Sub-Lethal Effects of Neonicotinoids on Butterfly Behavior and Physiology

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Experiment 1: Adult monarch exposure. In May 2019, we experimentally exposed adult monarchs to increasing doses of two neonicotinoids and quantified longevity, weight gain, mating and oviposition behavior, and behavior in flight cages with other monarchs. A total of 360 monarchs from 5 genetic lineages were reared singly in pint-sized containers through the larval stage and to adult eclosion. Caterpillars were fed cuttings of greenhouse-raised swamp milkweed. Once eclosed, we fed the adults 20% honey water with neonicotinoids added at the following concentrations: 0 (control), 25, 50, 100, and 500 ppb of clothianidin and imidacloprid (36 monarchs per treatment group). Adults were fed to satiation every other day for 10 days (5 feeding days), and were held at 24 C in glassine envelopes between feedings. Butterflies were weighed before and after each feeding to determine the volume they ingested and to track weight change over time. After feeding, butterflies were randomly assigned to one of six 0.6m mesh screen cages (fed ad libitum 20% honey water on sponges with no further pesticide treatment) and monitored twice a day (morning and evening) for deaths and mating pairs. One to two times per day, for 10-min intervals, monarchs were observed visually to record flying, feeding, mating and contact activities. After five days in cages, butterflies were removed and weighed. A subset of the mated females (n=40) were placed into $0.3m^3$ oviposition cages and allowed to lay eggs on milkweed stalks over the course of 3 consecutive days. Total numbers of eggs laid per female were recorded, and hatching success was quantified. All remaining butterflies were held in glassine envelopes at 12 C, and monitored for death daily. We conducted data analysis in R, with most models structured as: Response variable = lineage (as a random effect) + neonicotinoid type + dose + type*dose + sex + type*sex + dose*sex + type*dose*sex.

Results showed that only seven monarchs died during the 10-day feeding period, with no relationship to neonicotinoid treatment. The average nectar volume consumed per monarch was ~0.17g per feeding, and this did not vary significantly across experimental treatments. Adult mass upon entry and removal from mating cages was similar across experimental treatments. The number of times males and females mated differed significantly among treatments, with treatment groups mating less often than the control. The effect of the type of neonicotinoid was stronger in males than females, with clothianidin having stronger negative effects on male mating success. A negative effect of neonicotinoid concentration on mating behavior was only present in males. [Figure 1; Table 1]. We also found that the number of eggs laid by females, and egg hatching rates, were similar across experimental treatments. Behavioral observations (time spent flying, nectaring) were analyzed using a Principal Component Analysis, and no correlations could be found with treatment groups or by sex [Figure 2].

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Model: Matings ~ Neonic * Conc * Sex
+ (1 | Lin) + WeightPostCages
Data: Matings3
Df full model: 14
           Effect df   Chisq p.value
1          Neonic  1    0.53     .46
2            Conc  1 7.62 **    .006
3             Sex  2    4.46     .11
4 WeightPostCages  1    0.22     .64
5     Neonic:Conc  1    1.72     .19
6      Neonic:Sex  2  6.65 *     .04
7        Conc:Sex  2    0.92     .63
8 Neonic:Conc:Sex  2    4.35     .11
---
Signif. codes:  0 '***' 0.001 '**'
0.01 '*' 0.05 '+' 0.1 ' ' 1
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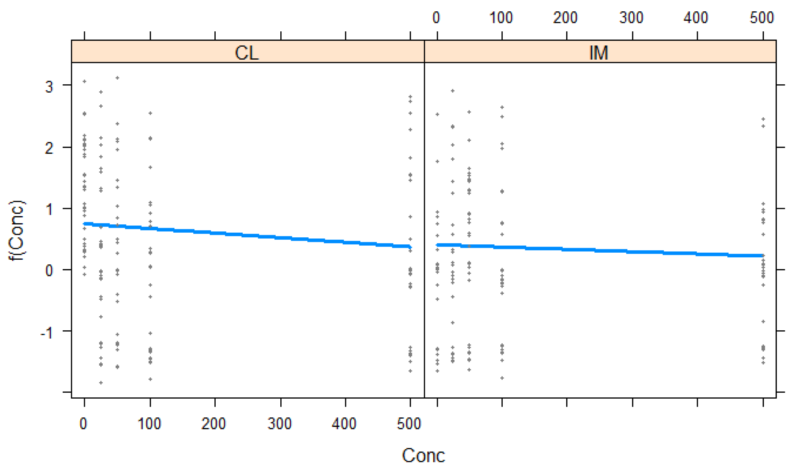

Table 1: The output from analyzing mating frequency data using a Poisson distribution. This analysis includes all individuals (male and female). Significant variables are highlighted.

Figure 1: The effect of neonicotinoid concentration on the number of matings for all individuals (males and females combined). (left) shows clothianidin and (right) imidacloprid. Pesticide dose is on the x-axis, and mating frequency is on the y-axis. Trendline is based on the analysis shown in Table 1.

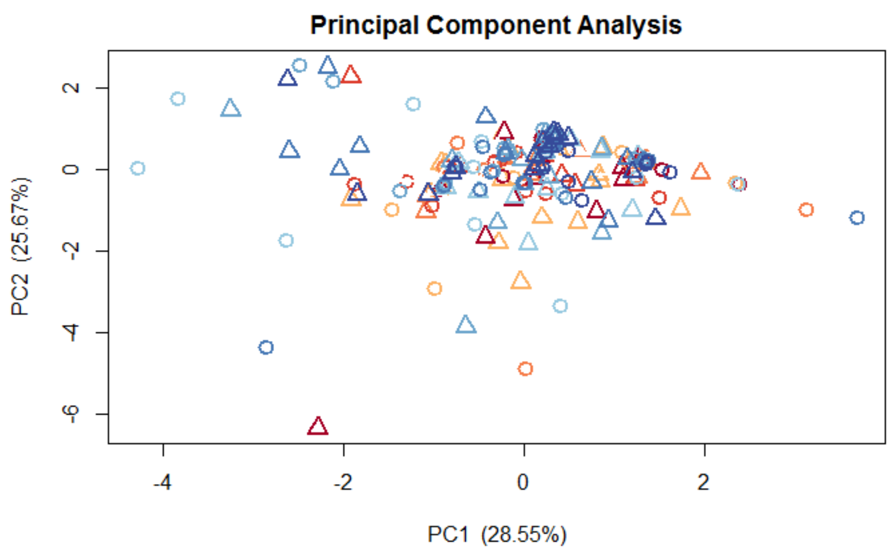

Figure 2: Principal component analysis of behavioral variables (time spent feeding, flying, mating, and contacting other monarchs). The blue gradient is imidacloprid (0-500 ppb) and the red is clothianidin (0-500 ppb). Circles and triangles are females and males. We found no association between behavioral observations and pesticide treatment.

Figure 3: ***Monarchs were fed neonicotinoids added to 20% honey water at increasing concentrations. Each treatment group had a unique feeding tray, and surfaces were sterilized with bleach solution and UV light between feedings. Monarchs were fed for an average of 10 minutes to satiation. Metal nuts were used to weigh monarchs down during feeding, and honey water was applied to a groove in a Plexiglas feeding tray.***

Field study: In June and July 2019, I traveled to Camp Dodge military base near Des Moines, Iowa, to conduct field experiments and assist with ongoing research with collaborators from Tuft's University. Our plan was to surface treat wild milkweed plants along transects with neonicotinoids, and observe monarch oviposition and larval development in the field. Owing to timing issues (monarch densities were low during June and July when the study was being conducted), we did not yet perform the outdoor portion of our research plan. We successfully established monitoring transects and constructed field cages, and when it became apparent that natural oviposition was too low, we captured wild females to attempt to collect eggs in cages. However, the eggs we obtained had low hatch rates, and did not provide suitable sample sizes to continue with this experiment. We are considering re-running this study in Athens, GA during spring or summer 2020.

Figure 4: Capturing and recording data from wild adult monarchs at Camp Dodge, Iowa.

Experiment 2: Larval monarch exposure. In October 2019, we reared monarch caterpillars on potted milkweed plants in the greenhouse, and exposed them to neonicotinoids on surface-treated leaves using three different plant species: *Asclepias incarnata* (swamp milkweed), *A. curassavica* (tropical milkweed), and *A. syriaca* (common milkweed). We used doses of 0, 50 or 500ppb of clothianidin or imidacloprid (Table 2). Plants were surface treated using a pump sprayer to administer 60 ml of solution across the tops and bottoms of leaves. Larvae were obtained from 5 genetic lineages and held on their natal stalks until they reached second instar. Plants were sprayed on day 0, and caterpillars were added to plants (as second instar larvae, one caterpillar per plant). We constructed plastic tubes (5mm thick, 0.5m wide, and 1m tall) and placed them over the top of each plant, with mesh secured at the top to contain the monarchs to their own plant. Owing to the known decay of neonicotinoids in response to UV light, plants were re-sprayed with 20 ml of solution per plant on day 5 post-larval application. We observed plants daily to record larval survival and pupation date. Most monarchs had pupated by 10 days post-application. Five days following pupation, we transferred pupae to our lab to weigh them and record any deformity or discoloration. This experiment is ongoing, with monarchs beginning to eclose now.

Results thus far show no evidence for larval mortality in response to experimental treatments. However, in the clothianidin 500 ppb treatment group, for both swamp and common milkweed, approximately 44% of monarchs failed to pupate properly. Additionally, individuals in this group on these milkweed species have also had pupal deformities, where the chrysalis is caved in and deformed near where the wings are forming, and they are often discolored, with brown tinting at the wingtips. So far, exposure on caterpillars appears more detrimental than exposure as adult monarchs, since we saw little to no mortality or behavioral changes for adult monarchs exposed to neonicotinoids in nectar. Analyses will continue through spring 2020. Once the remaining monarchs eclose, we will scan their wings on a flatbed scanner to quantify wing area and hue characteristics. We may collect additional data from the emerging adults as time permits.

Table 2: Experimental design for monarch larval neonicotinoid study. Each treatment 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 5: (left) A deformed pupa from the high dose clothianidin treatment. Indentations can be seen where wings are forming, and the pupal case is wrinkled and slightly discolored. A brown line can be seen where wingtips are forming. (right) A deformed pupa that was not able to finish shedding its larval skin and fully form the chrysalis (also from the high dose clothanidin treatment).

Figure 6: Experimental set up with plants in tubes. Each tube contains a single caterpillar. Plants were sprayed with neonicotinoids prior to introduction of monarchs, and were re-sprayed on day 5 following larval introduction.