

RESEARCH ARTICLE

Effects of early-life exposure to sublethal levels of a common neonicotinoid insecticide on the orientation and migration of monarch butterflies (*Danaus plexippus*)

Alana A. E. Wilcox^{1,*}, Amy E. M. Newman¹, Nigel E. Raine², Greg W. Mitchell^{3,4} and D. Ryan Norris^{1,5}

ABSTRACT

Migratory insects use a variety of innate mechanisms to determine their orientation and maintain correct bearing. For long-distance migrants, such as the monarch butterfly (*Danaus plexippus*), these journeys could be affected by exposure to environmental contaminants. Neonicotinoids are synthetic insecticides that work by affecting the nervous system of insects, resulting in impairment of their mobility, cognitive performance, and other physiological and behavioural functions. To examine how neonicotinoids might affect the ability of monarch butterflies to maintain a proper directional orientation on their ~4000 km migration, we grew swamp milkweed (*Asclepias incarnata*) in soil that was either untreated (0 ng g⁻¹: control) or mixed with low (15 ng g⁻¹ of soil) or high (25 ng g⁻¹ of soil) levels of the neonicotinoid clothianidin. Monarch caterpillars were raised on control or clothianidin-treated milkweed and, after pupation, either tested for orientation in a static flight simulator or radio-tracked in the wild during the autumn migration period. Despite clothianidin being detectable in milkweed tissue consumed by caterpillars, there was no evidence that clothianidin influenced the orientation, vector strength (i.e. concentration of direction data around the mean) or rate of travel of adult butterflies, nor was there evidence that morphological traits (i.e. mass and forewing length), testing time, wind speed or temperature impacted directionality. Although sample sizes for both flight simulator and radio-tracking tests were limited, our preliminary results suggest that clothianidin exposure during early caterpillar development does not affect the directed flight of adult migratory monarch butterflies or influence their orientation at the beginning of migration.

KEY WORDS: Insect migration, Pollinator conservation, Radio-tracking, Clothianidin

INTRODUCTION

Long-distance migrations occur in a wide range of taxa, with a variety of underlying mechanisms governing these movements (Mouritsen, 2003; Dingle, 2014). In some groups, such as ungulates, navigation is learned, and knowledge of routes and

locations of resources is transmitted between generations (Jesmer et al., 2018). In others [e.g. certain species of birds (Perdeck, 1958; Chernetsov et al., 2008); turtles (Putman et al., 2011); crustaceans (Boles and Lohmann, 2003)], innate mechanisms govern orientation and navigation capacity (Gould and Gould, 2012; Mouritsen, 2018). These ‘true navigators’ are able to assess their geographic location and/or orientation (e.g. assess the compass direction towards the final destination; Gould and Gould, 2012; Gould, 2014), while also correcting for displacement during migration (Mouritsen, 2003, 2018; Gould and Gould, 2012). ‘Map’ and ‘compass’ systems are assessed independently (Mouritsen, 2003, 2018; Gould and Gould, 2012), with location determined from, for example, the intensity and inclination of the Earth’s geomagnetic field (Mouritsen, 2003, 2018; Dingle, 2014). However, how animals compute their exact position, particularly longitude, is unclear (Mouritsen, 2018). Alternatively, ‘vector navigators’ orient in a fixed direction (e.g. compass systems) based on an internal clock and are unable to compensate for longitudinal displacement (Perdeck, 1958; Mouritsen, 2003; Mouritsen et al., 2013).

After summer breeding in the northeastern United States and southeastern Canada, the last generation of eastern monarch butterflies [*Danaus plexippus* (Linnaeus 1758)] migrate nearly 4000 km southwest towards Mexico (Urquhart, 1960; Urquhart and Urquhart, 1978; Brower, 1995). Monarch butterflies use a time-compensated sun compass (Reppert and Weaver, 2002; Reppert, 2006; Merlin et al., 2009; Reppert et al., 2010; Guerra et al., 2012) that integrates information on the solar azimuth, light intensity and spectral gradients to determine orientation (Dingle, 2014). The central complex in the monarch midbrain then transmits information on solar cues received by the eyes and antennae to the motor system to produce a directed flight response (Reppert et al., 2010; Dingle, 2014). As monarch butterflies are unable to compensate for a 2500 km westward displacement (Mouritsen et al., 2013), this suggests that they rely on a simple vector navigation system during long-distance migration. As a species at risk, with population declines of nearly 80% at overwintering sites in Mexico over the last two decades (Thogmartin et al., 2017a), it is critical to identify potential factors that could limit orientation and migratory capacity, and in turn, migration success.

Neonicotinoids are a class of widely used systemic insecticides (Bass et al., 2015), applied principally in agriculture as seed coatings or soil drenches (Jeschke and Nauen, 2010). The high water solubility of these insecticides (Simon-Delso et al., 2015) can often result in their movement in the environment and their rapid and significant uptake by surrounding non-crop plants (e.g. milkweed; Pecenka and Lundgren, 2015; Bargar et al., 2020; Halsch et al., 2020). Though environmental persistence varies among neonicotinoids, they can remain in the environment for years (DeCant, 2010; Goulson, 2013; Simon-Delso et al., 2015;

¹Department of Integrative Biology, University of Guelph, Guelph, ON N1G 2W1, Canada. ²School of Environmental Sciences, University of Guelph, Guelph, ON N1G 2W1, Canada. ³Wildlife Research Division, Environment and Climate Change Canada, National Wildlife Research Centre, 1125 Colonel By Drive, Ottawa, ON K1A 0H3, Canada. ⁴Department of Biology, Carleton University, 1125 Colonel By Drive, Ottawa, ON K1S 5B6, Canada. ⁵Nature Conservancy of Canada, 245 Eglinton Avenue East, Toronto, ON M4P 3J1, Canada.

*Author for correspondence (alanaawilcox@gmail.com)

 A.A.E.W., 0000-0001-9403-3812; A.E.M.N., 0000-0001-9750-7876; N.E.R., 0000-0001-6343-2829; G.W.M., 0000-0002-5009-9024; D.R.N., 0000-0003-4874-1425

Bonmatin et al., 2015; Wintermantel et al., 2020), exacerbating the risk of exposure for beneficial insect species that are susceptible to the chemical binding at the nicotinic acetylcholine receptors (nAChRs) in the brain (Bonmatin et al., 2015; Sánchez-Bayo et al., 2016). Both acute or chronic exposure to neonicotinoids can affect the sensory, cognitive and motor function and control of insects (Godfray et al., 2014; Williamson et al., 2014; Stanley et al., 2015; Stanley and Raine, 2016) and, although navigation in adult bees can be affected at high doses delivered orally (Fischer et al., 2014; Jin et al., 2015), impact likely varies depending on the extent and duration of exposure (Stanley et al., 2016). In fact, the sun compass, used to determine orientation relative to landscape features (Dovey et al., 2013), does not appear to be affected by neonicotinoid exposure in bees (Fischer et al., 2014). In monarch butterflies, the midbrain is key to integrating information on navigation and is also richly supplied with nAChRs (Heinze and Reppert, 2011, 2012; Cabirol and Haase, 2019). Given the dependence of monarch navigation on this neurological system, it is critical to determine whether neonicotinoid exposure leads to impaired orientation.

We conducted a controlled laboratory experiment to determine whether exposure to the neonicotinoid insecticide clothianidin during larval development might affect adult monarch butterfly orientation during autumn migration. Monarch butterflies rely on milkweed (*Asclepias* spp.) as their larval host plant, and females lay eggs on plants readily grown on agricultural landscapes (Oberhauser, 2004; Thogmartin et al., 2017b; Pitman et al., 2018), which may put them at risk of neonicotinoid exposure. We reared monarch caterpillars on milkweed grown in the laboratory in soil left untreated or treated with field-realistic low or high concentrations of clothianidin. We then tested whether these captive-reared monarchs differed in their orientation capacity as adults. Monarchs were either flown in a flight simulator or released and radio-tracked in the wild using an array of over 100 automated telemetry towers (Motus, <http://motus.org/about>; Taylor et al., 2017). Given previous evidence of negative effects of neonicotinoid exposure on insect navigation (Fischer et al., 2014; Jin et al., 2015), we hypothesized that clothianidin exposure during caterpillar development would negatively impact adult orientation capacity because of its potential physiological impact in the brain. We predicted that butterflies from insecticide treatment groups would not show a strong directional orientation to the southwest, and this effect would be particularly apparent for individuals in the higher concentration treatment group. As disorientation can lead to reduced flight and undirected movements through the same mechanisms, we also predicted that there would be a longer duration between telemetry tower detections for treated compared with control individuals. To test whether morphological (i.e. mass and forewing length) and environmental variables influenced flight behaviour, we also tested for an influence of butterfly sex, mass and forewing length, and the time of testing, wind direction and temperature when tests were conducted on orientation.

MATERIALS AND METHODS

Neonicotinoid treatment and milkweed growth

Stock solutions were made from a clothianidin standard (purity 99.9%; MDL no. MFCD06200753, Sigma-Aldrich, St Louis, MO, USA) diluted with distilled water and used to dose soil (LA4 Sunshine Loosefill, Sungro Horticulture, MA, USA) at concentrations of 15 ng g⁻¹ (i.e. 'low dose') and 25 ng g⁻¹ (i.e. 'high dose') of soil based on sub-lethal doses and field-realistic values from Ontario (Chan et al., 2019; Pecenka and Lundgren, 2015).

Swamp milkweed (*Asclepias incarnata*) was grown from seed (Richters Herbs, Goodwood, ON, Canada) in control (i.e. without clothianidin treatment), low dose or high dose soil treatments. Plants ($n=256$) were grown at a density of four plants per 6 in²/1.68 litre pot in environmental chambers (University of Guelph Phytotron) maintained at 29°C during the day and 23°C at night. Light intensity ranged from 11,914 to 16,280 lx (18 h:6 h light:dark) (Flockhart et al., 2012). Relative humidity, monitored hourly with a handheld hygrometer (Vaisala MI70 Measurement Indicator with HMP75 Humidity and Temperature Probe, Vaisala, Helsinki, Finland), was maintained at 77±10% (mean±s.d.). Plants were watered daily with reverse osmosis water and fertilized weekly with Plant-Prod Solutions fertilizer 17:5:17 N:P:K (Master Plant-Prod Inc., Brampton, ON, Canada). Predatory Swirski mites (*Amblyseius swirskii*) were introduced as a biocontrol (Bioline AgroSciences Swirskiline Biocontrol Agent and Biobest Swirskii-Breeding-System) to reduce the impact of thrips (Thysanoptera) (Flockhart et al., 2012).

Soil was collected at five time points for analytic quantification of clothianidin residues: (1) when the soil was dosed (day 0), (2) 14 days after dosing, (3) when eggs were transferred to the treatment leaves (day 28), (4) 2 weeks after egg transfer (day 43) and (5) when monarchs pupated (beginning on day 49). At each time point, at least 15 g of soil (sensitivity ±1.0 g; MyWeigh iBalance i500, HBI Technologies Canada, Vancouver, BC, Canada) was transferred to sterile polypropylene centrifuge tubes (High-Performance Centrifuge Tubes, catalogue no. 89039-656, VWR International LLC, Mississauga, ON, Canada). A leaf was randomly selected from each milkweed plant at 28, 43 and 49 days after the soil was dosed and combined to reach a minimum mass of 2 g for clothianidin detection, then stored in sterile polypropylene centrifuge tubes. To determine at what point during development neonicotinoids may be metabolized, a subset of instar 5 caterpillars and adult butterflies was haphazardly selected and combined to reach a 2 g minimum mass for clothianidin analysis. All samples were stored at -20°C prior to residue analysis at the University of Guelph Agriculture and food laboratory using the QUECHERS (i.e. Quick, Easy, Cheap, Effective, Rugged and Safe) method, which is appropriate for samples with high water content. In brief, a sample of the soil, plant or insect tissue was extracted and placed in a solution of 1% acetic acid in acetonitrile with anhydrous sodium and magnesium sulphate. The precipitate was then diluted with methanol and 0.1 mol l⁻¹ ammonium acetate. High-performance liquid chromatography/electrospray ionization tandem mass spectrometry (LC/ESI-MS/MS) and gas chromatography-tandem mass spectrometry (GC-MS/MS) were used to assess concentration (Canadian Food Inspection Agency, 2008; Wang and Daniel, 2009), returned in parts per billion (1 ppb=1 ng g⁻¹; Boguski, 2006). The limit of quantification (LOQ) is the lowest concentration that can be accurately quantified, whereas the limit of detection (LOD) is the lowest concentration that can be distinguished from the assay background and, therefore, has a higher degree of error. The LOQ and LOD depend on the sample type: LOQ/LOD for soil, 20 ppb/7 ppb; leaf, 30 ppb/10 ppb; monarch tissue, 2 ppb/0.7 ppb.

Monarch capture and rearing

We raised monarch caterpillars from eggs laid by wild females obtained from untreated properties at the Guelph Lake Conservation Area (43.61°N, 80.26°W; ♂ $n=7$, ♀ $n=11$). After capture, wild monarch butterflies were held in coin envelopes (6.35×10.8 cm) inside an animal carrier and kept at ambient temperature. Humidity was maintained with a damp cloth at the bottom of the carrier to avoid the wings drying out during transport to the University of

Guelph. Butterflies were weighed (PI-602 scale, Denver Instrument, Bohemia, NY, USA) to the nearest 0.01 g and hand-fed a 10% honey-water solution daily until satiation. Wild monarchs were mated in mesh enclosures (60×60×60 cm, height×depth×width) inside an incubator set at temperatures between 23 and 29°C, relative humidity 77±10% (mean±s.d.) with a light intensity between 11,914 and 16,280 lx (18 h:6 h light:dark). Enclosures contained untreated milkweed (grown in soil dosed with reverse osmosis water) and an *ad libitum* artificial nectar source (i.e. 10% honey-water). Monarchs were mated for two nights and eggs were collected each morning. Wild monarchs were released where they were captured.

We collected 192 eggs ($n=64$ per treatment) by gently pressing a fine-tipped paintbrush along the edge of the egg and transferring to a milkweed leaf with residual latex holding the egg in place. Leaves with eggs were placed haphazardly in large plastic containers arranged by treatment, enclosed using a finely perforated mosquito netting (Bulk Mosquito Netting, catalogue no. 09A04.73, Lee Valley Tools, Ottawa, ON, Canada), and containers that were cleaned daily with mild soap and water. Ambient conditions were maintained to represent those during the late autumn at 43.53°N, 80.23°W (13 h:11 h light:dark, 21°C by day, 11°C at night, mean±s.d. 87±6% relative humidity) to encourage development of migratory monarch butterflies. Caterpillars hatched within 3–5 days and were fed milkweed grown in treated or control soil *ad libitum* until pupation, when chrysalids were then transferred to mesh enclosures (120×120×120 cm; Popadome Plant Dome, catalogue no. XC515, Lee Valley Tools) separated by treatment in the laboratory (~19.5°C), where lighting cycle was variable, but supplemented by negligible foyer lighting. After eclosion, adult monarchs were hand-fed daily and provided dishes containing a 10% honey-water solution within the enclosures (Flockhart et al., 2012). All monarchs were measured and weighed in captivity. We also examined each individual for *Ophryocystis elektroscirrha* parasites by applying clear tape to the abdomen and analysing the tape for spores under a microscope at 400× (Altizer and Oberhauser, 1999); if an individual tested positive, it was removed from the study ($n=2$ from the low dose treatment group). All procedures were conducted under an Ontario Ministry for Natural Resources Wildlife Scientific Collectors Permit (no. 1090000).

Flight simulation

From 17 to 23 September 2018, a subset of monarch butterflies (control: ♂, $n=5$, ♀, $n=10$; low dose ♂, $n=8$, ♀, $n=8$; high dose ♂, $n=10$, ♀, $n=13$; tested 2–5 days after eclosion) was used to assess orientation during seasonal migration using flight simulators following methods developed by Mouritsen et al. (2013). Flight simulators were set up on the roof of the University of Guelph Phytotron and arranged so that no buildings were visible that could influence the direction of orientation while in the flight cylinder (Mouritsen et al., 2013). Tests occurred during daylight (09:30–15:46 h) when the sun was fully visible in the simulator to ensure consistency of polarized light cues (Reppert et al., 2004; Mouritsen et al., 2013). Individual butterflies were tethered to an L-shaped rod (modified to approximately 2.5 cm; catalogue no. 718000, 0.05×15.2 cm Tungsten Rods, A-M Systems, Sequim, WA, USA) inserted at the front of the dorsal thorax, avoiding flight muscle, and secured with super glue (All Purpose Krazy Glue No Run Gel, Elmer's Products, High Point, NC, USA; Mouritsen et al., 2013). Each tether was attached to a digital encoder that allowed 360 deg rotation and recorded orientation at 3 deg intervals (Mouritsen et al., 2013). The encoder was adhered to a plexiglass rod supported within a large cylinder (height: 67.9 cm, diameter: 59.1 cm) and

attached to a laptop computer to record directional data (Mouritsen et al., 2013). A fan at the base of the flight simulator provided airflow to encourage flight. Each monarch was flown in the flight simulator once for 12 min (5 direction recordings s^{-1}), with 2 min provided for acclimation before data collection to avoid a stress-induced flight response (Perez et al., 1999). Monarchs were removed (control: ♂, $n=2$, ♀, $n=1$; low dose ♂, $n=3$, ♀, $n=4$; high dose ♂, $n=2$, ♀, $n=3$) from the study if they were not demonstrating migratory flight behaviour (i.e. strong flapping with intermittent gliding).

Radio-telemetry tracking

Between 28 September and 7 October 2018, we tracked a separate subset of monarch butterflies (control: ♂, $n=8$, ♀, $n=6$; low dose: ♂, $n=8$, ♀, $n=6$; high dose: ♂, $n=7$, ♀, $n=8$; tested 8–12 days after eclosion) during early migration using radio-telemetry. Monarchs were outfitted with 200 mg NanoTags (Lotek Wireless Fish & Wildlife Monitoring, Newmarket, ON, Canada), programmed at a 166.380 MHz frequency with pulses emitted every 4.7 s to maximize the probability of detection and allow for individual identification (Taylor et al., 2017). Large monarchs (>0.3 g) were selected to minimize weight limitations imposed by the tags and maximize the capacity for long-distance flight. Monarchs were then released on a hill, above the tree line, at the base of the Cambridge-RARE Motus tower (43.38°N, 80.35°W) in Cambridge, ON, Canada. Detected signals could potentially be received at more than 100 independent VHF telemetry towers across southern Ontario and the northern United States, with towers in all directions around the release site (Taylor et al., 2017). Data were received by the Motus Wildlife Tracking System and made available later for download (<http://motus.org/about>; Taylor et al., 2017). We ran preliminary filters to remove detections with run lengths (i.e. number of detections) <2 and false detections as a result of noise (e.g. detections prior to release or beyond the species range, towers recording spurious detections). We also examined ambiguous detections manually using contextual information to identify true detections (Crewe et al., 2018); for instance, removing detections that bounced between multiple towers and/or countries. We removed detections recorded on the day of release at adjacent towers with signals overlapping with other nearby towers to avoid inaccurately assigning a direction of flight when the monarchs had not yet left the area. This resulted in true detections for 20 monarchs (control: ♂, $n=4$, ♀, $n=2$; low dose: ♂, $n=3$, ♀, $n=2$; high dose: ♂, $n=3$, ♀, $n=6$).

Statistical methods

North American monarch butterflies originating in Ontario orient in a south–southwest direction during autumn migration. For monarchs flown in the flight simulator, we calculated the mean direction (0–359 deg) and vector strength (r : 0–1), a measure of the concentration of data around the mean, for each monarch butterfly flight (Batschelet, 1981; Pewsey et al., 2013) using Oriana version 4.02 (<https://www.kovcomp.co.uk/>). Then, using the data for each individual, in separate tests we calculated the group mean direction and vector strength within each of the treatments for monarchs flown in the flight simulator and released with radio-tracking tags. Subsequently, a v -test, suitable for small sample sizes (Landler et al., 2018), was used to compare individual vector strengths among treatment groups in order to determine whether monarchs showed differences in directional flight.

To complement the above analysis, but for flight simulator monarchs only, we also tested for an effect of neonicotinoid

Table 1. Concentrations of the neonicotinoid clothianidin (CLO; ppb) in soil, swamp milkweed (*Asclepias incarnata*), instar 5 monarch caterpillars (*Danaus plexippus*) and adult butterflies for control (0 ng g⁻¹), low dose (15 ng g⁻¹) and high dose (25 ng g⁻¹) treatments applied to soil

Time of sampling	Treatment	Mean (ppb)	s.d. (ppb)	Range	Median	DET (<i>n</i>)	ND (<i>n</i>)
[CLO] in soil							
Soil dosing	Control	–	–	–	–	0	3
	Low dose	4.70	1.14	3.90–6.00	4.20	3	0
	High dose	7.90	0.66	7.30–8.60	7.80	3	0
14 days after dosing	Control	–	–	–	–	0	3
	Low dose	4.83	1.18	4.10–6.20	4.20	3	0
	High dose	8.00	0.87	7.00–8.50	8.50	3	0
28 days after dosing	Control	–	–	–	–	0	5
	Low dose	6.64	0.54	7.40–6.90	6.60	5	0
	High dose	10.18	0.60	9.20–10.60	10.50	5	0
43 days after dosing	Control	–	–	–	–	0	5
	Low dose	1.06	0.35	0.70–1.60	0.90	5	0
	High dose	1.84	0.29	1.40–2.20	1.90	5	0
49 days after dosing	Control	–	–	–	–	0	5
	Low dose	0.60	–	–	–	1	4
	High dose	1.12	0.22	0.80–1.30	1.20	5	0
[CLO] in milkweed leaves							
28 days after dosing	Control	–	–	–	–	0	3
	Low dose	–	–	–	–	0	3
	High dose	2.23	0.12	2.10–2.30	2.30	3	0
43 days after dosing	Control	–	–	–	–	0	3
	Low dose	1.60	0.57	1.20–2.20	1.60	2	2
	High dose	2.00	0.59	1.40–2.80	1.90	4	0
49 days after dosing	Control	–	–	–	–	0	5
	Low dose	1.05	0.07	1.00–1.10	1.05	2	1
	High dose	2.60	1.47	1.70–4.30	1.80	3	0
[CLO] in instar 5 monarch larvae							
	Control	–	–	–	–	0	7
	Low dose	1.26	0.59	0.30–2.20	1.2	7	0
	High dose	2.24	0.56	1.50–3.10	2.2	7	0
[CLO] in adult monarch butterflies							
	Control	–	–	–	–	0	5
	Low dose	–	–	–	–	0	3
	High dose	–	–	–	–	0	5

For samples with clothianidin detected [DET (*n*)], the mean, standard deviation, range and median concentrations are provided. The number of samples where no clothianidin detected [ND (*n*)] is provided as summary statistics could not be calculated (–).

treatment on vector strength using a general linear model, and examined the effects of morphological and environmental factors on orientation using separate circular-linear regressions for each variable in the *circular* package (v0.4-93, <https://CRAN.R-project.org/package=circular>) in R version 3.4.1 (<https://www.r-project.org/>). Monarch butterfly mass (mg), forewing length (mm), time of testing (i.e. minutes after 09:00 h), wind speed and temperature at the beginning of the test were included as predictors in separate models, with the mean flight direction as the response variable. Ambient temperature was obtained from Environment and Climate Change Canada in Guelph, ON (43.5°N, 80.2°W; Environment and Climate Change Canada, 2018). We then ran an ANOVA with *post hoc* Tukey's HSD using the *stats* package (<https://stat.ethz.ch/R-manual/R-devel/library/stats/html/stats-package.html>) to determine whether there was a difference in the body mass and forewing length among treatments. Lastly, for monarchs released with radio-tracking tags, we used a general linear model to investigate whether the neonicotinoid treatment affected the rate of travel to the first detection at a Motus tower.

RESULTS

Clothianidin residues

We detected no clothianidin in the soil for the control group at any of the time points. Clothianidin was detected in soil from both insecticide treatments at lower concentrations than originally applied to the soil (15 or 25 ng g⁻¹; Table 1). The concentration

of clothianidin remained consistent 14 and 28 days after soil dosing, before the concentration dropped at day 43 (Table 1). Clothianidin was found in a single sample of soil at the last time point (i.e. day 49 after soil dosing) in the low dose treatment (Table 1).

Table 2. Directional flight in eastern North American migratory monarch butterflies (*Danaus plexippus*)

Group	<i>n</i>	Mean direction (deg)	Cardinal direction	<i>r</i>	<i>v</i>	<i>P</i>
Flight simulator						
Control	12	358	N	0.13	–0.13	0.73
Low dose	9	183	S	0.18	0.18	0.23
High dose	18	355	N	0.16	–0.16	0.83
Radio-tracked						
Control	6	151	SSE	0.98	0.86	<0.001
Low dose	5	154	SSE	0.99	0.90	<0.001
High dose	9	158	SSE	0.99	0.93	<0.001

Butterflies were reared in environmental chambers simulating autumn conditions until pupation, then tested in an outdoor flight simulator to record flight orientation (between 0 and 359 deg) or radio-tracked during autumn migration. Separate *v*-tests (expected mean of 180 deg) were used to determine whether monarchs in each treatment group showed directional flight (*P*<0.05). Mean and cardinal directions and vector strength (*r*), representing the spread of the data between 0 (evenly spread) to 1 (concentrated around the mean), are shown.

Table 3. Mean vector direction of eastern North American migratory monarch butterflies (*Danaus plexippus*)

Group	Mean vector direction (μ ; deg)	Median (deg)	Concentration	Variance	s.d. (deg)	s.e.m. (deg)	95% CI low	95% CI high
Flight simulator								
Control	358	6	0	0.87	117	–	–	–
Low dose	183	137	0	0.82	106	–	–	–
High dose	355	343	0.32	0.84	110	59	–	–
Radio-tracked								
Control	151	156	15.61	0.02	11	6	139	163
Low dose	154	156	145.83	0.002	3	2	150	158
High dose	158	156	205.18	0.002	3	1	155	160

Butterflies were reared in environmental chambers simulating autumn conditions until pupation, then tested in an outdoor flight simulator to record flight orientation (between 0 and 359 deg) or radio-tracked during autumn migration. The mean vector direction (deg), median (deg), concentration parameter, circular variance, circular standard deviation and standard error of the mean, provided alongside the 95% confidence intervals of the mean vector direction (μ). Parameters were calculated in Oriana version 4.02 (www.kovcomp.co.uk). A dash (–) indicates that results could not be calculated because of low concentration.

We detected no clothianidin in milkweed leaves for the control group at any of the time points, but the insecticide was detected in leaves from both treatments (Table 1). Though the concentration of clothianidin remained consistent in leaves from the high dose treatment, clothianidin was only detected in leaves 43 and 49 days after soil dosing in the low dose treatment (i.e. clothianidin was not detected 28 days after soil dosing; Table 1). Clothianidin was also not detected in instar 5 caterpillars raised on control milkweed, but was detected at a concentration of >1 ppb in caterpillars from the low and high dose treatment groups (Table 1). As expected, the concentration of clothianidin was higher in the high dose treatment group relative to the low dose treatment group (Table 1). No clothianidin was detected in the tissue from adult monarch butterflies irrespective of treatment group (Table 1).

Flight simulation

When tested in the flight simulator, monarchs showed no consistency of flight direction in any of the treatment groups, with different individuals concentrating their flights in a variety of directions (Tables 2, 3, Fig. 1; Figs S1, S2, S3). Insecticide treatment groups also did not differ from the control in their vector

strength (Table 4). Given the lack of directional flight for all treatment groups, we pooled all individuals together to test the effects of morphological and environmental factors on orientation. There was no evidence that adult body mass, forewing length, time of the flight simulation test, wind speed or temperature influenced mean flight direction for either males or females (Table 4). Though body mass did not differ between the treatment groups and controls (Table 5; Fig. S4), monarchs from the high dose treatment had shorter forewings than monarchs from the low dose treatment and controls (Table 5; Fig. S4).

Radio-telemetry tracking

Similar to wild migratory monarch butterflies (Mouritsen et al., 2013), treatment and control monarchs that were reared in captivity and then released into the wild did not differ in their direction of flight (Tables 2, 3). However, unlike the flight simulator results, the direction of flight was strongly concentrated in a southern direction, which is as expected if they were migrating to their overwintering grounds (Table 2, Figs 1, 2). We found no evidence that the rate of travel differed among treatment and control groups (Table 4).

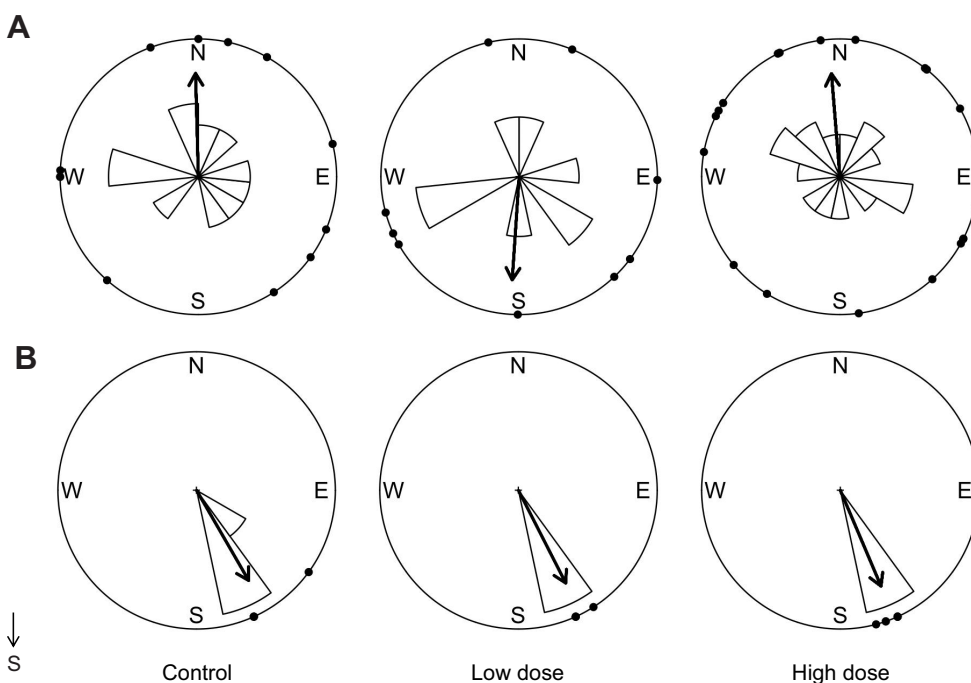


Fig. 1. Orientation of captive-reared eastern North American migratory monarch butterflies (*Danaus plexippus*) reared on swamp milkweed (*Asclepias incarnata*) grown in untreated control (0 ng g^{-1} ; $n=12$), 15 ng g^{-1} (i.e. low dose; $n=9$) or 25 ng g^{-1} (i.e. high dose; $n=18$) soil. Butterflies were (A) flown in a flight simulator for 10 min in Guelph, ON, Canada, in September 2018 or (B) released in Cambridge, ON, Canada, in October 2018 for radio-tracking (control: $n=6$; low dose, $n=5$; high dose, $n=9$). The direction of flight for individual monarchs (black dots) is shown in a circular plot, where multiple observations are shown overlapping. Each section of the central windrose indicates the proportion of individuals with directional flight, with the arrow inside the circles indicating the group mean direction. The arrow adjacent to the circles (\downarrow) indicates the expected mean direction of flight (S, 180 deg).

Table 4. Vector strength, flight direction and rate of travel in eastern North American migratory monarch butterflies (*Danaus plexippus*)

		Flight simulator						
Contrast		Estimate	s.e.	CI	<i>t</i>	<i>P</i>		
Vector strength	Control–Low dose	–0.02	0.14	–0.29;0.26	–0.11	0.91		
	Control–High dose	–0.10	0.12	–0.33;0.13	–0.87	0.39		
Variable		Estimate	s.e.	<i>t</i>	<i>P</i>	logLik	μ	κ
Morphological and environmental factors	Mass	–0.001	0.002	0.58	0.28	0.21	–157.1	0.15
	Forewing length	0.02	0.02	0.86	0.20	0.37	182	0.20
	Time of testing	–0.006	0.004	1.35	0.09	1.26	–146.5	0.36
	Temperature	–0.06	0.06	0.97	0.17	0.51	–196.7	0.23
	Wind speed	–0.30	0.49	0.62	0.27	0.27	–277.7	0.17
		Motus radio-tracking field data						
Contrast		Estimate	s.e.	CI	<i>t</i>	<i>P</i>		
Rate of travel	Control–Low dose	1.49	2.93	–4.70; 7.69	0.51	0.62		
	Control–High dose	0.81	3.37	–6.3; 7.93	0.24	0.81		

Butterflies were reared in environmental chambers simulating autumn conditions until pupation, then tested in an outdoor flight simulator to record flight orientation (between 0 and 359 deg). Linear models were used to determine the effect of neonicotinoid treatment on individual vector strength, and circular-linear models were used to determine whether morphological and environmental factors influenced the direction of flight ($P < 0.05$). For captive-reared monarchs radio-tracked during autumn migration, a linear model was used to test the effect of neonicotinoid treatment on rate of travel ($P < 0.05$). The estimate, standard error, *t*-values and *P*-values are presented alongside the 95% confidence intervals for linear models and log-likelihood (logLik), mean vector direction (μ) and concentration parameter (κ) for circular-linear models

DISCUSSION

Our results demonstrate that early exposure to clothianidin at field-realistic concentrations of 15 and 25 ng g^{–1} in soil during monarch caterpillar development had no apparent measurable effect on the orientation of adult monarch butterflies either flown in a flight simulator or released and radio-tracked in the wild. We also found no evidence to support the hypothesis that exposure to clothianidin affected the rate of travel or that morphological traits and environmental conditions affected flight behaviour. Although other studies indicate negative impacts of neonicotinoids on caterpillar development (Pecenka and Lundgren, 2015; Wilcox, 2020), we did not find evidence that exposure to clothianidin during development carries over to influence the orientation of adult migratory monarch butterflies. However, given that our sample sizes were limited for both the flight simulator and radio-tracking tests, it is possible that less pronounced yet biologically significant sublethal effects of clothianidin exposure occurred but were not detected in our study.

Southward orientation during migration is essential for monarch butterflies to reach their destination in the Cerro Pelón and Sierra Madre Oriental mountains of Mexico (Urquhart, 1960; Urquhart and Urquhart, 1978; Brower, 1995). Monarchs visually perceive solar cues and also have a light-dependent molecular clock in the antennae used for a sun compass (Reppert and Weaver, 2002; Reppert, 2006; Merlin et al., 2009; Guerra et al., 2012). Information on orientation from the time-compensated sun compass, as well as visual cues and timing information from the brain circadian clock, are likely integrated in the midbrain (Reppert et al., 2010). Though neonicotinoid insecticides, such as clothianidin, could bind to nAChRs in the midbrain, we did not find evidence to suggest that this has an effect on directed flight, as indicated by a high vector strength (i.e. strong concentration of directionality around the mean for radio-tracked individuals; Table 2). Given that clothianidin was not detected in adult monarch butterflies (Table 1), it is possible that it was metabolized prior to flight-testing. Although no studies have yet investigated the metabolism of neonicotinoids in monarch butterflies,

Table 5. Effect of neonicotinoid treatment on body mass and forewing length in eastern North American migratory monarch butterflies (*Danaus plexippus*)

		ANOVA			
Group	<i>F</i>	<i>P</i>	Sum of squares	d.f.	
Body mass	2.33	0.11	27,855	2,36	
Forewing length	7.04	0.003	242.5	2,35	
		Tukey's HSD			
Group	Difference in observed means	Lower	Upper	<i>P</i>	
Body mass					
Control–Low dose	–49.72	–132.97	33.53	0.33	
Control–High dose	–60.83	–131.19	9.53	0.11	
Low dose–High dose	11.11	–65.97	88.19	0.94	
Forewing length					
Control–Low dose	–0.76	–5.23	3.72	0.91	
Control–High dose	–5.37	–9.20	–1.54	0.004	
Low dose–High dose	4.62	0.42	8.80	0.03	

Butterflies were reared in environmental chambers simulating autumn conditions until pupation, then tested in an outdoor flight simulator to record flight orientation (between 0 and 359 deg). ANOVAs with *post hoc* Tukey's HSD were calculated for the effect of neonicotinoid treatment on body mass and forewing length using the *stats* package in R version 3.4.1

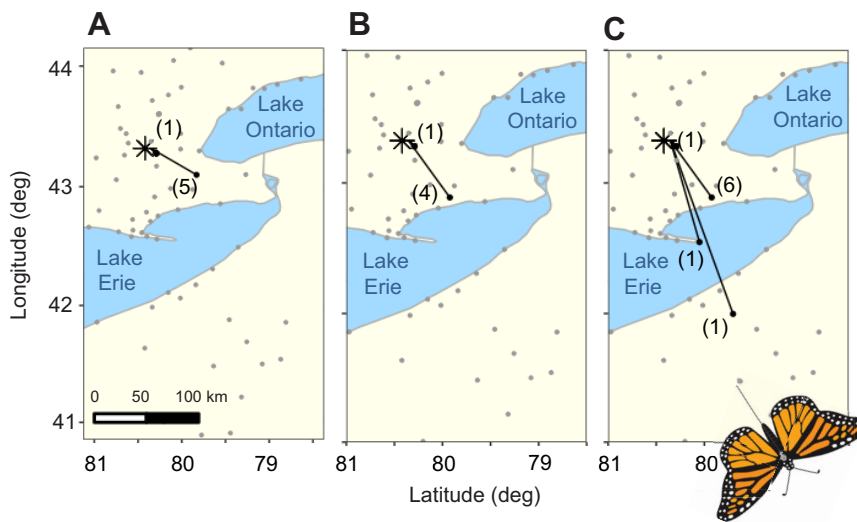


Fig. 2. Orientation of captive-reared eastern North American migratory monarch butterflies (*Danaus plexippus*) reared on swamp milkweed (*Asclepias incarnata*) released in Cambridge, ON, Canada, in October 2018. Butterflies were grown in (A) untreated control (0 ng g^{-1}), (B) 15 ng g^{-1} (low dose) or (C) 25 ng g^{-1} clothianidin-treated (high dose) soil. Symbols indicate the release site (stars) and location of first detection at a Motus tower (black dots) (1–16 days after release; Wilcox, 2020). The number of monarchs detected at each tower is shown in parentheses, and grey dots indicate Motus towers that were active at the time of releases but did not detect any monarchs.

the cytochrome P450 superfamily is responsible for metabolism of neonicotinoids (Manjon et al., 2018) at a rate of 2.0 ng day^{-1} in honeybees (Godfray et al., 2014). Therefore, our results are consistent with the hypothesis that clothianidin is metabolized during caterpillar development and/or metamorphosis. There is also some evidence that neonicotinoid exposure may affect forewing length (Wilcox, 2020). However, genetic variation may also contribute to differences in the size (i.e. based on forewing and hindwing length) of adult monarch butterflies exposed to neonicotinoids (Kobiela and Snell-Rood, 2020). For instance, in contrast with previous evidence (Wilcox, 2020), monarchs in our study had shorter forewings than controls (Table 5; Fig. S4). Nonetheless, the influence of early exposure to clothianidin on forewing length could indicate a potential impact of exposure on development.

Our results also suggest that morphological characteristics do not affect flight direction. The autumn migratory generation of monarch butterflies is characterized by a physiological shift during metamorphosis that drives the development of long, thin wings to reduce loading and drag, as well as increases in flight muscle (Dingle, 2006), resulting in butterflies that are larger than those from earlier reproductive generations. Though previous research suggests that more elongated wings may be related to migratory status (Li et al., 2016), our results suggest that monarch forewing length did not affect flight orientation.

Previous studies using flight simulators have found monarchs reared in captivity showed random flight orientation (Tenger-Trolander et al., 2019; Wilcox, 2020), but when monarchs are released into the wild they regain expected southward orientation for migration (Wilcox, 2020). Therefore, although we found no evidence of a difference in the flight orientation among treatment groups in the flight simulator, we are cautious about inferring migratory directionality from these data. Moreover, because we were unable to release and radio-track monarchs originally tested in the flight simulator, we could not account for potential differences in flight behaviour between flight simulator and radio-tracking assays. Lastly, monarchs were suspended in the flight simulator using a tungsten rod inserted into the front of the dorsal thorax. This resulted in a temporary impairment and, as such, monarchs showed visible signs of exhaustion (lethargy) after testing.

There are some limitations in this study that restrict our ability to fully assess the concentration-dependent effects of clothianidin exposure. First, the concentration of clothianidin in the soil decreased from the levels at dosing (15 and 25 ng g^{-1}) to between

4 and 8 ng g^{-1} after 2 weeks, potentially as a result of settling after dosing the soil and leaching during watering. Despite the levels detected at day 49 after dosing being similar to those found in field (Chan et al., 2019) and in milkweed leaves (Pecenka and Lundgren, 2015), it is possible that higher concentrations of insecticide may result in greater impacts on the monarch butterflies and provide insight into dose-dependent effects on behaviour. Moreover, though the results of this study suggest that clothianidin has a negligible effect on monarch flight orientation, we were unable to submit samples of monarchs that were released and radio-tracked for neonicotinoid analysis. Radio-transmitters have a limited battery lifespan and once data transmission ceased, we were unable to identify the location of the tagged monarchs. Despite these challenges, our study provides the opportunity to examine the effect of clothianidin exposure during caterpillar development and any subsequent impact on orientation for radio-tracked monarchs. Future work using metabolomics at each instar could reveal fine-scale developmental profiles of neonicotinoid assimilation in monarchs.

Conclusions

Although we had small sample sizes for both the flight simulator and radio-tracking tests, our preliminary findings suggest that the orientation of captive-reared migratory monarch butterflies flown in a flight simulator or released and radio-tracked was not affected by clothianidin exposure (applied to soil at 15 or 25 ng g^{-1}) during development. Our results also showed no measurable effect for morphological traits, including body mass and forewing length, or environmental conditions on migratory flight. The results from our study contribute to the understanding of the potential impacts of insecticide exposure on monarch butterflies and suggest that exposure to field-realistic levels of clothianidin at the larval stage is unlikely to impair migratory flight.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: A.A.E.W., A.E.M.N., N.E.R., G.W.M., D.R.N.; Methodology: A.A.E.W., D.R.N.; Formal analysis: A.A.E.W.; Investigation: A.A.E.W.; Resources: G.W.M.; Writing - original draft: A.A.E.W.; Writing - review & editing: A.A.E.W., A.E.M.N., D.R.N.; Visualization: A.A.E.W.; Supervision: A.E.M.N., D.R.N.; Project administration: A.A.E.W., A.E.M.N., D.R.N.; Funding acquisition: A.E.M.N., N.E.R., G.W.M., D.R.N.

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Data availability

Data presented in the main text are available from the figshare digital repository: 10.6084/m9.figshare.13123244 (flight simulation data) and 10.6084/m9.figshare.13123262 (radio-tracking data). Motus data are available at <https://motus.org/data/downloads> (project ID 209).

Supplementary information

Supplementary information available online at <https://jeb.biologists.org/lookup/doi/10.1242/jeb.230870.supplemental>

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