

Experimental field evidence shows milkweed contaminated with a common neonicotinoid decreases larval survival of monarch butterflies

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Abstract

1. Neonicotinoid insecticides are the most widely used class of insecticides in the world and can have both lethal and sub-lethal effects on non-target organisms in agricultural areas. Monarch butterflies *Danaus plexippus* have experienced dramatic declines in recent decades and, given that a large proportion of milkweed on the landscape grows in agricultural areas, there is concern about the negative effects of neonicotinoids on this non-target insect.
2. In the field, we exposed common milkweed *Asclepias syriaca*, an obligate host plant of monarch butterflies, to agriculturally realistic levels of clothianidin, a widely used neonicotinoid insecticide. We tested whether this treatment influenced the number of eggs laid and larval survival over 2 years.
3. Milkweeds were transplanted into 60 experimental plots alongside a corn crop planted with a clothianidin seed coat and 60 control plots alongside an untreated corn crop. The number of eggs, larvae at each stage (first to fifth instar), and the presence of other arthropods were recorded weekly from June to the end of August and survival from egg to fifth instar was estimated using a Bayesian state-space statistical model.
4. We counted more eggs in treated plots compared to control plots, suggesting a preference for treated milkweed. The number of plots with arthropods did not differ between treatments, but within treated plots, there was a greater decrease in the number of arthropods throughout the season. There was no evidence that monarchs selected plots with fewer arthropods for oviposition. Larval survival was lower in clothianidin-treated plots compared to control plots.
5. Our results suggest milkweed near clothianidin-treated crops can reduce larval survival of monarch butterflies. While we provide some evidence that clothianidin could also act as an ecological trap for this species, further work is needed to identify additional components of fitness, including individual egg-laying rates and survival beyond the pupal stage. Our findings add to a growing body of evidence that neonicotinoids can negatively affect non-target organisms.

KEYWORDS

agriculture, *Asclepias syriaca*, clothianidin, *Danaus plexippus*, ecological trap, non-target effect, oviposition

1 | INTRODUCTION

Neonicotinoids have become the most widely used class of insecticides in the world (Wood & Goulson, 2017) since first introduced in the early 1990s (Jeschke et al., 2010; Sur & Stork, 2003). They primarily target sucking and chewing insects (e.g. aphids, leafhoppers and whiteflies) and are applied to a wide variety of agricultural crops (e.g. cotton, corn, cereals, sugar beet, oilseed rape, rice, potato, citrus and soybean; Jeschke et al., 2010). Neonicotinoids have versatile application methods but are most commonly applied as a seed coat (Jeschke et al., 2010), with the active ingredient being systemically taken up through plant roots and distributed to all tissues for long-lasting effect (Elbert et al., 2008). However, only about 5% (as low as 1.5%; Alford & Krupke, 2017) of the active ingredient in seed coats is absorbed by the crop and most enters the soil where it can spread to the wider environment (reviewed in Sur & Stork, 2003).

The half-life (DT_{50}) of neonicotinoids in soil varies among compounds, soil composition and soil moisture, but the active ingredient can remain in the soil for years (reviewed in Goulson, 2013), accumulating over time with successive applications (Bonmatin et al., 2005). Most neonicotinoids are highly water soluble (Jeschke et al., 2010) and may leach down or laterally into groundwater if there is heavy rainfall (Gupta et al., 2008), a steep slope (Goulson, 2013) or high sand content and low soil organic content (Mörtl et al., 2016). These properties make neonicotinoids both mobile and persistent, and there has been growing concern about effects on non-target organisms through environmental contamination. In one study, the concentration of neonicotinoids detected in leachate from treated corn seeds in soil exceeded the concentration considered toxic to aquatic invertebrates by the US Environmental Protection Agency (Radolinski et al., 2018). Furthermore, neonicotinoids have been detected in a high proportion (63%) of US streams within agricultural and urban areas (Hladik & Kolpin, 2016). Plants bordering treated crops can also be contaminated with neonicotinoids (Botías et al., 2016; Krupke et al., 2012; Pecenka & Lundgren, 2015) through lateral movement in the soil or surface contamination by crop dust (reviewed in Bonmatin et al., 2015), posing a risk to non-target terrestrial invertebrates.

There is growing evidence that neonicotinoids can have both lethal and sub-lethal effects on non-target organisms at current levels of exposure in the environment. Bees have been the focus of many studies, with the risks posed by neonicotinoids occurring through various routes of exposure (e.g. pollen and nectar of treated flowering crops and non-treated plants surrounding crops). Sub-lethal effects of neonicotinoids on bees include reduced foraging efficiency (Feltham et al., 2014; Gill et al., 2012), decreased nutritional status (glycogen, lipid, protein; Mogren & Lundgren, 2016),

suppressed immune response (Di Prisco et al., 2013) and reduced reproductive success (Rundlöf et al., 2015; Sandrock et al., 2014). Lethal effects have been reported when foraging bees are powdered with fragments of seed coat by drilling machines sowing neonicotinoid-treated seeds (Girolami et al., 2012). Relatively few studies have investigated the effects of neonicotinoids on other non-target invertebrates, but lethal and sub-lethal effects have also been documented in invertebrates such as butterflies (Basley & Goulson, 2018; Krischik et al., 2015; Krishnan et al., 2020; Pecenka & Lundgren, 2015), beetles (Krischik et al., 2015; Kunkel et al., 2001), earthworms (Kreutzweiser et al., 2008) and amphipods (Nyman et al., 2013).

Monarch butterflies *Danaus plexippus* are an iconic species whose annual multi-generational migration between Eastern North America and Mexico (Brower, 1995; Flockhart et al., 2013) inspires awe among researchers and the public alike. Monarchs oviposit exclusively on milkweeds (mostly *Asclepias* spp.) and their larvae are monophagous on these plants. This population has experienced dramatic declines in the past few decades (Brower et al., 2012; Vidal & Rendón-Salinas, 2014) and is at risk of extirpation (Flockhart et al., 2015; Semmens et al., 2016). Threats to monarchs include loss of milkweed (Flockhart et al., 2015; Pleasants, 2017), pesticides (Halsch et al., 2020), overwintering habitat loss (Brower et al., 2002) and climate change (Batalden et al., 2007; Oberhauser & Peterson, 2003). Agricultural chemicals such as neonicotinoids may also pose a threat to breeding monarchs, with agricultural areas contributing a large proportion of monarch breeding habitat (estimated 67% of milkweed stems; Flockhart et al., 2015). Adults may be exposed by feeding on contaminated nectar or as larvae while feeding on contaminated milkweed that is adjacent to treated crops. In one study, first instar larvae exposed to neonicotinoids at field-realistic levels experienced sub-lethal effects on growth rate and body length, and lethal effects occurred at high levels of exposure (Pecenka & Lundgren, 2015). However, results from another study suggested these effects only occur at much higher levels of exposure (Krishnan et al., 2020).

In this study, we investigated the effects of milkweed contaminated with clothianidin on monarch butterfly oviposition and larval survival. Clothianidin has become the most common neonicotinoid pesticide applied (Goulson, 2013) to a variety of crops (e.g. rice, cereals, corn, oilseed rape, fruit, potatoes, sugar beets and vegetables) with a broad set of target pests (Coleoptera, Diptera, Hemiptera and Lepidoptera; Elbert et al., 2008). In 2011, over 80% of corn crops were planted with neonicotinoid-treated seeds (clothianidin or thiamethoxam; Douglas & Tooker, 2015). As a result of this large-scale use of clothianidin, between 15% and 25% of milkweeds sampled in agricultural areas in Indiana tested positive for clothianidin (Olaya-Arenas & Kaplan, 2019).

To address how clothianidin might influence monarch butterfly reproductive success, we established plots of milkweed within the outer borders of a corn crop, half of which were planted with a clothianidin seed coat, and the other half planted as an untreated control. We hypothesized that ovipositing monarchs would be attracted to milkweed that was contaminated with clothianidin because clothianidin is toxic to insects (Elbert et al., 2008) and, therefore, would have fewer arthropod egg predators. Previous studies have shown that aphids, pests that are specifically targeted by clothianidin (Elbert et al., 2008), attract other arthropods, including predators of developing monarchs, to milkweed (Haan & Landis, 2019; Prysby, 2004) and that predators are significantly less abundant on clothianidin-treated plants (Disque et al., 2018). Following this hypothesis, we tested three predictions: (a) more eggs would be counted on milkweed in experimental plots bordering clothianidin-treated corn, (b) the number of treated plots with arthropods present on milkweed would be fewer than the number of control plots and (c) fewer eggs would be counted in plots with arthropods present. We also examined the hypothesis that exposure to clothianidin would reduce survival of developing monarchs because it is toxic to insects (Elbert et al., 2008). Following this hypothesis, we predicted lower survival of developing monarchs observed in experimental plots alongside treated corn compared to plots alongside control corn.

2 | MATERIALS AND METHODS

2.1 | Growing milkweed

Common milkweed *Asclepias syriaca* seeds were collected from plants in Sarnia and Markham, Ontario in autumn 2016 and 2017. Seeds were stratified (cold shocked and several rubbed with

sand) to induce germination. Following stratification, seeds were planted in germination trays with soil, watered and placed in a growth chamber in the University of Guelph Phytotron, Guelph, ON. The chamber was kept at a programmed light level with a humidity of 60% and temperature of 24/22°C during the day/night. After 21–28 days, milkweeds were transplanted into individual pots and transferred to a greenhouse. Plants remained in the greenhouse for 3–4 weeks before being taken outside to the roof of the Phytotron to acclimatize to outdoor levels of radiation and wind. Milkweeds remained on the roof for at least 1 week before being transplanted into the field. Plants were watered daily from seeding through planting in the field and were fertilized weekly while in the greenhouse.

2.2 | Field preparation

The experiment was conducted in summer 2017 and 2018 in a 6.43-acre agricultural field in the Halton Region of Ontario, Canada (43.448°N, 79.816°W; Figure 1b). Corn seeds were mechanically planted by a local farmer in late May of each year. One half of the field was planted with seed (Dekalb DKC39-97RIB) that was only treated with fungicide and the other half with the same variety of corn that was also treated with a clothianidin seed coat (Figure 1a). The two sides of the field were separated by an approximately 3 m margin void of corn. The northeast corner of the field, where the treated corn was planted, was bordered by another agricultural field. The southwest corner of the field, where the control corn was planted, was bordered by a wide field margin with shrubs and trees. The southwest corner of the field was slightly downhill from the northeast corner and the south side of the field would likely have had more sun exposure. While our study was conducted in a single field, female monarchs traverse a wide area while laying eggs (Fisher

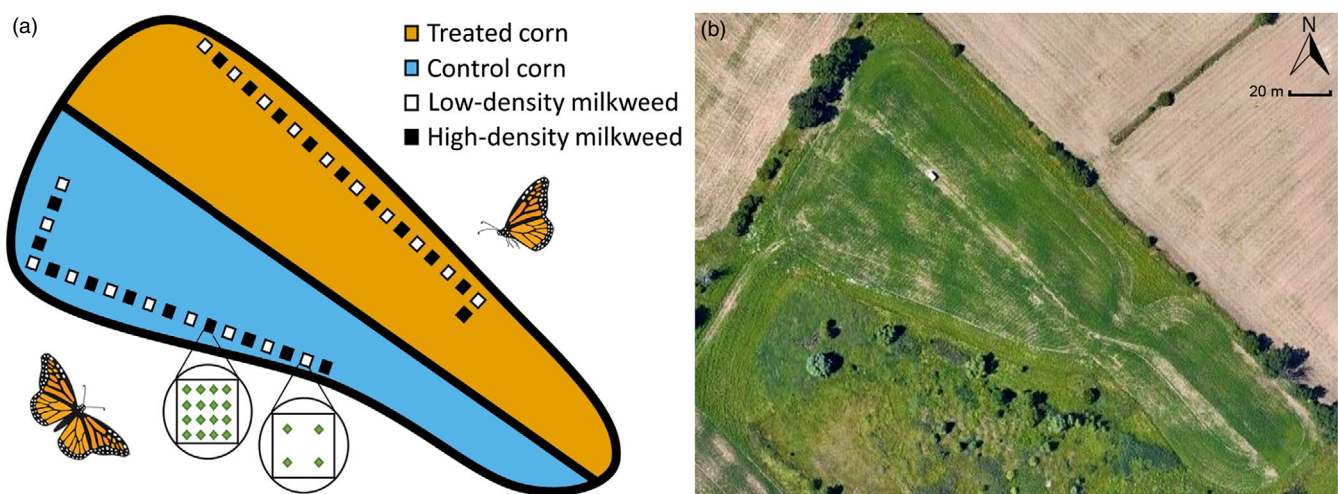


FIGURE 1 (a) Schematic of the experimental design and (b) aerial photograph of field site from summer 2018 (Google Maps). The irregular-shaped 6.43-acre agricultural field was half planted with clothianidin-treated corn and half with control corn. Approximately 3 m of buffer separated the two treatment areas. Within the outer borders of the field, 60 1 m^2 plots were established in each treatment ($n = 120$ total, not all plots shown in panel a). Within each treatment, the plots alternated between low (4 plants/ m^2) and high (16 plants/ m^2) density of planted milkweed

et al., 2020) and, therefore, we assume that female egg-laying choice was within the context of the larger landscape of available milkweed.

In early June, 60 1 m² plots were established in each treatment within the outer borders of the corn ($n = 120$). The treated plots were separated from the control plots by at least 40 m. Milkweed plants were transported from the Phytotron roof and transplanted into the experimental plots in early–mid June. Half of the plots were planted at a low density (4 plants/m²) and the other half at a high density (16 plants/m²). Beside each milkweed, a plastic label with an individual identifier was wedged into the soil. Milkweeds were watered when possible throughout the season and were replaced if they died in the first half of the season. The corn was not harvested, nor the field tilled, between the 2017 and 2018 growing seasons.

2.3 | Data collection

Each plot was monitored weekly between the end of June and early September 2017 and 2018 (10 weeks). We counted all monarchs (eggs, first to fifth instar larvae, adults) observed on each plant within each plot. We also recorded whether other arthropods were present on each plant, including aphids, ants, beetles, grasshoppers and spiders.

To measure the concentration of clothianidin in the soil, we collected 5 g soil samples from each plot. A hand trowel was dug as deep as ~10 cm, within the boundary of the plot. In 2017, each plot was sampled once in August, whereas in 2018, each plot was sampled twice between the end of June and end of August. Additionally, one leaf was collected from the top half of one plant within each plot at the same time soil samples were collected. Fifth instar larvae were collected between the end of July and mid-August 2018 to measure clothianidin concentrations in larval tissue. To measure clothianidin concentrations in adults from each treatment, we erected enclosures around fifth instar larvae starting in mid-August of both years. In 2017, butterflies emerged from pupae in the enclosures and were then removed from the field. During 2018, pupae were collected from enclosures and brought to the laboratory to eclose in containers. Adults and fifth instar larvae were euthanized by placing them in a –80°C freezer.

2.4 | Neonicotinoid sample analysis

Soil and monarchs were stored at –80°C until sample analysis. Milkweed leaves were immediately pressed and then dried in an oven at 65°C prior to sample analysis. Soil, leaf, larvae and adult tissue samples were analysed for presence and concentration of clothianidin and six other common neonicotinoids by liquid chromatography–mass spectrometry by the University of Guelph Agriculture Food Lab (LC-MS/MS; Xu et al., 2016). Thiamethoxam was the only other pesticide detected in some of our samples. The limits of detection (LOD)/limits of quantification (LOQ) for clothianidin in soil were 7/20 ppb, in leaves were 10/30 ppb and in tissue were 0.7/2 ppb, but valid detections slightly below the LOD were possible. Soil samples

were sent as collected from the field (2017: $n = 120$; 2018: $n = 240$). Leaf samples were pooled within each neonicotinoid treatment to reach a minimum weight of 2 g (2017: $n = 4$, 2018: $n = 16$). Both larvae (2018: $n = 8$) and adults (2017: $n = 2$, 2018: $n = 2$) were each pooled by neonicotinoid treatment to reach a minimum weight of 2 g. Pooling samples to reach the minimum weight for pesticide detection allowed us to have multiple samples per treatment in most cases. We note that our ability to accurately make direct comparisons of clothianidin concentrations among samples is reduced because samples likely came from soils with different moisture levels (e.g. dry soil vs. wet soil) but this was not measured.

2.5 | Statistical analysis

First, we tested whether treatment (clothianidin treated or control) or the presence of arthropods (present or absent) influenced the number of eggs counted in a plot in a given week. Because there were several instances where zero eggs were counted in plots, we used a zero-inflated generalized linear mixed-effects model with a Poisson distribution (GLMMTMB R package; Brooks et al., 2017). The total number of eggs counted in a plot was the response variable and fixed effects included in the model were treatment, arthropods, plant density (low density: 4 plants/m² or high density: 16 plants/m²), survey week and year. A quadratic term for survey week was included in the model to test for a parabolic relationship. Plot ID was included as a random effect. The model included plant density as an offset to scale the response variable to the number of eggs per plant.

To determine whether there was a difference in the presence of arthropods in plots between treatments, we modelled arthropod presence/absence using a generalized linear mixed-effects model with a binomial distribution (LME4 R package; Bates et al., 2015). Fixed effects included in the model were treatment (clothianidin treated or control), plant density (low; 4 plants/m² or high; 16 plants/m²), the week a plot was surveyed and year. We tested for two- and three-way interactions among survey week, treatment and year, and dropped interactions from the model that were not significant. An interaction between survey week and treatment, as well as survey week and year were retained in the analysis. Plot ID was included as a random effect. All statistical analyses were completed using R version 3.4.4 (R Core Team, 2018).

2.6 | Survival model

Survival of developing monarchs from oviposition to pupation in each plot was estimated using a Bayesian state-space statistical model (Grant et al., 2020). The model estimates the probability of survival for stage-structured populations where individuals cannot be marked (e.g. moulting arthropods) using cohort counts of the focal organism and estimated stage durations. In this study, the cohort counts were weekly counts of eggs and individuals in each larval stage. As developmental timing is temperature

dependent in monarchs (Zalucki, 1982), the model used ambient temperature from the field site to estimate the duration of each stage in days from egg to fifth instar larvae. Daily mean temperatures were extracted from the nearest weather station, which was 19 km south of the study site, and used to calculate accumulated degree days over time.

The state-space model included an observation model, which was conditioned on a state model. In the observation model, the data were the field counts (number of individuals of each cohort for each week) assumed to be drawn from a Poisson distribution. The state model consisted of three parameters: stage duration, daily survival probability for each stage and number of eggs initially laid in each cohort. The stage durations were calculated using the ancillary weather station data and known development schedules from laboratory studies (Zalucki, 1982). The ability to predict the stage durations improves the estimates of stage survival rates necessary for count data and small samples sizes. The number of eggs initially laid in each cohort was given a Poisson prior and was conditional on the data. The model estimates the parameters based on the count data and the primary parameter of interest is the cumulative survival probability of eggs to pupation (Grant et al., 2020). Using Markov Chain Monte Carlo (MCMC) simulations (RUNJAGS R package; Denwood, 2016), we sampled three independent Markov chains to estimate model parameters. Model convergence was estimated visually. After discarding the first 5,000 iterations from each chain for burn-in and adaptation, we retained the next 10,000 iterations from each chain (30,000 total) to summarize the posterior distribution. For further details on model development and the robustness of parameter estimates, see Grant et al. (2020).

The complexity of the survival model renders inclusion of multiple plots in a single analysis difficult (Grant et al., 2020). To overcome this challenge and test effects of year, milkweed density and treatment on larvae survival, we used a meta-analysis following Grant et al. (2020). This approach used the survival probability from each plot in each year ($n = 240$) as the response variable in a Bayesian linear regression. The cumulative larval survival probabilities were assumed to be drawn from a normal distribution $\pi_i \sim N(\mu_i, \sigma^2)$ where $\mu_i = \delta_1 X_{1i} + \delta_2 X_{2i} + \dots + \delta_8 X_{8i}$, and δ are the estimated regression coefficients and X_1, X_2, \dots, X_8 are variables indicating year, milkweed density and treatment (in all multiplicative combinations) associated with plot i . We assessed the probability that mean survival rates were higher on control plots than treatment plots by subtracting mean survival estimates (control - treatment) for each MCMC iteration and calculating the proportion of iterations that were positive. All model code for the larval survival analysis and regression meta-analysis is provided in the Supplementary Materials.

3 | RESULTS

Clothianidin concentrations varied by treatment and the type of sample. Within treated plots, the median clothianidin concentration was

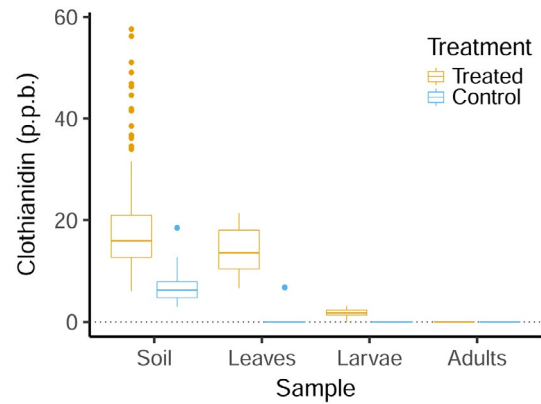


FIGURE 2 Boxplots showing the concentration of clothianidin (ppb) in soil, common milkweed leaves, monarch butterfly larval tissue and monarch butterfly adult tissue between clothianidin treated and control plots. Two outlier measurements of treated soil are not shown (85 and 106 ppb)

16 ppb in soil (range: 6–106 ppb), 14 ppb in leaves (range: 7–21 ppb) and 2 ppb in monarch larvae (range: 0–3 ppb; Figure 2). Clothianidin was not detected in adult monarchs originating from treated plots. Within control plots, lower levels of clothianidin were detected in soil (median = 6 ppb, range = 3–19 ppb) and leaves (median = 0 ppb, range = 0–7 ppb), but not detected in larvae or adults (Figure 2).

The number of eggs counted per plant within a plot depended on the year, week and treatment. Significantly more eggs were counted in 2018 (total = 1,536) compared to 2017 (total = 378; Table 1). There was a parabolic (opening down) relationship between the week a plot was surveyed and the number of eggs counted in a plot (Table 1). The total number of eggs counted peaked in late July in 2017 and early August in 2018 (Figure 3). There were significantly more eggs counted in clothianidin-treated plots (Table 1). On average, there were 0.09 eggs/plant/week in treated plots (total 1,116 eggs) compared to 0.07 eggs/plant/week in untreated control plots (total 798 eggs). There were more eggs counted on plots with arthropods present than on plots where arthropods were absent (Table 1). There was no evidence for a difference in the number of eggs/plant between plots with high milkweed density (mean = 0.08 eggs/plant/week, total 1,521 eggs) and low milkweed density (mean = 0.08 eggs/plant/week, total 393 eggs).

The factors influencing arthropod presence on milkweed were more complex. In 2018, there were more plots recorded with arthropods over the season (471/1,200 observations; 39%) than in 2017 (184/1,200 observations; 15%; Table 2). There was a negative relationship between the week a plot was monitored and arthropod presence, but the strength of this relationship varied by both treatment and year (Table 2). Arthropods declined faster throughout the season in treated plots than in control plots. Arthropods also had a greater decrease in presence throughout the season in 2018 compared to 2017 (Figure 4). A higher proportion of control plots had arthropods present throughout the season (397/1,200; 33%) compared to clothianidin-treated plots (258/1,200; 22%), but this was not a statistically significant difference (Table 2). A significantly

Parameter	Estimate \pm SE	z value	p value	Variance \pm SD
Random effects				
Plot ID				0.22 \pm 0.47
Fixed effects				
Intercept	-6.22 \pm 0.25	-25.10	<0.001	
Treatment (control)	-0.32 \pm 0.09	-3.51	<0.001	
Arthropods (present)	0.16 \pm 0.06	2.52	0.01	
Milkweed density (high)	-0.13 \pm 0.10	-1.30	0.19	
Survey week	1.21 \pm 0.07	18.02	<0.001	
Survey week ²	-0.10 \pm 0.01	-18.46	<0.001	
Year (2018)	1.34 \pm 0.10	13.45	<0.001	
Zero-inflated model				
Intercept	-0.57 \pm 0.10	-5.61	<0.001	

TABLE 1 Model summary of parameter estimates from a zero-inflated generalized linear mixed model (Poisson) explaining the number of monarch butterfly eggs counted per milkweed in a plot based on the treatment (clothianidin treated or control), arthropods (present or absent), milkweed density (high or low), survey week, year and plot ID (random effect). Fixed effects are on the log scale and the zero-inflated intercept is on the logit scale. Milkweed density was included in the model as an offset

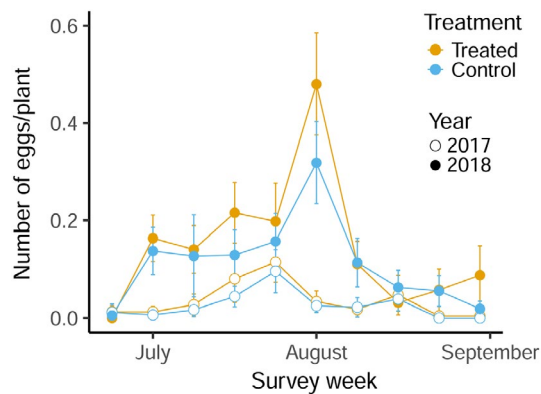


FIGURE 3 Mean number of monarch butterfly eggs counted per plant each week in 2017 and 2018 by treatment (clothianidin treated and control). Error bars show the 95% confidence interval around the estimate of the mean

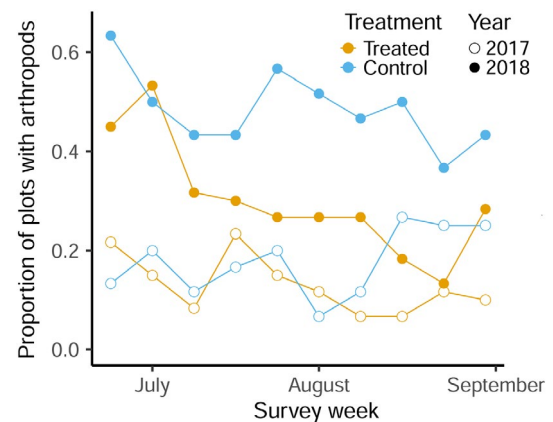


FIGURE 4 Proportion of plots with arthropods present on common milkweed throughout the season, depending on treatment (clothianidin treated and control) and year

Parameter	Estimate \pm SE	z value	p value	Variance \pm SD
Random effects				
Plot ID				0.85 \pm 0.92
Fixed effects				
Intercept	-3.02 \pm 0.31	-9.62	<0.001	
Treatment (control)	-0.16 \pm 0.30	-0.54	0.59	
Milkweed density (high)	1.91 \pm 0.18	10.73	<0.001	
Survey week	-0.09 \pm 0.04	-2.39	0.02	
Year (2018)	2.43 \pm 0.31	7.82	<0.001	
Survey week : treatment (control)	0.16 \pm 0.04	3.97	<0.001	
Survey week : year (2018)	-0.13 \pm 0.04	-3.20	0.001	

TABLE 2 Model summary of parameter estimates from a generalized linear mixed model (binomial) explaining the presence of arthropods in a plot based on the treatment (clothianidin treated or control), milkweed density (high or low), survey week, year and plot ID (random effect). Two- and three-way interactions among survey week, treatment and year were tested and dropped from the final model if interactions were not significant. Fixed effects are on the logit scale

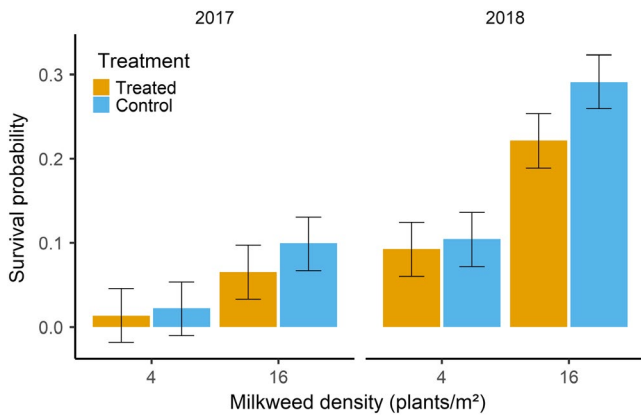


FIGURE 5 Survival probabilities from egg to pupa by milkweed density, treatment and year estimated using a meta-analysis of the plot-level survival probabilities from a Bayesian state-space statistical model. Error bars represent the 95% credible intervals

higher proportion of plots with high milkweed density had arthropods present (492/1,200; 41%) compared to plots with low milkweed density (163/1,200; 14%; Table 2).

Mean cumulative larval survival from egg to pupation during the study was 0.114 (95% CI = 0.102, 0.125) but survival estimates varied by treatment, year and milkweed density (Figure 5). There was a 0.9954 probability that survival was lower in treated plots (mean = 0.098, 95% CI = 0.082, 0.114) compared to control plots (mean = 0.129, 95% CI = 0.113, 0.145). Survival was significantly higher in 2018 (mean = 0.177, 95% CI = 0.161, 0.194) compared to 2017 (mean = 0.050, 95% CI = 0.034, 0.066). Survival was also higher in high-density plots (mean = 0.169, 95% CI = 0.152, 0.184) compared to low-density plots (mean = 0.058, 95% CI = 0.042, 0.074).

4 | DISCUSSION

Our experimental results demonstrated that milkweed growing directly alongside clothianidin-treated corn had clothianidin concentrations in leaves (7–21 ppb) that were greater than the LC₅₀ (15.63 ppb) reported for monarch larvae in a 36-hr toxicity assay (Pecenka & Lundgren, 2015). Furthermore, larvae feeding on contaminated milkweed in this study would have been exposed to these concentrations for not just 36 hr, but the entirety of larval development (approximately 10–20 days; Zalucki, 1982). We show that, as a result, larval survival was lower on milkweed growing alongside clothianidin-treated corn compared to milkweed growing alongside untreated corn across both years of the study. In addition, egg-laying females chose milkweed contaminated with clothianidin, as indicated by higher egg counts in treated plots.

While we found a higher number of eggs on milkweed growing in treated plots, suggesting that clothianidin-treated milkweed may act as an ecological trap for monarch butterflies, we caution that this, combined with our finding of lower larval survival in treated plots, is insufficient for concluding that clothianidin acts as an ecological trap

for this species. First, we lack data on individual female egg laying. We have shown higher egg density on treated plots compared to control plots but we do not know how this relates to individual egg-laying rates and, because of this, cannot conclusively state whether it was because more females laid eggs in treated plots or because each individual female laid more of their eggs in treated plots. Second, for those individuals that survived to the pupa stage, we do not have subsequent estimates of their adult longevity that predict lifetime fecundity (Oberhauser, 1997). Short of raising these pupae into the laboratory, which has its own obvious drawbacks, getting estimates of adult survival and egg laying in the wild is exceptionally difficult.

Given that agricultural areas contain the highest proportion of milkweed on the landscape (Flockhart et al., 2015; Thogmartin et al., 2017) and up to 25% of milkweeds sampled in agricultural areas have tested positive for clothianidin (Olaya-Arenas & Kaplan, 2019), neonicotinoids have the potential to negatively affect a large proportion of breeding monarchs. The survival model indicates, on average, 3% lower survival in treated plots compared to control plots after controlling for milkweed density and year. While this is a small difference, it could result in a measurable effect on the monarch population. However, milkweeds in this study were located within the treated crop. Due to the current extensive application of glyphosate in corn and soybean crops, fewer milkweeds are likely present within crops compared to before the introduction and widespread use of glyphosate-resistant crops (Hartzler, 2010). Now milkweeds are more likely to grow adjacent to crops (Olaya-Arenas & Kaplan, 2019), where uptake of neonicotinoids from crop seed coats is often, but not always (Olaya-Arenas & Kaplan, 2019), lower. In addition, Krishnan et al. (2020) recently estimated an LC₅₀ of 800 ppb for fifth instar monarch larvae, which was the larval stage most sensitive to clothianidin exposure. These LC₅₀ estimates are much greater than the Pecenka and Lundgren (2015) study and would require exposure to clothianidin at levels that are much higher than is typically recorded in milkweeds (Olaya-Arenas & Kaplan, 2019; Pecenka & Lundgren, 2015).

Insect population abundance varies considerably among years in large part due to variation in weather, density dependence and predators (Cornell & Hawkins, 1995; Haridas et al., 2016). Our results showed dramatic year-to-year differences in larvae survival as well as between low- and high-density milkweed plots. Annual variation in larvae survival is dramatic in monarchs and while the mechanisms underlying this relationship are not well understood, local weather conditions, predators and host plant abundance are considered to be the main driving factors (Nail et al., 2015; Prysby, 2004). Local weather conditions and growing conditions likely played a significant role at our study area given that we controlled for many other factors in our controlled experiment. However, we also found a significant effect of milkweed density which implies there may be higher competition for host plants that occur in low densities (Flockhart et al., 2012; Nail et al., 2015).

Butterflies may avoid laying eggs on host plants that are occupied by arthropods that could depredate their eggs or offspring

(Freitas & Oliveira, 1996; Sendoya et al., 2009). We hypothesized that clothianidin would reduce arthropod abundance in the treated area, and monarchs would subsequently select those patches where arthropods were absent for oviposition. However, opposite to our prediction based on this hypothesis, we counted more eggs in plots where predators were present. This may be because arthropods were attracted to plots with more eggs and, when plots were monitored, the presence of arthropods did not reflect those present at the time of oviposition. While there was no significant difference in the number of plots with arthropods present between clothianidin-treated and untreated control plots, there was a greater decrease in arthropod presence throughout the season in treated plots. In 2018, the decreasing number of arthropods in treated plots coincided with an increasing concentration of clothianidin in treated leaves between July and the end of August (approximately 8 to 20 ppb). Leaves sampled in August 2017 had an average concentration of 13 ppb, which was lower than the estimates from August 2018 and may explain why there was a less dramatic decline in the number of arthropods throughout the 2017 season compared to 2018. However, it is important to note that the clothianidin concentrations in leaves were all below the limits of quantification so there is some uncertainty in these estimates. It is also worth noting that though there were not significant differences in occupancy of arthropods between clothianidin-treated and untreated plots, there may have been differences in abundance between treatments. We did not take any measures of arthropod abundance nor the arthropod community; thus, this may have restricted our ability to detect a treatment effect on arthropod populations (Sendoya et al., 2009).

Clothianidin was detected in all control soil samples in both years of the study, and in leaf samples from 2017. The half-life of clothianidin in soils sampled in southern Ontario has been estimated at approximately 6 months (Schaafsma et al., 2016), so it is unlikely that the levels of clothianidin detected in the control soil are a result of clothianidin residues from prior to the start of this experiment. However, clothianidin concentrations in control plots were lower in 2018 compared to 2017, and they decreased further throughout the season in 2018. Yet we have no evidence of clothianidin accumulating in the treated soil between years. With clothianidin's very high leaching potential (Bonmatin et al., 2015), it is more likely that leaching occurred from the treated to the control side of the field following planting. The soil at the study site is a clay loam with lower leaching potential than sandy soils, but the control plots were on a downhill slope from the treated plots and in 2018 there was a heavy rainfall between corn planting and the first soil sample.

Despite being detected in control soil in both years, clothianidin was only detected in control leaves in 2017. It is unclear whether the concentrations in 2018 were too low to be detected since soil concentrations were slightly lower in 2018, or if there was no clothianidin uptake by the plants. Pecenka and Lundgren (2015) measured clothianidin concentrations of up to 4 ppb in milkweeds that were on average 1.5 m from the edge of a corn field, so we could expect concentrations in the control milkweeds that were >10 m away from treated corn to have been lower than this and below the

limits of detection. However, Olaya-Arenas and Kaplan (2019) also found that, although clothianidin was detected in untreated soil near agricultural sites throughout the summer, it was not detected in milkweed leaves past June. Clothianidin was also not detected in the control larvae (only sampled in 2018), which could be because there was no uptake by milkweed that year or concentrations were also too low to detect. If there were low levels of clothianidin in control milkweed that we could not detect, we would expect the true effect of clothianidin on larval survival to be stronger than that measured in this study. However, clothianidin was detected in leaves at as low as 7 ppb, so for it to not be detected in control milkweed, the concentrations would have been below the estimated LC_{10} of 7.72 ppb (Pecenka & Lundgren, 2015).

Our study also shows that there were more eggs in high-density plots of milkweed compared to low-density plots, but there was no significant difference in the number of eggs per plant between densities. This contrasts with previous studies that have documented higher egg densities in low-density milkweed plots (Zalucki & Suzuki, 1987, Pitman et al., 2018, but see Knight et al., 2019), but there was a greater range of milkweed densities in those studies compared to ours. Larval survival was also higher at higher milkweed densities. However, we show that there were more high-density milkweed plots with arthropods present than low-density plots. This may be because high-density patches of milkweed are easier to spot on the landscape. These results do not support our hypothesis that monarchs selected milkweeds for oviposition that had fewer arthropods present to avoid predators, although we could not directly measure this without observing oviposition. It is surprising that survival increased with higher milkweed densities given there were more arthropods (and likely predators) present in high-density plots. One possibility is that lower predation pressure on monarch larvae may arise if populations of other prey can regenerate quickly enough to sustain higher predator feeding rates. These results imply that when planting milkweed for habitat restoration patch densities of 16 plants/m² will provide greater benefits per plant for ovipositing monarchs compared to 4 plants/m².

Due to the nature of the field site, higher egg-laying rates and differential arthropod presence in treated plots could also be partly attributed to different habitat bordering the field near treated plots (an agricultural field) compared to control plots (a field margin). The field site was a single field which presented constraints to experimental design when comparing treated and control plots. There could have been more arthropods accessing the control plots from close field margin than accessing the treated plots from the other agricultural fields. Conversely, there may have been increased oviposition in treated plots near the other agricultural fields, where monarchs have been shown to prefer to lay eggs compared to natural habitat (Pitman et al., 2018). Examining within treatment differences in egg-laying rates, survival and arthropod presence in relation to soil clothianidin concentration would have helped elucidate these differences. Unfortunately, soil samples were not all taken on the same day and samples from the same plots at two different time points in the season did not have consistent clothianidin

concentrations. Furthermore, variable moisture content in the soil could have accounted for some plot-level variation in clothianidin concentration, but this was not accounted for in this study. Future studies should address these limitations by assessing more than one field and employ a block-design approach to ascertain the effects of neonicotinoid exposure while controlling for variation in the surrounding landscape.

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AUTHORS' CONTRIBUTIONS

D.T.T.F., D.R.N. and R.D. designed the research; S.M.K. and R.D. collected the data; S.M.K., D.T.T.F. and M.G.B. analysed the data; S.M.K. led the writing of the manuscript, although all authors contributed to the writing and subsequent revisions and all approved the final version of the manuscript.

DATA AVAILABILITY STATEMENT

Data available from the Dryad Digital Repository <https://doi.org/10.5061/dryad.1vhhmgqsk> (Knight et al., 2021).

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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