POTENTIAL THREATS TO THE CONSERVATION OF EASTERN NORTH AMERICAN MONARCH BUTTERFLIES (DANAUS PLEXIPPUUS) AND A TOOL FOR POPULATION RECOVERY

by

Alana A. E. Wilcox

A Thesis
presented to
The University of Guelph

In partial fulfilment of requirements for the degree of Doctor of Philosophy in Integrative Biology

Guelph, Ontario, Canada

© Alana Ashleigh Elizabeth Wilcox, May 2020
The eastern North American monarch butterfly (*Danaus plexippus*) has undergone large-scale declines over the last two decades and no clear single cause to explain the decline has yet been identified. In Chapter 2, I assess the contribution of potential threats on eastern population of monarch butterflies. I found that declines in suitable environmental conditions and overwintering and breeding habitat loss are likely the leading threats to the eastern population of monarch butterflies, but that risks imposed by contaminants, predation, and parasitism may also contribute. Agrochemical exposure, specifically from neonicotinoids, is a leading concern due to the potential lethal and sublethal effects on development and behaviour on non-target species, such as monarchs. I also conducted two experimental studies on monarchs reared on milkweed grown in soil treated with field-realistic levels of clothianidin at a low (15 ng/g of clothianidin) or high (25 ng/g of clothianidin) dose levels, or in a control (0 ng/g of clothianidin). In Chapter 3, I provide experimental evidence that late instar caterpillars reared on clothianidin-treated milkweed were smaller and weighed less than controls. Nonetheless, clothianidin treatment was also associated with larger adult monarch
butterflies, but did not influence the egg size or the number of eggs laid. In Chapter 4, I show that clothianidin did not influence orientation, vector strength, or the rate of travel of adult migratory monarch butterflies. Lastly, in Chapter 5, I investigate whether captive rearing, often used as a conservation and educational tool for this species, influences migratory behaviour. While captive-reared migratory monarchs tested in the flight simulator did not show a normal directional flight response, individuals released in the wild and radio-tracked showed proper orientation and flew in a southward direction towards their Mexican overwintering grounds. Collectively, my thesis contributes to a broader understanding of the potential causes underlying declines in monarch butterflies, most notably from neonicotinoids, and offers important insight into viability of captive rearing as an important conservation and educational tool.
Acknowledgements

I would like to thank my advisors Dr. Ryan Norris and Dr. Amy Newman for their mentorship and encouragement. Your guidance has helped me find and create my own niche in science and grow professionally during my time in your labs.

Thank you to my Advisory Committee Members, Dr. Nigel Raine and Dr. Tyler Flockhart for your insights and advice throughout my PhD. Your support and direction have greatly helped me in the creation of this thesis.

The experiments presented here would not have been possible without the technical support and coordination of the staff at the University of Guelph Phytotron, especially Mike Mucci and Tannis Slimmon. I would also like to extend my thanks to Darlene Burgess, Jim and Dave Ellis, Don Davis, and Jessica Linton for helping me locate catching sites and to Ivan McIlory, Kathryn Boothby, and staff at The Arboretum for offering their land to catch monarchs. I would also like to thank Stu Mackenzie, Zoe Crysler, and Tara Crewe for their support with Motus. Also, thank you to the staff at the rare Charitable Research Reserve, including Jenna Quinn, and to Mike Vandentillaart and Dave Gambin from Lotek Wireless Inc., who assisted in the monarch release. Lastly, I cannot express my gratitude for the time learning from the late Dr. Barrie Frost. Thank you for teaching me about insect migration and for sharing the many stories of your adventures and your love for the Limestone City.

I cannot thank enough my team of assistants and volunteers who dedicated their time to ensure the experiments ran smoothly. I am especially grateful to Angela Demarse,
Taylor Van Bell, Ava Chaplin, Vivian Forte-Perri, and Sara Vezina for their enthusiasm and hard work. A special thank you to Samantha Knight, your thoughtful approach to science has always been an inspiration.

To my labmates, thank you for your advice and friendship over the last 3 years. Thank you as well to my friends in Winnipeg, Guelph, and Toronto who have provided constant encouragement and especially to Heather Mayberry for your support and guidance during our many pizza nights.

Lastly, I am incredibly grateful to my parents, Bev and Grant Wilcox, for their encouragement and caring. Your constant patience and advice have helped guide me personally and in my studies. Thank you for your unwavering love and support.

I was provided funding from a Natural Sciences and Engineering Research Council (NSERC) Alexander Graham Bell Canada Graduate Scholarship (CGS D) and an Ontario Graduate Scholarship. The work in this thesis was also supported by a grant from the Ontario Ministry of Agriculture, Food and Rural Affairs (OMAFRA) to A. E. M. N and D. R. N. and Discovery Grants to D. R. N. and N. E. R.
# TABLE OF CONTENTS

Abstract .............................................................................................................................................. ii

Acknowledgements .......................................................................................................................... iv

Table of Contents ............................................................................................................................. vi

List of Tables ..................................................................................................................................... viii

List of Figures ................................................................................................................................... xi

Chapter Publications and Author Contributions ............................................................................. 1

Chapter 1: Introduction ...................................................................................................................... 3

  Introduction ....................................................................................................................................... 19
  Methods ........................................................................................................................................... 23
  Results ............................................................................................................................................. 25
  Discussion ....................................................................................................................................... 35
  Conclusion ................................................................................................................................. 39

Chapter 3: Developmental and Reproductive Effects of Neonicotinoid Exposure in Monarch Butterflies ..................................................................................................................... 45
  Introduction ................................................................................................................................. 46
  Methods ....................................................................................................................................... 49
  Results .......................................................................................................................................... 56
## Table of Contents

Discussion .......................................................................................................................... 59
Conclusion .......................................................................................................................... 63

Chapter 4: Effects of Early-life Exposure to Sublethal Levels of a Common Neonicotinoid Insecticide on the Orientation and Migration of Monarch Butterflies (*Danaus plexippus*) ........................................................................................................ 72

Introduction ....................................................................................................................... 73
Methods ............................................................................................................................... 76
Results ................................................................................................................................ 83
Discussion .......................................................................................................................... 85
Conclusion .......................................................................................................................... 88

Chapter 5: Captive-reared Migratory Monarch Butterflies Show Natural Orientation When Released in the Wild ........................................................................................................... 95

Introduction ....................................................................................................................... 96
Methods ............................................................................................................................... 97
Results ................................................................................................................................ 101
Discussion .......................................................................................................................... 101

Chapter 6: Conclusion ........................................................................................................ 108
References .......................................................................................................................... 114
Supplementary Material: Chapter 2 .................................................................................. 167
Supplementary Material: Chapter 5 .................................................................................. 210
List of Tables

Table 2.1 Summary of search terms associated with the declines in the eastern migratory North American population of monarch butterflies (*Danaus plexippus*) used in the comprehensive literature review using Web of Science™ ........................................... 41

Table 2.2 The proportion of peer-reviewed research papers classified by the type of study, effect on monarchs, and potential risk imposed by the threat to the eastern migratory North American population of monarch butterflies (*Danaus plexippus*) ............................................................................................................................. 42

Table 3.1 Concentration of the neonicotinoid clothianidin (ppb) in soil, swamp milkweed (*Asclepias incarnata*), instar 5 caterpillars, and adult monarch butterflies (*Danaus plexippus*) for insecticide applications in control, 15 ng/g (low dose) and 25 ng/g (high dose) treatments. For samples with clothianidin detected (DET (n)), the mean (x̄), standard deviation (s), range, and median are provided. The number of samples where no clothianidin detected (ND (n)) is provided as summary statistics could not be calculated (-) ........................................................................................................................................ 64

Table 3.2 Physiological effects of sublethal concentrations of the neonicotinoid insecticide clothianidin on monarch caterpillar (*Danaus plexippus*) reared on swamp milkweed (*Asclepias incarnata*) grown in control (no insecticide), 15 ng/g (low dose) and 25 ng/g (high dose) soil. Measurements are reported as the mean ± SD (n), except where no data was available (-) ................................................................................................................................. 66
Table 3.3 Top models for caterpillar development and adult butterfly reproductive output for monarchs (*Danaus plexippus*) reared on swamp milkweed (*Asclepias incarnata*) grown in control (no insecticide), 15 ng/g (low dose) and 25 ng/g (high dose) soil. Model estimates, upper and lower 95% confidence intervals are indicated, as well as Akaike’s information criterion (AIC) or the corrected AIC for small sample sizes, log likelihood (logLik), ΔAIC or ΔAICc, and Akaike weights. Variables included as fixed effects or predictors in the top models include instar, treatment, mass of both male and female monarchs (mass), male age (ageM), and female age (ageF) ............................................................ 67

Table 4.1 Concentration of the neonicotinoid clothianidin (ppb) in soil, swamp milkweed (*Asclepias incarnata*) and instar 5 monarch caterpillars (*Danaus plexippus*) for insecticide applications in control, 15 ng/g (low dose) and 25 ng/g (high dose) treatments. For samples with clothianidin detected (DET (n)), the mean (\( \bar{x} \)), standard deviation (s), range, and median are provided. The number of samples where no clothianidin detected (ND (n)) is provided as summary statistics could not be calculated (-) ............................................................ 89

Table 4.2 Eastern North American migratory monarch butterflies (*Danaus plexippus*) reared in environmental chambers simulating autumn conditions until pupation, then tested in an outdoor flight simulator to record flight orientation (between 0° to 359°) or radio-tracked during fall migration. Rayleigh test was 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 .......................... 92
LIST OF FIGURES

Figure 3.1 Body (a) length (mm) at instars 1, 3, and 5 and (b) width (mm) at instars 3 and 5 for control and monarch caterpillars (*Danaus plexippus*) exposed to the neonicotinoid pesticide clothianidin at 15 ng/g (low dose) and 25 ng/g (high dose) of the neonicotinoid pesticide clothianidin. Violin plots show the distribution of the raw data presented with median (⋆) and range of observations (whiskers) .......................... 69

Figure 3.2 Body (a) volume (mm$^3$) at instars 3 and 5 and (b) mass (g) at instar 5 for control (CO) and monarch caterpillars (*Danaus plexippus*) exposed to the neonicotinoid pesticide clothianidin at 15 ng/g (low dose) and 25 ng/g (high dose) of the neonicotinoid pesticide clothianidin. Violin plots show the distribution of the raw data presented with median (⋆) and range of observations (whiskers) ...................................................... 70

Figure 3.3 Forewing length (mm) by (a) treatment for adult monarch butterflies (*Danaus plexippus*) reared exposed to the neonicotinoid pesticide clothianidin at 15 ng/g (low dose) and 25 ng/g (high dose) of the neonicotinoid pesticide clothianidin. Violin plots show the distribution of the raw data presented with median (⋆) and range of observations (whiskers) ............................................................... 71

Figure 4.1 Orientation of captive-reared eastern North American migratory monarch butterflies (*Danaus plexippus*) reared on swamp milkweed (*Asclepias incarnata*) grown in control (0 ng/g; n=12), 15 ng/g (i.e., low dose; n=9) and 25 ng/g (i.e., high dose; n=18) soil and (a) flown in a flight simulator for 10 minutes in Guelph, ON in September 2018 or (b) released in Cambridge, ON in October 2018 for radio-tracking (control: n=6;
low dose, n=5; high dose, n=9). The direction of flight for individual monarchs (•) is shown in a circular plot, where each section of the central windrose indicates the proportion of individuals with directional flight …………………………………………………………………………………………………………

**Figure 4.2.** Orientation of captive-reared eastern North American migratory monarch butterflies (*Danaus plexippus*) reared on swamp milkweed (*Asclepias incarnata*) grown in (a) control (0 ng/g), (b) 15 ng/g (i.e., low dose) and (c) 25 ng/g (i.e., high dose) soil and released in Cambridge, ON in October 2018. Symbols indicate the sites of release (★) and location of first detection at a Motus tower (◆), with the relative size referring to the number of detections at that tower. The number of monarchs detected at each tower is shown in brackets and grey dots indicate Motus towers that were active at the time of releases but did not detect any monarchs …………………………………………………………………………………………………………

**Figure 5.1.** Orientation of captive-reared eastern North American migratory monarch butterflies (*Danaus plexippus*) (a) flown in a flight simulator for 10 minutes in Guelph, ON in September 2018. The (b) direction of flight (σ = 188°, n = 39; r = 0.30) of flight for individual monarchs (•) with the group mean direction (◆) is shown in a circular plot, where each section of the central windrose indicates the proportion of individuals with directional flight. Group mean direction is indicated as a solid line and each section of the windrose indicates the proportion of individuals with directional flight. (c) Map of the direction of flight for monarch butterflies released in Guelph, ON in September 2017 (n = 9, green lines) and Cambridge, ON in October 2018 (n = 20, orange lines). Symbols indicate the sites of release (★) and location of first detection at a Motus tower (◆), with the relative size referring to the number of detections at that tower (lowest number of
detections at a tower = 1, highest number of detections at a tower = 5). Grey dots indicate Motus towers that were active at the time of releases. (d) Circular plot shows the direction of flight for radio-tracked monarch butterflies ($\sigma = 145^\circ$, $n = 29$, $r = 0.71$)
Chapter Publications and Author Contributions

The four main research chapters were prepared for submission to, or are published in, academic journals. These submissions include multiple authors which were involved in the development, research, and writing phases.

Chapter 2 is published in *Frontiers in Ecology and Evolution* (Wilcox et al. 2019) and was co-authored by Amy E. M. Newman, D. T. Tyler Flockhart, and D. Ryan Norris. The study was designed and conceived by all authors. A. A. E. W. wrote the initial draft of the manuscript and all other authors contributed to writing.

Chapter 3 is in review in *Journal of Insect Conservation* (Wilcox et al. in review a) and was co-authored by Amy E. M. Newman and D. Ryan Norris. All authors conceived of and designed the study. A. A. E. W. conducted statistical analyses and wrote the manuscript. All authors provided comments and feedback on the manuscript.

Chapter 4 is in review in *Journal of Experimental Biology* (Wilcox et al. in review b) and was co-authored by Amy E. M. Newman, Nigel E. Raine, and D. Ryan Norris. All authors conceived and designed the project. A. A. E. W. conducted the experimental work, analyzed the data, and drafted the original manuscript. All authors contributed to writing and revising the manuscript.

Chapter 5 is in review in *Communications Biology* (Wilcox et al. in review c) and was co-authored by Amy E. M. Newman, Nigel E. Raine, and D. Ryan Norris. All authors conceived of and designed the study. A. A. E. W. conducted the experimental work,
analyzed the data, and drafted the original manuscript. All authors provided comments and feedback on the manuscript.
Chapter 1

Introduction

*Life history of the monarch butterfly*

The majority of monarch butterflies (*Danaus plexippus*) in North America originate in the eastern US (Flockhart et al. 2013; Oberhauser et al. 2017) and readily use agricultural landscapes for breeding and oviposition (Nail et al. 2015; Pitman et al. 2018; Thogmartin et al. 2017b). Monarch butterflies rely on milkweed species (*Asclepias* spp.) as an obligate host plant (Oberhauser 2004), principally laying eggs on stems grown in corn and soy fields (Nail et al. 2015), but over 850 million milkweed stems have been lost from agricultural landscapes and grasslands in the Midwestern US since 1999 (Pleasants 2017). As such, conservation management plans have focused on restoring milkweed abundance on agricultural plots in an effort to increase habitat substrate for egg deposition and availability of milkweed for developing caterpillars (Thogmartin et al. 2017b).

Female monarch butterflies accurately identify the phytochemical properties of milkweed (i.e., glycosides, Haribal and Renwick 1996; Zalucki et al. 1990) via chemoreceptors on their antennae and legs (Chew and Robbins 1984). These oviposition stimulants trigger deposition of eggs, which can number up to 400 during the breeding season, on the ventral side of milkweed leaves (Fischer et al. 2015; Haribal and Renwick 1996; Oberhauser 2004; Woodson 1954; Zalucki and Kitching 1982; Zalucki and Rochester 2014). Once hatched, monarch caterpillars consume their egg
casing and nearby foliage, rapidly increasing in size and gaining mass over 5 successive moults (Oberhauser 2004). The final instar then forms a chrysalid and emerges 8-15 days later as an adult monarch butterfly (Oberhauser 2004).

Physiological changes occurring during metamorphosis in the late fall results in a migratory generation of monarch butterflies characterized by the development of long, thin wings to reduce loading and drag and an increase in flight muscle (Dingle 2006). Females, however, commonly have physical features (i.e., smaller body size and thicker wings) that results in lower loading and greater mechanical strength compared to males, explaining their higher migratory success (Davis and Holden 2015). A suite of hormonal, molecular, and genetic signals trigger the morphological changes from the caterpillar to adult monarch life stage (Zhan et al. 2011; Zhu et al. 2008). After eclosion, the eastern migratory North American population of migratory generation of monarch butterflies migrates nearly 4,000 km southwest from southern Canada and the northern United states to the Sierra Madre Mountains of Mexico where they overwinter in large colonies for 4-5 months, remaining in reproductive diapause, before returning northward for breeding over another 3 generations (Brower 1995; Urquhart 1960; Urquhart and Urquhart 1978).

Animal migration is a natural phenomenon that draws together shared and divergent mechanisms that enable a wide-range of taxa to complete long-distance journeys in response to fluctuating environmental conditions (Dingle 2014). The ability of ‘true navigators’ to assess their geographic location and orientation (i.e., direction of travel, Gould 2014; Gould and Gould 2012) allows them to correct for displacement
during travel (Gould and Gould 2012; Mouritsen 2003, 2018). In comparison, an internal clock enables ‘vector navigators’ to orient in a fixed direction, unable to correct for longitudinal displacement (Mouritsen 2003; Mouritsen et al. 2013; Perdeck 1958). The monarch butterflies southwest vector navigation strategy relies on solar cues received by the eyes (i.e., solar azimuth, light intensity, and spectral gradients) and at the antennae, then integrated in the midbrain, to orient movement (Dingle 2014; Reppert et al. 2010). This time-compensated sun compass ensures directed flight, yet monarch butterflies are unable to correct for displacement (Mouritsen et al. 2013), exposing them to potential threats during their nearly two month long migration to Mexico.

*Declines in monarch butterflies and threats to population viability*

Monarch butterflies are one of North America’s most iconic species and are facing rapid declines (Thogmartin 2017b). The species’ complex life history and expansive migratory range, extending from southern Canada to the Oyamel fir forest in central Mexico (Urquhart 1960; Urquhart and Urquhart 1978; Brower 1995), poses a unique challenge to assessing population dynamics for the development of transnational conservation management plans for the recovery of this species at risk (Inamine et al. 2016).

Monarch butterfly populations vary substantially interannually (Swengel 1995; Rendón-Salinas et al. 2014) and counts taken during summer do not always correlate with those taken in the southern portion of the migratory range (Badgett and Davis 2015; Davis 2012; Inamine et al. 2016; Pleasants et al. 2017; Ries et al. 2015b). Perhaps the most reliable estimate comes from the mass aggregations of monarchs in their overwintering area in Mexico (Davis 2012; Ries et al. 2015b). Area occupied at overwintering sites is
used as an indirect measure of monarch abundance (Brower et al. 2011a), which over the 2 decades has shown an increasingly rapid downward trend (1997-2006: 6.7 ha ± 2.66 ha; 2007-2016: 2.84 ha ± 1.56 ha; Ries et al. 2015b; Vidal and Rendón-Salinas 2014), equating to a loss of more than 80% of the population (Thogmartin 2017b) and, most recently, there was a 53% decline in overwintering monarchs during 2018-2019 (i.e., a 4.02 ha loss in monarch butterfly abundance in the tree canopy; WWF México 2020). Though conservation efforts may partially contribute to the rise in monarch population abundance, fluctuations in counts at overwintering sites suggest potential differences in monitoring techniques (Badgett and Davis 2015, Davis 2012, Inamine et al. 2016, Pleasants et al. 2017, Ries et al. 2015b) and spatiotemporal trends in threats may also disproportionally affect monarch survival.

Conservation threats vary across the range of the monarch butterfly and contribute to declines at different stages of the monarch life cycle. Environmental conditions and habitat degradation and/or destruction at both breeding (Pleasants 2017; Thogmartin et al. 2017b,c) and overwintering (Oberhauser et al. 2017; Thogmartin et al. 2017b) sites are thought to be among the leading causes of monarch declines. In addition to milkweed being a nuisance plant on agricultural fields, genetically modified crops (e.g., high yield maize, soybean, cotton, and canola) grown to be herbicide resistant have resulted in severe declines in the abundance of the monarch’s host plant (Pleasants and Oberhauser 2013; Oberhauser et al. 2001). This significant loss of breeding and larval habitat is argued is to be one of the main causes for declines (Brower et al. 2012; Pleasants et al. 2017), with as many as 1.8 billion additional stems of milkweed plants needed in North America to increase monarch population size to
sustainable levels (Thogmartin et al. 2017a). Suboptimal environmental conditions (e.g.,
 extreme cold or warmth; Hunt and Tongen 2017) and long-term changes in climate
 (Barve et al. 2012; Opdam and Wascher 2004) are also thought to negatively affect
 overwintering monarch populations, coupled with habitat fragmentation and
deforestation (Brower et al. 2012; Vidal et al. 2014) which can alter population structure
(Ramírez et al. 2003) and limit resource (González et al. 1995) and habitat availability
(Zalucki and Lammers 2010; Zalucki et al. 2015). Environmental contaminants,
including exposure to the bacteria *Bacillus thuringiensis* (Anderson et al. 2004, 2005),
herbicides (Thogmartin et al. 2017b), and insecticides (Oberhauser et al. 2006, 2009;
Pecenka and Lundgren 2015), as well as potential pathogen exposure (Thogmartin et
al. 2017b), likely contributes to declines. Though it is imperative to assess the foremost
threats to monarch butterflies, it is also vital to recognize the non-exclusive nature of
each threat and the potential that risks likely interact synergistically across the
monarchs range.

It is vital to evaluate the impact of threats on biological systems and to develop
practical approaches for management. Understanding the drivers of species declines
critical requires a multi-disciplinary approach that integrates findings from statistical
methods, field studies, and experimental research to thoroughly inform decision-makers
when developing conservation plans for species protection and recovery (Wilcox et al.
2019). Statistical methods are valuable for the development of predictive models to
assess uncertainty in approaches to conservation. These computational tools are
developed from ecological, demographic, and genetic studies, among other scientific
fields, conducted in-field and/or laboratory. Field studies can be used to assess
ecologically relevant scenarios, evaluating the impact of large-scale habitat loss and contamination (Brower et al. 2015, 2017; Krischik et al. 2015), predation and parasitism (Alonso-Mejía et al. 1998), and behaviour (Bartholomew and Yeargan 2001). For instance, tracking devices (e.g., radio-transmitter and GPS tracking tags) can record species spatial movements, potentially providing detailed information on potential contact with threats. However, experimental research within a laboratory offers the opportunity to finely control environmental conditions and manipulate variables independently in order to assess the relative contribution of threats in a manner not feasible in-field. Nonetheless, though researchers can conduct a wide range of studies to better understand the underlying mechanisms driving losses, limited research is available to assess the extent of the contribution some threats play in the declines of monarch butterflies and rarely are the threats evaluated for their impact synergistically. More, assessing potential threats collectively is critical to disentangling the underlying mechanism of declines, allowing conservation practitioners to identify priorities for evidence-based stewardship and environmental management.

*Synthetic insecticides and ecological effects of neonicotinoids*

Neonicotinoids are the most widely used class of insecticides in the world for pest management (Jeschke et al. 2011; Simon-Delso et al. 2015). Though many neonicotinoid active compounds exist, over 40% of the global market value of nearly $2.6 billion USD (in 2009; Jeschke et al. 2011; Simon-Delso et al. 2015) is attributed to the sale of imidacloprid, clothianidin, and thiamethoxam (Bass et al. 2015). The physiochemical properties, chiefly high water solubility, provides pest control with an
ease of application (Simon-Delso et al. 2015) and broad range for use in veterinary medicine (Merck Manuals 2016), home gardening (Craddock et al. 2019), aquaculture (Simon-Delso et al. 2015), and crop protection (Craddock et al. 2019; Simon-Delso et al. 2015; US Geological Survey 2018). As an agrochemical, neonicotinoids have a low toxicity to people (compared to other insecticides) that reduces operator and consumer risk during application in irrigation systems, as a foliar spray, soil treatment, or as a seed dressing to decrease pest density, defoliation, and crop damage while increasing crop yield (Koch et al. 2005; Kuhar et al. 2002; Maienfisch et al. 2001; Nault et al. 2004; Simon-Delso et al. 2015; except Krupke et al. 2017; Mourtzinis et al. 2019). However, their ubiquitous use on agricultural landscapes has had the consequence of environmental accumulation in soil and waterbeds, with chemical persistence lasting up to several years (Bonmatin et al. 2015; DeCant 2010; Simon-Delso et al. 2015). As a result of the environmental prevalence of these insecticides, highly sensitive non-target species of insects and aquatic invertebrates (Sánchez-Bayo et al. 2016) are at an increased risk of exposure and time-cumulative toxicity (Bonmatin et al. 2015; Sánchez-Bayo et al. 2016).

Neonicotinoids effectively target arthropod nicotinic acetylcholine receptors (nAChRs), binding to the insect nAChRs to elicit depolarizing currents that lead to an excitatory response in the postsynaptic neuronal membrane (Bonmatin et al. 2015; Brown et al. 2006; Déglise et al. 2002; Palmer et al. 2013; Simon-Delso et al. 2015); yet, conversely, neonicotinoids can also exhibit nerve-blocking activity (Kagabu et al. 2008; Toshima et al. 2008). In addition to the variation in excitatory and inhibitory responses, differences in receptor specificity for particular neonicotinoids explain some
of the variation in toxicity profiles and the potency of different chemical formulations (Tomizawa and Casida 2011). The molecular mechanisms underlying physiological and behavioural responses to neonicotinoids exposure depends on the substance, organism, and the environment (Simon-Delso et al. 2015) and in many cases the physiological and molecular pathways have yet to be fully elucidated. For instance, neonicotinoid exposure increases cellular stress responses (Ayyanath et al. 2014) and may interact with the insect growth regulator juvenile hormone affecting the molting process, development, and reproductive maturation (Schelling and Jones 1996). The location of the nAChRs is also critical to understanding potential downstream effects of neonicotinoid exposure. Receptors are located predominantly in the insect midbrain (Bonmatin et al. 2015; Sánchez-Bayo et al. 2016), a region that processes information on navigation (Fischer et al. 2014) and orientation (Cabirol and Haase 2019; Heinze and Reppert 2011, 2012). For instance, the monarch butterfly midbrain has a high concentration of nAChRs, important for the integration of navigational information from the time-compensated sun compass (Heinze and Reppert, 2010, 2012; Cabirol and Haase, 2019), but currently no information is available on the effect of the insecticides on migratory orientation. Though insects appear to have the ability to metabolize neonicotinoids (Manjon et al. 2018), early and repeated exposure could result in detrimental effects on motor coordination and navigation, making it imperative to understand the species- and context-specific effects of neonicotinoids exposure.

Beneficial non-target species of insects and invertebrates that spend significant proportions of their time in the soil or waterbeds near agricultural fields or are dependent on landscape features with potential contact with neonicotinoid residues
could have a high degree of cumulative risk for sublethal and toxic effects. Phytophagous insects (e.g., lepidopteran larvae) may consume plant matter contaminated by foliar spray or translocated from the seed coatings and the soil to the plant leaves and fruits (Olaya-Arenas et al. 2019). Similarly, samples of pollen (Long et al. 2016; Tsvetkov et al. 2017), honeydew (Calvo-Agudo et al. 2019), beebread and royal jelly (Giroud et al. 2013) in regions contaminated with neonicotinoids are shown to have trace levels of the chemical and pose a risk to developing Hymenoptera that feed on the carbohydrates. Studies have also shown honeybees (Apis mellifera) and bumblebees (Bombus terrestris) preferentially feed from sucrose solutions containing imidacloprid and thiamethoxam (Arce et al. 2018; Kessler et al. 2015). Problems in ontogenetic development arise with early exposure to neonicotinoids. The cotton bollworm (Helicoverpa armigera; Ahmad et al. 2013) experiences accelerated growth with exposure to sublethal levels of imidacloprid, while monarch (Pecenka and Lundgren 2015) and cabbage (Pieris brassicae; Whitehorn et al. 2018) butterflies have reduced body size compared to controls reared without exposure to neonicotinoids. Fecundity is also negatively impacted in a number of insect species (Laycock et al. 2012, 2014; Rundlöf et al. 2015; Sandrock et al. 2014a,b; Woodcock et al. 2017) and, the adverse effects of neonicotinoids on the insect immune system (Di Prisco et al. 2013), could result in reduced reproductive capacity and colony growth, in the case of honeybees, if disease resistance is impaired (Brandt et al. 2017). Lastly, large acute doses of neonicotinoid can reduce insect activity (Stanley et al. 2016) and limit motor control (Williamson et al. 2014), while sublethal exposure levels can prolong foraging bouts (Gill and Raine 2014; Stanley et al. 2016), partially due to deficits memory and the
capacity for navigation (Fischer et al. 2014; Jin et al. 2015). Despite accumulating research on the profound physiological and behavioural effects of neonicotinoid exposure, the risk imposed by neonicotinoids on monarch butterflies is limited. Given the wide range of potential threats to monarch butterflies, it is critical to evaluate the impact neonicotinoids may have at different stages of the life cycle.

Captive rearing for species conservation

Wildlife conservation efforts have focused on the detection of population declines and the identification of factors driving the loss of biodiversity (Caughley 1994). Once identified, the threats imposing a risk to populations can be mitigated and actions taken to improve population recovery (Young 2000). Removal of individuals from the wild (i.e., ex situ conservation) can help reduce the risk imparted by potential threats and, if individuals are later reintroduced into the wild, may help improve population recovery (Lacy 2010). Captive rearing and reintroduction programs have been used extensively to support conservation efforts and, in some cases, successfully mitigate the risk of threats to species at risk and facing rapid declines. For instance, several species of endangered wētā (Hemideina spp.) have been reared in captivity and introduced to predator-frees islands near New Zealand (Gullan and Cranston 2014). In many cases, the rearing and release of endangered butterflies has also been successful. In 2019, the Oregon Zoo released 1,600 captive-reared endangered Taylor’s checkerspot butterflies (Euphydryas editha taylori) to their native habitat on the prairies of central Washington (Oregon Zoo 2019). Despite these successes, other species can be notoriously difficult to maintain in captivity (Mason 2010), so assessing factors that could impact long-term
fitness for captive-reared animals is important to ensure successful reintroduction in the wild.

Recognition of monarch declines, as well as their potential underlying causes, has garnered intense support for monarch conservation efforts and trilateral protection efforts of the three countries in the eastern North American monarch butterfly range (i.e., Mexico, Canada and the United States). Dramatic headlines on monarch declines (Wines 2013; Hannibal 2019) have prompted citizen science efforts that aims to raise awareness, increasing the planting of plants for nectaring, and milkweed (Diffendorfer et al. 2013; Hannibal 2019; however, Penn et al. 2018), and contributed to scientific studies estimating population abundance of caterpillars, butterflies, milkweed, and disease dynamics (Diffendorfer et al. 2013; Zimakas 2019). In fact, a survey of US households found that, as an iconic insect and pollinator, monarchs were valued at levels similar to charismatic vertebrate species at risk (Diffendorfer et al. 2013). Thus, the contribution of citizen science efforts highlights the need for a variety of management tools to address the conservation of this species.

The immense popularity of monarchs in North America makes them an incredibly valuable tool to engage the public in efforts to protect pollinators and help restore monarch populations to sustainable levels. Households and classrooms across North America readily rear monarchs in captivity as an educational tool and to enhance conservation efforts by protecting developing monarchs and reintroducing them into the wild in efforts to increase population size (Tenger-Trolander et al. 2019). However, controversy exists over exposure to pathogens (Journey North 2015) and potential
physiological changes that may occur in captivity (Tenger-Trolander et al. 2019). Tenger-Trolander et al. (2019) found that monarchs reared in autumn-like conditions in captivity were unable to orient properly in a southward direction toward Mexico when flown in a flight simulator. However, given that monarchs rely on a time-compensated sun compass during migration (Heinze and Reppert, 2010, 2012), it is unclear if prolonged exposure to sunlight cues when release into the wild could reset the physiological mechanism governing directed flight. Therefore, there is still the potential that if proper care is taken to minimize risks, the release of monarch butterflies in southern Canada and the US could potentially be used as a tool for the conservation of the species.

Research objectives

In my thesis, I explore the sublethal effect of neonicotinoid insecticides and evaluate a potential solution for the recovery of the eastern North American monarch butterfly. Proposed and current restrictions in Canada (Health Canada 2018, 2019) and internationally on neonicotinoids aims to reduce potential exposure and toxicity from the insecticides for non-target insect species. Understanding the contribution neonicotinoids and other insecticides play in the decline of monarch butterflies has also been identified as a priority by the Commission for Environmental Cooperation (CEC 2019), an intergovernmental (i.e., between Canada, US, and Mexico) organization with the aim to identify challenges and conservation priorities to improve environmental protection within each country (CEC 2018). Further, identifying the role neonicotinoids play at different stages in the monarch life cycle is key to the development of multinational
policies for management and recovery planning to mitigate population declines of this multi-generational and migratory species at risk. The overall objective of the research was to determine the relative impact of threats to monarch butterflies, assessing how prolonged and sublethal exposure to the neonicotinoid insecticide clothianidin during early monarch caterpillar influences development and behaviour, and the potential use of captive rearing as a tool for monarch conservation.

_Dissertation outline_

My thesis identifies the potential risk imposed by various threats, including neonicotinoids, to the eastern North American population of monarch butterflies and establishes whether a popular conservation tool (i.e., captive rearing) can aid in species recovery. In **Chapter 2**, I evaluate the strength of evidence concerning the potential causes of decline, including environmental contaminants like neonicotinoids, in the eastern population of monarch butterflies. I conducted a thorough literature review and classified five categories of threats to monarch butterflies: (1) change in suitable abiotic environmental conditions; (2) deforestation in the overwintering range; (3) exposure to contaminants including the bacteria _Bacillus thuringiensis_, herbicides, and insecticides; (4) loss of breeding habitat; and (5) predation, parasitism, and species-specific pathogens. I found that, though threats vary spatially and temporally across the monarchs range, published papers suggest that the decline in suitable environmental conditions and overwintering (i.e., deforestation) and breeding habitat loss are the principal threats to population viability of the eastern North American migratory monarch population. Next, I use a series of experimental studies on a captive population of
monarch butterflies reared from caterpillars on milkweed grown in soil treated with field-realistic levels of clothianidin at a low (15 ng/g of soil) or high (25 ng/g of soil) dose levels, or in a control (0 ng/g of soil). In Chapter 3 I evaluate the effect of clothianidin on early caterpillar development and adult female monarch reproductive output. I measured caterpillars during development and, after adults emerged, monarchs were mated to quantify egg size and the number of eggs laid. Though clothianidin did not appear to affect individual size, mass, or volume of instar 1 caterpillars, instar 5 caterpillars were shorter, thinner, had a smaller overall volume, and weighed less than controls. Adult monarchs from the treatment groups were larger, but this did not influence the egg size or the number of eggs laid. Our results reveal that early exposure to clothianidin can negatively impacts monarch development, but it is unlikely that these effects carry forward to reduce reproductive output in adult female monarch butterflies.

In Chapter 4 I explore the effect of early exposure to clothianidin during monarch caterpillar development on adult monarch orientation and migration. Butterflies were tested for orientation in a flight simulator or radio-tracked in the wild during the fall migratory period. There was no evidence that clothianidin influenced orientation, vector strength, or the rate of travel, nor was directionality affected by morphological traits (i.e., mass and forewing length), the time of testing, wind direction, or temperature, suggesting that clothianidin does not affect the migratory capacity of adult migratory monarch butterflies. Finally, Chapter 5 assesses whether captive rearing influences monarch orientation using a standardized laboratory flight simulation or radio-tracked in the wild using array of automated telemetry towers. I found that monarchs tested in the flight simulator did not show a directional flight response, but those released and radio-
tracked regained proper orientation and flew in a southward direction. The results suggest that, though captive rearing results in temporary disorientation, within days of release and exposure to natural skylight cues, monarchs can re-establish proper orientation.
Chapter 2


Abstract

The migratory monarch butterflies (*Danaus plexippus*) of eastern North America have undergone large-scale declines, which may be attributable to a variety of underlying causes. The uncertainty about the primary cause of declines and whether individual threats are likely to increase in the future presents challenges for developing effective conservation management and policy initiatives that aim to improve population viability. This paper identifies five potential threats and classifies these threats according to the types of studies (observational, experimental, simulation/models) and their current impact and anticipated risk. Broadly, the threats can be classified into five categories: (1) change in suitable abiotic environmental conditions; (2) deforestation in the overwintering range; (3) exposure to contaminants including the bacteria *Bacillus thuringiensis*, herbicides, and insecticides; (4) loss of breeding habitat; and (5) predation, parasitism, and species-specific pathogens. The vast distribution of the monarch butterfly makes it likely that population declines are attributed to a suite of interacting factors that vary spatially and temporally in their contribution. Nonetheless, the published papers we reviewed suggest the decline in suitable environmental
conditions in addition to overwintering (i.e., deforestation) and breeding habitat loss are the most likely threats to continue to affect the population viability of monarch butterflies.

**Introduction**

Insect populations are experiencing rapid declines globally (Dirzo et al. 2014; Stork et al. 2015) that may have implications for ecosystem function and contributions to economic services (Allen-Wardell et al. 1998; Potts et al. 2010). Changes in the suitability of environmental conditions driven by extreme weather and climate change (Batalden et al. 2007; Barve et al. 2012; Brower et al. 2017), habitat loss (Didham et al. 1996; Fattorini 2011; Thogmartin et al. 2017b), exposure to contaminants (Stanley-Horn et al. 2001; Thogmartin et al. 2017b), and changes in species interactions (e.g., invasive species, Burghardt and Tallamy 2015) can have profound effects on biodiversity. The substantial loss of biodiversity within the insect taxon emphasizes their sensitivity to environmental perturbations, but also makes them ideal bioindicators (Lenhard and Witter 1977; Nummelin et al. 2007) for testing the impact of threats and their downstream effects. By integrating an array of research methods and disciplines, the ramifications of declines on biological systems can be better-anticipated and incorporated into conservation management plans and policy initiatives (Vanbergen and Insect Pollinators Initiative 2013).

A multi-disciplinary approach is needed to address the loss of insect diversity and to understand the potential mechanisms driving declines. Extending research initiatives beyond traditional economically significant species may allow for identification of shared
mechanisms behind observed declines. In doing so, it is important to consider the type and strength of evidence for threats to a population, emphasizing varying susceptibility at different life stages and across the species range. Therefore, the dynamic and synergistic nature of potential threats can be evaluated and considered when developing conservation strategies, especially for animals that cross national and international boundaries.

The eastern migratory North American population of monarch butterflies (*Danaus plexippus*) undergoes an annual migration between the Sierra Madre Mountains of Mexico and the northern United States and southern Canada (Urquhart 1960; Urquhart and Urquhart 1978; Brower 1995). In Mexico, monarchs overwinter in large colonies for 4–5 months and remain in a reproductive diapause. In spring, individuals begin mating and migrate north to lay eggs on emerging milkweed (*Asclepias* spp.) in the southern United States (Brower 1995). Over successive generations the population colonizes much of the eastern and central United States and parts of southeastern and south central Canada (Brower 1995; Flockhart et al. 2013). The complex nature of the annual cycle and habitat specialization provides a rare opportunity to investigate ecological pressures across a variety of temporal and geographical scales. Moreover, such an understanding can improve our knowledge of the international cooperation required to preserve this flagship species at risk.

Conservation management plans rely on the accurate estimation of species abundance. Over the last 2 decades the eastern population of monarch butterflies has declined more than 80% at overwintering sites (Thogmartin et al. 2017b). Arguably
overwintering population size determined by the occupied surface area represents the most reliable estimates and denotes the effective population size (i.e., number of individuals contributing to the next generation, Davis 2012; Ries et al. 2015b). However, counts taken in the northerly portion of the range during pre-migration do not always correspond with those at overwintering sites, which may suggest either methodological issues in estimating population size or high mortality during migration (Davis 2012; Badgett and Davis 2015; Ries et al. 2015b; Inamine et al. 2016; Pleasants et al. 2017). Regardless, despite substantial interannual variation in monarch population size (>10-fold, Swengel, 1995; Rendón-Salinas et al. 2014), summer and winter counts show consistent year-to-year fluctuations (Ries et al. 2015a,b). The discrepancy between population estimates in winter and summer highlights the need to distinguish independent threats contributing to potential declines observed throughout the annual cycle and at different developmental stages.

Changes in suitable environmental conditions (Barve et al. 2012; Thogmartin et al. 2017b) and habitat degradation/loss at both breeding (Pleasants 2017; Thogmartin et al. 2017a) and overwintering (Oberhauser et al. 2017) sites, as well as contaminant exposure (Oberhauser et al. 2006, 2009; Pecenka and Lundgren 2015), are thought to be foremost threats to monarch butterfly populations. Suboptimal environmental conditions during the overwintering period, such as unseasonably warm temperatures (Hunt and Tongen 2017) or cold and wet microclimates that pose a risk of freezing (Anderson and Brower 1996), can accelerate lipolysis that quickly depletes lipid stores needed for overwinter survival (Alonso-Mejía et al. 1997). Overwintering lipid stores may be further reduced by limited availability of nectar sources due to habitat loss and a
northward shift in monarch movements expected with climate change (Batalden et al. 2007; Brower et al. 2015; Lemoine 2015). The introduction and widespread adoption of glyphosate resistant corn and soybean has greatly increased the use of herbicide, causing up to 68% loss of milkweed in some areas of the central United States (Pleasants and Oberhauser 2013; Flockhart et al. 2015; Zaya et al. 2017), and logging and rural development have limited suitable habitat for overwintering sites (Brower et al. 2011b). Finally, environmental contaminants, including genetically-modified (GM) pollen (i.e., Bacillus thuringiensis (Bt), Anderson et al. 2004, 2005) and insecticides (e.g., neonicotinoids, Pecenka and Lundgren 2015; pyrethroids, Oberhauser et al. 2006, 2009), as well as pathogens [notably, Ophryocystis elektroscirrha (OE)], could contribute to elevated rates of mortality. As a species at risk (SARA 2017, US Fish & Wildlife Service 2017) it is imperative that research identifies the foremost threat(s) resulting in decreased fitness and survival and the relative contribution of each threat to the cumulative population decline.

To better understand the main drivers of monarch decline it is important to evaluate the type of study investigating the threat and strength of evidence (i.e., support) for each potential threat in terms of their effect on monarch butterflies and possible future risk to population viability. Though it is probable that other threats exist beyond those identified in this review, we focus on five broad potential threats commonly reported for the eastern migratory North American population of the monarch butterfly: (1) change in suitable abiotic environmental conditions, (2) deforestation in the overwintering range, (3) exposure to contaminants including the bacteria Bt, herbicides, and insecticides, (4) loss of breeding habitat, and (5) predation, parasitism, and
pathogens. We modify a previously established scoring system (Godfray et al. 2013, 2014), sorting and summarizing peer-reviewed research papers into three groups based on the type of study. For each research paper we then assign the level of support for the potential threat affecting monarch butterflies and assess the likelihood of the threat persisting and/or increasing. Though a recent review highlighted anthropogenic impacts on monarch populations (Malcolm 2017), our aim is to evaluate the strength of evidence available for each threats and highlight key gaps in research needed to guide conservation management plans and policy development.

Methods

A systematic review of available peer-reviewed literature was performed following the procedure outlined in Bechshoft et al. (2017), but using monarch-specific terms associated with hypotheses regarding population declines in the Web of Science™ (Reuters 2016). An initial list of papers was established through a literature search of all databases, including the Web of Science Core Collection, Current Contents Connect, FSTA (The Food Science Resource), KCI (Korean Journal Database), MEDLINE, Russian Science Citation Index, SciELO Citation Index, and Zoological Record, with no limitations placed on the publication date. This list was later refined to retain only peer-reviewed research papers on the eastern migratory North American population of monarch butterflies in English. Search terms (“topic”) were combined in pairs using the Boolean operator “and,” with select terms having wildcard truncation (∗) to allow for various word endings (Table 2.1).
We sorted and summarized individual peer-reviewed research papers \((n = 115)\) published up to December 2018 by potential threat and assessed the type of study (e.g., [Control_data], [Field_data], [Mod]) using a scoring system modified from Godfray et al. (2013, 2014) with the following unranked categories:

[Control_data] evidence involving controlled experimental studies.

[Field_data] evidence involving data collected in the field but without experimental control.

[Mod] indirect evidence based on previously collected experimental and/or field collected data to assess the impact of threats on the population or make projections of future environmental and conservation scenarios. Models reflect a degree of uncertainty that cannot be fully accounted for as conditions encountered in the future may deviate from those defined in the model.

After classifying the type of study, we then identified whether research papers independently provided support (i.e., whether a threat was supported [S] or not [N]) for the threat as having a current impact on monarch butterflies and whether there was potential for the threat to pose continued risk to population viability. Support for future risk to the eastern migratory North American population of monarch butterflies was assigned based on when impacts were identified from multi-year and/or historical data or from model projections. Studies where no conclusion could be drawn about the future risk to the population were identified by [-]. We then calculated the percentage of research papers (i.e., number of research papers of the total available studies within
each type of threat) for: (1) the type of study, (2) whether the study provided support for
a threat to monarch butterflies, and (3) the future risk posed to the population. Papers
classified in multiple categories (e.g., [Control_data] and [Field_data]) contributed an
equivalent number of times to the total number of available studies. Where described in
the Results, we also calculated the percentage of represented papers for subcategories
within each threat category for the type of study or level of support. For example, within
the category of changes in suitable environmental conditions, we calculated the
percentage of studies addressing periodic, adverse weather conditions (relative to long-
term climate change).

Results

The summary of peer-reviewed literature, sorted by threat and type of study, assessing
the impact, and future risk imposed to the eastern migratory North American monarch
butterfly population is presented in Supplementary Material: Chapter 2 Table S2.1. The
percentage of research papers assigned to each type of study and their support for the
specific threat on monarch butterflies and potential risk to the population is presented
in Table 2.2.

Change in Suitable Abiotic Environmental Conditions

Field studies constituted one of the principal methods documenting the effects of
sudden changes in environmental conditions and adverse weather patterns in the
decline of monarch butterflies (50% of total studies on adverse weather events, Brower
et al. 2015, 2017), but only a single study considered the effect of extreme weather
patterns before fall migration (25% of total studies on adverse weather events, Hunt and Tongen 2017). Field studies examined the physiological response of monarchs to changes in environmental conditions in the southern portion of the migratory range (Brower et al. 2015), but controlled studies that assessed field-realistic, short-term changes in environmental variables such as temperature, humidity, precipitation, or solar radiation on the physiological condition and survival during the breeding season were absent. Similarly, few studies applied modeling techniques that evaluated the impact of weather extremes on monarch population viability (Flockhart et al. 2015; Hunt and Tongen 2017).

The peer-reviewed literature suggested a negative impact of adverse weather patterns on monarch butterflies (50% of total studies on adverse weather events, Brower et al. 2017; Hunt and Tongen 2017) and these conditions could impact monarchs at each stage of their life cycle (Hunt and Tongen 2017). Though sporadic events may result in considerable losses, the timing of the events is also suggested to alter the severity of the impact. Brower et al. (2015) noted that nectar sources available in the southern portion of the migratory range might offset the energetic cost of adverse conditions experienced earlier in migration, therefore having less impact than extreme weather on overwintering populations. Further, though Hunt and Tongen (2017) showed a negative effect of increasing extreme weather events on monarch butterflies, no studies evaluated the effect of adverse weather patterns in long-term datasets or the extent to which populations are capable of recovering afterwards.
Few controlled experiments investigated the effects of predicted long-term climatic conditions on the condition, growth, and reproduction of monarch butterflies (9% of total studies on climate change) and only a single study explored how rising temperatures impacted host plants at different latitudes (9% of total studies on climate change, Couture et al. 2015). Though no multi-year field studies exist, a substantial number of predictive models (82% of total studies on climate change) attempted to disentangle the effects of long-term climate change on breeding habitat (Zipkin et al. 2012; Lemoine 2015; Zalucki et al. 2015; Thogmartin et al. 2017b), overwintering conditions (Oberhauser and Peterson 2003; Barve et al. 2012; Sáenz-Romero et al. 2012; Zalucki et al. 2015; Thogmartin et al. 2017b), and overall distribution (Batalden et al. 2007).

Climatic conditions are anticipated to change drastically overtime. In line with the temperature-dependent growth of monarchs (Zalucki 1982), elevated temperatures are likely to positively affect larval growth and survival during the breeding season (Couture et al. 2015). Couture et al. (2015) predicted that larvae growth will increase under temperature- and water-stressed conditions, though it is unclear whether the shorter generation time will result in a greater number of generations overall during the breeding season. Beyond the direct effects on larval growth, models suggest climate change is anticipated to result in a northward expansion of the breeding range (Batalden et al. 2007; Lemoine 2015) and that elevated temperatures (Zipkin et al. 2012) are likely to facilitate population growth. As such, other threats likely have a greater potential to drive monarch declines (Flockhart et al. 2015; Zalucki et al. 2015). Nonetheless, the effect of climate change on monarchs at overwintering sites in Mexico
may contribute to lower population viability as rising temperatures may generate unsuitable conditions for diapause (Oberhauser and Peterson 2003; Barve et al. 2012; Sáenz-Romero et al. 2012). Taken together, the majority of studies implied that the threat of climate change is likely to continue (73% of total studies on climate change, Oberhauser and Peterson 2003; Batalden et al. 2007; Barve et al. 2012; Sáenz-Romero et al. 2012; Flockhart et al. 2015; Lemoine 2015; Thogmartin et al. 2017b) and the analysis of multi-year datasets suggests that it may also affect population viability (Zipkin et al. 2012, 2015; Flockhart et al. 2015).

**Deforestation in the Overwintering Range**

The level and effect of deforestation is quantified in the peer-reviewed literature primarily by means of field studies (82%, Table 2.2A). Field observations and aerial surveys assessed the extent of forest canopy loss (Brower et al. 2002; Ramírez et al. 2003; García 2001; Honey-Rosés et al. 2011; Navarrete et al. 2011; Champo-Jiménez et al. 2012; Vidal and Rendón-Salinas 2014; Vidal et al. 2014), microclimate suitability (Anderson and Brower 1996; Alonso-Mejía et al. 1997; Brower et al. 2009, 2011b), and predation levels (Alonso-Mejía et al. 1998) under changing forest conditions. Models (18%, Table 2.2A), relative to controlled studies (no studies, Table 2.2A), were used to quantify the likelihood that forest loss contributed to monarch butterfly declines.

All field studies and models suggested that forest loss is a likely contributor to declines in individual condition and population viability of monarch butterflies through its effect on available overwintering habitat (Brower et al. 2002; Ramírez et al. 2003; García 2001; Honey-Rosés et al. 2011; Navarrete et al. 2011; Champo-Jiménez et al. 2012; Vidal and Rendón-Salinas 2014; Vidal et al. 2014).
et al. 2012; Vidal and Rendón-Salinas 2014; Vidal et al. 2014; Flockhart et al. 2015; Hunt and Tongen 2017; Oberhauser et al. 2017) and suitable environment conditions (Anderson and Brower 1996; Alonso-Mejía et al. 1997 1; Brower et al. 2009, 2011b). However, studies varied on their assessment of the potential future risk to monarch populations. Multi-year data sets and modeling experiments showed continued forest loss within the Monarch Butterfly Biosphere Reserve (Brower et al. 2002; Ramírez et al. 2003; García 2001; Navarrete et al. 2011; Champo-Jiménez et al. 2012; Vidal and Rendón-Salinas 2014; Vidal et al. 2014; Flockhart et al. 2015) that may increase the exposure of monarchs and therefore the probability of a mass mortality event (53%, Table 2.2C). Improving habitat protection and availability at overwintering sites in Mexico is also considered a potential means to reduce losses (Oberhauser et al. 2017). Yet, other studies suggested that, though illegal logging and deforestation likely contributes to monarch declines, it is not the primary driver (Flockhart et al. 2015; Hunt and Tongen 2017) and further forest canopy losses would be required to significantly impact monarch populations (Hunt and Tongen 2017). The results from the remaining studies (Brower et al. 2009, 2011b; Honey-Rosés et al. 2011; 35%, Table 2.2C, Anderson and Brower 1996; Alonso-Mejía et al. 1997, 1998) did not suggest a continued threat from deforestation.

**Exposure to Contaminants**

The type of study assigned to peer-reviewed research papers investigating the effects of environmental contaminants varied depending on the nature of the contaminant. Control (56% of total studies on Bt) and field-based (44% of total studies on Bt) studies
were principally used to assess the effects of Bt exposure from GM crops. Control studies (75% of total studies on insecticides) pre-dominated for work on insecticides and modeling experiments examined the effect of insecticides (13% of total studies on insecticides) and herbicides (100% of total studies on herbicides) on population abundance. Though, certain types of studies are notably absent for each contaminant, perhaps most importantly the lack of field-based studies on the effect of contaminants limits extrapolation of results to field-realistic scenarios. Further, the majority of studies did not investigate multi-year datasets (57%, Table 2.2C) and control and field studies were limited to individual chemicals without considering the wide-range of potential agrochemicals or their interactions.

Controlled laboratory experiments showed a negative effect of Bt on larval development and survival (Losey et al. 1999; Hansen Jesse and Obrycki 2000; Stanley-Horn et al. 2001; Anderson et al. 2004, 2005; Dively et al. 2004) and reproduction (Tschenn et al. 2001), but effects were dependent on age (Hansen Jesse and Obrycki 2000; Hellmich et al. 2001) and Bt-transformation event (i.e., specific occurrence of the uptake of genetic material via transformation of cells of Bt, Stanley-Horn et al. 2001). Studies also confirmed that range overlap with Bt-exposed fields (Oberhauser et al. 2001; Pleasants et al. 2001) could contribute to lower reproductive output (Stenoien et al. 2015), though larval mortality is not always associated with proximity to Bt-exposed fields (Zangerl et al. 2001). Similarly, insecticide use showed effects on individual survival (pyrethroids, Oberhauser et al. 2006, 2009; Stanley-Horn et al. 2001; clothianidin, Pecenka and Lundgren 2015) and herbicide application (i.e., glyphosate) is known to influence population size (Thogmartin et al. 2017b). However, the strength of
the effects varied depending on the agrochemical (i.e., significant effects not shown for dicamba and 2,4-dichlorophenoxyacetic acid use, Thogmartin et al. 2017b), geographic location (Thogmartin et al. 2017b), and life stage (Pan et al. 2017). Overall, few studies suggested a potential future risk to monarch population viability from Bt-exposure (31% of total studies on Bt, Hansen Jesse and Obrycki 2000; Pleasants et al. 2001; Dively et al. 2004; Stenoien et al. 2015), dicamba (33% of total studies on herbicides, Thogmartin et al. 2017b), 2,4-dichlorophenoxyacetic acid (33% of total studies on herbicides, Thogmartin et al. 2017b), and glyphosate (33% of total studies on herbicides, Thogmartin et al. 2017b) applications. Neonicotinoids also did not contribute significantly to monarch declines in a population model (25% of total studies on neonicotinoids, Thogmartin et al. 2017b).

**Loss of Breeding Habitat**

The effects of habitat availability and the influence of urbanization are rarely examined for their impacts on monarch butterflies. In fact, habitat fragmentation was only evaluated in the context of field studies of vehicular collisions or roadsides serving as ecological traps (McKenna et al. 2001; Mueller and Baum 2014) and through 2 modeling experiments that examined the impact of fragmented landscapes on movement rate (Collingham and Huntley 2000) and path (Grant et al. 2018). Likewise, only a single study investigated how the availability of nectar resources affected monarch population growth (Oberhauser et al. 2017). Projections on the threat of habitat fragmentation and urbanization were absent, but the reduction in nectar availability (Oberhauser et al. 2017) was suggested to pose a sustained risk.
The literature on optimal breeding habitat for monarch butterflies is dominated by studies on the potential declines in survival on different host plants (35% of total studies on breeding habitat loss) and the declines in common milkweed (*Asclepias syriaca*) in agricultural fields (47% of total studies on breeding habitat loss). Controlled laboratory experiments investigated the oviposition tendencies on different host plants and the effect on larval growth (DiTommaso and Losey 2003; Mattila and Otis 2003; Yeargan and Allard 2005; Casagrande and Dacey 2007; Pocius et al. 2017a,b), except for two studies that found higher oviposition on common and swamp milkweed (*A. incarnata*, Pocius et al. 2018) and greater numbers of immature larvae on tropical milkweed (*A. curassavica*; Malcolm and Brower 1986) relative to other milkweed species. The effect of the loss of milkweed, principally on agricultural plots, was limited principally to field studies (40% of total studies on milkweed loss, Hartzler 2010; Pleasants and Oberhauser 2013; Inamine et al. 2016; Kasten et al. 2016; Zaya et al. 2017) and modeling experiments (60% of total studies on milkweed loss) relating overwintering population abundance to milkweed availability (Zalucki and Lammers 2010; Flockhart et al. 2015; Zalucki et al. 2016; Hunt and Tongen 2017; Oberhauser et al. 2017; Pleasants 2017; Thogmartin et al. 2017a,c).

Studies provide competing evidence that select species of plants (e.g., dog-strangler vine (*Cynanchum rossicum*), swallow-worts (*Vincetoxicum* spp.) resulted in changes in oviposition tendencies (DiTommaso and Losey 2003; Mattila and Otis 2003; Casagrande and Dacey 2007), and larval survival (Mattila and Otis 2003). Larval survival also varied across milkweed species and was generally higher on common milkweed (Yeargan and Allard 2005; Pocius et al. 2017a,b). Tropical milkweed posed a
more substantial threat as a greater number of larvae are found on this species relative
to common milkweed (Malcolm and Brower 1986) and year-round availability may alter
migration patterns (Satterfield et al. 2015, 2018). At the same time, declines in common
milkweed was almost unanimously agreed upon as a threat to monarchs, with the
exception of a 22-year study that showed monarch population size is predictable along
the migratory route and monarchs are capable of recovering during the breeding season
(Inamine et al. 2016; but see Pleasants et al. 2017). Studies did not evaluate the
sustained risk of the use of different host plant species during oviposition. The threat
imposed by milkweed loss (Hartzler 2010; Pleasants and Oberhauser 2013; Flockhart et
al. 2015; Zalucki et al. 2016; Hunt and Tongen 2017; Oberhauser et al. 2017; Pleasants
2017; Pleasants et al. 2017; Thogmartin et al. 2017a,c; Zaya et al. 2017) to the eastern
migratory North American population of monarch butterflies is anticipated to continue
(80% in support from the total studies on milkweed loss), though present-day loss may
occur at a lower rate than historical levels (Pleasants 2017).

**Predation, Parasitism, and Species-Specific Pathogens**

Predation and parasitism events experienced by monarch butterflies were recorded in
controlled experiments (34%, Table 2.2A) and in field observations (60%, Table 2.2A).
A variety of species were noted to prey upon monarch butterflies (e.g., flies, Arnaud
1978; spiders, Borkin 1982; orioles and grosbeaks, Fink and Brower 1981). Moreover, a
single quantitative risk assessment model determined the impact of the Asian lady
beetle (*Harmonia axyridis*) on monarch butterflies (Koch et al. 2006). The effect
of *OE* on body condition (Altizer and Oberhauser 1999), flight capacity (Bradley and
Altizer 2005), reproduction (Altizer and Oberhauser 1999), survival (Altizer and Oberhauser 1999), and virulence, spore load and transmission (Leong et al. 1997; de Roode et al. 2008a,b, 2009; de Roode and Altizer 2010; Satterfield et al. 2015) was principally quantified using controlled experiments (63% of total studies on OE), but OE detection was also available through in-field observations (32% of total studies on OE, Urquhart 1966; Leong et al. 1992). Thogmartin et al. (2017b) provided the sole instance modeling the effect of OE on population size. In-field observations based on counts during migration determined the potential for migratory culling (80% of total studies on migratory culling, Altizer et al. 2000; Bartel et al. 2011; Badgett and Davis 2015) and a single model determined that there was not a disconnect between monarch population estimates on summering and overwintering grounds, as would be predicted for migratory culling (20% of total studies on migratory culling; Pleasants et al. 2017).

A negative effect of predation and parasitism was found in all peer-review literature studying such events (94%, Table 2.2B), but few papers examined multi-year datasets or modeled the potential for the threat to pose future risk to the eastern migratory North American population of monarch butterflies (85%, Table 2.2C). Of those available, parasitism by OE was likely to have a continued negative effect, particularly with the increased availability of year-round tropical milkweed in the South (Satterfield et al. 2015, 2018; Thogmartin et al. 2017b). Though migratory culling due to OE infection may reduce population abundance at overwintering sites (Altizer et al. 2000; Bartel et al. 2011), other studies suggested this is unlikely the case (Pleasants et al. 2017). Badgett and Davis (2015) also highlight that monarch population abundance at survey sites in Michigan remained constant from 1996 to 2014, potentially due to the high
concentration of monarchs in this region that were born in the Upper Peninsula and Canada, but also suggesting that larval survival during the breeding season could offset losses observed at overwintering sites.

**Discussion**

Our review focused on five broad threats to the eastern migratory North American population of monarch butterflies and highlights the dynamic factors that influence monarch butterfly reproduction and survival at different stages of their life cycle and throughout their range. Though evidence exists in support of each threat contributing to the declines in the eastern population of monarch butterflies, based on the potential future risk, we suggest that the change in suitable environmental conditions, specifically that related to climate change, and habitat loss on overwintering (i.e., via deforestation) and breeding grounds are likely the greatest threats.

For each threat, the most common methodology applied was somewhat different. Projections on the decline in the availability of suitable environmental conditions were evaluated using models (73%, Table 2.2A) estimating range expansion and optimal abiotic conditions under climate change scenarios for both monarchs and their host plant. Studies quantifying deforestation, as expected, principally used observational field methods (82%, Table 2.2A). Models (18%, Table 2.2A) were then used to associate rates of loss and degradation to declines in monarch abundance. The toxicological effects of contaminants on monarch butterflies were principally evaluated using controlled designs (55%, Table 2.2A) and models (14%, Table 2.2A), though few
studies were conducted and only 9 agrochemicals (i.e., herbicides and insecticides) were assessed. Study type was equally weighted in evaluating the impact and requirements needed for restoration of breeding habitat, though variability existed when assessing the influence of different host species ([Control_data]: 82%, [Field_data]: 18% of total studies on host plant species). More, field studies and models contributed the most in research on the effects of milkweed loss ([Field_data]: 40%, [Mod]: 60% of total studies on milkweed loss). Finally, the effect of predation, parasites, and pathogens on monarch butterflies was determined primarily through field observations (60%, Table 2.2A), though the effect OE was quantified through controlled experiments (63% of total studies on OE).

Based on the current literature on potential threats in the declines of the eastern migratory North American population of monarch butterflies, the availability of suitable environmental conditions (i.e., climate change) and overwintering and breeding habitat loss arguably have the greatest impact on population viability and potential for continuing risk to monarch populations (Brower et al. 2011a). However, some threats are understudied and should not be discounted in their potential impact to the population. Contaminant exposure may also potentially drive declines based on evidence of the toxicological effects and potential for cumulative sublethal effects, but it is unknown whether the threat will rise given current high level of agrochemical use (Thogmartin et al. 2017b). Risk imposed from predation is also likely to continue given the interaction with climate warming and potential year-round residency by monarchs in the southern US (Sáenz-Romero et al. 2012; Satterfield et al. 2015, 2018).
The five threats highlighted in our review vary temporally (e.g., early vs. late migrants) and spatially (e.g., migrants vs. breeding populations) in their imposed risk. For instance, while exposure to \textit{Bt} pollen generally reduces survival (Hansen Jesse and Obrycki 2000; Dively et al. 2004), threat level may be minimized if larval populations do not occur at the same time as pollen shedding (Bartholomew and Yeargan 2001) and/or contact with toxins is reduced during early development (Hansen Jesse and Obrycki 2000; Sears et al. 2001). Each factor could interact synergistically, with the strength of effects varying over time. As the availability of milkweed declines around crops, the risk imposed by exposure to agricultural chemicals (e.g., \textit{Bt}, insecticides, herbicides) is likely to decline in tandem, though no studies have assessed this long-term change. Interactions between threats may also vary in accordance with the pre-dominant threats in a particular region. For instance, climate change may result in phenological mismatch (e.g., milkweed availability during oviposition and nectar sources during breeding and migration) if environmental conditions drive changes in plant growth or the pattern of monarch butterfly migration. Simultaneously, if, as in other species (i.e., honeybees, Mason et al. 2013; Sánchez-Bayo et al. 2015), agrochemical exposure reduces immune system function, the potential elevated risk of exposure to pathogens with climate warming (Altizer et al. 2011) may reduce survival. Thus, a complex array of factors and their interactions must be examined with different methodological protocols to resolve how each potential threat contributes to declines.

Our results are based on the available published peer-reviewed literature, but bias may exist in the publication and dissemination of research that may unintentionally affect meta-analyses and systematic reviews. Though we conducted an extensive
literature review, recently completed, unpublished literature may not yet be accessible and thus is unable to be accounted for in the results (Møller and Jennions 2001). Further, publication bias during the submission, review, and editorial processes may also influence the likelihood of research being available and accessed (Møller and Jennions 2001). Not only are significant results more likely to be submitted, but novel research with large sample sizes and greater statistical power are more likely to be published (Tregenza 2002; Joober et al. 2012; Mlinarić et al. 2017). Interestingly, publication record and identity of the author (e.g., gender, nationality, non-English surnames, alphabetical position of the surname in reference list) can also affect the likelihood of publication and subsequent citation rate (Tregenza and Wedell 1997; Kothiaho 1999a,b; Møller and Jennions 2001; Einac and Yariv 2006). The purpose of the research (e.g., natural history or multi-year modeling experiments) may also influence results. For instance, natural history studies on the effects of predators, parasites, and pathogens highlight the threat to monarchs, but were not intended as long-term studies and we therefore cannot extrapolate from these initial results. As research continues to expand reviews will need to incorporate new knowledge to properly evaluate the strength of evidence and potential threats to the eastern migratory North American population of monarch butterflies.

The threats examined in this review also pose considerable risk to other insect species. We suggest that the monarch butterfly is an ideal candidate to evaluate the contribution and the spatiotemporal interactions of each threat at different stages along the migratory route. Research should contribute to effective conservation management plans aimed at protecting habitat and raising population abundance, while also
emphasizing the importance of international cooperation in the protection of species at risk. To accomplish this, studies should tackle questions in an interdisciplinary manner, taking a whole-systems approach, and integrate multiple biological disciplines that address major gaps in methodological procedures (i.e., type of study) and knowledge. An integrated approach to understanding the mechanisms underlying declines will be important in mitigating further losses under escalating and interacting threats and will be vital to developing management responses.

**Conclusion**

In this review, we sort and summarize 115 peer-reviewed research papers based on the type of study existing within five broad potential threats, evaluating the effect and potential risk imposed by each threat to the eastern migratory North American population of monarch butterflies. We recommend that research initiatives address hypotheses examining the spatiotemporal nature of each risk and how each factor interacts by integrating fields spanning a range of biological disciplines including, though not limited to, ecology, physiology, endocrinology, and conservation management. Without thorough knowledge and management action plans, declines in monarch butterflies may have unintended downstream effects. For example, butterflies are valued for their cultural economic services and socioeconomic benefits in terms of ecotourism in the southern portion of their range (Semmens et al. 2018). We encourage more detailed studies on the mechanisms driving declines, particularly those evaluating the relative contribution of each threat throughout the monarch life cycle and its
geographic distribution. We also suggest that studies investigate potential interacting factors that may limit capacity to implement conservation management plans.
Table 2.1 Summary of search terms associated with the declines in the eastern migratory North American population of monarch butterflies (*Danaus plexippus*) used in the comprehensive literature review using Web of Science™.

<table>
<thead>
<tr>
<th>Topic</th>
<th>Search term</th>
</tr>
</thead>
</table>
| Species-related             | "monarch butterfly" OR " *Danapus plexippus*"  
"milkweed" OR " *Asclepias*" |
| Population declines        | decline OR abund*                                                                                                                           |
| Climate change              | climate change OR weather OR storm* OR oscill* OR temp*                                                                                   |
| Deforestation               | deforest* OR forest* OR log* OR industrial* OR fire OR clear* OR storm*                                                                  |
| GM                          | crop mod* OR genetically modif* OR trangenic* OR agri* OR *Bt* OR *Bacillus thuringiensis*                                                  |
| Contaminants                | pollut* OR contamin* OR insectic* OR pestic* OR metabolit* OR permethrin OR lead OR neonic* OR imidaclo* OR acetami* OR clothian* OR thiamethox* OR dinofur* OR nithiaz* OR nitenpyr* OR herbic* OR glyphosate |
| Habitat loss                | habitat loss OR habitat degradation OR fragment* OR loss* OR abund* OR eutroph* OR phospho* OR nitro* OR nectar OR distrib* OR invas*         |
| Predation, parasites, and   | pest* OR parasit* OR patho* OR prey* OR bird* OR mite* OR wasp* OR virus* OR ant* OR beetle* OR aphid* OR *Ophryocystis*               |
| pathogens                   |                                                                                                                                           |
Table 2.2 The proportion of peer-reviewed research papers classified by the type of study, effect on monarchs, and potential risk imposed by the threat to the eastern migratory North American population of monarch butterflies (*Danaus plexippus*).

<table>
<thead>
<tr>
<th>(a) Type of study</th>
<th>No. of studies</th>
<th>Percentage of total studies</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Availability of suitable environmental conditions</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>[Control_data]</td>
<td>1</td>
<td>7</td>
</tr>
<tr>
<td>[Field_data]</td>
<td>3</td>
<td>20</td>
</tr>
<tr>
<td>[Mod]</td>
<td>11</td>
<td>73</td>
</tr>
<tr>
<td><strong>Deforestation</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>[Control_data]</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>[Field_data]</td>
<td>14</td>
<td>82</td>
</tr>
<tr>
<td>[Mod]</td>
<td>3</td>
<td>18</td>
</tr>
<tr>
<td><strong>Contaminants</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>[Control_data]</td>
<td>16</td>
<td>55</td>
</tr>
<tr>
<td>[Field_data]</td>
<td>9</td>
<td>31</td>
</tr>
<tr>
<td>[Mod]</td>
<td>4</td>
<td>14</td>
</tr>
<tr>
<td><strong>Breeding habitat loss</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>[Control_data]</td>
<td>10</td>
<td>31</td>
</tr>
<tr>
<td>[Field_data]</td>
<td>10</td>
<td>31</td>
</tr>
<tr>
<td>[Mod]</td>
<td>12</td>
<td>38</td>
</tr>
</tbody>
</table>
### Predation, parasites, and pathogens

<table>
<thead>
<tr>
<th></th>
<th>Control data</th>
<th>Field data</th>
<th>Mod</th>
</tr>
</thead>
<tbody>
<tr>
<td>[S]</td>
<td>17</td>
<td>30</td>
<td>3</td>
</tr>
<tr>
<td>[N]</td>
<td></td>
<td>60</td>
<td>6</td>
</tr>
</tbody>
</table>

### (b) Is there an effect on monarch butterflies?

#### Availability of suitable environmental conditions

<table>
<thead>
<tr>
<th></th>
<th>S</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>[S]</td>
<td>9</td>
<td>56</td>
</tr>
<tr>
<td>[N]</td>
<td>7</td>
<td>44</td>
</tr>
</tbody>
</table>

#### Deforestation

<table>
<thead>
<tr>
<th></th>
<th>S</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>[S]</td>
<td>17</td>
<td>100</td>
</tr>
<tr>
<td>[N]</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

#### Contaminants

<table>
<thead>
<tr>
<th></th>
<th>S</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>[S]</td>
<td>20</td>
<td>65</td>
</tr>
<tr>
<td>[N]</td>
<td>11</td>
<td>35</td>
</tr>
</tbody>
</table>

#### Breeding habitat loss

<table>
<thead>
<tr>
<th></th>
<th>S</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>[S]</td>
<td>30</td>
<td>91</td>
</tr>
<tr>
<td>[N]</td>
<td>3</td>
<td>9</td>
</tr>
</tbody>
</table>

### Predation, parasites, and pathogens

<table>
<thead>
<tr>
<th></th>
<th>S</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>[S]</td>
<td>45</td>
<td>94</td>
</tr>
<tr>
<td>[N]</td>
<td>3</td>
<td>6</td>
</tr>
</tbody>
</table>
(c) Is there evidence the threat will persist/increase?

### Availability of suitable environmental conditions

<table>
<thead>
<tr>
<th></th>
<th>S</th>
<th>N</th>
<th>-</th>
</tr>
</thead>
<tbody>
<tr>
<td>[S]</td>
<td>9</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>[N]</td>
<td>3</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>[-]</td>
<td>3</td>
<td>20</td>
<td></td>
</tr>
</tbody>
</table>

### Deforestation

<table>
<thead>
<tr>
<th></th>
<th>S</th>
<th>N</th>
<th>-</th>
</tr>
</thead>
<tbody>
<tr>
<td>[S]</td>
<td>9</td>
<td>53</td>
<td></td>
</tr>
<tr>
<td>[N]</td>
<td>2</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>[-]</td>
<td>6</td>
<td>35</td>
<td></td>
</tr>
</tbody>
</table>

### Contaminants

<table>
<thead>
<tr>
<th></th>
<th>S</th>
<th>N</th>
<th>-</th>
</tr>
</thead>
<tbody>
<tr>
<td>[S]</td>
<td>8</td>
<td>31</td>
<td></td>
</tr>
<tr>
<td>[N]</td>
<td>3</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>[-]</td>
<td>15</td>
<td>57</td>
<td></td>
</tr>
</tbody>
</table>

### Breeding habitat loss

<table>
<thead>
<tr>
<th></th>
<th>S</th>
<th>N</th>
<th>-</th>
</tr>
</thead>
<tbody>
<tr>
<td>[S]</td>
<td>14</td>
<td>44</td>
<td></td>
</tr>
<tr>
<td>[N]</td>
<td>2</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>[-]</td>
<td>16</td>
<td>50</td>
<td></td>
</tr>
</tbody>
</table>

### Predation, parasites, and pathogens

<table>
<thead>
<tr>
<th></th>
<th>S</th>
<th>N</th>
<th>-</th>
</tr>
</thead>
<tbody>
<tr>
<td>[S]</td>
<td>5</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>[N]</td>
<td>2</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>[-]</td>
<td>41</td>
<td>85</td>
<td></td>
</tr>
</tbody>
</table>

44
Chapter 3

Developmental and Reproductive Effects of Neonicotinoid Exposure in Monarch Butterflies

Abstract

Neonicotinoid insecticides are a class of chemicals used to reduce crop damage caused by insect pests, but even sublethal levels can negatively affect development and reproduction in non-target insects. Monarch butterflies (*Danaus plexippus*), a species at risk, lay eggs on milkweed (*Asclepias* spp.) grown on agricultural landscapes, so it is important to understand how exposure to field-realistic concentrations of neonicotinoids could impact their fitness. We grew swamp milkweed (*Asclepias incarnata*) in either low (15 ng/g of soil) or high (25 ng/g of soil) levels of neonicotinoid clothianidin, or in a control (0 ng/g). Caterpillars were measured during development, and after adults emerged, they were measured and then mated to quantify egg size and mass, and the number of eggs laid. While there was no evidence that treatment influenced size, mass, or volume of instar 1 caterpillars, there was strong support for a treatment effect on late instar caterpillars. Generally, treated caterpillars, particularly those in the high dose, were smaller with a shorter length (except at instar 5), thinner width, smaller volume, and weighed less than controls. Adult monarch butterflies from both treatment groups were larger than controls, but exposure to clothianidin did not affect the number of eggs laid or egg size. Our results suggest that exposure to clothianidin during early life
negatively impacts monarch development, but that the magnitude of the effect depends on clothianidin concentration. While clothianidin may impact subsequent survival, our results also show that it is unlikely to reduce reproductive output of adult females.

**Introduction**

Neonicotinoids are a class of synthetic insecticides that are used in veterinary medicine (Merck Manuals 2016), home garden treatments (Craddock et al. 2019), and to protect crops (Craddock et al. 2019; Simon-Delso et al. 2015; US Geological Survey 2018). Neonicotinoids represent more than 25% of the global insecticide market (Bass et al. 2015) and, though hundreds of neonicotinoid compounds have been synthesized (Simon-Delso et al. 2015), 85% of worldwide neonicotinoid sales in 2014 were represented by clothianidin, thiamethoxam, and imidacloprid (Bass et al. 2014). As a systemic, water-soluble insecticide, the chemical is readily absorbed by plants, taken up by the roots and translocated to the foliage (Simon-Delso et al. 2015). After application, residual neonicotinoids can persistent in the environment for up to several years (Simon-Delso et al. 2015; Bonmatin et al. 2015; DeCant 2010). For species with high sensitivity to neonicotinoids, such as insects and aquatic invertebrates (Sánchez-Bayo et al. 2016), environmental persistence increases the likelihood of the chemical binding to the nicotinic acetylcholine receptors in the brain and time-cumulative toxicity (i.e., accumulating with the duration and frequency of exposure in comparison to bioaccumulative chemicals; Bonmatin et al. 2015; Sánchez-Bayo et al. 2016). Thus, exposure early in life may magnify detrimental effects and it is important to assess how early exposure across a concentration gradient impacts development.
Neonicotinoid application may impact insect development timing and body size and these effects could carry over to influence reproductive output. Exposure to sublethal levels of imidacloprid, for example, has been associated with accelerated development in both the cotton bollworm (*Helicoverpa armigera*; Ahmad et al. 2013). Monarch butterfly caterpillars (*Danaus plexippus*) fed swamp milkweed (*Asclepias incarnata*) treated with a 5 ppb aqueous solution of clothianidin had a shorter body length, but not a lower mass, at instar 1 and instar 2 (Pecenka and Lundgren 2015). In adult cabbage butterflies (*Pieris brassicae*), exposure up to 200 ppb of imidacloprid also resulted in reduced forewing length (Whitehorn et al. 2018). Exposure to neonicotinoid insecticides is also known to negatively impact fecundity in a number of insects, including red mason bees (*Osmia* spp.; Sandrock et al. 2014a), honeybees (Sandrock et al. 2014b; Woodcock et al. 2017), and bumblebees (*Bombus terrestris*; Baron et al. 2017b; Laycock et al. 2012, 2014). However, oral administration of imidacloprid at a field-realistic rate did not affect oocyte development in monarch butterflies (James 2019). Though the direct mechanism driving reduced fecundity is unknown, it is possible that the negative effects on development carry over to impact reproductive output (Baron et al. 2017a,b; Sandrock et al. 2014b; Williams et al. 2015; Wu-Smart and Spivak 2017). If neonicotinoids speed up the timing of moulting (Ahmad et al. 2013) and reduce individual size (Pecenka and Lundgren 2015; Whitehorn et al. 2018), a smaller maternal size could correspond with reduced egg size, mass, and a lower number of eggs produced (García-Barros 2000; Oberhauser 1997). Thus, it is important to not only understand whether neonicotinoid exposure could influence development, but also subsequent reproductive output.
Monarch butterflies are an iconic species facing severe declines (Thogmartin et al. 2017b), in part because of their dependence on milkweed (Asclepias spp.) during early caterpillar development (Oberhauser 2004). Documented population declines of more than 80% (Thogmartin et al. 2017b) have been attributed to a variety of causes including exposure to insecticides (Thogmartin et al. 2017b; Wilcox et al. 2019). Prophylactic applications have the potential to increase crop yield (Goulson 2013), but may harm non-target species. Monarch butterflies rely on milkweed as an obligate host plant (Oberhauser 2004) and readily use milkweed in agricultural landscapes for oviposition (Thogmartin et al. 2017c; Pitman et al. 2018). Female monarch butterflies lay up to 400 eggs on a plant during the breeding season (Zalucki and Rochester 2014) and, once hatched, instar 1 caterpillars consume the nearby foliage, increasing the potential for exposure to insecticides. After five successive moults, caterpillars form a chrysalid and emerge as butterflies after 8-15 days (Oberhauser 2004). Despite dependence on agricultural milkweed, few studies have quantified the potential risk imposed from neonicotinoids at field-relevant concentrations and the potential contribution of this class of insecticides to overall monarch declines (Pecenka and Lundgren 2015; James 2019).

We conducted a controlled laboratory experiment to assess how exposure to the neonicotinoid insecticide clothianidin during rearing influences monarch development and subsequent adult female reproductive output. Monarch caterpillars were reared on milkweed grown in soil treated with a field-relevant low or high concentration of the neonicotinoid clothianidin, or a zero concentration control group. Morphological measurements and mass were recorded during larval development and, after pupation,
then pairs of adult butterflies were mated to quantify female reproductive output (i.e., egg size and the total number of eggs laid). Given there is evidence that neonicotinoids affect monarch caterpillar development (Pecenka and Lundgren 2015), we hypothesized that clothianidin would negatively impact caterpillar size and predicted that individuals exposed to clothianidin would have a shorter body length, smaller body width, smaller body volume and lower mass compared to controls. We also hypothesized that female reproductive output would be negatively affected by clothianidin because neonicotinoids have been shown to impact fecundity in other invertebrate species (Baron et al. 2017a, b; Laycock et al. 2012, 2014; Sandrock et al. 2014a, b; Williams et al. 2015; Wu-Smart and Spivak 2017; although see James 2019). Thus, we predicted that this would result in a smaller egg size, mass, as well as fewer eggs being deposited.

Methods

Neonicotinoid Treatment, Milkweed Growth, and Chemical Analysis

A clothianidin standard (purity 99.9%; MDL # MFCD06200753, Sigma-Aldrich, St. Louis, Missouri, USA) was used to make stock solutions that were then diluted with distilled water to dose commercial soil (LA4 Sunshine Loosefill, Sungro Horticulture, Massachusetts, USA) at concentrations of 15 ng/g (hereafter ‘low dose) and 25 ng/g (hereafter ‘high dose’) of soil. The two treatment levels were chosen to represent field-realistic values recorded in Ontario and established standard sublethal doses (Chan 2019).
Swamp milkweed (*Asclepias incarnata*) was grown from seed in soil from either the low or high dose treatments or a control (no clothianidin). A total of 256 milkweed plants per treatment were grown at a density of 4 plants per 15.2 cm²/1.68 L pot (total 64 pots per treatment) in environmental chambers at the University of Guelph Phytotron. Temperature was maintained at 29°C during the day and 23°C at night, with 500 mol light (18L: 6D), and 60% RH based on conditions outlined in Flockhart et al. (2012). Plants were watered daily with reverse osmosis water and fertilized weekly with Plant-Prod Solutions fertilizer 17:5:17 NPK (Master Plant-Prod Inc., Brampton, ON, Canada). *Amblyseius swirskii* was 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).

To establish the temporal variation in clothianidin concentration throughout the experiment, a minimum 15 g of soil (sensitivity ± 1.0 g; MyWeigh iBalance i500, HBI Technologies Canada, Vancouver, BC, Canada) was collected for analysis in sterile polypropylene centrifuge tubes (VWR High-Performance Centrifuge Tubes, CAT # 89039-656, VWR International LLC, Mississauga, ON, Canada) when the soil was dosed, 14 days after dosing, 18 days after dosing when monarch eggs were transferred to the treatment leaves (see below), and 32 days after dosing when monarchs pupated. A single leaf was randomly selected from each milkweed plant at 18 and 32 days after the soil was dosed. These leaves were then combined to reach a minimum mass of 2.0 g required for clothianidin detection and stored in sterile polypropylene centrifuge tubes. A subset of instar 5 caterpillars were haphazardly selected and combined (\( \bar{x} = 2.4 \) caterpillars per sample tube) to reach minimum mass for neonicotinoid analysis to
determine if neonicotinoids were metabolized prior to metamorphosis. At the completion of the experiment, adult monarch butterflies were combined (\( \bar{x} = 5.2 \) butterflies per sample tube) and submitted for neonicotinoid analysis. All samples were stored at -20°C prior to analysis for clothianidin concentration at the University of Guelph Agriculture and Food Laboratory. Analysis was done using the AOAC 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 or tissue was extracted and placed in a solution of 1% acetic acid in acetonitrile with anhydrous sodium and magnesium sulfate. The precipitate is then diluted with methanol and 0.1 M ammonium acetate. High performance liquid chromatography coupled with electrospray ionization tandem mass spectrometry (LC/ESI-MS/MS) was used to assess neonicotinoid concentration and the concentration was returned in parts per billion (1 ppb = 1 ng/g; Boguski 2006). The limit of quantification (LOQ) is the lowest concentration that can be accurately quantified, whereas the limit of detection (LOD) is 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 (soil: LOQ/LOD 20 ppb/7 ppb; leaf: 30 ppb/10 ppb; monarch tissue: 2 ppb/0.7 ppb).

**Monarch Capture and Rearing**

Caterpillars and butterflies used during the experiments were reared from eggs laid by wild monarchs obtained from untreated properties in Long Point, ON (42.58°N, 80.43°W; \( \varnothing \ n = 2, \varphi \ n = 4 \)) and the Guelph Lake Conservation Area (43.61°N, 80.26°W; \( \varnothing \ n = 1, \varphi \ n = 5 \)). Butterflies were held in coin envelopes (6.35 cm x 10.8 cm) inside an
animal carrier kept at ambient temperature and lined with a damp cloth to maintain humidity to avoid drying of the wings and limit mortality during transport to the University of Guelph. Upon arrival, butterflies were weighed (Denver Instrument 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. All monarchs were tagged with a small, uniquely numbered, adhesive sticker (~0.65 cm) on the left hindwing near the abdomen that allowed us to visually identify individuals and monitor body condition. Breeding took place in large mesh enclosures (60 cm height x 60 cm depth x 60 cm width) inside an incubator set at temperatures fluctuating between 29°C and 23°C with 500 mol light (18L: 6D) and 60% RH to simulate conditions common during summer breeding. Each enclosure was outfitted with untreated milkweed (i.e., not exposed to clothianidin) and an artificial nectar source (i.e., sucrose water), changed daily, until all eggs were collected and the reproductive monarchs were released.

We removed 201 eggs (n = 67 per treatment) by gently rubbing the eggs off the milkweed leaf with a fine-tipped paintbrush onto a damped cloth before being adhered to the milkweed leaf using residual latex. Leaves with eggs were placed in large plastic containers enclosed using a finely perforated mosquito netting (Bulk Mosquito Netting, CAT # 09A04.73, Lee Valley, Ottawa, ON, Canada) and cleaned daily. Caterpillars were fed experimental milkweed (i.e., control, 15 ng/g or 25 ng/g clothianidin) ad libitum, to minimize risk of nutritional stress from food limitation. Light cycle and temperature mimicked ambient conditions during the early breeding season in Guelph, ON (43.5°N, 80.2°W; 16 hours light: 8 hours dark; Flockhart et al. 2012) at 28°C during the day, 22°C at night, and 60% RH to reduce the risk of monarchs entering reproductive diapause.
(Flockhart et al. 2012; Goehring and Oberhauser 2002). Given observed differences in the size of caterpillars reared on milkweed treated with neonicotinoid (Pecenka and Lundgren 2015), we collected body measurements (i.e., length and width) using digital calipers to the nearest 0.01 mm at instar 1, 3, and 5 as the stage of development could be reliably identified. Previous methods to measure body volume involved hemolymph extraction (Lin et al. 2011). As a non-lethal measure of body volume, we calculated the volume of a cylinder \((V = \pi r^2h)\) to estimate the body volume of instar 3 and 5 caterpillars. We also recorded mass to the nearest 0.1 g for instar 5 caterpillars.

Chrysalids were transferred to mesh enclosures (120 cm x 120 cm x 120 cm; Popadome Plant Dome, CAT # XC515, Lee Valley, Ottawa, ON, Canada) within 2 days of pupation until eclosion when adult monarchs were fed \textit{ad libitum} a sucrose solution from dishes placed at the bottom of the enclosures (Flockhart et al. 2012). All monarchs were measured and weighed in captivity and examined for \textit{Ophryocystis elektroscirrhra} (OE) parasites by applying clear tape to the abdomen and analyzing tape for spores under a microscope at 400x (Altizer and Oberhauser 1999). Individuals testing positive for OE were removed from the study and euthanized due to the potential for developmental deformities. All procedures were conducted under the Ontario Ministry for Natural Resources Wildlife Scientific Collectors Permit (#1090000).

**Assessing Reproductive Output**

To assess the influence of neonicotinoid exposure on adult female monarch butterfly reproductive output, a subset of female monarchs reared from caterpillars was provided untreated milkweed as substrate for oviposition in mesh enclosures (40.6 cm x 63.5 cm)
and fed a 10% honey-water solution. Virgin reproductive pairs (control: n = 7, low dose: n = 8, high dose: n = 7) were selected because prior matings can reduce the size of subsequent spermatophores being transferred (Oberhauser 1988). Once mated, males were removed and females remained in the enclosures to complete oviposition. We removed female monarchs when they died or after egg deposition ceased for a maximum of 7 days (Oberhauser 1997). For all females, we counted the number of eggs deposited and stored dead female butterflies for approximately 2 weeks until we could count the number of mature oocytes remaining in the ovarioles (Oberhauser 1997). Due to the small size of the eggs, we calculated the average mass in groups of 10 to the nearest ± 0.001 mg (XP26 Micro Balance, Mettler-Toledo Inc., Mississauga, ON, Canada) and measured the height and width using digital calipers to the nearest 0.01 mm using handheld digital calipers. Damaged eggs were included in the total count of the number of eggs deposited, but were not measured. The small size of monarch eggs did not permit digital measurement, so we followed the procedures outlined in García-Barros (2000) to calculate the egg size despite deviations from spherical shapes potentially inducing slight measurement error. Therefore, the cubic root of the volume of an ellipsoid,

\[ \text{Egg size} = (0.5236 \times d^2 \times h)^{1/3} \]

was used to estimate monarch egg size, where \( d = \) egg diameter and \( h = \) egg height (García-Barros 2000).
Statistical Analyses

Outliers, potentially as a result of measurement or transcription error, were identified and removed using rm.outlier in the outliers package (v0.14, Komsta 2015) and removed from analysis. Four outliers (control: n = 1, low dose: n = 2, high dose: n = 1) were identified for body length and two outliers (control: n=2) for body width. To test for the effect of neonicotinoids on caterpillar body length, body width, and body volume, we applied series of generalized linear mixed models (GLMM) using the nlme package (v3.1-147, Pinheiro et al. 2020) in R version 3.4.1 (Bolker et al. 2009; R Core Team 2015). The models included instar stage, treatment (i.e., control, low dose, and high dose) and the interaction between stage and treatment as fixed effects and individual identity as a random effect. We only used general linear model to test whether treatment affected the mass because only instar 5 caterpillars were measured for mass.

A general linear model was used to assess whether the neonicotinoid treatment affected adult monarch butterfly size. Forewing length was square root transformed to improve the normality of the distribution and included as a response variable to estimate body size (Miller 1977, 1991). Date of eclosion, mass, and sex were also included as predictors. We also used a generalized linear model with Poisson distribution and log link function to determine whether treatment affected the total number of eggs laid. The total number of eggs laid was included as the response variable, with treatment, forewing length, mass and age as predictors. Age of the male monarch in the mated pair was included as a proxy for spermatophore size and the amount of resources
transferred to the female during mating that may contribute to egg production (Oberhauser 1997).

We applied a series of GLMMs to determine if early neonicotinoid exposure affected the size of eggs. We used the average egg size and mass as response variables in separate models with treatment and total number of eggs laid as fixed effects. To account for potential differences in resource investment by older and larger female monarch butterflies (Oberhauser 1997), we included age, mass, and forewing length as fixed effects, as well as the age of the male monarch in the mated pair to account for the male contribution to the overall resource budget (Oberhauser 1997). Female identity was included as a random effect.

For all models, each possible combination of parameters was tested in a separate model, as well as a null model that included only the intercept. Akaike’s information criterion (AIC) or an AIC corrected for small sample sizes (AICc) was then used to rank models and those with a ΔAIC or ΔAICc ≤ 2 were considered to have support (Burnham and Anderson 2002). Support for a model was also provided by the log likelihood and Akaike weights (ωi).

Results

Analysis of Neonicotinoid Insecticides

We quantified whether clothianidin was detected in the soil, milkweed leaf, monarch caterpillar, and adult butterfly tissue. As expected, no clothianidin was detected in the soil for the control group. Clothianidin was detected in the soil for both treatments, but...
the concentration was lower than at dosing and the concentration decreased throughout the experiment (Table 3.1). Clothianidin was not detected in instar 5 monarch caterpillars raised on control milkweed, but < 1 ppb of the insecticide was detected in caterpillars from both low and high dose treatments (Table 3.1). No insecticide was detected in the tissue of the adult monarch butterflies, regardless of treatment (Table 3.1).

**Monarch Caterpillar Development**

The only model that held support to predict variation in caterpillar body length included an interaction between instar stage and treatment ($\omega_i = 1.00$; Table 3.2; Table 3.3). At instar 1, caterpillar body length was similar between treatments (control: 2.2 mm ± 0.3 SD, low dose: 2.3 mm ± 0.4 SD, high dose: 2.1 mm ± 0.5 SD Table 3.2), but, at instar 3, body length of caterpillars from the high dose treatment (9.8 mm ± 1.8 SD) were shorter than caterpillars from other groups (control: 11.6 mm ± 1.4 SD, low dose: 10.7 mm ± 1.7 SD; Table 3.2; Figure 3.1a). At instar 5, caterpillars from the high dose treatment (40.6 mm ± 5.8 SD) were longer relative to caterpillars from the control group (35.8 mm ± 4.6 SD) and from the low dose treatment (36.3 mm ± 4.8 SD; Table 3.2; Figure 3.1a).

Similarly, the only model that predicted variation in caterpillar body width included an interaction between instar stage and treatment ($\omega_i = 1.00$; Table 3.2; Table 3.3). At instar 3, body width of caterpillars from the high dose treatment (1.5 mm ± 0.2 SD) was narrower than controls (2.0 mm ± 0.3 SD) and the low dose treatment (1.9 mm ± 0.5 SD; Table 3.2; Figure 3.1b). At instar 5, caterpillars from the high dose treatment (3.5
mm ± 0.5 SD) were narrower than caterpillars from the control group (5.3 mm ± 0.5 SD) and the low dose treatment (5.0 mm ± 0.6 SD; Table 3.2; Figure 3.1b).

A single model predicted the variation in caterpillar body volume and included an interaction between instar stage and treatment ($\omega_i = 1.00$; Table 3.2; Table 3.3). At instar 3, body volume was similar between treatments (control: 38.0 mm$^3$ ± 14.0 SD, low dose: 33.3 mm$^3$ ± 18.2 SD, high dose: 18.0 mm$^3$ ± 6.3 SD), but, at instar 5, body volume of caterpillars from the high dose treatment (397.2 mm$^3$ ± 124.2 SD) was, on average, less than half that of other groups (control: 807.9 mm$^3$ ± 209.1 SD, low dose: 725.6 mm$^3$ ± 205.9 SD; Table 3.2; Figure 3.2a). Furthermore, the only model that predicted mass of instar 5 caterpillars included treatment ($\omega_i = 1.00$; Table 3.2; Table 3.3). Caterpillars exposed to milkweed grown in either low (1.3 g ± 0.2 SD) or high concentration of clothianidin (1.2 g ± 0.3 SD) had a lower mass than controls (1.3 g ± 0.3 SD; Table 3.2; Figure 3.2b).

**Monarch Butterfly Reproductive Output**

One of the three top models ($\Delta$AIC$_c$ ≤ 2) that predict adult monarch forewing length (Figure 3.3) was the null model ($\omega_i = 0.27$; Table 3.3). The remaining two models included clothianidin treatment and monarch butterfly mass, both of which were positively correlated with forewing length (Table 3.3).

We did not find evidence that clothianidin affected female reproductive output. Three models ($\Delta$AIC ≤ 2) explained the variation in the number of eggs laid, with the top
model being the null model ($\omega_l = 0.52$; Table 3.3). The top ranked model to predict egg size and egg mass was the null model ($\omega_l = 0.95$ and $\omega_l = 0.52$, respectively; Table 3.3).

**Discussion**

Our study provides evidence that exposure to clothianidin negatively impacts monarch caterpillar size, with caterpillars overall being smaller compared to controls. The shorter caterpillar length (except at the instar 5), smaller width, and volume we observed may be the result of a stress response impacting development. Neonicotinoid exposure can lead to an upregulation of the cellular stress response system (Ayyanath et al. 2014), but it is unclear whether this could have a downstream effect on the insect growth regulator juvenile hormone (JH) and the enzyme responsible for its degradation, juvenile hormone esterase (JHE). Nonetheless, evidence of a relationship between other stressors, specifically heat shock, and JHE expression exists (Schelling and Jones 1996), and suggests a potential effect on the molting process. Therefore, measuring the concurrent response of JH, JHE, and heat shock protein expression during development in insects would provide evidence for this cellular level response to neonicotinoid-induced stress. Alternatively, it is possible that milkweed grown in neonicotinoid-treated soil is less palatable for consumption, though this is unlikely given that honeybees show a preference for neonicotinoid-treated food sources (Arce et al. 2018; Kessler et al. 2015). Measuring leaf consumption would expose whether monarch caterpillars preferentially feed or have an aversion response to neonicotinoid-treated milkweed. At instar 5, monarch caterpillars from the high dose treatment group were longer than those from the low dose treatment and control. Though measuring JH
response during caterpillar development and at pupation could provide further insight into these developmental patterns, it is also possible that accelerated growth in the later development stages could have occurred, as found in the common blue butterfly (Polyommatus icarus; Basley and Goulson 2018).

Though clothianidin was detected in caterpillar tissue, it was not found in adult monarch butterflies. There was no effect of treatment on egg size and egg mass, despite the larger size of the adult monarch butterflies relative to controls. Previous studies in honeybees suggest a reduced capacity to lay fertilized eggs (Baron et al. 2017a,b; Sandrock et al. 2014b; Williams et al. 2015; Wu-Smart and Spivak 2017) and that this effect persists for a year after neonicotinoid exposure (Sandrock et al. 2014b). Despite this, we did not find any impact of early exposure to field-realistic levels of neonicotinoids on the number of eggs laid that would suggest problems in egg production. Our results agree with James (2019) who found that orally administered imidacloprid did not affect oocyte production. Combined, this suggests that neonicotinoids may have minimal impact on adult monarch butterfly reproductive output. Alternatively, because clothianidin was not detected in our samples, and the levels detected in the soil were quite low, it is still possible that exposure to a higher concentration of clothianidin could reduce reproductive output.

Neonicotinoids are highly water-soluble, which could affect chemical absorption and translocation into plant tissue. The concentration of clothianidin detected at analysis was lower than that applied to the soil, ranging between 0.40 ppb to 2.80 ppb (Table 3.1). The retention of clothianidin in soil is dependent on soil composition and
clothianidin may have not absorbed into the soil at dosing. The retention rate in soil is greatest with high levels of organic matter relative to sand and pumice (Mörtl et al. 2016). The soil used for potting milkweed contained a high concentration of sphagnum peat moss (60-70%) that reduces water drainage from the soil. Despite this, it is possible that the high water solubility of neonicotinoids and low sorption to surrounding organic matter (Bonmatin et al. 2015) resulted in the leaching of the insecticide and a higher concentration of insecticide at the bottom of the pots due to daily watering. Leaching of clothianidin may have also contributed to a lack of the chemical available for uptake to milkweed. Only a single sample of milkweed leaf had a detectable level of clothianidin, though the concentration was comparable to field studies assessing the concentration of clothianidin in milkweed (i.e., $\bar{x} = 0.48$-1.14 ppb; Olaya-Arenas and Kaplan 2019; Pecenka and Lundgren 2015). However, it is also possible that clothianidin was not distributed equally throughout the milkweed and may have accumulated in specific regions of the plant, as is the case with other neonicotinoids (Bonmatin et al. 2015). Therefore, it is important that we understand how to control leaching and how this process could affect uptake and translocation of clothianidin.

Though we show a negative effect on caterpillar development, the concentration of clothianidin used in this study, did not result in detrimental effects on adult monarch reproductive output. At the concentration of clothianidin used in this study, there are unlikely to be long-term detrimental effects on the life history of monarch butterflies. The concentration of clothianidin detected in monarch caterpillars (i.e., $< 1$ ppb) was far below field concentrations and no insecticide was detected in adult monarch butterflies. Honeybees (Apis mellifera) metabolize neonicotinoid at a rate of 2.0 ng/day (Cresswell
et al. 2013), with metabolism dependent on the availability of protein coding genes for detoxifying enzymes (Claudianos et al. 2006), most notably for enzymes in the cytochrome P450 superfamily (Manjon et al. 2018). No studies to date investigate the metabolism of neonicotinoids in Lepidoptera, but our results suggest the potential metabolism of the insecticide during the caterpillar stage or the elimination of the chemical during metamorphosis. However, there are a few limitations to our study that should be considered when extrapolating our results to field-realistic scenarios. First, the concentration of insecticide used to dose the soil (i.e., 15 ng/g and 25 ng/g) was near the mean detected in field, but by the time of planting the concentration had fallen to ~1 ppb (1 ppb = 1 ng/g; Boguski 2006). Though similar low concentrations have been detected in field (Chan et al. 2019), it is possible that impacts of clothianidin on monarch life history may become more apparent at higher concentrations of the insecticide. Therefore, we suggest monitoring caterpillars on agricultural plots to determine the effect of neonicotinoid treatment in realistic environmental scenarios. It is also critical to understand whether neonicotinoid treatment impacts mortality under these conditions. Oviposition tendencies also differ between lab and field conditions, with adult female monarch butterflies are known to lay fewer eggs in captivity relative to the wild (Oberhauser 2004). This, in addition to the small sample size of reproductive pairs, may have reduced our ability to detect differences in reproductive output between treatments. Therefore, though our laboratory experiment suggests that there are minimal long-term effects of clothianidin on monarch butterflies, further work is needed to understand the potential impacts to this species at risk in field-realistic scenarios.
Conclusion

We showed that exposure to field-realistic concentrations of the insecticide negatively impacts caterpillar development and, therefore, encourage investigation into the underlying mechanism driving the physiological changes. We did not, however, find evidence of an effect of neonicotinoid exposure on the reproductive output (i.e., egg size and the number of eggs deposited) of adult female monarch butterflies. This study contributes to the critical work assessing the impact of neonicotinoids on monarch butterflies and emphasizes the importance of understanding how environmental contaminants impact fitness at different life stages in a multigenerational species at risk. Overall, our results provide further insights for the development of species management plans and future research.
Table 3.1 Concentration of the neonicotinoid clothianidin (ppb) in soil, swamp milkweed (*Asclepias incarnata*), instar 5 caterpillars, and adult monarch butterflies (*Danaus plexippus*) for insecticide applications in control, 15 ng/g (low dose) and 25 ng/g (high dose) treatments. For samples with clothianidin detected (DET (n)), the mean (\(\bar{x}\)), standard deviation (s), range, and median are provided. The number of samples where no clothianidin detected (ND (n)) is provided as summary statistics could not be calculated (-).

<table>
<thead>
<tr>
<th>Time of sampling</th>
<th>Treatment</th>
<th>(\bar{x}) (ppb)</th>
<th>s (ppb)</th>
<th>Range</th>
<th>Median</th>
<th>DET (n)</th>
<th>ND (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soil dosing</td>
<td>Control</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Low dose</td>
<td>1.03</td>
<td>0.60</td>
<td>0.40 – 1.60</td>
<td>1.10</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>High dose</td>
<td>2.67</td>
<td>0.15</td>
<td>2.50 – 2.80</td>
<td>2.70</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>14 days after dosing</td>
<td>Control</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Low dose</td>
<td>1.07</td>
<td>0.31</td>
<td>0.80 – 1.40</td>
<td>1.00</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>High dose</td>
<td>1.83</td>
<td>0.25</td>
<td>1.60 – 2.10</td>
<td>1.80</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>18 days after dosing</td>
<td>Control</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Low dose</td>
<td>0.28</td>
<td>0.04</td>
<td>0.20 – 0.30</td>
<td>0.30</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>High dose</td>
<td>1.02</td>
<td>0.38</td>
<td>0.60 – 1.10</td>
<td>1.00</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>32 days after dosing</td>
<td>Control</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Low dose</td>
<td>3.48</td>
<td>0.08</td>
<td>3.40 – 3.60</td>
<td>3.50</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>High dose</td>
<td>6.34</td>
<td>0.43</td>
<td>5.90 – 6.80</td>
<td>6.20</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>----------------</td>
<td>-----------</td>
<td>------</td>
<td>------</td>
<td>-------------</td>
<td>------</td>
<td>-----</td>
<td>-----</td>
</tr>
<tr>
<td><strong>[CLO] in milkweed leaves</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>18 days after dosing</strong></td>
<td>Control</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Low dose</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>High dose</td>
<td>1.20</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td><strong>32 days after dosing</strong></td>
<td>Control</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Low dose</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>High dose</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td><strong>[CLO] in instar 5 monarch larvae</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>Low dose</td>
<td>0.46</td>
<td>0.15</td>
<td>0.30 – 0.70</td>
<td>0.4</td>
<td>5</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>High dose</td>
<td>0.79</td>
<td>0.19</td>
<td>0.60 – 1.00</td>
<td>0.7</td>
<td>9</td>
<td>0</td>
</tr>
<tr>
<td><strong>[CLO] in adult monarch butterflies</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Low dose</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>High dose</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0</td>
<td>4</td>
</tr>
</tbody>
</table>
Table 3.2 Physiological effects of sublethal concentrations of the neonicotinoid insecticide clothianidin on monarch caterpillar (*Danaus plexippus*) reared on swamp milkweed (*Asclepias incarnata*) grown in control (no insecticide), 15 ng/g (low dose) and 25 ng/g (high dose) soil. Measurements were reported as the mean ± SD (n), except where no data was available (-).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Body length (mm)</th>
<th>Body width (mm)</th>
<th>Body volume (mm$^3$)</th>
<th>Mass (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Instar 1</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>2.2 ± 0.3 (64)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Low dose</td>
<td>2.3 ± 0.4 (62)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>High dose</td>
<td>2.1 ± 0.5 (56)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>Instar 3</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>11.6 ± 1.4 (65)</td>
<td>2.0 ± 0.3 (66)</td>
<td>38.0 ± 14.0 (65)</td>
<td>-</td>
</tr>
<tr>
<td>Low dose</td>
<td>10.7 ± 1.7 (63)</td>
<td>1.9 ± 0.5 (64)</td>
<td>33.3 ± 18.2 (63)</td>
<td>-</td>
</tr>
<tr>
<td>High dose</td>
<td>9.8 ± 1.8 (52)</td>
<td>1.5 ± 0.2 (51)</td>
<td>18.0 ± 6.3 (51)</td>
<td>-</td>
</tr>
<tr>
<td><strong>Instar 5</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>35.8 ± 4.6 (65)</td>
<td>5.3 ± 0.5 (63)</td>
<td>807.9 ± 209.1 (63)</td>
<td>1.3 ± 0.3 (62)</td>
</tr>
<tr>
<td>Low dose</td>
<td>36.3 ± 4.8 (61)</td>
<td>5.0 ± 0.6 (62)</td>
<td>725.6 ± 205.9 (61)</td>
<td>1.3 ± 0.2 (62)</td>
</tr>
<tr>
<td>High dose</td>
<td>40.6 ± 5.8 (52)</td>
<td>3.5 ± 0.5 (53)</td>
<td>397.2 ± 124.2 (52)</td>
<td>1.2 ± 0.3 (52)</td>
</tr>
</tbody>
</table>
Table 3.3 Top models for caterpillar development and adult butterfly reproductive output for monarchs (*Danaus plexippus*) reared on swamp milkweed (*Asclepias incarnata*) grown in control (no insecticide), 15 ng/g (low dose) and 25 ng/g (high dose) soil. Model estimates, upper and lower 95% confidence intervals are indicated, as well as Akaike’s information criterion (AIC) or the corrected AIC$_c$ for small sample sizes, log likelihood (logLik), $\Delta$AIC or $\Delta$AIC$_c$, and Akaike weights. Variables included as fixed effects in the top models include instar, treatment, mass of both male and female monarchs (mass), male age ($age_M$), and female age ($age_F$).

<table>
<thead>
<tr>
<th>Monarch caterpillar development</th>
</tr>
</thead>
<tbody>
<tr>
<td>Variables</td>
</tr>
<tr>
<td>-----------</td>
</tr>
<tr>
<td><strong>Length</strong></td>
</tr>
<tr>
<td>instar * treatment</td>
</tr>
<tr>
<td><strong>Width</strong></td>
</tr>
<tr>
<td>instar * treatment</td>
</tr>
<tr>
<td><strong>Volume</strong></td>
</tr>
<tr>
<td>instar * treatment</td>
</tr>
<tr>
<td><strong>Mass</strong></td>
</tr>
<tr>
<td>treatment</td>
</tr>
</tbody>
</table>
### Adult monarch size

<table>
<thead>
<tr>
<th>Variables</th>
<th>est</th>
<th>CI</th>
<th>logLik</th>
<th>AICc</th>
<th>ωi</th>
</tr>
</thead>
<tbody>
<tr>
<td>null model</td>
<td>7.17</td>
<td>7.13 – 7.22</td>
<td>24.75</td>
<td>-45.41</td>
<td>0.27</td>
</tr>
<tr>
<td>mass</td>
<td>6.85</td>
<td>-0.0001 – 0.001</td>
<td>26.07</td>
<td>-45.84</td>
<td>0.34</td>
</tr>
<tr>
<td>mass +</td>
<td>3.94</td>
<td>-0.0003 – 0.001</td>
<td>28.57</td>
<td>-46.11</td>
<td>0.38</td>
</tr>
<tr>
<td>treatment</td>
<td>0.05</td>
<td>0.0002 – 0.10</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Adult female monarch reproductive output

<table>
<thead>
<tr>
<th>Variables</th>
<th>est</th>
<th>CI</th>
<th>logLik</th>
<th>AIC</th>
<th>ωi</th>
</tr>
</thead>
<tbody>
<tr>
<td>null model</td>
<td>14.79</td>
<td>12.57 – 17.01</td>
<td>-67.44</td>
<td>138.88</td>
<td>0.52</td>
</tr>
<tr>
<td>null model</td>
<td>0.71</td>
<td>0.70 – 0.72</td>
<td>5292.07</td>
<td>-10578.14</td>
<td>0.95</td>
</tr>
<tr>
<td>null model</td>
<td>4.56</td>
<td>4.39 – 4.73</td>
<td>-147.06</td>
<td>300.13</td>
<td>0.52</td>
</tr>
</tbody>
</table>
Figure 3.1 Body (a) length (mm) at instars 1, 3, and 5 and (b) width (mm) at instars 3 and 5 for control and monarch caterpillars (*Danaus plexippus*) exposed to the neonicotinoid pesticide clothianidin at 15 ng/g (low dose) and 25 ng/g (high dose) of the neonicotinoid pesticide clothianidin. Violin plots show the distribution of the raw data presented with median (•) and range of observations (whiskers).
Figure 3.2 Body (a) volume (mm³) at instars 3 and 5 and (b) mass (g) at instar 5 for control (CO) and monarch caterpillars (*Danaus plexippus*) exposed to the neonicotinoid pesticide clothianidin at 15 ng/g (low dose) and 25 ng/g (high dose) of the neonicotinoid pesticide clothianidin. Violin plots show the distribution of the raw data presented with median (•) and range of observations (whiskers).
Figure 3.3 Forewing length (mm) by (a) treatment for adult monarch butterflies (*Danaus plexippus*) reared exposed to the neonicotinoid pesticide clothianidin at 15 ng/g (low dose) and 25 ng/g (high dose) of the neonicotinoid pesticide clothianidin. Violin plots show the distribution of the raw data presented with median (•) and range of observations (whiskers).
Chapter 4

Effects of Early-life Exposure to Sublethal Levels of a Common Neonicotinoid Insecticide on the Orientation and Migration of Monarch Butterflies (*Danaus plexippus*)

Abstract

Migratory insects use a variety of innate mechanisms, coupled with environmental cues, to determine their orientation and maintain correct bearing. For long-distance migrants, like the monarch butterfly (*Danaus plexippus*), these journeys could be affected by exposure to environmental contaminants that could disrupt the processes critical for successful migration. Neonicotinoids are a class of synthetic insecticides that can accumulate in insects’ bodies over time and impair mobility. However, currently little is known about how these chemicals might affect the ability of monarch butterflies to maintain a proper orientation on their nearly 4,000 km southwest migration to Mexico each fall. We grew swamp milkweed (*Asclepias incarnata*) in soil that was either untreated (0 ng/g: control), or mixed with low (15 ng/g of soil), or high (25 ng/g of soil) levels of the neonicotinoid clothianidin (low and high treatments). 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 fall migratory period. Despite clothianidin being detectable in milkweed tissue consumed by caterpillars, there was no evidence that clothianidin influenced orientation, vector strength (i.e., concentration of the data around the mean direction), or the rate of travel
of adult butterflies and there was no evidence that morphological traits (i.e., mass and forewing length), the time of testing, wind speed, or temperature impacted directionality. Our results suggest that exposure to clothianidin during early caterpillar development does not measurably affect the directed flight of adult migratory monarch butterflies or influence the orientation at the beginning of migration.

**Introduction**

Long-distance migrations occur in a wide-range of taxa, with a variety of underlying mechanisms governing these movements (Dingle, 2014; Mouritsen, 2003). In some groups, such as ungulates, navigation is learned and knowledge of routes and locations of resources are 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, 2014; Gould and Gould, 2012), while also correcting for displacement during migration (Gould and Gould, 2012; Mouritsen, 2003, 2018). ‘Map’ and ‘compass’ systems are assessed independently (Gould and Gould, 2012; Mouritsen, 2003, 2018), with location assessed, for example, from the rotation of the stars or intensity of inclination of the earth’s geomagnetic field and compass orientation determined by using magnetic and/or geographic north or the position of the sun (Dingle, 2014; Mouritsen, 2003, 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 (Mouritsen, 2003; Mouritsen et al., 2013; Perdeck, 1958).

After summer breeding in the north-eastern United States and south-eastern Canada, the last generation of eastern monarch butterflies (*Danaus plexippus*) migrate nearly 4,000 km southwest towards Mexico (Brower, 1995; Urquhart, 1960; Urquhart and Urquhart, 1978). Monarch butterflies use a time-compensated sun compass (Guerra et al., 2012; Merlin et al., 2009; Reppert, 2006; Reppert and Weaver, 2002; Reppert et al., 2010) 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 (Dingle, 2014; Reppert et al., 2010). As monarch butterflies are unable to compensate for a 2,500 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.

Neonicotinoids are a class of widely used systemic insecticides (Bass et al., 2015), applied principally in agriculture as seed coatings or soil drenches (Jeschke et al., 2011), resulting in the rapid uptake by surrounding plants due to the insecticides’ high water solubility (Simon-Delso et al., 2015). Though environmental persistence varies among neonicotinoids, they can remain in the environment for years (Simon-
Delso et al., 2015; Bonmatin et al., 2015; DeCant, 2010; Winternantel et al. 2020) compounding the risk to 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). Large acute doses of neonicotinoids or chronic exposure can affect motor function and control (Williamson et al., 2014) and, while navigation in bees can be affected at high doses (Fischer et al., 2014; Jin et al., 2015), impact likely varies depending on exposure level (Stanley et al. 2016). In fact, the bees’ sun compass, used to determine orientation relative to landscape features (Dovey et al., 2013), does not appear to be affected by neonicotinoid exposure (Fischer et al., 2014). In monarch butterflies, the midbrain is a prominent region of nAChRs concentration and key to integrating information on navigation (Heinze and Reppert 2010, 2012; Cabirol and Haase 2019). Given the dependence of monarchs on this neurological system, it is critical to determine if neonicotinoid exposure leads to impaired orientation.

We conducted a controlled laboratory experiment to determine if exposure to the neonicotinoid insecticide clothianidin during larval development might affect adult monarch butterfly orientation during fall 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, 2017; 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 to 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, forewing length, and the time of testing, wind direction, and temperature when tests were conducted on orientation.

Methods

Neonicotinoid treatment and milkweed growth

Stock solutions were made from a clothianidin standard (purity 99.9%; MDL # MFCD06200753, Sigma-Aldrich, St. Louis, Missouri, USA) diluted with distilled water and used to dose soil (LA4 Sunshine Loosefill, Sungro Horticulture, Massachusetts, USA) at concentrations of 15 ng/g (i.e., “low dose”) and 25 ng/g (i.e., “high dose”) of soil based on sub-lethal doses and field-realistic values from Ontario (Chan et al. 2019).
Swamp milkweed (*Asclepias incarnata*) was grown from seed (Richters Herbs, Goodwood, ON) in control (i.e., without clothianidin treatment), low, or high dose soil treatments. Plants \((n = 256)\) were grown at a density of 4 plants per \(15.2\text{ cm}^2/1.68\text{ L pot}\) in environmental chambers (University of Guelph Phytotron) maintained at 29°C during the day and 23°C at night. Lights were maintained at 500 mol (18L:6D) (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 a 77% (SD ±10%) average. Plants were watered daily with reverse osmosis water and fertilized weekly with Plant-Prod Solutions fertilizer 17:5:17 NPK (Master Plant-Prod Inc., Brampton, ON, Canada). *Amblyseius swirskii* was introduced as 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 four timepoints for clothianidin residue quantification, (1) when the soil was dosed (day 0), (2) 14 days after dosing, when eggs were transferred to the treatment leaves (day 28), (3) two weeks after egg transfer (day 43), and (4) when monarchs pupated (day 49). At each timepoint 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 (VWR High-Performance Centrifuge Tubes, CAT # 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 reach a 2
minimum mass for clothianidin detection to determine at what point during
development neonicotinoids may be metabolized, a subset of instar 5 caterpillars and
adult butterflies were haphazardly selected and combined. All samples were stored at -
20°C prior to residue analysis at the University of Guelph Agriculture and Food
Laboratory using the AOAC 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 M ammonium acetate. High
performance liquid chromatography coupled with electrospray ionization tandem mass
spectrometry (LC/ESI-MS/MS) was used to assess neonicotinoid concentration,
returned in parts per billion (1 ppb = 1 ng/g; Boguski, 2006). The limit of quantification
(LOQ) is the lowest concentration that can be accurately quantified, whereas the limit of
detection (LOD) is 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 cm x 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 (Denver Instrument 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 cm height x 60 cm depth x 60 cm width) inside an incubator set at temperatures between 23-29°C, relative humidity 77% (SD ±10%) with 500 mol light (18L: 6D). Enclosures contained untreated milkweed (i.e., grown in soil dosed with reverse osmosis water) and an artificial nectar source (i.e., 10% honey-water provided ad libitum). 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, CAT # 09A04.73, Lee Valley, Ottawa, ON, Canada), and cleaned daily with mild soap and water. Ambient conditions were maintained to represent those during the late fall at 43.5°N, 80.2°W (13L:11D, 21°C by day, 11°C at night, mean 87% (SD ± 6%) relative humidity) to encourage development of migratory monarch butterflies. Caterpillars were fed milkweed grown in treated or control soil ad libitum until pupation when chrysalids were then transferred to mesh enclosures (120 cm x 120 cm x 120 cm; Popadome Plant Dome, CAT # XC515, Lee Valley, Ottawa, ON, Canada) separated by treatment in the laboratory (c. 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 with 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 analyzing tape for spores under a microscope at 400x (Altizer and Oberhauser, 1999) and, if testing positive, were removed from the study. All procedures will be conducted under the Ontario Ministry for Natural Resources Wildlife Scientific Collectors Permit (#1090000).

Flight simulation

From September 17-23, 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) were 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 EST) when the sun was fully visible in the simulator to ensure consistency of polarized light cues (Mouritsen et al., 2013; Reppert et al., 2004). Individual butterflies were tethered to an L-shaped rod (modified to approximately 2.5 cm; CAT # 718000, 0.020 in x 6 in Tungsten Rods, A-M Systems, Washington, 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° rotation and recorded orientation at
3° 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 minutes (5 samples/min), with 2 minutes provided for acclimation before data collection to avoid a stress-induced unidirectional flight response (Perez and Taylor, 2014). Monarchs were removed (control: ♀, n = 1, ♂, n = 3; low dose ♂, n = 3, ♀, n = 3; high dose ♂, n = 2, ♀, n = 2) from the study if they did not show a characteristic pattern of flight (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 frequency 166.380 MHz with pulses emitted every 1.5 seconds 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 tree line, at the base of the Cambridge-RARE Motus tower (43.4°N, 80.4°W) in Cambridge, ON. 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 (Motus, 2017; Taylor et al., 2017). Data were received by the Motus Wildlife Tracking System and made available later for download (Motus, 2017; 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., 2019); 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 southward direction during fall migration. For monarchs flown in the flight simulator we calculated the mean direction (0°-359°) and vector strength (r, ranges from 0-1), a measure of the concentration of data around the mean, for each monarch butterfly flight using Oriana version 4.02 (Kovach Computing Services 2020). Using the data for each individual, we then calculated the group mean direction and vector strength within each of the treatments. Subsequently, a Rayleigh test was used to assess vector strength in order to determine if monarchs showed directional flight.
We also examined the effects of morphological and environmental factors on orientation in a circular-linear regression in R version 3.4.1 (R Core Team 2015). Monarch butterfly mass, forewing length, time of testing (i.e., minutes after 09:00 EST), wind speed, and temperature at the beginning of the test were included as predictors, with the mean flight direction for each individual 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, climate.weather.gc.ca/historical_data/search_historic_data_e.html).

To test possible effects of the treatments on monarchs released with radio-tracking tags, we calculated the group mean direction and vector strength for monarchs tracked using the Motus telemetry array using Oriana version 4.02. We used a Rayleigh test to determine whether monarchs in each treatment group showed directional flight. We then used a general linear model to investigate whether the neonicotinoid treatments 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 timepoints. Clothianidin was detected in soil from both treatments but at a lower concentration than originally applied to the soil (15 or 25 ng/g; Table 4.1). The concentration of clothianidin remained consistent 14 and 28 days after soil dosing, before the concentration dropped
at day 43 (Table 4.1). Clothianidin was found in a single sample of soil at the last time

timepoint (i.e., day 49 after soil dosing) in the low dose treatment (Table 4.1).

We detected no clothianidin in the milkweed leaves for the control group at any of
the timepoints, but the insecticide was detected in leaves from both treatments (Table
4.1). Though the concentration of clothianidin remained consistent in leaves from the
high dose treatment (Table 4.1), 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 4.1). 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 4.1). As expected,
the concentration of clothianidin was higher in the high dose treatment group relative to
the low dose treatment group. No clothianidin was detected in the tissue from adult
monarch butterflies irrespective of treatment group (Table 4.1).

*Flight simulation*

Individual monarchs concentrated in a variety of directions when tested in the flight
simulator (Table 4.2; Figure 4.1a). Thus, within the control and treatment groups, there
was no evidence for a concentration around a mean direction (Table 4.2; Figure 4.1a).
Treatment groups also did not differ from the control in their vector strength ($F_{1,37} =
0.84, p = 0.37$; estimate (CI): -0.052 [-0.17 – 0.063]). Given the lack of directional flight
for all groups, we pooled the groups together for subsequent analysis. Adult body mass,
forewing length, time of the flight simulation test, wind speed, or temperature did not
influence mean flight direction for either male \((t = 1.024, p = 0.15, \text{estimate (s. e.): } -0.001 \pm 0.001))\).

Radio-telemetry tracking

Similar to wild migratory monarch butterflies (Mouriten et al., 2013), captive-reared treatment and control monarchs that were released and radio-tracked did not in their direction of flight and flew southward (Table 4.2). More, the direction of flight was strongly concentrated around the mean (Table 4.2; Figure 4.1b, 4.2). We found no evidence the rate of travel \((F_{1,18} = 0.27, p = 0.61, \text{estimate (CI): } 0.74 [-2.24 – 3.73])\) differed among treatment and control groups.

Discussion

Our results demonstrate that early exposure to clothianidin at field-realistic concentrations of 15 and 25 ng/g of soil during monarch caterpillar development had no effect on the orientation in 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 measured in this study affected flight behaviour. Though studies suggest a negative impact of neonicotinoids on caterpillar development (Pecenka and Lundgren, 2015), we did not find conclusive evidence that exposure to clothianidin during development carries over to influence the orientation of adult migratory monarch butterflies.
Southward orientation during migration is essential for monarch butterflies to reach their destination in the Cerro Pelón and Sierra Madre Oriental mountains of Mexico (Brower, 1995; Urquhart, 1960; Urquhart and Urquhart, 1978). Monarchs visually perceive solar cues and also have a light-dependent molecular clock in the antennae responsible for the sun compass (Guerra et al., 2012; Merlin et al., 2009; Reppert, 2006; Reppert and Weaver, 2002). 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, like clothianidin, could bind at nAChRs in the midbrain, we did not find evidence to suggest that this has an effect on directed flight, as indicated by the weak vector strength (i.e., weak concentration of directionality around the mean; Table 4.2). Given that clothianidin was not detected in adult monarch butterflies (Table 4.1), it is possible that it was metabolized prior to flight-testing. Although no studies have yet investigated the metabolism of neonicotinoids in monarch butterflies, the cytochrome P450 superfamily is responsible for metabolism of neonicotinoids (Manjon et al., 2018) at a rate of 2.0 ng/day in honeybees (Godfray et al., 2014). Therefore, our results are compatible with clothianidin being metabolized during caterpillar development and/or metamorphosis. However, there is some evidence that adult monarch butterflies reared as caterpillars on neonicotinoid-exposed milkweed have longer forewings than controls (Chapter 3), suggesting a potential impact of exposure on developmental outcomes for adults.

Our results also suggest that variation in body mass and forewing length does not drive migratory orientation. The fall 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 (Yiwen et al., 2016), our results indicate that monarch size (i.e., forewing length) is not an accurate indicator of migratory orientation.

While our results suggest no difference between the clothianidin treatment groups and controls, we caution drawing conclusions about flight directionality (or lack thereof) from monarchs flown in the flight simulator. Previous studies using flight simulators have found monarchs reared in captivity show semi-random flight orientation (Chapter 5; Tenger-Trolander, 2019), but when monarchs are released into the wild they regain expected southward orientation for migration (Chapter 5). Therefore, while 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 the individual monarchs tested in the flight simulator, we are unable to account for potential individual differences in flight behaviour between flight simulator and radio-tracking assays. Monarchs were suspended in the flight simulator using a tungsten rod inserted into the front of the dorsal thorax, resulting in temporary impairment, and showed visible signs of exhaustion (lethargy) after testing. Despite these challenges, our study provides the opportunity to examine differences between groups exposed to lower and higher clothianidin concentrations 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.

**Conclusion**

We found that the orientation of captive-reared migratory monarch butterflies was not affected by exposure as feeding larvae to field-realistic levels of the neonicotinoid clothianidin (applied at 15 ng/g and 25 ng/g to soil) when tested in either a tethered flight simulator or released and radio-tracked during the initial stage of early migration. Our results also showed no measurable effect for traits, including mass and forewing length, or environmental conditions having an effect on migratory flight. The results from our study contribute to the understanding of the potential impacts of insecticide exposure on monarch butterflies and suggests that exposure to field-realistic levels of clothianidin is unlikely to impair migratory flight.
Table 4.1 Concentration of the neonicotinoid clothianidin (ppb) in soil, swamp milkweed (*Asclepias incarnata*) and instar 5 monarch caterpillars (*Danaus plexippus*) for insecticide applications in control, 15 ng/g (low dose) and 25 ng/g (high dose) treatments. For samples with clothianidin detected (DET (n)), the mean (\(\bar{x}\)), standard deviation (s), range, and median are provided. The number of samples where no clothianidin detected (ND (n)) is provided as summary statistics could not be calculated (-).

<table>
<thead>
<tr>
<th>Time of sampling</th>
<th>Treatment</th>
<th>(\bar{x}) (ppb)</th>
<th>s (ppb)</th>
<th>Range (ppb)</th>
<th>Median (ppb)</th>
<th>DET (n)</th>
<th>ND (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Soil dosing</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14 days after dosing</td>
<td>Control</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Low dose</td>
<td>4.70</td>
<td>1.14</td>
<td>3.90–6.00</td>
<td>4.20</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>High dose</td>
<td>7.90</td>
<td>0.66</td>
<td>7.30–8.60</td>
<td>7.80</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>28 days after dosing</td>
<td>Control</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Low dose</td>
<td>6.64</td>
<td>0.54</td>
<td>7.40–6.90</td>
<td>6.60</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>High dose</td>
<td>10.18</td>
<td>0.60</td>
<td>9.20–10.60</td>
<td>10.50</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>43 days after dosing</td>
<td>Control</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Low dose</td>
<td>1.06</td>
<td>0.35</td>
<td>0.70–1.60</td>
<td>0.90</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>High dose</td>
<td>1.84</td>
<td>0.29</td>
<td>1.40–2.20</td>
<td>1.90</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>Days after dosing</td>
<td>Control</td>
<td>Low dose</td>
<td>High dose</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-------------------</td>
<td>---------</td>
<td>----------</td>
<td>-----------</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>49 days after dosing</td>
<td>- - - - 0 5</td>
<td>0.60 - - - 1 4</td>
<td>1.12 0.22 0.80–1.30 1.20 5 0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

[CLO] in milkweed leaves

<table>
<thead>
<tr>
<th>Days after dosing</th>
<th>Control</th>
<th>Low dose</th>
<th>High dose</th>
</tr>
</thead>
<tbody>
<tr>
<td>28 days after dosing</td>
<td>- - - - 0 3</td>
<td>- - - - 0 3</td>
<td>2.23 0.12 2.10–2.30 2.30 3 0</td>
</tr>
<tr>
<td>43 days after dosing</td>
<td>- - - - 0 3</td>
<td>1.60 0.57 1.20–2.20 1.60 2 2</td>
<td>2.00 0.59 1.40–2.80 1.90 4 0</td>
</tr>
<tr>
<td>49 days after dosing</td>
<td>- - - - 0 5</td>
<td>1.05 0.07 1.00–1.10 1.05 2 1</td>
<td>2.60 1.47 1.70–4.30 1.80 3 0</td>
</tr>
</tbody>
</table>

[CLO] in instar 5 monarch larvae

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Low dose</th>
<th>High dose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>- - - - 0 7</td>
<td>1.26 0.59 0.30–2.20 1.2 7 0</td>
<td>2.24 0.56 1.50–3.10 2.2 7 0</td>
</tr>
</tbody>
</table>

[CLO] in adult monarch butterflies

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Low dose</th>
<th>High dose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>- - - - 0 5</td>
<td>1.05 0.07 1.00–1.10 1.05 2 1</td>
<td>2.60 1.47 1.70–4.30 1.80 3 0</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>----------</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Low dose</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High dose</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 4.2 Eastern North American migratory monarch butterflies (*Danaus plexippus*) reared in environmental chambers simulating autumn conditions until pupation, then tested in an outdoor flight simulator to record flight orientation (between 0° to 359°) or radio-tracked during fall migration. Rayleigh test was 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>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Cardinal direction</th>
<th>r</th>
<th>z</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>12</td>
<td>N</td>
<td>0.13</td>
<td>0.19</td>
<td>0.83</td>
</tr>
<tr>
<td>Low dose</td>
<td>9</td>
<td>S</td>
<td>0.18</td>
<td>0.29</td>
<td>0.76</td>
</tr>
<tr>
<td>High dose</td>
<td>18</td>
<td>N</td>
<td>0.16</td>
<td>0.46</td>
<td>0.64</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Mean direction (°)</th>
<th>Cardinal direction</th>
<th>r</th>
<th>z</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6</td>
<td>151</td>
<td>SSE</td>
<td>0.98</td>
<td>5.78</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Low dose</td>
<td>5</td>
<td>154</td>
<td>SSE</td>
<td>0.99</td>
<td>4.98</td>
<td>0.001</td>
</tr>
<tr>
<td>High dose</td>
<td>9</td>
<td>158</td>
<td>SSE</td>
<td>0.99</td>
<td>8.97</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>
Figure 4.1 Orientation of captive-reared eastern North American migratory monarch butterflies (*Danaus plexippus*) reared on swamp milkweed (*Asclepias incarnata*) grown in control (0 ng/g; n=12), 15 ng/g (i.e., low dose; n=9) and 25 ng/g (i.e., high dose; n=18) soil and (a) flown in a flight simulator for 10 minutes in Guelph, ON in September 2018 or (b) released in Cambridge, ON in October 2018 for radio-tracking (control: n=6; low dose, n=5; high dose, n=9). The direction of flight for individual monarchs (•) is shown in a circular plot, where each section of the central windrose indicates the proportion of individuals with directional flight.
Figure 4.2 Orientation of captive-reared eastern North American migratory monarch butterflies (*Danaus plexippus*) reared on swamp milkweed (*Asclepias incarnata*) grown in (a) control (0 ng/g), (b) 15 ng/g (i.e., low dose) and (c) 25 ng/g (i.e., high dose) soil and released in Cambridge, ON in October 2018. Symbols indicate the sites of release (*) and location of first detection at a Motus tower (•), with the relative size referring to the number of detections at that tower. The number of monarchs detected at each tower is shown in brackets and grey dots indicate Motus towers that were active at the time of releases but did not detect any monarchs.
Chapter 5

Captive-reared Migratory Monarch Butterflies Show Natural Orientation When Released in the Wild

Abstract

Eastern North American migratory monarch butterflies (*Danaus plexippus*) have faced sharp declines over the past decade. Although captive rearing has been used as tool for engaging the public and supplementing conservation efforts, recent evidence using a flight simulator suggests that captive-reared monarchs may lose their capacity to orient southward during fall migration to their Mexican overwintering sites. We raised offspring of wild-caught monarchs on swamp milkweed (*Asclepias incarnata*) and, after eclosion, individuals were either tested in a flight simulator or radio-tracked in the wild using array of automated telemetry towers. While 26% (10/39) of monarchs tested in the flight simulator showed a weakly concentrated southeast to southwest orientation, 97% (28/29) of the radio-tracked individuals were detected by automated towers southeast to southwest of the release site, up to 200 km away. Our results suggest that, though captive rearing of monarch butterflies may cause temporary disorientation, proper orientation is likely re-established after exposure to natural skylight cues.
Introduction

Captive rearing and reintroduction of animals into the wild can be an effective tool for mitigating the decline of wild populations (Hughes and Bennett 1991). Capacity for acclimation in captivity varies among species (Chamove et al. 1988; Mettke 1995; Mason 2010), with some species, such as cheetahs (*Acinonyx jubatus*; Caro 1993), being notoriously difficult to maintain or having lower fitness in captivity. Animal behaviour is known to differ between captive and wild populations of mammals (Blanchard et al. 1986), fish (Salvanes et al. 2006), and insects (Fisher et al. 2015), and abnormal behaviour in captive populations of mammals is well documented (Birkett et al. 2011; McPhee 2004). However, only a single study, conducted on monarch butterflies (*Danaus plexippus*), has shown the potential for long-term impacts of behavioural changes in captive-reared insects intended to be released in the wild (Tenger-Trolander et al. 2019).

In late fall, monarch butterflies undergo up to a 4,000 km migration from the mid-western and north-eastern United States and south-eastern Canada to Cerro Pelón and Sierra Madre Oriental mountains in Mexico (Brower 1995; Urquhart 1960; Urquhart and Urquhart 1978). Monarch butterflies are often reared in captivity by hobbyists and conservationists aiming to contribute towards population recovery of this species at risk. However, a recent study provided evidence that chamber-reared monarch butterflies (i.e., reared from eggs indoors in autumn-like conditions until adult emergence (eclosion)) did not show normal southern orientation (Tenger-Trolander et al. 2019). These results were obtained when individual adult butterflies were tested immediately
after eclosion in a confined flight simulator that measured directional orientation. The authors concluded that the activity of rearing captive monarchs, notably from commercial sources, for release would be an ineffective conservation practice to help boost migratory populations. However, the possibility remains that monarch butterflies released in the wild are able to show proper orientation if they can recalibrate their internal compass with exposure to natural skylight cues, an external cue known to be critical to the functioning of the molecular clock that governs directional flight (Reppert et al. 2010).

In this study, we reared monarch butterflies in captivity and tested them in a confined flight simulator. Then, using an array of over 100 automated telemetry towers (Motus 2017; Taylor et al. 2017), butterflies reared in the same captive conditions were released and subsequently radio-tracked in the wild to evaluate whether captive-reared monarchs can reorient in a natural southward direction.

Methods

This study was part of a larger project testing the effect of exposure to the neonicotinoid insecticide clothianidin on orientation of fall migratory monarch butterflies. Monarch butterflies were reared in captivity on swamp milkweed (*Asclepias incarnata*) grown in commercial soil either treated with 4, 8, 15 or 25 ng/g of clothianidin or an untreated control (see Supplementary Material Chapter 5). Despite these rearing conditions, there was no effect of neonicotinoid exposure on orientation (Chapter 4). After eclosion, we tested the migratory orientation of adult monarchs in either a flight simulator or radio-
Flight simulator testing

For a subset of monarch butterflies (n = 54, tested 2-5 days after eclosion), we assessed monarch orientation during the fall migratory period (17-23 September 2018) using flight simulators (Figure 5.1a). Flight simulators were set up on the roof of the University of Guelph Phytotron, Guelph, ON, and arranged so that no surrounding buildings could obstruct the view of individuals while in the flight cylinder (Mouritsen et al. 2013). Tests occurred during daylight (9:30-16:00 EST) when the sun was fully visible from the simulator to ensure consistency of polarized light cues (Mouritsen et al. 2013; Reppert et al. 2004). Individual butterflies were tethered to an L-shaped rod (modified to approximately 2.5 cm; CAT # 718000, 0.020 in x 6 in Tungsten Rods, A-M Systems, Washington, 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). Each tether was attached to a digital encoder that allowed 360° rotation and recorded orientation at 3° intervals and there was no detectable resistance in the tether that would bias the direction of flight (Mouritsen et al. 2013). The encoder was adhered to a plexiglass rod supported within a large cylinder and attached to a nearby 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 once for 12 minutes (5 direction recordings/sec), with the initial 2 minutes for acclimation and to minimize the impacts of stress-induced unidirectional flight response (Perez et al. 1999). Monarchs were removed (n = 15) from the study if they did not show...
a characteristic pattern of flight (i.e., strong flapping with intermittent gliding).

**Radio-telemetry tracking**

We tracked monarch butterflies using radio-transmitters during early migration. Monarchs were outfitted with 200 mg NanoTags (Lotek Wireless Fish & Wildlife Monitoring, Newmarket, ON, Canada), each programmed to emit unique 166.380 MHz pulses every 1.5 seconds to maximize the probability of detection and allow individual identification (Taylor et al. 2017). Large individuals (> 0.3 g) were selected to minimize weight restrictions imposed by the tags and maximize the capacity for long-distance flight. On 5 October 2017, 41 monarch butterflies were released in an open field in Guelph, ON (43.6°N, -80.2°W), centered between adjacent Motus towers. On 27 September 2018, 43 monarchs were released on a hill, above tree line, at the base of the Cambridge RARE Motus tower (43.4°N, -80.4°W) in Cambridge, ON. The Motus telemetry array consists of more than 100 independent VHF telemetry towers across southern Ontario and the northern United States, with towers in all directions around the site of release (Motus 2017; Taylor et al. 2017). False detections were removed from analysis following the procedures outlined by Crewe et al. (2019). 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. 2019); for instance, removing detections that bounced between multiple towers and/or countries. We also removed detections recorded on the day of release at adjacent towers with
overlapping detection ranges to the site of release to avoid inaccurately assigning a direction of flight when the monarchs had not left the area. This resulted in true detections for 9 monarch butterflies in 2017 and 20 monarchs in 2018.

**Statistical analysis**

North American migratory monarch butterflies orient in a southward direction when flown in a flight simulator (Froy et al. 2003; Guerra and Reppert 2013; Mouritsen and Frost 2012). We calculated the mean direction (0° to 359°) and vector strength (r: 0 – 1) for each monarch butterfly flight using Oriana version 4.02 (Kovach Computing Services 2020). Vector strength is a measure of concentration for circular data with high values indicating a tighter grouping around the mean direction (Mouritsen et al. 2013; Pewsey et al. 2013).

To compare orientation between monarchs flown in the flight simulator and tracked using the Motus Wildlife Tracking System (Taylor et al. 2017). We calculated the mean group direction and vector strength separately for monarchs flown in the flight simulator and tracked using the Motus telemetry using Oriana. We also ran a Rayleigh test to assess the significance of the vector strength, allowing us to determine if monarchs flown in showed directional flight.

Finally, we calculated the percentage of individual monarch butterflies that flew in the southward direction (i.e., in the southeast to southwest direction) and calculated Spearman's Rank correlation coefficient in R version 3.4.1 (R Core Team 2015) to assess the relationship between distance travelled with time (i.e., greater distance
travelled with a longer duration of time since release).

Results

Although the mean direction for monarchs flown in the flight simulator was $\sigma = 352^\circ$ (N), individuals showed strong orientation in a variety of directions, resulting in the sample only being weakly concentrated around the mean ($n = 39, r = 0.07$, Rayleigh test, $z = 0.2, p = 0.82$; Figure 5.1b). Only 26% of monarchs tested in the flight simulator oriented in the southeast to southwest direction (Supplementary Material Chapter 5 Table S5.1). In contrast, 97% of radio-tracked monarchs (28/29) flew south to southeast ($\sigma = 147^\circ$; Figure 5.1c-d; Supplementary Material Chapter 5 Table S5.1). The direction of flight for radio-tracked monarchs was strongly concentrated around the mean (Rayleigh test, $n = 29, r = 0.93$, Rayleigh test, $z = 24.93, p < 0.001$; Figure 5.1d). Monarchs were first detected 1 to 16 days after release (Supplementary Material Chapter 5 Table S5.2) at towers from 12 km (52%, 15/29) up to ~200 km (3%, 1/29) from the site of release and the number of days to first detection was correlated with distance from the release site (Spearman’s rank correlation, $n = 29, r_s = 0.70, p < 0.001$).

Discussion

Our results provide evidence that monarch butterflies raised in captivity, but later exposed to natural conditions (i.e., sunlight and photoperiod), can reset the mechanism governing directional flight, allowing them to properly orient southward towards Mexico after they are released into the wild. Monarch butterflies tested in the flight simulator generally oriented in the southward direction, but the data were widely distributed in all
directions and only 10 flew in the southeast to southwest direction. When released into natural conditions 97% of monarchs flew in the south to southeast direction. Thus, while our study confirms the results from Tenger-Trolander et al. (2019) that most captive-reared monarchs tested in a flight simulator do not show proper orientation towards their Mexican wintering grounds, we also demonstrate that monarchs released in the wild are capable of recalibrating the mechanism responsible for directional flight. Therefore, we provide strong support for the practice of captive rearing as a conservation tool to supplement populations of monarch butterflies and improve recovery of this species at risk.

The results of our experiment suggest that outdoor environmental conditions are required for proper directional flight during migration. The sun’s position in the sky may act as a cue for the direction of migratory flight. In addition to this, sunlight cues are perceived through the eyes and monarchs also have a light-sensitive molecular clock in the antennae (Guerra et al. 2012; Merlin et al., 2009; Reppert and Weaver 2002; Reppert 2006), with information from these two systems likely integrated in the midbrain (Reppert et al. 2010). Disruption of this molecular mechanism by restricting natural light results in disoriented flight (Guerra et al. 2012; Merlin et al., 2009), providing evidence that sunlight is required for monarchs to calibrate flight orientation. A similar recalibration with environmental cues was found in Catharus thrushes (Cochran et al. 2004). After exposure to experimental magnetic fields, Gray-cheeked thrushes (C. minimus) and Swainson's thrush (C. ustulatus) were released and their flight patterns tracked using radio transmitters (Cochran et al. 2004). On the first night, birds flew westward, but corrected their orientation by the second night after they were exposed to
'normal' twilight cues and flew in the proper northward direction (Cochran et al. 2004). Though mechanisms underpinning flight orientation differ between birds and insects (Mouritsen 2018), it is possible that monarchs also recalibrate the direction of flight using information obtained via skylight and other natural cues. Alternatively, subtle differences in the photoperiod between our study and Tenger-Trolander et al. (2019) could underlie the observed differences in orientation. We used a slightly shorter photoperiod, with varying temperature, to approximate conditions experienced by monarch in fall. Photoperiod is a critical cue for animal migration and, in some species, has been implicated in triggering the change in migratory direction (Dingle 2014). Therefore, it is also possible that this variation in photoperiod is required for monarch butterflies to orient in a southward direction during fall migration.

Captive rearing of monarch butterflies for wildlife education, commercial breeding programs or by hobbyists can enhance conservation efforts if precautions are taken to rear monarchs in conditions that allow exposure to natural environmental conditions. Though commercially reared monarchs tested by Tenger-Trolander et al. (2019) showed a random orientation, the authors contrast their findings with a successful tag and release by Maeckle (2018) where released monarchs were re-sighted in Mexico and our results clearly demonstrate that upon release monarchs regain proper orientation. We forward that under proper rearing conditions, particularly exposure to sunlight, loss of orientation capacity may be negligible and future studies should determine the minimum duration of sunlight required to establish southward directional flight. Though the practice of captive rearing is contentious due to the potential for disease transmission (Journey North 2015; Monarch Joint Venture 2018; Tenger-
Trolander et al. 2019) and concerns around genetic viability (Journey North 2015; Monarch Joint Venture 2018; Tenger-Trolander et al. 2019; Willoughby et al. 2017), when these risks are minimized, reintroduction of monarch butterflies to the wild could contribute towards reversing the declines of migratory populations. Captive rearing of monarchs is not only a tool for conservation, but is also an extraordinary educational opportunity for the public to interact with nature and engage in conservation. The incredible social appeal of monarch butterflies and captive rearing for educational purposes encourages interactions between the public, educators, and scientists (Gustafsson et al. 2015). Thus, under proper conditions, captive rearing offers an opportunity for the public to engage in the conservation of this beloved and iconic species.

Although our results suggest that sunlight reestablishes southward directional flight in a North American fall migratory population of monarch butterflies, our experimental design did not allow us to investigate the duration of exposure to solar cues required for recalibration of the molecular clock mechanism. Nor were we able to test individual monarch butterflies in the flight simulator and then release the same individuals in the wild. Monarchs tested in the flight simulator were temporarily compromised due to the insertion of a rod into the front of the dorsal thorax and showed visible signs of exhaustion (i.e., lethargy) after testing. With the continued development of tracking technology, it is likely that we will soon have the ability to track monarchs and other insects at finer spatial resolutions and over multiple days during their migratory journey. When that occurs, our understanding of the proximate mechanisms that govern orientation and effects of captive rearing will likely improve.
Eastern North American monarch butterflies have undergone declines of over 80% in the last 2 decades (Thogmartin et al. 2017b). These astonishing declines serve as a reminder of the challenges faced in conserving biodiversity, particularly of insects, and in the conservation of this species at risk. Moreover, with increasing awareness of numerous threats to monarch butterfly populations (Thogmartin et al. 2017b), extensive support has been garnered across Canada, the US, and Mexico for monarch conservation. Our results confirm studies on the impact of captive rearing on monarch butterflies (Tenger-Trolander et al. 2019), but also strongly contrasts previously published research (Tenger-Trolander et al. 2019). Captive-reared monarchs regain proper flight orientation when released into the wild, demonstrating that the popular activity of rearing monarch butterflies from caterpillars in captivity can be a viable conservation tool and important education element to conserve this species.
Figure 5.1. Orientation of captive-reared eastern North American migratory monarch butterflies (*Danaus plexippus*) (a) flown in a flight simulator for 10 minutes in Guelph, ON in September 2018. The (b) direction of flight (σ = 352°, n = 39; r = 0.07) of flight for individual monarchs (●) with the group mean direction (◆) is shown in a circular plot, where each section of the central windrose indicates the proportion of individuals with directional flight. Group mean direction is indicated as a solid line and each section of the windrose indicates the proportion of individuals with directional flight. (c) Map of the direction of flight for monarch butterflies released in Guelph, ON in September 2017 (n = 9, green lines) and Cambridge, ON in October 2018 (n = 20, orange lines). Symbols indicate the sites of release (●) and location of first detection at a Motus tower (◆), with the relative size referring to the number of detections at that tower (lowest number of detections at a tower = 1, highest number of detections at a tower = 5). Grey dots
indicate Motus towers that were active at the time of releases. (d) Circular plot shows the direction of flight for radio-tracked monarch butterflies ($\sigma = 147^\circ$, $n = 29$, $r = 0.93$).
Chapter 6

Conclusion

Conservation management aims to protect species by mitigating the risk imposed by threats that result in a reduction in biodiversity and habitat availability. The dramatic loss of monarch butterflies (*Danaus plexippus*) from the eastern North American population is attributed to a variety of threats. To effectively mitigate these threats and accelerate the recovery of monarch abundance requires identification of the underlying causes driving underlying declines. I performed a systemic literature review on the potential threats to monarch butterflies suggests that the availability of suitable environmental conditions and habitat, both on breeding and overwintering grounds, are key factors negatively affecting monarch population viability; though, contaminant exposure may also play a role during early caterpillar development. Experimental studies that control for the influence of abiotic and biotic factors are a critical research approach that aims to disentangle and understand how interacting threats contribute to the decline of species at risk. As part of my thesis, I aimed to identify how prolonged and sublethal exposure to the neonicotinoid insecticide clothianidin during early monarch caterpillar influences development, reproductive output, and migration. I found that clothianidin had an effect on early caterpillar development at the fifth instar, yet exposure to clothianidin resulted in larger adult monarch butterflies and did not carryover to affect reproductive output. Similarly, clothianidin had no effect on the orientation, vector strength (i.e., concentration of the data around the mean direction), or rate of travel of adult migratory
monarch butterflies. Given the susceptibility of monarchs to threats during early life, research should focus on the impact of and interaction between threats during monarch development in order to help clarify the degree of and location(s) where conservation efforts that should be maximized. Lastly, I found that, contrary to previously published research (Tenger-Trolander et al. 2019), captive rearing has the potential to be used as a conservation management tool to supplement monarch populations. By integrating findings derived from different types of studies, decision-makers and planners can be better equipped to create conservation management plans to address potential large-scale impact of monarch declines on ecosystem function and economic services and improve recovery of this multigenerational species at risk.

Monarchs represent one of many migratory species whose complex multigenerational life cycle requires coordinated conservation actions between stakeholders and governments (Behrens et al. 2008). A multi-disciplinary approach is required for the development of conservation management plans that includes monitoring threats crossing international boundaries, tracking individuals, and integrating information across species to identify shared mechanisms behind observed declines (Diffendorfer et al. 2013). In Mexico, over 560 km² of overwintering habitat is protected in the Monarch Butterfly Biosphere Reserve, which, alongside more than 10 additional sister sites designated protected habitat for monarch butterflies (Shahani et al. 2015). Moreover, in the United States and Canada, coordinated work at multiple levels of government, NGOs, and private citizens augments conservation efforts. Despite habitat protections, threats from environmental contaminants, like neonicotinoids, persist. Mexico currently does not have a ban on neonicotinoids
(RAPAM 2018) and, despite a 2014 ban on neonicotinoids on US National Wildlife Refuges (BBC News 2018), the restrictions were lifted in 2018 (BBC News 2018). However, the US Environmental Protection Agency revoked approvals for a number of pesticides, including those containing clothianidin and thiamethoxam (Allington 2019). In 2019, Health Canada announced restrictions on applications and the timing of application for imidacloprid, clothianidin, and thiamethoxam to protect pollinators and is currently evaluating the risk of neonicotinoids in other ecological systems (Health Canada 2019).

Neonicotinoids can have dramatic and detrimental effects on pollinators including declines in physiological functions and immunity and impaired behavioural responses (Belzunces et al. 2012). Initially designed as a control method for pests, including some lepidopterans (Kundoo et al. 2018), the negative effects on non-target and beneficial species is not unexpected given the presence of nicotinic acetylcholine receptors, the main target of neonicotinoids, in insects (Bonmatin et al. 2015; Sánchez-Bayo et al. 2016). However, studies have often focused on species with considerable economic importance, so investigation into whether neonicotinoids play a role in reduced fitness and survival of other insects is required. No doubt neonicotinoids negatively impact monarch caterpillar development (Chapter 3; Pecenka and Lundgren 2015), but questions remain to whether these impacts carry forward to influence adult butterfly fitness. As a nectar-feeding insect, it will also be key to understand the degree to which this route of exposure could influence survival in the wild. Though my results do not show an effect of early-life neonicotinoid exposure on monarch migration (Chapter 4), it is still possible that high concentrations could impair physiological function and
behaviour. While likely a weak contributor relative to other threats to monarch population viability (Thogmartin et al. 2017b), more recent studies suggest that neonicotinoid exposure from nectar at concentrations > 20 ng/g could impact survival (James 2019). However, other studies have found that concentrations of neonicotinoids in nectar vary widely (i.e., < 0.5 – 16 ng/g in nectar) depending on agricultural crop and environmental conditions (Wood and Goulson 2017). Therefore, more research is needed to determine the degree to which neonicotinoids play a role, if any, in monarch declines.

Beyond government regulations to limit the impact of environmental contaminants on pollinators, captive rearing and reintroduction programs may enhance the possibility for population recovery. The rearing of butterflies in captivity can be done for a variety of purposes including, but not limited to, providing animals for research, large-scale conservation efforts, and interest from hobbyists. Captive rearing for reintroduction into the wild has long been suggested as a tool to supplement populations, particularly where species are extirpated (Herms et al. 1996), and has been successfully conducted for a number of insects including the Karner blue (Plebejus melissa samuelis; Herms et al. 1996) and Poweshiek skipperling (Oarisma Poweshiek; Smith 2018). Though risk from disease, inbreeding and its associated detriments could lead to reduced fitness remain concerns (Journey North 2015; Monarch Joint Venture 2018), captive rearing can be a cost-effective method to raise large numbers of butterflies, particularly when host plants or suitable habitat is unavailable (Morton 1983). Both large-scale captive rearing to protect at risk species and citizen-led operations can enhance recovery efforts, but the latter also encourages
public engagement and understanding of the wildlife-related issues. Citizen scientists also play a critical role in bolstering support for conservation efforts, even securing funding to help address questions and solve environmental problems (Ellwood 2018; EPA 2019; McKinley et al. 2017). In fact, public education, increased awareness, and capacity building, especially in terms of data gathering, has been identified as a key for insect conservation and recovery (Harvey et al. 2020). Collectively, coordinated efforts for monarch reintroduction, coupled with restoration and protection of ideal monarch habitat, has the potential to mitigate declines and boost population abundance.

A recent report on monarch population abundance at overwintering sites in Mexico has showed an increase in the overwintering population for the 2018-2019 season (Rendón-Salinas et al. 2019), calling into question whether threats to monarch populations have waned and/or whether conservation efforts have successfully slowed population declines. Though it is too early to gauge the long-term trends in monarch abundance or the degree independent conservation efforts have played in restoring monarch numbers (Thogmartin et al. 2020), efforts are underway to protect monarch habitat against degradation, most notably by increasing milkweed availability on the breeding range, and institute regulations to protect monarchs and other pollinators, notably from environmental contaminants such as neonicotinoids (Health Canada 2019). Moreover, with international recognition and increasing awareness of the declines of the eastern North American population of monarch butterflies, citizen scientist efforts in captive rearing and providing habitat in the form of pollinator gardens can positively enhance recovery efforts. My thesis research advances our understanding of the conservation threats to monarch butterflies and supports captive
rearing as a practical method to mitigate declines and serve as a viable educational tool. Overall, these insights are valuable for the development of conservation management plans and will help guide future research aimed to enhance conservation of this multigenerational species at risk.
REFERENCES


Bartholomew, C. S., and K. V. Yeargan. 2001. Phenology of milkweed (Asclepiadaceae) growth and monarch (Lepidoptera: Nymphalidae) reproduction in Kentucky and
ovipositional preference between common and honeyvine milkweed. Journal of the Kansas Entomological Society 74:211–220.


(*Danaus plexippus* L.): avian predation at five overwintering sites at

Calvert, W. H. 1996. Fire ant predation on monarch larvae (Nymphalidae: Danainae) in

Monarch Butterfly: Biology and Conservation* (K. S. Oberhauger and M. J.

Calvo-Agudo, M., J. González-Cabrera, Y. Picó, P. Calatayud-Vernich, A. Urbaneja, M.
insects kill beneficial insects. Proceedings of the National Academy of Sciences
of the United States of America 116:16817-16822.

Climatic signal in earlywood and latewood in conifer forests in the monarch
butterfly biosphere reserve, Mexico. Tree Ring Research 74:63–75.

Caro, T. M. 2013. Behavioral solutions to breeding cheetahs in captivity: Insights from

Casagrande, R. A., and J. E. Dacey. 2007. Monarch butterfly oviposition on swallow-


Chan, D. S. W., R. S. Prosser, J. L. Rodríguez-Gil, and N. E. Raine. 2019. Risks of exposure to systemic insecticides in agricultural soil in Ontario, Canada for the hoary squash bee (Peponapis pruinosa) and other ground-nesting bee species. Scientific Reports 9:11870.


Cresswell, J. E., F. X. Robert, H. Florance, and N. Smirnoff. 2014. Clearance of ingested neonicotinoid pesticide (imidacloprid) in honey bees (Apis mellifera) and
bumblebees (*Bombus terrestris*). Pest Management Science 70:332-337.


DeCant, J. 2010. *Clothianidin registration of Prosper T400 seed treatment on mustard seed (oilseed and condiment) and Poncho/Votivo seed treatment on cotton*. Washington, DC: U.S. Environmental Protection Agency.


Hochkirch, A. C. Hughes, S. Jepsen, T. H. Jones, B. M. Kaydan, D. Kleijn, A. M.
Klein, T. Latty, S. R. Leather, S. M. Lewis, B. C. Lister, J. E. Losey, E. C. Lowe,
Painting, T. H. Pham, S. G. Potts, A. Rauf, T. L. Roslin, M. J. Samways, F.
Sanchez-Bayo, S. A. Sar, C. B. Schultz, A. O. Soares, A. Thancharoen, T.
Vujic, D. L. Wagner, M. F. WallisDeVries, C. Westphal, T. E. White, V. L. Wilkins,
International scientists formulate a roadmap for insect conservation and

safety/pesticides-pest-management/public/consultations/proposed-re-evaluation-
decisions/2018/imidacloprid/document.html

Health Canada. 2019. Health Canada releases final pollinator re-evaluation decisions
for neonicotinoid pesticides (News Release). www.canada.ca/en/health-
canada/news/2019/04/some-cancellations-and-new-restrictions-to-protect-bees-
and-other-pollinators.html

Heinze, S., and S. M. Reppert. 2011. Sun compass integration of skylight cues in


journeynorth.org/tm/monarch/conservation_action_release.pdf


Citizen science can improve conservation science, natural resource management, and environmental protection. Biological Conservation 208:15-28.


   monarchjointventure.org/news-events/news/revised-handout- raising-monarchs- 
   why-or-why-not

   implications for community structure and indirect plant defense. American 

   Study on soil mobility of two neonicotinoid insecticides. Journal of Chemistry 
   2016:4546584.

   Biological Conservation 25:19-33.


   butterflies reveals their orientation mechanisms. Proceedings of the National 


Oberhauser, K., R. Wiederholt, J. E. Diffendorfer, D. Semmes, L. Ries, W. E.


Pleasants, J. 2017. Milkweed restoration in the Midwest for monarch butterfly recovery: estimates of milkweeds lost, milkweeds remaining and milkweeds that must be added to increase the monarch population. Insect Conservation and Diversity 10:42–53.


RAPAM. 2018. Highly Hazardous Pesticides in Mexico (Fernando Bejarano, ed). Mexico: IPEN.
ipen.org/sites/default/files/documents/HHHP%20in%20Mexico%202018REV.pdf


WWF México. 2020. La mariposa Monarca redujo en un 53% su ocupación en los bosques mexicanos de hibernación (Press release).

www.wwf.org.mx/noticias/?uNewsID=361041


**Table S2.1.** Evaluation of the literature classified based on whether results in peer-reviewed research does [S] or does not [N] support a threat impacting the eastern North American population of monarch butterflies (*Danaus plexippus*) and whether the threat may increase. Studies identified as [-] do not draw conclusion on the future risk to monarchs.

<table>
<thead>
<tr>
<th>Conservation threat</th>
<th>Type of study</th>
<th>Results</th>
<th>Effect on the summer breeding or overwintering population</th>
<th>Is there an effect on monarch butterflies?</th>
<th>Is there evidence the threat will persist or increase?</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adverse weather events</td>
<td>[Field_data]</td>
<td>Drought occurring in Texas in 2010-2011 thought to limit nectar sources, but had minimal impact on butterfly lipid stores, potentially due to alternative sources available at overwintering sites in Mexico.</td>
<td>overwinter</td>
<td>[N]</td>
<td>[-]</td>
<td>Brower et al., 2015</td>
</tr>
<tr>
<td>Adverse weather events</td>
<td>[Field_data]</td>
<td>Storm damage in 2016 at the Monarch Butterfly</td>
<td>overwinter</td>
<td>[S]</td>
<td>[-]</td>
<td>Brower et al., 2017</td>
</tr>
</tbody>
</table>
Biosphere Reserve (MBBR) resulted in declines of 38% in Pelón, 31% in Chincua, and 7.4% in Rosario.

[Mod] In a population model, included weather-induced mortality events in Mexico on monarch butterfly rates. Threat of severe weather events was predicted to decline at overwintering sites.

[Mod] Increasing frequency of extreme weather events likely to have negative impact on survival of monarchs at all stages in the life cycle.

Climate change [Control_data] Growth rate of common milkweed (Asclepias syriaca) was increased with elevated temperature and decreased under water stress. Greater growth and tolerance to water stress observed at higher latitudes in Wisconsin,
USA. Larval growth rates increased by 38% under elevated temperatures and by 13% when reared on water stress plants.

**[Field_data]** Precipitation during winter and spring positively influences, while mean maximum temperatures negatively affected, growth of sacred fir (*Abies religiosa*) and the smooth-bark Mexican pine (*Pinus pseudostrobus*). The sacred fir was more sensitive to temperature variations than the smooth-bark Mexican pine, suggesting that drier climate projections could negatively impact sacred fir growth rates in monarch butterfly overwintering habitat.

**[Mod]** Climate projections integrated with breeding niche models predict northward range overwinter

Carlón-Allende et al., 2018

Batalden et al., 2007
expansion over the next 50 years.

<table>
<thead>
<tr>
<th>Source</th>
<th>Prediction</th>
<th>Season</th>
<th>Note</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Barve et al., 2012</td>
<td>Optimal conditions at overwintering sites will reduce the area occupied by 73-100% by 2050.</td>
<td>overwinter</td>
<td>[S]</td>
<td>[S]</td>
</tr>
<tr>
<td>Flockhart et al., 2015</td>
<td>Population abundance sensitive to climate change on non-breeding grounds, but models predicted higher population sizes relative to the influence of milkweed loss on breeding grounds.</td>
<td>summer</td>
<td>[N]</td>
<td>[S]</td>
</tr>
<tr>
<td>Lemoine, 2015</td>
<td>Northward progression of <em>Asclepias</em> spp. likely to affect availability of suitable breeding habitat.</td>
<td>summer</td>
<td>[S]</td>
<td>[S]</td>
</tr>
<tr>
<td>Oberhauser and Petersen, 2003</td>
<td>Ecological niche models predicted that precipitation and diurnal temperature range predicted habitat use in Mexico and that conditions would be deemed unsuitable across the overwintering</td>
<td>overwinter</td>
<td>[S]</td>
<td>[S]</td>
</tr>
</tbody>
</table>
range in 50-year climate projections.

\[ M_{\text{bio}} \] Bioclimate model was driven by mean temperature in the warmest month, winter temperature, and precipitation that resulted in an estimated 96.5% loss of the sacred fir by 2090, restricting overwintering habitat.

\[ M_{\text{bio}} \] Population size was negatively associated with early warm temperatures and positively associated with later warm temperatures.

\[ M_{\text{bio}} \] Daily temperature and rainfall were determined for 25 locations across the breeding range from 1970-2010 and applied in a CLIMEX model, but no trends of climate on monarch population abundance was observed. Suggests other factors, including

\[ S\text{áenz-Romero et al., 2012} \]

\[ S\text{thogmartin et al., 2017b} \]

\[ Zalucki et al., 2015 \]
deforestation and loss of milkweed, may contribute to declines.

[Mod] Long-term environmental data obtained from the National Oceanic and Atmospheric Administration from 1996-2008 in Ohio and Texas were used to assess climatic factors on population abundance. High precipitation in Texas drives monarch abundance in Ohio, but conditions in Ohio do not affect the timing of arrival. Abundance on summer breeding grounds highest in warm temperatures and influenced minimally by precipitation.

<table>
<thead>
<tr>
<th>Deforestation</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>[Field_data]</strong></td>
</tr>
<tr>
<td>Source</td>
</tr>
<tr>
<td>--------</td>
</tr>
<tr>
<td>Alonso-Mejía et al., 1998</td>
</tr>
<tr>
<td>Anderson and Brower, 1996</td>
</tr>
<tr>
<td>Brower et al., 2002</td>
</tr>
<tr>
<td>Williams et al., 2007</td>
</tr>
</tbody>
</table>
Firs were 1.4°C warmer than ambient forest temperature, reducing the energetic cost of overwintering and mortality.

Dense forests increase overwintering survival by providing a suitable microclimate for monarchs as butterflies select least hazardous temperatures (i.e., intermediate tree heights that are warm at night and in early morning). This emphasizes the risk imposed by logging at overwintering sites.

Loss of 2,227 ha of firs within the MBBR at Michoacán, México, from 2006-2010, corresponding to 556.75 ha year\(^{-1}\).

Deforestation between 2003-2009 resulted in 1,105 ha of loss and 881
ha of degradation, partly attributed to logging, reducing vital closed canopy cover by 968 ha and semi-closed canopy cover by 413 ha.

**[Field_data]** Though conservation measures protected 3-16% of high quality habitat, only a maximum of 2.5% of forest with >70% canopy cover was protected.

**[Field_data]** Deforestation was greater in regions without Forest Management Protection Programs in MBBR at Michoacán state and forest disturbance was partly attributed to logging.

**[Field_data]** Aerial photographs and satellite images were examined between 1971-1994 and determined annual deforestation rate of 0.1% and annual disturbance (e.g., from

---

overwinter  [S]  [S]  Honey-Rosés et al., 2011

overwinter  [S]  [S]  Navarrete et al., 2011

overwinter  [S]  [S]  Ramírez et al., 2003
timber harvesting) rate of 1.3%.

[Field_data] Aerial photographs and satellite images of the MBBR from 2001-2012 showed clearing of 1254 ha of forest.

[Field_data] Surface area occupied by overwintering monarchs decreased by 44% in 2013-2014 from the previous season, likely caused by forest loss and degradation.

[Mod] Population models including forest loss as the primary driver showed higher population sizes relative to when milkweed loss was identified as the primary mechanism of declines. Though, reduction in forest cover resulted in greater risk of a high mortality event.
Deforestation a primary obstacle to the conservation of monarchs, but current levels of illegal logging and deforestation unlikely to be primary driver for decline.

Demographic models suggest that monarch population growth rates could increase with improved breeding and overwintering habitat.

### Contaminants

<table>
<thead>
<tr>
<th>Exposure to GM crops and <em>Bacillus thuringiensis (Bt)</em></th>
<th>Larvae exposed to Bt anthers had increased development time and reduced feeding, resulting in lower mass and survival, compared to controls. Late instars were more tolerant to Bt. Anther density present on common milkweed was not found at toxic levels.</th>
<th>summer</th>
<th>[S]</th>
<th>[N]</th>
<th>Anderson et al., 2004</th>
</tr>
</thead>
</table>

Hunt and Tongen, 2017

Oberhauser et al., 2017
### Control_data

<table>
<thead>
<tr>
<th>Study</th>
<th>Treatment</th>
<th>Result</th>
<th>Summer</th>
<th>Notes</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anderson et al., 2005</td>
<td>Monarch larvae consumed minimal amounts of <em>Bt</em> anther, but exposure to anthers and pollen resulted caused delayed development time (1.8 days longer) and lower mass (64% reduction) in pupae relative to controls.</td>
<td>summer</td>
<td>[S]</td>
<td>[-]</td>
<td>Anderson et al., 2005</td>
</tr>
<tr>
<td>Dively et al., 2004</td>
<td>Monarch larvae exposed to <em>Bt</em> corn pollen as larvae were 23.7% less likely to reach adulthood. When examined over the breeding range in the Corn Belt region of the US, exposure to <em>Bt</em> corn pollen prolonged development time (1.8 days), lower mass (5.5% reduction), and increased mortality by 0.6%.</td>
<td>summer</td>
<td>[S]</td>
<td>[S]</td>
<td>Dively et al., 2004</td>
</tr>
<tr>
<td>Hellmich et al., 2001</td>
<td><em>Bt</em> toxins Cry9 and Cry1F are non-toxic to first instar larvae. Larvae are sensitive to Cry1Ab and Cry1Ac proteins, but this declined with age.</td>
<td>summer</td>
<td>[N]</td>
<td>[-]</td>
<td>Hellmich et al., 2001</td>
</tr>
</tbody>
</table>
Results suggest pollen from Cry1Ab, Cry1F, and Cry9C Bt corn has minimal effects on monarchs.

[Control_data] Monarch larvae feeding on common milkweed exposure to Bt corn pollen had 20% mortality after 20 hours and up to 70% after 120 hours of exposure. No sub-lethal effects observed in adults.

[Control_data]/[Field_data] Monarchs were observed on 21 of 35 common milkweed (A. syriaca) plants in Bt corn fields. No difference in survival curves for monarchs between Bt and non-Bt corn fields, but, after excluding predators found a trend for higher rates of mortality among larvae exposed to Bt corn pollen and anthers (but it was not significant at a 0.05 level).

Jesse and Obrycki, 2000

Jesse and Obrycki, 2004
<table>
<thead>
<tr>
<th>Control_data</th>
<th>Summary</th>
<th>Data Type</th>
<th>Summer</th>
<th>Notes</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exposure to <em>Bt</em> pollen reduced feeding and mass in monarch larvae.</td>
<td>[Control_data]</td>
<td></td>
<td>summer</td>
<td>[S]</td>
<td>Losey et al., 1999</td>
</tr>
<tr>
<td>Instars exposed to Event-176 (i.e., low dose pollen) weighed 18% less than those exposed to Bt11 or Mon810. Exposure to Event-176 at 67 pollen grains per cm² on milkweed resulted in 60% lower survivorship in larvae, but no effect on development and survival were observed with Bt11 or Mon810.</td>
<td>[Control_data]</td>
<td></td>
<td>summer</td>
<td>[S]/[N]</td>
<td>Stanley-Horn et al., 2001</td>
</tr>
<tr>
<td>Higher proportions of eggs were recovered from milkweed patches in isolation of <em>Bt</em> corn and without contamination from corn pollen. No effects were observed on oviposition behaviour.</td>
<td>[Control_data]</td>
<td></td>
<td>summer</td>
<td>[S]</td>
<td>Tschenn et al., 2001</td>
</tr>
<tr>
<td>Monarch larvae mortality not associated with proximity to and exposure to pollen from <em>Bt</em> corn.</td>
<td>[Control_data]/[Field_data]</td>
<td></td>
<td>summer</td>
<td>[N]</td>
<td>Zangerl et al., 2001</td>
</tr>
</tbody>
</table>
Monarch butterflies laid eggs equally on common milkweed and honeyvine milkweed. Though monarchs use honeyvine milkweed in Kentucky, migratory monarchs are unlikely to be impacted by the presence of honeyvine milkweed as migration through the region occurs after \( Bt \) pollen shedding.

Survival was similar on milkweed near \( Bt \) corn and non-\( Bt \) corn. Larval mortality was lowest close to \( Bt \) corn.

Monarchs use milkweed throughout the breeding season, commonly on agricultural land, which results in exposure to \( Bt \) corn predominantly in the northern portion of the range.

\( Bt \) pollen density in cornfields ranged from
600-1400 grains per cm$^2$ and decreased towards the field edge.

Natural areas and sites with higher milkweed density had lower egg density based on records from 1997-2014 obtained by the Monarch Larva Monitoring Project. Area occupied on the overwintering grounds was positively correlated with egg density and negatively related to the percent crop area planted in herbicide-resistant crops.

**Herbicides**

Dicamba use was correlated with population size positively in the northern, but negatively in the southern, portion of the breeding range.

2,4-dichlorophenoxyacetic acid application is correlated with population size positively in the

**References**

Stenoien et al., 2015

Thogmartin et al., 2017b
northern, but negatively in the southern, portion of the breeding range.

<table>
<thead>
<tr>
<th>Source</th>
<th>Action</th>
<th>Result</th>
<th>Duration</th>
<th>Data Type</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>[Moo]</td>
<td>Across the breeding range, population size was negatively correlated with cumulative glyphosate use. Population declines likely related to loss of milkweed as a result of high prevalence of herbicide-resistant crops.</td>
<td>summer</td>
<td>[S]</td>
<td>Thogmartin et al., 2017b</td>
<td></td>
</tr>
<tr>
<td>Insecticides</td>
<td>Permethrin application resulted in longer development time and lower survival in larvae compared to controls and decreased survival in ovipositing females exposed to plants sprayed 1-day prior.</td>
<td>summer</td>
<td>[S]</td>
<td>Oberhauser et al., 2006</td>
<td></td>
</tr>
<tr>
<td>[Control_data]/[Field_data]</td>
<td>Adult monarchs fed syrup containing imidacloprid lived longer than free-ranging individuals, but larval survival was significantly reduced.</td>
<td>summer</td>
<td>[S]/[N]</td>
<td>Krischik et al., 2015</td>
<td></td>
</tr>
<tr>
<td>Control_data</td>
<td>Monarchs exposed to resmethrin spray developed into smaller adults and exposure soon after application (i.e., 1 day) resulted in larval mortality.</td>
<td>summer</td>
<td>[S]</td>
<td>[-]</td>
<td>Oberhauser et al., 2009</td>
</tr>
<tr>
<td>Control_data</td>
<td>RNA interference (RNAi) provides a tool to knockdown genes and provides the basis for alternative pest control treatments, particularly by means of ingestion. Dietary ingestion of V-ATPase A dsRNA resulted in variation in growth times depending on the instar stage, but no overall differences were observed in development time and survival.</td>
<td>summer</td>
<td>[N]</td>
<td>[-]</td>
<td>Pan et al., 2017</td>
</tr>
<tr>
<td>Control_data</td>
<td>LC\textsubscript{50} of the neonicotinoid clothianidin in monarch butterflies at 15.63 and sublethal effects on larval size at 1 ppb.</td>
<td>summer</td>
<td>[S]</td>
<td>[-]</td>
<td>Pecenka and Lundgren, 2015</td>
</tr>
<tr>
<td>Control_data</td>
<td>Instars survivorship and mass were reduced with exposure to $\lambda$-cyhalothrin.</td>
<td>summer</td>
<td>[S]</td>
<td>[-]</td>
<td>Stanley-Horn et al., 2001</td>
</tr>
<tr>
<td>Mod</td>
<td>Population size was negatively correlated with neonicotinoid use across the breeding range, but best fit models predicting declines did not include total neonicotinoid application. Neonicotinoids weakly determined population abundance.</td>
<td>summer</td>
<td>[S]</td>
<td>[N]</td>
<td>Thogmartin et al., 2017b</td>
</tr>
</tbody>
</table>

**Breeding habitat loss**

| Control_data | Instar 5 monarch larvae exposed to simulated traffic noise for 2 hours exhibited an increase in heart rate, suggesting a potential stressor, but this effect was not present after 7-12 days of chronic exposure. | summer | [S/N] | [-] | Davis et al., 2018 |
| Field_data | Data collected from transects along roadsides in Illinois was used to | summer | [S] | [-] | McKenna et al., 2011 |
assess mortality from vehicular collision. Peak mortality occurred during migration, with an estimated 500,000 individuals killed per week.

<table>
<thead>
<tr>
<th>Source</th>
<th>Summary</th>
<th>Season</th>
<th>Notes</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>[Field_data]</td>
<td>Roadsides may act as an ecological trap, concentrating butterflies in roadside prairies and increasing OE infections. However, there was no difference in the proportion of infected monarchs between roadside prairies and managed prairies.</td>
<td>summer</td>
<td>[N]</td>
<td>Mueller and Baum, 2014</td>
</tr>
<tr>
<td>[Mod]</td>
<td>Migration rate slowed when habitat area dropped below 25%, with migration rates showing greater reduction in fragmented landscapes.</td>
<td>summer</td>
<td>[S]</td>
<td>Collingham and Huntley, 2000</td>
</tr>
<tr>
<td>[Mod]</td>
<td>Movement paths in a spatially-explicit agent-based model show preferential use of high density milkweed areas in</td>
<td>summer</td>
<td>[S]</td>
<td>Grant et al., 2018</td>
</tr>
</tbody>
</table>
a fragmented landscape. Increased perceptual range and decreased spatial memory reduced the area used.

| Nectar sources | Improving nectar availability during migration in Mexico and the Southern US and during breeding in the Southern and Northeastern US may increase monarch population growth rates. |
| Host plants | 10.5-21.7% oviposition on swallow-wort plants (*Vincetoxicum* spp.) relative to common milkweed in choice tests. 15.4% of monarch eggs were found on plots containing a low proportion of swallow-warts relative to common milkweed and was over five times greater when milkweed abundance was low. |

Oberhauser et al., 2017

Casagrande and Dacey, 2007
Monarchs showed no preference for swallow-wort plants (*Vincetoxicum nigrum* and *Vincetoxicum rossicum*) during egg deposition in laboratory choice and no-choice tests, but larvae consumed a greater proportion of leaves from common milkweed. Mass was lower on swallow-wort and survival decreased by 44% on *V. rossicum* and 14% on *V. nigrum* compared to milkweed.

Swamp milkweed (*Asclepias incarnata*) had the greatest preference for oviposition, whereas narrowleaf milkweed (*A. fascicularis*) had the lowest preference. Larval survival was high on both species and preference did not differ between eastern and western populations of monarch butterflies.
<table>
<thead>
<tr>
<th>Source</th>
<th>Summary</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mattila and Otis, 2003</td>
<td>Monarchs showed no preference for dog-strangler vine (<em>Cynanchum rossicum</em>) during egg deposition in laboratory choice and no-choice tests and larvae preferentially consumed leaves from common milkweed. Larvae reared on dog-strangler vine died within 96 hours.</td>
</tr>
<tr>
<td>Pocius et al., 2017a</td>
<td>Monarch larvae can survive on poke milkweed (<em>A. exaltata</em>), tall green milkweed (<em>A. hirtella</em>), swamp milkweed, showy milkweed (<em>A.speciosa</em>), prairie milkweed (<em>A. sullivantii</em>), common milkweed, butterfly milkweed (<em>A. tuberosa</em>), whorled milkweed (<em>A. verticillata</em>), honeyvine milkweed (<em>Cynanchum laeve</em>) with probability of survival between 30-75%.</td>
</tr>
<tr>
<td>Pocius et al., 2017b</td>
<td>Monarch larvae can survive on poke</td>
</tr>
</tbody>
</table>
milkweed, tall green milkweed, swamp milkweed, showy milkweed, prairie milkweed, common milkweed, butterfly milkweed, whorled milkweed, honeyvine milkweed, but larvae reared on C. leave were an instar behind and those fed whorled milkweed had a higher mass.

Egg counts from 2015-2017 in Iowa showed free-flying wild monarchs preferentially oviposited on common and swamp milkweed, while fewer eggs were recorded on poke, butterfly, tall green, whorled, and honeyvine milkweed.

Monarchs suffered lower OE parasite infection when reared on tropical milkweed (A. curassavica) than on swamp milkweed, though...
Cardenoloide induction was unlikely to influence resistance.

### Control data

| Larvae developed faster on honeyvine milkweed than common milkweed and, though not significant, larval mass was lower on honeyvine milkweed. Survival was 14% higher on common milkweed. |
|---|---|---|---|---|
| Larvae developed faster on honeyvine milkweed than common milkweed and, though not significant, larval mass was lower on honeyvine milkweed. Survival was 14% higher on common milkweed. | summer | [S] | [-] | Yeargan and Allard, 2005 |

### Field data

| Immature larvae (5.7x) were found in greater numbers on tropical milkweed (A. curassavica) compared to common milkweed, likely due to the higher cardenolide concentration that would provide defence against predators after consumption. |
|---|---|---|---|---|
| Immature larvae (5.7x) were found in greater numbers on tropical milkweed (A. curassavica) compared to common milkweed, likely due to the higher cardenolide concentration that would provide defence against predators after consumption. | summer | [S] | [-] | Malcolm and Brower, 1986 |

| Migrants at sites with year-round residents were more likely to have OE infection. Exotic milkweed may attract |
|---|---|---|---|---|
| Migrants at sites with year-round residents were more likely to have OE infection. Exotic milkweed may attract | summer | [S] | [-] | Satterfield et al., 2018 |
migrants, allowing them to break reproductive diapause and increasing risk of infection.

**Milkweed** [Field_data] Glyphosate resistant crops have resulted in a decrease in milkweed, which was present in 50% of corn and soybean crops in Iowa in 1999 and only 8% in 2009. Summer [S] [S] Hartzler, 2010

Field_data] No statistically significant declines observed over 22 years in the northern portion of the breeding range, suggesting that populations were able to recover during the breeding season, contrary to the milkweed limitation hypothesis. Summer [N] [N] Inamine et al., 2016

Field_data] Milkweed was found on about 60% of roadsides surveyed, with common milkweed being the most common species observed. At low densities, milkweed presence per unit area summer [S] [-] Kasten et al., 2016
was positively correlated with monarch density.

[Efield_data] Egg densities were highest on small (<16 m$^2$) and low-density (0.1-2 milkweed per m$^2$) patches in agricultural landscapes. Predators were most abundant on medium-sized milkweed patches.

[Efield_data] Estimated a 58% decline in milkweed in the Midwestern US, coinciding with an increase in glyphosate-tolerant crops, and an 81% decline in monarch butterfly production between 1999-2010, which, in turn, was correlated with the overwintering population size in Mexico in the subsequent year.

[Efield_data] Observed declines of 68% in milkweed abundance in Illinois between 1997-2016,
likely resulting from changes in agricultural practices, and likely contributing to population declines.

<table>
<thead>
<tr>
<th>Method</th>
<th>Description</th>
<th>Season</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>[M_{mod}]</td>
<td>Predicted a loss of 1.49 billion milkweed plants from 1995-2013 (21% decline) with the highest loss occurring in the Central region of the United States and monarch breeding range. Considered to be the primary driver of monarch butterfly declines.</td>
<td>summer</td>
<td>Flockhart et al., 2015</td>
</tr>
<tr>
<td>[M_{mod}]</td>
<td>Reduced milkweed availability increases caterpillar density on host plants and reduces survival.</td>
<td>summer</td>
<td>Hunt and Tongen, 2017</td>
</tr>
<tr>
<td>[M_{mod}]</td>
<td>Improving milkweed availability during breeding in the Southern and Northeastern US may increase monarch population growth rates.</td>
<td>summer</td>
<td>Oberhauser et al., 2017</td>
</tr>
<tr>
<td>Source</td>
<td>Text</td>
<td></td>
<td></td>
</tr>
<tr>
<td>--------</td>
<td>------</td>
<td></td>
<td></td>
</tr>
<tr>
<td>[Mod]</td>
<td>From 1999 to 2014, an estimated 850 million milkweed were lost from corn and soybean fields and, from 2008-2014, 11 million were lost from grasslands, resulting in a decline of nearly 40%. 1.6 million milkweed plants are required to increase monarch populations to 6 ha conservation goal.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>summer</td>
<td>[S]</td>
<td>[S]</td>
<td>Pleasants, 2017</td>
</tr>
<tr>
<td>[Mod]</td>
<td>Milkweed limitation is the likely cause of decline as population size on the summer breeding range corresponds with overwintering populations declines.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>summer</td>
<td>[S]</td>
<td>[S]</td>
<td>Pleasants et al., 2017</td>
</tr>
<tr>
<td>[Mod]</td>
<td>Mean density at overwintering sites in Mexico estimated at 27.9 million monarch butterflies ha(^{-1}), resulting in a deficit in milkweed availability to support the population during the breeding season based</td>
<td></td>
<td></td>
</tr>
<tr>
<td>summer</td>
<td>[S]</td>
<td>[S]</td>
<td>Thogmartin et al., 2017a</td>
</tr>
</tbody>
</table>
on calculations from Pleasants (2017b).

<p>| $[M_{\infty}]$ | Greater than 1.3 million new milkweed stems required to reach monarch population conservation goal. | summer | [S] | [S] | Thogmartin et al., 2017c |
| $[M_{\infty}]$ | Reduction in milkweed can affect fecundity (i.e., number of eggs laid) when plants are present at low density. However, individuals that are good searchers can improve success by exploring outside a habitat patch. | summer | [S] | [S] | Zalucki and Lammers, 2010 |
| $[M_{\infty}]$ | Habitat restoration along roadways, increasing the presence of milkweed, increased milkweed cover from 1-2% and resulted in an increase in egg production at an average of 51% when included an agent-based model. | summer | [S] | [S] | Zalucki et al., 2016 |</p>
<table>
<thead>
<tr>
<th>Predation and parasitism</th>
<th>Control_data</th>
<th>Survival increased to nearly 73% from predation by Asian lady beetle (<em>Harmonia axyridis</em>) when alternate prey, <em>Aphis nerii</em>, was presented.</th>
<th>summer</th>
<th>[S]</th>
<th>[-]</th>
<th>Koch et al., 2005</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control_data</td>
<td>Milkweed genotype did not affect on predatory arthropods, but impact ants (<em>Formica podzolica</em>) via a negative influence on <em>Aphis</em> and <em>Myzocallis</em> aphids. Negative genetic correlation between aphids (in the presence of ants) and ants, independently, and monarch survival.</td>
<td>summer</td>
<td>[S]</td>
<td>[-]</td>
<td>Mooney and Agrawal, 2008</td>
</tr>
<tr>
<td></td>
<td>Control_data</td>
<td>Predation on eggs by green lacewing larvae (<em>Chrysoperla rufilabris</em>) in the lab.</td>
<td>summer</td>
<td>[S]</td>
<td>[-]</td>
<td>Oberhauser et al., 2015</td>
</tr>
<tr>
<td></td>
<td>Control_data/ Field_data</td>
<td>Monarch survival was 18% for aerial and terrestrial predators and</td>
<td>summer</td>
<td>[S]</td>
<td>[S]</td>
<td>Prysby, 2004</td>
</tr>
</tbody>
</table>
5% for ants and aphids. Up to 23% of monarchs were parasitized by tachnids.

<table>
<thead>
<tr>
<th>Control_data</th>
<th>Wasps (<em>Polistes dominulus</em>) consumed monarch larvae in the lab.</th>
<th>summer</th>
<th>[S]</th>
<th>[-]</th>
<th>Rayor, 2004</th>
</tr>
</thead>
<tbody>
<tr>
<td>Field_data</td>
<td>Higher rates of bird predation observed in open forest canopies (i.e., low tree density, low basal area, and low canopy coverage) compared to closed canopies.</td>
<td>overwinter</td>
<td>[S]</td>
<td>[-]</td>
<td>Alonso-Mejia et al., 1998</td>
</tr>
<tr>
<td>Field_data</td>
<td>Observations of parasitism by tachnid flies on monarch butterflies.</td>
<td>summer</td>
<td>[S]</td>
<td>[-]</td>
<td>Arnaud, 1978</td>
</tr>
<tr>
<td>Field_data</td>
<td>Observation of predation on monarch butterflies by turtle bugs (Pentatomidae), jumping spiders (Salticidae), ants (Formicidae), and tachnid flies (Tachinidae).</td>
<td>summer</td>
<td>[S]</td>
<td>[-]</td>
<td>Borkin, 1982</td>
</tr>
</tbody>
</table>
On average 15,067 butterflies per day are killed on average by black-backed orioles (*Icterus abeillei*) and black-headed grosbeaks (*Pheucticus melanocephalus*) at overwintering colonies in Mexico and select male monarchs, potentially due to differences between the sexes in fat or cardenolide content.

Predation on monarch butterflies by a variety of species of birds at overwintering sites in Mexico is negatively related to colony size.

Predation on monarch butterflies by fire ants (*Solenopsis invicta*).

Fire ants (*Solenopsis invicta*) predated on monarch butterflies and reproductive success was greater inside exclosures.

Predation by black-backed orioles and black-headed grosbeaks resulted in over 60% mortality at overwintering sites in Mexico.

Tachnid fly (*Lespesia archippivora*) parasitism

---

Fink et al., 1983

Fink and Brower, 1981

Geest et al., 2018
was >30% in conservation sites and gardens. Density of milkweed did not affect parasitism.

[Field_data] Observations from Los Angeles and Texas on predation by ants and crab spiders (Thomisidae).

[Field_data] Parasitism by *Pteromalus* spp. on pupae (rate up to 20%) in Minnesota.

[Field_data] Approximately 13% of monarch larvae were parasitized by tachnid flies as determined from 7 years of wild monarch collection by the Monarch Larva Monitoring Project.

[Field_data] Monarch larvae were collected and reared (n = 654), from which 12 tachnid flies emerged. Positive correlation between monarch density in the previous year and
the proportion parasitized.

| Field_data | Predation on pupae by wasps in Minnesota. | summer | S | - | Oberhauser et al., 2015 |
| Field_data | Chinese mantids (*Tenodera sinensis*) consumed larvae in the field and lab. | summer | S | - | Rafter et al., 2013 |
| Field_data | Predation on monarch butterflies by birds observed at overwintering sites. | overwinter | S | - | Sakai, 1994 |
| Field_data | Observed predation by ants on monarch larvae. | summer | S | - | Watts, 2000 |
| Field_data | Observations of predation on monarch butterflies by dragonflies (*Hagenisu brevistylus*). | summer | S | - | White and Sexton, 1989 |
| Mod | Predation by the Asian lady beetle (*Harmonia axyridis*) was predicted to be high in soybean fields relative to cornfields. | summer | S | - | Koch et al., 2006 |
| **Ophryocystis elektroscirrha (OE)** | **[Control_data]** | **Monarchs inoculated with a high concentration of OE spores had lower survival and emerged with smaller wingspans and body mass. Lifespan and reproductive success was also affected in males and activity levels in females. In wild populations, parasite load was negatively associated with size and condition (e.g., wing tatter and scale loss), and positively associated with mating activity.** | **summer** | **[S]** | **[-]** | **Altizer and Oberhauser, 1999** |
| **[Control_data]** | **Experimental infection with OE led to shorter flight distances, slower flight speeds, and a greater loss of body mass relative to controls.** | **summer** | **[S]** | **[-]** | **Bradley and Altizer, 2005** |
| **[Control_data]** | **Positive correlation between parasite load and adult larva longevity.** | **summer** | **[S]** | **[-]** | **de Roode et al., 2007** |
| **[Control_data]** | **OE infection, replication, and virulence depends on** | **summer** | **[S]** | **[-]** | **de Roode et al., 2008a** |
host plant cardenoloide level. OE-infected monarchs had lower lifespan on low cardenoloide milkweed.

[Control_data] Positive correlation between within-host replication of OE, virulence, and transmission. 

Monarchs infected with OE had shorter lifespans, lower fecundity. Greater spore loads resulted in higher transmission to eggs, mating partners, and the host plants.

OE virulence was positively related to transmission and higher infection levels. Higher virulence was observed in Western North American compared to eastern North American strains.

Aphids can reduce the levels of cardenoloides in

summer [S] [-] de Roode et al., 2008b

summer [S] [-] de Roode et al., 2009

summer [S] [-] de Roode and Altizer, 2010

summer [S] [-] de Roode et al., 2011
tropical milkweed (*Asclepias curassavica*) that is used by larvae to decrease the likelihood of *OE* infection. This results in an increased virulence and likelihood of transmission of *OE*.

**Control_data** Positive correlation between inoculum concentration and spore loads, while inoculation of first and third instars resulted in higher spore concentration in adults relative to when infection initially presented in fifth instars.

**Control_data** Greater larval density results in increased *OE* infection probability, with impacts on body size, development rate, and longevity in monarchs in moderate- and high-density treatments.

**Control_data**/ **Field_data** Availability of tropical milkweed (*A. curassavica*) could alter

summer [S] [-] Leong et al., 1997

summer [S] [-] Lindsey et al., 2009

summer [S] [S] Satterfield et al., 2015
migration patterns, resulting in higher OE infection prevalence among sedentary individuals.

[Control_data] Monarchs suffered lower OE parasite infection when reared on tropical milkweed (A. curassavica) than on swamp milkweed (A. syriaca), though cardenoloide induction was unlikely to influence resistance.

[Field_data] OE parasitism was detected in < 2% of larvae collected from conservation sites and gardens.

[Field_data] Up to 66.7% of monarchs were detected with OE on their wings in overwintering populations in Mexico.

[Field_data] Roadsides may act as an ecological trap, concentrating butterflies
in roadside prairies and increasing OE infections. However, there was no difference in the proportion of infected monarchs between roadside prairies and managed prairies.

**[Field_data]** Migrants at sites with year-round residents were more likely to have OE infection. Exotic milkweed may attract migrants, allowing them to break reproductive diapause and increasing risk of infection.

**[Field_data]** Monarchs collected during summers of 1964-1965 exhibited viral infection resulting in significant larval mortality.

**[Mod]** Parasitism by OE negatively correlated population size across the breeding range.

**Migratory culling** **[Field_data]** Presence of OE unlikely to contribute to

---

[Satterfield et al., 2018]

Summer

[S]

[-]

Satterfield et al., 2018

Summer

[S]

[-]

Urquhart, 1966

Summer

[S]

[S]

Thogmartin et al., 2017b

Summer

[S]

[S]

Altizer et al., 2000
overwintering mortality as average parasite load in breeding monarchs decreased with greater distance from overwintering sites.

<table>
<thead>
<tr>
<th>Field_data</th>
<th>No evidence for declines from censuses taken at Peninsula Point, MI, USA. Observed declines in Mexico may be attributed to reduced migratory success.</th>
<th>summer</th>
<th>[S]</th>
<th>[N]</th>
<th>Badgett and Davis, 2015</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Field_data</th>
<th>OE prevalence increased over the breeding season and was positively related to breeding activity. Prevalence decreased as monarchs progressed southward and was lower at overwintering sites in Mexico.</th>
<th>summer</th>
<th>[S]</th>
<th>[S]</th>
<th>Bartel et al., 2011</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Field_data</th>
<th>Infection prevalence was similar regardless of distance travelled, thereby not supporting the migratory culling hypothesis. Monarchs at</th>
<th>summer</th>
<th>[N]</th>
<th>[-]</th>
<th>Flockhart et al., 2018</th>
</tr>
</thead>
</table>
southern latitudes had higher infection prevalence and infections increased over the breeding seasons, supporting migratory escape hypothesis.

| \(M_{ad}\) | Migratory culling would predict a disconnect between population estimates in summering and overwintering monarch populations, but egg counts conducted by the Monarch Larva Monitoring Project were positively correlated with overwintering population size. | summer | [N] | [N] | Pleasants et al., 2017 |
Supplementary Material: Chapter 5

Methods

Milkweed

Swamp milkweed (Asclepias incarnata) was grown in commercial soil (LA4 Sunshine Loosefill, Sungro Horticulture, Massachusetts, USA) treated with either at 4, 8, 15 or 25 ng/g of clothianidin (neonicotinoid insecticide) or a control (i.e., distilled water) with 4 plants per 15.2 cm$^2$/1.68 L pot in environmental chambers at the University of Guelph Phytotron. Milkweed was watered twice daily until the soil was saturated. Room temperature was set at 29°C during the day and 23°C at night, 500 mol light (18L:6D) as outlined in Flockhart et al. (2012). Humidity was monitored hourly via a handheld thermohygrometer (Vaisala MI70 Measurement Indicator with HMP75 Humidity and Temperature Probe, Vaisala, Helsinki, Finland) with an average 77% (SD ±10%) RH. Plants were watered daily with reverse osmosis water and fertilized weekly with Plant-Prod Solutions fertilizer 17:5:17 NPK (Master Plant-Prod Inc., Brampton, ON, Canada). Amblyseius swirskii were introduced as a biocontrol (Bioline AgroSciences Swirskiline Biocontrol Agent and Biobest Swirskii-Breeding-System) measure to reduce the impact of thrips (Thysanoptera) (Flockhart et al. 2012).

Capture and maintenance

We raised monarch caterpillars from eggs laid by wild monarchs obtained from Gowanstown, ON (43.52°N, -81.08°W; male, n = 7; female, n = 13) on 14 August 2017
and the Guelph Lake Conservation Area (43.61°N, -80.26°W; male, n = 7; female, n = 11) from 2-6 August 2018. Wild monarch butterflies were held in coin envelopes (6.35 cm x 10.8 cm) inside an animal carrier kept at ambient temperature and 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 (Denver Instrument PI-602 scale, Denver Instrument, Bohemia, NY, USA) to the nearest 0.01 g and individually hand-fed a 10% honey-water solution daily until satiation. Wild monarchs were mated in large mesh enclosures (60 cm height x 60 cm depth x 60 cm width) inside an incubator set at temperatures fluctuating between 29°C and 23°C with 500 mol light (18L:6D) and an average 77% (SD ±10%) RH. Enclosures contained untreated milkweed (i.e., grown in soil dosed with reverse osmosis water) and an artificial nectar source (i.e., 10% sucrose water) provided ad libitum.

We collected 192 eggs each year (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. In 2017, monarch caterpillars were reared directly on the milkweed plants with pots enclosed with finely perforated mosquito netting (Bulk Mosquito Netting, CAT # 09A04.73, Lee Valley, Ottawa, ON, Canada). Light, temperature and humidity in the University of Guelph Phytotron were maintained according to ambient conditions during the early fall in Guelph (43.5°N, -80.2°W; 13 hours light: 11 hours dark) at 21°C day:11°C night and an average 87% (SD ±6%) RH. Caterpillars were fed milkweed ad libitum until pupation when chrysalids were then transferred to mesh enclosures (60 cm height x 60 cm depth x 60 cm width) in the University of Guelph Phytotron separated by treatment after eclosion from 19
September – 3 October 2017. In 2018, leaves with eggs were placed in large plastic containers and enclosed with finely perforated mosquito netting. Environmental conditions were replicated from those used in 2017, except that chrysalids were transferred to mesh enclosures (120 cm x 120 cm x 120 cm; Popadome Plant Dome, CAT # XC515, Lee Valley, Ottawa, ON, Canada) in the laboratory (c. 19.5°C) where lighting cycle was variable, but supplemented by negligible foyer lighting. Eclosion occurred from 14-19 September 2018. Adult monarchs were hand-fed daily and provided dishes with a sucrose solution within the enclosures (Flockhart et al. 2012). We examined each individual for *Ophryocystis elektroscirrha* protozoan parasites by applying clear tape to the abdomen and analyzing tape for spores under a microscope at 400x (Altizer and Oberhauser 1999) and infected butterflies were removed from the study. All procedures will be conducted under the Ontario Ministry for Natural Resources Wildlife Scientific Collectors Permit (2017: #1086793; 2018: #1090000).
Table S5.1. Eastern North American migratory monarch butterflies (*Danaus plexippus*) reared in environmental chambers simulating autumn conditions (day: 29°C, night 23°C and 77% RH) until pupation. Monarchs (n = 39) were tested in an outdoor flight simulator that recorded orientation between 0° to 359°. The mean direction (° ± SD) and vector strength (r), representing the concentration of the data between 0 (data are evenly spread) to 1 (data are concentrated around the mean), was calculated for each individual. Rayleigh test was used to determine whether monarchs showed directional flight (p < 0.05); however, the large sample size (n=3000) from the flight simulator tests results in low p values without practical significance. Each row represents an individual monarch butterfly.

<table>
<thead>
<tr>
<th>Cardinal direction</th>
<th>Mean direction (°)</th>
<th>SD (°)</th>
<th>r</th>
<th>z</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>E</td>
<td>91</td>
<td>36</td>
<td>0.82</td>
<td>2030</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>ENE</td>
<td>76</td>
<td>51</td>
<td>0.68</td>
<td>1367</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>ENE</td>
<td>60</td>
<td>57</td>
<td>0.61</td>
<td>1104</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>ESE</td>
<td>119</td>
<td>74</td>
<td>0.43</td>
<td>561</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>ESE</td>
<td>117</td>
<td>56</td>
<td>0.62</td>
<td>1168</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>ESE</td>
<td>117</td>
<td>75</td>
<td>0.42</td>
<td>537</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>ESE</td>
<td>112</td>
<td>21</td>
<td>0.93</td>
<td>2613</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>N</td>
<td>352</td>
<td>42</td>
<td>0.76</td>
<td>1735</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>N</td>
<td>12</td>
<td>90</td>
<td>0.29</td>
<td>250</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Direction</td>
<td>Frequency</td>
<td>Duration</td>
<td>Probability</td>
<td>Significance</td>
<td></td>
</tr>
<tr>
<td>-----------</td>
<td>-----------</td>
<td>----------</td>
<td>-------------</td>
<td>--------------</td>
<td></td>
</tr>
<tr>
<td>N</td>
<td>6</td>
<td>41</td>
<td>0.78</td>
<td>&lt; 0.001</td>
<td></td>
</tr>
<tr>
<td>N</td>
<td>0</td>
<td>0</td>
<td>1.00</td>
<td>&lt; 0.001</td>
<td></td>
</tr>
<tr>
<td>NE</td>
<td>39</td>
<td>79</td>
<td>0.38</td>
<td>&lt; 0.001</td>
<td></td>
</tr>
<tr>
<td>NNE</td>
<td>38</td>
<td>93</td>
<td>0.27</td>
<td>&lt; 0.001</td>
<td></td>
</tr>
<tr>
<td>NNE</td>
<td>30</td>
<td>124</td>
<td>0.10</td>
<td>&lt; 0.001</td>
<td></td>
</tr>
<tr>
<td>NNE</td>
<td>23</td>
<td>144</td>
<td>0.04</td>
<td>0.004</td>
<td></td>
</tr>
<tr>
<td>NNW</td>
<td>347</td>
<td>13</td>
<td>0.98</td>
<td>&lt; 0.001</td>
<td></td>
</tr>
<tr>
<td>NNW</td>
<td>340</td>
<td>13</td>
<td>0.97</td>
<td>&lt; 0.001</td>
<td></td>
</tr>
<tr>
<td>NNW</td>
<td>334</td>
<td>95</td>
<td>0.25</td>
<td>&lt; 0.001</td>
<td></td>
</tr>
<tr>
<td>NNW</td>
<td>333</td>
<td>91</td>
<td>0.29</td>
<td>&lt; 0.001</td>
<td></td>
</tr>
<tr>
<td>S</td>
<td>181</td>
<td>142</td>
<td>0.05</td>
<td>0.001</td>
<td></td>
</tr>
<tr>
<td>S</td>
<td>172</td>
<td>41</td>
<td>0.78</td>
<td>&lt; 0.001</td>
<td></td>
</tr>
<tr>
<td>SE</td>
<td>138</td>
<td>76</td>
<td>0.42</td>
<td>&lt; 0.001</td>
<td></td>
</tr>
<tr>
<td>SE</td>
<td>137</td>
<td>57</td>
<td>0.61</td>
<td>&lt; 0.001</td>
<td></td>
</tr>
<tr>
<td>SE</td>
<td>127</td>
<td>43</td>
<td>0.75</td>
<td>&lt; 0.001</td>
<td></td>
</tr>
<tr>
<td>SE</td>
<td>126</td>
<td>105</td>
<td>0.19</td>
<td>&lt; 0.001</td>
<td></td>
</tr>
<tr>
<td>SSE</td>
<td>147</td>
<td>77</td>
<td>0.41</td>
<td>&lt; 0.001</td>
<td></td>
</tr>
<tr>
<td>SSW</td>
<td>212</td>
<td>153</td>
<td>0.03</td>
<td>0.08</td>
<td></td>
</tr>
<tr>
<td>SW</td>
<td>230</td>
<td>1</td>
<td>1.00</td>
<td>&lt; 0.001</td>
<td></td>
</tr>
<tr>
<td>SW</td>
<td>221</td>
<td>42</td>
<td>0.76</td>
<td>&lt; 0.001</td>
<td></td>
</tr>
<tr>
<td>W</td>
<td>280</td>
<td>68</td>
<td>0.50</td>
<td>&lt; 0.001</td>
<td></td>
</tr>
<tr>
<td>W</td>
<td>273</td>
<td>30</td>
<td>0.87</td>
<td>&lt; 0.001</td>
<td></td>
</tr>
<tr>
<td>W</td>
<td>270</td>
<td>33</td>
<td>0.85</td>
<td>&lt; 0.001</td>
<td></td>
</tr>
<tr>
<td>W</td>
<td>270</td>
<td>117</td>
<td>0.13</td>
<td>&lt; 0.001</td>
<td></td>
</tr>
<tr>
<td>WNW</td>
<td>302</td>
<td>45</td>
<td>0.74</td>
<td>&lt; 0.001</td>
<td></td>
</tr>
<tr>
<td>Direction</td>
<td>Observations</td>
<td>Frequency</td>
<td>Mean</td>
<td>SD</td>
<td>p-value</td>
</tr>
<tr>
<td>-----------</td>
<td>--------------</td>
<td>-----------</td>
<td>------</td>
<td>----</td>
<td>---------</td>
</tr>
<tr>
<td>WNW</td>
<td>299</td>
<td>81</td>
<td>0.37</td>
<td>414</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>WNW</td>
<td>296</td>
<td>90</td>
<td>0.29</td>
<td>248</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>WSW</td>
<td>255</td>
<td>52</td>
<td>0.66</td>
<td>1320</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>WSW</td>
<td>246</td>
<td>2</td>
<td>1.00</td>
<td>2997</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>WSW</td>
<td>241</td>
<td>87</td>
<td>0.32</td>
<td>306</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>
Table S5.2. Eastern North American migratory monarch butterflies (*Danaus plexippus*) reared in environmental chambers simulating autumn conditions (day: 29°C, night 23°C and 77% RH) until pupation. Monarch butterflies (*n = 29*) were then released with radio-telemetry tags and tracked using the Motus telemetry array. The number of days and distance to first detection, as well as the direction (°) of flight after release in Guelph (2017) and Cambridge, ON (2018) was recorded. Each row represents an individual monarch butterfly.

<table>
<thead>
<tr>
<th>Year</th>
<th>No. days after release</th>
<th>Distance (km)</th>
<th>Cardinal direction</th>
<th>Direction (°)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2017</td>
<td>4</td>
<td>4</td>
<td>N</td>
<td>47</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>43</td>
<td>ESE</td>
<td>117</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>14</td>
<td>SE</td>
<td>125</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>14</td>
<td>SE</td>
<td>125</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>14</td>
<td>SE</td>
<td>125</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>63</td>
<td>SSE</td>
<td>149</td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>201</td>
<td>SSW</td>
<td>174</td>
</tr>
<tr>
<td>2018</td>
<td>3</td>
<td>52</td>
<td>SE</td>
<td>126</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>64</td>
<td>SSE</td>
<td>147</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>12</td>
<td>SSE</td>
<td>156</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>12</td>
<td>SSE</td>
<td>156</td>
</tr>
<tr>
<td>2</td>
<td>12</td>
<td>SSE</td>
<td>156</td>
<td></td>
</tr>
<tr>
<td>-----</td>
<td>----</td>
<td>------</td>
<td>-----</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>12</td>
<td>SSE</td>
<td>156</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>12</td>
<td>SSE</td>
<td>156</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>12</td>
<td>SSE</td>
<td>156</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>12</td>
<td>SSE</td>
<td>156</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>12</td>
<td>SSE</td>
<td>156</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>12</td>
<td>SSE</td>
<td>156</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>12</td>
<td>SSE</td>
<td>156</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>12</td>
<td>SSE</td>
<td>156</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>12</td>
<td>SSE</td>
<td>156</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>12</td>
<td>SSE</td>
<td>156</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>12</td>
<td>SSE</td>
<td>156</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>12</td>
<td>SSE</td>
<td>156</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>162</td>
<td>SSE</td>
<td>161</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>162</td>
<td>SSE</td>
<td>161</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>95</td>
<td>ESE</td>
<td>165</td>
<td></td>
</tr>
</tbody>
</table>