Do pollinators prefer pesticide-free plants? An experimental test with monarchs and milkweeds

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Abstract

1. Hundreds of recent studies have voiced concern over the negative impacts of non-target pesticides on pollinator health. However, pesticide loads are highly variable across agricultural landscapes and it is unclear whether pollinators exhibit behavioural responses (e.g. aversion) that mediate their exposure risk under realistic foraging environments.

2. We tested whether monarch butterfly Danaus plexippus adults and larvae base their oviposition and foraging decisions, respectively, on the presence and concentration of pesticide residues on their milkweed host-plant, Asclepias syriaca. To do so, we experimentally simulated field-realistic exposure for six of the most commonly detected pesticides—one insecticide, two herbicides and three fungicides—either alone or in combination. These laboratory and greenhouse manipulations experimentally paired an untreated control with the pesticides at their mean or maximum concentrations. Last, we used a two-year field survey to correlate pesticide concentration on milkweed leaves with monarch oviposition.

3. Butterflies placed fewer eggs on milkweeds treated with a cocktail containing all six pesticides at their maximum concentrations, resulting in c. 30% less oviposition compared to the solvent control. Neonate (1st instar) larvae also showed a preference for pesticide-free leaves in paired disc assays for four out of six compounds tested, with feeding aversion observed at both mean and maximum concentrations. Later instars did not show a comparable behavioural reaction to pesticide presence or concentration, but this could be partially due to the feeding-deterrent properties of the acetone solvent used. In the field, per plant egg load was negatively correlated with increasing leaf concentration for one of the herbicides, metolachlor; although all pesticides tested showed similar oviposition trends.

4. Synthesis and applications. Our data provide evidence that monarchs are capable of adaptively adjusting their oviposition and foraging behaviours based on which pesticides are present on their host-plants. Importantly, this preference was observed at field-relevant concentrations, suggesting that monarchs behaviourally regulate pesticide exposure risk for their offspring by avoiding contaminated...
plants. Variability in pesticide identity and/or load among milkweeds within/between habitat patches should be considered in future restoration efforts aimed at attracting butterflies for larval development, as well as the relative importance of pesticides compared with other plant factors (e.g. genotype or species, developmental stage) known to affect oviposition. More broadly, this work illustrates some of the limitations of no-choice trials and suggests that experimental designs embracing field-scale heterogeneity in pesticide presence and concentration will lead to more realistic non-target impact assessments and improved management.

**KEYWORDS**

agroecosystems, behavioural response, butterfly, milkweed, monarchs, pesticides, pollinators

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1 | INTRODUCTION

Insect abundance and biodiversity have been declining for at least the past several decades across human-dominated landscapes (Eisenhauer, Bonn, & Guerra, 2019; Wagner, 2020). Multiple anthropogenic factors are usually implicated in these declines (e.g. disease, climate or land use change), but due to their close association with agriculture, pesticides are one of the most commonly cited factors (Hallman et al., 2017; Homburg et al., 2019; Sánchez-Bayo & Wyckhuys, 2019; Wood, Gibbs, Graham, & Isaacs, 2019). Thus, determining whether, and to what degree, pesticides impact the health of insect communities has become the focus of hundreds of studies over the past decade, with most focusing on bees and other pollinators. However, the method of exposure to pesticides in such experiments strongly affects the interpretation of those data. Many studies, for example, employ no-choice assays that force individuals to develop at a fixed pesticide concentration, quantifying the lethal and sublethal effects of such exposure compared to a pesticide-free control. A benefit of this approach is that it allows for a relatively straightforward measure of individual and population-level impacts. The drawback is that under natural conditions, insects encounter highly variable pesticide loads over their lifetime and may simply choose to avoid foraging on pesticide-contaminated resources if given the option. Even within a limited foraging radius, proximity to cropland will likely create a complex mosaic of food resources that vary from trace pesticide amounts to those exceeding the species’ LD₅₀ (Krupke, Holland, Long, & Eitzer, 2017; Olaya-Arenas & Kaplan, 2019; Tsvetkov et al., 2017).

An alternative approach is to conduct a more realistic choice study whereby insects are simultaneously presented with pesticide-treated versus untreated plants. In this case, it would be expected that individuals preferentially forage on pesticide-free plants (i.e. avoiding poisonous food should be highly adaptive). Indeed, this expectation underlies the rationale that the detrimental effects of pesticides are overestimated. The few controlled choice studies to date, however, report counterintuitive findings, e.g. honeybees and bumblebees prefer nectar containing neonicotinoid insecticides, as well as some herbicides and fungicides (Kessler et al., 2015; Liao, Wu, & Berenbaum, 2017; but see Muth & Leonard, 2019). Yet, field experiments still report observations of pesticides deterring pollinators. For instance, fungicide application during cranberry bloom dramatically increased honeybee foraging on non-crop pollens (Jaffe, Lois, & Guedot, 2019). Disentangling preference in social bees is further complicated by behavioural regulation and feedbacks that occur at the colony level (Dolezal, Carrillo-Tripp, Miller, Bonning, & Toth, 2015).

Unlike bees, pesticide-mediated food plant preferences are poorly studied in other groups such as butterflies, even though evidence is accumulating for the role of pesticides in butterfly declines. This is particularly true for threatened or endangered species on wild host-plants (Forister et al., 2016; Gilburn et al., 2015; Muratet & Fontaine, 2015; Wepprich, Adrion, Ries, Wiedmann, & Haddad, 2019), since pesticide responsiveness is widely reported in crops for the many Lepidoptera that act as agricultural pests. Butterflies ecologically differ from bees in several ways that could affect their behavioural response to pesticides. For one, choice can occur at two levels; initially, oviposition preference by gravid adult females (i.e. between plants), followed by larval feeding choices within and across leaves, which typically occurs on an individual plant. Either stage could result in behaviours leading to aversion or attraction. In monarch butterflies Danaus plexippus, for example, preference for cardenolides—milkweed toxins that cure individuals from parasite infection—only occurs during oviposition. Caterpillars do not display an analogous feeding preference, despite the medicinal benefits of consuming cardenolide-rich leaf tissue (Lefèvre et al., 2012). In North America, monarchs are in the midst of a long-term population decline and pesticides are one of several factors that are thought to be contributing to this decline (Agrawal & Inamine, 2018; Inamine, Ellner, Springer, & Agrawal, 2016; Malcolm, 2018; Rogmarten et al., 2017). Their milkweed host-plants commonly grow on crop field margins, potentially exposing them to a range of agrochemicals such as neonicotinoid insecticides used in corn and soybean production (Olaya-Arenas & Kaplan, 2019; Pecenka & Lundgren, 2015). This is especially the case for the eastern migratory population, which traverses several agricultural zones in its summer breeding range east of the Rocky Mountains as part of its
annual cycle. However, monarch ovipositional responses to pesticides are almost entirely unknown. In one of the only studies to date, oviposition by monarch females was unaffected by the pyrethroid insecticide, permethrin, used for mosquito control when sprayed on milkweeds, even though larval survival was reduced on permethrin-treated plants (Oberhauser et al., 2006).

In this study, we quantified the oviposition and feeding behaviours of monarch butterflies and caterpillars, respectively, to variation in leaf pesticide residues on their primary host-plant in North America, the common milkweed Asclepias syriaca. To do so, we simulated field-realistic pesticide residues using previously published data from a two-year field survey that measured the presence and concentration of a diversity of pesticides on leaves of milkweeds bordering cropland (Olaya-Arenas & Kaplan, 2019). Our central objective was to determine whether monarchs avoid pesticides on milkweed when given an option. Secondly, we aimed to isolate the life stage (i.e. adult oviposition vs. larval foraging) during which preference occurs in this species.

2  | MATERIALS AND METHODS

2.1  | Plants and insects

Milkweed used for all experiments were either germinated from seed or propagated from stem cuttings (Prairie Moon Nursery) in a climate-controlled greenhouse (26–30°C) on the Purdue University campus (West Lafayette, Indiana, USA). Plants were cultivated in 366 ml pots using SunGro professional growing mix with two teaspoons of time-released NPK fertilizer (Scotts Osmocote Classic®). Monarch larvae and adults used in tests derived from crosses among wild individuals captured in 2017 from the eastern North American migratory population in Georgia and Indiana. Although we were unable to track the specific origin of adults or larvae for each experiment, all individuals used were recent (F1 or F2) offspring from wild-caught individuals and thus we do not anticipate that inbreeding or other issues associated with colony rearing affected our results. Adults were maintained in a greenhouse in two large (4.8 m3) mesh cages with six A. syriaca plants and 12 petri dishes, four with water, four with Gatorade and four with two pieces of banana and two tangerines cut longitudinally. The temperature in the greenhouse was maintained at 26–30°C. Cages were occasionally misted with water for evaporative cooling to avoid temperature spikes on warm days. Approximately 100 adults were kept in each cage. Butterflies were used in oviposition experiments 6–8 days after emerging from cocoons to ensure that some mating had occurred (Oberhauser, 1997; Zalucki, 1981).

2.2  | Adult oviposition preference

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

1. Pesticide-free control. Did not receive any experimental manipulation.
2. Pesticide solvent and surfactant control. Plants were sprayed from a stock solution containing water (465.3 ml), acetone (29.7 ml) and tween (0.5 ml). Acetone (99.5%, Sigma-Aldrich) was used in pesticide dilutions. Tween® 20 (Fisher Scientific) is a surfactant that helps pesticides to be absorbed by plants; these are commonly added to pesticide mixtures for field application. This second or ‘true’ control was used to evaluate whether the non-pesticide components of the pesticide treatments contributed to observed effects on monarch behaviour.
3. Mean pesticide treatment. Plants were treated from a 480 ml solution containing six of the most commonly encountered pesticides on milkweeds in the field, combined into a single treatment (Table 1). The target concentrations applied to the plants are based on field data collected in Olaya-Arenas and Kaplan (2019). These levels correspond to actual concentrations of each pesticide measured on milkweed leaves for individual sample dates, even if the compounds were applied to crops in different applications separated over time. We used a pesticide cocktail rather than individual compounds (unlike the larval trials described below) because it represents the actual blend that monarchs encounter in

| TABLE 1  | Pesticide concentrations measured on plants for the adult oviposition experiment. Values were recorded using QuEChERS method for pesticide extraction, followed by LC-MS for quantification |
|-----------------------------------------------|--------------------------------------------------|--------------------------------------------------|--------------------------------------------------|--------------------------------------------------|--------------------------------------------------|--------------------------------------------------|--------------------------------------------------|--------------------------------------------------|--------------------------------------------------|
| **Pesticide type** | **Active ingredient** | **Concentration (ng/g) in leaves** | **Mean** | **SE** | **Max** | **SE** | **Mean** | **SE** | **Max** | **SE** | **Mean** | **SE** | **Max** | **SE** |
|-----------------|-----------------------|------------------------------------|----------|--------|---------|--------|----------|--------|---------|--------|----------|--------|---------|--------|
| Insecticide     | Clothianidin          | 2 days after spray | 1.37     | 0.44   | 8.61    | 1.22   | 1.52     | 0.71   | 8.2     | 0.71   | 1.27     | 0.82   | 9.1     | 3.46   |
| Herbicides      | Atrazine              | 4 days after spray | 1.3      | 0.21   | 44.3    | 5.01   | 0.58     | 0.07   | 34.86   | 4.81   | 0.99     | 0.21   | 43.1    | 13.16  |
|                 | S-metolachlor         |                      | 0.11     | 0.01   | 0.52    | 0.13   | 0.13     | 0.01   | 0.25    | 0.07   | 0.08     | 0.01   | 0.16    | 0.04   |
| Fungicides      | Azoxyostrobin         | 7 days after spray | 0.14     | 0.03   | 3.3     | 0.63   | 0.09     | 0.02   | 2.15    | 0.54   | 0.06     | 0.02   | 2.17    | 0.83   |
|                 | Pyraclostrobin        |                      | 2.46     | 0.37   | 95.22   | 4.44   | 2.14     | 0.17   | 110.8   | 36.75  | 4.43     | 1.24   | 204.7   | 145.67 |
|                 | Trifloxystrobin       |                      | 0.87     | 0.16   | 39.2    | 0.92   | 0.6      | 0.02   | 24.6    | 7.57   | 0.47     | 0.13   | 31.5    | 15.80  |
the field. For this treatment, we used the mean concentrations recorded for plants on which we detected each of the targeted pesticides.

4. Max pesticide treatment. Same as treatment 3 above, except here we used the maximum concentrations observed for pesticides detected in the field (see Table 2 for targeted mean and max values). This was used as a ‘worst-case scenario’ for monarchs, while remaining within biologically plausible levels.

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

Potted milkweed plants were standardized by age and size (mean height: 33.2 cm) and each plant was sprayed with an average of 23.3 ml (min 21–max 26) volume of solution on the abaxial and adaxial leaf surfaces to ensure complete coverage. Before spraying experimental plants, we used a beaker to estimate the per spray volume to control subsequent application rates across plants. Each plant was treated with the prepared solution using a hand sprayer (Equate™ spray bottle, 236 ml). Plants were sprayed within a cardboard box outside and left to dry overnight in the greenhouse. It is important to note that, although plants were sprayed with a pesticide mixture, this was not intended to simulate the actual exposure route in the field. It is likely that systemic pesticides (e.g., clothianidin) are taken up by the roots; however, administering pesticides via the soil sacrifices the precision necessary for recreating field-relevant target values.

TABLE 2 Pesticide concentrations in milkweed leaves collected in the field (i.e. the target level; see Olaya-Arenas & Kaplan, 2019), as well as leaf discs treated in the laboratory to corroborate that the targeted concentrations were successfully achieved

| Pesticide type | Active ingredient | Concentration in the field (ng/g) | Concentration in 1 g of leaf discs from HPLC analysis (ng/g) |
|---------------|------------------|-----------------------------------|-------------------------------------------------------------|
| Insecticide   | Clothianidin     | Mean: 15.28 Max: 56.55             | Mean: 14.36 SE: 0.48 Max: 54.47 SE: 6.45                  |
| Herbicides    | Atrazine         | Mean: 8.59 Max: 238.7              | Mean: 9.7 SE: 1.40 Max: 329.5 SE: 1.09                    |
|               | S-metolachlor    | Mean: 1.23 Max: 15.31              | Mean: 1.36 SE: 0.14 Max: 15.6 SE: 2.46                    |
| Fungicides    | Azoxystrobin     | Mean: 0.67 Max: 31.06              | Mean: 0.48 SE: 0.03 Max: 23.2 SE: 1.57                    |
|               | Pyraclostrobin   | Mean: 8.51 Max: 211.75             | Mean: 11.7 SE: 1.69 Max: 236.6 SE: 26.01                  |
|               | Trifloxystrobin  | Mean: 4.48 Max: 164.25             | Mean: 6.17 SE: 0.52 Max: 157.9 SE: 7.10                   |

2.3 Larval feeding preference

To test the effects of pesticides on larval feeding behaviour, we measured the foraging preferences of early instar monarch caterpillars comparing pesticide-free versus pesticide-treated milkweed leaves using a paired leaf disc assay enclosed in a series of Petri dishes. To do so, we paired untreated with pesticide-treated milkweed discs for each of the six pesticides tested in the above oviposition trial, except here we assessed each pesticide individually, rather than in combination. This was done each for the mean and maximum concentrations recorded, resulting in 12 comparisons (i.e. 6 pesticides × 2 concentrations).

Using this set-up, we performed two separate trials. One trial used an untreated leaf disc as the control and 1st instars, while the...
second trial used acetone as the control, since pesticides were diluted in acetone, and 2nd instars. Two instars were tested because gustatory receptors in the mandibles of larval Lepidoptera respond differently to the same compounds at the same concentrations (e.g. amino acids and sugars; Panzuto & Albert, 1997, 1998; Sandoval & Albert, 2007). Feeding preferences also vary with time of the day, age of the larvae within stadium and their interactions (Simmonds, Schoonhoven, & Blaney, 1991). In general, young instars are more susceptible to deterrent/toxic compounds than later instars (Ahmad & Forgash, 1975; Hedin, Parrott, & Jenkins, 1991; Parrot, Jenkins, & McCarty, 1983). The increase in tolerance with age has been associated with higher levels of detoxification enzymes (e.g. microsomal oxidases, glutathione s-transferase and esterase) in older instars in Lepidoptera (Yu, 1983) and honeybees (Gilbert & Wilkinson, 1974). The increases in detoxification enzymes have also been related to larval size (Ahmad & Forgash, 1975), which changes through instars and since larger instars eat more this can account for less discrimination of what they are eating (Moreau & Bauce, 2003).

Both trials included 30 experimental replicates of each treatment combination except for mean concentrations in trial 1, which used only 25 replicates. Pesticides and concentrations are listed in Table 2. In trial 1, we did not include a mixed pesticide treatment, whereas we did so in trial 2.

Leaf cores (20 mm diameter) were taken from the leaves of potted milkweed plants using a cork borer. We avoided major leaf veins to provide a standardized amount of leaf tissue and avoid variation in leaf thickness. Although leaf cores exhibit reduced latex flow compared with an intact leaf, making it easier for caterpillars to feed, we used this commonly employed bioassay because it allowed us to deliver precise pesticide concentrations compared with whole-plant manipulations. Cores were randomly assigned to treatments and treated with 20 μl of each individual pesticide using an Eppendorf repeater pipette. Pesticides were applied to the underside of the leaf, which had fewer trichomes, resulting in less dispersion away from the disc surface. To standardize pesticide concentrations per unit leaf, we first calculated the weight of an average milkweed leaf disc (n = 18 discs; 65.69 mg/disc) and adjusted accordingly through a process of serial dilutions of pure compounds in acetone as a solvent. Discs were treated inside of a fume hood and allowed to dry for 30 min, before moving them to Petri dishes.

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

### 2.4 Relationship between monarch colonization and pesticide concentration in the field

As part of a published two-year study (2015–2016) aimed at quantifying pesticide residues on milkweeds in the field (see Olaya-Arenas & Kaplan, 2019), we also collected previously unpublished data on monarch oviposition from the same plants. Thus, we were able to use those data to correlate concentration of each of the six pesticides with egg load per plant to determine if pesticides deter oviposition in the open field environment. Because egg counts were extremely low in 2016 (only 5 out of 234 sampled plants had eggs), we only used 2015 data for which we visually surveyed 290 A. syriaca plants across five sites in Indiana, USA. Each plant was searched for monarch eggs and leaves were simultaneously collected for pesticide residue analysis at three times; once each in June, July and August. In our region, monarchs do not colonize until July so virtually all of the egg data reported are from July and August 2015. For additional detail on the field sites and pesticide residue analysis, see Olaya-Arenas and Kaplan (2019).

### 2.5 Statistical analysis

#### 2.5.1 Adult oviposition choice test

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

#### 2.5.2 Larval feeding choice test

We paired each treatment with its control and used a two-proportions z-test to assess the effect of pesticides on monarch larval feeding preferences. The two trials were analysed independently since they used different controls (untreated vs. acetone) and caterpillars varied in starting age (1st vs. 2nd instar). We tested the effect of position (right or left) of the control discs in the second trial to test a possible effect of right- or left-biased asymmetries in movement behaviours that could affect preferences between treatments. Petri dishes where larvae did not feed on either leaf disc were removed from the analysis. To test differences in leaf tissue eaten among treatments, we counted the number of instances that each treatment was chosen.
relative to the control per pesticide tested. Counts were then compared among pesticides with a Pearson’s chi-squared test.

2.5.3 | Field colonization

Because we had no way to distinguish between sites and/or plants that were never encountered by monarchs compared with those evaluated and subsequently rejected by gravid females, we only used plants for which at least one egg was counted (i.e. we are certain that a female interacted with the plant). Since most of our plant counts had zero eggs, this allowed us to use egg load—number per plant—as a measure of female preference, assuming preferred plants received >1 egg. We then used a Poisson regression on the count $Y - 1$ to test the effect of pesticide concentration on monarch egg number ($n = 49$ plants with eggs and pesticides measured). The test was conducted individually for five of the six pesticides assessed in this study. Unfortunately, clothianidin could not be evaluated because it was primarily detected on plants in June, before monarchs colonized our field sites. However, it should also be noted that most of these pesticides are highly correlated with one another (see Figure S1), as would be expected since factors such as nearest linear distance to cropland affects pesticide presence and concentration on milkweeds (Olaya-Arenas & Kaplan, 2019).

R 3.5.1 and the packages STATS, CAR, lme4, multcomp, ggplot2, vgam, boot and matrix were used for statistical analysis (R Core Team, 2018).

3 | RESULTS

3.1 | Verification of experimental pesticide treatments

Data from our LC-MS analysis showed variation in the efficacy of treatments designed to mirror those values recorded from the field. Specifically, whole-plant applications used for oviposition trials tended to have lower pesticide residues across both mean and maximum concentration treatments, compared with targeted applications on leaf discs using a pipette for larval choice (compare Tables 1 and 2). Whole-plant spray treatments were a fraction of their target values in most cases; for example, the mean targeted concentration for the fungicide pyraclostrobin (8.51 ng/g) was approximately two to four times higher than those recorded from sprayed plants (2.1–4.4 ng/g). However, concentrations in most cases remained relatively stable over the course of the experiment, with few showing evidence of substantial (>50%) degradation between days 2 and 7.

In all cases the maximum concentrations were far higher than mean concentrations, as intended. Unlike whole-plant assays, leaf disc treatments much more closely mimicked field-relevant concentrations, in many cases with the applied amounts showing <5% difference compared with the targeted value (Table 2). For instance, we aimed for 15.28 and 56.55 ng/g clothianidin in the mean and maximum treatments; actual measured values from leaf discs simulating these values were 14.36 and 54.47 ng/g, respectively. Thus, leaf discs for larval choice closely matched the intended field dose.

3.2 | Adult oviposition preference

There were no differences in the number of eggs on plants comparing those sprayed with the acetone/tween/water solution versus the unsprayed control. Similarly, plants treated with the mean concentration of pesticides did not differ from the control, but there were fewer eggs laid on milkweeds initially treated with pesticides at their maximum field concentration (Figure 1; Table 3). Additionally,

![Figure 1](image-url) Mean (+SE) total eggs laid by monarch females per treatment; sample size ($n = 9$ cages). Control (unsprayed plants), mix mean (plants sprayed with a pesticide mix at mean concentrations) and mix max (plants sprayed with a pesticide mix at maximum concentrations) are compared to the acetone/tween control. Mean and maximum concentrations refer to concentrations found in milkweed leaves in margins close to corn or soybean fields.

| TABLE 3 The effects of pesticide mixtures at their mean and maximum concentrations on monarch oviposition preference. A Dunnett test was used to compare treatments against the control acetone/tween (A/T). Significance at ***$p < 0.001$, **$p < 0.01$ and *$p < 0.05$.

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

Multiple comparisons of means: Dunnett contrasts

| Linear hypotheses      | Estimate | SE    | Z value | p       |
|------------------------|----------|-------|---------|---------|
| Control–A/T == 0       | -0.1384  | 0.1204| -1.149  | 0.5311  |
| Mix max–A/T == 0      | -0.346   | 0.1276| -2.712  | 0.0189* |
| Mix mean–A/T == 0     | -0.1076  | 0.1194| -0.901  | 0.7043  |
oviposition increased in relation to leaf number, especially on plants with 20–25 leaves ($F_{1,34} = 5.57, p = 0.024, R^2 = 0.14$). However, the number of leaves did not vary among treatments and thus this oviposition preference did not affect the experimental design ($F_{3,32} = 0.193, p = 0.90$; average number of leaves per treatment: control = 20.2, A/T = 20.5, mix max = 20.8, mix mean = 21.5).

### 3.3 Larval feeding preference

In the first trial using 1st instar larvae and untreated leaf disc controls, mean concentrations of clothianidin, s-metolachlor and trifloxystrobin significantly influenced caterpillar feeding preference; namely, there was reduced feeding on pesticide-treated leaf discs compared to untreated controls (Figure 2a; Table 4a). Importantly, the untreated control was not different from the acetone control in this trial. When testing higher concentrations, we found even stronger negative effects of pesticides on larval feeding behaviour for three of the six pesticides tested (Figure 2b; Table 4b).

In the second trial using 2nd instars, we found deterrent effects of the acetone treatment on larval feeding behaviour at both mean (Figure 3a; Table 5a) and maximum (Figure 3b; Table 5b).

**TABLE 4** Effects of mean (a) and maximum (b) pesticide concentrations on 1st instar larval feeding preference. Significance at ***$p < 0.001$, **$p < 0.01$ and *$p < 0.05$. Control = pesticide-free milkweed leaf discs

| Treatments     | $\chi^2$ | df | 95% CI        | $p$  |
|----------------|----------|----|---------------|------|
| (a) Mean concentrations |         |    |               |      |
| Acetone        | 0.13     | 1  | $-0.531$      | 0.281| 0.7237 |
| Clothianidin   | 7.53     | 1  | $-0.873$      | $-0.185$| 0.0061** |
| Atrazine       | 1.88     | 1  | $-0.674$      | 0.086| 0.1701 |
| Metolachlor    | 15.13    | 1  | $-1.000$      | $-0.458$| 0.0001*** |
| Azoxystrobin   | 2.46     | 1  | $-0.816$      | 0.047| 0.1167 |
| Pyraclostrobin | 1.13     | 1  | $-0.148$      | 0.648| 0.2888 |
| Trifloxystrobin| 8.00     | 1  | $-1.000$      | $-0.376$| 0.0047** |
| (b) Max concentrations |       |    |               |      |
| Acetone        | 2.88     | 1  | $-0.586$      | 0.026| 0.0897 |
| Clothianidin   | 2.08     | 1  | $-0.566$      | 0.066| 0.1489 |
| Atrazine       | 7.41     | 1  | $-0.688$      | $-0.127$| 0.0065** |
| Metolachlor    | 6.23     | 1  | $-0.674$      | $-0.095$| 0.0126* |
| Azoxystrobin   | 0.07     | 1  | $-0.226$      | 0.368| 0.7893 |
| Pyraclostrobin | 14.52    | 1  | $-0.814$      | $-0.297$| 0.0001*** |
| Trifloxystrobin| 1.39     | 1  | $-0.543$      | 0.108| 0.2382 |

**FIGURE 2** First instar larval feeding preferences ($M + SE$) when exposed to the pesticides clothianidin (insecticide), atrazine and s-metolachlor (herbicides), and azoxystrobin, pyraclostrobin and trifloxystrobin (fungicides), at mean (a) and maximum (b) concentrations. The pesticide solvent (acetone) was also tested. NS, non-significant; significance at *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. I, insecticides, H, herbicides, F, fungicides

**FIGURE 3** Second instar larval feeding preferences ($M + SE$) when exposed to the pesticides clothianidin (insecticide), atrazine and s-metolachlor (herbicides), azoxystrobin, pyraclostrobin and trifloxystrobin (fungicides), or the mix of all of them (mix), at mean (a) and maximum (b) concentrations. Leaf discs treated with acetone were used as the control. Untreated leaf discs were designated as the pesticide treatment in comparison among "control" groups (i.e. far left pair of black vs. grey bars). Significance at **$p < 0.01$ or ***$p < 0.001$, everything else was not significant (NS). I, insecticides, H, herbicides, F, fungicides
concentrations. Because acetone was used as the control in this trial, none of the pesticide treatments had an impact on foraging behaviour.

All larvae survived in both trials under the two concentration levels tested. Pesticide type did not affect whether larvae made a decision between either the control or treatment disc (Table S1). Similarly, the amount of tissue consumed from the pesticide-treated disc across treatments was not different in any of the trials (Trial 1, mean: $\chi^2 = 6.29, p = 0.392$; Trial 1, max: $\chi^2 = 3.18, p = 0.786$; Trial 2, mean: $\chi^2 = 2.48, p = 0.870$; Trial 2, max: $\chi^2 = 0.60, p = 0.996$).

TABLE 5 Effects of mean (a) and maximum (b) pesticide concentrations on 2nd instar larval feeding preference. Significance at *** $p < 0.001$ and ** $p < 0.01$. Control = leaf discs treated with the pesticide solvent acetone.

| Treatments     | $\chi^2$ | df | 95% CI    | p       |
|----------------|----------|----|-----------|---------|
| (a) Mean concentrations |          |    |           |         |
| Control        | 11.00    | 1  | 0.2523    | 0.8386  | 0.0009*** |
| Clothianidin   | 0.00     | 1  | -0.4532   | 0.3355  | 1.0000    |
| Atrazine       | 0.10     | 1  | -0.4583   | 0.2583  | 0.7518    |
| Metolachlor    | 2.78     | 1  | -0.6969   | 0.0302  | 0.0956    |
| Azoxystrobin   | 3.79     | 1  | -0.7166   | -0.0202 | 0.0516    |
| Pyraclostrobin | 3.79     | 1  | 0.0202    | 0.7166  | 0.0516    |
| Trifloxystrobin| 0.00     | 1  | -0.3570   | 0.4904  | 1.0000    |
| Mix            | 0.13     | 1  | -0.5285   | 0.2785  | 0.7216    |

(b) Max concentrations

| Treatments     | $\chi^2$ | df | 95% CI    | p       |
|----------------|----------|----|-----------|---------|
| Control        | 8.70     | 1  | 0.1810    | 0.7755  | 0.003**   |
| Clothianidin   | 0.00     | 1  | -0.2770   | 0.3570  | 1.0000    |
| Atrazine       | 0.82     | 1  | -0.1542   | 0.5178  | 0.366     |
| Metolachlor    | 0.00     | 1  | -0.4228   | 0.3175  | 1.0000    |
| Azoxystrobin   | 2.50     | 1  | -0.0456   | 0.6456  | 0.114     |
| Pyraclostrobin | 0.00     | 1  | -0.3465   | 0.3465  | 1.0000    |
| Trifloxystrobin| 0.11     | 1  | -0.2691   | 0.4913  | 0.739     |
| Mix            | 0.00     | 1  | -0.3973   | 0.3021  | 1.0000    |

FIGURE 4 Field oviposition by wild monarch females on Asclepias syriaca ($n = 49$ plants) varying in leaf concentrations of five pesticides (a–e) in 2015. Note the different x-axis scale across active ingredients. Vertical dashed lines show the mean value from our broader field survey. The neonicotinoid clothianidin was not included because it was detected primarily in June before monarchs colonized field sites.
3.4 | Field colonization

In the field, monarchs tended to place fewer eggs on plants with increasing pesticide concentrations (i.e. negative trend on all slopes), but only metolachlor showed a significant negative effect on oviposition (Figure 4; Table 6). Plants with metolachlor concentrations below the mean had higher numbers of eggs. Curiously, for atrazine only plants with concentrations below the mean had eggs so it was not possible to test against plants with higher pesticide concentrations.

4 | DISCUSSION

The experimental methodology employed here was largely successful in simulating pesticide residues occurring on field-grown milkweed plants. However, we noted important differences comparing sprayed plants versus leaf discs. These differences can likely be explained as follows. First, the method of pesticide application was vastly different. In the larval experiment, leaf discs were precisely treated with a 20 μl droplet of pesticide solution at almost contact distance, whereas plants for the oviposition experiment were sprayed outdoors. Although we attempted to evenly coat the full plant across all leaves, this methodology is surely less accurate than pipetting onto small leaf discs where full coverage is ensured. We also suspect there was pesticide biotransformation (Gavrilescu, 2005) in the oviposition experiment due to processes such as plant and microorganism metabolism (Van Eerd, Hoagland, Zabloutowicz, & Hall, 2003), and abiotic factors such as pesticide volatilization, or UV exposure and higher temperatures in the greenhouse compared to the laboratory. Nevertheless, pesticide values were relatively stable over the experiment and thus butterflies were exposed to approximately the same concentrations throughout, even if they were somewhat lower than field values, resulting in a more conservative evaluation for butterfly oviposition. The fact that maximum concentrations always produced substantially higher leaf values than mean concentrations, as intended, confirms the validity of this approach.

4.1 | Oviposition preference

Plants treated with pesticides at their maximum concentrations had a clearly deterrent effect on oviposition. The number of eggs placed on plants treated with a mix of pesticides at their maximum concentrations was 29.2% lower than plants receiving the solvent control. An important caveat to the oviposition trial is that the measured maximum concentrations were far lower than the expected field-targeted ones (as noted in the above section) and were in fact closer to the mean values for most of the six pesticides in the blend. Thus, despite their treatment label, monarchs clearly avoided pesticide-treated plants at submaximal levels that are more typical of the range of concentrations encountered in the field.

Oviposition deterrence behaviour has been similarly studied in the lepidopteran pest Plutella xylostella when exposed to the insecticides gamma-cyhalothrin and spinetoram (Nansen, Baisac, Nansen, Powis, & Baker, 2016) and extracts from different parts of the plant Strychnos nux-vomica (Loganiaceae) (Selvaraj, Kennedy, & Suganthy, 2017). Chemosensory structures located in the tarsi and antennae of butterflies vary in their susceptibility to different concentrations of natural compounds used for host recognition, but monophagous species feeding exclusively on one plant family often detect stimulant or deterrent phytochemicals with great precision (Honda, 1995; Nishida, Ohsugi, & Fukami, 1990). Therefore, it is not surprising that monarch females, which are specialists on milkweeds, can detect the synthetic pesticides applied on milkweeds and adjusted oviposition behaviour accordingly.

A limitation of the caged design of our oviposition bioassay is that gravid females may become less selective among plant options when presented with a small number of choices in a small arena, particularly when paired with a male butterfly that can cause mating-induced stress. However, correlative data from our field survey also indicate that females avoid placing multiple eggs on plants with relatively high pesticide loads, even when presented in light of variation in numerous additional factors impacting oviposition decisions. For example, monarch butterfly oviposition is affected by the spatial configuration of plants (Baker & Potter, 2019), habitat type (Myers, Bahlai, & Landis, 2019), patch size (Pitman, Flockhart, & Norris, 2018), plant size or phenology (Cohen & Brower, 1982; Haan & Landis, 2019), milkweed species (Ladner & Altizer, 2005; Pocius et al., 2018) and the presence of conspecific caterpillars (Jones & Agrawal, 2019).

4.2 | Larval feeding preference

In our study, second instar monarch larvae were deterred by acetone, but pesticides did not affect their feeding preference. Unlike with second instars, three pesticides at mean concentrations (Figure 2a) and three pesticides at maximum concentrations (Figure 2b) had a deterrent effect on first instar larvae. S-metolachlor is the only pesticide that acted as an antifeedant at both concentrations; interestingly, this was also the only pesticide that significantly reduced oviposition in the field. The lack of deterrence in 2nd instars could be related to instar-mediated variation in response to insecticides, which is consistent with other studies showing more susceptibility of early instars to clothianidin in Agrotis ipsilon (Hufnagel) (Lepidoptera: Noctuidae) (Ding, Zhao, Zhang, Xu, & Mu, 2018) and to pyrethroids in Pieris brassicae (Linnaeus, 1758) (Lepidoptera: Pieridae) (Tan, 1981). The susceptibility of young instars varies with both the insecticide and Lepidoptera species tested (Rodriguez-Saona, Wanumen, Salamanca, Holdcraft, & Kryczenko-Roth, 2016).

In two of the four larval groups tested, acetone acted as a feeding deterrent. This outcome is interesting because experiments testing effects of pesticides on bees (Taylor, Waller, & Crowder, 1987) and other Lepidoptera (Bhattacharyya, Barua, & Ghosh, 1995; Gist & Pless, 1985) using acetone as a control have not shown any negative effects. We used acetone because it dissolves all test materials and evaporates
quickly, but the residues on (and within) the leaves can deter monarch feeding under certain conditions. One explanation is that acetone removes amino acids or secondary chemicals from the leaf surface that are important for monarch larvae; for example, flavonoids for which acetone is an excellent extraction solvent (Munhoz et al., 2014). This outcome highlights the fact that a dual-control approach should be employed in future pesticide/pollinator studies, as many experiments dissolve pesticides in acetone and use an acetone control.

5 | CONCLUSIONS

In Lepidoptera, female oviposition preference is assumed to be correlated with larval offspring performance (Gripenberg, Mayhew, Parnell, & Roslin, 2010). This is particularly the case for monarch butterflies, which are host-plant specialists. The few studies that evaluated preference-performance relationships in the monarch/milkweed system, however, showed that the two were poorly correlated and alternative factors (e.g., learning, disease status, presence of competitors) affected host selection behaviours (Jones & Agrawal, 2019; Ladner & Altizer, 2005; Lefèvre et al., 2012). It is largely unclear how novel factors introduced to native co-evolved systems such as pesticide presence and concentration alter female choice, but our data indicate that females can effectively detect and avoid pesticide-treated plants at field-relevant doses. Assuming those pesticides are harmful to monarch caterpillars, this suggests that monarchs adaptively shift their oviposition in agricultural landscapes to maximize offspring fitness. Of the six pesticides tested, only one of them (clothianidin) has been experimentally evaluated for developmental effects on monarch caterpillars and the range tested in this study (8.2–9.1 ng/g) exceeds the level shown to cause sublethal effects on growth (Pecenka & Lundgren, 2015). Thus, our findings indicate that female choice can lessen the negative effects of pesticide exposure on monarch health, but this depends in large part on the degree of variation in pesticide presence across neighbouring milkweeds within or across patches, which is poorly known.

Despite the expectation that female choice alone dominates avoidance behaviours, our data show that early instar larvae also avoid certain pesticides when given the option. Unlike adults, young caterpillars are typically confined to a single plant and thus the benefits of displaying choice at the plant-level are contingent on leaf-to-leaf differences in pesticide concentration. Similar to inter-plant differences, this is not well-documented, either in milkweeds or other wild plant systems. Documenting neonate survival and behaviour on plants treated with pesticides in combination with differences in other natural attributes like trichomes, latex and cardenolides, will provide a better understanding of ecological pressures that pesticides place on this iconic butterfly. For example, monarch larvae grow slower on Asclepias syriaca and A. speciosa than on A. incarnata and A. fascicularis (Ladner & Altizer, 2005). It would be instructive for future studies to consider whether pesticide-mediated stress exacerbates the negative effects due to existing host-plant differences among milkweed species. The first generation of migratory monarchs develop on the spider milkweed A. viridis, which is far less palatable than the common milkweed A. syriaca, used in this study. Future experiments should compare the consequences of pesticide exposure for monarch caterpillars on these alternative Asclepias species. While still highly speculative, it is possible that plant defense reactions affect pesticide detoxification by the plant itself, e.g. herbivory could induce a plant to detoxify certain pesticides. This possibility is worth investigating given the mechanistic links between plant defense and pesticides, and the variation in defense reactions observed across related plant species. Milkweeds, for instance, show a wide range of plant defense syndromes within the genus (Agrawal & Fishbein, 2006).

Given that monarchs are part of a relatively specialized system consisting of a host-plant specialist interacting with a highly toxic plant (i.e. cardenolides), these outcomes will ultimately need to be compared with more generalized plant-pollinator systems. Specialists are thought to be better at distinguishing relatively minor variations in host-plant quality and the toxicity of milkweeds could make monarchs more attune to differences in leaf chemistry. Both features lead us to predict that generalist pollinators are less likely to detect and exhibit preference based on pesticide residues, which could result in greater developmental consequences when foraging in agricultural or urban landscapes. This prediction, however, remains to be tested.

Last, we strongly encourage studies assessing pollinator responses to pesticides to allow the opportunity for choice, integrating the spatial-temporal variation that is characteristic of the landscape. Doing so requires better documentation of the range of pesticide values naturally encountered by a foraging insect across and within plants in the field. This will ultimately allow for a more accurate estimate of the population-level impacts of pesticide on non-target species.

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AUTHORS’ CONTRIBUTIONS

P.O.-A. and I.K. conceived the idea; P.O.-A. conducted the experiment, collected the data and performed the statistical analyses; all authors (P.O.-A., M.E.S., I.K.) co-designed the methodology and contributed to the writing and revising of the manuscript.

DATA AVAILABILITY STATEMENT

Data available from the Purdue University Research Repository (PURR), https://doi.org/10.4231/XA35-0802 (Olaya-Arenas, Scharf, & Kaplan, 2020).
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**SUPPORTING INFORMATION**

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

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