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Non-target Effects of Clothianidin on Monarch Butterflies

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SHORT COMMUNICATION

Non-target effects of clothianidin on monarch butterflies

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Abstract Monarch butterflies (*Danaus plexippus*) frequently consume milkweed in and near agroecosystems and consequently may be exposed to pesticides like neonicotinoids. We conducted a dose response study to determine lethal and sublethal doses of clothianidin using a 36-h exposure scenario. We then quantified clothianidin levels found in milkweed leaves adjacent to maize fields. Toxicity assays revealed LC_{10}, LC_{50}, and LC_{90} values of 7.72, 15.63, and 30.70 ppb, respectively. Sublethal effects (larval size) were observed at 1 ppb. Contaminated milkweed plants had an average of 1.14 ±0.10 ppb clothianidin, with a maximum of 4 ppb in a single plant. This research suggests that clothianidin could function as a stressor to monarch populations.

Keywords *Asclepias* · *Danaus plexippus* · Neonicotinoid · Non-target · Seed treatment

Introduction

Over the last 15 years, populations of the monarch butterfly, *Danaus plexippus* L. (Lepidoptera: Nymphalidae), have declined (Brower et al. 2012; Pleasants and Oberhauser 2012). *D. plexippus* migrates over multiple generations from winter breeding grounds in Mexico to summer habitats that reach to Canada (Zipkin et al. 2012). Recent changes in agroecosystems, particularly the drastic reduction of weeds in glyphosate-tolerant field crops, have resulted in major habitat changes throughout much of the monarch's North American range (Pleasants and Oberhauser 2012). Monarch larvae are monophagous on milkweed (*Asclepias* spp.) foliage, and populations of this plant have experienced substantial reductions linked to these cropping changes. One estimate is that the reproductive potential of *D. plexippus* populations from the Midwestern USA have declined by 81 %, which is in part linked to habitat reductions and a 58 % reduction in milkweed (Pleasants and Oberhauser 2012). As nearly weed-free cropland dominates a landscape, non-crop habitats that harbor the remaining milkweed are often in close proximity to agricultural fields (Johnston 2014; Wright and Wimberly 2013), which potentially exposes the plants and monarchs to agrichemicals like pesticides.

Neonicotinoid insecticides have become the most widely used pesticide class in the world (Goulson 2013) and are one potential hazard associated with cropland that monarchs may be exposed to. These highly water-soluble pesticides are readily taken up by roots and are systemically transported throughout a developing plant (Tomizawa and Casida 2005). Plants within or adjacent to crops whose seeds are treated with neonicotinoids can unintentionally take up excess neonicotinoids (Krupke et al. 2012). The recent population declines in North American monarchs, and their potential exposure to neonicotinoids, prompted an experiment to 1) determine the toxicity of clothianidin to monarch larvae and 2) measure the quantities of clothianidin in milkweed associated with maize fields. The result is a first risk assessment of this common agrichemical as a potential contributing factor to monarch declines.

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Methods

Hazard assessment

Plants and insects Swamp milkweed, *Asclepias incarnata* L. (Gentianales: Apocynaceae) (Victory Seed Company, Molalla, OR), was produced in a greenhouse. Within 1 h of the assay, 1-cm-diameter leaf discs were excised and randomly assigned to a treatment. *D. plexippus* eggs were purchased from Butterfly Workx (Dunnellon, FL, 34430) or were reared from locally collected monarch females (N 44.341, W 96.793). Preliminary assessments revealed that both populations responded identically to the toxin, and so blocks were pooled across populations. Resulting neonates were randomly assigned upon hatching to a treatment within 8 h of hatching.

Experimental procedures Prior to the exposure assessment, we hypothesized that monarch larvae in the field would be exposed to short pulses of clothianidin coincidental with planting (Krupke et al. 2012) or maize anthesis (e.g., as with Bt maize pollen; Dively et al. 2004). We attempted to conservatively reflect this predicted exposure level by feeding neonate monarchs the toxin for 36 h rather than a continuous exposure over the entire larval stage. Availability of monarch eggs necessitated that the experiment be conducted in seven independent blocks with sample sizes per block ranging from three to ten individuals per treatment (sample size was consistent across treatments within a block). Based on the toxicity observed in early blocks, we adjusted the doses to best capture the LC_{10}, LC_{20}, LC_{50}, and LC_{90}, so not all dietary treatments are reflected in each block.

Larvae were randomly assigned to one of 11 dietary treatments. Nine of these treatments received a concentration of aqueous clothianidin: 1000 (n=40), 500 (n=10), 100 (n=40), 50 (n=10), 25 (n=40), 10 (n=40), 5 (n=30), 1 (n=30), and 0.5 (n=30) parts per billion (ppb). The other two treatments were fed a water control (n=30) or were unfed (n=40). All fed treatments received a single 1-cm-diam leaf disc with aqueous 10 μL of the designated test substance administered individually on a base of 3 % agarose gel (Hellmich et al. 2001). Following the 36-h exposure to the test substance (milkweed discs were replaced if they were completely consumed), all surviving larvae were fed excess milkweed leaf tissue until the third stadium. Mortality was recorded daily throughout the experiment. Larval body length, mass, and head capsule width were measured prior to the experimental treatment and upon eclosion to the second and third stadia.

Exposure assessment

Leaf tissue analysis The presence of clothianidin was tested in milkweed (*Asclepias syriaca*) plants that were a mean (SEM) 1.47±0.39 m from maize fields in Brookings County, SD, soon after maize planting (June 20, 2014; plants were 5–15 cm tall; n=8 sites), and when monarch populations were most abundant (July 25, 2014; n=10 sites; Lyons et al. 2012). The late planting date in our area was the result of a very wet spring. At each site, five plants were chosen randomly, and two 1-cm-diam tissue samples were removed and frozen at −18 °C. Simultaneously, we counted the number of monarch eggs and larvae per plant on ten plants at each site. We acknowledge that *A. syriaca* has thicker leaves than *A. incarnata* (used in the toxicity assay above), and this may affect the final dose consumed by larvae in the field.

Enzyme-linked immunosorbent assay (ELISA) kits (Product #500800, Abraxis, Warminster, PA) were used to quantify clothianidin content in the leaf tissue, following kit instructions. Nearly 100 % of corn in our region is seed-treated with thiamethoxam or its metabolite, clothianidin. There are currently no commercial corn hybrids treated with imidacloprid, thus strengthening our assertion that any toxin detected with this ELISA in milkweed adjacent to cornfields was clothianidin. The two leaf discs from each plant were combined and crushed in 258 μL of water. The resulting supernatant (50 μL) was incubated on a pre-coated ELISA plate. A negative control series (n=3 aliquots from a single untreated plant sample) and three standard curves were run on each plate. The negative control series consisted of the supernatant of macerated, greenhouse-produced milkweed leaf discs (two 1-cm-diam discs) treated identically to field samples. The same sample for the negative control series was used to create three standard curve series on each plate (concentrations ranged from 0.03 to 2 ppb clothianidin). The absorbance values at 450 nm were recorded for each sample and control using a microplate reader (μQuant, Biotek Instruments, Winooski, VT 05404). On each plate, the mean and standard deviation of the three negative controls were calculated. Any samples that had a lower optical density than the mean negative minus 2.5 times the standard deviation of this series were considered to be positive for clothianidin. The absorbance of these positive samples was then transformed into ppb clothianidin using the plate-specific standard curve series. Based on these measurements, the mean concentration in two leaf discs was calculated per milkweed plant at each site.

Data analysis To test the effects of concentration on these metrics of monarch fitness, data blocks were pooled (no effects of blocks were discovered) and comparisons were made using independent ANOVAs. Significantly different means were separated using Fisher's LSD test. A scatterplot was created that contrasted the proportion of larvae surviving at the onset of the third stadium (transformed into probits) with insecticide concentration (log+1 ppb), and linear regression was fitted to the resulting data. This curve was used to generate LC_{10}, LC_{20}, LC_{50}, and LC_{90} values. The mean clothianidin

Table 1 Sublethal effects of clothianidin on monarch (*Danaus plexippus*) larvae (following a 36-h exposure during the first stadium)

Treatment (concentration, ppb; *n*)	Head capsule width (mm)	Body length (mm)	Weight (mg)	Duration of stadium (days)
1st instar (measured after molt to 2nd stadium)				
0 (39)	1.41±0.01	8.70±0.30A	23.23±1.70B	3.77±0.13A
0.5 (30)	1.72±0.33	7.74±0.13BC	17.39±0.78A	4.28±0.16BC
1 (30)	1.40±0.01	7.87±0.18BC	19.61±1.00AB	4.06±0.07AB
5 (28)	1.40±0.01	7.65±0.11AB	18.72±0.87A	4.33±0.09BC
10 (18)	1.42±0.01	7.17±0.38BC	21.62±2.68AB	4.27±0.18BC
25 (22)	1.40±0.01	11.55±0.39AB	32.40±3.50AB	4.66±0.19C
2nd instar (measured after molt to 3rd stadium)				
0 (39)	1.68±0.02A	12.78±0.21A	72.68±3.17	1.28±0.07
0.5 (30)	1.65±0.01AB	13.08±0.21A	60.57±1.33	1.16±0.05
1 (30)	1.62±0.02BC	12.47±0.23AB	77.28±17.16	1.19±0.06
5 (28)	1.59±0.02C	11.95±0.26BC	59.25±1.71	1.20±0.06
10 (18)	1.55±0.03D	11.85±0.38BC	55.35±4.36	1.18±0.09
25 (22)	1.64±0.03ABC	11.29±0.27C	53.77±2.69	1.04±0.03

Values (mean±SEM) followed by different letters differ significantly (α=0.05) by ANOVA tests

content per plant and proportion of contaminated plants per site were compared between the early season and late season leaf discs using an ANOVA. All statistics were conducted using Systat 13 (SYSTAT Software, Inc; Point Richmond, CA).

Results

Hazard assessment Based on the probit analysis, the LC_{10}, LC_{20}, LC_{50}, and LC_{90} concentrations were found to be 7.72, 9.89, 15.63, and 30.70 ppb, respectively (Fig. 1). Significant differences were seen in development time ($F_{5,\ 162}$=6.32, P<0.001), body length ($F_{5,\ 163}$=3.20, P=0.009), and weight ($F_{5,\ 163}$=2.28, P=0.049) for newly eclosed second instars (Table 1). Body length and development rate were significantly affected at 0.5 ppb, but these fitness effects did not extend through the second stadium. There was no significant difference in second instar head capsule width ($F_{5,\ 163}$=0.86, P=0.51). Body length ($F_{5,\ 163}$=6.82, P<0.001) and head capsule width ($F_{5,\ 163}$=4.10, P=0.002) of newly eclosed third instars were significantly affected by treatment (Table 1). Specifically, body length was significantly reduced (compared to the 0-ppb treatment) at 5 ppb, and head capsules were reduced at 1 ppb of clothianidin. Weights of new third instars ($F_{5,\ 163}$=1.49, P=0.20) and the duration of the second stadium ($F_{5,\ 163}$= 1.765, P=0.12) were unaffected by treatment.

Exposure assessment The sites sampled earlier in the season had a mean (SEM) of 1.29±0.29 eggs and 0.57±0.20 larvae per plant. In July, 1.40±0.27 eggs and 0.3±0.15 larvae were found per plant on the ten sites used. Mean (SEM) clothianidin per plant was 0.58±0.07 ppb, with a maximum amount of 4.02 ppb in one plant. Twice the proportion of plants per site were contaminated in July compared to June (36.57±0.08 and 64.50±0.08 % of plants contaminated; seasonal mean was 51.3 % of plants) ($F_{1,\ 15}$=5.51, P=0.03). In June, mean (SEM) clothianidin content per plant was 0.40± 0.09 ppb, and in July, it was 0.69±0.09 ppb ($F_{1,\ 15}$=3.96, P= 0.07); the clothianidin content per contaminated plant was 1.24±0.12 and 1.11±0.15 ppb in June and July, respectively ($F_{1,\ 15}$=0.11, P=0.75).

Discussion

This experiment documents sublethal effects of the toxin at exposure levels observed under field conditions, and indicates that neonicotinoids could negatively affect larval monarch populations. The toxicity assay revealed both lethal and

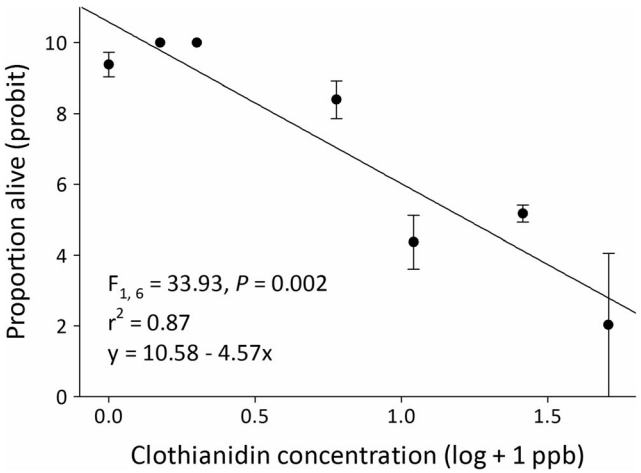

Fig. 1 Cumulative mortality (to the third instar) of monarchs (*Danaus plexippus*) fed clothianidin. *Bars* represent SEM

sublethal effects of clothianidin on young monarch larvae. Previous risk assessments have shown that clothianidin and other neonicotinoids typically have a LD_{50} of 3.7–81 ppb for non-target insects (Schmuck et al. 2001; Nauen et al. 2003). The LC_{90} values for monarch larvae are higher than those reported for some other taxa; this is likely because of our curtailed exposure in the laboratory (only 36 h exposure). The toxicity assay revealed that, even at this short exposure period, sublethal effects of clothianidin were observed at much lower concentrations (1 ppb) than were lethal effects. The larger mean size of larvae fed the 25-ppb concentration (Table 1) is the result of smaller individuals in the population being killed by the toxin. Only two individuals fed 50 ppb survived (and none survived at higher doses), so analyses on sublethal effects were focused on individuals fed 25 ppb or less. Smaller individuals are more susceptible to predation (Kingsolver and Huey 2008) and may succumb to other stressors (e.g., pesticides, pathogens, etc.) more readily (Altizer et al. 2000) than larger conspecifics. Combined or taken individually, these stressors may contribute to population declines in a non-target insect.

In the field, clothianidin was found more frequently in milkweed adjacent to maize fields when monarch larvae are typically at their peak numbers (Zipkin et al. 2012), compared to early in the season. This prolonged presence of clothianidin (beyond just when maize is planted) indicates that monarch larvae are likely exposed to clothianidin throughout their larval lives and that the 36-h exposure event in our toxicity assay is an underestimate of field exposures. We found up to 4 ppb of clothianidin in a plant (mean of 1.14±0.10 ppb per contaminated plant), which is sufficient to reduce larval size in our 36-h assay. Field populations of monarch eggs and neonate larvae suffered high rates of mortality. In Brookings County, SD, 50–80 % of the eggs died before or soon after hatching. Although high larval mortality is frequently recorded in young monarch larvae (Oberhauser et al. 2001), the fact that these eggs were laid on milkweed plants with clothianidin suggests that this systemic insecticide may be contributing to the mortality of neonates. Cropping patterns in Brookings County are similar to those experienced throughout the Corn Belt, and we expect the experimental results to be transferable to other areas. Although preliminary, this study clearly shows that monarch larvae are exposed to clothianidin in the field at potentially harmful doses of the toxin. Additional work that investigates wider geographic and seasonal ranges is necessary to firmly substantiate whether this insecticide is contributing to monarch declines.

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