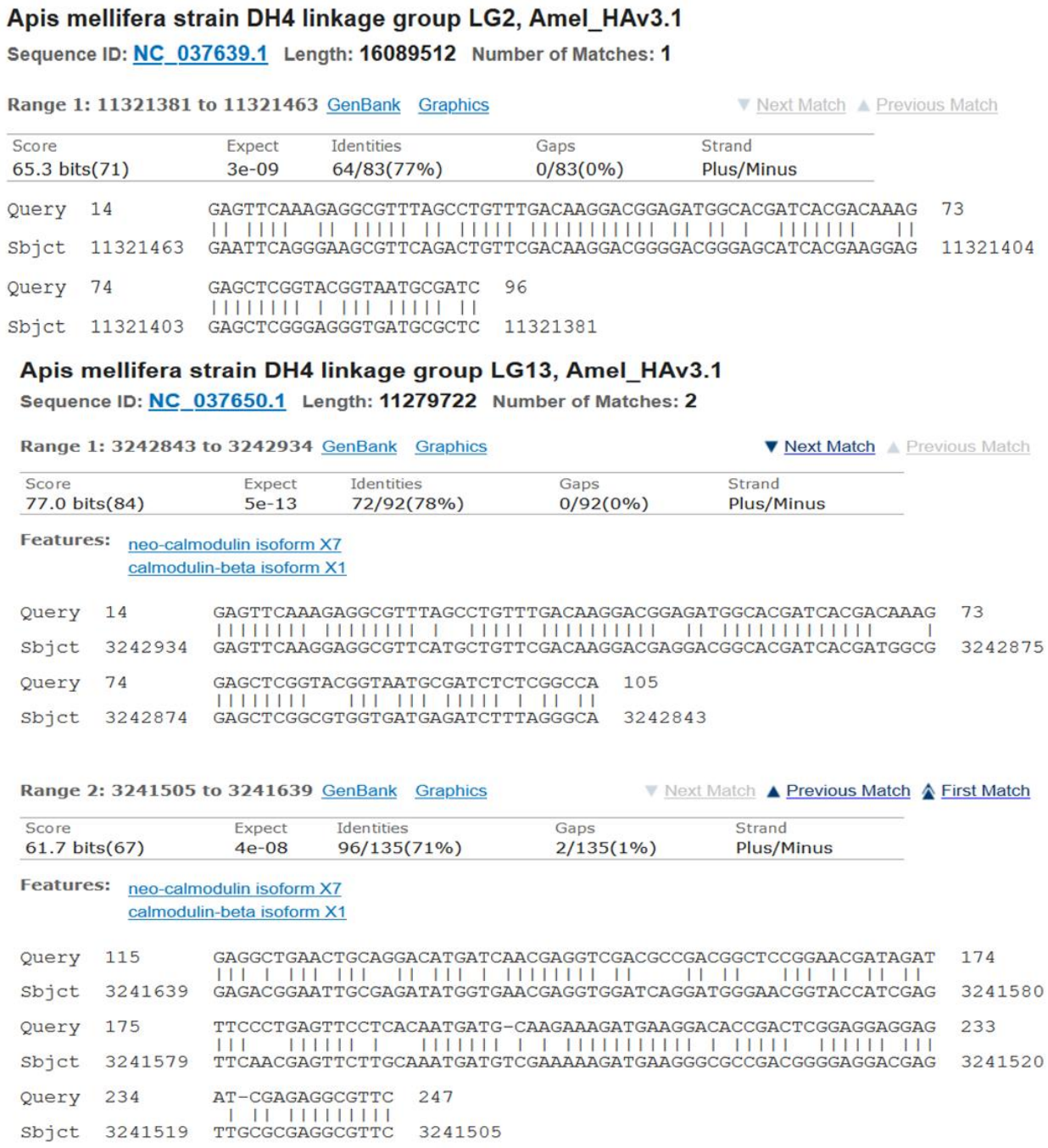
Sequence ID: NC_037645.1 **Length:** 12717210 **Number of Matches:** 1

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--CAACGGTTTCATTTCGGCG  285
                |||||||||||||  ||| |||||||||||||||
Sbjct  7596118  ATTCGACAAGGATGGGCAA-GGTTTCATTTCGGCG  7596085
```

Figure S5. Varroa dsRNA comparison to honeybee sequences.



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

Figure S5 continued

PREDICTED: Apis mellifera calmodulin (LOC551859), mRNA

Sequence ID: XM_006565254.3 **Length:** 1161 **Number of Matches:** 2

Range 1: 401 to 774 GenBank Graphics ▼ Next Match ▲ Previous I

Score	Expect	Identities	Gaps	Strand
224 bits(247)	3e-62	277/374(74%)	4/374(1%)	Plus/Plus

```
Query  2    GAACAGATCGCCGAGTTCAAAGAGGCGTTTAGCCTGTTTGACAAGGACGGAGATGGCACG  61
            ||||| ||||| || || || || || |||   || ||||| || || ||||||||  ||
Sbjct  401  GAACAAATCGCAGAATTTAAGGAAGCATTTTCACTATTTGATAAAGATGGAGATGGTACC  460

Query  62   ATCACGACAAAGGAGCTCGGTACGGTAATGCGATCTCTCGGCCAGAA-CCCACTGAGGCT  120
            ||||| || || ||| | ||||| || |||||||| || || || || ||||| || |||
Sbjct  461  ATCACAACTAAAGAGTTGGGTACAGTTATGCGATCACTAGGTCAAAATCCCACAGAAGCT  520

Query  121  GAACTGCAGGACATGATCAACGAGGTCGACGCCGACGGCTCCGGAACGATAGATTTCCCT  180
            || || ||||| ||||| || || || || || || ||    || || || ||||| ||
Sbjct  521  GAGCTTCAGGATATGATTAATGAAGTTGATGCAGATGGTAATGGCACAATCGATTTTCCG  580

Query  181  GAGTTCCTCACAATGATG-CAAGAAAGATGAAGGACACCGACTCGGAGGAGGAGATC-GA  238
            || ||| | || |||||| |  | || ||||| || || ||    ||||| || ||  |
Sbjct  581  GAATTCTTAACTATGATGGCTCGTAAAATGAAAGATACTGATAGTGAGGAAGAAATTAGG  640

Query  239  GAGGCGTTCCGCGTATTCGACAAGGATGCAAC-GGTTTCATTTCGGCGGCCGAGCTCAGG  297
            ||||| ||| | ||||| || ||||||| ||  |||||||| || || || || |||||
Sbjct  641  GAGGCCTTCAGAGTATTTGATAAGGATGGAAATGGTTTCATATCCGCAGCAGAACTCAGA  700

Query  298  CACGTTATGACCAACCTTGGCGAGAAGCTTACGGACGAGGAGGTAGATGAGATGATTCGG  357
            || |||||||| || |||||||||| || || || || || || ||||| |||||||||
Sbjct  701  CATGTTATGACAAATCTTGGCGAGAAACTCACTGATGAAGAAGTTGATGAAATGATTCGG  760

Query  358  GAGGCAGATATTGA  371
            ||||| || |||||
Sbjct  761  GAGGCTGACATTGA  774
```

B. Varroa dsRNA (query) overlap in the honeybee calmodulin mRNA (subject).

Figure S5 continued

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.

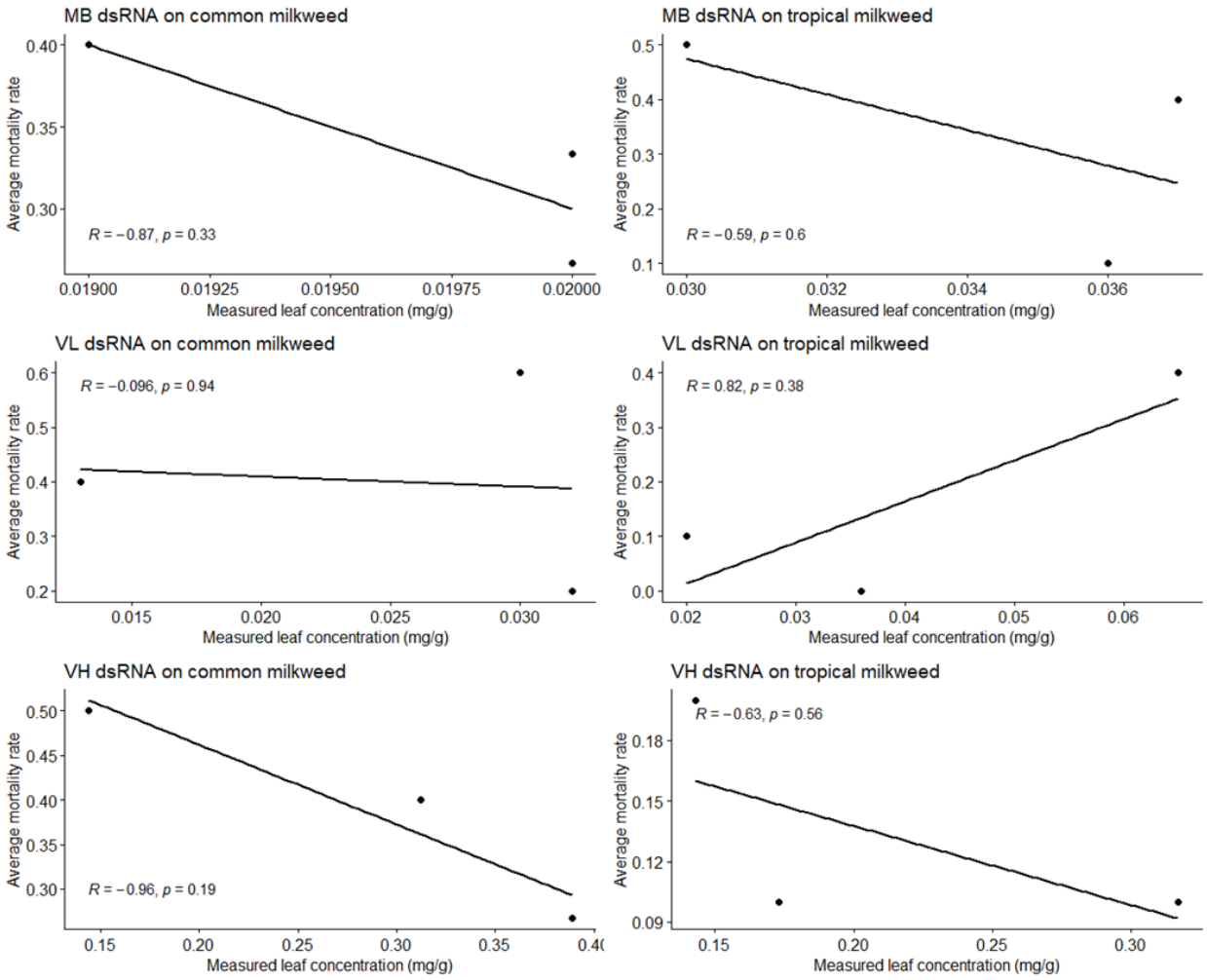


Figure S6. Correlation between measured leaf concentration and mortality for monarch butterfly (MB) dsRNA, 1X Varroa (VL) dsRNA, and 10X Varroa (VH) dsRNA treatments. Data were analyzed separately for common and tropical milkweed. Each point on the graph indicates a bioassay run.

CHAPTER 5. EFFECT OF NEONICOTINOID INSECTICIDES ON LEPIDOPTERAN PUPAL ECDYSIS

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Abstract

Previously we had shown that neonicotinoid insecticides cause an arrest in pupal ecdysis in final instar monarch butterfly (*Danaus plexippus*; Nymphalidae) larvae. This novel neonicotinoid mode of action has not been previously reported in the literature. In this paper, we explore arrested ecdysis in greater detail and propose adverse outcome pathways to explain how neonicotinoid exposure leads to this effect. Using imidacloprid as a model compound, we determined that susceptibility to arrested ecdysis varies across Lepidoptera final instars. Monarchs, corn earworms (*Helicoverpa zea*; Noctuidae), and wax moths (*Galleria mellonella*; Pyralidae) showed high susceptibility while painted ladies (*Vanessa cardui*; Nymphalidae) and red admirals (*Vanessa atalanta*; Nymphalidae) had low susceptibility. Fall armyworms (*Spodoptera frugiperda*; Noctuidae) and European corn borers (*Ostrinia nubilalis*; Crambidae) did not exhibit arrested ecdysis; the recalcitrant response in fall armyworms could be due to its efficient metabolism of imidacloprid. All larvae with arrested pupal ecdysis developed pupal cases, but with incomplete shedding of larval tracheal lining and unexpanded appendages. Corn earworm larvae with arrested pupal ecdysis could successfully develop into adults but with unexpanded appendages. Time-course studies indicate that expression of arrested ecdysis is highly dependent on the timing of imidacloprid exposure; neonicotinoids do not cause failure in

larval ecdysis and, in corn earworms, they disrupt pupal ecdysis if applied at least 26 hours prior to pupal ecdysis. In addition, delayed initiation of pupal ecdysis was seen in neonicotinoid-treated larvae. These observations suggest neonicotinoids may disrupt the function of crustacean cardioactive peptide neurons, either by directly acting on them or by acting on inhibitory neurons that regulate their function. Further studies are needed to test this hypothesis.

Keywords: Arrested pupal ecdysis (AE), crustacean cardioactive peptide (CCAP), adverse outcome pathway (AOP), imidacloprid, neonicotinoid.

Introduction

Neonicotinoids are among the most widely used insecticides in the world. In the United States, nearly all corn acres and the majority of soybean and cotton acres are planted with neonicotinoid-treated seeds, accounting for over 80% of their total use (Douglas and Tooker 2015). In addition, nearly 680,000 kilograms of imidacloprid, thiamethoxam, and clothianidin, the three most commonly used neonicotinoids, are applied as spray, soil drench, or injection (soil/tree) formulations in agricultural land, non-crop land, and urban areas (U.S. Geological Survey 2017; U.S. Environmental Protection Agency 2020). Not surprisingly, studies have reported potential neonicotinoid exposure to target and non-target insect species, including Lepidoptera larvae (Van Timmeren et al. 2012; Basley et al. 2018; Olaya-Arenas et al. 2019; Peterson et al. 2019; Halsch et al. 2020).

In insects, neonicotinoids exert their neurotoxic effects by binding to the α4β2 subunits of the nicotinic acetylcholine receptors (nAChR) in the central nervous system. At toxic doses, receptor binding results in neuronal overstimulation, paralysis, and death (Christen et al. 2016). Adverse outcome pathways (AOPs; Ankley et al. 2010) that postulate steps that lead to an adverse effect in bees at sublethal doses have been proposed. In the honey bee, *Apis mellifera*,

LaLone et al. (2017) proposed that nAChR could be desensitized if exposed to prolonged but relatively low neonicotinoid doses. Alternatively, sublethal exposures could cause mitochondrial dysfunction, which would lead to alteration of Ca^{2+}-calmodulin-activated signal transduction. Altered Ca^{2+} transduction could, in turn, prevent translation of proteins involved in long-term memory and thereby cause abnormal foraging behavior that eventually leads to colony failure (LaLone et al. 2017). Camp and Lehmann (2020) proposed that neonicotinoids could exert similar cellular effects in bumble bees (*Bombus terrestris* and *Bombus impatiens*).

In the present paper, we investigated a unique adverse outcome, termed arrested pupal ecdysis (AE), which was first reported following neonicotinoid exposure to final instars of the monarch butterfly (*Danaus plexippus*; Nymphalidae). Larvae died during pupation following topical or dietary exposure to imidacloprid, clothianidin, and thiamethoxam at doses that did not elicit prior signs of intoxication (Krishnan et al. 2020; Krishnan et al. 2021). Arrested ecdysis was not observed during larval-to-larval molts. These observations suggest that neonicotinoids are disrupting signaling during pupal ecdysis through a novel toxicity pathway (Krishnan et al. 2020).

To characterize this symptomology more fully, we undertook a series of experiments and analyses to formulate potential AOPs that can help guide future research and understanding of neonicotinoid-mediated developmental effects and the lepidopteran metamorphosis process. More specifically, we used imidacloprid as a model compound to determine:

1. The extent to which AE is conserved across Nymphalidae, Noctuidae, Crambidae, and Pyralidae.
2. Larval susceptibility to AE and adult eclosion at different times of exposure.
3. Differences in the ecdysis process in control and treated larvae.

4. The toxicokinetics of the parent compound in AE-sensitive and AE-insensitive species.

To formulate proposed AOPs, results from these studies were interpreted in light of scientific literature elucidating neuroendocrine control of pupal ecdysis.

Materials and methods

Insect rearing

Monarch butterfly and European corn borer (*Ostrinia nubilalis*; Crambidae) larvae were obtained from colonies established by the U.S. Department of Agriculture in Ames, IA. Monarch larvae were fed on greenhouse-grown tropical milkweed (*Asclepias curassavica*) leaves (Krishnan et al. 2020), while European corn borer larvae fed were on an artificial diet (Lewis et al. 1969; Brindley et al. 1975; Guthrie 1989). Larvae of both insects was reared in incubators maintained at 26.6ºC, 65% relative humidity, and 16:8 light: dark cycle.

Corn earworm (*Helicoverpa zea*; Noctuidae) and fall armyworm (*Spodoptera frugiperda*; Noctuidae) eggs were purchased from Benzon Research, Carlisle, PA, and painted lady (*Vanessa cardui*; Nymphalidae) eggs were purchased from Carolina Biological Supply Co., Burlington, NC. Larvae were fed *ad libitum* on an artificial diet (Stonefly Heliothis Diet, Ward's Science) and were reared in an incubator maintained at 26ºC and 14:10 light: dark cycle.

Ovipositing female red admiral butterflies (*Vanessa atalanta*; Nymphalidae) were collected from common nettle (*Urtica dioica*) in June 2020 from prairies in Story and Boone counties, IA. Larvae that hatched from eggs laid by the captured females were fed common nettle leaves. Wax moth (*Galleria mellonella*; Pyralidae) larvae were purchased from a commercial store in Ames, IA. Larvae were fed multigrain baby cereal (Gerber brand) that was mixed with water to achieve consistency of thick peanut butter. Red admirals and wax moths were reared in an incubator at 26.6ºC, 80% relative humidity, and 16:8 light: dark cycle.

Chemicals and insecticide solutions

Analytical grade imidacloprid (IUPAC name: *N*-[1-[(6-chloropyridin-3-yl)methyl]-4,5-dihydroimidazol-2-yl]nitramide; CAS number: 138261-41-3; Percentage purity: 100%) was purchased from Sigma-Aldrich. To prepare insecticide stock solutions for topical and dietary bioassays, certified ACS reagent grade acetone, certified ACS reagent grade dimethylformamide (DMF), and Silwet L-77 were purchased from Fisher Scientific.

For the topical bioassays, 20 and 10 mg/mL imidacloprid stock solutions were prepared in acetone. Ten-fold serial dilutions through 0.01 mg/mL imidacloprid were made with the 10 mg/mL solution. For the dietary bioassay, 10 mg of imidacloprid was dissolved in 10 mL of DMF; the stock solution was diluted ten-fold to 0.1 mg/mL with DMF. This concentration was serial diluted with a suspension of 0.1% silwet : water to obtain a 0.01 mg/mL (or 0.01 μg/μL) imidacloprid suspension.

Toxicity bioassays

All bioassays were conducted from January 2020 to January 2021 at the environmental conditions specified in *Insect rearing*.

Species sensitivity experiments: Studies were conducted on 1- or 2-day-old final-instar larvae of European corn borers (fifth instar), corn earworms (sixth instar), fall armyworms (sixth instar), painted ladies (fifth instar), red admirals (fifth instar), and wax moths (sixth instar). All larvae were placed in individual rearing containers and randomly assigned a treatment prior to starting a bioassay. One μL of acetone or an imidacloprid-acetone solution was placed on the dorsal prothorax using a 10-μL pipette. Five imidacloprid concentrations and an acetone control were used. At least ten larvae were treated in each of the six groups and five control larvae were

weighed prior to treatment. Daily observations for mortality were taken until pupation and at adult eclosion. Signs of larval intoxication (e.g., spasms, paralysis) and formation of malformed pupae were recorded. Dissections were performed on a subset of larvae that had AE. Photographs were taken with a Nikon DS-Ri2 digital camera connected to a Nikon SMZ1270 stereo microscope. To provide a more complete description of AE in fifth-instar monarch larvae following topical exposure to imidacloprid, we re-analyzed our previously published papers (Krishnan et al. 2020; 2021) and preliminary range-finding data.

Temporal dosing experiments: Fifth-instar corn earworm larvae (penultimate instar) were topically treated with the same concentrations and volume (i.e., one μL) of imidacloprid-acetone solutions as the sixth-instar larvae used in the first set of experiments. Ten larvae were assigned to each concentration; all controls were weighed prior to treatment. In a second set of experiments, sixth-instar corn earworm larvae were topically treated with 1 μL of 20 μg/μL imidacloprid-acetone solution at four different stages of molting as measured by stemmata pigment movement during head capsule slippage (HCS): 1) stemmata intact (several hours prior to HCS), 2) start of HCS, 3) 8 to 14 hours after HCS (average 12 hours), and 4) 20 to 24 hours after HCS (average 23 hours). A minimum of eight larvae were treated per stage. Acetone controls (n = 4 to 6) at each stage were employed. During the course of the experiments, signs of intoxication, daily mortality until pupation, pupal development, and adult eclosion were recorded. A subset of AE larvae was dissected and observed with the dissecting microscope. In addition, we re-analyzed our published papers (Krishnan et al. 2020; 2021) to further characterize timing of dietary imidacloprid exposures and mortality and AE rates in different monarch instars.

Observational experiments: Two-day old sixth-instar corn earworms were treated with acetone and 20 µg imidacloprid and observed continually, except from 20:00 to 06:00, for initiation of apolysis (denoted by HCS; observed under a dissecting microscope) and the beginning of ecdysis (denoted by commencement of tracheal shedding at the posterior end). This information was obtained for 24 larvae treated with acetone or imidacloprid (n = 12 each). Time to landmark events were recorded.

Toxicokinetic experiments: Fifth-instar monarch larvae and sixth-instar fall armyworm larvae were topically treated with a nominal dose of 20 µg imidacloprid, as outlined in previous experiments. Five larval and pupal samples, each with a minimum mass of 400 mg for armyworms and 800 mg for monarchs were collected at the start of an experiment and at the following time points post-treatment: 4 hours (armyworms), 24 hours (monarchs and armyworms), and after pupation or arrested ecdysis (monarchs and armyworms). Fifth-instar monarch larvae were also fed an average concentration of 0.5 µg of imidacloprid per g tropical milkweed leaf for 24 hours. This concentration was obtained by applying 100 or 150 µL of a 0.01 µg/µL imidacloprid suspension on approximately 2 or 3 g of leaf material, respectively. Leaves were air-dried and photographs of leaves were taken prior to larval feeding and 24 hours after feeding to obtain the surface area consumed by each larva, similar to methods described in Krishnan et al. (2020). Based on the estimated leaf concentration and surface area of leaf consumed, an oral imidacloprid dose consumed by each larva was estimated (note that use of silwet ensured that one side of a leaf surface was entirely coated). Five to six larval/pupal samples, with each sample corresponding to a minimum of 700 mg at the start of experiment, were collected at the following time points after treatment: 24 hours, following arrested ecdysis (larvae fed on treated leaves for 24 hours and were not provided any untreated leaves), and

following pupation (larvae fed on treated leaves for 24 hours and untreated leaves for another 24 hours). All larval/pupal samples were stored individually in plastic containers at -20 ºC until analysis.

Quantification of imidacloprid and metabolites in larvae

All samples were analyzed for imidacloprid and its two metabolites, 5-hydroxy imidacloprid and imidacloprid olefin, which are toxic to insects (Suchail et al. 2001). Samples were individually homogenized using a mortar and pestle. A 0.2-g portion of the sample was extracted with acetonitrile. Approximately 1 mL of the extract was transferred to a dispersive solid phase extraction (dSPE) tube containing 150 mg $MgSO_4$, 50 mg PSA, and 50 mg C18. Extracts were diluted with 50:50 methanol : water and internal standards (5-OH imidacloprid-d4, ^{13}C, ^{15}N imidacloprid olefin, and imidacloprid-pyridine-d4-methylene-d2) were added prior to LC-MS analysis. An injection volume of 2 µL was used. A Vanquish Flex LC pump interfaced with a TSQ Altis triple-quadrupole mass spectrometer (Thermo Fisher Scientific) were used for the analysis. All insecticides were analyzed in positive electrospray ionization mode. The MS ionization 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. MS acquisition was performed in selected reaction monitoring (SRM) mode with argon used as the collision gas. Data analysis was performed using Xcalibur 4.2 software (Thermo Fisher Scientific).

Chromatographic separation was achieved using an AccucoreaQ column (100 x 2.1 mm, 2.6µm). The column compartment temperature was 30°C. Mobile Phase A was 2% methanol, 5mM ammonium formate, and 0.1% formic acid in water and Mobile Phase B was 2% water, 5mM ammonium formate, and 0.1% formic acid in methanol. The gradient conditions were as

follows: start at 0% B, linear ramp to 100% B at 8 min, hold at 100% B for 2 min, drop to 0% B in half a min, and hold at 0% B for 1.5 min. The flow rate was 0.3 mL/min and the total run time of the method was 12 min. For both species, a calibration curve that ranged from 0.02-2.5 µg/g was prepared with control larvae. Quality control (QC) samples were prepared in triplicate at 0.3 µg/g and analyzed with experimental samples. All QC samples had a calculated concentration within 15% of the nominal value. The percent recovery was 99% for imidacloprid, 94% for 5-hydroxy imidacloprid, and 95% for imidacloprid olefin.

Results

Species sensitivity experiments

AE was observed in monarchs, corn earworms, painted ladies, red admirals, and wax moths that were topically treated with imidacloprid at doses ranging from 0.01 to 20 µg. All corn earworm and painted lady larvae that did not pupate expressed AE. Similar observations were seen in red admirals treated with 0.01 and 1.0 µg and wax moths treated with 1 and 10 µg imidacloprid (Table 1). Wax moth larvae treated with the 20-µg dose either had AE on the fifth or sixth day or died by imidacloprid's primary mode of action (i.e., paralysis, reduced feeding, and death prior to pupation) on the second- or third-day post exposure; a single pupa that formed on the sixth day died prior to eclosion. At the 1 to 20 µg imidacloprid dose, AE was observed in 70 to 100% of treated corn earworms, 20 to 40% of painted ladies, 10% of red admirals, and 25 to 53% of wax moths. On average, both AE and pupation occurred three to five days following treatment. With respect to controls, AE and/or pupation was delayed by one day at the 20-µg dose in painted ladies and wax moths, while it was accelerated by one day at the 0.01 to 10 µg doses in red admirals.

Final-instar larvae of European corn borers and fall armyworms did not exhibit AE and, despite being smaller than some of the other species tested, had low (27%) to no mortality at the 20-µg dose. Consequently a 100-µg dose was tested, which caused no mortality in fall armyworms and 50% mortality in European corn borers through imidacloprid's primary mode of action (Table 1). Both the 20- and 100-µg exposure delayed initiation of ecdysis in the European corn borers. In concentrations where at least two larvae had successfully pupated, the pupae had 61 to 100% adult eclosion across all six species.

Data from monarch butterfly toxicology studies published by Krishnan et al. (2020) and associated range-finding experiments showed that all mortality at doses ≤ 20 µg imidacloprid occurred through AE within two to three days following treatment. Treated larvae continued feeding or demonstrated no signs of intoxication prior to AE. However, when treated with 60 µg imidacloprid, larvae ceased feeding for one to two days and exhibited repetitive head motions. By the fourth day, 70% had died before ecdysis; the remaining larvae resumed feeding and gained weight but died four to six days after treatment through AE. All ten fifth instars treated with 100 µg imidacloprid showed signs of intoxication at the 24-h observation period and died through paralysis within four days of treatment (Krishnan et al. 2020).

For all three butterfly species, larvae expressing AE either died following emergence of the pupal cuticle on the thorax or at the "J" stage. Some of the AE monarch larvae also bled externally or melanization of the hemolymph was seen internally. The moth species that had AE typically did not die during ecdysis and had a visible pupal cuticle on the dorsal and posterior sides of the thorax. Removal of larval cuticle from AE butterfly larvae and a closer examination of AE moth larvae showed incomplete shedding of the tracheal lining, a retracted larval at the posterior cuticle, a partially complete pupal case (i.e., larval cuticle of the legs could not be removed

to determine if there was an underlying pupal cuticle), and untanned cuticle on the ventral side where the pupal wing cuticle would normally expand (Figures 1 and 2). Surviving corn earworm larvae developed into adults within the old larval and pupal cuticle. After approximately two weeks, careful removal of the old cuticles revealed a completely formed adult except the appendages (proboscis, antennae, wings, and legs) were not expanded (Figure 3). In wax moths, AE larvae died within their pupal cases; their mortality had likely been delayed as several had initiated adult development.

Temporal dosing experiments

When fifth-instar corn earworm larvae were treated with the same doses as sixth instars, all larvae successfully molted to the sixth instar. Two larvae in the 10-µg-imidacloprid dose showed signs of poisoning and died soon after the molt. However, the remaining 20 larvae that did not successfully pupate, and which received 1, 10, and 20 µg doses as fifth instars, exhibited AE (Table 2). On average, fifth-instar larvae treated with ≤ 1 µg dose pupated or exhibited AE on the sixth day after treatment, while larvae treated with 10 and 20 µg imidacloprid exhibited AE on the seventh day. For the subset of AE larvae that were dissected, two-thirds underwent adult development. Approximately 30% of these adults tried to emerge, but only their abdomen lost the pupal cuticle. All fifth-instar larvae treated with 0, 0.01, and 0.1 µg imidacloprid pupated successfully and eclosed normally. Larvae that pupated following treatment at higher doses either did not emerge as adults (25%), emerged with deformed and uninflated wings (50%), or emerged normally (25%) (Table 2).

Following topical treatment with 20 µg imidacloprid, sixth-instar corn earworms treated prior to HCS had the highest level of AE (90%). Larvae treated just after HCS, which was approximately 26 hours before pupation, had 75% AE. Larvae that were treated ca. 12 and 23

hours post-HCS, had 20 and 8% AE, respectively, with the rest successfully undergoing pupal ecdysis (Table 2). Nearly 90% of the AE larvae survived and developed to the adult stage, and a third of these tried to emerge but only their abdomen lost the pupal cuticle. Of those larvae that successfully pupated, a small percentage had wrinkled appendages, hemolymph loss, and cuticle around the wings that was bloated (Figure 4A). Larvae that successfully pupated emerged as adults one to three days later than control larvae, with only 23% emerging normally. The rest either did not emerge, only the abdomen emerged, or emerged with deformed and uninflated wings (Figure 4B).

Previously, we had published dietary imidacloprid toxicity data for monarch larvae provided treated tropical milkweed leaves for 24-to-48 h. Studies were undertaken with second-, third-, and early (ca. 1-day old) fifth-instar monarchs (Krishnan et al. 2020). All instars were provided similar concentrations of imidacloprid (i.e., 0.71 to 0.76 µg/g tropical milkweed leaf), and mortality ranged from 0 to 5%, following corrections for control mortality (6 to 16%). However, when late fifth instars (ca. 3-day old) were provided a similar imidacloprid concentration (0.80 µg/g leaf), 100% mortality was observed, all through AE. Chronic dietary imidacloprid exposures to 0.5 µg/g leaf concentrations also produced similar results; 91% mortality occurred, of which 82% was attributed to AE (Krishnan et al. 2021) (Table 3). When comparing estimated internal doses following consumption of treated leaves, acute studies showed that 0.76 µg/g larva was adequate to cause 100% AE mortality in late fifth instars; 124, 25, and 14 µg/g larva caused similar levels of non-AE related mortality in early fifth, third, and second instars, respectively. On a µg imidacloprid basis, early fifth instars were least sensitive (92 µg caused 91% mortality, with no AE), followed by third instars (0.96 µg caused 100%

mortality), late fifth instars (0.78 µg caused 100% mortality with AE), and second instars (0.14 µg caused 100% mortality).

Observational experiments

On average, both acetone-treated and imidacloprid-treated sixth instar corn earworms initiated HCS 42 hours after exposure. The pupal ecdysis was initiated 68 (± 3.7) and 73 (± 7.5) hours post-exposure, respectively (Table 4). Following HCS, the imidacloprid-treated larvae initiated the ecdysis motor process approximately five hours later than the controls, i.e., at 31 (± 6.4) vs. 26 (± 1.7) hours for controls. This difference was statistically significant at $\alpha = 0.05$ level ($t = -2.859$; $p = 0.0139$; Welch two sample t-test).

Acetone-treated corn earworm larvae took ca. 10 minutes to complete pupal ecdysis , from initiation of tracheal shedding to completion of larval cuticle shedding. After ca. 1 to 2 minutes following initiation of the posterior abdomen tracheal shedding, the larval cuticle at the dorsal thorax split along the ecdysial line, and the pupal case emerged; ca. 1 minute later the larvae turned over, exposing the ventral side and began shedding its larval cuticle from the anterior. Expansion of the appendages began just before ecdysis and continued during and after ecdysis. Immediately following ecdysis, the wing length was ca. 5 mm and expanded to 10 mm over the next 15 minutes prior to cuticle hardening and tanning.

Imidacloprid-treated corn earworm larvae initiated, but did not complete, tracheal shedding. While the larval cuticle at the thorax did not split in most larvae, some did split the dorsal thoracic cuticle along ecdysial lines ca. 5 minutes after initiation of tracheal shedding. None of the imidacloprid-treated larvae progressed beyond this point, i.e., they did not turn over to expose the ventral side and begin shedding their larval cuticle. These larvae also did not expand their appendages. In two AE corn earworm larvae that continued the ecdysis process past

the abdominal tracheal shedding, the larval cuticle was removed from the abdomen, but the thoracic and head larval cuticle remained. About 30 minutes afterwards we could carefully remove the larval cuticle from the thorax and head. The larval cuticle over the legs and head could be successfully pulled off and revealed a complete pupal cuticle except the appendages were not expanded (Figure 5).

Toxicokinetic experiments

Sixth-instar fall armyworm that were topically treated with 20 µg imidacloprid and collected within five minutes of treatment contained a mean (± SD) of 59 (± 11) µg/g imidacloprid; two of the four larval samples also had detectable 5-hydroxy imidacloprid amounts but were below the limit of quantification (Table 5). Larvae collected 4 hours after treatment had a similar imidacloprid concentration (56 ± 23 µg/g) and three of the five samples had one or both imidacloprid metabolites. Larvae collected 24 hours after treatment had four-fold lower imidacloprid concentrations (13 ± 7.9 µg/g) and four of the five samples contained either or both metabolites. Pupation occurred approximately four days after treatment and only two of the five pupal samples had detectable concentrations of imidacloprid; in both samples the concentrations were lower than the limit of quantification (< 0.02 µg/g). Neither of the metabolites were detected in the pupal samples.

Fifth-instar monarch butterflies that were topically treated with 20 µg imidacloprid and collected within five minutes of treatment contained a mean of 24 (± 10) µg/g imidacloprid. Fifth instars collected 24 hours (larval stage) and 48 hours (AE stage) after treatment contained similar imidacloprid concentrations (31 ± 12 and 20 ± 3.4 µg/g, respectively). Following one-day feeding on leaves treated with 0.5 µg/g imidacloprid, the 24-h (larval) and 38-h (AE) fifth-instar samples contained 0.11 (± 0.04) and 0.14 (± 0.06) µg/g imidacloprid, respectively. Pupal

samples from larvae that fed on milkweed leaves with 0.5 µg/g imidacloprid for 24 hours, followed by consumption of untreated leaves for another 24 hours, contained ≤ 0.02 µg/g imidacloprid (note: pupae were collected 72 hours after larval treatment because larvae took ca. a day to pupate). The two imidacloprid metabolites, 5-hydroxy imidacloprid and imidacloprid olefin, were not found in any of the monarch samples (Table 5).

Discussion

In the following sections, we interpret and integrate results from our experiments to gain a better understanding of AE symptomology and differences in interspecies susceptibility to this effect. Based on our observations and review of the insect ecdysis literature, we propose two adverse outcome pathways (AOP) for neonicotinoid-induced AE. We do note AE-like effects were reported by Bargar et al. (2020) when they chronically exposed monarch larvae to clothianidin-treated milkweed plants, and by Heneberg et al. (2020) when they treated crabronid wasp prepupae with neonicotinoids. While these studies are broadly concordant with our observations, they do not provide detailed descriptions of the symptoms or their time course. There are currently no published papers that attempt to elucidate the potential mechanism(s) by which neonicotinoids could cause AE.

Conservation of neonicotinoid-induced AE across Lepidoptera

Arrested pupal ecdysis was observed in monarchs, painted ladies, red admirals, corn earworms, and wax moths, but not in European corn borers or fall armyworms. These findings suggest susceptibility to AE may not be consistent with phylogenetic similarity, given corn earworms and fall armyworms belong to the Noctuidae family but exhibit opposite responses.

In monarchs, all AE larvae died within hours of expressing the symptomology, often due to loss of hemolymph. Arrested pupal ecdysis caused 100% of the mortality at lower doses (≤ 20 µg) and 15% of all mortality at higher doses (60 and 100 µg). High doses killed most monarchs prior to the start of ecdysis through imidacloprid's primary neurotoxic mode of action, with symptoms including cessation of feeding, paralysis, and loss of hemolymph. In wax moths, all larvae that failed to pupate at doses up to 10 µg had AE while larvae treated with the 20-µg dose either exhibited AE or died through the primary mode of action. Of the ten AE larvae we dissected, four were found to have initiated adult development. In corn earworms, all treated larvae that did not successfully pupate had AE; however, this often did not lead to mortality and adults developed within the unshed larval and pupal cuticles. In all three species, higher imidacloprid doses (up to 20 µg) typically elicited higher rates of AE.

In painted ladies and red admiral fifth instars, lower rates (10 to 40%) of AE were observed. In red admirals, the symptomology was observed across all doses. As the larval cuticle of both species are densely covered with hairs, it is possible imidacloprid was not completely absorbed. With dietary exposures, which includes cuticular and oral uptake, painted ladies are approximately 70-fold less sensitive to the neonicotinoid clothianidin when compared to monarchs (Peterson et al. 2019; Krishnan et al. 2021). While observations following removal of larval cuticle show unshed tracheal lining and unexpanded appendages similar to those observed in monarchs, corn earworms, and wax moths, the lack of a dose-response for the effect and the low mortality rates indicate that more replicates and doses are necessary to fully understand imidacloprid's effect on painted ladies and red admirals.

European corn borers and fall armyworm were the least sensitive species tested. The 20-µg per larva dose used across the tested species corresponded to 330 µg/g for European corn

borers (27% mortality with no AE); 130 µg/g for red admirals (20% mortality with half attributed to AE); 83 µg/g for wax moths (94% mortality with half attributed to AE); 54 µg/g for fall armyworms (0% mortality); 41 µg/g for corn earworms (100% AE); 30 µg/g for painted ladies (30% mortality all attributed to AE); and 18 µg/g for monarch butterflies (100% mortality all attributed to AE). At the 100 µg per larva dose, which corresponded to 1600 µg/g larva in European corn borers and 270 µg/g larva in fall armyworms, 50 and 0% mortality was observed, respectively. Fall armyworm larvae treated with higher concentrations (up to 100 µg imidacloprid) pupated successfully within three or four days. However, when we treated ten fall armyworms that were within two days of pupation with 20 µg imidacloprid, nine of the ten successfully pupated, while one larva exhibited AE. We also observe low rates of AE-like symptomology in our fall armyworm colony. Consequently, we are unable to draw any conclusions on fall armyworms' susceptibility to AE. These data do, however, clearly demonstrate that final instar lepidopteran species generally have low sensitivity to imidacloprid's primary mode of action but, if they are susceptible to AE, their sensitivity increases significantly.

To assess if interspecies variability in AE sensitivity could be due, in part, to differences in toxicokinetics, we topically treated monarch and fall armyworm larvae and quantified internal concentrations of imidacloprid and two of its toxic metabolites. In monarchs, internal imidacloprid concentrations remained stable over the observation periods; 24 h (larval) and 48 h (AE) post-exposure internal concentrations were, on average, 1.3 and 0.83-fold lower than the imidacloprid concentration at 0 h (larval; 24 µg/g), respectively. Neither of the two imidacloprid metabolites were detected in any of the sampled larvae or pupae. In fall armyworms, two of the 0 h and three of the 4-h larval samples had detectable concentrations of the metabolites, suggesting rapid metabolism (note: the 0-h larvae were sampled 5 minutes post treatment and then stored at

-20 ºC). The 24- h larval samples had, on average, 4.3 times less parent imidacloprid than the 0-h samples (59 µg/g) and 4 of the 5 samples had detectable concentrations of both metabolites. No imidacloprid was found in three of the five fall armyworm pupal samples collected approximately four days after treatment, while it was detected at concentrations below the level of quantification (< 0.02 µg/g) in two larvae. While the time course of imidacloprid residues suggests fall armyworms metabolize and excrete imidacloprid more efficiently than monarchs, examination of insecticide uptake and distribution in the central nervous system and endocrine-active tissues over time is needed to more fully assess the extent of toxicokinetic differences between the species. We also cannot rule out toxicodynamic considerations that could explain the differences in species susceptibility, e.g., interspecies differences in imidacloprid binding potential to nAChR.

Rationale for a novel mode of action

There are several observations that suggest imidacloprid-induced AE occurs through a novel toxicity pathway. These observations include a delayed effect, expression of AE at doses lower than those that cause neurotoxic symptoms, successful larval ecdysis, increased sensitivity of final instars, and differential susceptibility to AE before versus after HCS.

Delayed adverse outcome with no prior symptoms: Monarchs, corn earworms, painted ladies, and red admirals that exhibited AE at doses ≤ 20 µg and wax moths that exhibited AE at doses ≤ 10 µg showed no detectable symptoms prior to ecdysis. Most AE larvae had initiated pupal ecdysis on the same day as control larvae. These time frames ranged from 2 to 3 days for monarchs, 3 to 5 days for painted ladies and red admirals, and 4 to 6 days for corn earworms and wax moths (Table 1). In fifth-instar corn earworms treated with imidacloprid, no symptoms were observed until AE occurred, which was 6 to 8 days after treatment.

Effect at relatively low doses: In monarch larvae, the minimum measured internal concentration (based on MS/MS analyses) and the minimum calculated internal concentration (based on amount of imidacloprid-treated leaves consumed or amount of topically applied imidacloprid) that caused AE were 0.08 and 0.3 µg of imidacloprid/g body weight, respectively. The average calculated internal concentrations that caused over 90% mortality through AE ranged from 0.76 (dietary exposure) to 9.1 (topical exposure) µg/g. To cause similar rates of mortality through imidacloprid's primary mode of action requires estimated internal concentrations of 124 (dietary exposure) and 91 (topical exposure) µg/g (Tables 1, 3, and 4). This indicates that the concentrations of imidacloprid that cause AE in monarchs are 10- (topical exposure) to 160- (dietary exposure) fold lower than concentrations that cause larval mortality through the primary neurotoxic mode of action.

Successful larval ecdysis: All ten corn earworms that were topically treated with 20 µg imidacloprid in the fifth-instar stage successfully molted to sixth instar but exhibited AE six to eight days later. Successful larval ecdysis has also been observed in monarch first, second, and third instars that were treated with neonicotinoids either topically or through their diet (Krishnan et al. 2020). In chronic dietary bioassays, second-instar monarch larvae successfully molted through to the fifth-instar stage but then exhibited AE (Krishnan et al. 2021).

Increased sensitivity of final instars: In monarchs, topical imidacloprid doses that caused 90% mortality in second (17 µg/g) and third instars (56 µg/g) were two and six times greater than the dose that caused 90% mortality in fifth instars (9 µg/g), respectively (Krishnan et al. 2020). Dietary imidacloprid doses that caused 100% mortality in second (14 µg/g) and third (25 µg/g) instars were 20 to 30 times greater than the dose that caused 100% mortality in late fifth instars (0.76 µg/g; Krishnan et al. 2021). Increased sensitivity of final instars was also seen in corn

earworms; fifth instars treated with 1 µg of imidacloprid had 30% AE and sixth instars provided the same dose had 70% AE. This response likely reflects declining insecticide concentration in larvae over time which, at the time of ecdysis, may drop below the threshold concentration that elicits AE. The internal imidacloprid concentration drops more rapidly following dietary exposure. For example, monarch larvae that exhibited AE following 24-h consumption of milkweed leaves treated with 0.5 µg/g imidacloprid had an internal concentration of 0.14 µg/g body weight, while pupae from fifth-instar larvae that fed for 24 h on leaves treated at the same imidacloprid concentration, followed by 24 h feeding on untreated leaves, had concentrations below the level of quantification (≤ 0.02 µg/g body weight; Table 5).

Differential susceptibility to AE before and after HCS: In fifth-instar monarchs, we found that dietary exposures caused AE symptomology when larvae were provided imidacloprid ca. 38 hours before pupation; earlier exposures at the same concentration followed by feeding on untreated leaves caused no effect (Table 5). In sixth-instar corn earworm larvae, we observed high rates of AE (75 to 90%) when topical imidacloprid exposures occurred ca. 1 day before HCS or immediately after HCS; if larvae were dosed 12 and 23 hours after HCS, 80 and 92% formed complete pupae, respectively (Table 2). Of note, HCS occurs after decline of the second ecdysteroid peak, which leads to the release of ecdysis-triggering hormone (ETH) peptides that initiate the ecdysis process (Zitnan and Adams 2012). While toxicokinetic factors that account for insecticide absorption and distribution need to be resolved, these findings nevertheless suggest that neonicotinoids disrupt pupal ecdysis within a relatively narrow time window.

Interpretation of AE symptomology

As described previously, AE typically does not lead to mortality in corn earworms; consequently, an analysis of these larvae provided useful insights on the symptomology. All AE

larvae initiated, but failed to complete, pupal ecdysis. Careful removal of larval cuticle about 30 minutes after ecdysis apparently stopped, revealed a completely developed pupa (previous failure to remove larval cuticle at the unexpanded legs could be attributed to hardening and tanning of the pupal cuticle as the attempt was made about 12 hours after AE). When the pupal cuticle was removed from AE larvae ca. 2 weeks after initiation of pupal ecdysis, a completely developed adult was seen approximately 90% of the time (Table 2). These observations suggests that neonicotinoids do not disrupt pupal or adult development. However, at both the pupal and adult stages, appendages, i.e., antennae, proboscis, wings, and legs, were not expanded (Figures 2 and 3). The lack of expansion could be due to incomplete shedding of larval cuticle although, even in larvae that successfully pupated following imidacloprid treatment, the developed pupae and adults often had reduced expansion and inflation of the wings. Larvae that were treated ca. 12 hours after HCS occasionally displayed unexpanded wings at the pupal stage, while larvae that were treated ca. 12 and 23 hours after HCS often emerged with deformed, uninflated wings (Figure 4). These symptoms suggest that imidacloprid prevents complete expansion/inflation of the appendages at both the pupal and adult stage, with more severe effects seen if exposure occurs before HCS.

Imidacloprid-treated larvae that successfully emerged as adults also had incomplete or delayed eclosion when compared to acetone-treated larvae (Table 2). While two-day old sixth-instar corn earworms treated either with acetone or imidacloprid had started HCS (or apolysis) at approximately the same time, imidacloprid-treated larvae had delayed initiation of the ecdysis by ca. 5 hours and had incomplete ecdysis (Table 4). These observations suggest imidacloprid may modulate a neuroendocrine signal that is involved in the ecdysis motor process in pupal ecdysis.

All these lines of evidence point towards interference of crustacean cardioactive peptide (CCAP) neuronal function. CCAP neurons are responsible for initiating the ecdysis motor program. In fruit flies (*Drosophila melanogaster*), CCAP neurons are necessary for pupal leg and wing expansion, timely eclosion, and adult wing inflation (Park et al. 2003; Veverytsa and Allan 2012). These neurons are also needed for successful pupal, but not larval ecdysis; failed pupal ecdysis following ablation of CCAP neurons is characterized by incomplete shedding of larval tracheal lining (Park et al. 2003). In the hemimetabolous insect *Rhodnius prolixus*, knockdown of CCAP has also been reported to delay initiation of the ecdysis motor process (Lee et al. 2013).

Different subsets of CCAP neurons release CCAP, myoinhibitory peptides (MIPs), and bursicon peptides during ecdysis and after ecdysis (Zitnan and Adams 2012). Bursicon is responsible for cuticle sclerotization and tanning; however, the pupal and adult cuticle of neonicotinoid-treated larvae have coloration and hardness similar to control cuticle (note: the unsclerotized cuticle on the ventral side of AE larvae can be attributed to unexpanded appendages). In fruit flies, bursicon subunits are also believed to be necessary for successful pupal ecdysis and adult wing inflation (Lahr et al. 2012). Also, of note, just prior to pupal ecdysis in fruit flies, there is an emergence of 12 ‘late’ CCAP neurons that differentiate to express CCAP and bursicon. These neurons alone are sufficient to initiate pupal ecdysis (Veverytsa and Allan 2012). These ‘late’ CCAP neurons are also necessary for pupal leg extension, while the ‘early’ CCAP neurons are necessary for adult wing inflation. In total, these observations in fruit flies suggest neonicotinoids could disrupt signaling from both ‘early’ and ‘late’ CCAP neurons, including certain subsets of bursicon-expressing CCAP neurons. However,

it is important to note that studies to date have not reported the extent to which the roles of CCAP neurons are conserved across insect orders.

Development of an adverse outcome pathway

We propose two AOPs that provide testable hypotheses to elucidate the molecular initiating event and key events associated with neonicotinoid-induced AE. We reviewed AOPs for neonicotinoid-induced effects on honeybee colony death (LaLone et al. 2017) and an AOP for ecdysone agonists that leads to lethal molting disruption (Song et al. 2017). We also reviewed the literature addressing the neuroendocrine pathways that initiate and regulate ecdysis. Our proposed AOPs are depicted in Figure 6.

Molecular initiating event: While neonicotinoid's primary mode of action involves their agonism of acetylcholine at nAChRs, it is possible AE could be due to interactions at other receptor binding sites. However, we previously reported increased rates of AE in monarch fifth instars topically treated with chlorpyrifos (Krishnan et al. 2020), which is an acetylcholine esterase inhibitor that has the net effect of increasing acetylcholine signaling. To determine if this response could be replicated in another susceptible species, we treated sixth-instar corn earworm larvae with chlorpyrifos. Of the ten corn earworms topically treated with 140 µg chlorpyrifos, seven exhibited AE (data not shown). These findings suggest that disruption of acetylcholine signaling pathways may be central to AE.

In holometabolous insects, several steps occur sequentially to initiate pupal ecdysis. In moths, following the release of ETH, pre-ETH (PETH), and ETH-associated peptide (ETH-AP) from Inka cells, various cells are activated including the L3, 4 neurons that release diuretic hormones and kinins, VM neurons that release eclosion hormone (EH), and the CCAP neurons (Park et al. 2003; Zitnan and Adams 2012; Lahr et al. 2012). In fruit flies, subsets of CCAP

neurons possess different receptors including ETHR, EHR, nAChR, muscarinic acetylcholine receptors (mAChR), GABA, and glutamate receptors (Zitnan and Adams 2005; Ewer 2005; Kim et al. 2006; Vömel and Wegener 2007). Perturbation of normal ETH, EH, acetylcholine, GABA, and glutamate signaling could interfere with CCAP activation and/or secretion and cause symptoms similar to the neonicotinoid-induced AE. In this regard, Vömel and Wegener (2007) reported that in fruit flies, the vast majority of CCAP neurons respond to acetylcholine (which binds to nAChR and mAChR) and nicotine (which binds to nAChR). Desensitization of response was observed at higher nicotine concentrations. Subsets of CCAP neurons that were inhibited by GABA were stimulated by acetylcholine. *In vivo* studies indicated nicotine could activate presynaptic inhibitory neurons, which are likely GABA neurons.

Key events: Studies in tobacco hornworm (*Manduca sexta*) have shown that cGMP production can take place in certain nitric oxide (NO)-sensitive CCAP neurons only during pupal ecdysis (Zayas et al. 2000). In turn, elevated cGMP can lead to increased excitability of CCAP neurons (Gammie and Truman 1997). Zayas et al. (2002) and Mannai et al. (2016) showed that nicotinic acetylcholine receptors can control cGMP levels by coupling to NO production. While cGMP elevation in CCAP neurons mark the activation of the ecdysis circuitry, release of CCAP neuropeptides and initiation of ecdysis behavior takes place 30 minutes later. This delay in release of peptides is likely caused by a descending inhibitory input (Zitnan and Adams 2000; Fuse and Truman 2002). In fruit flies, GABA neurons are partly responsible for this inhibition (i.e., delayed activation) of CCAP neurons (Mena et al. 2016).

Neonicotinoids could initiate AE through two pathways (see Figure 6). They could directly bind to nAChRs on a subset of CCAP neurons and cause receptor overstimulation and desensitization (LaLone et al. 2017). Alternatively, neonicotinoids could bind to nAChRs on

GABA neurons that inhibit CCAP neurons prior to ecdysis. Continued activation of GABA-releasing neurons could delay or diminish disinhibition of a subset of CCAP neurons. Both these AOPs would have a net effect of reduced cGMP elevation and reduced activation of CCAP neurons. In turn, reduced release of CCAP neuropeptides would attenuate the ecdysis motor process (weaker ecdysis movements and abdominal contractions) and prevent expansion of appendages, consistent with observed AE symptomology and mortality.

Conclusions

We describe a unique adverse effect caused by neonicotinoids in butterflies and moths. Specifically, we show that neonicotinoids cause failure of pupal (but not larval) ecdysis at doses below those that cause mortality through neurotoxicity. We propose that neonicotinoids at lower doses potentially interfere with the release of neuropeptides that initiate the ecdysis motor program and expand pupal appendages. While the majority of lepidopteran species we studied were susceptible to AE, some species were recalcitrant to the effect, which may, in part, be due to species' differences in neonicotinoid toxicokinetics. Further investigation of the mechanisms through which neonicotinoids induce AE will likely aid in better understanding of the insect molting and metamorphosis processes and help refine the neonicotinoid risk assessment process. In this regard, we propose two AOPs that hypothesize different molecular initiating events through which neonicotinoids could attenuate signaling from CCAP neurons that initiate the ecdysis motor process and extend appendages during the larval to pupal molt.

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Data availability

Data and metadata pertaining to this manuscript will be made publicly available at this GitHub repository following acceptance of manuscript:

https://github.com/Niranjana296/Arrested-pupal-ecdysis

References

Ankley GT, Bennett RS, Erickson RJ, Hoff DJ, Hornung MW, Johnson RD, Mount DR, Nichols JW, Russom CL, Schmieder PK, Serrrano JA, Tietge JE, Villeneuve DL. 2010. Adverse outcome pathways: a conceptual framework to support ecotoxicology research and risk assessment. Environ Toxicol Chem. 29 (3): 730-741.

Bargar TA, Hladik ML, Daniels JC. 2020. Uptake and toxicity of clothianidin to monarch butterflies from milkweed consumption. PeerJ, 8, e8669.

Basley K, Goulson D. 2018. Effects of field-relevant concentrations of clothianidin on larval development of the butterfly *Polyommatus icarus* (Lepidoptera, Lycaenidae). Environ Sci Technol. 52 (7): 3990-3996.

Brindley TA, Sparks AN, Showers WB, Guthrie WD. 1975. Recent research advances on the European Corn Borer in North America. Ann Rev Entomol. 20: 221-239.

Camp AA, Lehmann DM. 2020. Impacts of neonicotinoids on the bumble bees *Bombus terrestris* and *Bombus impatiens* examined through an Adverse Outcome Pathway framework lens. Environ Toxicol Chem. Accepted Author Manuscript.

Christen V, Mittner F, Fent K. 2016. Molecular Effects of Neonicotinoids in Honey Bees (*Apis Mellifera*). Environ Sci Technol. *50* (7), 4071–4081.

Douglas MR, Tooker JF. 2015. Large-Scale Deployment of Seed Treatments Has Driven Rapid Increase in Use of Neonicotinoid Insecticides and Preemptive Pest Management in U.S. Field Crops. Environ Sci Technol 49 (8): 5088–97.

Ewer J. 2005. Behavioral actions of neuropeptides in invertebrates: Insights from Drosophila. Horm Behav. 48:418–429.

Fuse M, Truman JW. 2002. Modulation of Ecdysis in the Moth Manduca Sexta: The Roles of the Suboesophageal and Thoracic Ganglia. J Exp Biol. 205 (Pt 8), 1047–1058.

Gammie SC, Truman JW. 1997. Neuropeptide Hierarchies and the Activation of Sequential Motor Behaviors in the Hawkmoth, *Manduca Sexta*. J. Neurosci. 17 (11), 4389–4397.

Guthrie WD. 1989. Advances in rearing the European corn borer on a meridic diet. In International Symposium on Methodologies for Developing Host Plant Resistance to Maize Insects, Mexico, DF (Mexico), 9-14 Mar 1987. CIMMYT.

Halsch CA, Code A, Hoyle SM, Fordyce JA, Baert N, Forister ML. 2020. Pesticide Contamination of Milkweeds Across the Agricultural, Urban, and Open Spaces of Low-Elevation Northern California. Front Ecol Evol. 8, 162.

Heneberg P, Bogusch P, Astapenková A, Řezáč M. 2020. Neonicotinoid Insecticides Hinder the Pupation and Metamorphosis into Adults in a Crabronid Wasp. Sci Rep, *10* (1), 7077.

Kim YJ, Žitňan D, Cho KH, Schooley DA, Misoguchi A, Adams ME. 2006. Central peptidergic ensembles associated with organization of an innate behavior. Proc Natl Acad Sci. 103 (38): 14211-14216.

Krishnan N, Yang Z, Bidne KG, Hellmich RL, Coats JR, and Bradbury SP. 2020. Assessing Field-Scale Risks of Foliar Insecticide Applications to Monarch Butterfly (Danaus Plexippus) Larvae. Environ Toxicol Chem 39 (4): 923–941.

Krishnan N, Yang Z, Aust M, Hellmich RL, Coats JR, and Bradbury SP. 2021. Monarch Butterfly (Danaus Plexippus) life stage risks from foliar and seed-treatment insecticides. Environ Toxicol Chem. *Under Review.*

Lahr EC, Dean D, Ewer J. 2012. Genetic Analysis of Ecdysis Behavior in Drosophila Reveals Partially Overlapping Functions of Two Unrelated Neuropeptides. J Neuroscience. *32* (20), 6819–6829.

LaLone CA, Villeneuve DL, Wu-Smart J, Milsk RY, Sappington K, Garber KV, Housenger J, Ankley GT. 2017. Weight of evidence evaluation of a network of adverse outcome pathways linking activation of the nicotinic acetylcholine receptor in honey bees to colony death. Sci Total Environ. 584-585:751-775.

Lee, D, Orchard I, Lange AB. 2013. Evidence for a Conserved CCAP-Signaling Pathway Controlling Ecdysis in a Hemimetabolous Insect, Rhodnius Prolixus. Front Neurosci 7.

Lewis LC, Lynch RE. 1969. Rearing the European corn borer, Ostrinia nubilalis, on diets containing corn leaf and wheat germ. Iowa State J. Sci. 44: 9-14.

Mannai S, Bitri L, Thany SH. 2016. CGMP/CGMP-Dependent Protein Kinase Pathway Modulates Nicotine-Induced Currents through the Activation of α-Bungarotoxin-Insensitive Nicotinic Acetylcholine Receptors from Insect Neurosecretory Cells. J Neurochem. 137 (6), 931–938.

Mena W, Diegelmann S, Wegener C, Ewer J. 2016. Stereotyped Responses of Drosophila Peptidergic Neuronal Ensemble Depend on Downstream Neuromodulators. *eLife*. *5*, e19686.

Olaya-Arenas P, Kaplan I. 2019. Quantifying pesticide exposure risk for monarch caterpillars on milkweeds bordering agricultural land. Front Ecol Evol. 7 (6):223.

Park JH. 2003. Targeted Ablation of CCAP Neuropeptide-Containing Neurons of Drosophila Causes Specific Defects in Execution and Circadian Timing of Ecdysis Behavior. Development. *130* (12), 2645–2656.

Peterson EM, Shaw KR, Smith PN. 2019. Toxicity of Agrochemicals Among Larval Painted Lady Butterflies (*Vanessa cardui*). Environ Toxicol Chem. 38 (12): 2629-2636.

Song Y, Villeneuve DL, Toyota K, Iguchi T, Tollefsen KE. 2017. Ecdysone Receptor Agonism Leading to Lethal Molting Disruption in Arthropods: Review and Adverse Outcome Pathway Development. Environ Sci Technol. 51 (8), 4142–4157.

Suchail S, Guez D, Belzunces LP. 2001. Discrepancy between Acute and Chronic Toxicity Induced by Imidacloprid and Its Metabolites in Apis Mellifera. Environ Toxicol Chem. *20* (11), 2482–2486.

U.S. Geological Survey. 2017. Estimated Annual Agricultural Pesticide Use Maps [cited 2020 October 18]. Available from: https://water.usgs.gov/nawqa/pnsp/usage/maps/

U.S. Environmental Protection Agency. 2020. EPA actions to protect pollinators. Available at: https://www.epa.gov/pollinator-protection/epa-actions-protect-pollinators

Van Timmeren S, Wise JC, Isaacs R. 2012. Soil Application of Neonicotinoid Insecticides for Control of Insect Pests in Wine Grape Vineyards. Pest Manag Sci. *68* (4), 537–542.

Veverytsa L, Allan DW. 2012. Temporally Tuned Neuronal Differentiation Supports the Functional Remodeling of a Neuronal Network in Drosophila. Proceedings of the National Academy of Sciences. *109* (13), E748–E756.

Vömel M, Wegener C. 2007. Neurotransmitter-Induced Changes in the Intracellular Calcium Concentration Suggest a Differential Central Modulation of CCAP Neuron Subsets In Drosophila. Devel Neurobio. *67* (6), 792–808.

Zayas RM, Qazi S, Morton DB, Trimmer BA. 2000. Neurons Involved in Nitric Oxide-Mediated CGMP Signaling in the Tobacco Hornworm, Manduca Sexta. J Comp Neurol, *419* (4), 422–438.

Zayas RM, Qazi S, Morton DB, Trimmer BA. 2002. Nicotinic-Acetylcholine Receptors Are Functionally Coupled to the Nitric Oxide/CGMP-Pathway in Insect Neurons: Nicotinic Evoked NO/CGMP-Signaling. J Neurochemistry. 83 (2), 421–431.

Zitnan D, Adams ME. 2000. Excitatory and inhibitory roles of central ganglia in initiation of the insect ecdysis behavioural sequence. J Exp Biol. 203:1329–1340.

Zitnan D, Adams ME. 2005. Neuroendocrine regulation of insect ecdysis. In: Gilbert LI, Iatrou K, Gill SS, editors. Coprehensive Molecular Insect Science. Vol. 3. pp. 1–60.

Zitnan D, Adams ME. 2012. Neuroendocrine Regulation of Ecdysis. In Lawrence IG, ed., Insect Endocrinology. Academic Press, San Diego, CA, USA, pp. 253-309.

Tables and Figures

Table 1. Rates of arrested pupal ecdysis following topical imidacloprid treatment of seven species of final-instar lepidopteran larvae[a]

Dose (µg/ larva)	Endpoint measured	European corn borer (100)	Corn earworm (60)	Fall armyworm (80)	Painted lady (60)	Red admiral (60)	Wax moth (92)	Monarch butterfly[b]
Mean (± SD) weight in g[c]		*0.06 (±0.02)*	*0.49 (±0.11)*	*0.37 (±0.07)*	*0.66 (±0.18)*	*0.15 (±0.04)*	*0.24 (±0.06)*	*1.1 (± 0.25)*
0	% mortality[d]	0	0	0	0	0	0	0
	% AE	0	0	0	0	0	0	0
	Days to ecdysis[e]	5 to 6	4 to 5	3 to 4	3 to 4	4 to 5	4 to 5	2 to 3
	% adult eclosion	87	90	100	100	100	87	90
0.01	% mortality[d]	0	0	10	0	22[f]	0	0
	% AE	0	0	0	0	22	0	0
	Days to ecdysis[e]	5 to 6	4 to 5	3 to 4	3 to 4	3 to 4	4 to 5	2 to 3
	% adult eclosion	87	100	90	100	56	93	95
0.1	% mortality[d]	7	0	0	0	11[f]	0	9
	% AE	0	0	0	0	11	0	9
	Days to ecdysis[e]	5 to 6	4 to 5	3 to 4	3 to 4	3 to 4	4 to 5	2 to 3
	% adult eclosion	93	100	100	100	78	93	85
1.0	% mortality[d]	0	70	0	20	20	25	15
	% AE	0	70	0	20	10	100	15
	Days to ecdysis[e]	5 to 6	4 to 5	3 to 4	3 to 4	3 to 4	4 to 5	2 to 3
	% adult eclosion	87	20	100	80	80	50	76
10	% mortality[d]	20	90	0	40	10	53	91
	% AE	0	90	0	40	10	53	91
	Days to ecdysis[e]	5 to 6	4 to 5	3 to 4	3 to 4	3 to 4	4 to 5	2 to 3
	% adult eclosion	91	10	100	60	90	60	9
20	% mortality[d]	27	100	0	30	20	94	100
	% AE	0	100	0	30	10	50	100
	Days to ecdysis[e]	7 to 8	4 to 5	3 to 4	4 to 5	4 to 5	5 to 6	2 to 3
	% adult eclosion	67	0	100	70	80	0	0

Table 1 continued

60	% mortality[d]	NT	NT	0	NT	NT	NT	100
	% AE	NT	NT	0	NT	NT	NT	30
	Days to ecdysis[e]	NT	NT	3 to 4	NT	NT	NT	4 to 6
	% adult eclosion	NT	NT	100	NT	NT	NT	NA
100	% mortality[d]	50	NT	0	NT	NT	NT	100
	% AE	0	NT	0	NT	NT	NT	0
	Days to ecdysis[e]	6 to 7	NT	3 to 4	NT	NT	NT	
	% adult eclosion	10	NT	100	NT	NT	NT	0
Total AE rate in dead larvae[d]		*0*	*1.0*	*0*	*1.0*	*0.75*	*0.71*	*0.66*

[a] Based on treating 10 to 16 larvae per imidacloprid-acetone concentration or acetone alone (see b for exception). One µL of imidacloprid-acetone solution (or acetone alone) was applied on the dorsal prothorax of one to two-day old final instar (fifth or sixth instar) larvae.

[b] Data obtained from Krishnan et al. (2020) and range-finding experiments. Between 22 to 33 larvae were treated per dose up to the 10-µg dose. Ten larvae per dose were treated at the 20, 60, and 100 µg imidacloprid doses.

[c] Based on weight of five to ten control larvae at the time of treatment. For monarchs, weights based on treating 18 control larvae.

[d] Mortality in the larval stage or due to AE. In corn earworms, mortality often indicates AE as most larvae developed into adults.

[e] Average days to pupa/AE.

[f] One larva each in the 0.01 and 0.1 µg imidacloprid doses went missing.

SD: standard deviation; AE: arrested (pupal) ecdysis; NA: not available; NT: not treated.

Table 2. Effect of timing of topical imidacloprid exposures to fifth- (penultimate instar) and sixth (ultimate instar) corn earworm

Instar	Stage of treatment	Dose (µg/ larva)[a]	# larvae treated	% pupated (% AE)[b]	Average days to pupa/AE	Results from observations/dissections[c]
5th	ca. 2-day old fifth instars with a mean (± SD) weight of 0.22 (± 0.11) g at the time of treatment (n = 10 larvae)	0	10	100 (0)	6	Adults: 10 emerged normally (~ 11 days as pupae)
		0.01	10	100 (0)	6	Adults: 10 emerged normally (~ 11 days as pupae)
		0.1	10	100 (0)	6	Adults: 10 emerged normally (~ 11 days as pupae)
		1	10	70 (30)	6	AE larvae: 2died with some adult development; 1 not dissected. Adults: 1 emerged normally, 2 emerged with deformed wings; 4 not analyzed (~ 11 days as pupae)
		10	10	10 (70)[d]	7	AE larvae: 2 died with some adult development. 4 had complete adult development but did not eclose; 1 not dissected. 1 died as pupa.
		20	10	0 (100)	7	AE larvae: 1 died with some adult development. 9 had complete adult development but did not eclose.
6th	Prior to HCS	0	6	100 (0)	2 to 3	Adults: 6 emerged normally (~ 13 days as pupae)
		20	10	10 (90)	2 to 3	AE larvae: 1 died with some adult development. 8 had complete adult development but did not eclose. Adults: 1 emerged with deformed wings (16 days as pupa)
	Just after HCS	0	4	100 (0)	1 to 2	Adults: 4 emerged normally (~ 10 days as pupae)
		20	8	25 (75)	1 to 2	AE larvae: 6 had complete adult development but did not eclose, . Adults: 2 emerged with deformed wings (~11 days as pupae)
	ca. 12 hours post HCS	0	6	83 (0)[e]	< 1	Adults: 5 emerged normally (~11 days as pupae)
		20	10	80 (20)	< 1	AE larvae: 1 died with some adult development. 1 had complete adult development but incomplete eclosion. Adults: 2 died as pupae, 1 incomplete eclosion, 4 emerged with deformed wings, 1 emerged normally (~ 13 days as pupae)
	ca. 23 hours post HCS	0	5	100 (0)	< 0.5	Adults: 5 emerged normally (~ 11 days as pupae)
		20	12	92 (8)	< 0.5	AE larva: 1 had adult development but did not emerge. Adults: 1 died as pupa, 3 incomplete eclosion, 3 emerged with deformed wings, 4 emerged normally (~ 12 days as pupae)

[a] The µg/larva dose was obtained following application of 1 µL of imidacloprid-acetone solution (or acetone alone) on the dorsal

prothorax of the larva.
[b] Percentage of pupated larvae and percentage of larvae that had AE.
[c] Larvae that had AE were dissected and larvae that successfully pupated were observed for eclosion.
[d] Remaining larvae (i.e., 2 fifth instars treated with 10 µg) died prior to ecdysis.
[e] Remaining larva (i.e., 1 sixth instar treated with acetone) died prior to ecdysis.
AE: arrested (pupal) ecdysis; SD: standard deviation; HCS: head capsule slippage.

Table 3. Timing of exposure responses following dietary imidacloprid exposure on monarch butterfly larvae[a]

Instar	Exposure duration	# larvae treated	Exposure concentration (µg/g leaf)[b]	Mean (± SD) dose consumed (µg/larva)[b]	Average dose consumed (µg/g larva)	Percent of effect[c]		Mean (± SD) days to pupa/AE following treatment
						Mortality	AE	
Fifth	24-h treated leaves +	39	0.71	1.1 (± 0.30)	1.5	5	25	3.2 (± 0.53)
(early)	untreated leaves till pupation[d]	11	540	92[d]	124	91	0	NA
(late)	24-h treated leaves[e]	13	0.80	0.78 (± 0.53)	0.76	100	100	1.6 (± 0.51)
Third	48-h treated leaves +	22	0.75	0.27 (± 0.02)	7.0	3	NA	NA
	48-h untreated leaves	11	240	0.96[d]	25	100	NA	NA
Second	48-h treated leaves +	33	0.76	0.067 (± 0.01)	6.7	0	NA	NA
	48-h untreated leaves	33	66	0.14 (± 0.11)	14	100	NA	NA
Second to pupa	Chronic exposure to treated leaves	30	0.50	NC	NC	91	82	NA

[a] Data were obtained from Krishnan et al. (2020) and Krishnan et al. (2021).
[b] Based on measured stock solution concentrations and known weights and surface areas of leaves.
[c] Imidacloprid mortality was corrected for control mortality (Abbott's formula; see Krishnan et al. 2020 and Krishnan et al. 2021). AE mortality was the percentage of dead larvae that showed AE symptomology.
[d] Data from a single bioassay run.
[e] Data included from Krishnan et al. (2020), preliminary experiments, and larvae treated for internal dose analyses (see Table 4).
AE: arrested (pupal) ecdysis; SD: standard deviation; NC: not calculated; NA: not applicable for study design.

Table 4. Time to HCS and pupation or AE for corn earworm sixth-instar larvae topically treated with acetone or 20 µg imidacloprid[a].

Treatment (n)	Percentage	Mean (± SD) hours to start of HCS [A][b]	Mean (± SD) hours to start of pupation/AE [B][b]	Mean (± SD) hours from start of HCS to start of pupation/AE [B-A]
Acetone (n = 12 larvae)	Pupation: 100%	42 (± 3.7) (range: 39 to 49 hours)	68 (± 3.7) (range: 64 to 75 hours)	26 (± 1.7) (range: 22 to 29 hours)
Imidacloprid (n = 12 larvae)	AE: 100%	42 (± 3.4) (range: 39 to 49 hours)	73 (± 7.5) (range: 68 to 94 hours)	31 (± 6.4) (range: 26 to 49 hours)

[a] Larvae were approximately 2-day old sixth instars at the time of treatment.
[b] Hours to HCS and pupation/AE were calculated from time of treatment.
HCS: head capsule slippage; AE: arrested (pupal) ecdysis; SD: standard deviation.

Table 5. Internal concentrations of imidacloprid and its metabolites in final instar larvae of fall armyworms and monarch butterflies following topical or dietary exposures.

Species	Exposure route	Collection endpoint/time following treatment	# of samples analyzed[a]	Sample mean (± SD) weight at treatment	Mean (± SD) measured internal dose (µg/g insect)[b]
Fall armyworm (sixth instar)	Topical: single application of 20 µg imidacloprid[c]	0 h	4[d]	0.65 (± 0.07)	59 (± 11) parent IMI (n = 4) 0.4 (± 0.05) 5-hydroxy IMI (n = 2)
		4 h	5	0.55 (± 0.07)	56 (± 23) parent IMI (n = 5) 0.6 (± 0.2) 5-hydroxy IMI (n = 3) 2.1 (± 0.9) IMI olefin (n = 2)
		24 h	5	0.54 (± 0.12)	13 (± 7.9) parent IMI (n = 5) 0.3 (± 0.2) 5-hydroxy IMI (n = 2) 1.4 (± 0.4) IMI olefin (n = 3)
		Pupa (~ 96 h)	5	0.55 (± 0.15)	< 0.02 parent IMI (n = 2)
Monarch butterfly (fifth instar)	Topical: single application of 20 µg imidacloprid[c]	0 h	5	1.1 (± 0.09)	24 (± 10) parent IMI (n = 5)
		24 h	5	0.87 (± 0.07)	31 (± 12) parent IMI (n = 5)
		AE (~ 48 h)	5	1.0 (± 0.04)	20 (± 3.4) parent IMI (n = 5)

Table 5 continued

Monarch butterfly (fifth instar)	Dietary: 24 h exposure to 0.5 µg of imidacloprid/g leaf[e]	24 h	5	0.81 (± 0.07)	0.11 (± 0.04) parent IMI (n = 5)
		AE [24 h feeding of treated leaves (~ 38 h)]	5	0.99 (± 0.08)	0.14 (± 0.06) parent IMI (n = 5)
		Pupae [24 h feeding of treated leaves + 24 h feeding of untreated leaves (~ 72 h)]	6	0.74 (± 0.03)	< 0.02 parent IMI (n = 6)

[a] A fall armyworm sample consisted of two larvae and a monarch butterfly sample consisted of a single larva.
[b] Imidacloprid parent and metabolite doses in larval/pupal samples were measured through LC/MS-MS. Limit of quantification was 0.02 µg/g.
[c] One µL of 20 µg/µL imidacloprid-acetone dose was applied on the dorsal prothorax of each larva.
[d] A sample was excluded from analyses (3X greater concentration provided).
[e] Each larva was provided known weights and surface areas of tropical milkweed leaves that were treated with known volumes of 0.01 µg/µL imidacloprid in a 1:9 dimethylformamide : 0.1% silwet suspension. The larvae in the 24-h, AE, and pupa groups consumed a mean oral dose of 0.68 (± 0.48), 1.2 (± 0.40), and 0.86 (± 0.23) µg of imidacloprid, respectively.
SD: standard deviation; IMI: imidacloprid; n = number of samples with detectable doses; AE: arrested (pupal) ecdysis.

Figure 1. Arrested pupal ecdysis in representative fifth-instar butterfly larvae treated with imidacloprid (left column). Careful removal of old larval cuticle showed complete pupal case on the dorsal and posterior side and unshed tracheal lining (right two columns). The appendages had not expanded and the ventral side of the first abdominal segments were not sclerotized. Top to bottom rows: monarch butterfly, painted lady, and red admiral.

Figure 2. Arrested pupal ecdysis in representative sixth-instar moth larvae treated with imidacloprid. AE individuals had complete pupal case on the dorsal and posterior side and unshed tracheal lining. Note that the appendages had not expanded and the ventral side of the first abdominal segments are not tanned. Top row are corn earworms and bottom row are wax moths.

Figure 3. Corn earworm larvae that exhibited AE were allowed to develop as adults. Careful removal of larval and pupal cuticle after adult development shows an adult with appendages (labeled) that did not expand.

Figure 4. Representative corn earworms that had pupated following imidacloprid treatment. A. Treatment occurred 12 hours after head capsule slippage. Pupa had wrinkled appendages, some blood loss, and bloated wings. B. Treatment occurred 23 hours after head capsule slippage resulting in a normal looking pupa. Removal of pupal cuticle ca. 2 weeks later showed a completely developed adult and mostly expanded appendages, except for deformed/uninflated wings (right panels). All panels showing the ventral side.

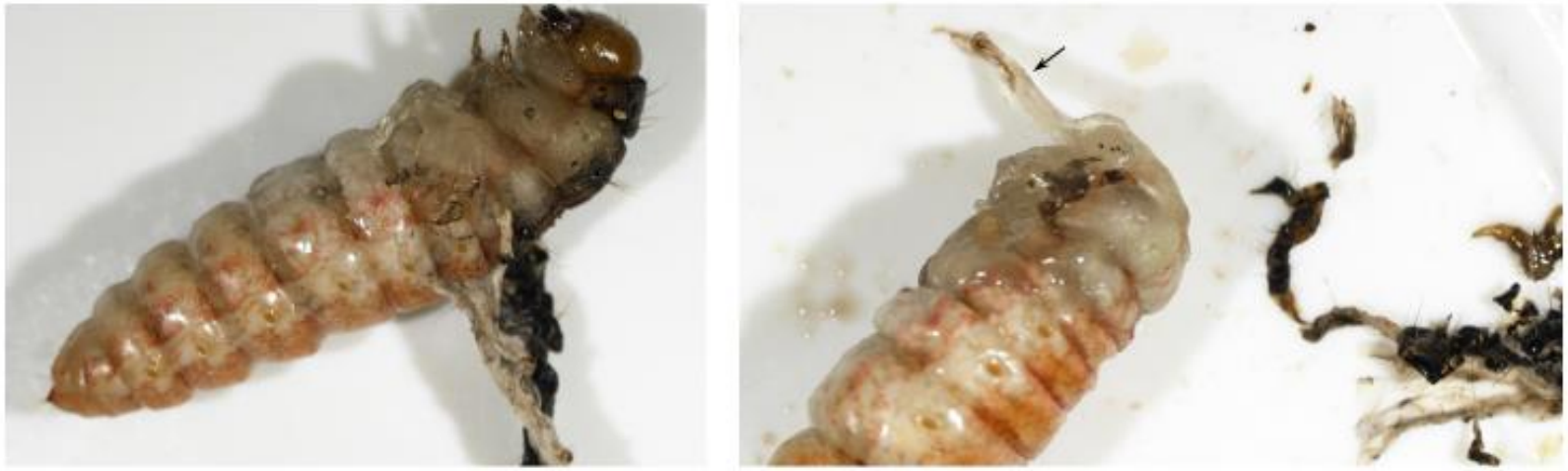

Figure 5. Careful removal of unshed larval cuticle from imidacloprid-treated corn earworm sixth-instar larvae that had just exhibited arrested ecdysis showed a completely developed pupal case throughout the body. Abdominal segments were starting to tan. Appendages were unexpanded and untanned. Arrow points to antennae.

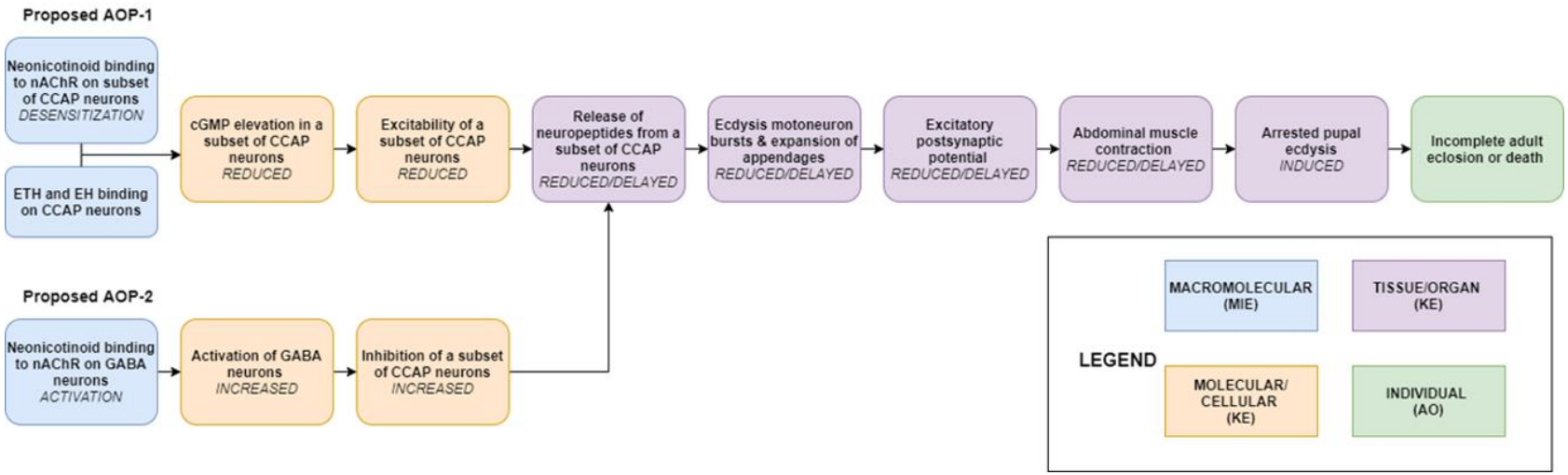

Figure 6. Proposed adverse outcome pathways that elucidate how neonicotinoid treatment could lead to arrested pupal ecdysis. MIE: molecular initiating event; KE: key event; AO: adverse outcome.

CHAPTER 6. COMPARING INSECTICIDE SENSITIVITY OF MONARCH BUTTERFLIES (LEPIDOPTERA; *DANAUS PLEXIPPUS*) TO OTHER LEPIDOPTERAN SPECIES AND HONEY BEES (*APIS MELLIFERA*)

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Abstract

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. In December 2020, the USFWS designated the monarch butterfly (*Danaus plexippus*) as a candidate species. In addition to loss of habitat, exposure to pesticides, particularly insecticides, is considered a threat to population recovery. As many lepidopteran ‘at-risk’ species, most of which are butterflies, are difficult if not impossible to rear in the laboratory, generating insecticide toxicity data to inform risk assessments for these species are generally not possible. One exception is the monarch butterfly. While challenging to rear, an increasing body of insecticide toxicity information has been generated for this species. These data, combined with the limited toxicity data available for other butterfly species, could be sufficient to generate species sensitivity distributions models to evaluate interspecies variability. An evaluation of toxicity studies that provided dosimetry information and employed similar testing methods as those described for monarch larval bioassays indicated comparable sensitivity of butterfly species to pyrethroid and organophosphate insecticides. A brief review of the moth toxicity literature indicated the same. Monarchs, however, are less sensitive to double-stranded RNA (dsRNA) products following dietary exposure. Compared to honey bees (*Apis mellifera*), another insect species for which decline has been attributed, in part, to insecticide exposure,

monarchs have lower sensitivity to the nitroguanidine neonicotinoids and the organophosphate chlorpyrifos, similar sensitivity to the pyrethroid cyfluthrin, and greater sensitivity to the anthranilic diamide chlorantraniliprole. Strategic testing across insecticide classes with representative lepidopteran species using standardized testing methods would support advancement of species sensitivity distributions that could aid in the toxicity assessment of 'at-risk' species, as well as those listed under the Endangered Species Act.

Keywords: 'At-risk' species, surrogate species, Lepidoptera, Hazard assessment, Species Sensitivity Distributions.

Introduction

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 (USFWS 2019). While many insect populations have declined over the last decade, species from Lepidoptera, which include butterflies and moths, are among the most impacted (Sanchez-Bayo and Wyckhuys 2019). Under the Endangered Species Act, there are currently 25 lepidopterans (24 butterflies and one moth) that are listed as endangered and five (four butterflies and one moth) that are listed as threatened (USFWS 2020a). In addition, six butterflies [monarch butterflies (*Danaus plexippus*), Jamaican kite swallowtail (*Eurytides marcellus*), Hahnel's Amazonian swallowtail (*Parides hahneli*), Fluminense swallowtail (*Parides ascanius*), Harris' mimic swallowtail (*Eurytides lysithous harrisianus*), and Kaiser-I-Hind swallowtail (*Teinopalpus imperialis*)] have been designated as candidate species, and several more [including Duke's skipper (*Euphyes dukesi calhouni*), Palatka skipper (*Euphyes pilatka klotsi),* Linda's Roadside-skipper (*Amblyscirtes linda), and* Regal fritillary (*Speyeria idalia)]* are currently being considered for listing (USFWS 2020b; USFWS 2020c).

There are several factors that contribute to the decline and/or threaten recovery of species. For lepidopterans, these include habitat loss, climatic changes, susceptibility to diseases, and insecticide exposure. While insecticide toxicity studies have been conducted with several lepidopterans, the only ‘at-risk’ species for which data are available is the monarch. In addition, the biology of monarchs is well understood, and rearing methods, while challenging, have been published (Krishnan et al. 2020). Other lepidopteran ‘at-risk’ species are difficult, if not impossible, to rear in the laboratory and generation of insecticide toxicity data to inform risk assessments for these species is not possible, at least on a routine basis. Thus, the question is raised as to whether the monarchs could be considered a surrogate organism for other ‘at-risk’ lepidopterans. That is, do monarchs, other ‘at-risk’ lepidopteran species, and abundant lepidopteran species show similar sensitivity to insecticide mode of action classes? This question can be reasonably answered by strategically testing monarchs and representative abundant lepidopteran species with different insecticide classes using standardized testing methods, including the generation of full dose-response curves for different life stages. The data obtained from such bioassays can be input into species sensitivity distribution (SDD) models, which are statistical distributions that describe variations in toxicity among species to a compound or mixture. The distributions can be used to help set environmental standards and predict sensitivity for untested species, including ‘at-risk’ species, as well as those listed under the Endangered Species Act.

Methods

SSDs require that the species employed are from a similar taxon, of a similar stage, and exposed to similar compounds and testing methods, which include generation of a consistent toxicity endpoint and dosimetry information. A minimum of 8 to 10 species are needed to

generate a reliable SSD (Posthuma et al. 2000). Toxicity data for monarch butterfly was obtained from Chapter 2, and a literature search was performed to obtain data for other butterfly species (see Table 1). This included review of a recent butterfly toxicology paper (Braak et al. 2018) and employment of the following search terms on Google Scholar: “butterfly” AND “larva” OR “caterpillar” AND “topical” OR “cuticular” OR “dietary” OR “oral” AND “LD50” AND “pyrethroid” OR “organophosphate” OR “diamide” OR “neonicotinoid”. As dosimetry information was unavailable for many dietary toxicity studies reported in the literature, larval topical toxicity studies, most of which had reported LD_{50} values in µg of insecticide/larva and had provided larval weights, were used to develop SSDs. Distributions were generated for two classes of chemical insecticides with the most species data, pyrethroids and organophosphates. The U.S. Environmental Protection Agency’s Species Sensitivity Distribution Generator v1 (USEPA 2020) was used to generate the SSDs.

To evaluate monarch sensitivity to insecticides employing an RNA-interference (RNAi) mechanism, data from Chapter 4 was compared to published Lepidoptera, mostly moth, data that used similar testing methods; monarchs are the only butterfly species that have been treated with dsRNA molecules. Comparisons were made to dietary bioassay studies in which larvae were exposed to dsRNA molecules that had sequences homologous to their own mRNA. Studies that utilized an artificial diet or a vehicle to deliver the dsRNA were excluded as both have been shown to influence toxicity (Peng et al. 2019; Christiaens et al. 2020). Studies that did not provide a leaf/plant concentration or a larval dose also were excluded (see Table 2).

Honey bee (*Apis mellifera*) adult and larval topical and dietary LD values for chemical insecticides were obtained from literature (see Table 3). While larval weights were often not

provided, we assumed an adult honey bee weighs 0.1g (Thompson 2015). Monarch larval and adult topical and dietary doses were obtained from Chapters 2 and 3.

Results

Butterfly SSDs for organophosphate and pyrethroid insecticides can be found in Figures 1, 2, and 3. The organophosphates were separated based on whether they had an oxon group (P=O; active form) or a thion group (P=S; inactive form) since the former are at least 10-fold more toxic. Data from both type I and type II pyrethroids were combined due to the limited number of available studies. Overall, the distributions show that, for the insecticides tested, monarch butterfly larvae have susceptibility similar to other butterfly larvae. Butterflies showed greater susceptibility to pyrethroid than organophosphate insecticides. Atala hairstreak (*Eumaeus atala*) and long-tailed skipper (*Proteus urbanus*) were generally the most sensitive butterflies, while painted lady (*Vanessa cardui*) was among the least sensitive butterflies.

Most species had two or more LD_{50} values generated for each insecticide class/subclass; the distributions provide the average LD_{50} for each species. The spread in toxicity for all three distributions is 2 to 2.5 orders of magnitude. Organophosphates belonging in the phosphate subclass have LD_{50}s ranging from 0.1 to 10 µg/g; the slope of the distribution is 1.2 with an R^2 of 0.94. Organophosphates belonging in the phosphorothioate and phosphorodithioate subclasses have LD_{50}s ranging from 1.0 to 100 µg/g with a slope and R^2 of 1.4 and 0.95, respectively. For pyrethroids, the LD_{50}s range from 0.01 to 1.0 µg/g and the slope and R^2 are 1.2 and 0.92, respectively.

A brief review of the moth toxicity literature revealed sensitivity similar to monarchs and other butterflies. For example, cotton bollworm (*Helicoverpa armigera*), corn earworm (*Helicoverpa zea*), and tobacco budworm (*Heliothis virescens*) larvae produced topical LD_{50}

values ranging from 0.029 to 1.2 µg/g for eight pyrethroid insecticides (Luttrell et al. 1987; Roush and Luttrell 1989; Abd-Elghafar et al. 1993; Kranthi et al. 2001; Martin et al. 2003; Tan and McCaffery 2007; Jacobson et al. 2009; Bird 2018). The butterfly larval LD_{50}s for pyrethroid insecticides range from 0.0009 to 0.79 µg/g. When treated with two organophosphates in the phosphorothioate and phosphorodithioate subclass, cotton bollworm, corn earworm, and cotton cutworm (*Spodoptera litura*), had topical LD_{50}s ranging from 0.73 to 26 µg/g (Abd-Elghafar et al. 1993; Martin et al. 2003; Huang et al. 2006). Butterfly LD_{50}s for the same subclass were more variable and ranged from 0.26 to 2400 µg/g (see Table 1 for references).

With regard to insecticides that employ the RNAi mechanism, we find that monarchs are less sensitive than other tested lepidopteran species, which are all moths in the Plutellidae, Crambidae, and Noctuidae families (Table 2). In the moth studies, a dsRNA concentration of 1 to 2 µg/cm^2 leaf was often adequate to cause at least 40% mortality. A higher concentration (up to 18 µg/cm^2 leaf) was needed to target the function of juvenile hormone (JH) and ecdysone. A dsRNA dose of 3 µg/larva (leaf/plant concentration was not provided) was adequate to silence the chitin synthase mRNA and result in ≥ 50% mortality in diamondback moth (*Plutella xylostella*), legume pod borer (*Maruca vitrata*), spotted stalk borer (*Chilo partellus*), and tobacco cutworm (*Spodoptera litura*) (Rana et al. 2020). In monarchs, a dsRNA that targeted the V-ATPase mRNA did not cause increased mortality even at a dose of 255 µg/larva, which corresponded to a concentration of 0.9 µg/cm^2 leaf provided over the entire larval cycle (Krishnan et al. 2021b). In addition, a two-day exposure to the same dsRNA at a much higher concentration of 100 µg/cm^2 leaf also did not cause any effect in monarchs (Pan et al. 2017).

Table 3 provides LD_{50} estimates, in µg of insecticide/g for monarch butterfly larvae and adults and honey bee adults. We find that both monarch stages are typically less sensitive to the

neonicotinoids imidacloprid, thiamethoxam, and clothianidin, and the organophosphate chlorpyrifos. Monarchs and honey bees have similar sensitivity to the pyrethroid cyfluthrin/beta-cyfluthrin, while monarch larvae are at least 10-fold more sensitive to the diamide chlorantraniliprole. Additional data for monarch adults are needed to better discern interspecies sensitivity.

Dietary studies on honey bee larvae produced LD_{50} values of 0.46 and 4.2 µg/larva for chlorpyrifos and imidacloprid, respectively; 37% mortality was seen in larvae exposed to 1.0 µg of chlorantraniliprole (Dai et al. 2017; Wade et al. 2019). In contrast, doses that caused approximately 50% mortality in monarch larvae exposed to chlorpyrifos- and imidacloprid-treated milkweed leaves ranged from 0.41 to 11 and 0.26 to 4.5 µg/larva, respectively; 20 to 40% mortality was seen in larvae exposed to 0.00013 to 0.18 µg of chlorantraniliprole (Krishnan et al. 2021a). Thus, monarch and honey bee larvae show similar sensitivity to imidacloprid and chlorpyrifos, while monarchs show greater sensitivity to chlorantraniliprole. Honey bee larvae, like monarch larvae, are also recalcitrant to dsRNA products that target their V-ATPase mRNA (Velez et al. 2015).

Discussion

Challenges in rearing monarch butterflies

At Iowa State University, the U.S. Department of Agriculture − Agricultural Research Service maintains one of the largest monarch butterfly (*Danaus plexippus*) colonies in the country. While the colony is capable of producing 300 eggs and neonate larvae per week, rearing monarch butterflies is labor-intensive and challenging. From 2016 to 2020, several pathogen outbreaks were encountered (preliminary diagnosis provided by Denny Bruck at Corteva Agriscience), including bacteria (*Serratia marcescens*, *Enterobacter cloacae*, and *Pseudomonas*)

and possible viruses (e.g., Nuclear Polyhedrosis Virus), which resulted in elevated background (control) larval mortality (75% survival from neonate to pupation without disease symptomology is considered acceptable). In addition, infestation of host plant milkweed *(Asclepias curassavica* and *Asclepias syriaca*) by oleander aphids (*Aphis nerii*), western flower thrips (*Frankliniella occidentalis*), greenhouse whiteflies (*Trialeurodes vaporariorum*), and spider mites (*Tetranychus urticae*) can reduce the amount of diet and/or the quality of the diet to maintain high levels of monarch production. To maintain the quality of milkweed and/or prevent pest infestations, expensive biological controls agents, e.g., parasitic wasps (*Aphidius colemani*), predatory mites (*Neoseiulus californicus* and *Phytoseiulus persimilis*), and rove beetles (*Dalotia coriaria*), were employed (Krishnan et al. 2020), as the use of insecticides or miticides could harm monarch larvae.

Given these rearing challenges, significant labor and resources would be needed to maintain a sustained production of monarchs to provide toxicity data for new insecticides to support their registration and for currently registered insecticides undergoing re-evaluation. For example, over the course of five years, the generation of monarch toxicology data reported in this dissertation for five different insecticide modes of action [pyrethroids, organophosphates, diamides, neonicotinoids, and RNAi], across all life stages (egg, various larval instars, pupae, and adults), and different routes and durations of exposure (topical and dietary, and acute and chronic), required approximately 10,000 healthy individuals. The data reported in this dissertation, combined with related data in the literature, can provide a framework to develop SSD models (Posthuma et al. 2000) to predict monarch and other ‘at-risk’ lepidopteran species responses to insecticides without the need for an extensive testing program.

Monarch butterfly insecticide toxicity comparisons with other species

While the SSD graphs indicate that monarch larvae show sensitivity to organophosphate and pyrethroid insecticides that is similar to other butterfly larvae, it is important to note that there are several problems with the toxicity data used to develop the distributions. For example, some of the insecticides were applied as formulated products, and an appropriate control was not utilized. Although toxic doses are expressed in units of active ingredient, larval responses could have been influenced by inert ingredients (studies that employed a synergist were not included in the SSDs). Most studies only reported toxicity responses based on nominal solution concentrations, and some did not adequately describe the preparation of solutions. In addition, toxicity studies with different carrier solvents (including acetone, olive oil, and diesel fuel), insecticides (five pyrethroids, three organophosphates in the phosphate subclass, and nine organophosphates in the phosphorothioate and phosphorodithioate subclasses), larval instars (all instars), and lengths of observation (24 hours to 96 hours) were included in the SSDs. If further data quality attributes were used to filter the studies, the paucity of remaining data would preclude the means to generate SSDs. Therefore, standardized and well-designed studies are necessary to refine understanding of butterfly interspecies variability and generate robust SSDs. Inclusion of moth data, which displays toxicity results similar to butterflies for the classes of insecticides tested, can also lead to more robust distributions. Also, additional toxicity data are needed for newer classes of chemical insecticides, e.g., diamides and sulfoximines, as both pyrethroids and organophosphates are being phased out, either due to development of pest resistance or high mammalian toxicity.

In Chapter 5 of this dissertation, I evaluated the susceptibility of seven final-instar lepidopteran larvae to neonicotinoid-mediated arrested pupal ecdysis. Following topical

application of imidacloprid, monarchs were found to be the most sensitive on a µg/g basis. While painted ladies (*Vanessa cardui*) and red admiral (*Vanessa atalanta*) larvae were considerably smaller than monarch larvae, a 20-µg dose killed only 20 to 30% of these treated butterflies. The same dose killed 100% of tested monarchs. Corn earworms (*Helicoverpa zea*) and wax moths (*Galleria mellonella*) were 2 to 5 times less sensitive, while European corn borers (*Ostrinia nubilalis*) and fall armyworms were at least 100 times less sensitive (Krishnan et al. 2021c).

The lower sensitivity of monarchs to dsRNA insecticides could be due to less effective silencing of the V-ATPase mRNA. Reduced silencing and lower mortality rates also were observed when cotton bollworm (*Helicoverpa armigera*) larvae were provided V-ATPase small interfering RNA (siRNA), which are molecules that result from dsRNA processing and are directly involved in mRNA silencing. When a leaf concentration of 10 µg/cm^2 V-ATPase siRNA was provided to 4-day-old larvae for 10 days, only ~ 10% mortality occurred (Mao et al. 2015). However, when an artificial diet (which could potentially reduce dsRNase expression; Peng et al. 2019), was used to chronically expose tobacco hornworm (*Manduca sexta*) larvae to a v-ATPase dsRNA that was homologous to its own mRNA, a LC_{50} of 11 µg/g diet was obtained (Whyard et al. 2009). In our study, monarch larvae were unaffected by chronic exposure to 34 µg/g leaf. Reduced susceptibility of monarchs could also be due to the presence of higher dsRNase activity in the saliva and/or gut. Thus, our studies indicate that while monarchs may serve as a good surrogate species for the classes of chemical insecticides tested, they may not be an ideal non-target lepidopteran test species for dsRNA insecticides.

Honey bee is another insect species of agronomic concern and for which there is sufficient toxicity data available for comparisons with monarch bioassay results. Most studies have focused on adult honey bees that forage on plants within and outside agricultural fields.

Compared to monarchs, they show increased sensitivity to neonicotinoid and organophosphate insecticides; this could be because of low levels of detoxification enzymes (Claudianos et al. 2006). Honey bees also show reduced sensitivity to chlorantraniliprole, possibly due to the insecticide's weak binding potential to their ryanodine receptors (Qi and Casida 2013).

Conclusions

While SSDs can be useful in predicting toxicity for hard-to-rear species, selecting surrogate species for toxicity testing guidelines, and setting environmental standards (Posthuma et al. 2000), there are additional biological and ecological factors that need to be considered. For example, allometric scaling, which relates toxicity to species' body mass, are often recommended for interspecies comparisons (Foureman and Kenyon 2006). Some toxicokinetic and toxicodynamic properties that influence toxicity may not scale with mass (Schneider et al. 2004). For example, we found that two lepidopteran species, corn earworms and fall armyworms, that belong to the same family and have similar mass show very different susceptibilities to imidacloprid-mediated arrested pupal ecdysis (see Chapter 5). In addition, SSDs do not account for the bioavailability of an insecticide in the environment and a species' likelihood of exposure. Distributions also do not consider interactions within ecosystems or the combined effects of multiple stressors. Thus, it is important to assess SSDs in combination with other factors to accurately estimate a species' risk to a chemical exposure.

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References

Abd-Elghafar SF, Knowles CO, Wall ML. 1993. Pyrethroid Resistance in Two Field Strains of Helicoverpa Zea (Lepidoptera: Noctuidae). J Econ Entomol. 86 (6), 1651–1655.

Arena M, Sgolastra F. 2014. A Meta-Analysis Comparing the Sensitivity of Bees to Pesticides. Ecotoxicology. 23 (3), 324–334.

Bird LJ. 2018. Pyrethroid and Carbamate Resistance in Australian *Helicoverpa Armigera* (Lepidoptera: Noctuidae) from 2008 to 2015: What Has Changed since the Introduction of Bt Cotton? Bull Entomol Res. 108 (6), 781–791.

Braak N, Neve R, Jones AK, Gibbs M, Breuker CJ. 2018. The effects of insecticides on butterflies – A review. Environ Pollut. DOI: 10.1016/j.envpol.2018.06.100.

Çilgi T, Jepson PC. 1995. The risks posed by deltamethrin drift to hedgerow butterflies. Environmental Pollution 87, 1-9.

Claudianos C, Ranson H, Johnson RM, Biswas S, Schuler MA, Berenbaum MR, Feyereisen R, Oakeshott JG. 2006. A Deficit of Detoxification Enzymes: Pesticide Sensitivity and Environmental Response in the Honeybee. Insect Mol Biol. 15 (5), 615–636.

Chaitanya BN, Asokan R, Sita T, Rebijith KB, Ram Kumar P, Krishna Kumar NK. 2017. Silencing of JHEH and EcR Genes of Plutella Xylostella (Lepidoptera: Plutellidae) through Double Stranded RNA Oral Delivery. J Asia-Pacific Entomol. 20 (2), 637–643.

Christiaens O, Whyard S, Vélez AM, Smagghe G. 2020. Double-Stranded RNA Technology to Control Insect Pests: Current Status and Challenges. Front Plant Sci. 11, 451.

Dai P, Jack CJ, Mortensen AN, Ellis JD. 2017. Acute Toxicity of Five Pesticides to *Apis Mellifera* Larvae Reared *in Vitro*: Acute Toxicity of Five Pesticides to *Apis Mellifera* Larvae. Pest Manag Sci. 73 (11), 2282–2286.

Davis BNK, Lakhani KH, Yates TJ. 1991. The hazards of insecticides to butterflies of field margins. Agriculture, Ecosystems & Environment 36, 151-161.

Davis BNK, Lakhani KH, Yates TJ, Frost AJ, Plant RA. 1993. Insecticide drift from ground-based, hydraulic spraying of peas and brussels sprouts: bioassays for determining buffer zones. Agriculture, Ecosystems & Environment 43, 93-108.

Eliazar PJ, Emmel TC. 1991. Adverse impacts to non-target insects. Mosquito control pesticides: Ecological impacts and management alternatives. Scientific Publishers Inc., Gainesville, Florida, pp. 17-19.

Ellango R, Asokan R, Chandra GS, Kumar NKK, Mahmood R, Ramamurthy VV. 2018. Tyrosine Hydroxylase, a Potential Target for the RNAi-Mediated Management of Diamondback Moth (Lepidoptera: Plutellidae). Florida Entomologist. 101 (1), 1–5.

Foureman and Kenyon. 2006. Harmonization in Interspecies Extrapolation: Use of BW 3/4 as Default Method in Derivation of the Oral RfD. USEPA External Review Draft.

Hoang TC, Pryor RL, Rand GM, Frakes RA. 2011. Use of butterflies as non-target insect test species and the acute toxicity and hazard of mosquito control insecticides. Environ Toxicol Chem. 30, 997-1005.

Huang SJ, Xu JF, Han ZJ. Baseline toxicity data of insecticides against the common cutworm *Spodoptera litura* (Fabricius) and a comparison of resistance monitoring methods. Int J Pest Manag. 2006; 52:209–213.

Iwasa T, Motoyama N, Ambrose JT, Roe RM. 2004. Mechanism for the Differential Toxicity of Neonicotinoid Insecticides in the Honey Bee, Apis Mellifera. Crop Protection 23 (5), 371–378.

Jacobson A, Foster R, Krupke C, Hutchison W, Pittendrigh B, Weinzierl R. 2009. Resistance to Pyrethroid Insecticides in <I>Helicoverpa Zea</I> (Lepidoptera: Noctuidae) in Indiana and Illinois. ec 102 (6), 2289–2295.

Kadala A, Charreton M, Charnet P, Collet C. 2019. Honey Bees Long-Lasting Locomotor Deficits after Exposure to the Diamide Chlorantraniliprole Are Accompanied by Brain and Muscular Calcium Channels Alterations. Sci Rep. 9 (1), 2153.

Kranthi KR, Jadhav D, Wanjari R, Kranthi S, Russell D. 2001. Pyrethroid Resistance and Mechanisms of Resistance in Field Strains of <I>Helicoverpa Armigera</I> (Lepidoptera: Noctuidae). ec 94 (1), 253–263.

Krishnan N, Yang Z, Bidne KG, Hellmich RL, Coats JR, Bradbury SP. 2020. Assessing field-scale risks of foliar insecticide applications to monarch butterfly (*Danaus plexippus*) larvae. Environ Toxicol Chem. 39 (4), 923–941.

Krishnan N, Yang Z, Aust M, Hellmich RL, Coats JR, Bradbury SP. 2021a. Monarch butterfly (*Danaus plexippus*) life stage risks from foliar and seed treatment insecticides. Environ Toxicol Chem. *In revision.*

Krishnan N, Hall MJ, Hellmich RL, Coats JR, Bradbury SP. 2021b. Assessing the toxicity of varroa-active dsRNA on monarch butterfly (*Danaus plexippus*) larvae. *PLOS ONE. Submitted.*

Krishnan N, Jurenka RA, Bradbury SP. 2021c. Effects of neonicotinoid insecticides on lepidopteran pupal ecdysis. *In Preparation.*

Laurino D, Manino A, Patetta A, Porporato M. 2013. Toxicity of neonicotinoid insecticides on different honey bee genotypes. Bull. Insectol. 66, 119–126.

Luttrell RG, Roush RT, Ali A, Mink JS, Reid MR, Snodgrass GL. 1987 Pyrethroid Resistance in Field Populations of Heliothis Virescens (Lepidoptera: Noctuidae) in Mississippi in 1986. J Econ Entomol. 80 (5), 985–989.

Mao J, Zhang P, Liu C, Zeng F. 2015. Co-Silence of the Coatomer β and v-ATPase A Genes by SiRNA Feeding Reduces Larval Survival Rate and Weight Gain of Cotton Bollworm, Helicoverpa Armigera. Pesticide Biochemistry and Physiology. 118, 71–76.

Martin T, Ochou OG, Vaissayre M, Fournier D. 2003. Organophosphorus Insecticides Synergize Pyrethroids in the Resistant Strain of Cotton Bollworm, Helicoverpa Armigera (Hübner) (Lepidoptera: Noctuidae) from West Africa. J Econ Entomol. 96 (2), 468–474.

Mohamed AAM, Kim Y. 2011. A Target-Specific Feeding Toxicity of B1 Integrin DsRNA against Diamondback Moth, Plutella Xylostella. Arch Insect Biochem Physiol. 78 (4), 216–230.

Pan H, Yang X, Bidne K, Hellmich RL, Siegfried BD, Zhou X. 2017. Dietary Risk Assessment of V-ATPase A DsRNAs on Monarch Butterfly Larvae. Front Plant Sci. 8.

Peng Y, Wang K, Zhu G, Han Q, Chen J, Elzaki MEA, et al. Identification and Characterization of Multiple DsRNases from a Lepidopteran Insect, the Tobacco Cutworm, Spodoptera Litura (Lepidoptera: Noctuidae). Pestic Biochem Physiol. 2020; 1. 162 (1): 86–95.

Posthuma L, Traas TP, Suter GW. 2000. General introduction to species sensitivity distributions. In Species sensitivity distributions in ecotoxicology, Lewis, Boca Raton, FL, USA.

Qi S, Casida JE. 2013. Species Differences in Chlorantraniliprole and Flubendiamide Insecticide Binding Sites in the Ryanodine Receptor. Pesticide Biochem Physiol. 107 (3), 321–326.

Rana S, Rajurkar AB, Kumar KK, Mohankumar S. 2020. Comparative Analysis of Chitin SynthaseA DsRNA Mediated RNA Interference for Management of Crop Pests of Different Families of Lepidoptera. Front Plant Sci. 11, 427.

Roush RT, Luttrell RG. 1989. Expression of Resistance to Pyrethroid Insecticides in Adults and Larvae of Tobacco Budworm (Lepidoptera: Noctuidae): Implications for Resistance Monitoring. J Econ Entomol. *82* (5), 1305–1310.

Salvato M. 2001. Influence of mosquito control chemicals on butterflies (Nymphalidae, Lycaenidae, Hesperiidae) of the lower Florida Keys. Journal of The Lepidopterists Society 55, 8-14.

Sanchez-Bayo F, Goka K. 2014. Pesticide Residues and Bees – A Risk Assessment. PLoS ONE. *9* (4), e94482.

Sánchez-Bayo F, Wyckhuys KAG. 2019. Worldwide Decline of the Entomofauna: A Review of Its Drivers. Biological Conservation. 232, 8–27.

Schneider K, Oltmanns J, Hassauer, M. 2004. Allometric Principles for Interspecies Extrapolation in Toxicological Risk Assessment—Empirical Investigations. Regul Toxicol Pharmacol. *39* (3), 334–347.

Sharath Chandra G, Asokan R, Manamohan M, Krishna Kumar N. 2019. Enhancing RNAi by Using Concatemerized Double-Stranded RNA: Enhancing RNAi Efficiency. Pest Manag Sci. 75 (2), 506–514.

Sinha S, Lakhani K, Davis B. 1990. Studies on the toxicity of insecticidal drift to the first instar larvae of the large white butterfly *Pieris brassicae* (Lepidoptera: Pieridae). Annals of Applied Biology 116, 27-41.

Thompson H. 2016. Extrapolation of Acute Toxicity across Bee Species: Extrapolation of Acute Toxicity across Bee Species. Integr Environ Assess Manag. 12 (4), 622–626.

Tan J, McCaffery AR. 2007. Efficacy of Various Pyrethroid Structures against a Highly Metabolically Resistant Isogenic Strain of Helicoverpa Armigera (Lepidoptera: Noctuidae) from China. Pest. Manag. Sci. 63 (10), 960–968.

USEPA 2020. Species Sensitivity Distribution Generator. Available at: https://www.google.com/url?sa=t&rct=j&q=&esrc=s&source=web&cd=&ved=2ahUKEwj5sMfJuI3uAhXZX80KHQOvB6EQFjAAegQIARAC&url=https%3A%2F%2Fwww.epa.gov%2Fsites%2Fproduction%2Ffiles%2F2017-10%2Fssd_generator_v1.xlsm&usg=AOvVaw3vEYfUspuimupcbPrDnExY

USFWS 2019. At-risk species conservation. Available at: https://www.fws.gov/Southeast/endangered-species-act/at-risk-species/

USFWS 2020a. Listed Species. Available at: https://ecos.fws.gov/ecp0/reports/ad-hoc-species-report?kingdom=V&kingdom=I&status=E&status=T&status=EmE&status=EmT&status=EXPE&status=EXPN&status=SAE&status=SAT&mapstatus=3&fcrithab=on&fstatus=on&fspecrule=on&finvpop=on&fgroup=on&header=Listed+Animals

USFWS 2020b. Candidate Species Report. Available at: https://ecos.fws.gov/ecp/report/table/candidate-species.html

USFWS 2020c. At-Risk Species Finder. https://www.fws.gov/southeast/finder/#/species/list

Vélez AM, Jurzenski J, Matz N, Zhou X, Wang H, Ellis M, Siegfried BD. 2016. Developing an in Vivo Toxicity Assay for RNAi Risk Assessment in Honey Bees, Apis Mellifera L. Chemosphere 144, 1083–1090.

Wade A, Lin CH, Kurkul C, Regan ER, Johnson RM. 2019. Combined Toxicity of Insecticides and Fungicides Applied to California Almond Orchards to Honey Bee Larvae and Adults. Insects *10* (1), 20.

Wahla MA, Gibbs RG, Ford JB. 1976. Diazinon poisoning in large white butterfly larvae and the influence of sesamex and piperonyl butoxide. Pesticide Science 7, 367-371.

Whyard S, Singh AD, Wong S. Ingested Double-Stranded RNAs Can Act as Species-Specific Insecticides. Insect Biochem Mol Biol. 2009; 11. 39 (11): 824–832.

Tables and Figures

Table 1. Butterfly studies employed to generate pyrethroid and organophosphate species sensitivity distributions[a]

Common name (Scientific name)	Class and Insecticide tested
Atala hairstreak (*Eumaeus atala*)	Pyrethroid (type I): Permethrin; Organophosphate (oxon): Dichlorvos, Naled
Common blue (*Polymornatus icarus*)	Organophosphate (thion): Fenitrothion
Common buckeye (*Junonia coenia*)	Pyrethroid (type I): Permethrin; Organophosphate (oxon): Naled, Dichlorvos
Giant swallowtail (*Papilio cresphontes*)	Organophosphate (oxon): Naled; Organophosphate (thion): Fenthion, Malathion
Green-veined white (*Pieris napi*)	Organophosphate (thion): Dimethoate, Fenitrothion, Phosalone
Gulf fritillary (*Agraulis vanilla*)	Organophosphate (oxon): Naled; Organophosphate (thion): Malathion
Hedge brown (*Pyronia Tithonus*)	Organophosphate (thion): Fenitrothion, Phosalone
Large cabbage white (*Pieris brassicae*)	Pyrethroid (type II): Cypermethrin, Fenvalerate, Deltamethrin; Organophosphate (oxon): Diazinon, Diazoxon, Dimethoate, Fenitrothion, Phosalone, Pirimiphos-methyl, Triazophos
Long-tailed skipper (*Proteus urbanus*)	Organophosphate (oxon): Naled; Organophosphate (thion): Malathion
Monarch butterfly (*Danaus plexippus*)	Pyrethroid (type II): Beta-cyfluthrin; Organophosphate (thion): Chlorpyrifos
Painted lady (*Vanessa cardui*)	Pyrethroid (type I): Permethrin; Organophosphate (oxon): Dichlorvos, Naled; Organophosphate (thion): Fenthion, Malathion
Small cabbage white (*Pieris rapae*)	Pyrethroid (type II): Deltamethrin
Tropical-checkered skipper (*Pyrgus oileus*)	Organophosphate (oxon): Naled
White peacock (*Anartia jatrophae*)	Pyrethroid (type I): Permethrin; Organophosphate (oxon): Dichlorvos, Naled
Zebra longwing (*Heliconius charithonia*)	Pyrethroid (type I): Permethrin; Organophosphate (oxon): Dichlorvos, Naled; Organophosphate (thion): Fenthion, Malathion

[a] Data obtained from Wahla et al. (1976); Sinha et al. (1990); Eliazar and Emmel (1991); Davis et al. (1991); Davis et al. (1993); Cilgi and Jepson (1994); Salvato (2001); Huang et al. (2006); Hoang et al. (2011); Krishnan et al. (2020).

Table 2. Summary of Lepidopteran studies in which larvae were provided known concentrations or doses of dsRNA that targeted one of their mRNA sequences. The unadulterated dsRNA solutions were applied on fresh leaves or plant tissue[a]

Species	Stage and duration of exposure	Target site activity	Concentration/dose and larval mortality
Diamondback moth (*Plutella xylostella*)	5-day-old larvae for 5 days	Tyrosine hydroxylase	1 to 3 µg/cm^2 leaf (6 to 9 µg/larva) ≥ 55% mortality
	4-day-old larvae till pupation	Acetylcholinesterase	2 µg/cm^2 leaf: 40% mortality
	Second instar for 4 days	JH epoxide hydrolase	18 µg/cm^2 leaf: 67% mortality
	Second instar for 4 days	Ecdysteroid receptor	18 µg/cm^2 leaf: 53% mortality
	Second instar for 0.5 days	ß1 integrin	0.12 µg/larva: 100% mortality
	Second instar till pupation	Chitin synthase	3 µg/larva: 70% mortality
Legume pod borer (*Maruca vitrata*)	Second instar till pupation	Chitin synthase	3 µg/larva: 50% mortality
Spotted stalk borer *(Chilo partellus)*	Second instar till pupation	Chitin synthase	3 µg/larva: 68% mortality
Tobacco cutworm (*Spodoptera litura*)	Second instar till pupation	Chitin synthase	3 µg/larva: 58% mortality
Monarch butterfly (*Danaus plexippus*)	Neonate for 2 days	V-ATPase	100 µg/cm^2 leaf (16 µg/larva): No effect
	Neonate to pupation	V-ATPase	0.9 µg/cm^2 leaf (255 µg/larva): No effect[b]

[a] Data obtained from Mohamed and Kim (2011); Chaitanya et al. (2017); Pan et al. (2017); Ellango et al. (2018); Sharath Chandra et al. (2018); Rana et al. (2020); and Krishnan et al. (2021b).

[b] Based on weight to surface area conversion for tropical milkweed leaves; 1 g corresponds to 38 cm^2 (Krishnan et al. 2021a). As the average monarch V-ATPase dsRNA concentration on tropical milkweed leaves is 34 µg/g, this corresponds to ca. 0.9 µg/cm^2.

Table 3. Toxicity estimates for monarchs and bees following topical and dietary exposures to four classes of insecticides[a]

Insecticide	Exposure route	Acute LD_{50} value estimates (µg of insecticide/g body weight)			Broad conclusions on sensitivity
		Monarch larvae[b]	Monarch adults	Honey bee adults	
Cyfluthrin	Topical	0.0092 to 0.048	0.08 to 1.2[c]	0.01 to 0.19	Similar sensitivity in both species
	Dietary	0.37 to 0.84	NA	0.51	
Chlorpyrifos	Topical	18 to 79	7.7 to 86[c]	0.59 to 0.72	Monarchs less sensitive than honey bees
	Dietary	15 to 56	NA	2.4	
Chlorantraniliprole	Topical	0.012 to 0.19	3.8 to 44[c]	>2.5 to 40	Honey bees less sensitive than monarchs
	Dietary	0.016 to 1.1	NA	>1040	
Imidacloprid	Topical	3.0 to 8.4	52 to 104[c]	0.026 to 0.4	Monarchs less sensitive than honey bees
	Dietary	6.0 to 26	No effect at 0.028	0.037 to 0.054	
Thiamethoxam	Topical	6.1 to 35	No effect at 32	0.061 to 0.3	Monarchs less sensitive than honey bees
	Dietary	9.0 to 17	No effect at 0.036	0.043 to 0.047	
Clothianidin	Topical	0.19 to 1.3	No effect at 83	0.22	Monarchs less sensitive than honey bees
	Dietary	1.0 to 16	No effect at 0.015	0.026 to 0.028	

[a] Data obtained from Iwasa et al. (2004); Laurino et al. (2013); Sanchez-Bayo and Goka (2014); Arena and Sgolastra (2014); Kadala et al. (2019); Wade et al. (2019); Krishnan et al. (2020); and Krishnan et al. (2021a).

[b] LD_{50} estimate ranges across three instars (first/second, third, fifth).

[c] LD_{50} not estimated but lies within the range of values described.

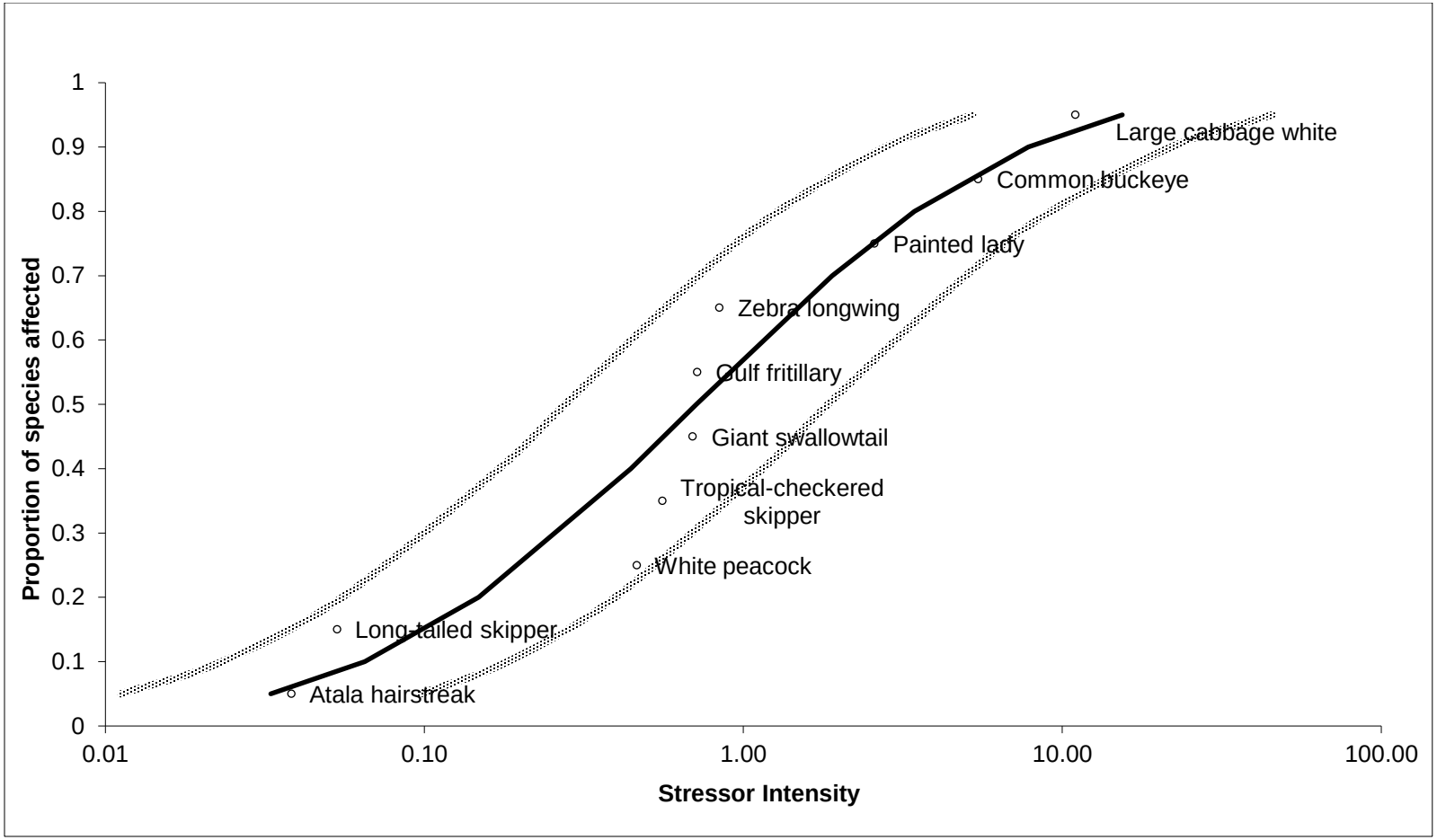

Figure 1. A species sensitivity distribution model depicting larval sensitivity of butterfly species to organophosphate insecticides (Table 1) in the phosphate subclass.

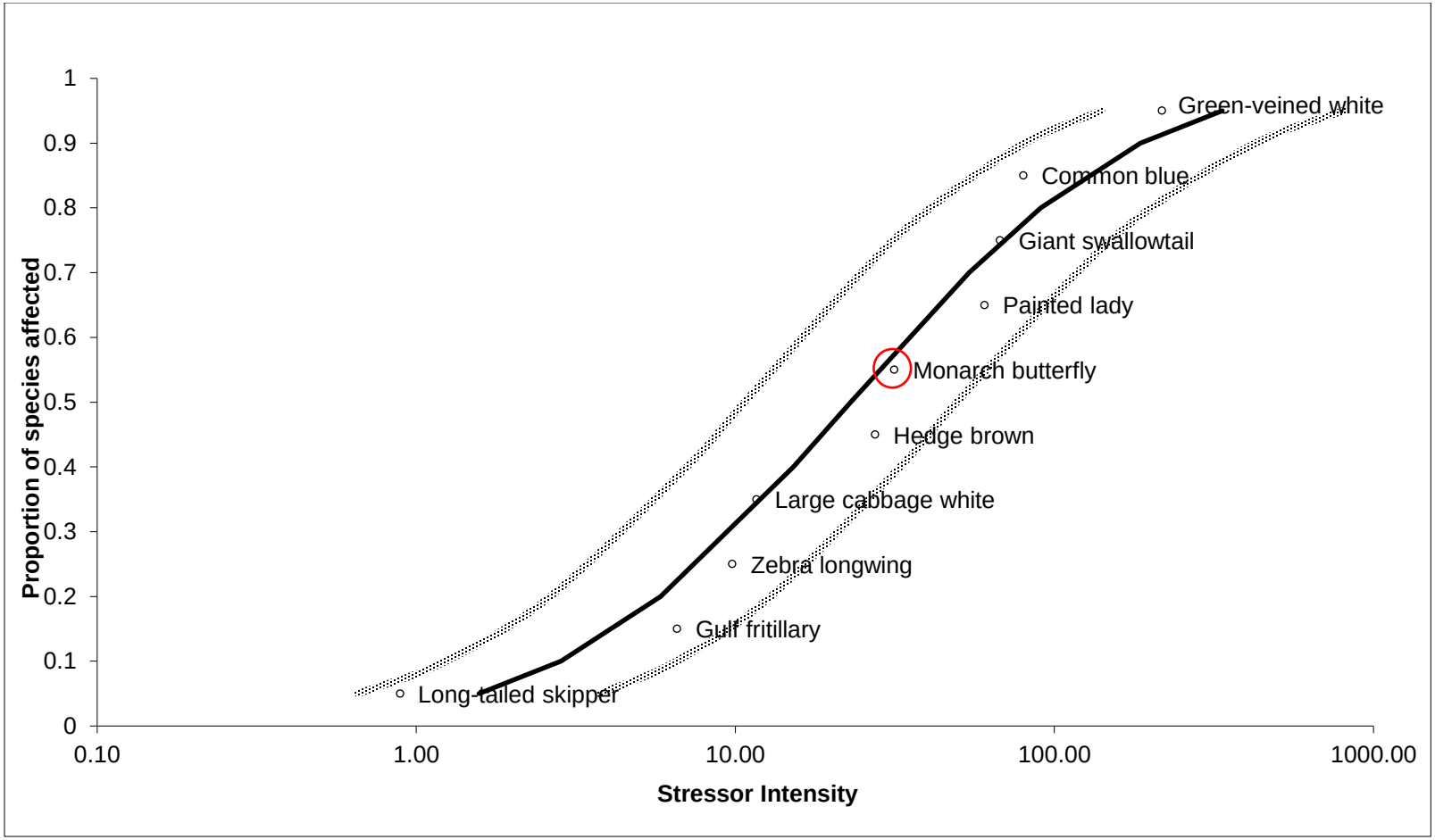

Figure 2. A species sensitivity distribution model depicting larval sensitivity of butterfly species to organophosphate insecticides (Table 1) in the phosphorothioate and phosphorodithioate subclasses. The red circle indicates the LD_{50} value of monarch butterflies.

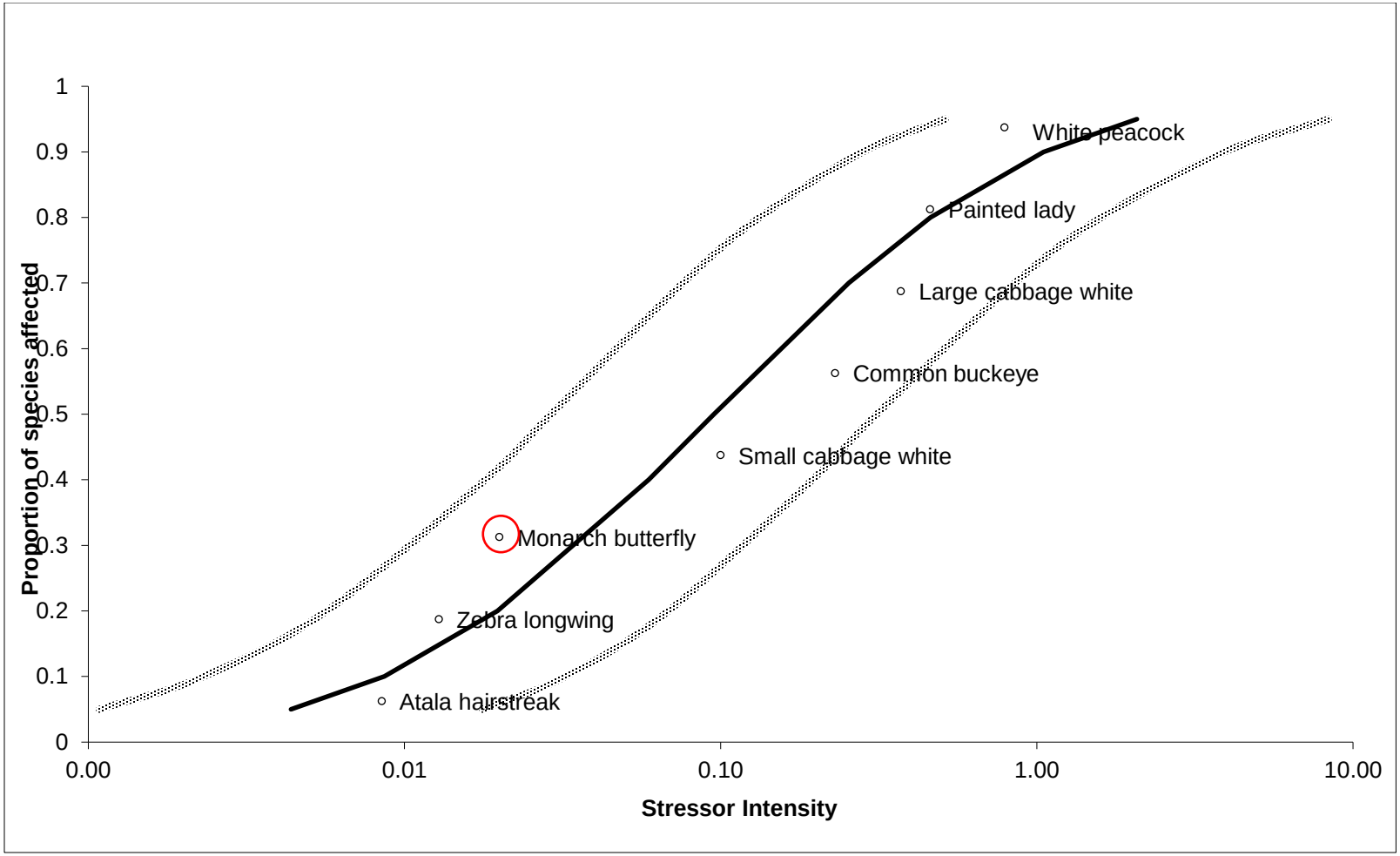

Figure 3. A species sensitivity distribution model depicting larval sensitivity of butterfly species to pyrethroid insecticides (type I and II; Table 1). The red circle indicates the LD_{50} value of monarch butterflies.

CHAPTER 7. GENERAL CONCLUSION

The research presented in this dissertation employed several steps in the insecticide risk assessment process for monarch butterflies (*Danaus plexippus*). These steps included a) design of conceptual models to elucidate likely pathways of insecticide exposure; b) generation of *in vivo* toxicity data with relevant routes of exposure and appropriate dose-metrics for comparison with measured or modeled exposure estimates to obtain field-scale probabilistic risks; c) development of adverse outcome pathways for a novel mode of action; and d) modelling of preliminary species sensitivity distributions for butterfly larvae to support species extrapolation. Of note, the work in Chapter 2 was cited in the candidate species listing decision made for monarch butterflies under the Endangered Species Act (U.S. Fish and Wildlife Service 2020).

Outside this dissertation, the field-scale risk estimates reported in Chapters 2 and 3 were incorporated into a spatially explicit population model that integrated monarch demographics, behavior, and natural survival rates to elucidate the conservation risks and benefits of establishing milkweed (*Asclepias spp.*) in close proximity to crop fields in Iowa agricultural landscapes (Grant et al. 2021).

Brief overview of chapter findings

Of the four classes of chemical insecticides studied, the anthranilic diamide chlorantraniliprole and the pyrethroid beta-cyfluthrin were the most toxic, while the neonicotinoid thiamethoxam and the organophosphate chlorpyrifos were the least toxic. Except for beta-cyfluthrin, both topical and dietary routes of exposure often produced similar toxicity in monarch larvae. On a µg/g larval basis, we found that other lepidopteran (butterfly and moth) species showed sensitivity similar to topical applications of pyrethroid and organophosphate insecticides; however, final-instar monarchs could be slightly more sensitive to neonicotinoids.

For the classes of insecticides tested, monarch larvae and eggs were generally more sensitive than monarch adults and pupae.

Comparison of toxicity data with exposure estimates revealed that foliar insecticide use posed significantly greater risks to monarchs; although neonicotinoid seed treatments are used ubiquitously in North Central USA, their concentrations in milkweed leaves and wildflower nectar are below that which cause an adverse effect. Incorporation of foliar insecticide field-scale risk estimates into a spatially explicit agent-based model allowed us to estimate landscape-scale risks to non-migratory monarch butterfly populations in Iowa. We found that while field-scale estimates for some insecticides indicated nearly 100% larval mortality up to 38 m downwind of treated fields, landscape-scale simulation model results indicated that more monarchs would be produced if milkweed were established in all available space in agricultural landscapes (Grant et al. 2021). This finding is related to a number of landscape-scale factors including pest species, levels of pest pressure above or below economic thresholds, wind direction at the time of insecticide application, and likelihood of monarch exposure.

Figure 1a illustrates findings from a central Iowa scenario with economically significant soybean aphid (*Aphis glycines*) outbreaks occurring, on average, three times over 10 years. Production of adult monarchs was estimated following aerial applications of chlorantraniliprole and thiamethoxam, which are estimated to cause the most and least larval mortality, respectively. Three scenarios for milkweed augmentation were considered: baseline, i.e., no new milkweed added to the landscape; milkweed established only outside the 38-m ‘no plant zone’; and milkweed established both within and outside the 38-m ‘no plant zone’. These model simulations indicated that establishing milkweed everywhere, including within 38 m of a crop field, would produce more adult monarchs than other scenarios with habitat establishment outside a 38-m

exclusion zone, even with insecticide exposure. Employment of additive mixture models for insecticide formulations that contain two active ingredients and assumptions of 100% downwind mortality reduced simulated adult production by less than 3%, compared to the no-insecticide exposure scenario (Grant et al. 2021). However, if integrated pest management (IPM) practices are not followed and an application is done every year regardless of pest pressure, 8% fewer monarchs were produced as compared to the no-insecticide exposure scenario (Figure 1b). Thus, our simulations suggest that conservation benefits of establishing milkweed everywhere in agricultural landscapes, along with implementation of IPM practices, would outweigh the risks of increased insecticide exposure near crop fields.

Dietary toxicity bioassays with double-stranded RNA molecules (dsRNA) that target the v-ATPase mRNA in monarchs (100% match) and Varroa mites (*Varroa destructor*; 21-nucleotide match) produced no adverse effects in monarch larvae. These findings suggest that monarch v-ATPase mRNA might be refractory to silencing by dsRNA or that monarch saliva or gut may contain high levels of dsRNase, which suggests this new insecticide technology might pose less risks to monarchs in comparison to target pests. Other lepidopteran species, however, show greater sensitivity to dsRNA molecules that target their own mRNA indicating additional research is needed to better understand species sensitivity and differences in silencing and/or degradation of dsRNA. In the course of this research, we found that neonate monarchs needed 7.5 to 10.5 g of fresh milkweed leaf tissue to reach a healthy pupal weight and that larger pupae were produced if larvae were reared on common milkweed (*Asclepias syriaca*) vs. tropical milkweed (*Asclepias curassavica*) leaves.

Finally, this dissertation reports discovery of a novel mode of action for neonicotinoid insecticides. Five of seven final-instar lepidopteran larvae displayed arrested pupal ecdysis (AE)

following treatment with imidacloprid. Larvae with AE had unexpanded appendages and delayed initiation of their ecdysis motor program; these observations, along with a narrow window of developmental susceptibility, suggest that neonicotinoids are disrupting the function of crustacean cardioactive peptide (CCAP) neurons. Adverse outcome pathways for this effect were proposed. The potential molecular initiating events could include neonicotinoids directly binding to CCAP neuron acetylcholine receptors leading to desensitization, or neonicotinoids acting via acetylcholine signaling pathways to prolong activation of inhibitory neurons which regulate CCAP neuron function.

Recommendations for future work

Currently, lepidopteran bioassays are not standardized. The vast majority are acute studies that do not employ sufficient observation periods and testing has been mostly done with early larval instars or adults. In addition, many dietary bioassays do not provide dosimetry information, and dose-response curves are not always generated. Sublethal effects are rarely studied, and 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.

Having said that, generation of extensive toxicity data is time-consuming and resource intensive. Some lepidopteran species, like the monarchs, are not easy to rear in large numbers, while others are nearly impossible to rear. In addition, every year, multiple new chemicals are being registered, and it is not possible to test these and the existing chemicals on the many non-target lepidopterans. Thus, there is a need to employ other methods, including *in vitro* and *in silico* methods that can hasten and improve testing (National Academy of Sciences 2007). For example, *in vitro* techniques like use of cell lines and ‘omics’ and imaging technology can lead to development of adverse outcome pathways that connect molecular and cellular events to

population-level effects. Use of agent-based computer models that incorporate different streams of information, from the basic biology of a species, its landscape, and likelihoods of exposure to stressors like chemicals and climate change, can simulate future population responses. In addition, chemical testing could be prioritized by use of models that predict how a chemical would move and behave in the environment and within organisms. These include quantitative structure-activity relationship (QSAR) models, ReadAcross techniques, physiologically based pharmacokinetic models (PBPK), and spray drift and aquatic and terrestrial fate and transport models. Recent advances in artificial intelligence also can be harnessed.

While *in vitro* and *in silico* techniques are being increasingly used in mammalian (human) toxicology, their use in invertebrate toxicology is very limited and provides a future avenue for research. And while better-quality *in vivo* data are needed in the short term and are necessary for validation of *in vitro* and *in silico* data, the future of insect conservation with respect to chemical exposure relies on reducing, replacing, and refining insect use in toxicity bioassays.

References

Grant TJ, Krishnan N, Bradbury SP. 2021. Conservation risks and benefits of establishing monarch butterfly (*Danaus plexippus*) breeding habitat in close proximity to maize and soybean fields in the U.S. Midwest: A landscape-scale analysis of foliar insecticide effect on non-migratory monarch butterfly populations. Integr Environ Assess Manag. *Under Review*.

National Academy of Sciences. 2007. Toxicity Testing in the 21st Century: A Vision and a Strategy. Available at: https://www.nap.edu/catalog/11970/toxicity-testing-in-the-21st-century-a-vision-and-a

U.S. Fish and Wildlife Service. 2020. Species Status Assessment Report. Available at: https://www.fws.gov/savethemonarch/pdfs/Monarch-SSA-report.pdf

Figure

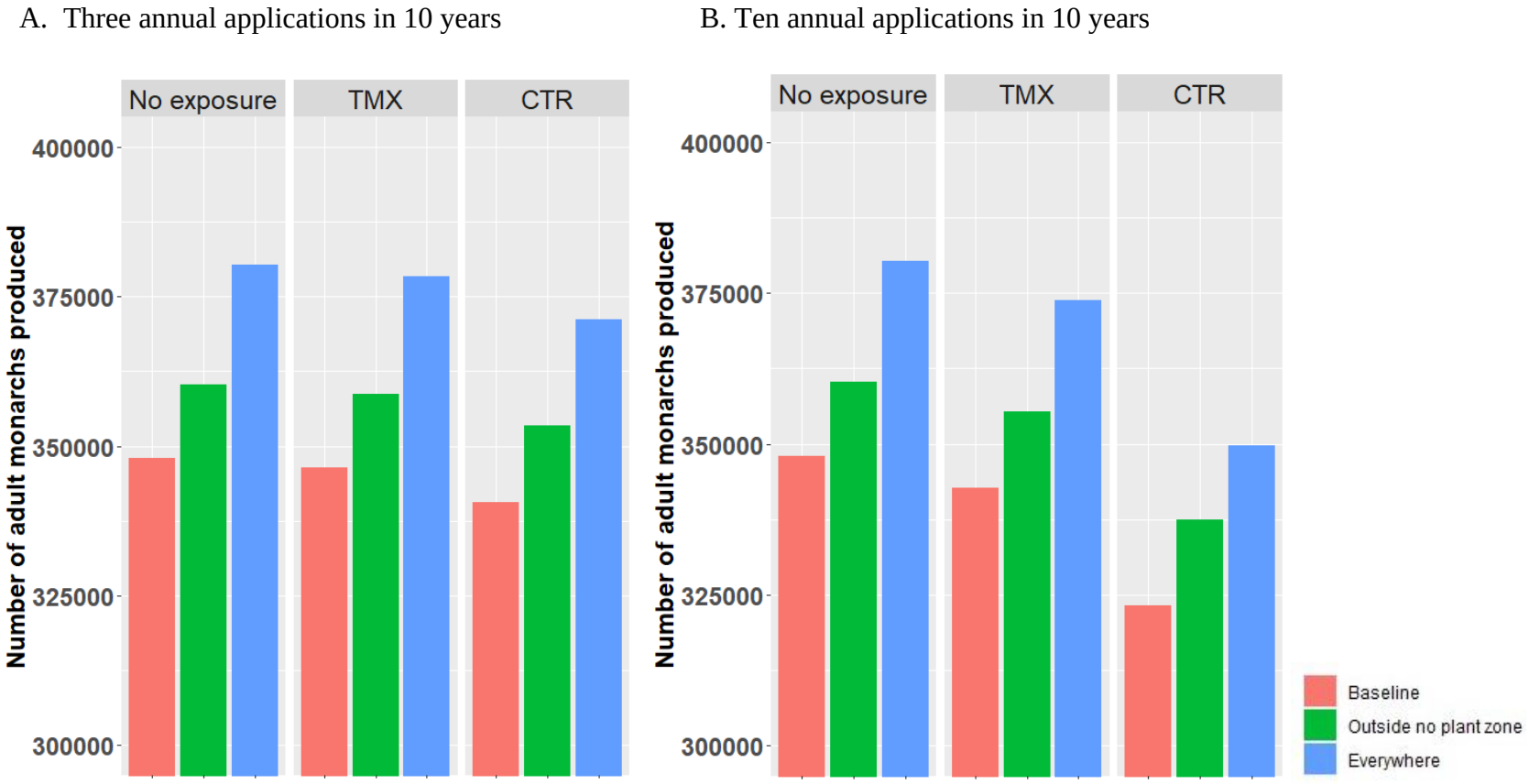

Figure 1. Simulated adult monarch recruitment over 10 years following three (A) and ten (B) annual aerial applications of thiamethoxam (TMX) and chlorantraniliprole (CTR) for soybean aphid management in soybean fields in Story County, Iowa. Comparisons were made with a no-insecticide exposure scenario and three scenarios for milkweed augmentation [baseline, new habitat established within and outside a 38-m habitat exclusion zone (everywhere) and new habitat established only outside the exclusion zone]. Adapted from Grant et al. (2021).