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Monarch Butterfly (*Danaus plexippus*) Life-Stage Risks from Foliar and Seed-Treatment Insecticides

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Abstract: Conservation of North America's eastern monarch butterfly (*Danaus plexippus*) population would require establishment of milkweed (*Asclepias* spp.) and nectar plants in the agricultural landscapes of the north central United States. A variety of seed-treatment and foliar insecticides are used to manage early- and late-season pests in these landscapes. Thus, there is a need to assess risks of these insecticides to monarch butterfly life stages to inform habitat conservation practices. Chronic and acute dietary toxicity studies were undertaken with larvae and adults, and acute topical bioassays were conducted with eggs, pupae, and adults using 6 representative insecticides: beta-cyfluthrin (pyrethroid), chlorantraniliprole (anthranilic diamide), chlorpyrifos (organophosphate), imidacloprid, clothianidin, and thiamethoxam (neonicotinoids). Chronic dietary median lethal concentration values for monarch larvae ranged from 1.6×10^{-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 nonmigratory monarchs, considering these results within a landscape-scale context suggests that 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 the north central United States. *Environ Toxicol Chem* 2021;40:1761–1777.

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

INTRODUCTION

Decline of North America's monarch butterfly (*Danaus plexippus*) populations, which was recently designated as a candidate species for listing under the US Endangered Species Act (US Fish and Wildlife Service 2020), has spurred collaborative conservation efforts that link federal and state agencies with a diversity of nongovernmental organizations and the public (e.g., Monarch Joint Venture 2010; US 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-km migratory path (Oberhauser et al. 2017). The north central United States is a 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 yr 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

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establishing new habitat in the north central states (Thogmartin et al. 2017). Maize and soybean fields account for 75% of this agricultural land cover (US Department of Agriculture 2019).

The percentage of maize and soybeans that are treated with foliar or soil-applied chemical insecticides ranges from 8 to 20% and 6 to 30% in the north central states, respectively (US 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 (Olaya-Arenas and Kaplan 2019) studies. 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. Potential risks of these exposures led the US Fish and Wildlife Service (2020) to identify insecticides as a factor threatening recovery of monarch populations.

Neonicotinoids and chlorantraniliprole used in maize and soybean seed treatments can move downslope in subsurface runoff, reach monarch habitat, and be systemically absorbed by milkweed and flowering forbs (Figure 1). Olaya-Arenas and Kaplan (2019) sampled common milkweed (*Asclepias syriaca*) plants, typically within 100 m of maize and soybean field edges in Indiana, USA. The percentage of sampled leaves that had detectable concentrations of clothianidin, thiamethoxam, and imidacloprid ranged from 0.2 to 4.6% in 2015 and from 0 to 75% in 2016. The mean and maximum concentrations of the compounds ranged from 0.01 to 1.87 ng/g and from 3.7 to 151.3 ng/g, respectively. The leaves were collected in the months of June, July, and August, suggesting that larvae could be chronically exposed to neonicotinoids through consumption of milkweed leaves. Botías 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 from 0 to 21%, with concentrations ranging from ≤0.10 to 1.8 ng/g. Because adult monarchs, with a life span of 2 to 8 wk (Oberhauser 1989), are vagile (i.e., they move extensively among milkweed patches in a landscape [Zalucki and Lammers 2010]), 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 (Table 1). 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 Supplemental Data, Table S1). The half-lives of these insecticides on growing plants range from 1 to 17 d (Mukherjee et al. 2000; Galietta et al. 2011; Banerjee et al. 2012; Chowdhury et al. 2012; 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 d (Rawlins and Lederhouse 1981; Zalucki 1982).

In the present study, we provide data to more rigorously test the hypothesis that the conservation benefits of establishing milkweed habitat close to maize and soybean fields outweigh the risk of insecticide exposure from foliar and seed-treatment applications. We evaluated 6 representative insecticides used

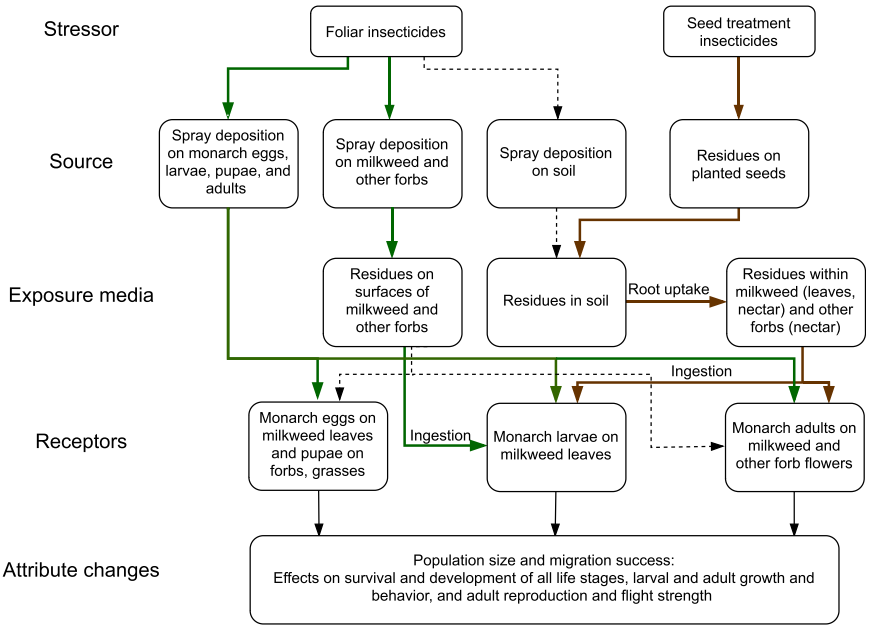

FIGURE 1: Conceptual model describing how different life stages of the monarch butterfly could be exposed to foliar (green arrows) and seed-treatment (brown arrows) insecticides and potential adverse effects that could occur from these exposure pathways. Dotted lines are minor exposure pathways.

TABLE 1: Summary of experiments conducted on different monarch life stages to assess toxicity of 6 foliar and seed-treatment insecticides used in maize and soybean fields in the north central United States

Study type	BCF	CFS	CTR	IMI	TMX	CDN	Endpoints assessed	Reference
Foliar insecticide: Spray drift exposure to monarchs and milkweed								
Acute egg topical	✓	✓	✓	✓	✓	✓	Egg mortality, days to hatch	Present study
Acute larva topical	✓	✓	✓	✓	✓	✓	Larval mortality, arrested ecdysis, days to instar/pupa, larval/pupal weight, adult eclosion	Krishnan et al. (2020)
Acute larva dietary	✓	✓	✓	✓	✓	✓	Larval mortality, arrested ecdysis, days to instar/pupa, larval/pupal weight, adult eclosion	Krishnan et al. (2020)
Chronic larva dietary		✓	✓	✓	✓	✓	Larval mortality, arrested ecdysis, days to instar/pupa/adult, adult eclosion, weight, wing span, sex	Present study
Acute pupa topical	✓	✓	✓	✓	✓	✓	Pupal mortality, days to adult, adult health, weight, sex	Present study
Acute adult topical	✓	✓	✓	✓	✓	✓	Adult mortality	Present study
Seed treatment: Runoff exposure to milkweed and other blooming forbs								
Chronic larva dietary			✓	✓	✓	✓	Larval mortality, arrested ecdysis, days to instar/pupa/adult, adult eclosion, weight, wing span, sex	Present study
Acute adult dietary				✓	✓	✓	Adult mortality	Present study

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

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), by undertaking the following studies (Table 1). 1) Chronic dietary toxicity bioassays with monarch larvae to assess their potential risk to consuming milkweed that contain foliar or seed-treatment insecticide residues. We estimate field-scale mortality and sublethal effects based on insecticide exposure estimated from a spray drift model (AgDRIFT; US Environmental Protection Agency 2011a) and milkweed residue data reported in the literature. 2) Acute topical toxicity bioassays 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 bioassays with monarch adults to assess their potential risks to consuming nectar that contains seed-treatment (systemic) insecticides. We compare the mortality results with 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. 2021), 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 US 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 median lethal concentration (LC50) values within 2- to 5-fold of those previously reported using the Iowa colony (Krishnan et al. 2020), suggesting comparable larval sensitivity across the colonies (see Supplemental Data, Table S2, and associated summary). Leaves from tropical milkweed (*Asclepias curassavica*) were used to feed larvae in all the bioassays, per Krishnan et al. (2020).

Insecticides

The following analytical-grade insecticides were used (International Union of Pure and Applied Chemistry name; Chemical Abstracts Service 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 American Chemical Society (ACS) reagent-grade acetone, certified ACS reagent-grade dimethylformamide, and Silwet L-77 were purchased from Fisher Scientific.

Toxicity bioassays

All toxicity bioassays were conducted between June 2019 and July 2020 in 2 laboratory rooms that were maintained at 21 to 29 °C, 20 to 50% relative humidity, and a 14:10-h 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, 4 to 6 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 dimethylformamide; 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% dimethylformamide) 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 are summarized in Supplemental Data, Table S3. The volume of insecticide suspension to mass ratio was kept constant to ensure that 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 h following treatment. Leaves were wrapped in aluminum foil and stored in Ziploc bags at –20 °C for residue analyses (see Supplemental Data, *Residue analyses*). Based on these analyses, nominal leaf concentrations at time 0 were used in the concentration–response analyses because they were within ±25% of the measured leaf concentrations (the chlorpyrifos 5×10^{-2} µg/g concentration was an exception; see Supplemental Data, Table S4).

Neonate larvae were individually plated onto Petri plates (60 × 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 2 d for the first 6 d and daily thereafter (see Krishnan et al. [2020] for methodological details). The average control mortality over all insecticide bioassays was 18% (range 13–28%). 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 the bioassay. Following eclosion, adults were weighed and sexed, and the 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:dimethylformamide stock solution that was employed in the adult topical bioassays (chlorantraniliprole solubility in acetone is 3.4 mg/mL at 20 °C [US Environmental Protection Agency 2008]). Acetone solution was used to treat control eggs and pupae, and acetone or dimethylformamide 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 Supplemental Data, *Residue analyses* and Table S5), and measured concentrations, 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 Supplemental Data, 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 3 to 4 h 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 h, 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 h. Unhatched eggs were observed for an additional 2 d; 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 1 or 2 d 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 Supplemental Data, Table S5) using a 50-µL Hamilton syringe to nonspiracle regions of the pupal cuticle; none of the 6 insecticides suppressed adult eclosion. The same concentrations (including acetone control) and volume were then spread over the 4 upper pupal spiracles (see Figure 2A) to enhance insecticide uptake. Within 5 d 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 d following treatment; day of adult emergence and coloration were recorded. One to 2 d 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 2 d 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 2 d with fresh Gatorade Glacier Cherry Frost Thirst

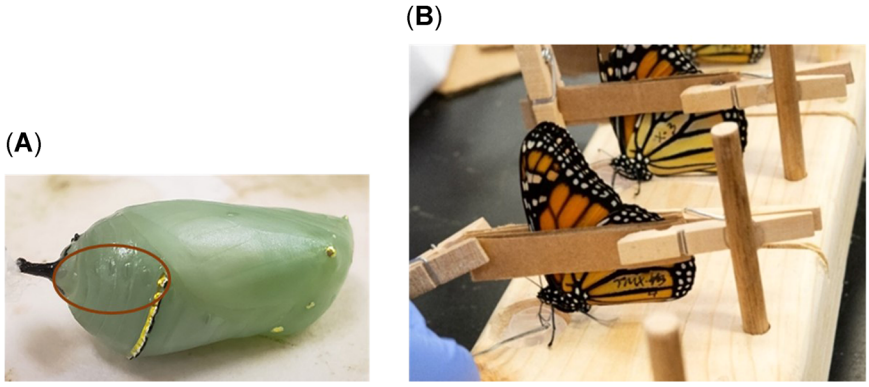

FIGURE 2: (**A**) A representative monarch pupa treated with an insecticide suspension; suspensions were applied to the 4 spiracles located within the red circle. (**B**) The experimental apparatus used to restrain monarch adults provided an artificial nectar containing imidacloprid, thiamethoxam, or clothianidin to assess acute dietary toxicity.

(Gatorade Company) that included sugar and dextrose as a nutritional source. The adults were treated within 5 d of emergence with 1.0 µL of the insecticide solution that was applied to the center of each of the 4 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 2 concentrations were tested for all insecticides except thiamethoxam and clothianidin, which caused no effects at the highest tested concentration (Supplemental Data, 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 100-fold higher than the highest concentration measured in nectar of wildflowers adjoining seed-treated fields (Botías 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 (Supplemental Data, Table S5).

Either 1 or 2 d 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 1 d prior to treatment. Diet was withheld 1 d prior to a bioassay to ensure that butterflies readily consumed the insecticide solution the following day. The age of butterflies at the time of treatment did not exceed 9 d, 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 microliters 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

Insecticide spray drift exposure to different monarch life stages was estimated using AgDRIFT (US Environmental Protection Agency 2011a). Neonicotinoid seed-treatment exposure to larvae and adults was estimated from milkweed leaf and wildflower nectar residue data (Olaya-Arenas and Kaplan 2019; Botías et al. 2015; M.J. Hall, Iowa State University, Ames, IA, USA, personal communication). See Supplemental Data, *Estimated insecticide exposure and field-scale risks* and Tables S6 and S7.

Statistical analyses

All statistical analyses were done in RStudio 1.1.383 (R, Ver 3.5.2; R Development Core Team 2018). All insecticides and monarch stages were analyzed independently. The "drc" package (Ver 3.0.1) was used to generate mortality concentration– and dose–response curves and lethal concentration and lethal dose values for monarch larvae and eggs. Based on Akaike information criterion estimates, a 3-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 of mortality to larvae and eggs from the dose– and concentration–response curves based on AgDRIFT outputs.

For analyzing sublethal effects, we excluded insecticide concentrations that had fewer than 3 surviving monarchs. Bioassay run was accounted for in the models whenever present. A binomial generalized linear model with type 3 analysis of variance (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. Because 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 LC10, LC50, and LC90 values and associated 95% confidence intervals for monarch larvae are provided in Table 2. Chlorantraniliprole was the most toxic insecticide (95% confidence intervals do not overlap with other insecticide confidence intervals), with an LC50 of 1.6×10^{-3} μg/g leaf. Imidacloprid and clothianidin were similarly toxic (LC50 values were 0.13 and 7.4×10^{-2} μg/g leaf, respectively, with overlapping confidence intervals), followed by thiamethoxam (LC50 of 0.94 μg/g leaf). Chlorpyrifos was the least toxic insecticide (LC50 of 5.3 μg/g leaf). Concentration–response curves expressed as micrograms per gram of leaf and micrograms per square centimeter of leaf generally had steep slopes that ranged from –1.5 (chlorantraniliprole) to –6.2 (chlorpyrifos; Figure 3; Supplemental Data, Figure S1).

The highest leaf concentration used for each insecticide caused between 88 and 100% larval mortality (percentage of mortality rates for all insecticide concentrations are provided in Supplemental Data, Table S8). The highest chlorpyrifos (25 μg/g) and chlorantraniliprole (5×10^{-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 d 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 (Supplemental Data, 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 to 11 d to pupate and 11 to 13 d to eclose (Supplemental Data, Figure S2), with no differences observed between concentrations ($p > 0.19$ and $p > 0.18$, respectively; see Supplemental Data, Table S9). Larvae that pupated successfully had a 71 to 100% eclosion success rate, again with no differences between concentrations ($p > 0.055$; see Supplemental Data, 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 from 0 to 43%, respectively, with no significant effects noted except in the 0.5 μg/g chlorpyrifos treatment group ($p = 0.045$; Supplemental Data, 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 (Supplemental Data, Figure S3). Butterflies in the 5 μg/g chlorpyrifos treatment had 8% smaller wings ($p = 0.0007$; Supplemental Data, Table S9). All chlorpyrifos-treated butterflies ($p < 0.036$ for all concentrations) and the 5×10^{-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

TABLE 2: Chronic dietary toxicity of 5 insecticides to monarch larvae following exposure to treated tropical milkweed leaves[a]

		LC values and 95% CIs		
Insecticide	Concentration unit	LC10	LC50	LC90
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]	1.1×10^{-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 to 40 larvae at each insecticide concentration. Larvae were fed leaf tissue treated with 0.1% Silwet:water/dimethylformamide suspensions (control) or one of 5 insecticides in 0.1% Silwet:water/dimethylformamide suspensions.
[b]Concentrations were calculated by dividing the nominal insecticide amount pipetted on each leaf by the approximate average weights of leaves used in the experiments.
[c]Derived from Supplemental Data, Table S7.
LC10/50/90 = lethal concentrations that kill 10, 50, and 90% of a treated population, respectively; CFS = chlorpyrifos; CTR = chlorantraniliprole; IMI = imidacloprid; TMX = thiamethoxam; CDN = clothianidin.

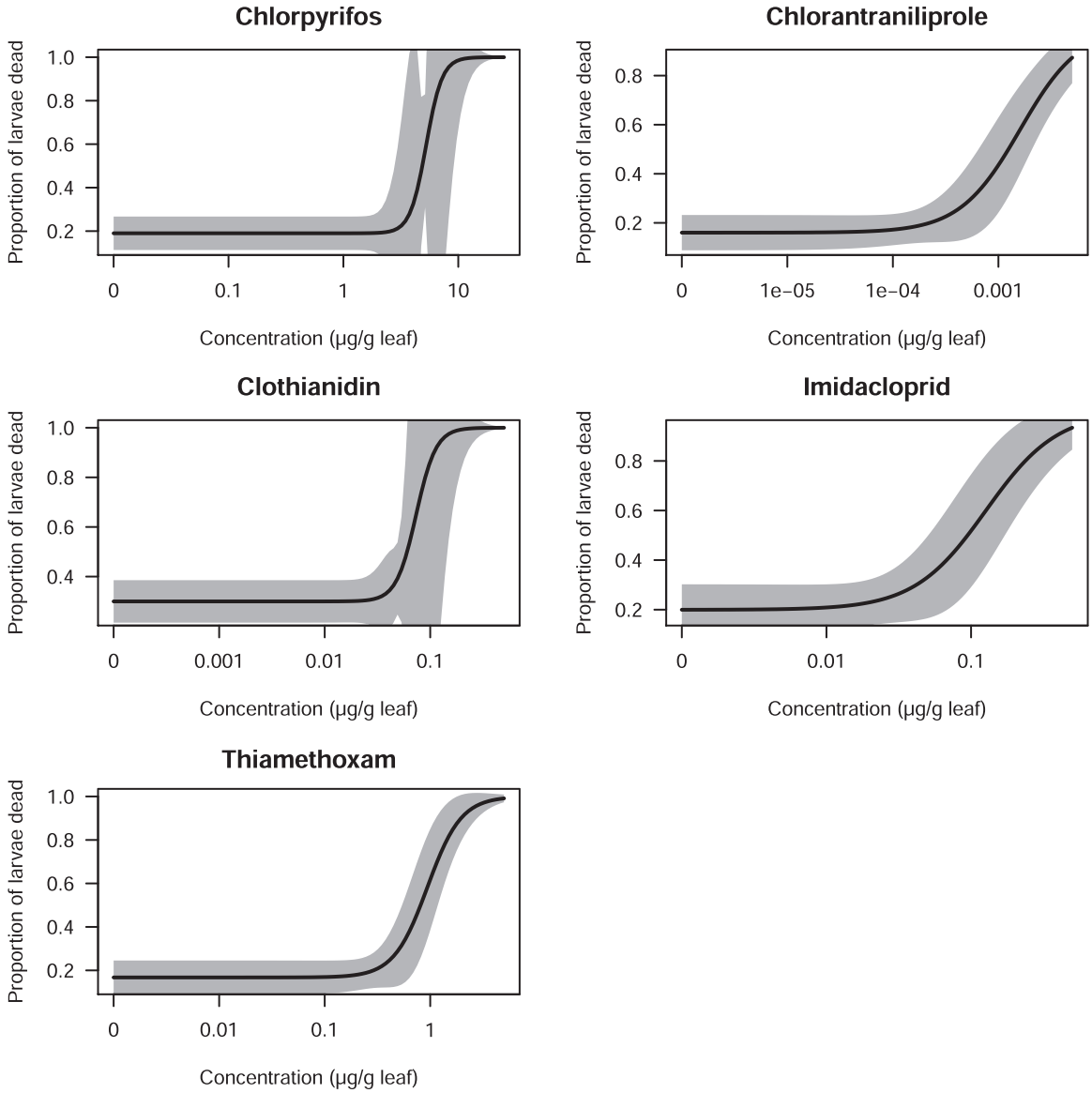

FIGURE 3: Mortality concentration–response curves for monarch butterfly larvae following chronic dietary exposure to tropical milkweed leaves treated with 5 insecticides in 0.1% Silwet:water/dimethylformamide suspensions. Larvae were exposed from the second instar through pupation.

treatments (Supplemental Data, Figure S4 and Table S9). The sex ratio, defined as the number of females divided by the 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 Supplemental Data, Table S10).

Acute topical toxicity studies with monarch eggs, pupae, and adults. Acute topical 10% lethal dose (LD10), LD50, and LD90 values and associated 95% confidence intervals for monarch eggs are provided in Table 3. Beta-cyfluthrin and chlorantraniliprole were the most toxic insecticides (overlapping 95% confidence intervals), with LD50 values of 7.3×10^{-3} and 1.8×10^{-2} µg/g egg, respectively. The neonicotinoids had LD50 values of 1.2 (clothianidin), 2.9 (imidacloprid), and 87 (thiamethoxam) µg/g egg. Chlorpyrifos was the least toxic insecticide, with an LD50 value of 3600 µg/g egg. Egg percentage of mortality rates for all insecticide concentrations are provided in Supplemental Data, Table S11. Dose–response curves in micrograms per gram of egg and micrograms per square centimeter of egg had slopes ranging from –0.040 (beta-cyfluthrin) to –6.4 (chlorpyrifos; Supplemental Data, Figures S5 and S6). The vast majority of eggs hatched on the third day following treatment (Supplemental Data, Table S11). No differences in days to hatch were observed, except for eggs treated with 4.3×10^{-2} µg/g beta-cyfluthrin (Supplemental Data, Table S12), which on average hatched on day 4.

Pupae treated on the spiracles with chlorpyrifos and neonicotinoids had 100% eclosion (Table 4), with no effects seen on pupal duration ($p > 0.068$; Supplemental Data, Table S12); adults that emerged appeared healthy. When pupal spiracles were treated with beta-cyfluthrin and chlorantraniliprole, no

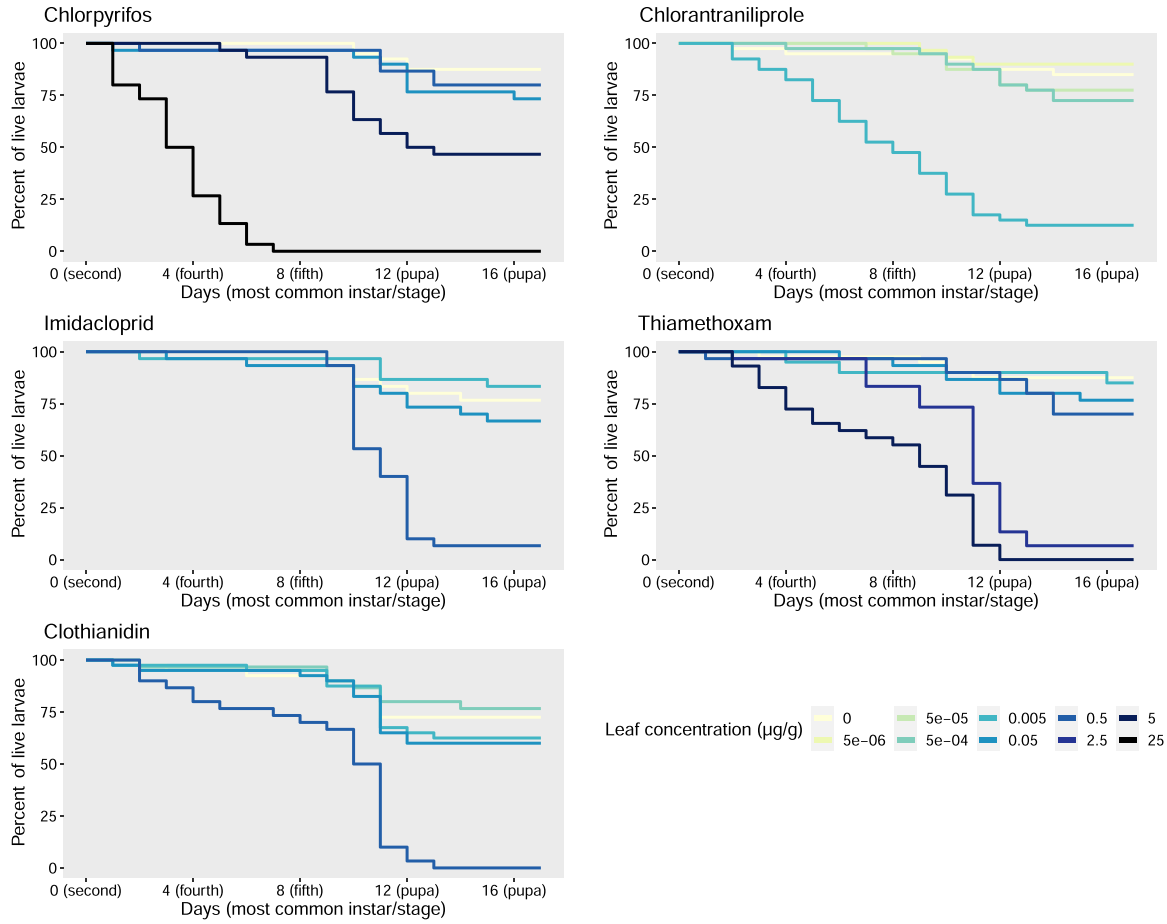

FIGURE 4: The time to mortality of monarch larvae chronically exposed to tropical milkweed leaves treated with 5 insecticides. The *y*-axis is the percentage of larvae living over time for each insecticide concentration. The *x*-axis is the number of days from initiation of the experiment. The most common instar/life stage observed on days 0, 4, 8, 12, and 16 are noted.

adults eclosed (Table 4) 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 (Table 4). 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 2 d. No adults emerged when pupae were treated with 4.0×10^{-2} µg/g chlorantraniliprole; a 10-fold lower dose had 30% emergence and a shorter pupal duration ($p = 0.034$; see Table 3; Supplemental Data, Table 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 (2- and 20-fold lower doses caused 26 and 0% mortality, respectively) and 0% with thiamethoxam and clothianidin (Table 5). Both the 86 µg/g chlorpyrifos dose and the 8×10^{-2} µg/g beta-cyfluthrin dose killed 100% of butterflies in 4 d. Doses that were 10-fold lower caused little to no mortality. The highest chlorantraniliprole dose killed approximately 60% of treated monarchs, whereas 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 3 times as susceptible to the 21 µg/g chlorantraniliprole dose.

Acute dietary toxicity studies with monarch adults. Butterflies in both the treatment and control groups typically consumed the 50 µL of insecticide-treated or untreated Gatorade solution in 2 to 3 min. Mortality rates (Supplemental Data, Table S13) across all treatments were <20%: control butterflies (18%), imidacloprid (5%), thiamethoxam (0%), and clothianidin (0%). The slightly higher mortality in controls (4 dead vs 1 dead in the imidacloprid treatment) is likely a chance occurrence. No other observable adverse effects occurred within the 96-h observation period.

Estimated insecticide exposure and field scale risks

Chronic dietary larval exposure to spray drift from foliar applications. When aerial applications of foliar formulations of chlorpyrifos, chlorantraniliprole, imidacloprid, and

TABLE 3: Acute toxicity of 6 insecticides to monarch eggs following topical exposure[a]

Insecticide	Concentration unit	LD values and 95% CIs		
		LD10	LD50	LD90
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 (46–140 000)	3600 (700–19 000)	5100 (2300–11 000)
	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} ($4.4 \times \times 10^{-5}$–$2.6 \times 10^{-2}$)	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 micrograms per 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 micrograms per egg with the average surface area of an egg, which was 0.5 ± 0.1 cm^2 ($n = 10$).
LD10/50/90 = lethal doses that kill 10, 50, and 90% of a treated population, respectively; CFS = chlorpyrifos; CTR = chlorantraniliprole; IMI = imidacloprid; TMX = thiamethoxam; CDN = clothianidin.

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 and 24% larval mortality from the field edge to 60 m downwind (Figure 5). High-ground boom applications for soybean aphid are expected to cause 100% mortality for all insecticides at the field edge; however, because of reduced off-site drift, lower mortality was predicted for chlorpyrifos and thiamethoxam at 15 (17–27% mortality), 30, and 60 m (17–19% mortality) downwind. Imidacloprid is expected to cause between 70 and 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 (Supplemental Data, Figure S7). Although exposure concentrations were based on the 50th percentile results for ground applications, 90th percentile results to capture worst-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 of 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 and 19% egg mortality from the edge of the field to 60 m downwind (Figure 5). High-ground boom applications for soybean aphid are expected to cause at least 95% mortality for all insecticides at the edge of the field. However, because of reduced off-site drift, lower mortality was predicted for the neonicotinoids at 15 (27–76% mortality), 30 (24–72% mortality), and 60 (23–68% mortality) m 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 and 93% egg mortality even 60 m downwind. Similar mortality patterns were seen for modeled high- and low-ground boom applications to manage true armyworm (Supplemental Data, Figure S7).

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 nonspiracular 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 the field, with little to no mortality occurring farther downwind. Chlorantraniliprole boom applications are expected to cause between 70 and 100% pupal mortality at all modeled distances.

No mortality is expected for adult monarchs from wing exposure to 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 the field, with no mortality anticipated at 15, 30, and

TABLE 4: Percentage of eclosion of monarch pupae following topical exposure to 6 insecticides[a]

Insecticide	Mean (±SD) pupal weight (g)	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 (g)[g]
Control	1.23 (±0.14)	0	0	0	42	100	11.3 (±0.7)	1.6	0.48 (±0.10)
CFS	1.17 (±0.15)	56	48	8.9	20	100	11.7 (±0.7)	1.2	0.50 (±0.08)
IMI	1.18 (±0.15)	14	12	2.2	20	100	11.5 (±0.7)	0.82	0.46 (±0.09)
TMX	1.20 (±0.21)	24	20	3.8	20	100	11.1 (±1.0)	1.2	0.45 (±0.17)
CDN	1.19 (±0.16)	7.9	6.6	1.3	20	100	11.5 (±0.8)	1.2	0.46 (±0.09)
Control	1.22 (±0.22)	0	0	0	10	100	12.5 (±0.7)	4.0	0.48 (±0.09)
BCF	1.16 (±0.20)	0.93	0.80	0.15	22	0	NA	NA	NA
BCF	1.11 (±0.15)	9.3×10^{-2}	8.3×10^{-2}	1.5×10^{-2}	10	40	12.5 (±0.6)	3.0	0.49 (±0.07)
BCF	1.19 (±0.15)	9.3×10^{-3}	7.8×10^{-3}	1.5×10^{-3}	10	100	12.4 (±0.7)	2.3	0.47 (±0.11)
BCF	1.19 (±0.09)	9.3×10^{-4}	7.8×10^{-4}	1.5×10^{-4}	10	100	12.0 (±0.7)	2.3	0.46 (±0.04)
CTR	1.20 (±0.17)	0.47	0.39	7.5×10^{-2}	21	0	NA	NA	NA
CTR	1.17 (±0.20)	4.7×10^{-2}	4.0×10^{-2}	7.5×10^{-3}	10	0	NA	NA	NA
CTR	1.13 (±0.13)	4.7×10^{-3}	4.2×10^{-3}	7.5×10^{-4}	10	30	12.7 (±0.6)	0.5	0.47 (±0.12)
CTR	1.17 (±0.17)	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 a 1-μL volume of acetone or insecticide-acetone solution on the spiracles at either 24 or 48 h 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 micrograms per pupa with the corresponding mean weight of the treated pupae (see second column).
[d]Calculated by dividing the micrograms per 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; CFS = chlorpyrifos; CTR = chlorantraniliprole; IMI = imidacloprid; TMX = thiamethoxam; CDN = clothianidin; NA = not available; SD = standard deviation.

TABLE 5: Percentage of mortality of monarch adults following topical exposure to 6 insecticides[a]

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

[a]Wings were topically treated with a 4-μL volume of acetone or dimethylformamide (controls) and insecticide-acetone or insecticide-dimethylformamide solution 1 to 5 d following eclosion.
[b]The ratio of number of females to 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 micrograms per adult with the corresponding mean weight of the treated adults (see third column).
[e]Calculated by dividing the micrograms per adult with the average surface area of an adult, which was 33 ± 5 cm^2 ($n = 9$).
[f]The adult percentage of mortality for each insecticide concentration was adjusted for control mortality from the same bioassay runs using Abbott's formula.
[g]Female butterflies had 3 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 doses, respectively).
Control-A = acetone treatment; Control-D = dimethylformamide treatment; BCF = beta-cyfluthrin; CFS = chlorpyrifos; CTR = chlorantraniliprole; IMI = imidacloprid; TMX = thiamethoxam; CDN = clothianidin; SD = standard deviation.

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 the field, with little to no mortality downwind.

Downslope chronic larval dietary and acute adult dietary 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 (Supplemental Data, Table S14). 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 (Supplemental Data, Table S14).

DISCUSSION

Insecticide exposure of monarchs and their habitat is considered a primary driver affecting the health of North America's migratory monarch populations (US Fish and Wildlife Service 2020). Monarchs in the north central US 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 nonmigratory monarchs in the region. In maize and soybean fields, insecticide-treated seeds are routinely used to manage early-season pests (Tooker et al. 2017), whereas foliar insecticides are used to varying degrees to manage early- and late-season pests (see Krishnan et al. 2020, Figure 1 and references therein). Assessment of potential risk of insecticide use on monarch productivity requires quantitative information on the nature and extent of insecticide exposure and toxicity of products to different monarch life stages.

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 LC50s were 1.1 (chlorpyrifos), 3.7 (thiamethoxam), 5.2 (chlorantraniliprole), 11 (clothianidin), and 39 (imidacloprid) times lower than acute LC50s for the most sensitive instars (Krishnan et al. 2020). Sublethal effects were largely absent, except for chlorpyrifos. Overall, mortality in 53 to 68% of fifth instars treated with neonicotinoids occurred as a result of arrested pupal ecdysis, with no symptoms observed prior to death. Following exposure to chlorpyrifos, chlorantraniliprole, and the control solvent, approximately 10 to 20% of fifth instar mortality occurred 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 LC50s 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 leaf.

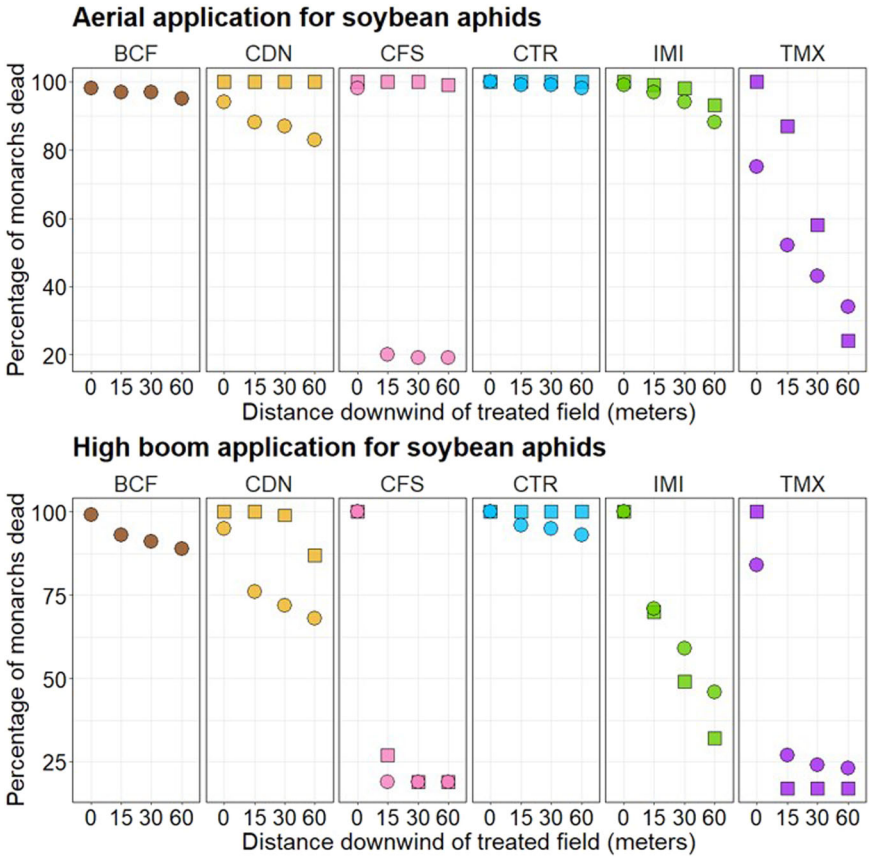

FIGURE 5: Estimated monarch egg and larval mortality due to insecticide spray drift at increasing distances downwind from a treated soybean field. Squares are predicted larval percentage of mortality following chronic dietary exposure to 5 insecticides. Circles are predicted egg mortality following acute topical exposure to 6 insecticides. Mortality rates were estimated using active ingredient–specific larval and egg concentration– and dose–response curves (Supplemental Data, Figures S1 and S6, respectively) and estimated 50th percentile, active ingredient–specific exposures using the AgDRIFT model (US Environmental Protection Agency 2011a) for aerial and ground boom applications for representative formulated products (see Table S5 in Krishnan et al. 2020 and the present Supplemental Data, Table S3). Note the *x*-axes are not proportionally spaced. BCF = beta-cyfluthrin; CDN = clothianidin; CFS = chlorpyrifos; CTR = chlorantraniliprole; IMI = imidacloprid; TMX = thiamethoxam.

We determined a chronic LC50 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 a microgram per gram basis. Assuming these swamp milkweed leaf discs weighed 16 mg (based on our independent measurements), their reported acute LC50 would be approximately 9.8×10^{-3} µg/g swamp milkweed leaf. Previously, we reported acute LC50s ranging from 0.80 to 7.8 µg/g tropical milkweed leaf (Krishnan et al. 2020). We also obtained a similar acute dietary LC50 with an artificial diet (see Supplemental Data, Tables S15 and S16 and *Artificial diet*). The 100- to 1000-fold greater sensitivity reported by Pecenka and Lundgren (2015) 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 3 different sources of monarchs and 3 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 that monarch larvae are approximately 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 LD50 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; MacBean 2012]) may facilitate greater diffusion into the egg, resulting in a higher delivered dose. Thiamethoxam and chlorpyrifos, both of which undergo metabolic activation, were 30 and 1600 times less sensitive, respectively, than imidacloprid (2.9 µg/g) and clothianidin (1.2 µg/g). Although 4.3×10^{-2} µg/g beta-cyfluthrin delayed larval emergence, this effect was not observed in the other insecticides. Comparisons of our results with prior insecticide toxicity studies with butterfly eggs was not possible because effect concentrations or doses were not provided (Braak et al. 2018).

One- to 2-d-old monarch pupae were unaffected when the highest modeled exposure concentration for each insecticide

was applied to nonspiracular regions of the pupal surface, presumably due to no or low diffusion across the cuticle. When the insecticides were applied to the spiracles, chlorpyrifos and neonicotinoids caused no adverse effect; however, at the highest tested beta-cyfluthrin (0.80 μg/g) and chlorantraniliprole (0.39 μg/g) doses, 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. As noted, 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 10-fold lower, caused 100% mortality. A further 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 LD50s ranging from 0.66 to 8.69 μg/g. Exposures to naled and dichlorvos (organophosphates) resulted in LD50s between 1.31 and 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, whereas the organophosphates were more toxic when applied to the wings. Although we only applied insecticides on the wings, our results suggest that monarchs, in general, are slightly more sensitive to pyrethroids and slightly less sensitive to organophosphates (beta-cyfluthrin LD50 is between 8×10^{-2} [1% mortality] and 1.2 [100% mortality] μg/g and chlorpyrifos LD50 is between 7.7 [6% mortality] and 86 [100% mortality] μg/g) compared with the other species. When compared with adult honeybees, adult monarchs are generally less sensitive to all classes of insecticides tested (Arena and Sgolastra 2014; Thompson 2015; Kadala et al. 2019; Wade et al. 2019).

Monarch adults exhibited no acute adverse effects when they consumed an artificial nectar source containing 140 μg/L clothianidin, 250 μg/L imidacloprid, or 330 μg/L thiamethoxam (see Supplemental Data, Table S5). Krischik et al. (2015) reported no increased mortality when monarchs were exposed to 15 and 30 μg/L imidacloprid for 29 d. James (2019) reported that a 22-d 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 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 2018). 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, 2 herbicides, and 3 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 micrograms of insecticide per gram of mass basis. The methods used to obtain the larval dietary doses and the results are described in Supplemental Data, Table S17.

Insecticide comparisons. Beta-cyfluthrin (pyrethroid) and chlorantraniliprole (diamide) are the most toxic insecticides, followed by the neonicotinoids. Typically, clothianidin is the most toxic neonicotinoid, whereas thiamethoxam is the least. The organophosphate chlorpyrifos is the least toxic insecticide tested. Because 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, are 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 micrograms per gram basis. Although full dose–response curves with monarch pupae and adults would provide a more extensive life-stage comparison, our findings indicate that 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. Although no comparable toxicity studies have been conducted on other butterfly eggs and pupae, topical exposure studies conducted by Hoang et al. (2011) suggest, in general, that butterfly larval stages are more sensitive than their adult stages, consistent with our findings.

Exposure-route comparisons. On a micrograms per gram 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 Supplemental Data, Table S17; Krishnan et al. 2020). Our data suggest that the dietary bioassays also resulted in topical uptake of insecticide; Olaya-Arenas et al. (2020) also noted the possibility of combined exposures in their dietary studies. In 2 butterfly species, Hoang et al. (2011; Hoang and Rand 2015) observed differential toxicity with the 2 exposure routes. For example, in Atala hairstreak larvae, permethrin was 9 times more toxic via the topical route, whereas in common buckeye larvae, naled and dichlorvos were 17 to 23 times more toxic via the dietary route. However, in white peacock larvae, the 3 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 dietary and topical routes.

Characterizing mortality risks from insecticide seed treatments

To estimate risks associated with insecticide seed treatments, we relied on residue data reported by Olaya-Arenas and Kaplan (2019), M.J. Hall (Iowa State University, Ames, IA, USA, personal communication), and Botías et al. (2015). Olaya-Arenas and Kaplan (2019) sampled plants up to 100 m from fields that may or may not have been planted with treated seeds. Hall (study details provided in Supplemental Data, Table S14) and Botías et al. (2015) sampled milkweed leaves and wildflower nectar at the edge of crop fields known to be planted with neonicotinoid-treated seeds. No larval and adult mortality is predicted at the highest neonicotinoid residue concentrations reported in milkweed and wildflower growing next to crop fields planted with neonicotinoid-treated seeds (Supplemental Data, Table S14). The lack of milkweed or wildflower monitoring studies at sites neighboring fields planted with chlorantraniliprole-treated seeds precludes estimation of its risk to monarchs.

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 approximately 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 d [Galietta et al. 2011; Szpyrka et al. 2017]; chlorantraniliprole, 3–17 d [Lee et al. 2019; Szpyrka et al. 2017]; imidacloprid, 2–5 d [Mukherjee and Gopal 2000; Banerjee et al. 2012]; thiamethoxam, 4–6 d [Rahman et al. 2015]; and clothianidin, 4 d [Chowdhury et al. 2012]), which are shorter than the length of the entire larval stage (12–13 d at 27 and 25 °C, respectively [Rawlins and Lederhouse 1981; Zalucki 1982]). Assuming an insecticide half-life of 4 d, 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 4 d later. This results in a significant reduction in larval mortality at 60 m downwind for chlorpyrifos, imidacloprid, and thiamethoxam applications. Because of 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 Supplemental Data, Table S18, and *Foliar insecticide degradation*).

We also compared our toxicity data to field-measured insecticide residues reported by Halsch et al. (2020), who quantified pesticide concentrations in 4 species of milkweed plants sampled from the Central Valley of California. The combined mean concentration of chlorantraniliprole in milkweed plants in 9 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; US 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 >80% egg mortality up to 60 m downwind; however, with ground boom, mortality falls to approximately 50%. Thiamethoxam and chlorpyrifos are expected to cause the least mortality (~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, whereas 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, whereas 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.

Although 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 2010) 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 because they could have simultaneous topical and dietary exposure to insecticides. Although 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 that these seed-treatment uses pose little risk to monarch larvae and adults, consistent with the 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 (US Environmental Protection Agency 2011b, 2020), and their use may increase in the future. 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; >190 000 kg were applied in Iowa in 2018 (US Department of Agriculture 2019). Neonicotinoids and diamides are also registered for foliar applications, though they are not as widely employed (Hodgson et al. 2012; Whalen et al. 2016). Less than a third of maize and soybeans in the north central United States are annually treated with foliar insecticides (US 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 population-level conservation risks and benefits of establishing monarch habitat in agricultural landscapes (Grant et al. 2021). This analysis accounts for several factors, including adult monarch vagile behavior (Zalucki and Lammers 2010; Grant et al. 2018) and population demographics (Grant et al. 2020); 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 that 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 United States (Grant et al. 2021).

Supplemental Data—The Supplemental Data are available on the Wiley Online Library at https://doi.org/10.1002/etc.5016.

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This article has earned an Open Data badge for making publicly available the digitally-shareable data necessary to reproduce the reported results. The data are available at https://github.com/Niranjana296/Monarch-butterfly-life-stage-risks-to-insecticides. Learn more about the Open Practices badges from the Center for Open Science: https://osf.io/tvyxz/wiki.

Data Availability Statement—Data, associated metadata, and calculation tools are available from the corresponding author (nkrish@iastate.edu).

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