EFFECTS OF NEONICOTINOIDS ON LARVAL AND ADULT MONARCH BUTTERFLIES

by

CODY S. PROUTY

(Under the Direction of Sonia Altizer)

ABSTRACT

Neonicotinoids are the most widely used insecticides in North America. Many studies have documented neonicotinoids' negative effects on bees, and there is evidence that neonicotinoids correlate with declines in monarch butterflies (*Danaus plexippus*). We examined how monarch development, survival, reproduction, and flight were affected by neonicotinoids, and how these effects depended on milkweed host plant species. Larval ingestion of low neonicotinoid doses did not affect monarch fitness traits. At the highest dose, neonicotinoids affected monarch pupation and survival for caterpillars that fed on the least toxic milkweed species; with differing effects on other species of milkweed. Adult ingestion of low and moderate neonicotinoid doses reduced reproductive activity only. At high doses, adult monarchs showed reduced flight performance and survival. Overall, monarchs tolerate low and moderate neonicotinoid doses, but experience detrimental effects at higher doses. These findings indicate that neonicotinoids are unlikely to cause widespread declines of monarchs at field-relevant levels.

INDEX WORDS: Insecticides, *Danaus plexippus*, clothianidin, imidacloprid, flight performance, migration

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CHAPTER 1

INTRODUCTION

Pollinators and other beneficial insects can become exposed to insecticides that are used for agriculture or gardening to reduce pests and increase crop yield. Of these insecticides, neonicotinoids are currently the most commonly used in North America to treat row crops, orchards, and ornamental plants (Bonmatin et al. 2015; van der Sluijs et al. 2013). Neonicotinoids are a class of synthetic neuroactive insecticides, the most common of which are clothianidin, imidacloprid, and thiamethoxam. Neonicotinoids can be applied using seed treatment, soil drenching and foliar application, with seed and soil application most commonly used in agricultural settings. However, studies have found that in typical agricultural applications, only around 5% of the active ingredient ends up in the target plant and the rest enters the environment (Hladik et al. 2018). Since neonicotinoids are systemic insecticides and highly water soluble, they can be incorporated into plant tissue through uptake in the roots and expressed persistently in leaves and flowers. The compounds persist in the environment for many months, with half-lives from hundreds to thousands of days in the absence of exposure to UV light (Mohapatra et al. 2019).

Neonicotinoids bind to central nervous system receptors in insects, causing paralysis and death (Simon-Delso et al. 2015), and are highly effective against many sucking, leaf chewing, and soil insects. Honeybees (*Apis mellifera*) and bumblebees (*Bombus* spp.) are extremely well studied in their responses to neonicotinoids, but the effects of neonicotinoids on butterflies have not been as well examined. At sub-lethal concentrations, bees suffer from reduced foraging performance, including lower efficiency, motivation, and nutritional status (Azpiazu et al. 2019,

Feltham et al. 2014, Lamsa et al. 2018, Morfin et al 2019, Phelps et al. 2020, Scholer and Krischik 2014, Stanley and Raine 2016). There is also evidence for low dose effects on social behavior, reproduction, navigation, and flight performance (Bryden et al. 2013, Crall et al. 2018, Fischer et al. 2014, Laycock et al. 2012, Scholer and Krischik 2014, Switzer and Combes 2016, Tosi et al. 2017, Whitehorn et al. 2017). The concentrations of neonicotinoids found in nectar vary widely from plant to plant, but the nectar of wildflowers in field margins have been shown to contain neonicotinoids. Up to 97% of neonicotinoids brought back to honeybee hives were from wildflowers, not crops, with the highest concentrations of neonicotinoids (86.02 ppb) in pollen (Botias et al. 2015).

Recent studies showed that butterflies appear to be more tolerant than bees to levels of neonicotinoid exposures found in the margins of agricultural fields. Krishik et al. (2015) used painted lady butterflies (*Vanessa cardui*) as well as monarchs (*Danaus plexippus*) in their experiments and found that painted lady butterflies were less sensitive to neonicotinoids than monarchs, with no mortality after 7 days. Additionally, Basley and Goulson (2018) found slower larval growth in common blue butterflies (*Polyommatus Icarus*), but no detectable increase in mortality at field relevant concentrations. Although more work needs to be done on butterfly and other lepidopteran responses to neonicotinoids, there is evidence that bees have much higher sensitivity in terms of both mortality and sub-lethal responses, including reduced cognitive ability and poor mobility.

Monarch butterflies are charismatic and iconic insects, with scientific interest in their biology stemming from their long-distance yearly migrations and ability to sequester cardenolide toxins from their milkweed (*Asclepias* spp.) host plants (e.g., Malcolm 1994; Zhan et al. 2014; Brower 1996; Oberhauser et al. 2015; Gustaffson et al. 2015). Monarchs occur worldwide, and

their migratory behaviors vary across regions (Ackery and Vane-Wright 1984). The eastern North American population of monarchs undergo a yearly migration, from as far north as Canada to Central Mexico, a 5,000-kilometer journey (Urquhart and Urquhart 1976). Declines in winter colonies of North American monarchs have caused concern for the persistence of their migration (Brower et al. 2012; Schultz et al. 2017). Insecticides, such as neonicotinoids, have been suggested as a potential cause of decreased monarch migration success (Tracy et al. 2019; Stenoin et al. 2018). Some studies further suggested that the increasing use of neonicotinoids is correlated with declines in eastern North American monarchs, (Thogmartin et al. 2017; Stenoien et al. 2018) and other work showed that both pesticide use and habitat loss predicted western monarch declines (Crone et al. 2019).

The route of exposure to neonicotinoids is an important consideration in determining their impacts on monarch fitness. There could be major differences in monarch responses to insecticides at the larval or adult stage. Larval monarchs feed on milkweed, a plant that is known for its toxic secondary chemicals, cardenolides (Agrawal et al. 2012). Many insects have evolved detoxification systems for coevolved plant compounds (Berenbaum and Johnson 2015), and monarchs can tolerate and incorporate cardenolides produced by milkweeds into their bodies to deter predation (Malcolm 1994). However, at high concentrations, these toxic cardenolides can lead to decreased fitness for monarchs, both as larvae and adults (Agrawal et al. 2012). Once monarchs emerge as adults, they no longer feed on toxic leaves, and instead consume the nectar of wildflowers. Thus, monarch diets switch from highly toxic (depending on the species of milkweed) to minimally toxic, and the amount of sequestered chemicals do not increase after their larval stage. Adding a synthetic insecticide, such as neonicotinoids, to these food resources at either stage of development could have implications for monarch fitness.

The goal of my thesis work was to ask how monarch development, reproductive behavior and migration respond to field-relevant concentrations of neonicotinoids. My first goal was to determine how larval monarch survival, development, and flight were affected by ingestion of imidacloprid and clothianidin applied to milkweed host plants. To achieve this goal, experiments were designed to answer (1) whether exposure to low field relevant doses at the larval stage lead to declines in survival, development, and adult flight ability, and (2) whether the effects on larval monarchs are moderated by milkweed species that differ in their cardenolide profiles. My second goal was to determine how adult monarchs respond to exposure to clothianidin and imidacloprid ingested via nectar (a 20% honey-water solution). This goal was achieved by determining (1) whether low-dose exposure at the adult stage led to decreased survival, reproduction, and viability of offspring or changes in behavior, and (2) whether higher dose exposure reduced adult monarch survival, flight ability and reaction time.

This thesis work expands knowledge of the impacts of neonicotinoids on monarch behavior and physiology, which can inform management issues for conservation. Work that quantifies monarch responses to chemical exposure is crucially needed to predict future changes in monarch migration and survival. In the face of habitat degradation, climate changes, and population loss, limiting future interactions between a diversity of threatening processes could be important for protecting North American monarch migration. In particular, if monarch survival, reproduction and flight performance are substantially reduced by field-relevant neonicotinoid exposure, this could motivate stricter insecticide regulations to ensure the future persistence of monarch migration. On the other hand, if monarchs tolerate moderate levels of neonicotinoid exposure, this indicates that other factors are more important conservation targets for protecting monarchs.

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CHAPTER 2

HOST PLANT SPECIES MEDIATES MONARCH BUTTERFLY RESPONSES TO LARVAL-STAGE NEONICOTINOID EXPOSURE[1]

[1] Prouty, C.S., P. Barriga, A.K. Davis, V. Krischik, and S. Altizer. Submitted to *Insects*, March 17, 2021.

Abstract

Neonicotinoids are the most widely used insecticides in North America. Numerous studies document the negative effects of neonicotinoids on bees, and it remains crucial to demonstrate if neonicotinoids affect other non-target insects, such as butterflies. Here we examine how two neonicotinoids (imidacloprid and clothianidin) affect the development, survival and flight of monarch butterflies (*Danaus plexippus*), and how these chemicals interact with secondary toxins in monarch host plants (milkweeds). Milkweed growing near agricultural areas can be contaminated with neonicotinoids, and a handful of studies to date show mixed results for the lethal dose of neonicotinoids for monarchs. We first fed caterpillars field-relevant low doses of neonicotinoids applied to milkweed leaves, and found no significant reductions in larval development rate, pre-adult survival, or adult flight performance. We next fed monarch larvae higher neonicotinoid doses and reared the larvae on milkweed species known to produce low, moderate, or high levels of secondary toxins (cardenolides). Monarchs exposed to the highest dose of clothianidin experienced pupal deformity, low survival to eclosion, smaller body size and weaker adult grip strength. This effect was most evident for monarchs reared on the lowest-cardenolide milkweed (*Asclepias incarnata*), whereas monarchs reared on the high-cardenolide *A. curassavica* showed no significant reductions. Findings here indicate that monarchs are more tolerant to neonicotinoids than bees, and that coevolved plant toxins could confer protective effects. Although neonicotinoid residues are ubiquitous on milkweeds in agricultural and ornamental settings, commonly encountered doses below 50 ppb are unlikely to cause substantial declines in monarch survival or migratory performance.

Introduction

Neonicotinoids are a class of synthetic neuroactive insecticides similar in structure to nicotine; they have come into widespread use since the late 1990s, and are presently the most widely used class of insecticide in the world (van der Sluijs et al. 2013). Neonicotinoids such as clothianidin, imidacloprid, and thiamethoxam are widely used in row crops in North America, as well as on orchards, vegetables, and ornamental plants, and can be applied through seed treatment, soil drenching and foliar application (Bonmatin et al. 2015). The compounds persist in the environment for many months, with half-lives from hundreds to thousands of days in the absence of UV exposure (Mohapatra et al. 2019). They can be incorporated into plant tissue through uptake in the roots and expressed persistently in leaves and flowers. Seed and soil treatments are commonly employed for application of neonicotinoids; with these methods, only about 5% of the active ingredient ends up in the target plant, while the rest enters the environment (Hladik et al. 2018).

Neonicotinoids bind to central nervous system receptors in insects, causing paralysis and death (Simon-Delso et al. 2015), and are highly effective against many sucking, leaf chewing, and soil insects (Gervais et al. 2010). These insecticides have many sublethal affects that alter movement, behavior, and navigation (Lu et al. 2020). Owing to strong negative effects on honeybees and bumblebees exposed through pollen and nectar (and sub-lethal effects detected as low as 2-20ppb; Yao et al. 2018; Scholer and Krischik 2014; Krischik et al. 2007; Blanken et al. 2015; Morfin et al. 2019), neonicotinoids are now banned in the European Union.

Monarchs (*Danaus plexippus*) are charismatic and iconic insects, with scientific interest in their biology stemming from their long-distance yearly migrations, and their ability to sequester cardenolide toxins produced by their milkweed host plants (e.g., Zhan et al. 2014;

Oberhauser et al. 2015; Gustaffson et al. 2015). Declines of winter colonies of North American monarchs caused concern for the persistence of their migration (Brower et al. 2012; Shultz et al. 2017). While some evidence has suggested these declines stem from issues related to habitat loss during summer (Thogmartin *et al.* 2017; Flockhart *et al.* 2015; Pleasants and Oberhauser 2013), other research points to problems faced during the fall migration (Ries *et al.* 2015; Inamine *et al.* 2016; Saunders et al. 2019). In particular, neurotoxic neonicotinoids have been suggested as a potential cause of decreased monarch migration success (Tracy et al. 2019; Stenoin et al. 2018). Some studies cited the increasing use of neonicotinoids as a correlational factor with declines in eastern monarchs, (Thogmartin et al. 2017; Stenoien et al. 2018) and another found that western monarch declines were greater where pesticide use and habitat loss were higher (Crone et al. 2019).

Monarchs could be exposed to neonicotinoids in agricultural environments through drift from foliar applications, or soil leaching from seed treatments at planting (Nuyttens et al. 2013). Monarch caterpillars often feed on milkweed in agricultural fields, and could be exposed to herbicide and insecticide foliar spraying (Oberhauser et al. 2001). A handful of studies to date tested effects of neonicotinoids on monarchs following larval exposure with mixed results. These studies differed in the insecticides used, application methods, exposure stage, milkweed species used as host plants, and response variables recorded [summarized in Table S1]. Whereas some studies show negatives effect of low doses of clothianidin (Lundgren and Pecenka, 2015), more studies showed moderate (Bargar et al. 2019; Olaya-Arenas, P. et al. 2020) or low toxicity (Krishnan et al. 2020) for neonicotinoid levels commonly reported in field surveys. Further work is needed to resolve the differences reported in studies to date, particularly in reference to effects

of different host plant species and methods of exposure (Krishik et al. 2015; Basley and Goulson 2018; Whitehorn et al. 2018).

Insects have evolved detoxification systems for coevolved plant compounds (Berenbaum and Johnson 2015). Monarchs in particular can tolerate and incorporate cardenolides produced by milkweeds into their bodies to deter predation (Malcolm 1994). This comes at a cost, as high cardenolide doses can reduce caterpillar survival and development (Agrawal et al. 2012). Both cardenolides and neonicotinoids oppose the transfer of cations, albeit through different modes of action. The pathway that monarchs and other species that feed on milkweed use to deal with cardenolides has been recently identified (Petschenka et al. 2013; Agrawal et al. 2012). Neonicotinoids and cardenolide toxins could potentially interact in ways that amplify the effects of insecticides, or dampen their overall effects. Because milkweed species have differing levels of cardenolides, the dose and types of cardenolide exposure differ with plant species range, abundance and phenology (Zalucki et al. 1990; Rasmann and Agrawal, 2011).

Here we examined how neonicotinoid consumption by caterpillars influences monarch development and survival, and whether insecticide impacts vary among milkweed species known to differ in cardenolides. We first exposed monarchs reared on less toxic swamp milkweed (*Asclepius incarnata*) to field-relevant doses of clothianidin and imidacloprid. After finding no effect of neonicotinoids at these low doses on monarch development, survival, or flight performance, we conducted a second experiment with higher neonicotinoid doses applied across three milkweed species representing low *(A. incarnata*) moderate (*A. syriaca*) and high (*A. curassavica*) average cardenolide content. We again examined monarch development and survival to the adult stage, and tested grip strength (Davis et al. 2020) as an indicator of physical performance. From the data in Krishnan et al. (2020), we predicted that monarchs would be more

tolerant of neonicotinoids (e.g. higher lethal doses) than previous studies indicate, such as Lundgren and Pecenka (2015). We predicted that monarchs would fly shorter distances and more slowly when exposed to neonicotinoids, due to their effects on insects' neurological functions. We also predicted that differences in milkweed cardenolide concentration would influence monarch tolerance of neonicotinoids, with more toxic plant species either upregulating monarchs' ability to tolerate other toxins, or intensifying the negative neonicotinoid effects.

Methods

Monarch, Plant, and Neonicotinoid Sources

We used captive-reared monarchs that were non-inbred F3 descendants of wild-caught fall migrants from Athens, GA and St. Marks, FL, USA in Oct 2017 (Experiment 1) and Oct 2018 (Experiment 2). Adult monarchs were mated in 0.6 m^3 mesh cages and fed *ad libitum* with a 20% honey water solution. Mated females oviposited onto *A. incarnata* cuttings, and larvae remained on natal stalks until second instar. We obtained 3-4 outcrossed genetic lineages of monarchs per experiment.

Milkweed plants were raised from seeds obtained from Prairie Moon nursery (swamp, *A. incarnata* and common, *A. syriaca*) and the vendor SEEDS2GO (tropical, *A. curassavica*) and planted into 12.5 cm diameter pots. Plants were pruned several times prior to each experiment, and received bi-monthly pelleted fertilizer and weekly spraying with insecticidal soap to control aphids and thrips. Greenhouse temperatures fluctuated between 15°C and 35°C, with a 16:8 light:dark cycle under broad-spectrum lights.

One mg each of clothianidin and imidacloprid (Sigma-Aldrich) was dissolved separately into 0.5 L of distilled water, to achieve a 2 ppm (mg/L) stock solution, which was further diluted to doses of 5, 15, 50 and 500 ppb (ng/mL). We measured out multiple dilutions throughout each experiment from a single stock solution mixed at the start of each experiment. Stock solutions were held at 4 °C for up to 12 days per experiment, and original (solid) chemicals were held at 22 °C for up to 18 months. Liquid aliquots and leaf samples were sent to the USDA Agricultural Marketing Service, laboratory in Gastonia, NC to estimate the concentration of clothianidin and imidacloprid through standard methods using HPLC-GC. The doses used in these experiments are ecologically relevant, given that field studies found up to 56.5 ng/g (ppb) of clothianidin in wild milkweed leaves on field margins (Olaya-Arenas, 2019; Basley and Goulson, 2018).

Experiment 1: Low Dose Larval Exposure and Monarch Flight

To test responses to low doses of clothianidin and imidacloprid, caterpillars were raised singly on cuttings of greenhouse-raised *A. incarnata*. Five treatments (N = 227) included a distilled water control (35 larvae), clothianidin 5 and 15 ppb (48 larvae/treatment), imidacloprid 5 and 15 ppb (48 larvae/treatment) applied directly to leaves. Larvae remained on natal milkweed stalks until they reached mid-second instar, and were then transferred to 0.5L plastic containers with mesh screen lids. Each day for five days, we painted milkweed cuttings (removing all but four leaves per container) with 15 µL insecticide solution per leaf. Solution was administered using a micro-pipetter and spread across the leaf surface with a small craft paintbrush. If monarchs consumed the treated cutting, they were fed *ad libitum* with untreated milkweed stalks until the next day. Tools used for feeding and applying insecticides were physically separated for each treatment, and were exposed to UV light daily for two hours to degrade residual neonicotinoids.

After five days of treatment application, monarchs were fed *A. incarnata* stalks *ad libitum* until pupation (3-4 additional days). Containers were checked twice daily for deaths or pupation. Monarch pupae were weighed five days post-pupation to the nearest 0.001g using an analytical balance. We recorded eclosion date, sex, checked for infection by the protozoan *Ophryocystis elektroscirrha,* and held monarchs in individual glassine envelopes at 24° C. We fed monarchs by hand a 20% honey-water solution each day for five days after eclosion.

We measured monarch flight indoors during May-Jun 2018 using a tethered flight mill in a 9 m^2 room at 29.7°C (range 27.8 - 31.4°C) between 1000 and 1730 h. Five to six days post-eclosion, we glued lightweight steel wires (15lb test) to the dorsal side of each monarchs' thorax using rubber cement, following Bradley and Altizer (2005). As per Schroeder et al. (2019), the average mass of the wire attachment was 0.19 g (range 0.10-0.33 g). Monarchs were placed into 0.6 m^3 mesh cages to adjust to the weight of the wire, with 20% honey water provided *ad libitum*. The flight mill was constructed as described in Bradley and Altizer (2005) and Fritzsche McKay et al. (2016) from a 120 cm lightweight carbon rod with a diameter of 3 mm (4.23 m circumference) attached to a nearly frictionless steel pivot (Fig. S1). We tethered monarchs to one end of the horizontal rod, and a flag at the opposite end passed through an infrared beam on a photo-gate to estimate flight velocity per revolution (m/s; software PASCO Capstone). Windows were covered with white paper to limit sun angle cues during flight, and we positioned floor lamps to provide an even distribution of light.

Monarchs were flown for a maximum of 1hr. Monarchs that stopped flying for more than 5s were agitated with a gust of air. If the monarch did not resume flight after three agitations, the flight was terminated. For each flight, we calculated total distance (km) flown based on the number of revolutions. We calculated average flight velocity (km/hr) by dividing the total

distance by time in flight. We non-destructively measured wing area (in mm^2) using Fovea Pro 4.0 plugins for Photoshop CS2 from scanned images of the adults acquired from a digital flatbed scanner, following Davis et al. (2012). Wing loading (g/mm^2) was calculated as body mass divided by wing area, and we used wing area and the weight of each wing to calculate wing thickness, following Davis and de Roode (2018).

Experiment 1 Analyses

Analyses were performed in R version 3.5.3. We first used general linear mixed models to test for relationships between neonicotinoid exposure and monarch development. Neonicotinoid type was categorized as a 5-level factorial variable (levels = control, 5 and 15 ppb imidacloprid, 5 and 15 ppb clothianidin). We analyzed pupal weight, larval growth rate (pupal mass / days to pupation), wing area, wing weight, wing thickness, and adult monarch weight as response variables, using GLMMs with normal error structures and the following model structure in the lme4 package (Bates et al. 2015): [response variable = insecticide group + genetic lineage (random effect)]. Sex was included as an additional additive predictor for adult response variables. For flight variables, we analyzed distance, average speed, time spent flying, and power (from the calculation described in Fritzsche McKay et al. (2016)). Flight distance and time were log-transformed to normalize the error variance. Models for flight variables included the following covariates: adult age on date flown, wire weight, weight before flight, and wing loading, together with insecticide group (5 levels), sex, and genetic lineage (random effect).

Experiment 2: High Dose Larval Exposure and Monarch Development

To test whether insecticide effects on monarch survival and development depended on host plant species, we reared monarchs on potted milkweeds inside a greenhouse, using *A. incarnata* (low cardenolide levels, 0 mg/g dry (pp thousand), *A. syriaca* (moderate cardenolide levels, 0.5 mg/g), and *A. curassavica* (high cardenolide levels,1 mg/g; Sternberg et al. (2012). For clothianidin and imidacloprid, five treatments (N=240) were used: control (water only), clothianidin 50 and 500 ppb, and imidacloprid 50 and 500 ppb for both *A. incarnata* (24 monarchs per treatment) and *A. curassavica* (15 monarchs per treatment). For *A. syriaca* (15 monarchs per treatment) only two treatments were used: control and 500 ppb (Table S2).

We used a pump sprayer to administer 60-70 mL/plant of clothianidin and imidacloprid solutions (distilled water for controls) on the tops and bottoms of leaves of pruned 0.6m tall plants. Second instar monarch larvae (from 5 outcrossed lineages) were reared singly on potted milkweed after plants were sprayed and dried. Larvae were enclosed in a clear acrylic tube (0.5mm thick, 1m tall, 12-13cm in diameter; Fig. S2) with mesh fabric fastened to the top. Pots were placed into solid-bottom trays to retain water and hydrate plants. Trays were randomly organized across four greenhouse benches in two adjacent rooms. Owing to the known decay of neonicotinoids in UV light, plants were re-sprayed with 20mL fresh solution per plant 5d after initial treatment. Monarchs remained on plants during the second spray, but we avoided the direct spraying of caterpillars.

Monarchs were observed daily to record survival and pupation. Five days post-pupation, pupae were weighed to the nearest 0.001g using an analytical balance. We recorded pupal deformity or discoloration on a 0-3 scale (0 = normal pupal color and shape; 1 = mild discoloration or deformity; 2 = moderate discoloration or deformity; 3 = failure to complete ecdysis; Fig. S3). We recorded monarch eclosion date and sex, and checked all monarchs for

infection by the protozoan *O. elektroscirrha*. As in Experiment 1, we scanned adult monarchs and measured wing area using digital image analysis.

Monarch grip strength was measured following Davis et al. (2020) using a device that detects how much force in newtons (nw) is exerted when monarchs pull on a rod attached to a force gauge. Briefly, an observer holds a monarch by the closed wings and lowers it to the rod until it grips with all four tarsi. The observer then gently pulls the monarch directly upwards until it releases from the rod. This was repeated five times per individual, to obtain an average measure of releasing force (i.e. grip strength). Strength trials were performed blind (we reassigned monarch identification numbers) to limit observer bias.

Experiment 2 Analysis

We analyzed outcomes of control, 50 ppb and 500 ppb applications across all three host plant species, coding neonicotinoid treatment as a 5-level fixed factor (control, 50 ppb imidacloprid, and 50 ppb clothianidin 500 ppb imidacloprid, and 500 ppb clothianidin). We tested the following response variables: larval growth rate (pupal mass / days to pupation), forewing area, grip strength, and pupal deformity (0-3 scale), using the following model: response variable = insecticide + milkweed species + insecticide*milkweed species + block (5 greenhouses) + monarch lineage (random effect). Analyses based on adult data (forewing area and grip strength) included sex as a main effect. We analyzed the proportion of monarchs that eclosed (0/1) using a GLM with binomial distributions for the error structure. In any instance with a non-significant block effect, the variable was removed.

Wild Caught Bee Neonicotinoid Bioassay

To confirm the toxicity of the neonicotinoid doses used here, wild caught *Bombus impatiens* were exposed to 0, 5, 50, and 500 ppb clothianidin and imidacloprid. Bees were captured while foraging at the UGA campus trail gardens in Athens, GA USA, and were exposed within 1 hr. On average, the bumblebees used for this bioassay were of similar mass to monarch butterflies. We used the same stock solutions as for Experiment 2, to make a 20% honey water solution with the specified neonicotinoid dose. Bees were placed into clear acrylic containers (15x10x10cm) with mesh lids, to which we added sponges soaked in the honey water solution placed inside petri dish bottoms. We placed 4 bees into each container, with 1 container for each treatment. Containers were checked every 40-60 mins over a 4hr period to record the activity of each bee as: flying, active/crawling, standing, twitching (while lying on side), or dead (Table S3). Bees were frozen at -20 °C after observations were concluded.

Results

Leaf and aliquot neonicotinoid assays

For Experiment 1, HPLC residue assays showed that only trace amounts (< 3ppb) of 15 and 50 ppb imidacloprid and clothianidin were detected on milkweed leaves on the day of application (Table S4). For Experiment 2, residue assays showed that *A. incarnata* and *A. curassavica* leaves had roughly 10% of the applied concentration of clothianidin and imidacloprid on the day plants were treated. On day 4 post-application, *A. incarnata* leaves maintained similar residues to those detected on day 0, with clothianidin concentrations at 5 and 25 ppb and imidacloprid at 4 and 31

ppb (Table S4). However, only trace levels were detected on *A. curassavica* leaves on day 4 post-application. Neonicotinoids were not detected on control leaves.

Experiment 1: Low Dose Larval Exposure and Flight

A total of 165 (72.7%) of the 227 monarchs placed on plants at second instar survived to eclosion. No larval or adult response variables differed significantly among the insecticide treatments (Table 1; Fig. 1A,B). We selected 139 of the 165 adult monarchs to measure flight (20 controls, and 21-31 per insecticide treatment; data were excluded from 2 control and 14 insecticide-treated monarchs that did not fly for a minimum of 3 min. Monarchs across all treatments flew an average of 0.745 km ± 0.06 SE, at a speed of 2.55 km/hr ± 0.05 SE, and for a duration of 16.92 min ± 1.25 SE. Flight measures were similar across all treatments (Table 1; Fig. 1C,D).

Experiment 2: High Dose Larval Exposure

Of the 240 monarchs placed onto plants in Experiment 2, 210 (87%) survived to pupation, 208 (86.67%) eclosed as adults, and 206 (86%) had wings scanned and participated in the grip strength test. Larval development rate (g/d) was significantly lower for monarchs reared on common milkweed (*A. syriaca)*, and was faster for monarchs that fed on tropical and swamp milkweed (*A. curassavica* and *A. incarnata)*. Larval development rate did not differ according to insecticide treatment (Table 2).

Nearly all monarchs fed on *A. incarnata* and exposed to the highest dose of clothianidin (500 ppb) experienced problems during pupation (Fig. 2B), with many failing to shed their larval integuments (failed ecdysis; Fig. S3D). Nearly half of all monarchs that fed on *A. syriaca* (low

cardenolide) and exposed to 500 ppb clothianidin also experienced problems during pupation, or showed pupal discoloration and deformity (Fig. 2A). In contrast, monarchs reared on *A. curassavica* pupated normally, irrespective of insecticide treatment (Fig. 2A). The interactive effects of milkweed species and insecticide treatment on pupal deformity were highly significant (Table 2).

In the high dose (500ppb) insecticide treatments, over 90% of monarchs that fed on *A. curassavica* survived to the adult stage, whereas 70% and 48% of monarchs that fed on *A. syriaca* and *A. incarnata* emerged successfully as adults. The interaction between host plant and insecticide treatment on adult survival was significant (Fig. 2B, Table 2). Adult wing area was highest for monarchs reared on *A. curassavica*, and lowest for monarchs reared on *A. incarnata* (Fig. 2C, Table 2). Larvae exposed to the highest dose of clothianidin (500 ppb) and fed *A. incarnata* were significantly smaller than control monarchs (Fig. 2C). The main effect of insecticide treatment on wing area was significant, but not the interaction between insecticide treatment and milkweed species (Table 2).

The grip strength of adults was lowest for monarchs reared on *A. incarnata* and treated with 500 ppb of clothianidin (Fig. 2D). However, analysis showed no significant main or interactive effects of insecticide treatment on grip strength (Table 2). Males showed significantly greater grip strength than females (Table 2).

Bee exposure

After 4 hrs, all four *B. impatiens* in the 0 ppb (honey water only) treatment remained actively flying, and had to be chilled at 14° C prior to removal. For imidacloprid 50 ppb, most bees

remained active for the first 2 hrs. By 4 hrs, the bees became inactive and showed signs of persistent twitching (Fig. S4). At the 500 ppb imidacloprid dose, all bees became inactive or were twitching after 2 hrs of exposure, and one bee died by the end of the 4 hr interval. For clothianidin, all bees from both the 50 and 500 ppb treatments died before the end of 4 hrs. For the 50 ppb dose, all bees remained alive until 1.5 hrs post-exposure, but for the 500 ppb treatment, most bees died after just 1 hr (Fig. S4). Collectively, these findings demonstrate the lethality of the neonicotinoid solutions prepared for this study towards bees.

Discussion

The impact of neonicotinoid insecticides on monarch butterflies, both in their breeding range and during the long-distance fall migration, is a potential concern (e.g. Tracy et al. 2019; Stenoin et al. 2018; Thogmartin et al. 2017; Lu et al. 2020). Based on our results, neonicotinoid doses of 5 and 15 ppb applied to leaves resulted in residues at or below the limits of detection on the day of treatment. Monarchs ingesting these trace amounts experienced no lethal or sub-lethal effects on development, size or flight. At the highest doses of 500 ppb (where residue is comparable to the upper-end of levels detected at in the agricultural and nursery industries), negative effects of clothianidin began with the onset of pupation, and depended strongly on host plant species and insecticide type. Negative effects on development and size were strongest for monarchs that fed on swamp milkweed treated with high dose clothianidin. In contrast, caterpillars that fed on the highest cardenolide milkweed, and those treated with imidacloprid, showed little to no negative response to neonicotinoid exposure. The same solutions used in Experiment 2 dramatically reduced bumble bee survival within a period of 4hr.

In Experiment 2, neonicotinoid residue on swamp milkweed was maintained at 10% the application dose for several days after treatment. At the 500ppb application of clothianidin (50ppb residual), nearly all caterpillars showed signs of pupal deformity, including failure to shed larval integuments, and only half of the monarchs in this treatment eclosed as adults. Similar rates of pupal deformity and ecdysis failure following exposure to high clothianidin doses were reported by another recent study (Krishnan et al. 2020). On tropical milkweed, residue was reduced to a trace amounts by day 4 post-application, and monarchs feeding on this high-cardenolide host plant showed normal pupation and over 90% eclosion success. Monarchs that fed on common milkweed (intermediate cardenolides) showed intermediate rates of pupal deformity (50%) and eclosion success (70%). To our knowledge, this is the first study to show that host plant species with higher constitutive residue of cardenolides potentially reduced the residue of neonicotinoid insecticides on the leaf surface. This raises the possibility that plant secondary compounds might leach onto the leaf surface and degrade other leaf surface chemicals, and this phenomenon warrants further research.

Importantly, we note that tropical milkweed should not be used to "protect" monarchs from the negative effects of neonicotinoids. Tropical milkweed has been shown to be an ecological trap for migrating monarchs, leading to increases in infection by a debilitating protozoan, reducing the induction of reproductive diapause prior to fall migration, and reducing wing elongation (Majewska and Altizer 2019; Faldyn et al. 2018, Satterfield et al. 2015, 2018, Davis et al. 2020). Thus, we argue that the negative effects of tropical milkweed for monarch health and migration outweigh the potential benefits shown in this study.

Past research on lethal doses of neonicotinoids varied between feeding intact stems, leaf discs, and treating entire plants with neonicotinoids. In our study, monarchs fed on whole plants

and intact stems showed few negative effects on survival or life history patterns. Krischik et al. (2015) showed significantly reduced survival in monarch larvae at 25 ppb imidacloprid (leaf residue measured by HPLC, USDA, Gastonia, NC) in *A. curassavica* leaves treated with a soil application. Bargar et al. (2019) found no effects on larvae fed plants treated with a soil application that resulted in around 11-15 ppb clothianidin. In leaf disc studies, Lundgren and Pecenka, 2015 found an LC50 of approximately 15 ppb for clothianidin (*A. incarnata*), whereas Krishnan et al. 2020 found a LC50 of 7 ppm for imidacloprid and 4.2 ppm clothianidin (larvae fed on *A. curassavica*). Additionally, Basley and Goulson (2018) found slower larval growth in common blue butterflies, but no detectable increase in mortality at 15 ppb of clothianidin. In summary, some feeding studies showed effects at around 15 ppb clothianidin or imidacloprid, but others, including our study, indicate that higher doses are needed to produce toxic effects in monarchs.

The absence of negative effects of neonicotinoids on monarch development, survival and flight (except for the highest doses of clothianidin, which is at the upper limit of residues found on field-collected plants) indicates that exposure to this class of insecticides might be less important for monarch migration than previously indicated (e.g., James 2019; Olay-Arenas et al. 2020). Grant et al. 2020 demonstrated that natural areas had higher monarch mortality compared to agricultural field edges. Some researchers have suggested that declines in monarch numbers reported at wintering sites in Mexico in recent years could reflect higher mortality during their fall migration (e.g., Agrawal and Inamine 2018). It appears unlikely, however, that declines in migratory success of monarchs are driven by neonicotinoid exposure at the larval stages.

There are contexts under which monarchs and other pollinators could become exposed to higher doses than field margins. Cowles and Eitzer (2017) found that milkweed treated with

products usually bought by gardeners contain neonicotinoids can lead to doses of up to 1,000 ppb in nectar. If gardeners use these products to deter pest insects, monarchs and other pollinators could be exposed to high doses via this route. Given reductions in bee mobility following insecticide exposure (e.g. Wood and Goulson 2017, Switzer and Combes 2016), and the known presence of neonicotinoids along agricultural field margins (e.g. Hladik et al. 2016, Mogren and Lundgren 2016, Krupke et al. 2012), it seems plausible that longer-term pesticide exposure at both larval and adult stages could lower monarch flight performance during the fall migration.

Findings here point to several areas for further investigation. First, neonicotinoids are not the only insecticides that migrating or feeding larvae encounter. Many studies showed numerous insecticides, herbicides, and fungicides on wild flowers which can act synergistically and increase toxicity. Second, it is important to ask whether neonicotinoid exposure could amplify the negative fitness consequences of other environmental stressors, such as food limitation, thermal stress, or parasite infection. For example, infection by the protozoan *O. elektroscirrha* is known to significantly reduce monarch survival, body size, and flight performance (Bradley and Altizer, 2005), which can lead to higher mortality of monarchs during migration (Bartel et al. 2011). It is possible that neonicotinoids could intensify negative effects of infection for monarch flight speed and duration, and that monarch orientation during flight could be affected by neonicotinoids (Wilcox et al. 2021). Our study also reared monarchs under low density, with ample food and ideal temperatures during development. An experiment that compares these effects in the field, under cases of food limitation or other sub-optimal conditions, would be important in addressing whether these effects hold up across a range of environmental circumstances. It is also important to investigate potential host plant effects on the

decay rate of neonicotinoids in the field, to explore mechanisms that underlie patterns observed in this study.

Author Contributions

CP, PB, VK, AD and SA conceived the ideas and designed methodology; CP collected the data with input from PB and AD; CP analyzed the data; CP and SA led the writing of the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

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Table 2.1. Results of general linear models investigating predictors of monarch development, survival and flight in Experiment 1. Monarch lineage was included as a random effect in all models. Neonicotinoid treatment was included as a fixed factor with 5 levels as described in the Methods text. Significant variables appear in bold.

Response Variable	Predictors	Mean Sq	DF	F Value	P Value
Pupal mass	Neonicotinoid treatment	3.56e-02	4	1.59	0.180
	Sex	**1.99e-01**	**2**	**8.93**	**<0.001**
Larval growth rate	Neonicotinoid treatment	3.21e-04	4	2.05	0.089
	Sex	**1.06e-03**	**2**	**6.8**	**0.002**
Adult mass	Neonicotinoid treatment	2.30e-03	4	0.21	0.934
	Sex	1.42e-07	1	0.00	0.991
Distance flown	Neonicotinoid treatment	6.01e-01	4	1.08	0.372
	Sex	**2.40e+00**	**1**	**4.31**	**0.040**
	Pre-flight weight	2.1e-04	1	0.00	0.984
	Wire weight	1.91e-01	1	0.34	0.560
	Age at flight (days)	1.90e-02	1	0.03	0.854
Flight duration	Neonicotinoid treatment	6.48e-01	4	1.40	0.240
	Sex	**1.83e+00**	**1**	**3.93**	**0.0498**
	Pre flight weight	5.09e-02	1	0.11	0.741
	Weight of wire	2.53e-01	1	0.54	0.462
	Age at flight (days)	7.01e-02	1	0.15	0.699
Flight speed	Neonicotinoid treatment	4.84e-02	4	1.74	0.145
	Sex	1.88e-02	1	0.68	0.413
	Pre flight weight	6.19e-02	1	2.23	0.138
	Weight of wire	3.66e-04	1	0.01	0.909
	Age at flight (days)	1.03e-01	1	3.73	0.056

Table 2.2. Results of general linear models investigating predictors of monarch development, survival and flight in Experiment 2, for the case of all 3 milkweed species (swamp, common and tropical) and 5 insecticide treatments (Table S2). Monarch lineage was included as a random effect in all models. Neonicotinoid treatment was included as a fixed factor with 3 levels as described in the Methods text. Survival to eclosion was treated as a binomial variable (binomal errors, logit link); all other variables were treated as normally distributed. Significant variables appear in bold. † indicates deviance values for variables analyzed using binomial error structures.

Response Variable	Predictors	Mean Sq	DF	F Value	P Value
Larval growth rate	Neonicotinoid treatment	6.30e-04	4	1.24	.294
	Milkweed species	**1.95e-03**	**2**	**7.69**	**<0.001**
	Block	3.30e-04	1	2.61	0.133
	Treatment:MWSpecies	2.53e-03	6	1.99	0.680
Pupal deformity (0-3)	**Neonicotinoid treatment**	**1.51e+01**	**4**	**39.59**	**<0.001**
	Milkweed species	**2.98e+00**	**2**	**7.82**	**<0.001**
	Genetic lineage	5.25e-01	4	1.38	0.242
	Block	2.58e-01	1	0.68	0.411
	Treatment:MWSpecies	**4.55e+00**	**6**	**11.95**	**<0.001**
Grip strength	Neonicotinoid treatment	4.83e-02	4	2.29	0.062
	Milkweed species	8.38e-03	2	0.4	0.673
	Sex	**3.65e-01**	**1**	**17.27**	**<0.001**
	Block	2.23e-03	1	0.11	0.770
	Treatment:MWSpecies	3.14e-02	6	1.49	0.185
Wing area	**Neonicotinoid treatment**	**9.15e+03**	**4**	**2.71**	**0.032**
	Milkweed species	**2.26e+04**	**2**	**6.69**	**0.002**
	Sex	**2.22e+04**	**1**	**6.57**	**0.011**
	Block	4.21e+03	1	1.25	0.281
	Treatment:MWSpecies	3.99e+03	6	1.18	0.318
Proportion eclosed	**Neonicotinoid treatment**	**NA**	**4**	**20.22†**	**<0.001**
	Milkweed species	NA	2	4.03†	1.335
	Genetic lineage	**NA**	**4**	**16.93†**	**0.002**
	Block	NA	1	0.39†	0.534
	Treatment:MWSpecies	**NA**	**6**	**14.05†**	**0.029**

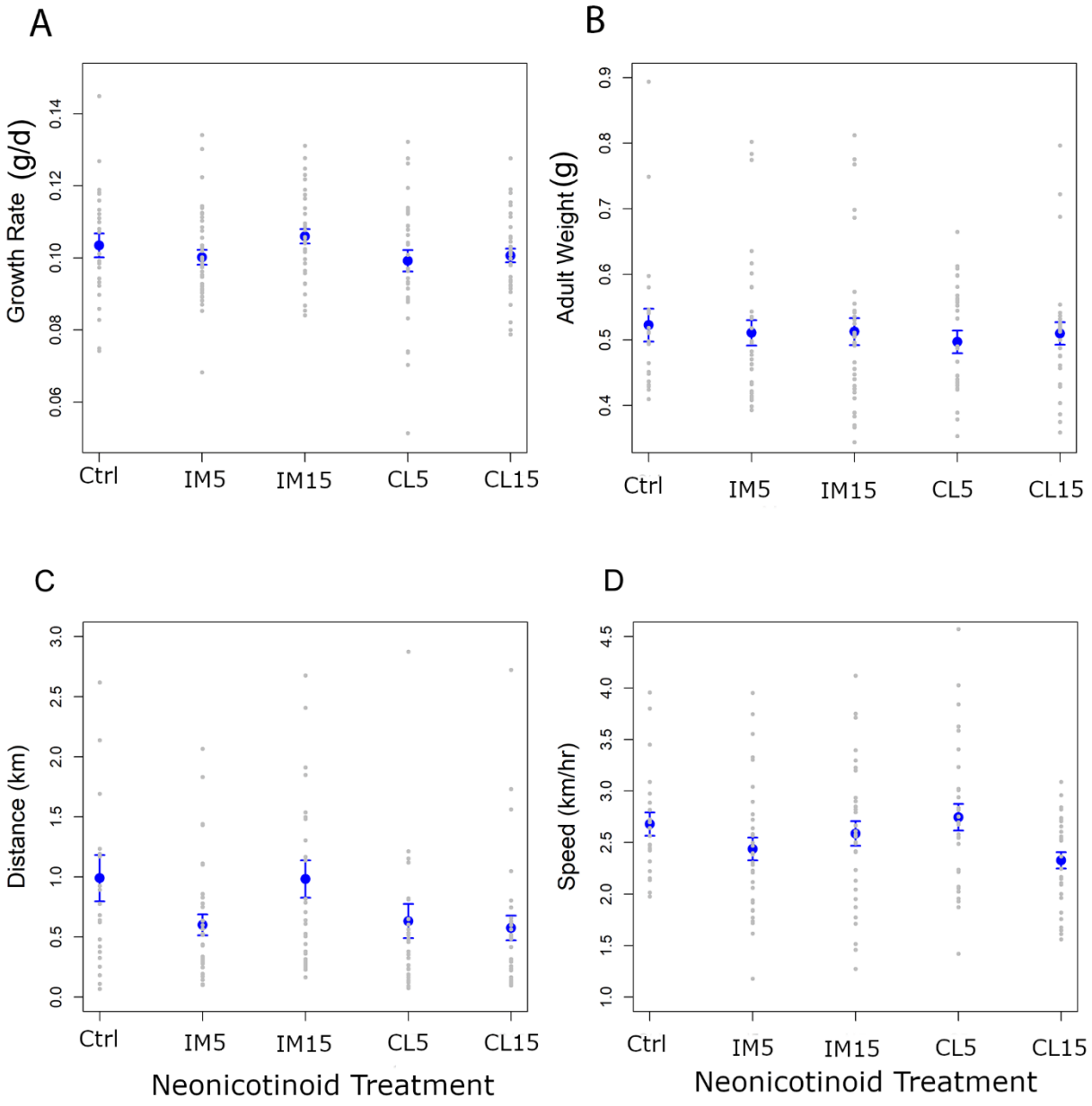

Figure 2.1: Monarch response variables shown for each neonicotinoid dose and type in Experiment 1, based on doses commonly found in field settings. (A) Growth rate (pupal weight / development time) for each neonicotinoid treatment, with I corresponding to imidacloprid and C to clothianidin; 5 and 15 represent the dose in ppb. (B) Weight of adult monarchs (g). Grey dots represent each individual monarch. Effects of neonicotinoids on monarch flight ability. (C) Distance in km travelled by monarchs with a minimum flight duration of 3 minutes (flights were terminated after a maximum of 1 hr). (D) Monarch flight speed, measured as total distance in km over flight duration in hr.

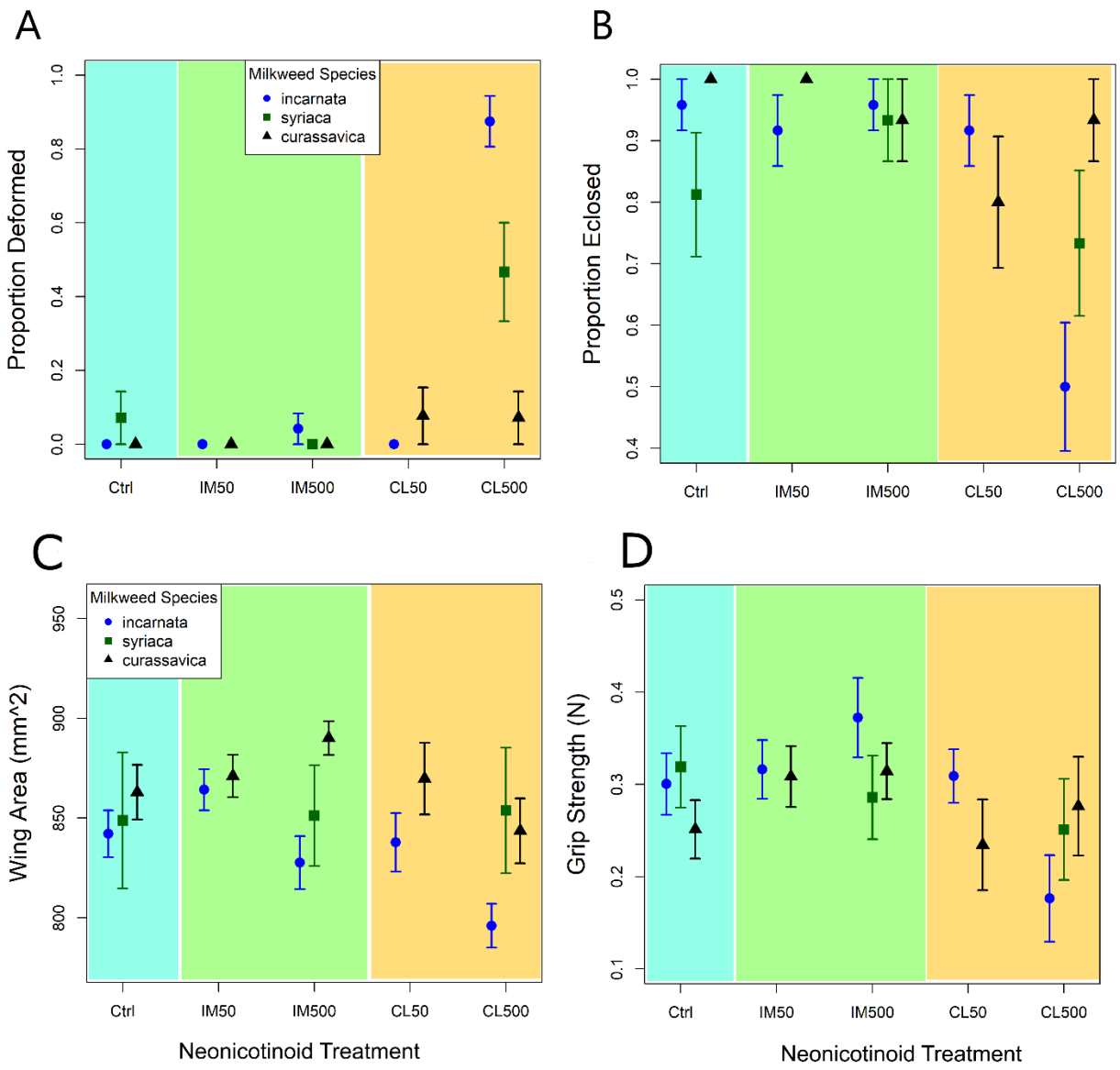

Figure 2.2: Effects of neonicotinoids and milkweed species on pre-adult development and survival. (A) The proportion of monarchs that showed any deformities. IM corresponds to imidacloprid and CL to clothianidin. Swamp milkweed (A. incarnata) is in blue circles, tropical (A. curassavica) is in black triangles, and common milkweed is in green squares. (B) The proportion of monarchs that eclosed successfully. (C) Wing area of monarchs in mm^2 for all treatments. (D) Grip strength as measured by testing monarchs' ability to pull a rod, in Newtons.

CHAPTER 3

ADULT MONARCH BUTTERFLIES SHOW HIGH TOLERANCE TO NEONICOTINOID INSECTICIDES CONSUMED IN NECTAR[2]

[2] Prouty, C.P., L. Bartlett, V. Krischik, and S. Altizer. To be submitted to *Ecological Entomology*.

Abstract

Neonicotinoids are the most widely used insecticides in North America. Numerous research papers have documented the negative effects of neonicotinoids on bees, but it remains crucial to examine how neonicotinoids affect other non-target insects, such as the monarch butterfly (*Danaus plexippus*). Wildflowers growing near agricultural areas can be contaminated with neonicotinoids that could harm nectar-feeding insects following ingestion. A handful of studies to date show mixed results for the lethal dose of neonicotinoids for monarchs. Here we examine how two neonicotinoids (imidacloprid and clothianidin) affect adult monarch fitness, flight, and behavior. We first fed adult monarchs field-relevant low doses (25, 50, 100, and 500 ppb) of imidacloprid and clothianidin in an artificial nectar solution and found no significant reductions in monarch survival, weight change, or activity levels, but weak negative effects on monarch reproduction. We next fed monarchs higher clothianidin doses (1,000 and 5,000 ppb), exceeding what they would experience in natural settings. These higher doses reduced nectar consumption, survival, flight performance, and response times. Results show that adult monarchs tolerate low-to-moderate neonicotinoid doses, with little evidence for lethal and sub-lethal effects for concentrations reported in the literature for contamination of nectar and pollen.

Introduction

Monarch butterflies (*Danus plexippus*) are charismatic and iconic insects, with scientific interest in their biology stemming from their long-distance yearly migrations (e.g., Malcolm 1994; Zhan et al. 2014; Brower 1996; Oberhauser et al. 2015; Gustaffson et al. 2015). Declines in winter colonies of North American monarchs have caused concern for the persistence of their migration (Brower et al. 2012; Shultz et al. 2017). While some evidence has suggested these declines stem from issues related to habitat loss during summer (Semmens et al. 2016; Thogmartin et al. 2017; Vidal and Rendon-Salinas 2014; Flockhart et al. 2015; Pleasants and Oberhauser 2013), other research points to problems faced during the fall migration (Ries et al. 2015; Inamine et al. 2016; Saunders et al. 2019). In particular, insecticides such as neonicotinoids have been suggested as a potential cause of decreased monarch migration success (Tracy et al. 2019; Stenoin et al. 2018). Some studies further cited the increasing use of neonicotinoids as a correlational factor with declines in eastern North American monarchs, (Thogmartin et al. 2017; Stenoien et al. 2018) and other work showed that both pesticide use and habitat loss predicted western monarch declines (Crone et al. 2019).

Neonicotinoids are the most widely used class of insecticide in the world, with high use throughout North America on row crops, orchards, vegetables, and ornamental plants (Bonmatin et al. 2015; van der Sluijs et al. 2013). Since neonicotinoids are systemic insecticides and are applied through seed treatment, soil drenching and foliar application, they can be incorporated into plant tissue through uptake in the roots and expressed persistently in leaves and flowers. The compounds persist in the environment for many months, with half-lives from hundreds to thousands of days in the absence of exposure to UV light (Mohapatra et al. 2019). This can lead to neonicotinoids occurring in the pollen and nectar of plants used by beneficial insects. The

concentrations of neonicotinoids found in nectar vary widely from plant to plant, but the nectar of wildflowers in field margins have been shown to contain neonicotinoids, and 97% of neonicotinoids brought back to honey bee hives were from wildflowers, not crops, with the highest concentrations of neonicotinoids (86.02 ppb) in pollen (Botias et al. 2015). In an experimental setting, the nectar of swamp milkweed has been shown to contain up to 1,000 ppb of neonicotinoids as late as 6 weeks after spray and drench exposure at label rates (Cowles and Eitzer, 2017).

Although monarch caterpillars have evolved to incorporate toxic cardenolides (plant secondary metabolites) via consumption of their milkweed host plants (Malcolm 1994), adult monarchs feed on the nectar of wildflowers (generally containing sugars and water, with traces of proteins and salts). During their fall migration, monarchs can be seen nectaring in large numbers near agricultural fields, roadsides, gardens and other habitats, on plants such as goldenrod and aster, and in fields of clover and alfalfa (Brower et al. 2006). Nectar resources acquired during the fall migration are crucial for building lipid reserves that monarchs use throughout their overwintering period (Alonso-Mejia et al. 1996). The conservation of nectar plants along migratory flyways is widely recognized as being important for sustaining the monarch's annual migration (Saunders et al. 2014),and factors that lower nectar abundance or that lead to the presence of toxins in nectar could lower monarch migration success.

Neonicotinoids bind to central nervous system receptors in insects, causing paralysis and death (Simon-Delso et al. 2015), and are highly effective against many sucking, leaf chewing, and soil insects (Gervais et al. 2010). The negative fitness effects of neonicotinoid insecticide ingestion by honey- and bumblebees has been well documented. At low concentrations (<50 ppb, readily found in field samples), bees suffer from foraging performance, such as efficiency,

motivation, and nutritional status (Azpiazu et al. 2019, Feltham et al. 2014, Lamsa et al. 2018, Morfin et al 2019, Phelps et al. 2020, Scholer and Krischik 2014, Stanley and Raine 2016). There is also evidence for low dose effects on social behavior, reproduction, navigation, and flight performance (Bryden et al. 2013, Crall et al. 2018, Fischer et al. 2014, Laycock et al. 2012, Scholer and Krischik 2014, Switzer and Combes 2016, Tosi et al. 2017, Whitehorn et al. 2017). More work is needed to examine neonicotinoid effects on other non-target insects such as butterflies. In recent years, a handful of studies tested neonicotinoid effects the larval stage of butterflies, where ingestion occurs via insecticide in host plant material (e.g. Krishnan et al. 2020, Olaya-Arenas et al. 2020, summarized in Prouty et al. 2021). A much smaller number of studies tested consumption of neonicotinoids as adults, with mixed results. One study on adult monarchs showed negative effects on survival, but not reproduction, at field relevant concentrations (23 ppb imidacloprid) fed *ad libitum* to butterflies held in outdoor cages over 22 days (James, 2019). Another study showed no reductions in survival for both adult monarchs and painted ladies (*Vanessa cardui*) at comparable concentrations (Krischik et al. 2015). Additionally, Krishnan et al. (2021) found no effects of clothianidin, imidacloprid or thiamethoxam up to 330 µg/L (ppm) on adult monarch consumption. Differences in findings between studies might be attributed to the percentage of sugar in the nectar solutions, butterfly sources and genetic background, and the source of insecticides used.

In the present study, we examined how neonicotinoid consumption by adult monarchs influences survival, behavior, reproduction, and flight performance. We first exposed monarchs to a range of concentrations of imidacloprid and clothianidin (0, 25, 50, 100, or 500 ppb). After finding no effect of neonicotinoids at these doses on monarch survival, and weak effects on reproduction, we conducted a second experiment with higher concentrations of clothianidin

(1000 and 5000 ppb), and observed monarch survival and flight performance. Following work by Krischik et al. (2015), we predicted that adult monarchs would better tolerate neonicotinoid exposure relative to bees. We further predicted that monarch reproduction would be reduced by high neonicotinoid doses, as shown in bees (e.g. Laycock et al. 2012), as mating and egg-laying require energetically expensive complex behaviors (e.g., Brower et al. 2007; Oberhauser et al. 1989). We also predicted that monarch flight speed and distance would be unaffected by low neonicotinoid doses, but reduced following high-dose neonicotinoid exposure (following work on bees, e.g., Tosi et al. 2017).

Methods

Neonicotinoid and Monarch Sources

Neonicotinoids were obtained in powder forms of clothianidin and imidacloprid from Sigma-Aldrich in 2018. One mg of each neonicotinoid was dissolved separately into 0.5 L of distilled water to achieve two 2 ppm (mg/L) stock solutions for experiment 1 and 5 mg were dissolved into 0.5 L to achieve a 10 ppm (mg/L) stock solution for experiment 2, which was further diluted with a distilled water and 20% honey solution to concentrations of 25, 50, 100 and 500 ppb (ng/mL) for experiment 1 and 1,000 and 5,000 ppb (ng/mL) for experiment 2. We measured multiple dilutions throughout each experiment from a stock solution. Stock solutions were held at 4 C for up to 7 days per experiment, and original (solid) chemicals were held at 22°C for up to 18 months. Liquid aliquots (6-10ml) from experimental nectar solutions were sent to the USDA Agricultural Marketing Service, laboratory approval and testing division in Gastonia, NC to

estimate the actual concentration of solutions using HPLC-GC. Limits of detection were: clothianidin, 3 ppb and imidacloprid, 2 ppb.

We used captive-reared monarchs from 5 outcrossed genetic lineages that were the descendants of ~100 wild-caught fall migrants from Athens, GA and St. Marks, FL, USA in Oct 2018 (Experiment 1). Adult monarchs were mated in 0.6 m^3 mesh cages and fed *ad libitum* with a 20% honey water solution. Mated females oviposited onto *A. incarnata* cuttings, and larvae remained on natal stalks until second instar. We reared monarch caterpillars individually in 0.6 L plastic containers with mesh screen lids on milkweed cuttings. Milkweed (*Asclepias incarnata*) used as larval food plants were raised from seeds obtained from Prairie Moon nursery and planted into 12.5 cm diameter pots in February 2019. Plants were cut back several times prior to the study and received bi-monthly Ozmocote fertilizer. The temperature range fluctuated between 15°C and 35°C in the climate-controlled greenhouse. We held larval monarchs between 25-28°C and exposed them to incident light in a windowed room. Containers were arranged on shelves and monitored twice daily for deaths, pupation, and eclosion. When adults emerged, we recorded monarch sex, and weighed monarchs to the nearest .001g 24 hours post-eclosion. We also checked for infection by the protozoan *Ophryocystis elektroscirrha* (which can negatively affect flight and survival) following methods described in Altizer et al. (2000).

For experiment 2, wild migratory adult monarchs were caught in Athens, Georgia in October and November 2019. Monarchs that tested positive for *O. elektroscirrha* were excluded from further study. Monarchs were kept in an incubator to simulate day length and temperatures at the Mexico overwintering sites to maintain reproductive diapause and simulate overwintering. They were fed a 20% honey water solution every 11 – 12 days for 4 months. Of the 100 captive monarchs, 40 individuals (20 males and 20 females) were randomly selected for neonicotinoid

exposure and flight trials. We used an additional 10 captive-reared monarchs (reared under conditions described above) to ask whether migrants and captive reared monarchs respond differently to neonicotinoids.

Experiment 1: Lab-Reared Monarchs and Sub-lethal Effects

To test the effect of neonicotinoid consumption in nectar on adult monarchs, we fed adults every other day for 10 days, using 20% honey water solutions mixed with the neonicotinoid treatment (0, 25, 50, 100, or 500 ppb of either clothianidin or imidacloprid). We used two control groups: one each for clothianidin 0 ppb and imidacloprid 0 ppb using distilled water in place of the neonicotinoid stock solution. Feeding was accomplished by restraining monarchs with steel nuts on plexiglass feeding trays (Figure S1) for 10 mins per group. We weighed monarchs to the nearest .001g before and after each feeding to determine volume ingested. We used separate trays for each dose-by-neonicotinoid type treatment to minimize cross-contamination, and sanitized trays by exposure to artificial UV light for 1hr at the end of each day followed by soaking overnight in 20% bleach solution. Controls (honey-water only) were fed in an adjacent room on separate trays. All adults were stored in individual glassine envelopes at 23°C (14hr daylength) in the same incubator between feedings. Nectar solutions were stored at 4°C for up to three days between use. Each treatment group initially contained 36 monarchs, for a total of 360 monarchs fed with neonicotinoid treatments.

Following the fifth and final feeding, we randomly assigned butterflies from each treatment group, including controls, to 0.6m cubed indoor mesh cages. We used a total of 16 cages with approximately 20 butterflies per cage. Butterflies were numbered using ultrafine

permanent marker on the discal cell of the hindwing, and were fed *ad libitum* using untreated 20% honey-water solution on small dish sponges. Cages were randomly distributed across the room (Figure S2). Twice per day, at 9:00 am and 4:00 pm, mating pairs and deaths were recorded. Each cage was also observed for a total of eight 10-min intervals (times dispersed throughout the day between 9am-4pm) to record the approximate duration of time that each monarch spent actively flying, mating, and feeding (as opposed to resting). After 5 days, all butterflies were removed from the cages and weighed immediately following removal.

To measure effects on reproduction, we placed 4 mated females per treatment (n=40) into individual oviposition cages for three days with a stalk of milkweed and 20% honey water on sponges provided *ad libitum*. The total number of eggs laid per female at the end of the 3 day interval was recorded, and the proportional hatching success of those eggs was quantified to the nearest 10%. All other butterflies (males and unselected females) were placed into an incubator at 12°C and held to record the time (in days) to death.

Experiment 1 Analyses

Analyses for both experiments were performed in R version 3.5.3. We first used general linear mixed models with normal error structures to test for relationships between neonicotinoid exposure and monarch weight. We analyzed monarch average and total nectar consumption throughout feedings (post- minus pre-feeding weight), weight before and after entering the flight cages, and the difference in weight between eclosion and exiting the mating cages, using the following model structures in the lme4 package (Bates et al., 2015): [response variable = insecticide type * concentration + sex + genetic lineage (random effect) + weight at eclosion

(only for consumption variables)]. The number of eggs laid by the subset of females, the number of times male monarchs mated, the total number of days monarchs survived, and the success of egg hatching were analyzed using generalized linear models with Poisson error distributions and the same model above, but with eclosion weight included as a continuous covariate. Residuals were checked for normality and equal variances.

Principal component analysis was used to reduce the behavioral observations (time spent flying, mating, or feeding) into a single response variable. 36.89% of the variation in the data was explained by PC-1. Variable loadings for each of the constituent components were: 0.369 for feeding, 0.332 for flying, and 0.299 for mating. We then examined how the first principal component (PC-1) depended on insecticide treatments (type * concentration) and monarch sex.

Experiment 2: Wild Monarchs and High-Dose Effects

We exposed 40 wild-caught fall migrant monarchs that overwintered in the lab, and 10 lab-reared monarchs, to high concentrations of neonicotinoids to test effects on weight gain, flight performance and survival. Using the same feeding method as in the previous experiment, we fed monarchs 20% honey-water solutions of either 1,000 or 5,000 ppb of clothianidin, with a control (0 ppb). Monarchs were fed every second day for 10 days. We weighed monarchs before and after each feeding to the nearest .001g. Between feedings, monarchs were held in an incubator set at 16-hour daylength and 23°C.

After the fifth and final feeding, we flew monarchs on a near-frictionless tethered flight mill to measure flight speed and distance (Figure S3). Flight trials were conducted indoors during March 2020 in a 9 m^2 room at 29.7°C (range 27.8-31.4°C) between 1000 and 1730 h. On

the last feeding day, we glued lightweight steel wires to the dorsal thorax of each monarch using rubber cement, following Bradley and Altizer (2005). As per Schroeder et al. (2019), the average mass of the wire attachment was 0.19 g (range 0.10-0.33 g). Monarchs were placed into 0.6 m^3 mesh cages to adjust to the weight of the wire for 12-24hr, with 20% honey water provided *ad libitum*. The flight mill was constructed as described in Bradley and Altizer (2005) and Fritzsche McKay et al. (2016) from a 120 cm lightweight carbon rod with a diameter of 3 mm (4.23 m circumference) attached to a nearly frictionless steel pivot (Figure S3). We tethered monarchs to one end of the horizontal rod, and a flag at the opposite end passed through an infrared beam on a photo-gate to estimate flight velocity per revolution (m/s; software PASCO Capstone). Windows were covered with paper to prevent monarchs from responding to sun angle cues during flight, and we set four floor lamps to provide an even distribution of light.

Monarchs were flown for a maximum of one hour. If monarchs stopped flying for more than five seconds, they were agitated with a gust of air for up to three times. If the monarch did not resume flight after three agitations, the flight was terminated. Monarchs were returned to mesh cages after flight. For each flight, we calculated distance (km) flown and total time in flight (hr). We calculated flight velocity by dividing the circumference of the flight path by the time to completion of a revolution. Average velocity across the entire trial was calculated as the mean of all velocities per revolution.

Roughly 2hrs after monarchs were flown, we performed a drop test to quantify the reaction time of each butterfly. Monarchs were grasped with 2 fingers (holding all 4 wings together close to the thorax) and dropped from chest height to the floor. The time in sec taken to open their wings was measured by stopwatch. If monarchs did not open their wings before

landing on the ground, drop time was recorded as one full second, as monarchs that successfully opened their wings did not exceed 0.8 seconds.

Experiment 2 Analyses

We analyzed average and total nectar consumption, and flight speed and distance using GLMs with normal error structures, with clothianidin dose (0, 1 or 5 ppm), monarch sex, and monarch source (wild or reared) as predictor variables. We used GLMs with Poisson error structure to analyze adult survival in days. Model residuals were checked for normality and equal variances. For flight variables, we included weight before first feeding and weight change between first and last feeding as continuous covariates.

Wild Caught Bee Neonicotinoid Bioassay

To confirm toxicity of the neonicotinoid doses used in our monarch study, wild individuals of *Bombus impatiens* were caught while foraging on flowers on the University of Georgia campus. Bees were exposed to the same solutions used Experiment 1 (0, 25, 50, 100, 500 ppb), mixed into a 20% honey water solution, within one hour of their capture. Bees were placed into clear acrylic aquaria (15x10x10cm) with mesh lids. We added sponges soaked in the honey water solution to petri dish lids. We placed 2-4 bees into each container, with 1 container for each treatment. Containers were checked every 20-60 minutes over a 5 hr period. Bee activity was recorded as flying, crawling, standing, twitching (while lying on side), or dead (Table S1). Bees were frozen at 20 °C after observations were concluded.

Results

Aliquot neonicotinoid assays

HPLC analyses of aliquots in experiment 1 showed that all samples were close to the intended concentration, with the lowest being 54% of the intended concentration. One aliquot was 6.2% higher than the intended concentration (Table S1). In experiment 2, liquid aliquots were within 80-90% of the intended dosage at 909 ppb instead of 1,000 ppb and 4,030 ppb instead of 5,000 ppb (Table S1).

Experiment 1: Lab-Reared Monarchs and Sub-Lethal Effects

A total of 319 (88.6%) of the 360 monarchs at the start of the experiment survived to the adult stage. The vast majority (97.8%) of the monarchs that survived to the adult stage survived the feeding treatment. The 7 monarchs that died were distributed across most of the feeding treatments, including controls. The proportional change in monarch mass over the course of the experiment ((final – initial)/ initial) did not differ among insecticide treatment groups (Figure 1A, Table 1). Weight change depended on monarch sex, with females gaining proportionately more weight than males.

The number of times monarchs mated significantly decreased with higher neonicotinoid concentrations ($X^2 = 8.69$, DF = 1, $p < 0.003$). No other variable predicted variation in mating, including the interaction between concentration and neonicotinoid type. *Post hoc* analyses showed that the change in number of times monarchs mated across insecticide treatments was driven primarily by males, for which neonicotinoids reduced the number of matings (Figure 1B, Table 1). The number of eggs females laid decreased with higher imidacloprid, but not

clothianidin, concentrations (Figure 1C). This interaction between neonicotinoid type and concentration was significant (Table 1). The hatching success of these eggs did not appear to differ in response to neonicotinoid type or concentration (Table 1). Amount of time monarchs spent mating, flying, and feeding as summarized in PC-1, showed no relationship with neonicotinoid treatment (Table 1; Figure S1).

Experiment 2: Wild Monarchs and High Dose Effects

A total of 46 of the 67 monarchs (68.7%) at the start of the experiment survived the 10-day feeding period. Monarchs treated with higher concentrations of clothianidin had significantly reduced survival (Figure 2; Table 2). The average amount of nectar monarchs consumed was significantly dependent on neonicotinoid treatment, sex, and the weight before exposure (Figure 3A, Table 2). Monarchs fed higher clothianidin doses lost more weight than control monarchs, males lost more weight than females, and monarchs that weighed more prior to feeding lost more weight during the 10-day feeding period. Before feeding, adult monarch mass did not differ significantly between treatment groups ($F_{1,62} = 0.045$; $p = 0.8327$).

Flight data were excluded from 2 controls and 9 treatment monarchs that did not fly for at least 2 mins. Across all treatments (n=35), monarchs flew for an average distance of 1.77 km ± 0.22 SE at a speed of 2.95 km/hr ± 0.15 SE. Monarchs treated with higher concentrations of clothianidin flew for significantly shorter distances at significantly slower speeds (Figure 3C,D, Table 2), with no effect from any variable other than concentration. The drop test showed that monarchs treated with higher concentrations of clothianidin also had slower reaction times (were more delayed in opening their wings; Figure 3B, Table 2).

Bumblebee bioassay

After 4 hours, all four *B. impatiens* in the 0 ppb (honey water only) treatment remained actively flying, and had to be chilled at 14° C prior to removal. For imidacloprid, the 25 ppb bees were immobile by 4 hr but still alive; moving the container did not cause flight. At 50 and 100 ppb imidacloprid, bees were also lethargic, and we were unable to induce flight after 4 hours. At the 500 ppb imidacloprid dose, all bees died within 2 hours of exposure. For clothianidin, all bees from both the 25 and 50 ppb treatments were lethargic and moving slower than controls, and unable to fly by 4 hr. For the 100 ppb dose of clothianidin, 1 of 2 bees were dead by 4 hrs, and the other was slow and lethargic. For the 500 ppb treatment, all bees died after 2 hours. Collectively, these findings demonstrate the lethality of the neonicotinoid solutions prepared for this study towards bees.

Discussion

The impact of neonicotinoid insecticides on monarch butterflies, both in their breeding range and during the long-distance fall migration, has been raised as a concern for the future persistence of migratory populations (e.g. Tracy et al. 2019; Stenoin et al. 2018; Thogmartin et al. 2017; Lu et al. 2020). Results here show that adult monarchs tolerate neonicotinoid doses within the range of concentrations reported in the literature for field contamination of nectar and pollen. Higher doses (500 ppb) negatively affected monarch reproduction, reducing male mating success and female oviposition. The same solutions used in Experiment 1 (50-500 ppb) dramatically reduced bumble bee survival within a period of <4 hours. Extremely high doses (1,000+ ppb) negatively affected monarch survival, reaction time, and flight.

To our knowledge, this is the first experiment to show negative reproductive effects on butterflies as a result of adult-stage exposure to neonicotinoids. In particular, female monarchs showed reduced fecundity at moderately high concentrations of imidacloprid. Bees and butterflies have major differences in their life histories and behavior, but some similarities may exist in their reproductive responses to neonicotinoids. Bumblebees were shown to have reduced fecundity following low dose exposure to imidacloprid, and at higher concentrations, bees' ovaries did not develop at all (Laycock et al. 2012). Authors speculate that this finding was likely due to the bees' inability to successfully feed following exposure to imidacloprid. Monarchs were not shown to have reduced weight change throughout the experiment, so it is unlikely that reduced fecundity in monarchs was a result of underfeeding. Male monarchs that were fed moderately high (500 ppb) concentrations of clothianidin and imidacloprid were shown to mate with females less. Reductions in male mating success and female oviposition activity could result from reduced neurological functions from the treatment, as these behaviors are complex and energetically demanding (Oberhauser and Frey 1999, Oberhauser 1989, Solensky 2004).

Because North American monarchs undertake an annual long-distance migration of up to 5000km, even small reductions in activity levels and flight performance could lower migratory success. In experiment 2, we showed that extreme exposure (1,000 and 5,000 ppb) to clothianidin lowers monarch flight performance (distance and speed) and reaction time. This experiment was conducted on wild monarchs captured during their migration that were stored in an incubator for several months prior to exposure to clothianidin. Wild monarchs were also compared to lab-reared monarchs, which showed similar trends. Importantly, exposure to lower doses did not appear to reduce monarch feeding or flight activity, and the extremely high doses that reduced flight in this study are unlikely to be encountered by monarchs in the wild.

Past studies of adult monarch consumption of neonicotinoids yielded contrasting results. The first, Krischik et al. (2015), found no reductions in survival or fecundity in monarch and painted lady butterflies following treatment with imidacloprid up to 30 ppb. A second study(James 2019) did find evidence for significant reductions in adult monarch survival at 23.5 ppb of imidacloprid mixed into 5% sugar water (fed ad libitum for up to 22 days), with no effect on oogenesis. Differences in findings between these two studies could be explained in the percentage of sugar water used for feeding (30% in Krischik et al. vs. 5% in James), the source of imidacloprid (pure 99% crystalline in Krischik et al. vs. store-bought mixture of 0.235% imidacloprid with adjuvant ingredients in James), and the conditions of exposure (indoor enclosures vs outdoor cages). Multiple replicates and more robust sample sizes and monarch sources were used in Krischik et al. (2015). Our methods were similar to Krischik et al. (2015). Pure (99%) imidacloprid and clothianidin were used, and a 20% honey water solution was used for feeding monarchs. It is possible that store-bought insecticide mixtures have other ingredients that interact with neonicotinoids and cause more detrimental effects. Additionally, food limitation could cause synergistic effects, such that monarchs with poorer body condition can better tolerate neonicotinoid exposure, but those treated with an insecticide and caloric restriction might be less able to tolerate toxins.

There are contexts under which monarchs and other pollinators could become exposed to higher doses than field margins. Cowles and Eitzer (2017) found that milkweed treated with neonicotinoids products commonly bought by gardeners can lead to doses up to 1,000 ppb in nectar. If gardeners use these products to deter pest insects, monarchs and other pollinators could be exposed to high doses via this route. Given reductions in bee mobility following insecticide exposure (e.g. Wood and Goulson 2017, Switzer and Combes 2016), and the known presence of

neonicotinoids along agricultural field margins (e.g. Hladik et al. 2016, Mogren and Lundgren 2016, Krupke et al. 2012), it seems plausible that longer-term pesticide exposure at both larval and adult stages could lower monarch flight performance during the fall migration.

Findings here point to several areas for further investigation. Nectar concentration in wildflowers vary significantly, and the quality of nectar resources monarchs feed on could change dramatically depending on the land use and regions monarchs travel. Investigating the effects of nectar limitation and exposure to neonicotinoids could prove to be useful. It is important to ask whether neonicotinoid exposure could amplify the negative fitness consequences of other environmental stressors, such as food limitation, thermal stress, or parasite infection. For example, bees that were parasitized by a Varroa mite and fed neonicotinoids had lower flight performance, but only when exposed to both and not when exposed to each individually (Blanken et al. 2015). Our study also reared monarchs under low density, with ample food and ideal temperatures during development. An experiment that compares these effects in the field, under cases of food limitation or other sub-optimal conditions, would be important in addressing whether these effects hold up across a range of environmental circumstances.

Author Contributions

CP, VK, and SA conceived the ideas and designed methodology; CP collected the; CP and LB analyzed the data; CP and SA led the writing of the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

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Table 3.1: Results from analyses from experiment 1. Variables that were analyzed using normal error structures included weight post cages, proportional weight change, average nectar consumed and the first PC. Count data (egg hatching success, days survived, number of times mated, number of eggs laid) were analyzed using Poisson error structures. Full models are described in the Methods text. Rows in bold indicate significant effects.

Response Variable	Predictors	Mean Sq	DF	F Value	P Value
Weight post cages	Concentration	4.12e-02	1	1.46	0.228
	Neonicotinoid treatment	1.77e-02	1	0.63	0.429
	Sex	**7.36**	**1**	**260.99**	**<0.001**
	Concentration:neonic	8.00e-04	1	0.03	0.865
Final-initial weight	Concentration	1.76e-02	1	1.51	0.220
	Neonicotinoid treatment	1.00e-02	1	0.86	0.354
	Sex	**2.93e+00**	**1**	**251.84**	**<0.001**
	Concentration:neonic	7.51e-04	1	0.06	0.800
Average nectar consumed	Concentration	1.66e-04	1	0.14	0.713
	Neonicotinoid treatment	1.57e-03	1	1.29	0.258
	Sex	**5.21e-03**	**1**	**4.26**	**0.040**
	Weight at eclos	**1.31e-02**	**1**	**10.67**	**0.001**
	Concentration:neonic	2.64e-04	1	0.22	0.642
Egg hatching success	Concentration	NA	1	0.24	0.624
	Neonicotinoid treatment	NA	1	0.12	0.732
	Weight at eclos	NA	1	0.29	0.592
	Concentration:neonic	NA	1	0.01	0.937
Days survived post expt	Concentration	NA	1	1.63	0.202
	Neonicotinoid treatment	NA	1	0.01	0.925
	Sex	**NA**	**1**	**174.81**	**<0.001**
	Weight at eclos	**NA**	**1**	**11.35**	**<0.001**
	Concentration:neonic	NA	1	0.07	0.792
Times males mated	**Concentration**	**NA**	**1**	**6.54**	**0.011**
	Neonicotinoid treatment	NA	1	0.50	0.482
	Weight at eclos	NA	1	1.64	0.200

Response Variable	Predictors	Mean Sq	DF	F Value	P Value
	Concentration:neonic	NA	1	0.00	0.980
Number of eggs laid	**Concentration**	**NA**	**1**	**207.64**	**<0.001**
	Neonicotinoid treatment	NA	1	0.64	0.425
	Weight at eclos	**NA**	**1**	**41.44**	**<0.001**
	Concentration:neonic	**NA**	**1**	**136.18**	**<0.001**
Principal	Concentration	9.60e-01	1	0.86	0.355
Component (PC-1)	Neonicotinoid treatment	3.55e-01	1	0.32	0.573
	Sex	4.46e-02	1	0.04	0.842
	Concentration:neonic	1.38e-01	1	0.12	0.726

Table 3.2: Results from the analyses of experiment 2. All variables were analyzed using normal error structures. Full models are described in the Methods text. Rows in bold indicate significant effects.

Response Variable	Predictors	Mean Sq	DF	F Value	P Value
Average nectar consumed	**Concentration**	**2.19e-02**	**1**	**21.22**	**<0.001**
	Monarch source	2.31e-04	1	0.22	0.638
	Sex	**9.60e-03**	**1**	**9.29**	**0.003**
	Weight pre-exposure	**6.77e-03**	**1**	**6.55**	**0.013**
Proportional weight	Concentration	3.13e-02	1	2.14	0.151
change during feeding	**Monarch source**	**1.11e-01**	**1**	**7.60**	**0.009**
	Sex	**1.03e-01**	**1**	**7.05**	**0.011**
Distance flown	**Concentration**	**1.41e+01**	**1**	**11.45**	**0.002**
	Monarch source	6.73e-01	1	0.54	0.466
	Sex	9.78e-02	1	0.08	0.780
	Weight change during flight	3.32e+00	1	2.69	0.112
Average flight speed	**Concentration**	**8.63e+00**	**1**	**13.78**	**<0.001**
	Monarch source	4.62e-01	1	0.74	0.400
	Sex	4.25e-01	1	0.68	0.417
	Weight change during flight	8.16e-02	1	0.13	0.721
Drop test	**Concentration**	**2.46e+00**	**1**	**83.52**	**<0.001**
	Monarch source	5.17e-02	1	1.75	0.195
	Sex	2.08e-04	1	0.01	0.934
	Weight pre-exposure	9.14e-03	1	0.31	0.582
Survival	**Concentration**	**NA**	**1**	**5.23**	**0.022**
	Monarch source	NA	1	0.02	0.879
	Sex	NA	1	0.00	0.964
	Weight pre-exposure	**NA**	**1**	**10.38**	**0.001**

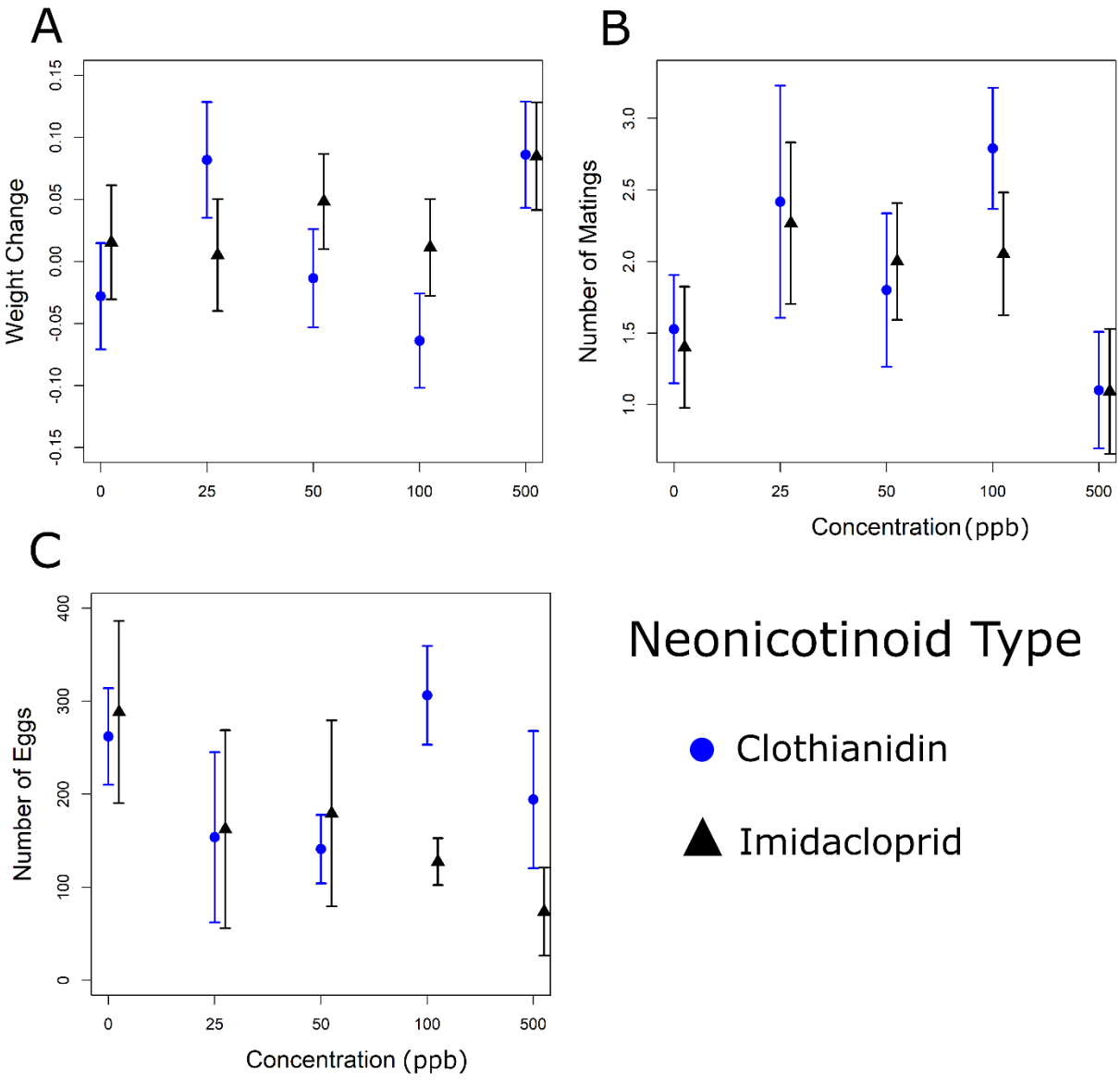

Figure 3.1: Results from experiment 1, where captive monarchs were fed sub-lethal doses of clothianidin (blue) and imidacloprid (black). Error bars represent standard error. (A) Monarch weight change from the start of the experiment to the end (weight final – weight initial / weight initial). (B) The number of times male monarchs mated over a 5-day period. (C) The number of eggs laid over a 3-day period by a subset of females, n= 4 individuals per treatment, with a total of 40 individuals.

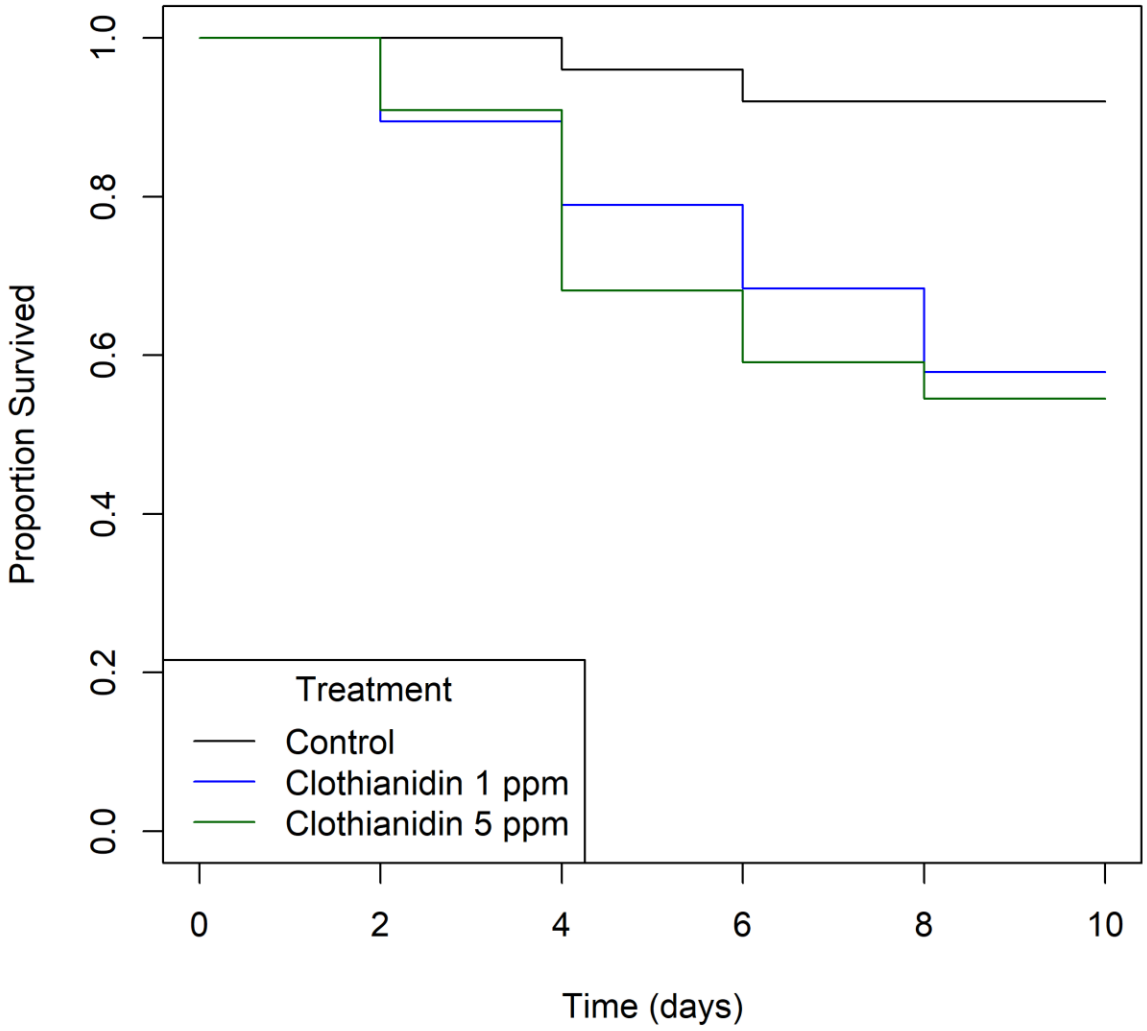

Figure 3.2: The proportion of adult monarchs that survived the high dose clothianidin feeding treatments in experiment 2. Each step indicates when monarchs were checked for survival and were found dead. Black represents the control group, blue is clothianidin 1,000 ppb, and green is clothianidin 5,000 ppb.

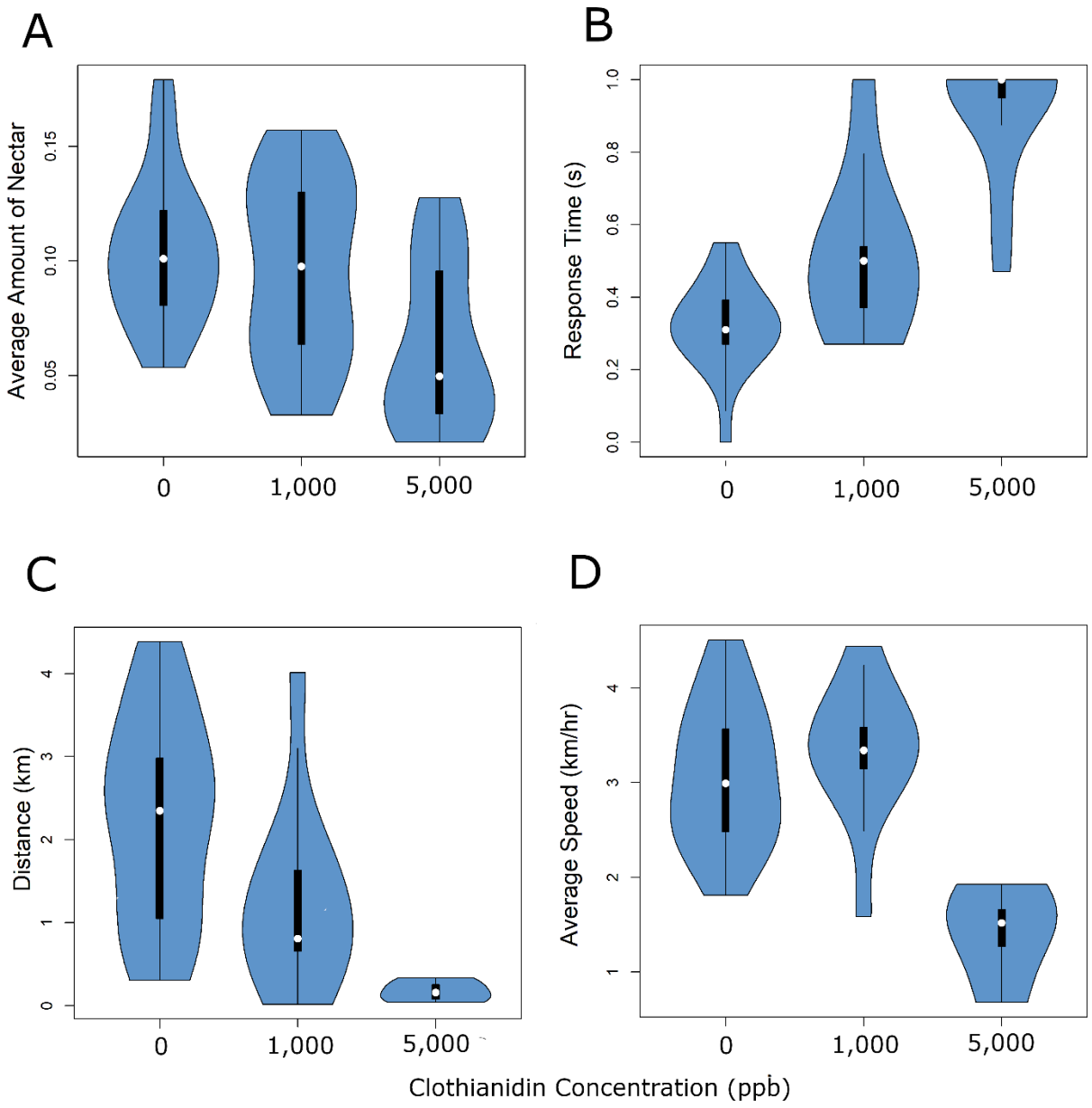

Figure 3.3: Results from experiment 2, where wild adult monarchs were fed high clothianidin doses. Violin plots show the distribution of data points (in the width of the blue shading), means and spread of data. (A) The average amount of nectar monarchs consumed throughout the experiment (g). (B) Time taken to respond to a dropping stimulus. Timer was stopped when monarchs opened their wings. (C) Distance (km) monarchs traveled during their flight. (D) Average speed (km/hr) monarchs flew during their flight testing.

CHAPTER 4

GENERAL CONCLUSIONS

Neonicotinoids have the potential to pose a threat to the survival, reproduction, and migration of many insects, including monarchs.. It is therefore important to determine the concentrations of neonicotinoids in the environment that pose negative impacts to various beneficial and charismatic insect species. Prior to this study, several studies have investigated bee performance in the face of neonicotinoid insecticides, but the implications for neonicotinoids' effects on other beneficial insects was not well understood at the sub-lethal level.

I found monarchs to have a greater tolerance to neonicotinoids than bees in their survival and sub-lethal effects. This could be explained by monarchs' ability to sequester toxins from their host plant species. In this study, we found larval monarchs that consumed milkweed species containing the least amount of toxic cardenolides responded poorly to neonicotinoids. Monarchs raised on this species of milkweed were shown to have reduced fitness and development following exposure to neonicotinoids. The concentration of clothianidin that was shown to cause negative effects was outside of what is commonly found in agricultural drift, but could be achieved in garden settings depending on timing and methods of application used. We do not recommend, however, that monarchs be preferentially reared on milkweed that contains more cardenolides in an attempt to protect them from negative effects of neonicotinoids. Consumption by monarchs of milkweeds containing higher cardenolides can lead to negative effects on fitness.

I found monarchs fed neonicotinoids at the adult stage to be much more tolerant of high concentrations. Monarchs that consume high concentrations of neonicotinoids, higher than those commonly found in nature, experience reduced survival, reproduction (in terms of the number of times males mate and the number of eggs females lay), and reduced flight performance. Lab-

reared and wild-caught monarchs were equally resilient to clothianidin and responded similarly. Monarchs that interact with lower-quality nectar resources could be at an increased risk for negative compounding effects, due to the energetic cost of maintaining their body condition, fueling reproduction, and utilizing their immune system.

Based on these experiments, declines in monarch overwintering numbers cannot be attributed to the use of commercial neonicotinoids alone. There is a need for research on monarch responses to combinations of chemicals, the interactions between insecticides and parasitism, and the interactions between insecticides and environmental stress. Additionally, monarchs in these experiments were fed *ad libitum*, kept at constant temperature, and did not receive pressures from predators or parasites. Experiments that incorporate these effects would have a clearer picture of how monarchs in the wild respond to neonicotinoids and other insecticides. If neonicotinoids are to be cited as a reason for monarch decline, I argue that they cannot be named in isolation. Monarchs face growing pressures from habitat fragmentation and degradation, disease, and population sinks from the planting of non-native milkweed. The limits of neonicotinoids use should be set using bee tolerance as the threshold, since at this point in time, they are the most sensitive beneficial insects to neonicotinoids.

APPENDICES

APPENDIX A

Table S1: Prior studies of neonicotinoid effects on monarchs following exposure at the larval stage. Studies are identified by the type of neonicotinoid that was used and the milkweed species. If a study included multiple species of milkweed or neonicotinoids it was separated into multiple rows. LD50s are listed in the units each paper represented them in, with ppb = ng/g and ppm = µg/g. Exposure method indicates the way insecticides were applied to monarchs. Overall, all studies differed in terms of their exposure method, milkweed species used, and the stage at which monarchs were exposed, which led to differences in LD50.

Author	Neonic	Milkweed	Stage Exposed	LD50	Survival	Sublethal effects	Exposure Method
Lundgren and Pecenka 2015	Clothianidin	Swamp A.incarnata	1st, 2nd instar + 36 hr	15.63 ppb (ng/g)	NA	.5 ppb +	solution on 1 cm diameter leaf discs
Krischik et al. 2015	Imidacloprid	Tropical A.curassavica	early instar until death (max 7 days)	NA	reduction at 8 ppm (µg/g) +	NA	Soil
Bargar et al. 2019	Clothianidin	Swamp A.incarnata	Newly hatched until death or pupation	47 to 205 ng/g	NA	177 ng/g +	Soil
Krishnan et al. 2020	Imidacloprid	Tropical A.curassavica	2nd, 3rd instar + 48 hrs	5.1,17 µg/g (2nd,3rd instar)	NA	0.75 µg/g (third ins.) +	Foliar
Krishnan et al. 2020	Clothianidin	Tropical A.curassavica	2nd, 3rd instar + 48 hrs	4.2,7.8 µg/g	NA	NA	Foliar
Krishnan et al. 2020	Thiamethoxam	Tropical A.curassavica	2nd, 3rd instar + 48 hrs	3.5,5.6 µg/g	NA	4.8 µg/g +	Foliar
Olay-Arenas et al. 2020	Clothianidin	Common A.syriaca	Newly hatched	N/A	Reduction at 56.55 ng/g	N/A	Foliar, leaf clippings
Wilcox et al. 2021	Clothianidin	Swamp Milkweed	From eggs to pupation	NA	NA	None up to ~10 ppb recovered	Soil

Table S2: Experimental design for monarch larval neonicotinoid study. Each treatment group included 5 genetic lineages.

Milkweed	Swamp					Tropical					Common		
Treatment	Control	Clothianidin		Imidacloprid		Ctrl	CL		IM		Ctrl	CL	IM
Conc(ppb)	0	50	500	50	500	0	50	500	50	500	0	500	500
# Monarchs	24	24	24	24	24	15	15	15	15	15	15	15	15

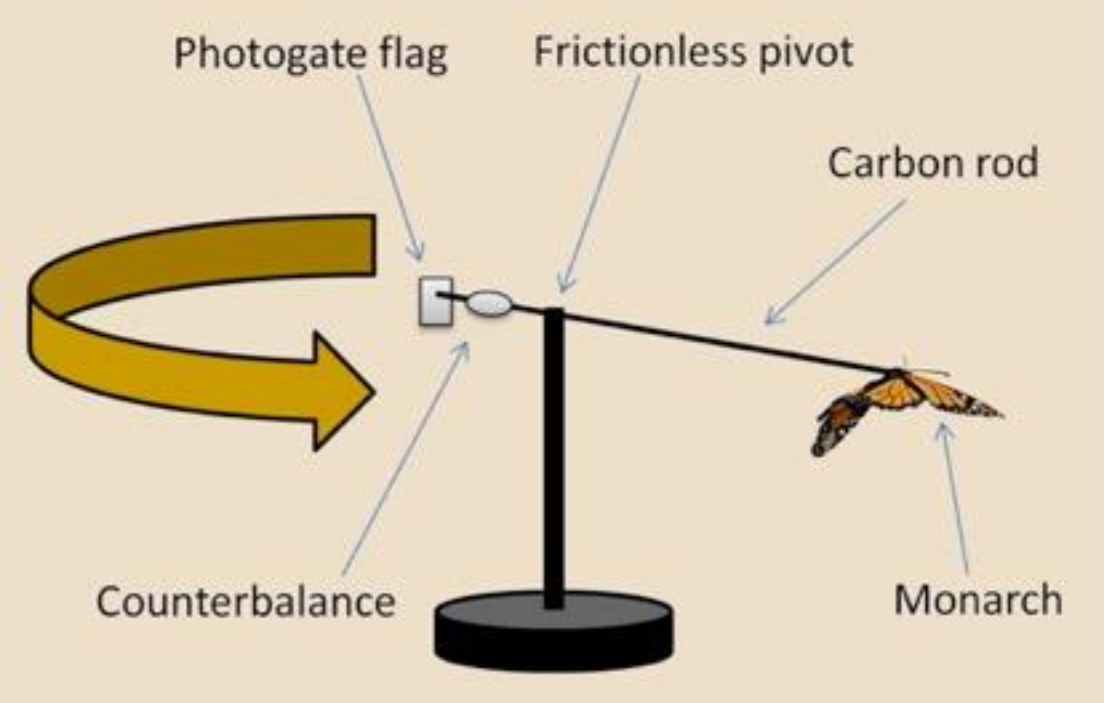

Figure S1: Diagram of the flight mill apparatus used to induce powered flight in experimental monarchs. Monarchs were attached to a 90-cm lightweight carbon rod with a diameter of 3 mm (4.23-m circumference flight path) balanced on a nearly frictionless steel pivot, and with a moveable counterbalance to account for variation in each monarch's weight. We tethered monarchs to one end of the horizontal rod using a lightweight steel fishing line. A 5-cm flag at the opposite end of the rod passed through an infrared beam on a photo-gate to estimate flight time for each revolution, using CAPSTONE Software (Pasco). Credit: Andrew K. Davis

Figure S2: Plants with tubes and mesh covering. Each tube contains one caterpillar. Tubes were roughly 1 meter tall, with a diameter of 15 cm, that were fit into the pots and covered with soil. Mesh screens were held on with rubber bands.

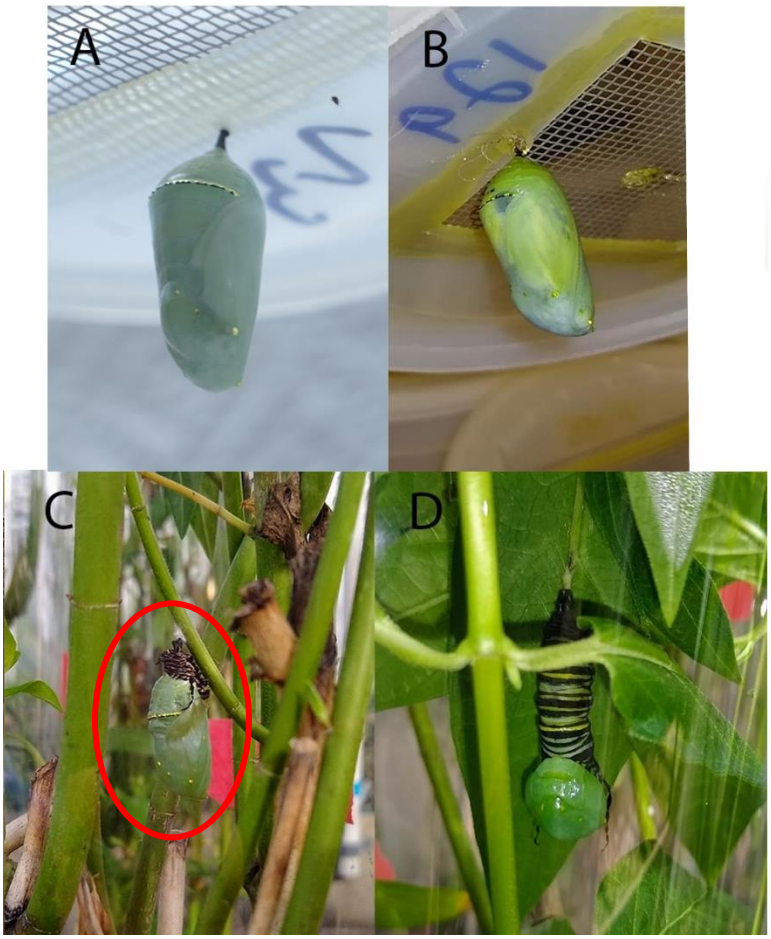

Figure S3: (A) Monarch pupa showing normal morphology with no sign of deformity. (B) Monarch pupa with slight discoloration and wrinkles (deformity score = 1). (C) Pupa showing substantial discoloration and wrinkles (deformity score = 2). (D) Pupa showing failed ecdysis (larval integuments remained on pupa as it hardened; deformity score = 3).

Table S3: Results from bee exposure as described in the methods of the main text. Data were collected hourly in the form of general observations. Time indicates minutes after exposure to neonicotinoids. IM corresponds to imidacloprid, CL to clothianidin and 50 and 500 are the concentrations in ppb. “Active” indicated that the bee was seen walking around the cage at a normal speed. Bees that were “twitching” were seen standing still with legs twitching or shaking.

Time	Control	IM 50	IM 500	CL 50	CL 500
10	4 active	4 active	4 active	4 active	2 dead,2 twitching
50	4 active	4 active	4 active	3 active, 1 immobile	3 dead, 1 twitching
90	4 active, flying	4 active	4 immobile/twitching	1 dead, 3 active	4 dead
140	4 active	1 twitching, 3 active	2 twitching, 2 slowly crawling	3 dead, 1 crawling slowly	-
190	4 flying	2 twitching, 2 not moving	1 dead, 2 twitching, 1 standing	3 dead, 1 on sponge	-
250	4 flying	3 twitching, 1 moving	1 dead, 3 twitching on backs	4 dead	-

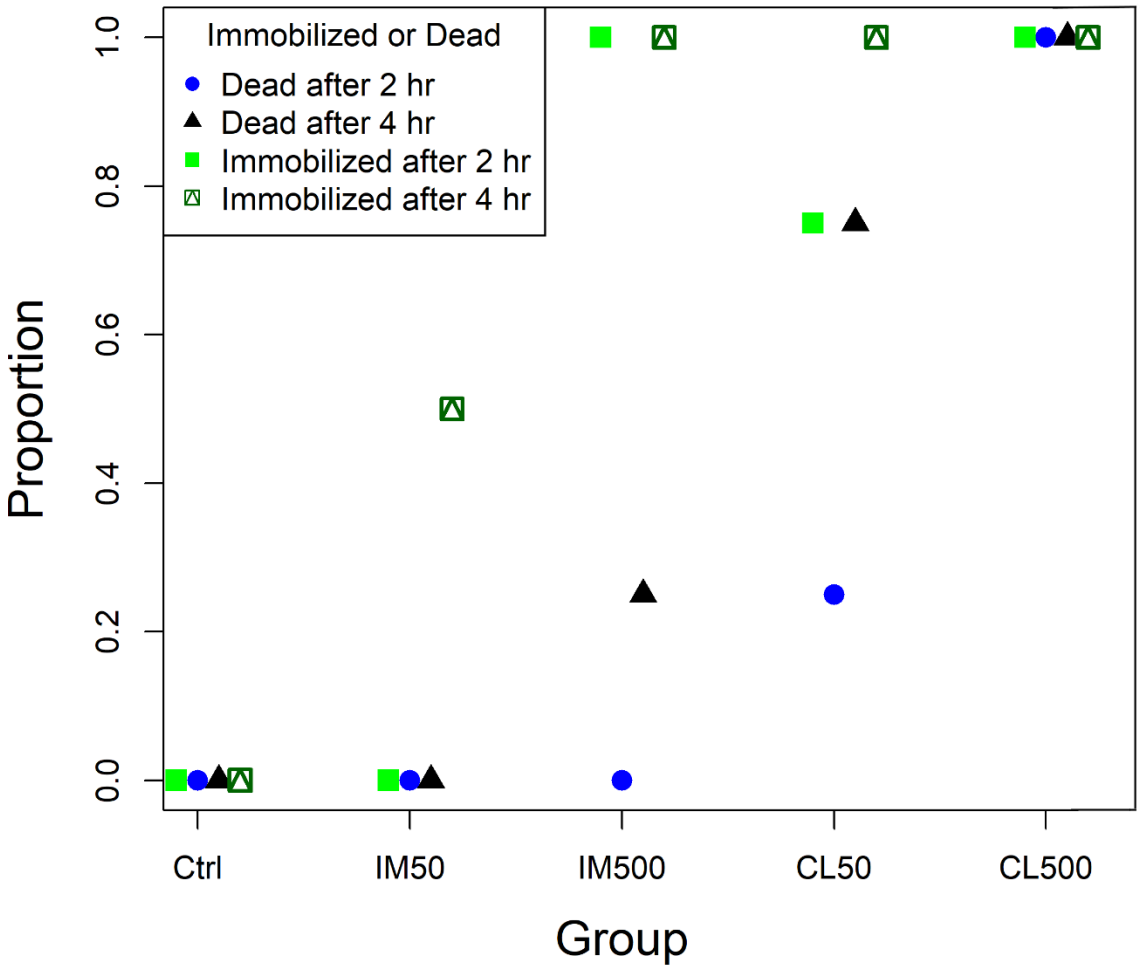

Figure S4: Summary of findings from observing bees' response to neonicotinoids. X-axis represents treatment groups, and each dot corresponds to the proportion of bees that were either dead or immobile at each given time.

Table S4: Results from assay of neonicotinoids in each leaf sample (experiments 1 and 2) or aliquot sample (experiment 2). Neonicotinoid type is coded as I = Imidacloprid, C = Clothianidin; dose column indicates intended concentration, and assay result shows the concentration following results of HPLC analysis. ND = Not detected. Collection day refers to the number of days after the sample was applied to a leaf that the leaf was collected and frozen. For experiment 1, leaves were painted with the solution and immediately bagged and frozen. Since monarchs only consumed freshly painted leaves, only one sample per concentration and chemical type was sent. For experiment 2, we selected leaf samples by choosing one plant each from swamp and tropical milkweed on days 0 (1 hr after spraying) and 4. Plants selected on each day were different from each other. Common milkweed was not tested, owing to limited funds. Leaves from the same individual plant were combined to reach a target minimum of 3 g per sample. Limits of detection for HPLC assays were: clothianidin 3 ppb and imidacloprid 2 ppb.

Experiment	Sample Type	Neonic type	Dose	Assay Result	Plant species	Collection Day
1	leaf	I	5	trace	swamp	0
1	leaf	I	15	trace	swamp	0
1	leaf	C	5	ND	swamp	0
1	leaf	C	15	trace	swamp	0
2	leaf	C	500	51	swamp	0
2	leaf	C	50	4	swamp	0
2	leaf	I	50	7	swamp	0
2	leaf	I	500	33	swamp	0
2	leaf	N/A	0	ND	swamp	0
2	leaf	N/A	0	ND	tropical	0
2	leaf	I	50	5	tropical	0
2	leaf	I	500	47	tropical	0
2	leaf	C	50	4	tropical	0
2	leaf	C	500	70	tropical	0
2	leaf	I	500	25	swamp	4
2	leaf	I	50	5	swamp	4
2	leaf	C	500	31	swamp	4
2	leaf	C	50	4	swamp	4
2	leaf	I	50	trace	tropical	4
2	leaf	I	500	trace	tropical	4
2	leaf	C	500	trace	tropical	4
2	leaf	C	50	trace	tropical	4
2	aliquot	C	50	36		
2	aliquot	I	50	28		
2	aliquot	C	500	531		

2	aliquot	I	500	386		

APPENDIX B

Figure S1: Monarchs fed on plexiglass feeding trays. A trough was cut along the base of the tray and monarchs are held down by nuts. Monarchs' proboscis are probed when they are placed on the tray and honey water is distributed in the trough.

Figure S2: Monarchs are randomly placed into mesh cages (1 m^2) after feeding was completed. Approximately 20 monarchs are in each cage, with a total of 16 cages. Monarchs are observed for behavior variables as shown.

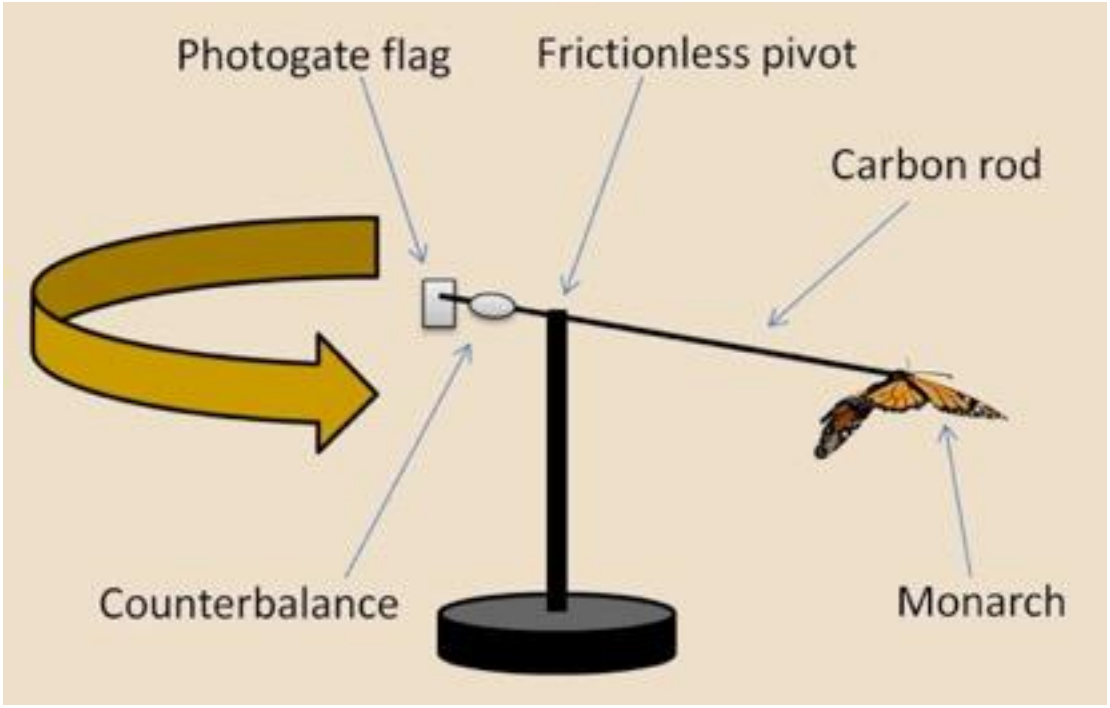

Figure S3: Diagram of monarchs fixed to a frictionless flight mill. Lightweight fishing wire is glued to monarchs' thorax using non-toxic glue and the wire is taped to the flight mill. Monarchs are encouraged to fly using flowers in a circle around the flight mill.

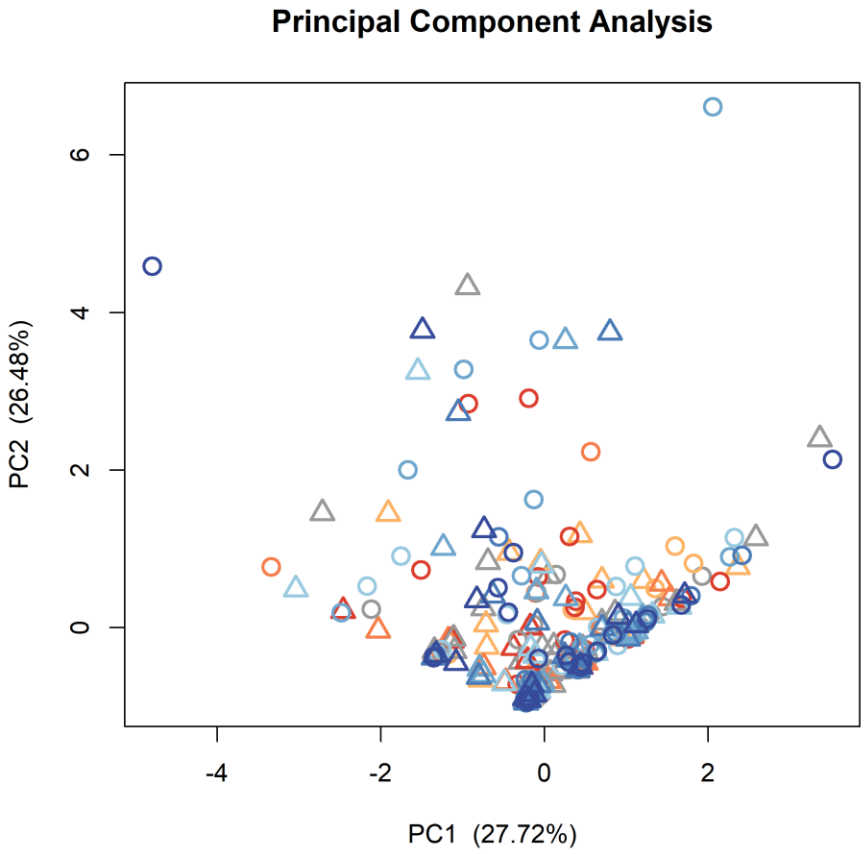

Figure S4: PCA for the five behavioral observations, grouped by neonicotinoid type with imidacloprid in the blue gradient (darker shades are higher concentrations) and clothianidin in the red gradient. Females and males are triangles and circles, respectively.

Table S1: Results from HPLC analysis. All samples in this experiment were liquid aliquots. The column “doseppb” is the intended dose, and “assayresult” is the result from the analysis.

Sample type	Neonicotinoid Type	Dose (ppb)	Assay result
aliquot	clothianidin	50	36
aliquot	imidacloprid	50	28
aliquot	clothianidin	500	531
aliquot	imidacloprid	500	386
aliquot	clothianidin	1000	909
aliquot	clothianidin	5000	4030
aliquot	imidacloprid	25	15
aliquot	clothianidin	25	19
aliquot	imidacloprid	100	54
aliquot	clothianidin	100	75