Uptake and toxicity of clothianidin to monarch butterflies from milkweed consumption

Timothy A Bargar [Corresp., 1] , Michelle L Hladik [2] , Jaret C Daniels [3, 4]

1 Wetland and Aquatic Research Center, U.S. Geological Survey, Gainesville, Florida, United States

2 California Water Science Center, U.S. Geological Survey, Sacramento, California, United States

3 Department of Entomology and Nematology, University of Florida, Gainesville, Florida, United States

4 Florida Museum of Natural History, Gainesville, Florida, United States

Corresponding Author: Timothy A Bargar
Email address: tbargar@usgs.gov

Recent concern for the adverse effects from neonicotinoid insecticides has centered on risk for insect pollinators in general and bees specifically. However, natural resource managers are also concerned about the risk of neonicotinoids to conservation efforts for the monarch butterfly (*Danaus plexippus*) and need additional data to help estimate risk for wild monarch butterflies exposed to those insecticides. In the present study, monarch butterfly larvae were exposed in the laboratory to clothianidin via contaminated milkweed plants from hatch until pupation, and the effects upon larval survival, larval growth, pupation success, and adult size were measured. Soils dosed with a granular insecticide product led to mean clothianidin concentrations of 10.8 – 2193 ng/g in milkweed leaves and 5.8 – 58.0 ng/g in larvae. Treatment of soils also led to clothianidin concentrations of 2.6 – 5.1 ng/g in adult butterflies indicating potential for transfer of systemic insecticides from the soil through plants and larvae to adult butterflies. Estimated LC_{50}s for total mortality (combined mortality of larvae and pupae) and EC50 for larval growth were variable but higher than the majority of concentrations reported in the literature for clothianidin contamination of leaves.

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2 # butterflies from milkweed consumption

4 Timothy A. Bargar[1], Michelle L. Hladik[2], Jaret C. Daniels[3]

6 [1]U.S. Geological Survey, Wetland and Aquatic Research Center, 7920 NW 71st Street,
7 Gainesville, FL, 32653, USA.
8 [2]U.S. Geological Survey, California Water Science Center, 6000 J Street, Placer Hall,
9 Sacramento, CA, 95819, USA.
10 [3]Florida Museum of Natural History, 3215 Hull Road, Gainesville, FL 32611; and Department of
11 Entomology and Nematology, University of Florida, 1881 Natural Area Drive, Steinmetz Hall,
12 Gainesville, FL, 32611, USA.

14 Corresponding Author:
15 Tim Bargar
16 7920 NW 71st Street, Gainesville, FL, 32653, USA
17 tbargar@usgs.gov

19 **Abstract**

20 Recent concern for the adverse effects from neonicotinoid insecticides has centered on risk for
21 insect pollinators in general and bees specifically. However, natural resource managers are also
22 concerned about the risk of neonicotinoids to conservation efforts for the monarch butterfly
23 (*Danaus plexippus*) and need additional data to help estimate risk for wild monarch butterflies
24 exposed to those insecticides. In the present study, monarch butterfly larvae were exposed in the
25 laboratory to clothianidin via contaminated milkweed plants from hatch until pupation, and the
26 effects upon larval survival, larval growth, pupation success, and adult size were measured.
27 Soils dosed with a granular insecticide product led to mean clothianidin concentrations of 10.8 –
28 2193 ng/g in milkweed leaves and 5.8 – 58.0 ng/g in larvae. Treatment of soils also led to
29 clothianidin concentrations of 2.6 – 5.1 ng/g in adult butterflies indicating potential for transfer
30 of systemic insecticides from the soil through plants and larvae to adult butterflies. Estimated
31 LC_{50}s for total mortality (combined mortality of larvae and pupae) and EC50 for larval growth
32 were variable but higher than the majority of concentrations reported in the literature for
33 clothianidin contamination of leaves.

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Introduction

42

43 Since their first development in the 1980s and initial registrations for use in agriculture in the

44 1990s, neonicotinoid insecticides have grown to comprise a major portion of the world's overall

45 insecticide market with a wide variety of uses in crops, livestock, lawn and garden, and

46 companion animals (Jeschke et al., 2011). They have replaced organophosphates and

47 carbamates in agriculture because they are generally considered less toxic for nontarget

48 organisms (Uneme et al., 2006; Simon-Delso et al., 2015). In the Midwestern United States, the

49 neonicotinoid pesticides clothianidin and its precursor thiamethoxam are registered for use on

50 corn and soybeans only as a seed coating. The high water solubility of these pesticides facilitates

51 their absorption by the seedling upon germination and translocation throughout the plant where

52 they are available for exposure to targeted herbivorous insect pests (Simon-Delso et al., 2015).

53 In addition, the combination of high-water solubility, relatively long half-lives, and the fact that

54 <20% of the neonicotinoids in the seed coating are taken up by target plants increases the

55 likelihood of environmental contamination (Hladik et al., 2014; Hladik, Main & Goulson, 2018)

56 as indicated by their presence in streams (Hladik and Kolpin, 2016), groundwater (Bradford et

57 al., 2018), and tap water (Klarich et al., 2017).

58

59 Recent concern for the adverse effects from neonicotinoid insecticides has centered on risk for

60 insect pollinators in general and bees specifically (e.g. Balfour et al., 2017; Woodcock et al.,

61 2017). Beekeepers began reporting in 2006 incidences of colony collapse disorder for honey

62 bees. The collapses were occurring unexpectedly and have led to a variety of explanations

63 including pesticides (vanEngelsdorp et al., 2010). While neonicotinoid insecticides are not the

64 only pesticides to which the bees may be exposed, their role in the collapses has received

65 considerable attention. The fact that bee colonies are used commercially to facilitate pollination

66 of agricultural crops targeted by the neonicotinoid insecticides indicates an increased likelihood

67 of exposure through pollen grains, which have been shown to contain the insecticides (Rolke et

68 al., 2016; Xu et al., 2016). In addition, the USEPA (2005) noted a major risk concern for honey

69 bees potentially exposed to clothianidin because of its toxicity to bees and recommended caution

70 during applications. However, concern for the environmental impacts of neonicotinoids extend

71 beyond the potential effects to bees (Chevillot et al., 2017; Han et al., 2018; Hallmann et al.,

72 2014; Morrissey et al., 2015; Zhang et al., 2018).

73

74 Natural resource managers are concerned about the risk of neonicotinoids to conservation efforts

75 for the monarch butterfly (*Danaus plexippus*). The long-term viability of North American

76 populations is threatened (Semmens et al., 2016; Schultz et al., 2017; Pelton et al., 2019). The

77 U.S. Fish and Wildlife Service was petitioned by The Center for Biological Diversity, The

78 Center for Food Safety, and The Xerces Society in 2014 to list the monarch under the

79 Endangered Species Act. Currently, the U.S. Fish and Wildlife Service is assessing the status of

80 the population and the potential impact of multiple threats, including insecticides, to determine if

81 listing is warranted. Simultaneously, monarch conservation practices and plans are being
82 developed and enacted by States and multiple partners across the monarch's range to address
83 threats and the immediate need to increase the population (Commission for Environmental
84 Cooperation, 2008; Caldwell et al., 2018). Monarchs face a multitude of threats including loss of
85 habitat quality throughout their breeding grounds due to insecticide use (Stenoien et al., 2018;
86 Crone et al., 2019) including neonicotinoids (Thogmartin et al., 2017). The systemic nature of
87 the neonicotinoid insecticides could lead to the contamination of milkweed plants and nectar
88 resources. From this contamination, there can be dietary exposure potentially impacting monarch
89 butterflies. The agricultural belt of the Midwestern United States is the breeding range for
90 approximately 38% of the monarch butterflies that winter in Mexico (Flockhart et al., 2017)
91 making the Midwestern United States a priority for monarch conservation efforts for the Fish and
92 Wildlife Service. This region is also where neonicotinoid pesticide (thiamethoxam,
93 imidacloprid, clothianidin) use is also high (USGS, 2014) increasing the exposure likelihood for
94 monarch butterflies.
95
96 In the present study, we exposed monarch butterfly larvae to clothianidin, taken up by milkweed
97 plants via application of a granular formulation to the soil, to evaluate its effect on monarch
98 development. Monarch larvae were exposed from the time they hatched from eggs until
99 pupation to the contaminated milkweed plants, and the effects of that exposure upon larval
100 survival, larval growth, pupation success, and adult mass were measured.
101

102 **Materials & Methods**
103 Three separate experiments were conducted during this study. Experiment 1 evaluated butterfly
104 response to dose levels based on label application rates for the pesticide product used in the
105 experiments. Experiment 2 was conducted at higher dose levels due to low butterfly response in
106 Experiment 1. Experiment 3 was conducted because aphids negatively affected the health of the
107 control plants in Experiment 2, which likely influenced butterfly response in the controls.
108 Information regarding the experiment start and end dates, temperature, photoperiod, and relative
109 humidity is reported in Table 1. All experiments occurred at the laboratory facilities of the
110 Wetland and Aquatic Research Center in Gainesville, Florida, USA (29.7261722⁰ N,
111 82.4188444⁰ W).
112
113 Swamp milkweed (*Asclepias incarnata*) was the host plant utilized in the experiments. The
114 swamp milkweed is a local species and is grown pesticide-free by a local vendor. During
115 Experiments 1 and 2, the plants were purchased from the local native plant vendor. The vendor
116 is surrounded by fallow fields and pine plantations that are no nearer to the vendor than
117 approximately 1 km. Due to heavy aphid infestation of those plants during the first two
118 experiments, plants used in Experiment 3 were grown at the laboratory under controlled
119 conditions.   The laboratory-propagated plants were started from seeds (American Meadows,

120   https://www.americanmeadows.com/) planted in organic seed-starter soil (Jiffy® Natural &
121   Organic Seed Starting Mix). The planted seeds were placed under growth lights indoors. Two
122   weeks after germination, the seedlings were transferred to larger pots and moved outside to be
123   exposed to natural conditions (photoperiod, temperature, humidity) during the remainder of the
124   growth period.

125

126   All plants were propagated in pots (one plant per pot) filled with organic potting soil (10% peat,
127   45% pine bark, 43% wood chips, 5% sand, and 2% NutriHold™) instead of natural or artificial
128   top soil to minimize the effect of top-soil compaction on plant health. A slow-release fertilizer
129   was added to the potting soil before planting. All plants were watered every 2-3 days with
130   enough water to moisten the soil, but typically not enough to result in water leaching from the
131   pot. Weekly, the water was fortified with a liquid fertilizer (Miracle-Gro® Liqua Feed® 12:4:8
132   N-P-K ratio). All plants were maintained outside in screened terraria (42 cm deep x 76.2 cm
133   wide x 121.9 cm tall; 1 plant per terrarium) before and during all experiments to prevent access
134   of wild monarch butterflies and predators to the plants, and to retain larvae and adults during the
135   experiments (Figure 1A). All terraria were maintained outside to ensure exposure to ambient
136   conditions, but under a translucent roof to enable moisture control in the pots while ensuring a
137   natural photoperiod and light intensity. After planting, each plant was assigned a unique number
138   to be used in their random assignment (Microsoft Excel©) among dose levels for the
139   experiments.

140

141   Monarch eggs were purchased from a local butterfly farm vendor (Shady Oak Butterfly Farm,
142   https://shadyoakbutterflyfarm.com/). The colony is continuously maintained by the vendor for
143   commercial purposes and is regularly supplemented by wild stock. The following procedures are
144   taken by the vendor to ensure the colony is not infected by the OE (*Ophryocystis elektroscirrha*)
145   parasite, which can adversely affect health and survival of larvae and pupae. All milkweed
146   plants are raised indoors to eliminate deposition of OE spores onto the colony plants by
147   potentially infected wild adults; colony breeders are regularly checked for the presence of spores;
148   all eggs are rinsed in a bleach solution to kill spores (Altizer & Oberhauser, 1999); and wild
149   stock captured for introduction for infusion of additional genetic diversity into the colony are
150   raised separately for several generations to monitor for eradicate OE and disease.

151

152   The pesticide product used to dose the soils with clothianidin was Arena® 0.25G, which is a
153   granular pesticide containing 25% clothianidin by weight. Arena ® 0.25G is used in commercial
154   and residential landscapes for control of a wide variety of herbivorous insect pests. Use of the
155   granular product facilitated treatment of the soils with a known clothianidin mass without
156   introduction of unwanted pesticides in the formulation that could be associated with the use of
157   treated seed. The label rate of this product for flower beds (1.63 kg per 92.9 m^2, converted from
158   the rate of 3.6 pounds per 1,000 square feet noted on the label). Since the soil surface area in

159 each pot during Experiments 1 and 2 was 0.085 m^2, the label rate would be 1.5 g per pot for
160 those experiments. Since smaller pots were used for Experiment 3 (soil surface area 0.0345 m^2),
161 the label rate for those pots would have been 0.6 g per pot.

162

163 Experiment 1 consisted of 6 replicates in each of 5 dose levels plus a control. Three plants were
164 randomly assigned to each replicate to ensure adequate host plant for the larvae (108 plants total
165 evenly divided among 36 replicate sets). Plant height in Experiment 1 ranged from 0.6 – 1.2 m.
166 The mean Arena 0.25G mass added to each pot was 0.08 (SD=0.004), 0.21 (0.004), 0.64 (0.004),
167 1.92 (0.004), and 5.77 (0.006) g for dose levels 1, 2, 3, 4, and 5, respectively. No granular
168 pesticide was added to the Control pots. All treated pots were dosed once on June 15, 2018 and
169 then watered and fertilized as noted earlier. Monarch eggs were received on July 18, 2018 and
170 divided among 36 pre-numbered plastic cups. The cups were randomly assigned to one of the
171 three plants in each of the 36 replicates (1 cup per replicate). When the eggs began to hatch, a
172 single 1 cm diameter leaf disc from the associated plant was placed into the cup (Figure 1B).
173 24h after the first egg within a cup hatched, the cup was attached to the associated plant to allow
174 the larvae to crawl onto the plant (Figure 1C). Larvae were transferred by hand from one plant
175 to the next in a replicate when the source plant was nearly defoliated. No plants within a
176 replicate were reused. The maximum number of larvae per replicate ranged from 1 – 8
177 depending on hatching success for the cup. The approximately 1-month delay between egg
178 purchase and dosing ensured adequate time for clothianidin uptake by and distribution within the
179 milkweed plants before introduction of the larvae to the plants. Each plant was inspected daily
180 to monitor larval growth. The length of each larva found on a plant was measured and recorded
181 daily (nearest 0.01 mm as determined by digital calipers). Mass was not utilized as the growth
182 indicator to minimize handling stress as a potential factor in larva growth. Dead larvae were
183 noted and collected when found. Small larvae (< 2 days of age) were most easily located by
184 searching for forage areas on the leaves, but sometimes they were not found on plants with heavy
185 foliage or with large numbers of aphids. Missing larvae (typically 1st instar) were not assumed
186 dead because of the difficulty of finding small (1st and 2nd instar) larvae. The day post-hatch on
187 which larvae began to pupate (prepupa) and on which pupae were first observed was recorded
188 for each replicate. The pupae were monitored daily until eclosion (adult emergence from pupa),
189 and the day post-pupation on which eclosion occurred was noted. The mass and forewing length
190 (thorax to distal wing tip, to the nearest 0.01 mm) was measured for each adult butterfly.
191 Prepupae were considered dead if they failed to completely form a pupa within 24h, while pupae
192 were considered dead if adult eclosion failed to occur.

193

194 Milkweed leaves, larvae, and adult butterflies were collected for clothianidin residue analysis.
195 Composite leaf sampling was conducted to ensure that all milkweed plants that hosted larvae
196 were sampled for analysis. Three composite leaf samples were collected from each dose level.
197 The 6 replicates within each dose level (e.g., 1, 2, 3, 4, 5, 6) were randomly divided into 3 pairs

198   (e.g., 2&5, 1&6, 3&4) from which each composite sample was collected. The paired replicates
199   were sampled twice (sampling A and B) during the experiment with the tissue from two
200   samplings (A + B) comprising the composite sample. The first sampling (A) that occurred when
201   larvae were < 7 days old represented exposure of the early developmental stage. The A sample
202   consisted of 3 x 1 cm leaf discs collected from each plant that had hosted larvae. The second
203   sampling (B) that occurred when larvae were > 7 days of age represented exposure of the later
204   developmental stage. Because the larger larvae consume more leaf material and would be
205   exposed to more pesticide, sampling B consisted of 6 x 1 cm leaf discs collected from each plant
206   that hosted larvae. The locations on the plant from which leaf discs were collected were chosen
207   arbitrarily, but when available, samples were collected from all portions (new growth to old
208   growth) of the plant. All leaf discs from a replicate pair were composited within a Ziploc bag
209   and placed into a freezer (-20° C) until analysis. Compositing the first and second sampling
210   resulted in 31 cm^2 of leaf sample with a mass that ranged from 0.173 – 0.306 g wet weight. All
211   residue data for leaf tissues represent clothianidin concentrations among plants in the replicate.
212   A single 5th instar larva (0.28 – 1.64 g per larva) and adult was collected for analysis from each
213   of 3 randomly chosen replicates in each dose level and control. The larvae were placed into
214   separate plastic culture tubes and placed into a freezer until analysis.
215
216   Based on the response observed during Experiment 1, a second experiment (Experiment 2) was
217   conducted. Fewer plants were used in this experiment because the plants received from the
218   vendor were in poor health due to heavy aphid infestation. The aphid populations were
219   addressed before the experiment by both physical removal and soap water sprays, but the amount
220   of time available to rehabilitate the plants was limited because of declining temperatures and
221   photoperiod associated with the change in season (summer to fall). Both factors reduced plant
222   growth and recovery meaning fewer plants suitable for the study were available for Experiment
223   2. As a result, the number of replicates was reduced to 3 per dose level and control. The dose
224   rates in Experiment 2 were higher relative to Experiment 1: 1.93 (SD=0.01), 3.86 (0.004), 7.71
225   (0.006), 15.40 (0.004), and 30.08 (0.004) g of product per pot. Other differences relative to
226   Experiment 1 include a shorter time period between pot dosing (September 4, 2018) and
227   monarch egg receipt (September 24, 2018), and newly hatched larvae (2 – 7 per replicate) were
228   placed onto random locations of the associated plant by using a camel-hair brush rather than by
229   attaching the cup to the plant. Leaf sampling in Experiment 2 was like that for Experiment 1
230   with the exception that the samples were not composited among replicates. The composite leaf
231   sample mass collected from each plant ranged from 0.266 – 0.363 g wet weight. All pupae were
232   transferred from the terraria to a separate rearing cage to shelter them from the sun and heat.
233   Pupae were moved by first tying dental floss to the cremaster, removing it from the attachment
234   surface by gripping the silk pad with tweezers and pulling, and then taping the dental floss to the
235   rearing cage interior ensuring the pupae hung naturally (Figure 1D). In addition, not all larvae
236   collected for analysis were in the 5th instar because larvae in some dose levels (4 and 5) died or

237   went missing before attaining the 5th instar. As a result, some of the larvae collected for analysis
238   were dead and much smaller (0.003 g) than those collected during Experiment 1. Also, no adult
239   butterflies were available from Dose Levels 4 and 5, and only a single adult was available for
240   Level 3. As a result, a single adult butterfly was chosen from each replicate that had adults
241   during Experiment 2.

242

243   It should be noted that aphid populations on plants in the control group of Experiments 1 and 2
244   were very high most likely due to those plants being clothianidin-free. This impacted plant
245   health, our ability to locate newly hatched larvae, and could affect larval behavior. The last
246   noted impact could result in larvae having difficulty finding suitable foraging locations. While
247   plants at the lower dose levels in Experiment 1 also had aphids, they were less abundant.

248

249   Due to concerns for the effect of aphids on results in the first two experiments, Experiment 3 was
250   conducted to ensure the milkweed plants were not infested with aphids. Rather than purchase
251   milkweed from the vendor, seeds were sown in a seed-starter soil (Miracle-Gro® seed starting
252   potting mix) and placed under plant growth lights in the laboratory. Approximately 2-3 weeks
253   after emergence, the seedlings were transplanted into larger pots containing a custom potting soil
254   mix (described previously) and placed into terraria outside to expose the seedlings to natural
255   environmental conditions (photoperiod, temperature, humidity) for continued growth. Watering
256   and fertilization of the plants were conducted for these plants as described previously for
257   Experiments 1 and 2. The plants were randomly assigned into the control group and 5 dose
258   levels. The dose rate (g product per pot) was 0.78, 1.55, 3.11, 6.2, and 12.11 for Dose Levels 1-
259   5, respectively. The rates for this experiment were lower relative to Experiment 2 due to smaller
260   soil surface area for Experiment 3 (0.0345 m^2) compared to Experiment 2 (0.0856 m^2). Control
261   plants were not treated with the granular pesticide product. Milkweed plant height and the
262   number of leaf pairs per plant at the start of the experiment were 53 cm (SD=5.2) and 33 pairs
263   (SD=5.2), respectively. As was done in Experiment 2, larvae were individually transferred to the
264   plants at hatch. In contrast to the first two experiments, each replicate was comprised of one
265   larva for each of two plants to ensure adequate availability of food and reduce competition
266   among larvae for available food. In addition, each larva was transferred to an apical leaf.
267   Leaves were sampled at three time points during larval growth (first, middle, and last instar)
268   from all plants on which the larvae fed. Whole leaves were taken from the portion of the plant
269   (top, middle, or lower third) on which the larva was feeding at the time and composited (3 leaves
270   per composite) together for analysis. Most of the leaves were taken from the top third of the
271   plants. Data for larval growth and pupation were collected as described previously in
272   Experiments 1 and 2. No larvae or adults were collected for analysis due to the reduced number
273   of larvae per replicate.

274

275   *Analytical Procedure*

276   All samples were weighed before analysis. Approximately 0.2 to 0.3 g of milkweed leaves or
277   individual larvae and adults were dried/homogenized with sodium sulfate. Samples were spiked
278   with a recovery surrogate (imidacloprid-d^4; Cambridge Isotope) and extracted using an ASE®
279   200 (Dionex) using a 50:50 mixture of acetone:dichloromethane (1500 psi; 100 ºC). The extracts
280   were solvent exchanged into acetonitrile and passed through solid-phase extraction cartridges
281   containing 500 mg graphitized carbon (Restek). The samples were then evaporated to 200 μL
282   and spiked with an internal standard (clothianidin-d$_3$; Sigma-Aldrich). Extracts were analyzed
283   on an Agilent 1260 bio-inert liquid chromatograph (LC) coupled to an Agilent 6430 tandem
284   mass spectrometer (MS-MS). Instrument details are given in Hladik & Calhoun (2012). The
285   theoretical limit of detection (LOD) was 3 ng/g for the leaves (0.3 g sample) and 1 ng/g for the
286   larvae and adults (1 g sample). All reported concentrations herein are for wet weight.
287
288   *Statistical Analysis*
289   Mortality and larval growth were the endpoints evaluated during this study. Length
290   measurements for all larva in a replicate were averaged each day throughout larval development
291   during Experiments 1 and 2. Larva length measurements for each replicate during Experiment 3
292   represent a single larva. A bivariate plot of larva age and length for each replicate in the dose
293   levels and control yielded an independent indication of larval growth in response to dietary
294   clothianidin exposure. Larval growth appeared to increase exponentially until larvae began to
295   pupate at which time larvae in the replicate dropped out of the average size calculation. An
296   exponential curve was fit (Microsoft Excel©) to growth data for larvae that had reached at least
297   the 4th instar (13 – 25 mm length) to estimate the growth rate as described by the exponential
298   growth equation $A_t = A_0 e^{kt}$ where A_t is larva length at time t, A_0 is the intercept, k is the growth
299   rate, and t is time in days. Since some larvae died during the early instars, the potential influence
300   of clothianidin on larval growth was assessed by comparing growth rate for larger larvae (at least
301   at the 4th instar) to clothianidin concentrations in leaves of plants consumed by the larger larvae.
302   Growth data were initially compared by ANOVA followed by the post-hoc Holm-Sidak test (α =
303   0.05).
304
305   Microsoft Excel© was used to estimate (by Probit Analysis, Finney, 1952) the LC$_{50}$ based on
306   clothianidin concentrations in leaves. An LC$_{50}$ was estimated for larvae and for total mortality
307   (sum of mortality for larvae, prepupae, and pupae) for Experiment 2. An LC$_{50}$ was estimated
308   only for larval mortality in Experiment 3 since 100% mortality occurred in Dose Levels 2
309   through 5 not enabling estimation of an LC$_{50}$ for total mortality.   No dose-response relation was
310   evident for mortality or larval growth during Experiment 1. The numbers of larvae that were
311   collected for residue analyses and that could not be found (i.e. missing larvae) were not included
312   in the Probit analyses.
313

314 Residue levels in leaves and larvae, as well as adult mass and larval mortality, were compared
315 among dose levels and control by ANOVA if the appropriate assumptions for the test were
316 satisfied, or by the nonparametric Kruskal-Wallis test if the assumptions were violated. Where
317 possible, the ratio of clothianidin concentrations in larvae and leaves (larva:leaf) was evaluated
318 as a means to estimate clothianidin concentrations in larvae based on analysis of leaves. If the
319 ratio is consistent regardless of exposure (i.e., concentrations in leaves), then the ratio may be a
320 useful tool for estimation of larval exposure.
321

Results

323 *Experiment 1*
324 The dose rates to soils in Experiment 1 did not result in measurable clothianidin concentrations
325 in leaves in Dose Levels 1 – 3 (method detection limit or MDL = 4.3 ng/g), but did result in
326 measurable concentrations in milkweed leaves in Dose Levels 4 (11 ng/g, SD = 3.6) and 5 (54
327 ng/g, SD = 27.2) (Table 2). Those concentrations did not differ significantly (χ^2 = 3.00, p =
328 0.083, 1 df). Concentrations in larvae were also below detection (0.9 ng/g) in Dose Levels 1 – 3
329 and in adults were below detection (1.4 ng/g) in Dose Levels 1 – 4 (Table 2). The concentrations
330 in larvae were marginally different between Dose Levels 4 and 5 (χ^2 = 3.857, p = 0.05, 1 df).
331 Two of the three adult butterflies from Level 5 analyzed for clothianidin had detectable
332 concentrations (3.1 and 5.2 ng/g).
333

334 The relation between clothianidin concentrations in the leaves and effects in monarch butterflies
335 was not clear in this experiment. Mortality of larvae and pupae was observed, but neither was
336 proportional to clothianidin exposure (Figure 2). Mean adult mass (F-ratio = 0.59, 5 df, p = 0.7)
337 and forewing length (F-ratio 1.19, 5 df, p = 0.35) did not vary with clothianidin exposure and did
338 not differ significantly among the dose levels and control (Table 3). The percentage of missing
339 larvae varied among the dose levels and control but did not trend with clothianidin
340 concentrations in leaves (Figure 2). Only the percentage of larvae reaching the adult stage
341 trended with clothianidin concentrations in leaves. However, the low percentage reaching the
342 adult stage in the control may have resulted from the high percentage of missing larvae and dead
343 pupae, both indicating stressful conditions for the larvae and pupae.
344

345 Larva growth for all dose levels and control increased from hatch until approximately 9 days
346 post-hatch when larvae began to pupate (Figure 3). The growth rates during that time (0.26 –
347 0.28, Table 4) did not differ significantly among the dose levels and control (F ratio = 0.7, 5 df, p
348 = 0.6) and was not proportional to clothianidin concentrations in the leaves.
349

350 *Experiment 2*
351 The greater clothianidin dose to soils during this experiment resulted in detectable concentrations
352 in leaves and larvae from all dose levels (Table 2). Average clothianidin concentrations in leaves

353   were 20 (SD = 3.4), 105 (80.4), 107 (16.3), 263 (152.5), and 843 ng/g (614.6) for Dose Levels 1,
354   2, 3, 4, and 5, respectively (Table 2). Clothianidin concentrations in larvae did not differ
355   significantly among the dose levels (Kruskal-Wallis statistic 6.255, 4 df, p = 0.18), but they did
356   peak at 58 ng/g when concentrations in leaves averaged 107 ng/g (Dose Level 3) and decline at
357   the higher dose levels despite higher concentrations in leaves (Table 2). Detectable clothianidin
358   concentrations in adults were found for only Level 3 (107 ng clothianidin/g leaf) – no adults
359   eclosed in Level 4 (263 ng/g leaf) and no pupae were formed in Level 5 (843 ng/g leaf).
360
361   Larval and total mortality (larval plus pupal mortality) were inversely proportional to
362   clothianidin concentrations in leaves (Figure 2). The LC_{50} for total mortality (47 ng/g, 95% CI =
363   29.3 – 75.8) was lower than the LC_{50} for larval mortality (205 ng/g, 95% CI = 117.4 – 357.0).
364   The lower total mortality LC_{50} (mortality at the larval and pupal stages) indicates that pupal stage
365   may be more sensitive to clothianidin exposure than larval stage.
366
367   A relation was also evident between clothianidin concentrations in leaves and both the
368   percentage of missing larvae and the percentage of larvae reaching the adult stage (Figure 2).
369   The percentage of missing larvae increased from 20 to 47% in Dose Levels 1 (20 ng/g leaf) and 5
370   (843 ng/g leaf), respectively. However, the high percentage of missing larvae in the control
371   (60%) indicates a factor other than clothianidin may have contributed to larval disappearance.
372   The percentage of larvae reaching the adult stage declined from a high of 50% in Dose Level 1 to
373   0% in Dose Levels 4 and 5 (263 and 843 ng/g leaf, respectively). The lower percentage in the
374   control (33%) is likely a reflection of the high percentage of missing larvae in the control.
375
376   Neither adult mass (Kruskal-Wallis statistic = 5.6, 2 df, p = 0.061) nor forewing length (Kruskal-
377   Wallis statistic 1.156, 2 df, p = 0.561) differed significantly among dose levels and control
378   (Table 3). Relative to Experiment 1, larvae growth was not as uniform among the dose levels
379   (Figure 3). Larvae in the Control and in Dose Levels 1 – 3 (20 – 107 ng/g leaf) grew faster than
380   larvae in Dose Levels 4 and 5. In fact, the growth rates declined significantly (F ratio = 5.7, 4 df,
381   p = 0.014) from Dose Levels 1 – 5 (Table 4). Relative to larvae in the control group, growth of
382   larvae consuming leaves with an average clothianidin concentration up to 155 ng/g (Dose Level
383   5) was not significantly reduced (Table 4). Clothianidin concentrations in leaves of 177 ng/g
384   (Dose Level 4) did significantly reduce larval growth (p = 0.034, Holm-Sidak post-hoc
385   comparison) (Table 4).
386
387   Clothianidin concentrations in the different matrices collected from a dose level consistently
388   declined from leaves to larvae to adults (Table 2). This consistency indicates estimation of larval
389   body burdens based on concentrations in leaves may be possible based on a ratio of
390   concentrations between larvae and leaves (larva:leaf). The ratio ranged from 0.14 – 0.74 in
391   Experiment 1, averaging 0.42 (SD = 0.230), and from 0.04 – 0.8 in Experiment 2, averaging 0.30

392   (SD = 0.233). However, a bivariate plot of the ratio relative clothianidin concentrations in leaves

393   revealed that the ratio was inversely proportional to clothianidin concentrations in the leaves

394   (Figure 4). The inverse relationship indicates that the larva:leaf ratio is not independent of

395   clothianidin concentrations in the leaves, and that a single ratio would not be sufficient to

396   estimate larval contamination. The decline of clothianidin in larvae despite increasing dietary

397   contamination may also mean larvae are consuming less food as clothianidin contamination

398   increases, which was indicated by the lower growth rates (Table 4 and Figure 3).

399

400   *Experiment 3*

401   Average clothianidin concentrations in leaves during Experiment 3 were 54 (SD = 42.3), 91

402   (41.9), 232 (94.2), 1,007 (461.3) and 1,545 ng/g (481.2) for Dose Levels 1, 2, 3, 4, and 5,

403   respectively (Table 2). Those concentrations increased with dose and differed significantly (χ^2 =

404   20.593, 4 df, p <0.001) (Table 2). Clothianidin concentrations in leaves in Dose Levels 4 (1,007

405   ng/g) and 5 (1,545 ng/g) were higher than in leaves from Dose Level 5 of Experiment 2 (843

406   ng/g). No adults were analyzed since 3 of the 4 were from the Control group while the 4th was

407   from Dose Level 1 in which clothianidin residues, based on results from Experiments 1 and 2,

408   would likely have been undetectable.

409

410   Larval consumption of the contaminated leaves negatively affected monarch butterflies. Larval

411   mortality ranged from 50% in Dose Levels 1 (54 ng/g leaf) and 2 (91 ng/g leaf), to 60% in Dose

412   Levels 3 (232 ng/g leaf) and 4 (1,007 ng/g leaf), to 100% in Dose Level 5 (1,545 ng/g leaf)

413   (Figure 2). Between 33 (Level 1) and 50% (Level 2) of the monarch butterflies died at the pupal

414   stage. The LC_{50} for larval mortality (66 ng/g, 95% CI 9.7 – 451.4) was lower than the LC_{50} for

415   larvae during Experiment 2 (205 ng/g) but approximated the LC_{50} for total mortality in

416   Experiment 2 (47 ng/g). An LC_{50} for total mortality was not estimated for Experiment 3 since

417   partial mortality was observed only at Dose Level 1 (Figure 2). Larval mortality reduced the

418   number of larvae that reached the adult stage from a high in the Control group (75%) to 17% in

419   Dose Level 1 and 0% in the higher dose levels.

420

421   Larval growth (Figure 3 and Table 4) differed significantly among dose levels (F-ratio = 4.6, 3

422   df, p = 0.037). Growth was unaffected by clothianidin up to a concentration of 355.6 ng/g in

423   leaves but was affected at 1,154 ng/g (Dose Level 4). No larvae in Dose Level 5 reach the pupal

424   stage. Only four butterflies successfully eclosed in this experiment, three in the control and one

425   in Dose Level 1 (54 ng/g leaf).

426

427   No data were collected for the adult butterflies since there was only a single adult from the

428   treated replicates.

429

430 All data generated from the present study and used in the preparation of this paper can be
431 accessed at https://doi.org/10.5066/P9QX4OJW.
432

433 **Discussion**
434 The goal for this study was to estimate effect levels relating clothianidin residues in milkweed
435 leaves to adverse effects in monarch butterflies. Such data would assist resource managers
436 focused on the risk of neonicotinoid contamination in general and clothianidin specifically to
437 monarch butterflies. Some investigations have reported on the correlations between increased
438 neonicotinoid use and butterfly population trends (Gilburn et al., 2015; Forister et al., 2016), but
439 they are limited in their utility for resource managers concerned with monarch butterfly
440 conservation because they did not directly evaluate neonicotinoid toxicity. Several studies have
441 directly investigated neonicotinoid toxicity to lepidopterans and reported adverse effects in
442 nontarget (Krischik et al., 2015; Pecenka & Lundgren, 2015; Basley & Goulson, 2018;
443 Whitehorn et al., 2018) and target (Ding et al., 2018) lepidopterans. Three of those studies report
444 dietary exposure levels that have utility for risk estimation (Basley & Goulson, 2018; Ding et al.,
445 2018, Krischik et al., 2015). One evaluated clothianidin toxicity for monarch butterflies
446 (Pecenka & Lundgren, 2015), but it did not report dietary exposure levels. The goal for the
447 present study was estimation of the dietary exposure level to clothianidin associated with adverse
448 effects in monarch butterflies.
449

450 Three separate experiments were conducted to attain this goal. The first experiment found no
451 significant effect (larval growth and survival, adult mass, pupal survival) at mean clothianidin
452 concentrations in leaves up to 59 ng/g wet weight. This concentration in leaves is generally
453 greater than what has been reported in the literature. Bredeson & Lundgren (2019) reported
454 concentrations of 0.6 – 9.7 ng/g in leaves of cover crops interseeded with thiamethoxam-treated
455 corn seed crops; Main et al. (2017) reported clothianidin concentrations from below detection
456 (0.002 – 0.66 ng/g) – 2.01 ng/g in plants from wetlands bordering treated canola crops; Botías,
457 Hill & Goulson (2016) reported concentrations ranging from below detection (detection limit =
458 0.12 ng/g) to 11.45 ng/g in foliage of plants bordering treated oilseed rape fields; Basley &
459 Goulson (2018) reported clothianidin concentrations ranging from below detection (0.2 ng/g) –
460 48 ng/g in plants from the margins of clothianidin treated wheat fields; and Olaya-Arena &
461 Kaplan (2019) reported concentrations in milkweed plants (median less than the detection limit,
462 1.06 ng/g) ranging from below detection – 56.5 ng/g. The results from these and Experiment 1
463 of the present study indicate that clothianidin concentrations in wild milkweed plants are
464 generally not high enough to adversely affect monarch butterflies. However, the amount of data
465 on clothianidin concentrations in nontarget plant foliage is limited.
466

467 The second and third experiments were conducted at a higher dose level to increase the
468 likelihood of finding an exposure level associated with observable effects; Experiment 3 was

469   conducted to eliminate the possible effect of aphids during Experiment 2. The elevated exposure
470   in both experiments led to adverse effects on survival and growth. Lethal effect levels (LC50 for
471   clothianidin in leaves) ranged from 47 – 205 ng/g, while larval growth was reduced at 177 ng/g
472   during Experiment 2 and 1,153.9 ng/g during Experiment 3. These results are in line with those
473   reported by others. Basley & Goulson (2018) reported that larva size for the common blue
474   butterfly (*Polyommatus icarus*) was affected at a relatively low clothianidin concentration in
475   leaves (14.5 ng/g). Results from the present study indicate no effect on monarch larval growth at
476   that exposure level. However, the same study reported that 439.1 ng/g in leaves significantly
477   decreased larval survival, an exposure level higher than the LC_{50} values estimated in the present
478   study. Ding et al. (2018) estimated a dietary LC_{50} of 27.77 μg/g (27,700 ng/g) for larvae of a
479   pest moth species (black cutworm, *Agrotis ipsilon*). That value was much greater than the
480   dietary LC_{50} values estimated in the present study and by Basley & Goulson (2018). Pecenka &
481   Lundgren (2015) reported an LC_{50} of 15.6 ng/g for monarch larvae, but it was unclear if that
482   value represents concentration in leaves or in the solution used to dose those leaves.
483
484   While effect levels were estimated for monarch butterflies based on data from the present study,
485   the application rates in the Experiments 2 and 3 exceeded those recommended on clothianidin
486   product use labels. Those application rates resulted in residue levels in the leaves largely higher
487   than what has been reported in the literature, but that were useful in generating exposure levels to
488   achieve effects on the measured endpoints and estimate effect levels.
489   During the present study, the application rates were 9.4 – 681.7 g a.i./acre (grams of active
490   ingredient [clothianidin] per acre) for Experiment 1 and 228.0 – 3,553.8 g a.i./acre for both
491   Experiments 2 and 3. No effect was observed at rates less than the label rate, during Experiment
492   1, neither were they evident at higher rates. The second and third experiments were conducted at
493   greater application rates to determine the residue level in the plants that leads to an adverse
494   effect. The rates in the Experiment 2 and 3 were much greater than those noted on the labels for
495   several pesticide products (Arena® 0.25G, Poncho®, Belay®, Sepresto 75 WS, Arena® 50 WG,
496   Clutch™ 50 WDG) with clothianidin (4.5 – 180.7 g a.i./acre). The results indicate monarch
497   butterflies may be relatively insensitive to clothianidin at label application rates, clothianidin
498   availability from potting soil may be low relative to that typical for top soil, or a combination of
499   the two were needed to elicit effects.
500
501   High aphid density on some milkweed species can induce production of the toxin cardenolide by
502   the plant (reviewed in Agrawal et al., 2012), and Zalucki et al. (1990) reported early instar
503   monarch larvae are negatively affected by high cardenolide concentrations. Since the milkweed
504   plants in the control and low dose groups of Experiment 1 and in the control group of
505   Experiment 2 suffered from high aphid densities, the aphids may have indirectly affected the
506   monarch larvae. Indeed, larval growth rate in the control group of Experiment 2 was low relative
507   to the controls in the other two experiments, but it was not significantly lower. On the other

508   hand, the percentage of missing larvae in the controls for Experiments 1 and 2 (40 – 60%)_was
509   an issue. However, toxin production by swamp milkweed, the species used in this study, is not
510   induced by high aphid densities (Martel & Malcolm, 2004; Zhender & Hunter, 2007). Therefore,
511   the effect of aphids on the results of this study may be behavioral resulting in the larvae dropping
512   from the plant leading the high percentage missing in the control plants.

513

514   To our knowledge, this is the first study to present data demonstrating that soil clothianidin
515   residues can result in clothianidin exposure for adult butterflies through larval consumption of
516   contaminated leaves. Krischik et al. (2015) conducted a similar study in which they tested the
517   possibility that adult butterflies and lady beetles may be affected as a result of feeding from
518   flowers contaminated by imidacloprid translocated from soil. While lady beetle survival was
519   affected, they reported no significant mortality for adult monarch or painted lady (*Vanessa*
520   *cardui*) butterflies exposed to flowering milkweed plants (*Asclepias curassavica*). While residue
521   levels in butterflies were not measured in that study, the lack of effect in the butterflies should
522   not be assumed to indicate no exposure since exposure was clearly adequate to affect lady
523   beetles. However, exposure for the lady beetles and butterflies was presumed in that study rather
524   than confirmed through analyses. Exposure of adult butterflies in the present study as a result of
525   larval consumption of leaves was assessed by analyses of larvae and adults. There is no
526   indication of bioaccumulation of clothianidin, but clothianidin is not completely metabolized and
527   eliminated by larvae allowing its transfer to adults. Also, while adult butterflies can be captured
528   and analyzed for clothianidin, doing so may not be the best method in field studies to determine
529   butterfly exposure to systemic insecticides. That is because in the present study, either adverse
530   effects occurred at earlier life stages when residues were not detectable in adults, or adverse
531   effects at earlier stages prevented complete development to the adult stage. The concentrations
532   detected in the adult butterflies would represent a sublethal exposure, the toxicity of which is
533   unknown. To our knowledge, the sublethal toxicity of clothianidin for adult butterflies has not
534   been reported. Whitehorn et al. (2018) reported sublethal toxicity (reduced size) of the
535   neonicotinoid insecticide imidacloprid for adult farmland butterflies (*Pieris brassicae*) exposed
536   during the larval stage. The present study did not find adult mass or forewing length were
537   significantly affected by larval consumption of clothianidin contaminated leaves.

538

539   Neonicotinoid insecticide mobility in soils is inversely related to soil organic carbon (Zhang et
540   al., 2018; Singh et al., 2018). Zhang et al. (2018) reported that sorption affinity of clothianidin
541   for four different soils declined with organic carbon content, while Singh et al. (2018) reduced
542   clothianidin concentrations in the leachate of soils by amending them with farm yard manure.
543   The organic carbon content for the potting soil used in the present study was measured to be
544   approximately 80%. The soil organic matter content in natural top soils of the Western (~1%,
545   Derner et al., 2019) and Northern Great Plains (~3%, Frank et al., 1995) are much lower, and
546   likely represent the expected organic content of soils on which the application rates for

547   clothianidin are based. Therefore, availability of clothianidin from the potting soil was likely
548   significantly reduced in the present experiment compared to what would be expected for natural
549   top soils where this pesticide is typically applied. As a result, the effects observed during the
550   present study are better associated with clothianidin concentrations in the leaves as opposed to
551   the soil application rates.
552

553   **Conclusions**
554   The present study was conducted to determine the effect of dietary clothianidin exposure upon
555   monarch butterfly development. This study is the first to report the transfer of a clothianidin
556   from soil to adult monarch butterflies only from larvae exposed through consumption of
557   contaminated leaves. Monarch butterfly survival and larval growth were adversely affected by
558   increased clothianidin concentrations in leaves. But the clothianidin concentration leading to
559   those effects was variable with LC_{50}s ranging from $47 - 205$ ng/g and effects upon growth at 177
560   and 1,154 ng/g. The measured effects levels were greater than most of the data for clothianidin
561   concentrations in leaves reported to date. When combined with data from field monitoring of
562   clothianidin concentrations in milkweed leaves, those effect levels will aid in risk estimation for
563   monarch butterflies exposed to that neonicotinoid in the field. Further research is needed to
564   determine if toxicologically relevant doses can be transferred from larvae to adult butterflies.
565

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572

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Figure 1

Photographs of swamp milkweed (*Asclepias incarnata*) in screened terraria during Experiment 1 (1A), newly hatched monarch larva in cup with 1 cm leaf disc from milkweed plant (1B), cup attached to milkweed plant (1C), and pupae in a rearing cage (1D)

1A   1B

1C   1D

Figure 2

Effect of clothianidin concentrations in leaves on larval and pupal survival during Experiments 1 (top), 2 (middle), and 3 (bottom).

The sum of % dead (larvae and pupae), % missing, and % reaching adult stage is 100%.

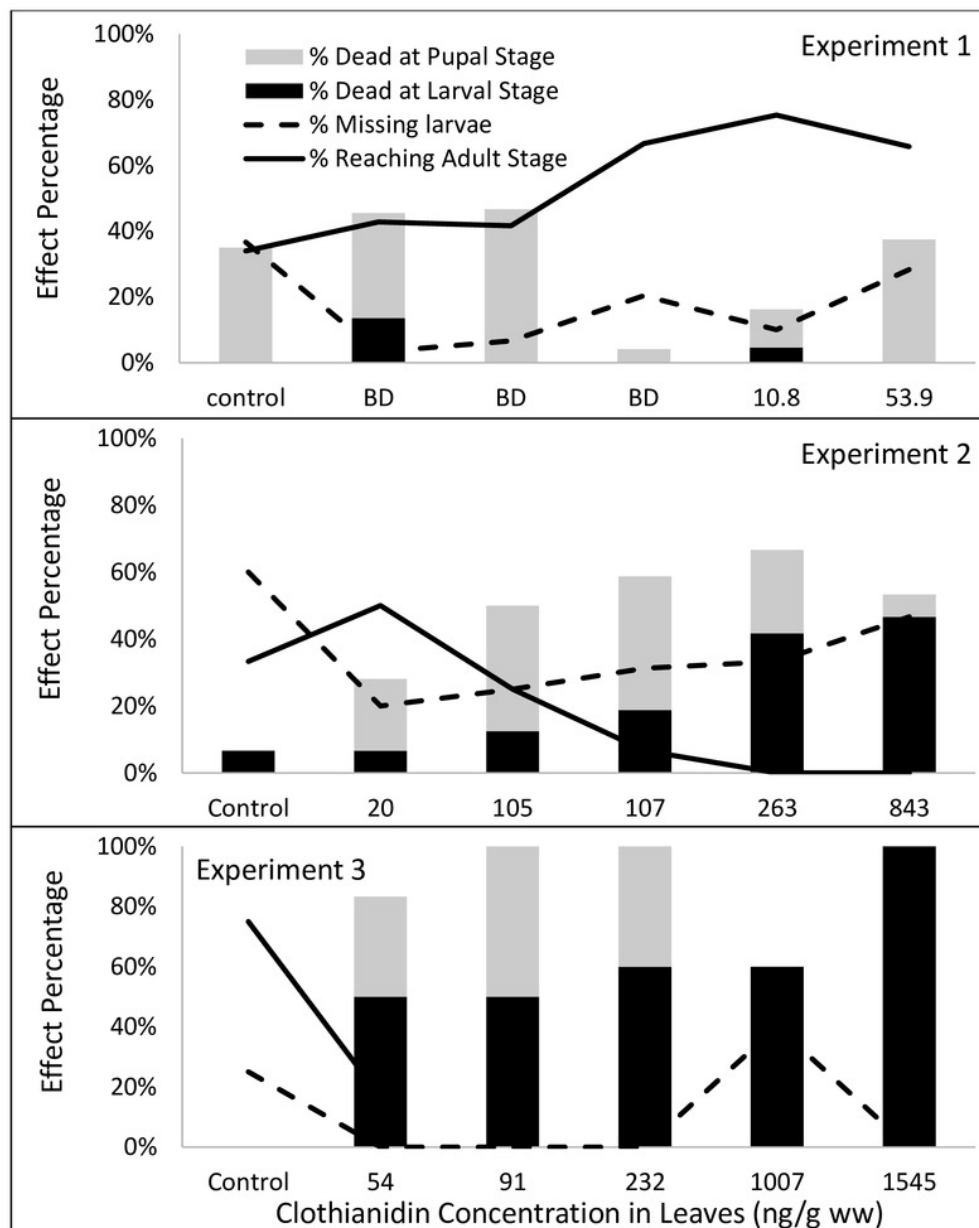



Figure 3

Larvae growth during Experiments 1 (top), 2 (middle), and 3 (bottom).

The shown lines are based on the mean larval length for replicates within the respective dose level or control and terminate on the last day prior to formation of pupae in the dose level or control. The concentrations shown in the legends indicate the mean for clothianidin in the leaves from all plants on which larvae had fed. Growth rates for larvae in Experiment 1 did not differ significantly among dose levels (F-ratio = 0.492, df = 5, p = 0.8), but they did differ significantly in Experiments 2 (F ratio = 5.7, 4 df, p = 0.014) and 3 (F ratio = 4.6, 3 df, p = 0.037). Asterisks shown in Experiment 3 denote dose levels (4 and 5) in which no larvae survived to the pupal stage.

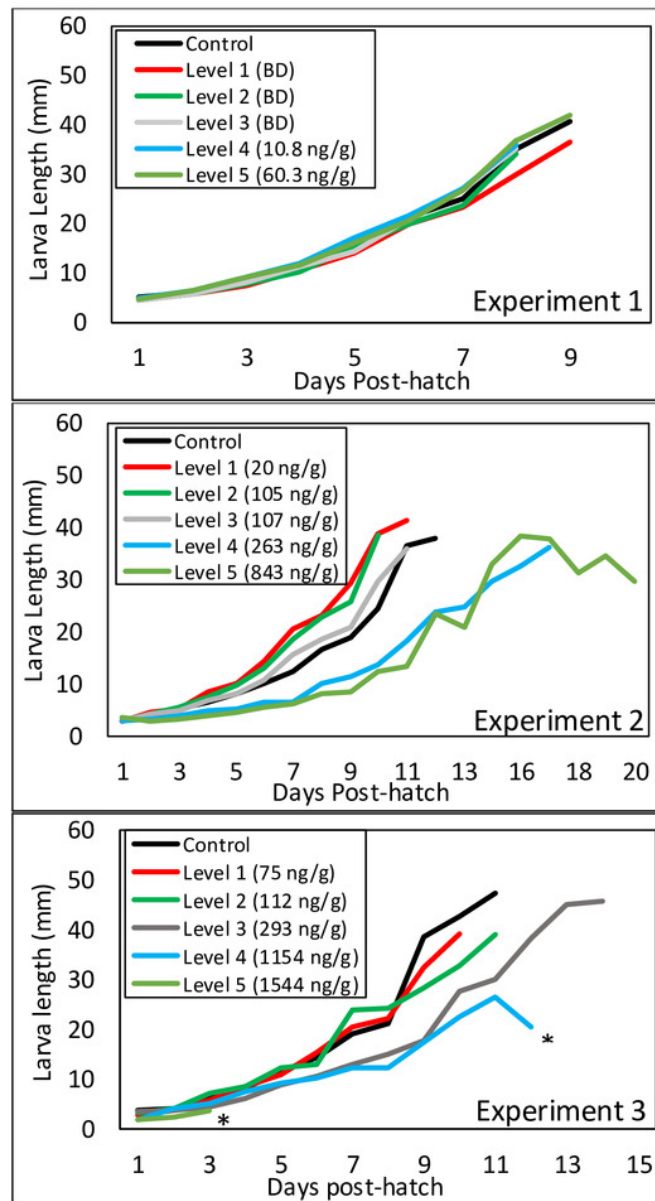
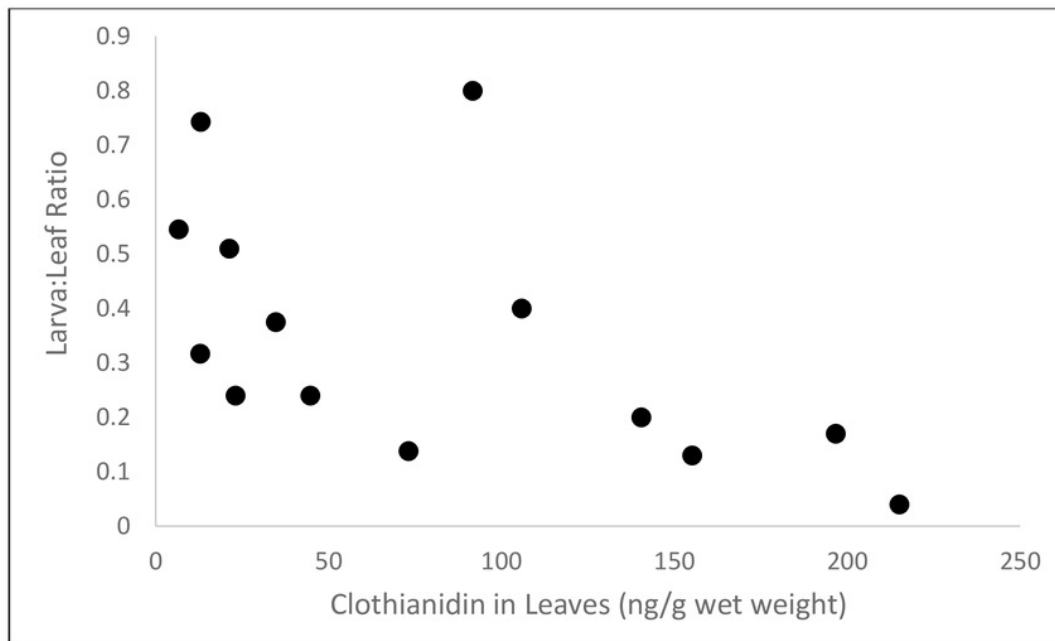



Figure 4

Relation between clothianidin concentrations in leaves and the ratio of clothianidin concentrations in larvae and the leaves (Larva:Leaf ratio) during Experiments 1 and 2.

Dates and environmental conditions for Experiments 1, 2, and 3.

Experiment	Start Date mm-dd-yyyy	End Date mm-dd-yyyy	Temperature (Celsius)		Relative Humidity (%)		Photoperiod (hh:mm)	
			Min	Max	Min	Max	Min	Max
1	07-15-2018	08-20-2018	20.5	35.0	50	79	13:05	13:52
2	09-30-2018	10-30-2018	8.3	34.4	40	82	11:30	11:54
3	09-20-2019	10-11-2019	13.9	35.0	41	86	11:35	12:12

Clothianidin concentrations in leaves, larvae, and adults during the three experiments.

Dose Level	Dose Rate [1] (g/pot)	Clothianidin Concentrations (ng/g) in Matrices [2] (mean±SD, n)		
		Leaves	Larvae	Adults
		Experiment 1		
Control	0	BD	BD	BD
1	0.08	BD	BD	BD
2	0.21	BD	BD	BD
3	0.64	BD	BD	BD
4	1.92	11±3.6, 3	6±3.3, 3	BD
5	5.77	54±27.2, 2	13±3.4, 3	3±2.6, 3
		Experiment 2		
Control	0	BD	BD	BD
1	1.93	20±3.4, 3 [a]	5±5.4, 3	BD
2	3.86	105±80.4, 3 [a,b]	22±16.3, 2	BD
3	7.71	107±16.3, 3 [a,b]	58±22.1, 2	5, 1
4	15.4	263±152.5, 3 [b,c]	18±13.5, 2	NP
5	30.08	843±614.6, 3 [c]	20, 1	NP
		Experiment 3		
Control	0	BD	NL	NS
1	0.78	54±42.3, 6 [a]	NL	NS
2	1.55	91±41.9, 4 [a,b]	NL	NP
3	3.11	232±94.2, 5 [a,b]	NL	NP
4	6.20	1007±461.3, 6 [b,c]	NL	NP
5	12.11	1545±481.2, 4 [c]	NL	NP

[1] Grams of product (clothianidin comprises 0.25% of product mass) added to the pot. Soil surface areas (m^2) in the pots was 0.0856 for Experiments 1 and 2, and was 0.0345 for Experiment 3.

[2] Significant differences between means were found for larvae in Experiment 1 ($\chi^2 = 3.857$, df = 1, p = 0.05), and for leaves in Experiments 2 (F-ratio = 10.236, 4 df, p = 0.001, post-hoc pair-wise comparison by Tukey's HSD) and 3 (Kruskal-Wallis test on the ranks H = 20.593, 4 df, p <0.001, post-hoc pair-wise comparisons by Dunn's method with $\alpha = 0.05$). Means with different superscript letters are significantly different. Significant differences were not found for the other matrices.

BD – below detection. Detection limit ~ 1 ng/g per gram of sample (leaf, larva, and adult mass (g) ranged from 0.173 – 0.781, 0.003 – 1.644, and 0.426 – 0.977, respectively); NP – no pupae in dose level; NL – no larvae available for sampling; NS – adults not sampled.

Larval growth rates and adult size in relation to clothianidin concentrations in leaves.

Dose Level	Clothianidin in leaves (ng/g) [1] (mean±SD)	Adult Mass (g) [2] (mean±SD, n)	Forewing Length (mm) [3] (mean±SD, n)
	Experiment 1		
Control	BD	0.65±0.197, 4	47.4±0.37, 4
1	BD	0.75±0.168, 5	46.7±5.33, 5
2	BD	0.73±0.118, 5	50.3±3.48, 5
3	BD	0.71±0.186, 6	49.4±3.02, 6
4	10.8±3.2	0.80±0.111, 5	50.8±0.73, 5
5	60.3±22.2	0.69±0.035, 4	49.3±0.64, 4
	Experiment 2		
Control	BD	0.51±0.068, 3	49.4±1.70, 3
1	20.2±3.4	0.67±0.065, 3	49.9±1.18, 3
2	105.4±80.4	0.57±0.012, 3	46.2±6.14, 3
3	107.2±16.3	0.52, 1	47.0, 1
4	177±52.8	NP	NP
5	155	NP	NP

[1] Mean clothianidin concentration in leaves of plants consumed by larvae that had grown to at least the 4th instar ($\leq$ 15 mm in length).

[2] Adult mass did not differ significantly among the dose levels and control for Experiments 1 (F-ratio = 0.59, 5 df, p = 0.7) and 2 (Kruskal-Wallis statistic = 5.6, 2 df, p = 0.061).

[3] Forewing length did not differ significantly among the dose levels and control for Experiments 1 (F-ratio 1.19, 5 df, p = 0.35) and 2 (Kruskal-Wallis statistic = 1.156, 2 df, p = 0.561).

NP – no pupae in dose level.

Monarch larvae growth in relation to clothianidin concentrations in leaves.

Dose Level	Growth Rate (k) [1,2] (mean±SD, n)	Intercept (A_0) (mean±SD)	Clothianidin in leaves [3] (ng/g ± SD)
	Experiment 1		
Control	0.26±0.040, 5	4.08±0.746	BD
1	0.26±0.026, 6	3.67±0.632	BD
2	0.26±0.021, 6	3.65±0.893	BD
3	0.28±0.022, 6	3.57±0.668	BD
4	0.27±0.032, 6	3.98±0.707	10.8±3.2
5	0.27±0.025, 5	3.87±0.872	60.3±22.2
	Experiment 2		
Control	0.24±0.023, 3 [a,b]	2.35±0.155	BD
1	0.27±0.007, 3 [a]	2.57±0.1258	20.2±3.4
2	0.26±0.029, 3 [a]	2.53±0.1903	105.4±80.4
3	0.23±0.042, 3 [a,b]	2.60±0.1046	107.2±16.3
4	0.17±0.035, 2 [c]	2.71±0.663	177±52.8
5	0.16, 1 [b,c]	2.63	155
	Experiment 3		
Control	0.27±0.027, 3 [a]	2.86±0.170	BD
1	0.27±0.035, 3 [a]	2.54±0.199	74.7±53.8
2	0.27±0.035, 3 [a]	2.58±0.375	111.8±11.6
3	0.22±0.018, 4 [a,b]	2.54±0.244	355.6±152.8
4	0.17, 1 [b]	3.19	1,153.9
5	--		

[1] Larval growth rate modeled by the exponential growth equation $A = A_0 e^{kt}$ where A is the estimated larva length at time t, A_0 is the intercept, k is the growth rate, and t is the day post-hatch. Growth rate estimated only for larvae that had grown to at least the 4th instar ($\leq$ 15 mm long).

[2] Mean larval growth rate was significantly different among dose levels and control in Experiments 2 (F ratio = 5.7, 4 df, p = 0.014) and 3 (F ratio = 4.6, 3 df, p = 0.037), but not in Experiment 1 (F ratio = 0.7, 5 df, p = 0.6). Means within Experiments 2 and 3 with different superscript letters are significantly different based on post-hoc comparisons (Holm-Sidak method). No larvae in Dose Level 5 of Experiment 3 survived to the 4th instar, meaning no growth rate was determined for that dose level.

[3] Mean clothianidin concentration for leaves of plants consumed by larvae that had survived to at least the 4th instar. The means do not include contamination in plants consumed by larvae that died in the earlier instars (Instars 1 – 3).

BD – below detection. Detection limit ~ 1 ng/g per gram of leaf sample (leaf sample mass ranged from 0.173 –0.781 g).