2018

Monarch butterfly preference and use of nine Midwestern milkweed species

Victoria Marie Pocius

Iowa State University

Follow this and additional works at: https://lib.dr.iastate.edu/etd

Part of the Biodiversity Commons, Ecology and Evolutionary Biology Commons, Entomology Commons, Natural Resources and Conservation Commons, and the Natural Resources Management and Policy Commons

Recommended Citation

https://lib.dr.iastate.edu/etd/16438

This Dissertation is brought to you for free and open access by the Iowa State University Capstones, Theses and Dissertations at Iowa State University Digital Repository. It has been accepted for inclusion in Graduate Theses and Dissertations by an authorized administrator of Iowa State University Digital Repository. For more information, please contact digirep@iastate.edu.
Monarch butterfly preference and use of nine Midwestern milkweed species

by

Victoria Marie Pocius

A dissertation submitted to the graduate faculty

in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Major: Ecology and Evolutionary Biology

Program of Study Committee:
Diane M. Debinski, Co-Major Professor
John M. Pleasants, Co-Major Professor
Dean C. Adams
Amy L. Toth
Sue L. Blodgett

The student author, whose presentation of the scholarship herein was approved by the program of study committee, is solely responsible for the content of this dissertation. The Graduate College will ensure this dissertation is globally accessible and will not permit alterations after a degree is conferred.

Iowa State University
Ames, Iowa
2018

Copyright © Victoria M. Pocius, 2018. All rights reserved.
DEDICATION

“Around here we don't look backwards for very long. We keep moving forward, opening up new doors and doing new things, because we're curious… and curiosity keeps leading us down new paths.”

-Walt Disney

To my family, friends, and mentors, thank you for inspiring me to always move forward.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>LIST OF FIGURES</td>
<td>v</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>vi</td>
</tr>
<tr>
<td>ACKNOWLEDGMENTS</td>
<td>vii</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>ix</td>
</tr>
<tr>
<td>CHAPTER 1: INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>Literature Cited</td>
<td>6</td>
</tr>
<tr>
<td>CHAPTER 2: PERFORMANCE OF EARLY INSTAR MONARCH BUTTERFLIES (DANAUS PLEXIPPUS L.) ON NINE MILKWEED SPECIES NATIVE TO IOWA</td>
<td>12</td>
</tr>
<tr>
<td>Abstract</td>
<td>12</td>
</tr>
<tr>
<td>Introduction</td>
<td>12</td>
</tr>
<tr>
<td>Methods and materials</td>
<td>16</td>
</tr>
<tr>
<td>Results</td>
<td>20</td>
</tr>
<tr>
<td>Discussion</td>
<td>25</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>31</td>
</tr>
<tr>
<td>Literature Cited</td>
<td>32</td>
</tr>
<tr>
<td>CHAPTER 3: MILKWEED MATTERS: MONARCH BUTTERFLY (LEPIDOPTERA: NYMPHALIDAE) SURVIVAL AND DEVELOPMENT ON NINE MIDWESTERN MILKWEED SPECIES</td>
<td>40</td>
</tr>
<tr>
<td>Abstract</td>
<td>40</td>
</tr>
<tr>
<td>Introduction</td>
<td>41</td>
</tr>
<tr>
<td>Materials and methods</td>
<td>44</td>
</tr>
<tr>
<td>Results</td>
<td>48</td>
</tr>
<tr>
<td>Discussion</td>
<td>54</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>60</td>
</tr>
<tr>
<td>Literature Cited</td>
<td>61</td>
</tr>
</tbody>
</table>
LIST OF FIGURES

Figure 2.1  Differences in mass (mg) among larvae fed excised leaves of nine native milkweed species. .......................................................... 22

Figure 2.2  Differences in head capsule width (mm) among larvae fed excised leaves.................................................................................. 23

Figure 2.3  Differences in mass among larvae fed whole plants ................. 24

Figure 3.1  Visualization of Kaplan-Meier survival probability....................... 50

Figure 3.2  Percent of monarchs surviving from 0-5 days as larvae (A), 0-10 days as larvae (B), 0-14 days during pupation (C pupation), and from neonate to adulthood (D).......................................................... 51

Figure 3.3  Differences in relative adult lipid content ........................................ 55

Figure 4.1  Pictures of each of the nine milkweed species.............................. 73

Figure 4.2  Average total eggs counted per female ........................................ 82

Figure 4.3  Average total eggs laid per female on each plant (A), the untransformed average number of eggs per centimeter of plant height (B), and the average percentage of eggs laid on each milkweed species (C) from the oviposition choice study. ...............83

Figure 4.4  Average total eggs laid per female on each plant........................ 84

Figure 5.1  Map of milkweed demonstration plot locations ......................... 107

Figure 5.2  Milkweed plot arrangement. ..................................................... 108

Figure 5.3  Milkweed demonstration plot at the Allee Research Farm ............ 109
LIST OF TABLES

Table 2.1 Cardenolide concentration of nine native milkweeds......................... 24
Table 3.1 Mean adult measurements...................................................................... 52
Table 3.2 Mean pupal measurements...................................................................... 53
Table 5.1 Pairwise Comparisons for total number of eggs laid on each milkweed species during June-August 2015 ........................................ 114
Table 5.2 Pairwise Comparisons for total number of eggs laid on each milkweed species during June-August 2016 ........................................ 116
Table 5.3 Pairwise Comparisons for total number of eggs laid on each milkweed species during June-August 2017 ........................................ 118
Table 6.1 Summary of the utility of nine milkweed species .................................. 145
Table A.1 Summary of milkweed species traits. ................................................. 147
ACKNOWLEDGMENTS

This dissertation is the product of many hours work under the guidance, patience, and support of my advisers, Dr. Diane Debinski and Dr. John Pleasants. They allowed me the freedom to explore questions that interested me and gain new knowledge about the preferences of an iconic butterfly. In addition, the expertise and support of my committee members Dr. Sue Blodgett, Dr. Amy Toth, and Dr. Dean Adams has expanded my research questions and my approach to answering them. They have also made the process less intimidating and a lot more fun.

My colleagues in both the Debinski Lab and the Iowa Monarch Working Group deserve much credit for assisting me throughout the years, especially Keith Bidne, Dana Schweitzer, Teresa Blader, David Stein, Royce Bitzer, Seth Appelgate, and Kelsey Fisher. The numerous and enthusiastic undergraduate assistants have provide invaluable assistance and kept me excited about coming back to the lab and field sites every day. Thank you Logan Crees, Nancy Shryock, Nicholas Oppedal, Ali Ford, Jackie Appelhans, Hannah Frye, Will Douglas, and Cory Haggard! This work would not have been possible without generous support from the USDA Agricultural Research Station, Corn Insects and Crop Genetics Research Unit, especially Keith Bidne who always made sure that we had every supply needed, even with short notice, during several grueling field seasons. In addition, my fellow EEB and EEOB colleagues have been reliable support in and outside the lab. Thank you Monica Cox, David Stein, Andrew Kaul, Jermaine Mahguib, Hilary Haley, Alex Walton, Hannah Carol,
Derek Houston, Sean Saterlee, Justin van Goor, Nick Lyon, Kaitlyn Holden, Emily Ernst, Sydney Lizotte-Hall, Kelsey Fisher, Niranjana Krishnan, and Teresa Blader. I am grateful to Dr. Nancy M. Waters, Jane Nagurney, and Marilyn Devine for being strong females mentors in the sciences; their encouragement made my research path possible. Thank you to Dr. Lincoln P. Brower for teaching me how to properly conduct research, inspiring me to always ask new questions, graciously welcoming me into the monarch research community, and for mentoring me throughout my graduate degrees. Your support has been invaluable to me over the past six years.

My academic achievements in the past four years would not have been possible without the support of my entire family, especially my parents Jim and Kris Pocius. They endured many panicked phone calls over the past four years, but always provided unwavering support. I am forever grateful that they have instilled curiosity, hard work, dedication, and fun into my approach to both research and life.
ABSTRACT

The population of monarch butterflies east of the Rocky Mountains has experienced a significant decline over the past twenty years. In order to increase monarch numbers in the breeding range, habitat restoration that includes planting milkweeds is essential. Milkweeds in the genus Asclepias and Cynanchum are the only host plants for larval monarch butterflies in North America, but larval performance, survival, and oviposition preference across milkweed species native to the Midwest, especially those with overlapping ranges, is not well documented. We examined early instar survival, survival and development from first instar to adult, oviposition inclination, and oviposition preference on nine milkweed species native to Iowa. The milkweeds included Asclepias exaltata (poke milkweed), A. hirtella (tall green milkweed), A. incarnata (swamp milkweed), A. speciosa (showy milkweed), A. sullivantii (prairie milkweed), A. syriaca (common milkweed), A. tuberosa (butterfly milkweed), A. verticillata (whorled milkweed), and Cynanchum laeve (honey vine milkweed).

We report: (1.) early instars that fed on C. laeve plants were an instar behind larvae that fed on any other species, while larvae that fed on A. verticillata weighed more than larvae that fed on any other species, but that larvae early in development can survive on all nine milkweeds tested, (2.) fewer larvae that fed on A. hirtella and A. sullivantii reached adulthood compared to larvae that fed on the other milkweed species in greenhouse experiments, but larval duration (days), pupal duration (days), pupal mass, pupal length, and adult wet mass were not significantly different although survival probability varied from 30% to
75% among the nine milkweed species, (3.) *A. incarnata* had the greatest egg counts of all species when female monarchs were presented with either a single milkweed species or multiple milkweed species in laboratory experiments, but females laid more total eggs when exposed to multiple milkweeds than when compared to a single milkweed species, and (4) *A. incarnata* and *A. syriaca* were preferred for oviposition in the field during the breeding seasons of 2015-2017, although eggs were observed on all nine milkweed species. This research highlights the utility of multiple native milkweed species as host plants for monarch butterflies, but that preferences for some species exist among the milkweed species tested. These preferences should inform further research about milkweed use as pollinator conservation efforts continue.
CHAPTER 1: INTRODUCTION

Monarch butterflies east of the Rocky Mountains are best known for their 2,000 mile annual migration each year from as far north as Southern Canada to Central Mexico (Brower 1985, Gustafsson 2015). Over the past twenty years the number of monarchs at the Mexican overwintering grounds has declined by almost 80% (Brower et al. 2012, Espeset et al. 2016, Stenoien et al. 2016). Several factors have contributed to this decline, including parasitism (Altizer et al. 2000, Bradley and Altizer 2005), climate change (Batalden, 2011), the loss of overwintering habitat (Calvert et al. 1992, Brower et al. 1992, Brower et al. 2016), the loss of nectar plants, which could make the migration more arduous (Inamine et al. 2016, Agrawal 2016), and the loss of milkweeds within the breeding range (Oberhauser et al. 2001, Pleasants and Oberhauser 2013, Pleasants 2015, Pleasants 2017, Zaya et al. 2017). Recent modeling efforts have identified habitat loss, especially milkweed loss, within the breeding range as the largest threat to the monarch population (Zalucki and Lammers 2010, Flockhart et al. 2015, Zalucki et al. 2016).

There are 3-4 generations of monarch butterflies per year, depending upon the geographic location (Oberhouser and Solensky 2004). In Iowa and the upper Midwest, there are usually two summer generations before the migratory generation emerges between the end of August and early September. While prior research has focused on the biology of the monarch migration, information about the summer generations is lacking, especially regarding a suite of issues associated with milkweed use. Most monarchs that overwinter in Mexico
originate in the Midwest (Wassenaar and Hobson 1998, Flockhart et al. 2017) and feed on common milkweed, *Asclepias syriaca*, as larvae (Seiber et al. 1986, Malcolm et al. 1989). Traditionally, row crop agriculture in the Midwest was a significant source of common milkweed (*Asclepias syriaca*), one of the most heavily used host plants by monarchs in the North American breeding range (Oberhauser 2001, Pleasants and Oberhauser 2013). Virtually all habitat restoration recommendations are based on *A. syriaca*, whereas the historic Midwestern grassland and wetland habitats contained several milkweed species (Hayden 1919, Woodson 1954, Pleasants 2015). Other milkweed species could potentially provide a broader resource base across a greater portion of the growing season. A diversity of milkweed species would also include plants adapted to a wider range of sites and weather conditions, providing more sustainable habitat restorations. However, more information is needed about monarch larval survival and oviposition on these milkweeds to understand how they contribute to population growth. Restoration of monarch habitat in the Midwest is essential to increase population numbers (Oberhauser et al. 2016). As organizations begin habitat restoration efforts specifically targeted at boosting monarch numbers, all conservation recommendations are currently based on common milkweed, ignoring many other native milkweed species.

Milkweeds are the only host plants for monarch butterfly larvae. Monarchs are able to sequester cardiac glycosides (cardenolides) from milkweed leaves and incorporate them into their own tissues primarily for defense against vertebrate predators (Roeske et al. 1976). Studies have elucidated the
phylogenetic relationships among milkweed species (Agrawal et al. 2009, Rasmann and Agrawal 2011, Agrawal 2016), tradeoffs between plant defenses and insect use (Agrawal 2016), and milkweed species identification and presence across large areas (Woodson 1954). Work by Roeske et al. (1976) showed that monarchs use 28 of over 100 milkweed species in North America; relative differences in monarch adult and larval utilization of milkweeds is not well understood when multiple milkweed species exist in close proximity on the landscape.

There are 18 native milkweed species in Iowa (USDA, 2017). These plants have different habitat requirements (Woodson 1954, Kaul et al. 1991, Eilers and Roosa 1994), concentrations of plant chemicals (Agrawal et al. 2009, Rasmann 2011, Agrawal 2016), phenologies, and distributions across the state (Woodson 1954, USDA 2017). The four most common milkweeds are *A. incarnata* (swamp milkweed), *A. syriaca* (common milkweed), *A. tuberosa* (butterfly milkweed), and *A. verticillata* (whorled milkweed) (Woodson 1954, USDA 2017). We identified these four species as a high priority for study because these are most likely to be used in habitat restoration. We included an additional five milkweed species (*A. exaltata, A. hirtella, A. speciosa, A. sullivantii, and C. laeve*) with narrower distributions across the state (Woodson 1954, USDA 2017). We were most interested in how monarch adults and larvae use these milkweed species in the field and laboratory.

Iowa is an ideal location to study both monarchs and milkweeds. Monarchs are present each summer from late May-mid September. Iowa is
dominated by agriculture; only a tenth of one percent of native tallgrass prairie remains (Jackson, 2002). Before the use of glyphosate herbicide, monarchs thrived on milkweeds within and along the edges of agricultural fields. Those milkweeds no longer exist within fields, and few milkweeds inhabit field margins (Oberhauser and Pleasants 2013). Iowa is a priority for habitat restoration efforts because it is in the core summer breeding rage of the monarch butterfly (Oberhauser et al. 2016). Additional information about how monarchs use each of these nine native milkweed species will be influential in choosing the best milkweed species for restoration efforts.

The main objective of this dissertation was to identify the native milkweed species that are best for monarch larval survival and most preferred by female monarchs for oviposition. As restoration efforts continue, we need more information about monarch use and survival on these species to make the best decisions regarding plant and seed mixes for habitat restoration. Both laboratory and field testing were used to understand monarch survival and preferences.

In chapter two, we investigated larval survival over the first five days of life. Larvae ranged from 1st-3rd instar by the end of this experiment. Larvae were reared on fresh excised leaves and on young milkweed plants of the nine native milkweed species named above. We also extracted lipids from larvae that fed on each milkweed species to examine potential differences in the nutritional value of the milkweed species. Leaf feeding trials offer the maximum potential survival rates, because larvae did not have to contend with the defenses of an intact milkweed plant. These experiments contributed to our understanding of early
instar survival on a variety of milkweed species and how these species can influence larval energy stores.

In chapter three, we examined monarch survival over a longer period of time, from first instar through adult eclosion, on the same nine native milkweed species. In this experiment, larvae fed on entire milkweed plants. The purpose of this study was to gain an estimate of monarch survival on each milkweed species in the absence of predation, and to see if there were any differences in development rates among larvae that fed on different milkweed species. Furthermore, we analyzed the lipid content of the resulting adults that fed on each of the nine species to compare the lipid content patterns of early instar larvae to those in adults.

In chapter three we investigated females’ oviposition preference when each of the nine milkweed species was presented in a no-choice environment. We then performed a choice experiment using the four most common milkweed species (A. incarnata, A. syriaca, A. tuberosa, and A. verticillata). We investigated the effect of different plant traits (plant height and leaf number) on the average total egg count. We also compared the average total egg count per female per plant in the preference studies to those recorded in the no choice study for A. incarnata, A. syriaca, A. tuberosa, and A. verticillata in an effort to better understand how monarchs could distribute their eggs on a variety of milkweed plants and milkweed species in close proximity to each other.

In chapter five we investigated similar questions in a three-year field trial using the same nine milkweed species planted at 15 locations across Iowa. This
experiment determined the phenology and geographic responses of all nine milkweed species and to identify broader geographic patterns of monarchs’ preference for milkweed species as adults. It also provided an opportunity to compare the distribution of eggs across different milkweed species and breeding seasons (2015, 2016, and 2017).

In summary, this dissertation offers a baseline for milkweed species selection within monarch habitat restorations across the Midwest by addressing questions about milkweed survival and monarch use. Is there a difference in survival percentages among larvae that fed on different milkweed species? Do females exhibit an oviposition preference for certain milkweed species? Building on these experiments, scientists and stakeholders will become better at restoring and managing land for monarchs and other pollinators, especially in close proximity to agriculture where such restorations are most needed.

**Literature Cited**


CHAPTER 2: PERFORMANCE OF EARLY INSTAR MONARCH BUTTERFLIES (*Danaus plexippus* L.) ON NINE MILKWEED SPECIES NATIVE TO IOWA

A manuscript published in the Journal of the Lepidopterist’s Society

**Abstract**

Over the past two decades, the population of monarch butterflies east of the Rocky Mountains has experienced a significant decline. Habitat restoration that includes milkweed plants is crucial to boost population numbers in the breeding range. Monarch butterfly larvae use milkweeds as their only host plant, but larval performance on different milkweed species is not well documented. We examined early instar survival and growth on nine milkweed species native to Iowa. These included *Asclepias exaltata* (poke milkweed), *A. hirtella* (tall green milkweed), *A. incarnata* (swamp milkweed), *A. speciosa* (showy milkweed), *A. sullivantii* (prairie milkweed), *A. syriaca* (common milkweed), *A. tuberosa* (butterfly milkweed), *A. verticillata* (whorled milkweed), and *Cynanchum laeve* (honey vine milkweed). In laboratory and greenhouse experiments, larval survival on all nine milkweed species did not differ. Larvae that fed on *C. laeve* plants were an instar behind larvae that fed on any other species, while larvae that fed on *A. verticillata* weighed more than larvae that fed on any other species. Our results show that early instar larvae can survive on all nine milkweed species.

**Introduction**

Over the last two decades, the populations of monarch butterflies (*Danaus plexippus* L., *Nymphalidae*) east and west of the Rocky Mountains have
experienced a significant decline in overwintering numbers (Brower et al. 2012, Espeset et al. 2016, Inamine et al. 2016). Although recent work has suggested that these declines may not be representative of monarch population size during other stages of monarch phenology or ontogeny (Davis 2012, Davis and Dyer 2015), this decline has been attributed to the loss of milkweed in agricultural fields resulting from the rise of genetically modified crops, increased agricultural herbicide spraying (Oberhauser et al. 2001, Pleasants and Oberhauser 2013), and potentially limited nectar resources (Inamine et al. 2016) as well as the loss of overwintering habitat (The Center for Biological Diversity 2014). Recent models have implicated the loss of milkweeds within the breeding range as the largest threat to the monarch population (Zalucki and Lammers 2010, Flockhart et al. 2015, Zalucki et al. 2016). Monarchs require milkweed species as larval host plants, but apparently feed indiscriminately on nectar from a variety of plants as adults (Brower et al. 2006). Restoration of monarch habitat within the breeding range is of utmost concern to boost population numbers (Oberhauser et al. 2016); roughly 29 milkweed plants will be needed to produce one adult monarch that will be part of the migratory generation (Nail et al. 2015). For that reason, there have been extensive efforts across federal, state, and non-profit groups to establish monarch habitat to boost monarch numbers. These restoration projects have focused on adding milkweeds to the landscape. Most monarchs found at the overwintering sites have originated in the Midwest (Wassenaar and Hobson 1998, Flockhart et al. 2017) and fed on common milkweed, *Asclepias syriaca* (*Asclepiadaceae*), as larvae (Seiber et al. 1986,
Malcolm et al. 1989). However, there are a number of milkweed species in the Midwest that were probably used by monarchs before agriculture dominated the landscape and increased the abundance of common milkweed. These other milkweed species could potentially provide important resources, but more information is needed about monarch larval performance on these milkweed species to ensure the most efficient and effective use of resources.

Since the advent of agriculture, milkweeds that grew in-between crop rows in the Midwest (A. syriaca) were among the most heavily used monarch host plants in the North American breeding range (Oberhauser 2001, Pleasants and Oberhauser 2013). Virtually all restoration recommendations to date are based on A. syriaca, whereas the historic Midwestern grassland and wetland habitats contained several (2-4) milkweed species (Hayden 1919, Pleasants 2015). There are surprisingly few studies that address larval survival on milkweed species with overlapping ranges. Of the studies comparing larval feeding on milkweed species in North America that do exist, Erickson (1973) measured larval performance and nutrition on four milkweed species, while Schroeder (1976) evaluated an energy budget for larvae that fed on A. syriaca. Ladner and Altizer (2005) examined growth differences between monarchs collected from eastern and western North America on widely distributed milkweed species; Yeargan and Allard (2005) examined growth differences of larvae that fed on A. syriaca and Cynanchum laeve; Zalucki et al. (2012) studied the survival and growth of first instars on milkweeds in southern California; Robertson et al. (2015) focused on larval preference among four desert milkweeds native to
California; and Agrawal et al. (2015) compared larval performance on a broad range of milkweed species, some of which were native to North America, to determine the impacts of evolutionary history and latex on milkweed defenses and monarch growth. Because most milkweeds native to the Midwest, especially those with narrow ranges, have not been tested, we examined larval survival on nine milkweed species native to Iowa, which is a high priority area for Midwestern conservation efforts (The Center for Biological Diversity 2014). The species we examined are: *A. syriaca*, *A. incarnata*, *A. tuberosa*, *A. verticillata*, *A. speciosa*, *A. exaltata*, *A. sullivantii*, *A. hirtella*, and *C. laeve*. These species have overlapping ranges (Woodson 1954), varying concentrations of both cardenolides (Woodson 1954, Roeske et al. 1976, Malcolm 1991, Agrawal et al. 2009, Rasmann and Agrawal 2011, Table 2.1) and quercetin glycosides (Haribal and Renwick 1996, Agrawal et al. 2009), and different habitat requirements (Woodson 1954, Kaul et al. 1991, Eilers and Roosa 1994, Table 2.2). We examined larval performance on excised leaves and whole plants of the nine species listed above. An investigation of larval performance on excised leaves separates differences in intrinsic leaf qualities, such as cardenolide content, from the latex found in intact plants, while the data from intact plants addresses latex and overall plant architecture as additional factors in larval performance. Understanding larval performance on each of these milkweed species will be useful in choosing milkweed species for monarch habitat restoration efforts across the Midwestern U.S.
Methods and materials

Monarch larvae used in experiments

A monarch butterfly colony was started by collecting 253 monarch eggs and young larvae on *A. syriaca* and *A. incarnata* plants from May 21 to June 9, 2014 from Boone and Story Counties in Iowa. Larvae were reared on *A. syriaca* through the summer growing season in 2014 and *A. curassavica*, a tropical milkweed, from greenhouse-grown plants through the fall and winter of 2014. Adults were allowed to mate and eggs were collected for propagation of the colony on a weekly basis. Twelve generations of colony breeding preceded the beginning of this experiment (Summer 2014- Spring 2015). All of the resulting larvae from colony matings were reared on *A. curassavica* prior to the beginning of this experiment in late spring 2015. Although the colony was exposed to *A. syriaca* in generations prior to this experiment, we do not think that the colony adapted to a particular host plant because monarchs collected from opposite coasts of the U.S. showed no host preference for milkweeds based on geographic location after colony breeding (Ladner and Altizer 2005).

Excised leaf feeding assay

Milkweeds of all nine species were grown from seed without the use of chemical pesticides in a greenhouse at Iowa State University (21.1- 35 °C, 16h photophase, and 56% relative humidity (rh)). During each trial, blocks of petri dishes were set up where each block contained 9 petri dishes, with one replicate of each milkweed species and one larva per petri dish. There were six sets of six blocks throughout this assay. For each group of six blocks, plants of each species were randomly selected, stems were cut, leaves were taken above the
cotyledon leaf, and the leaves were immediately placed in water. Leaf material was kept cool and transported to the laboratory in wet paper towels, surface sterilized in 10% bleach (sodium hypochlorite) solution for 10 min., and then rinsed 3 times for 10 minutes (30 minutes total) each with cool running water in order to remove potential pathogens, such as OE. Petri dishes (60 mm x 15 mm) were prepared with water-based agar (2.0% w/v agar to water) to keep plant material moist.

Plant species were randomly assigned within a block (each trial= 6 blocks; 6 trials were included for n=36 total blocks). Plant material was placed into each block of petri dishes and one first instar was added to each petri dish. Larvae were kept on trays in an incubator set at 28°C and 40% RH with a 16:8 hr. photophase. Larvae were monitored daily for survival and surface-sterilized leaf material was provided *ad libitum*; all leaf material was replaced daily. After five days, larvae were removed from the petri dishes. By conducting this assay over a short five-day period, we were able to avoid large reductions in sample size associated with early instar mortality on some host plants (Hódar et al. 2002). We harvested all larvae after five days throughout the study in order to compare the mass gain and developmental stage for each larva over a fixed amount of time (Agrawal et al. 2015). Larval mass was recorded to the nearest hundredth of a milligram (AND Model GR-202). Head capsule width was measured using a Nikon SMZ 1000 microscope (0.75 x objective, 10x eyepiece with eyepiece grid set with a stage picrometer) and was recorded to the nearest tenth of a
millimeter. Instar was determined from head capsule measurements (Oberhauser and Kuda 1997). All larvae were frozen (-28°C) immediately after weighing.

**Whole plant feeding assay**

Milkweeds of all nine species were grown from seed without the use of chemical pesticides in a greenhouse (21.1-35 °C, 16h photophase, and 56%rh) at Iowa State University. Seeds were sown into 128-cell plug trays (Landmark Plastics, Akron OH) and then at approx. 6 weeks from germination were transplanted into 3.5 inch square deep perennial pots (Kord, Ontario Canada). Plants ranged from 10-30cm in height depending on milkweed species; milkweeds were 8 weeks old when used in each trial. Each plant was watered and placed into a water-filled waxed-paper cup. One neonate was added to each plant. A mesh pop-up hamper cage (57x37x55 cm) was placed over the plant and neonate; a no-see-um netting bag was pulled up over the mesh cage and tied on the top with a wire tie. A block in this case included one whole plant of each of the 9 species growing in the pop-up cage. The total number of blocks was 6 per trial, 36 blocks total.

All blocks were kept on the same bench in the greenhouse (21.1-35 °C, 16h photophase, and 56%rh) positioned in a randomized complete block design (6 groups of 6 blocks as in the excised feeding assay). Greenhouse temperature was recorded hourly via Thermcron sensors (iButton, New South Wales Australia). Larval weight (mg), survivorship, and head capsule width (mm) were recorded after 5 days.
Lipid assay

Lipid content was quantified for larvae used in the excised leaf feeding and larvae used in the whole plant feeding assay. Lipid content was quantified using whole bodies of individual larvae that were 5 days old, a mixture of 2\textsuperscript{nd} and 3\textsuperscript{rd} instars, via colorimetric assays with a sulphophosphovanillin reagent, a method that has been demonstrated to provide consistent results for honey bees (Toth et al. 2005, Toth and Robinson 2005). We homogenized whole caterpillars (n=6 per milkweed species for both the excised leaf feeding assay and the whole plant feeding assay, for a total of 108 larvae analyzed) in 2:1 chloroform: methanol solvent in 12 mL glass vials using glass stirring rods to crush each individual. Samples were then left undisturbed for 17 hours to allow the lipids to be extracted into chloroform methanol. After 17 hours, samples were strained through glass wool to remove particulates and leave only lipids dissolved in chloroform methanol. Extracted lipids were then stored in 1mL of 2:1 chloroform: methanol at -20C. One hundred μL of lipid extract was used in each assay. Each sample was dried completely under a stream of air, then 200 μL of 100% sulfuric acid were added, and all samples were heated for 10 minutes in a bath of boiling water. Two ml of a sulfophosphovanillin reagent were added to each sample (Toth et al. 2005). Samples were then briefly vortexed and placed in the dark for 15 minutes to allow the reaction to proceed. Three technical replicates of 200 ul of the resulting solution from each sample were measured for absorbance in a Gen5 2.06 multiwell spectrophotometer at 525 nm. The average of the three replicates was used to estimate lipid quantity by treatment. Estimated quantities of lipids were calculated from standard curves, run alongside the samples, based
on known concentrations of cholesterol in petroleum ether (Toth and Robinson 2005, Toth et al. 2009).

**Statistical analysis**

Data were analyzed using R version 3.1.2 (R Core Team 2014). Data were combined across trials (36 blocks total) within each experiment, as blocks were not significantly different from one another. Differences in survival were determined using a log rank test on the Kaplan-Meier survival estimates for larvae fed each milkweed species. A one-way ANOVA was used to assess differences in larval mass and head capsule width between groups relative to the milkweed species they were fed in both excised feeding and whole plant experiments. A Tukey HSD test was used to assess pairwise differences in larval responses among milkweed species. A linear regression was used to assess the relationship between larval mass and cardenolide content, reported in Agrawal et al. 2009, in the excised feeding assay. Mass and head capsule width were not transformed prior to analysis. A one-way ANOVA was used to assess differences in total percent of lipids between groups relative to the milkweed species they were fed in both excised feeding and whole plant experiments. A Tukey HSD test was used to assess pairwise differences in larval lipid percentages.

**Results**

**Excised leaf feeding assay.**

Larval survivorship varied from 94-100% across milkweed species, averaging 96% across treatments. Survivorship did not differ among milkweed species ($\chi^2=9.8$, d.f. =8, p <0.05). Larval mass was significantly different among
milkweed species (F=11.65, d.f. =8, p<0.001). Larvae that fed on *C. laeve* weighed significantly less than those that fed on *A. incarnata* (p <0.01), *A. tuberosa* (p <0.01), and *A. verticillata* (p <0.01; Figure 2.1). Larvae that fed on *A. hirtella* weighed significantly less than those that fed on *C. laeve* (p <0.05), *A. incarnata* (p<0.001), *A. speciosa* (p <0.01), *A. sullivantii* (p<0.001), *A. syriaca* (p<0.001), *A. tuberosa* (p<0.001), and *A. verticillata* (p<0.001; Figure 2.1). Larvae that fed on *A. exaltata* weighed significantly less than those that fed on *A. incarnata* (p<0.001), *A. tuberosa* (p<0.001), and *A. verticillata* (p<0.001; Figure 2.1).

Larval head capsule width was significantly different among milkweed species (F= 2.56, d.f. =8, p <0.01) when all instars were pooled; head capsule width was positively correlated with larval weight. This relationship was significant (r=0.71; p<0.001). Larvae that fed on *A. incarnata* developed to 4th instars and had the largest head capsule width. Larvae that fed on *A. hirtella* developed to 3rd instars and had a head capsule width that was significantly smaller than those fed on *A. incarnata* (p<0.05) or *A. verticillata* (p <0.05; Figure 2.2). All other comparisons were not significantly different.
Figure 2.1  Differences in mass (mg) among larvae fed excised leaves of nine native milkweed species. This graph represents 6 trials (36 blocks, 315 larvae total). Each bar represents one milkweed species; error bars depict 95% confidence intervals. EXA= A. exaltata (n=34 larvae), HIR= A. hirtella (n=34 larvae), INC= A. incarnata (n=35 larvae), LAE= C. laeve (n=36 larvae), SPE= A. speciosa (n=34 larvae), SUL= A. sullivantii (n=35 larvae), SYR= A. syriaca (n=35 larvae), TUB= A. tuberosa (n=35 larvae), and VER= A. verticillata (n=36). Bars that share a letter are not significantly different from each other at p<0.05.
Figure 2.2  Differences in head capsule width (mm) among larvae fed excised leaves of nine native milkweed species. This graph represents 6 trials (36 blocks, 315 larvae total). Each bar represents one milkweed species; error bars depict 95% confidence intervals. EXA= A. exaltata (n=34 larvae), HIR= A. hirtella (n=34 larvae), INC= A. incarnata (n=35 larvae), LAE= C. laeve (n=36 larvae), SPE= A. speciosa (n=34 larvae), SUL= A. sullivantii (n=35 larvae), SYR= A. syriaca (n=35 larvae), TUB=A. tuberosa (n=36 larvae), and VER= A. verticillata (n=36 larvae). Bars that share a letter are not significantly different from each other at p<0.05.

**Whole plant feeding assay**

Larval survivorship varied from 81-100% across milkweed species, averaging 90% across treatments. Survivorship did not differ among milkweed species ($\chi^2=11.4$, d.f. =8, $p > 0.05$). Larval mass was significantly different among species ($F=6.956$, d.f. =8, $p<0.001$; Figure 2.3). Larvae fed *A. verticillata* weighed more than larvae fed any other species (Figure 2.3) and were significantly different from *C. laeve* ($p<0.001$), *A. incarnata* ($p<0.01$), *A. speciosa* ($p<0.05$), *A. sullivantii* ($p<0.01$), or *A. tuberosa* ($p<0.001$). Larvae that fed on *C. laeve* weighed the least. This difference was significant in comparison to *A.*
hirtella (p<0.001), A. exaltata (p<0.05), A. speciosa (p<0.05), A. sullivantii (p<0.05), A. syriaca (p<0.05), and A. verticillata (p<0.001). No other species showed differences in pairwise comparisons.

Larval head capsule width was significantly different among milkweed species (F=17.25, d.f. =8, p<0.001); head capsule width was positively correlated with larval weight. This relationship was significant (r=0.54; p<0.001). All larvae reached the third instar during the study, with the exception of those fed C. laeve. Larvae that fed on C. laeve did not reach the third instar. Larvae fed C. laeve

![Figure 2.3](image-url)
had a significantly smaller head capsule width in comparison with each of the other 8 milkweed species (p<0.001 for all species). No other species showed differences in pairwise comparisons.

**Lipid assay**

During excised leaf feeding trials, lipid concentration (lipids as a percentage of total larval mass) was not significantly different among caterpillars that fed on nine different milkweed species (F=0.475, d.f. =8, p>0.05). However, the percent lipid was different among larvae that fed on different species of milkweed plants in the whole-plant assay (F=3.707, d.f. =8, p<0.01). Larvae that fed on *A. incarnata* had a higher percentage of lipids than larvae that fed on *A. exaltata* (p<0.01), *A. hirtella* (p<0.05), *A. sullivantii* (p<0.05), *A. syriaca* (p<0.05), *A. tuberosa* (p<0.05), or *A. verticillata* (p<0.001). All other comparisons were not significantly different.

**Discussion**

Our findings suggest that young monarch larvae can survive on all nine milkweed species. Eight of the nine species could be used for monarch habitat restoration in the Midwest, provided that each species is planted within its native range and in its appropriate habitat (Table 2.2). *C. laeve* is not the best choice for such plantings because larvae did not grow as quickly when they fed on this species.

Larvae that fed on excised leaves reached the fourth instar in five days, while larvae that fed on whole plants only reached the third instar in five days in the greenhouse. On average, larval mass after 5 days for larvae that fed on whole plants was 33.4% that of larvae fed on excised leaves. Differences in
instar and larval mass are likely due in part to differing temperatures between excised leaf and whole plant experiments. Larvae fed leaf material in petri dishes in the laboratory experienced a stable temperature of 28°C in the controlled environmental chamber while those that fed on whole plants experienced fluctuating temperatures from 23°C to 28°C in the greenhouse. Given that larval growth rates are dependent on temperature (Zalucki and Kitching 1982), the lower temperature in the greenhouse probably resulted in less rapid growth during the whole-plant feeding assay. Larvae that fed on excised leaves also were not exposed to plant latex flow and pressure, which can slow larval growth by up to 50%; larvae in petri dishes also moved less due to a confined space and did not need to negotiate the architecture of the plants (Zalucki and Malcolm 1999, Zalucki et al. 2001a). Larval mortality was minimal throughout the study (96.6% survival excised leaf feeding; 90.4% survival plant feeding), well below ~50% reported elsewhere regardless of whether larvae fed on excised leaves or whole plants (Oberhauser and Solensky 2004).

Unlike Ladner and Altizer (2005), we found no difference in larval mass or instar size between larvae fed A. incarnata and A. syriaca (Figures 2.1 and 2.3). However, it is possible that differences in larval growth among milkweed plants may be more pronounced during the final instars. We did see evidence, as they did, that A. speciosa may produce lighter larvae, but only when larvae fed on excised leaves (Figure 2.1). This could suggest that young larvae have difficulty processing milkweed leaves with higher cardenolide content, as A. speciosa tends to have higher foliar cardenolides compared to some of the other milkweed
species (Table 2.1; Woodson 1954, Roeske et al. 1976, Agrawal et al. 2009, Rasmann and Agrawal 2011) or that these leaves are structurally difficult to eat. We also saw evidence that *A. hirtella* leaves produced lighter larvae than other species (Figure 2.1), but this could be the result of wilting of the excised leaves during larval feeding or larval difficulty processing leaf material with a high cardenolide content (Table 2.1; Agrawal et al. 2009, Rasmann and Agrawal 2011). Larvae that fed on *A. hirtella* plants were not significantly lighter than larvae that fed on other species (Figure 2.3).

Unlike Yeargan and Allard (2005), larvae reared on *C. laeve* plants were significantly smaller and did not grow as quickly as larvae fed other species; larvae fed *C. laeve* did not reach the third instar during the whole plant assay in our study. Our results suggest that larvae can survive on *C. laeve*, but those larvae may not mature as quickly as larvae feeding on other milkweeds. Larvae that fed on *A. verticillata*, a milkweed species that tends to have low cardenolide levels (Figures 2.1 and 2.3, Table 2.1), produced the heaviest larvae. Although we did not measure cardenolide content in our milkweed plants, *A. speciosa* and *A. hirtella* have higher average foliar cardenolides when compared to other milkweed species (Table 2.1, Woodson 1954, Roeske et al. 1976, Agrawal et al. 2009, Rasmann and Agrawal 2011). Cardenolide content is only one factor that could contribute to the variation in larval mass that we observed. Other factors such as differing latex content and flow, differing amounts of larval movement on various milkweed species, and differing plant architecture among milkweed
species also likely contributed to the observed differences in larval mass (Zalucki et al. 2001a,b).

Like Cookman et al. (1984), we observed differences in lipid concentration among larvae reared on different host plants. However, in our study larvae that fed on excised leaves did not show a difference in lipid concentration. Our results suggest that *A. incarnata* may be a more lipid-rich food source for monarch larvae, and that other milkweed species may not be as good a food source for lipid content. Alternatively, monarchs may be able to process toxins from *A. incarnata* more effectively, leading to higher lipid storage (Roeske et al. 1976).

In summary, all nine milkweed species can be used as host plants by the monarch butterfly. Larvae that fed on excised leaves at a controlled temperature weighed more and matured faster than those raised on whole plants in a greenhouse with more variable temperature. Larvae that fed on *A. incarnata* and *A. verticillata* weighed the most, while those that fed on *C. laeve* weighed the least. This is an important finding because milkweeds are needed to boost monarch numbers during the breeding season in the Midwestern U.S (Pleasants and Oberhauser 2013, Flockhart et al. 2015).

Although larvae that fed on *A. incarnata* and *A. verticillata* weighed the most, monarch habitat should include milkweed species with habitat needs that best match the potential restoration site (Table 2.2). *A. syriaca*, *A. incarnata*, and *A. verticillata* are found across the entirety of Iowa, but *A. syriaca* and *A.
Table 2.1 Cardenolide concentration of nine native milkweeds. Chemical concentrations from Woodson (1954), Roeske et al. (1976), Agrawal et al. (2009), and Rasmann and Agrawal (2011).

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>A. exaltata</td>
<td>0-0.70</td>
<td>0-0.70</td>
<td>.125</td>
<td>0.735</td>
</tr>
<tr>
<td>A. hirtella</td>
<td>n/a</td>
<td>n/a</td>
<td>.208</td>
<td>3.289</td>
</tr>
<tr>
<td>A. incarnata</td>
<td>0-0.28</td>
<td>0-0.28</td>
<td>.117</td>
<td>0.511</td>
</tr>
<tr>
<td>A. speciosa</td>
<td>0.149</td>
<td>0.15</td>
<td>.227</td>
<td>1.112</td>
</tr>
<tr>
<td>A. sullivantii</td>
<td>n/a</td>
<td>n/a</td>
<td>.123</td>
<td>2.149</td>
</tr>
<tr>
<td>A. syriaca</td>
<td>0.06-2.64</td>
<td>0.06-2.64</td>
<td>.113</td>
<td>1.573</td>
</tr>
<tr>
<td>A. tuberosa</td>
<td>0-0.06</td>
<td>n/a</td>
<td>.064</td>
<td>0.070</td>
</tr>
<tr>
<td>A. verticillata</td>
<td>0</td>
<td>n/a</td>
<td>.114</td>
<td>0.031</td>
</tr>
<tr>
<td>C. laeve</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
</tr>
</tbody>
</table>
Table 2.2. Summary of milkweed habitat preferences. Information compiled from Woodson (1954), Kaul et al. (1991), Eilers and Roosa (1994), and USDA-NRCS (2017).

<table>
<thead>
<tr>
<th>Milweed Species</th>
<th>Common Name</th>
<th>Habitat Preference</th>
<th>Blooming Period</th>
<th>Soil Moisture</th>
<th>Soil Type</th>
<th>Iowa Distribution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asclepias exaltata</td>
<td>Poke Milkweed</td>
<td>woodland edges, upland woods prairie remnants, fields wet meadows, floodplains,</td>
<td>May-August</td>
<td>Moist</td>
<td>n/a</td>
<td>Northeastern Iowa</td>
</tr>
<tr>
<td></td>
<td></td>
<td>riverbanks, pond shores, stream banks, wet woods, swamps, marshes, prairies, fields, and roadsides along canal banks and riparian sites and in sub-irrigated or occasionally flooded wetlands with sedges and rushes, roadside</td>
<td>May-September</td>
<td>Mesic to Dry</td>
<td>sandy and clayey soils</td>
<td>South Central Iowa</td>
</tr>
<tr>
<td>Asclepias incarnata</td>
<td>Swamp Milkweed; Rose Milkweed</td>
<td>May-August moist to Mesic clayey soils, neutral to slightly acidic pH</td>
<td>Entire State</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Asclepias speciosa</td>
<td>Showy Milkweed</td>
<td>ditches mesic prairie, alluvial meadows, floodplains, and level roadside</td>
<td>May-September</td>
<td>Moist to Dry</td>
<td>sandy, well-drained soils, neutral to slightly acidic pH</td>
<td>Western Iowa</td>
</tr>
<tr>
<td>Asclepias silvinitii</td>
<td>Prairie Milkweed; Sullivan's Milkweed</td>
<td>June-August moist to Mesic low, moist soils</td>
<td>Central and Western Iowa</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Asclepias syriaca</td>
<td>Common Milkweed</td>
<td>waste places prairies, open woodlands, roadsides, and disturbed areas</td>
<td>June-August</td>
<td>Moist to Dry</td>
<td>sandy, clayey, or rocky calcareous soils</td>
<td>Entire State</td>
</tr>
<tr>
<td>Asclepias tuberosa</td>
<td>Butterfly Milkweed</td>
<td>areas prairies, open woodlands, roadsides, and disturbed areas</td>
<td>April-September</td>
<td>Mesic to Dry</td>
<td>sandy, loamy, or rocky limestone soils</td>
<td>Entire State</td>
</tr>
<tr>
<td>Asclepias verticillata</td>
<td>Whorled Milkweed</td>
<td>areas alluvial woods, cities, waste areas, disturbed areas</td>
<td>April-August</td>
<td>Mesic to Dry</td>
<td>sandy soils</td>
<td>Entire State</td>
</tr>
<tr>
<td>Cynanchum laeve</td>
<td>Milkweed</td>
<td>alluvial woods, cities, waste areas, disturbed areas</td>
<td>July-October</td>
<td>Moist to Mesic</td>
<td>sandy soils</td>
<td>Southwestern Iowa</td>
</tr>
</tbody>
</table>

In order to provide a complete assessment of the value of different milkweed species, we need to examine adult female egg load and potential fecundity for individuals that have fed on different milkweed species from first instar through adult eclosion. These feeding trails should use mature milkweed plants. We also need to understand the oviposition response and preference of female monarchs for different milkweed species to gauge their potential value in habitat restoration.

**Acknowledgements**

This research was funded by the Iowa Monarch Conservation Consortium and Prairie Biotics Inc. The authors would like to thank Nicholas Oppedal, Brandon Ritland, Nicole Ozigbo, Ali Ford, Hannah Frye, and Will Douglas for
their help with experimental set up and collecting measurements. We thank the staff of the ISU Forestry Greenhouse for their help with plant care, and Dr. Lincoln Brower for insights on experimental design and monarch biology. We also thank John Pleasants, Amy Toth, Steve Malcolm and an anonymous reviewer for their comments, which greatly improved this manuscript.

**Literature Cited**


Flockhart, D.T., Brower, L.P., Ramirez, M.I., Hobson, K.A., Wassenaar, L.I.,
Altizer, S. and Norris, D.R., 2017. Regional climate on the breeding
grounds predicts variation in the natal origin of monarch butterflies
overwintering in Mexico over 38 years. Global Change Biology
Unravelling the annual cycle in a migratory animal: breeding season
habitat loss drives population declines of monarch butterflies. Journal of
Haribal, M., and J. A. Renwick. 1996. Oviposition stimulants for the monarch
butterfly: Flavonol glycosides from Asclepias curassavica. Phytochemistry
41:139-144.
Hayden, A. 1919. Notes on the floristic features of a prairie province in central
survival of pine processional caterpillar Thaumetopoea pityocampa in
relation to food quality in three Pinus species. Ecological Entomology 27:
292–301.
Inamine, H., S.P. Ellner, J.P. Springer, and A.A. Agrawal. 2016. Linking the
continental migratory cycle of the monarch butterfly to understand its
population decline. Oikos 125:1081-1091.


CHAPTER 3: MILKWEED MATTERS: MONARCH BUTTERFLY (LEPIDOPTERA: NYMPHALIDAE) SURVIVAL AND DEVELOPMENT ON NINE MIDWESTERN MILKWEED SPECIES

A manuscript published in the Journal of Environmental Entomology

Abstract

The population of monarch butterflies east of the Rocky Mountains has experienced a significant decline over the past twenty years. In order to increase monarch numbers in the breeding range, habitat restoration that includes planting milkweed plants is essential. Milkweeds in the genus *Asclepias* and *Cynanchum* are the only host plants for larval monarch butterflies in North America, but larval performance and survival across nine milkweeds native to the Midwest is not well documented. We examined development and survival of monarchs from first instar larval stages to adulthood on nine milkweed species native to Iowa. The milkweeds included *Asclepias exaltata* (poke milkweed), *A. hirtella* (tall green milkweed), *A. incarnata* (swamp milkweed), *A. speciosa* (showy milkweed), *A. sullivantii* (prairie milkweed), *A. syriaca* (common milkweed), *A. tuberosa* (butterfly milkweed), *A. verticillata* (whorled milkweed), and *Cynanchum laeve* (honey vine milkweed). In greenhouse experiments, fewer larvae that fed on *A. hirtella* and *A. sullivantii* reached adulthood compared to larvae that fed on the other milkweed species. Monarch pupal width and adult dry mass differed among milkweeds, but larval duration (days), pupal duration (days), pupal mass, pupal length, and adult wet mass were not significantly different. Both the absolute and relative adult lipids were different among milkweed treatments; these differences are not fully explained by differences in
adult dry mass. Monarch butterflies can survive on all nine milkweed species, but the expected survival probability varied from 30% to 75% among the nine milkweed species.

**Introduction**

The populations of monarch butterflies east and west of the Rocky Mountains have experienced a significant decline in overwintering numbers over the past twenty years (Brower et al. 2012, Espeset et al. 2016, Stenoien et al. 2016). Although this decline may not be representative of the monarch population size during other times of the year (Davis 2012, Davis and Dyer 2015), this decline has been attributed to multiple factors including the loss of milkweed (Oberhauser et al. 2001, Pleasants and Oberhauser 2013, Pleasants 2017, Zaya et al. 2017) and nectar sources (Inamine et al. 2016) within the breeding range. Recent modeling work has implicated the loss of habitat, including milkweeds, within the breeding range as the largest threat to the monarch population (Zalucki and Lammers 2010, Flockhart et al. 2015, Zalucki et al. 2016). A large proportion of the monarchs that overwintered in Mexico originated from the Midwest (Wassenaar and Hobson 1998, Flockhart et al. 2017) and fed on common milkweed, *Asclepias syriaca* (*Asclepiadaceae*), as larvae (Seiber et al. 1986, Malcolm et al. 1989). Restoration of monarch habitat in this region is essential to increase population numbers (Oberhauser et al. 2016) and federal, state, and non-profit groups have undertaken efforts to establish monarch habitat. These projects have focused on adding milkweed plants, the only host plants of monarch larvae, to the landscape.
Traditionally, row crop agriculture in the Midwest was a significant source of common milkweed (*Asclepias syriaca*), among the most heavily used host plants by monarchs in the North American breeding range (Oberhauser 2001, Pleasants and Oberhauser 2013). Virtually all habitat restoration recommendations are based on *A. syriaca*, whereas the historic Midwestern grassland and wetland habitats contained several milkweed species (Hayden 1919, Woodson 1954, Pleasants 2015). These other milkweed species could potentially provide a broader base of resources adapted to a wider range of sites and weather for a more sustainable approach to habitat restorations. More information is needed about monarch larval survival and performance on these milkweeds to understand how they contribute to population growth.

Several prior studies have addressed various aspects of monarch survival from larvae to adults, but few include comparative work on multiple milkweed species. Comparative studies on North American monarchs include Schroeder’s (1976) energy budget for larvae that fed on *A. syriaca*, larval performance and nutrition on four milkweed species (Erickson 1973) and growth differences between monarchs collected from eastern and western North America on widely distributed milkweed species (Ladner and Altizer 2005). Other studies have examined growth differences of larvae that fed on *A. syriaca* and *Cynanchum laeve* (Yeargan and Allard 2005) and on milkweeds native to Southern California (Zalucki et al. 2012) throughout development. Additional work has focused on the survival of early instar larvae on a range of North American species native to Florida (Zalucki and Brower 1992), the Midwest (Pocius et al. 2017), and across
the Eastern United States (Zalucki and Malcolm 1999). Furthermore, Robertson et al. (2015) investigated larval preferences among four milkweeds native to the California desert, while Agrawal et al. (2015) compared larval performance on a wide variety of milkweed species to determine the impacts of evolutionary history and latex on milkweed defenses and monarch growth.

Because most milkweeds native to the Midwest, especially those with narrow ranges, have not been tested, we examined larval survival on nine milkweed species native to Iowa, which is a high priority area for Midwestern conservation efforts (The Center for Biological Diversity 2014). The species we examined were: *Asclepias syriaca* (common milkweed), *Asclepias incarnata* (swamp milkweed), *Asclepias tuberosa* (butterfly milkweed), *Asclepias verticillata* (whorled milkweed), *Asclepias speciosa* (showy milkweed), *Asclepias exaltata* (poke milkweed), *Asclepias sullivantii* (prairie milkweed), *Asclepias hirtella* (tall green milkweed), and *Cynanchum laeve* (honeyvine milkweed). These species have overlapping ranges (Woodson 1954), varying concentrations of cardenolides (Woodson 1954, Roeske et al. 1976, Malcolm 1991, Agrawal et al. 2009, Rasmann and Agrawal 2011) quercetin glycosides, (Haribal and Renwick 1996, Agrawal et al. 2009), and adaptation to different habitats (Woodson 1954, Kaul et al. 1991, Eilers and Roosa 1994). We examined larval performance and survival on young plants of the nine species listed above to determine any differences in the resulting adults including mass, forewing length, and hindwing length, or development time (days) in the larval and pupal stages relative to the milkweed species on which the larvae fed. Our prior work suggested that there
were differences in both mass and lipid content in young larvae, 2\textsuperscript{nd}-3\textsuperscript{rd} instars, that fed on both leaves and young plants of different milkweed species (Pocius et al. 2017), although there were no differences in survival. We suspected that these differences could change as the monarch larvae develop to adulthood because there were no significant differences in pupal weight and development time among larvae that fed on \textit{A. syriaca} and \textit{C. laeve} (Yeargan and Allard 2005), although larval growth rates differed based on the host plant species (Ladner and Altizer 2005, Yeargan and Allard 2005). Understanding how milkweed species influence monarch development and survival will be critical in choosing milkweed species for monarch habitat restoration, and given the large number of acres that are being planted, this knowledge could also have significant economic implications.

**Materials and methods**

**Monarch Larvae Used in Experiments**

A monarch butterfly colony was started by collecting 253 monarch eggs and young larvae from May 21 to June 9, 2014 from Boone and Story Counties in Iowa. Larvae were reared on \textit{A. syriaca} through the summer growing season and \textit{A. curassavica}, a tropical milkweed, from greenhouse-grown plants through the fall and winter. Upon eclosion, adults were tested for \textit{Ophryocystis elektroscirrha} (OE). Adults that tested negative for OE were allowed to mate and eggs were collected for propagation of the colony on a weekly basis. Twelve generations of colony breeding preceded the beginning of this experiment; inbreeding should not affect monarch preferences as colony breeding for multiple
generations did not influence monarch growth or performance on different milkweeds (Ladner and Altizer 2005).

**Milkweed Feeding Assay**

Milkweeds of all nine species were grown from seed without the use of chemical pesticides in a greenhouse (21.1- 35 °C, 16h photophase, and 56%RH) at Iowa State University. Growing conditions represent a middle ground among the nine species tested. Seeds were sown into 128-cell plug trays (Landmark Plastics, Akron OH) and then at approximately 6 weeks following germination were transplanted into 8.9 cm square deep perennial pots (Kord, Ontario Canada). Plants ranged from 10-30cm in height depending on milkweed species. Milkweeds were 8 weeks old when used in each trial; all plants were healthy with undamaged leaves at the start of each trial. Each plant was watered and placed into a water-filled waxed- paper cup. One neonate was added to each plant. A mesh pop-up hamper cage (57x 37x 55 cm) was placed over the plant and neonate; a no-see-um netting bag was pulled up over the mesh cage and tied on the top with a wire tie. The experiment was arranged in a randomized complete block design with the block including one plant of each of the nine milkweed species growing in each pop up cage. Each trial (6 blocks) was replicated six times for a total of 36 blocks.

All blocks were kept on the same bench in the greenhouse (21.1- 35 °C, 16h photophase, and 56% RH) positioned in a randomized complete block design (six trials of six blocks). Greenhouse temperature was recorded hourly via Thermochron sensors (Embedded Data Systems, iButton, New South Wales Australia). Larvae were monitored for survivorship on days five, 10, and 14,
when the larvae ranged from 2nd-5th instar. Beginning at day 10, we monitored each cage for pupae in order to record the most accurate pupation date; we did not monitor young larvae daily in order to reduce stress on the larvae and young milkweed plants. Milkweed plants were watered daily, and additional milkweed plants were added on days six and 10 to provide adequate food for each larva. No larvae ran out of food over the course of this experiment. Larvae were monitored daily for pupation starting at day 12.

Following pupation, chrysalids were allowed to sclerotize in the greenhouse for 24 hours after which they were removed from each cage and transported to the laboratory. Hardened pupae were weighed to the nearest hundredth of a milligram on an AND GR-202 balance (A&D Company, Limited, Toshima-ku, Tokyo, Japan); pupal length and width were measured to the nearest hundredth of a millimeter with digital calipers (Neiko Tools, USA). Individual pupae were attached to wooden applicators with small beads of hot glue (AdTech Detailer Glue Gun), and hung inside individual plastic cups (227 ml, WNAT8T) for eclosion.

Upon eclosion, adult emergence date and sex were recorded. Live adults were weighed to the nearest hundredth of a milligram after allowing their wings to harden for 24 hours. Adult forewing length and hindwing length were measured to the nearest hundredth of a millimeter using digital calipers (Neiko Tools, USA); adults were then frozen for subsequent lipid extraction.

**Adult Lipid Assay**

Lipid content was quantified for half of the resulting adults at Sweet Briar College in July 2016. Lipids were extracted following the procedure outlined in
Brower (2006), which includes drying the butterflies, weighing them, extracting the lipid in petroleum ether, evaporating the petroleum ether, and then weighing the extracted lipid (Alonso-Mejia et al. 1997, Brower 2006, Brower et al. 2015). Because there were no significant differences in lipid content between the sexes, lipids from males and females were pooled for analysis (Alonso-Mejia et al. 1997, Brower 2006, Brower et al. 2015). Data are presented both as average milligrams of lipid and lipid as a percentage of butterfly mass for butterflies that fed on each milkweed species.

**Statistical Analysis**

Data were analyzed using R version 3.1.2 (R Core Team 2014). Within each experiment, data were combined across trials (36 blocks total), as blocks were not significantly different from one another. Differences in survival were determined using a log rank test on the Kaplan-Meier survival estimates for larvae that fed on each milkweed species. Pairwise log rank tests were used to compare species (Jokela et al. 2016) as this analysis allowed us to include individuals that spent different amounts of time as larvae and pupae; a Bonferroni correction was used to adjust the significance level for pairwise comparisons (adjusted $\alpha=0.0014$, Thieltges 2005). A one-way ANOVA was used to assess differences in pupal and adult responses (mass, pupal length, pupal width, forewing length, and hindwing length) among milkweed species. A Tukey HSD test was used to assess pairwise differences in larval development time among milkweed species. A one-way ANOVA was used to assess differences in total percent of lipids between groups relative to the milkweed species they were fed. A Tukey HSD test was used to assess pairwise differences in lipid percentages.
Sexes were pooled for all analyses, as there were no significant differences when males and females were analyzed separately.

**Results**

**Milkweed Feeding Assay**

Survivorship from first instar to adult varied from 30-70% across milkweed species, averaging 58% across all milkweeds species. Survivorship differed among milkweed species ($\chi^2=32.8$, d.f. =8, p<0.001, Figure 3.1, 3.2). Fewer monarchs that fed on *A. hirtella* survived than those that fed on *A. tuberosa* (p<0.001), or *A. exaltata* (p<0.001). Fewer monarchs that fed on *A. sullivantii* survived than those that fed on *A. exaltata* (p<0.001). No other pairwise differences in survival were significant. When survival was analyzed in five-day increments, there were no differences in the proportion of larvae that survived on each milkweed species (Figure 3.2), although there was lower survival on *C. laeve* during the first five days (Figure 3.2), on *A. sullivantii* for the first ten days (Figure 3.2), and both *A. hirtella* and *A. sullivantii* during the first 14 days (Figure 3.2). Between pupation and eclosion, there was high mortality in both *A. hirtella* and *A. sullivantii* (Figure 3.2). There were no differences in larval or pupal duration, defined by number of days as a larva (all instars combined), or as a pupa, among feeding treatments. Monarchs spent 14-15 days as larvae and 9-11 days as pupae across treatments. There were no differences in adult wet mass or hindwing lengths, but forewing length (F=4.12, d.f. =8, p<0.001, Table 3.1) and adult dry mass were significantly different among the resulting adults (F=4.17, d.f.=8, p<0.001, Table 3.1). When adults were dried before lipid analysis, adults that fed on *A. hirtella* weighed less than adults that fed on *A.
Adults that fed on *A. exaltata* (*p*<0.01), *A. incarnata* (*p*<0.01), *A. speciosa* (*p*<0.01), *A. syriaca* (*p*<0.001), *A. tuberosa* (*p*<0.001), *A. verticillata* (*p*<0.01), and *C. laeve* (*p*<0.05) as larvae had longer forewings than those that fed on *A. hirtella* (Table 3.2). No other species showed difference in pairwise comparisons in forewing length.

Pupal mass was significantly different across milkweed treatments (*F*=4.04, d.f. =8, *p*<0.001, Table 3.2). Pupae that consumed *A. hirtella* as larvae weighed less than those that fed on *A. exaltata* (*p*<0.001), *A. incarnata* (*p*<0.001), *A. speciosa* (*p*<0.01), *A. syriaca* (*p*<0.001), *A. tuberosa* (*p*<0.001), *A. verticillata* (*p*<0.01), and *C. laeve* (*p*<0.01). Pupal length was not different among milkweed treatments, but pupal width (*F*=3.08, d.f. =8, *p*<0.01, Table 3.2) was different among milkweed treatments. Pupae that consumed *A. exaltata* (*p*<0.05), *C. laeve* (*p*<0.05), *A. speciosa* (*p*<0.01), *A. tuberosa* (*p*<0.05), and *A. verticillata* (*p*<0.05), as larvae were wider than those that fed on *A. hirtella*. 
Figure 3.1 Visualization of Kaplan-Meier survival probability over time (days) of monarch butterflies from larvae to adults that fed on nine different milkweed species (EXA= A. exaltata, HIR=A. hirtella, INC=A. incarnata, LAE=C. laeve, SPE=A. speciosa, SUL= A. sullivantii, SYR=A. syriaca, TUB=A. tuberosa). At the beginning of the experiment, N=36 larvae for each milkweed species. Each line represents one milkweed species. Fewer monarchs that fed on A. hirtella survived than those that fed on A. tuberosa or A. exaltata; fewer monarchs that consumed A. sullivantii survived than those that consumed A. exaltata. Lines that do not share a letter are significantly different from each other at p<0.001.
Figure 3.1 Percent of monarchs surviving from 0-5 days as larvae (A), 0-10 days as larvae (B), 0-14 days during pupation (C pupation), and from neonate to adulthood (D). There are no significant differences among milkweed species when survivorship is examined at 5, 10, or 14 days. Survival is different among milkweed treatments from neonate to adulthood (D). More monarchs survived on *A. exaltata* and *A. tuberosa* than on *A. hirtella* ($p < 0.05$); more monarchs survived on *A. tuberosa* than on *A. sullivantii* ($p < 0.05$).
Table 3.1 Mean adult measurements (+/- 95% confidence intervals) from 6 trials (N= 168 butterflies total). Each measurement represents mean ± standard error. Adult mass and hindwing length were not different across milkweed species. Adult dry mass was significantly different across milkweed species at a significance level of p<0.001. **Forewing length was significantly different across treatments at a significance level of p<0.01. ***Milligrams of lipid were significantly different across treatments at a significance level of p<0.05. Log-transformed lipids were used for analysis; untransformed values are reported below. Cells within columns that do not share a letter are significantly different from each other.

<table>
<thead>
<tr>
<th>Milkweed Species</th>
<th>Milkweed Common Name</th>
<th>No. Of Adults Measured</th>
<th>Mean Adult Wet Mass</th>
<th>Mean Adult Dry Mass*</th>
<th>Mean Forewing Length</th>
<th>Mean Hindwing Length</th>
<th>Mean Lipid Content (mg)***</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. exaltata</td>
<td>Poke milkweed</td>
<td>22; 13 for</td>
<td>718.8 ±</td>
<td>177.3±21.1^</td>
<td>49.7±0.75^</td>
<td>33.9±0.61</td>
<td>13.0±1.69^</td>
</tr>
<tr>
<td>A. hirtella (HIR)</td>
<td>Tall green</td>
<td>6; 3 for</td>
<td>307.2</td>
<td>87.0±30.4^B</td>
<td>43.8±1.4^B</td>
<td>30.4±1.0</td>
<td>2.2±0.30^B</td>
</tr>
<tr>
<td>A. incarnata</td>
<td>Swamp</td>
<td>25; 12 for</td>
<td>543.8</td>
<td>193.6±9.2^A</td>
<td>50.6±0.44^A</td>
<td>33.7±0.45</td>
<td>15.9±5.8^AB</td>
</tr>
<tr>
<td>C. laeve (LAE)</td>
<td>Honeyvine</td>
<td>18; 11 for</td>
<td>502.7</td>
<td>152.4±19.8^AB</td>
<td>49.8±0.52^A</td>
<td>33.6±0.45</td>
<td>6.3±0.75^AB</td>
</tr>
<tr>
<td>A. speciosa</td>
<td>Showy</td>
<td>18; 10 for</td>
<td>529.3</td>
<td>174.4±8.6^A</td>
<td>50.7±0.69^A</td>
<td>37.5±3.6</td>
<td>7.2±1.6^AB</td>
</tr>
<tr>
<td>A. sullivantii</td>
<td>Prairie</td>
<td>9; 4 for</td>
<td>456.1</td>
<td>167.4±104.6^A</td>
<td>46.1±2.9^AB</td>
<td>31.7±1.8</td>
<td>8.3±1.9^AB</td>
</tr>
<tr>
<td>A. syriaca</td>
<td>Common</td>
<td>22; 13 for</td>
<td>552.4</td>
<td>174.3±22.8^A</td>
<td>50.8±0.41^A</td>
<td>33.9±0.29</td>
<td>12.5±1.4^</td>
</tr>
<tr>
<td>A. tuberosa</td>
<td>Butterfly</td>
<td>25; 12 for</td>
<td>529.2</td>
<td>161.9±20.2^A</td>
<td>50.9±0.60^A</td>
<td>34.8±0.41</td>
<td>16.7±8.2^</td>
</tr>
<tr>
<td>A. verticillata</td>
<td>Whorled</td>
<td>23; 11 for</td>
<td>513.6</td>
<td>171.0±14.1^</td>
<td>49.9±0.89^A</td>
<td>34.4±0.78</td>
<td>6.9±2.5^AB</td>
</tr>
</tbody>
</table>
Table 3.2  Mean pupal measurements (+/- 95% confidence intervals) from 6 trials (N= 188 pupae total). Milkweed abbreviations are the same as in Table 1. *Pupal mass was significantly different across milkweed treatments at a significance level of p<0.001. **Pupal width was different among milkweed treatments at a significance level of p<0.01. Cells within columns that do not share a letter are significantly different from each other.

<table>
<thead>
<tr>
<th>Milkweed Species</th>
<th>Number of Pupae Measured (N)</th>
<th>Pupal Mass (mg)*</th>
<th>Pupal Length (mm)</th>
<th>Pupal Width (mm)**</th>
</tr>
</thead>
<tbody>
<tr>
<td>EXA</td>
<td>28</td>
<td>1386.3±41.6(^{A})</td>
<td>23.7±0.29</td>
<td>10.9±0.17(^{A})</td>
</tr>
<tr>
<td>HIR</td>
<td>6</td>
<td>903.2±88.6(^{B})</td>
<td>21.5±1.0</td>
<td>9.5±0.31(^{B})</td>
</tr>
<tr>
<td>INC</td>
<td>27</td>
<td>1417.1±48.5(^{A})</td>
<td>24.2±0.27</td>
<td>10.6±0.13(^{AB})</td>
</tr>
<tr>
<td>LAE</td>
<td>18</td>
<td>1330.2±41.1(^{A})</td>
<td>23.7±0.36</td>
<td>10.7±0.12(^{A})</td>
</tr>
<tr>
<td>SPE</td>
<td>21</td>
<td>1395.7±36.0(^{A})</td>
<td>24.2±0.30</td>
<td>11.0±0.13(^{A})</td>
</tr>
<tr>
<td>SUL</td>
<td>13</td>
<td>1167.1±133.3</td>
<td>22.3±1.2</td>
<td>10.3±0.39(^{AB})</td>
</tr>
<tr>
<td>SYR</td>
<td>25</td>
<td>1379.2±37.5(^{A})</td>
<td>24.5±0.37</td>
<td>10.6±0.13(^{AB})</td>
</tr>
<tr>
<td>TUB</td>
<td>26</td>
<td>1365.1±43.5(^{A})</td>
<td>24.1±0.42</td>
<td>10.9±0.17(^{A})</td>
</tr>
<tr>
<td>VER</td>
<td>24</td>
<td>1313.1±40.4(^{A})</td>
<td>23.5±0.29</td>
<td>10.8±0.16(^{A})</td>
</tr>
</tbody>
</table>
Lipid assay

The total amount of lipid (mg) was significantly different among adults that fed on the nine different milkweed species (F=3.36, d.f. =8, p<0.01, Table 3.1). Adults that fed on A. exaltata (p<0.01), A. incarnata (p<0.01), and A. syriaca (p<0.01) had higher lipid content than those that fed on A. hirtella as larvae. Adults contained between 1.9-25.5 mg of lipid across species (For species averages, see Table 3.1). Lipid concentration (lipids as a percentage of total adult mass) was also significantly different among milkweed treatments (F=5.35, d.f. =8, p<0.0001, Table 3.1). Adults that fed on A. exaltata as larvae had higher lipid concentrations than those that fed on A. hirtella (p<0.05), A. speciosa (p<0.05), or A. verticillata (p<0.001). Adults that fed on A. syriaca as larvae had higher lipid concentrations than those that fed on A. hirtella (p<0.05), A. speciosa (p<0.05), A. tuberosa (p<0.001), and A. verticillata (p<0.05). All other comparisons were not significantly different.

Discussion

Monarchs can survive on and will consume all nine milkweed species tested, but survivorship throughout development is higher on some species compared to others (Figures 3.1 and 3.2). Seven of the nine species could be used for monarch habitat restoration in the Midwest provided that each species is planted within its native range and in its appropriate habitat. Our findings suggest that A. hirtella and A. sullivantii are not the best choice for these
Figure 3.2  Differences in relative adult lipid content (% total mass) among nine native milkweed species. This graph represents the lipid content from half of the resulting adults from 8 trials (N=89 total). Error bars depict 95% confidence intervals. EXA= A. exaltata (n= 13 butterflies), HIR= A. hirtella (n= 3 butterflies), INC= A. incarnata (n=12 butterflies), LAE= C. laeve (n= 11 butterflies), SPE= A. speciosa (n= 10 butterflies), SUL= A. sullivantii (n= 4 butterflies), SYR= A. syriaca (n=13 butterflies), TUB=A. tuberosa (n=12 butterflies), and VER= A. verticillata (n=11 butterflies). Bars that do not share a letter are significantly different from each other at p<0.05.

plantings because monarchs had a lower probability of reaching adulthood when fed young plants of these milkweed species. Only 30% of larvae that fed on A. hirtella and 36% that fed on A. sullivantii reached adulthood compared to 75% that fed on A. tuberosa and 72% that fed on A. exaltata.

On average, larval survival was above 50% for the entirety of the study when larvae fed on young plants, higher than larval survival recorded in the field (Nail et al. 2015, Oberhauser and Solensky 2004). Handling the larvae during plant replacements or increased larval stress due to feeding on fresh milkweeds with intact plant defenses such as latex may have contributed to mortality rates. Unlike Ladner and Altizer (2005), we found no difference in larval survival among
A. incarnata, A. speciosa, and A. syriaca (Figure 3.1), but they recorded larval survival to fifth instar on milkweed leaf cuttings, not plants. A. exaltata and A. tuberosa had the highest survivorship in our study, but these species were not tested by Ladner and Altizer (2005). We did not see highest larval mortality during early instars as Ladner and Altizer (2005) did, but rather during pupation and eclosion (Figure 3.2). We did see increased early instar mortality on C. laeve as in Pocius et al. (2017), but this difference was not significant (Figure 3.2). Unlike our previous work, there were no developmental lags in larvae that fed on C. laeve plants. Larvae that fed on C. laeve progressed through both larval and pupal stages in the same amount of time as larvae that fed on other species.

Differing water content in live butterflies most likely masked the differences in dry tissue weight when each adult was measured initially. Our prior work suggested that A. hirtella produced lighter larvae after day 5 than other milkweed plants (Pocius et al. 2017); this difference in mass was evident in the pupal stage (Table 3.2), but not when wet mass was compared in live adults. When adults were dried, those that fed on A. hirtella had a lower dry mass than adults that fed on other milkweed species (Table 3.1).

Given that larval development is driven by temperature, the similarities in development time across species were not surprising (Zalucki and Kitching 1982) although development can vary with food quality (Lavoie and Oberhauser 2004). Monarchs spent 14-15 days as larvae and 9-11 days as pupae across treatments. Unlike Yeargan and Allard (2005), we did not see any growth
differences between larvae, pupae, and adults that fed on *C. laeve* vs. *A. syriaca*. We did see differences in pupal mass, as did Yeargan and Allard (2005), but only *A. hirtella* pupae were significantly lighter than pupae that fed on other milkweeds as larvae (Figure 3.3). Fewer early instars reared on *C. laeve* plants survived during the first five days of this study, but those that did survive were the same size as other pupae and adults; this indicates that any early differences in mass, as in Pocius et al. (2017), can be overcome during later developmental stages (Table 3.1). In prior work, young larvae that fed on *A. verticillata*, a milkweed species that tends to have low cardenolide levels produced the heaviest larvae (Pocius et al. 2017); however this difference in mass did not carry into subsequent developmental stages (Table 3.1).

Cardenolide content is only one factor that could contribute to the variation in survival that we observed. Although we did not measure cardenolide content in our milkweed plants, *A. hirtella* has higher average foliar cardenolides when compared to other milkweed species in prior studies (Woodson 1954, Roeske et al. 1976, Agrawal et al. 2009, Rasmann and Agrawal 2011). This difference in cardenolide content may influence monarch survival (Malcolm 1994, Malcolm and Zalucki 1996) and persists whether cardenolides are induced or remain at constitutive levels (Rasmann and Agrawal 2011). Plants grown inside the greenhouse in smaller pots may not respond to larval feeding by inducing higher cardenolide concentrations (Baldwin 1987, 1988), but differences in constitutive cardenolide levels may have influenced larval performance in our experiment. *A. hirtella* had higher average published cardenolide content compared to other
species tested and those larvae struggled to pupate, but larvae that fed *A. speciosa*, a milkweed with published cardenolide content higher than most of the species tested (Roeske et al. 1976, Agrawal et al. 2009, Rasmann and Agrawal 2011), pupated without difficulty (Figure 3.2C). Other factors such as differing latex flow, differing amounts of larval movement on various milkweed species, and differing plant architecture among milkweed species also likely contributed to the observed differences in monarch survival (Malcolm and Zalucki 1996, Zalucki and Brower 1992, Zalucki and Malcolm, 1999, Zalucki et al. 2001a,b). We observed differences in adult forewing length among milkweed species, but these measures are within the range observed in wild monarchs (Altizer and Davis 2010). We do not know if there is an advantage of larger forewings for a breeding monarch, but autumn migrants usually have longer forewings (Altizer and Davis 2010).

We observed differences in pupal mass and length (Table 3.2). Some of these differences in mass did carry over to the adult stage, but only when the adults were dried (Tables 3.1 and 3.2). Although these data are noteworthy, we do not know how the measured parameters may influence monarch success.

The lipid content of freshly eclosed monarchs was similar to previous studies in which monarchs were collected in the field and reared in the laboratory (Beall 1948, Cohen 1985, James 1984, Brower et al. 2006, Brower et al. 2015). Lipids ranged from 2-50 mg across treatments; importantly, differences in dry adult mass do not entirely explain the differences in lipid content (Table 3.1). Like Cookman et al. (1984), we observed differences in lipid concentration
among larvae reared on different host plants. Our results suggest that *A. exaltata*, *A. incarnata*, and *A. syriaca* may be more lipid-rich food sources for monarch larvae, and that other milkweeds, such as *A. hirtella*, may not be as good a food source for lipid content (Table 3.1, Figure 3.3). Alternatively, monarchs may be able to process toxins from *A. exaltata*, *A. incarnata*, and *A. syriaca* more effectively, leading to higher lipid storage (Roeske et al. 1976).

Lipid content is only one potential indicator of host plant quality for monarch larvae; larvae that fed on *A. tuberosa* eclosed with lower lipid stores than larvae that fed on other milkweeds (Figure 3.3), but more larvae survived on *A. tuberosa* than any other milkweed in this experiment (Figures 3.1, 3.2). Although lipid stores are an important energy source for monarchs (Brower 2006), we do not know how these differences may affect breeding adults.

Although survivorship was highest on *A. exaltata* and *A. tuberosa*, monarch habitat should include milkweed species with habitat needs that best match the potential restoration site. Growing conditions used in this study represent middle ground for the nine species tested; some species may have grown better in more specialized conditions such as *A. incarnata* in a moist environment. All nine milkweeds favor different habitats. For example, *A. syriaca*, *A. incarnata*, *A. tuberosa*, and *A. verticillata* are found across the entirety of Iowa, but *A. syriaca* and *A. verticillata* are found in drier locations than *A. incarnata* (Woodson 1954, Eilers and Roosa 1994, USDA-NRCS 2017). While *A. exaltata* had the second highest survival, this species tends to favor woodland
edges and is rare across the state (Woodson 1954, Eilers and Roosa 1994, USDA-NRCS 2017).

Future research should investigate adult female egg load and potential fecundity for individuals that have fed on different milkweed species in order to further assess the value of different milkweeds on the landscape. These trials should use mature, hardened milkweed plants so that monarchs encounter both buds and blooms. We acknowledge that our experiment was conducted under artificial conditions; feeding choices made by monarchs in the wild may differ from the results presented here. More information is needed about how monarchs respond to milkweeds grown in conditions mirroring native habitat and both the oviposition response and preference of female monarchs for different milkweed species to gauge their potential value in habitat restoration.

Acknowledgements

This work was partially funded by the USDA National Institute of Food and Agriculture, Hatch project number 1009926 (IOW05478) and by the USDA, Natural Resources Conservation Service’s Conservation Innovation Grant program under Agreement Number 69-3A75-16-006. Additional support was provided by Prairie Biotics Inc. and the Iowa Monarch Conservation Consortium. Mention of a proprietary product does not constitute an endorsement or a recommendation by Iowa State University or USDA for its use. The authors would like to thank Nicholas Oppedal, Jaclyn Appelhans, Cory Haggard, and Nancy Shryock for their help with experimental set up and collecting measurements. We thank the staff of the ISU Forestry Greenhouse for their help.
with plant care, as well as Dean Adams, Sue Blodgett, and two anonymous
reviewers for their feedback, which improved this manuscript.

**Literature Cited**

trends in the defense of milkweeds against monarchs: latex, cardenolides,
and tolerance of herbivory, pp 47-59. In Oberhauser, K.S., Altizer, S., and
Nail K. (ed.), Monarchs in a Changing World: Biology and Conservation of

phenolic metabolism of milkweeds (Asclepias): evidence for escalation.
Evolution 63:663-673.

1997. Use of lipid reserves by monarch butterflies overwintering in Mexico:

Altizer, S. and Davis, A. K. 2010. Populations of monarch butterflies with different
migratory behaviors show divergence in wing morphology. Evolution 64:

Baldwin, I.T. 1987. Damage-induced alkaloids in tobacco: pot-bound plants are

Baldwin, I.T.1988. Short-term damage-induced increases in tobacco alkaloids

Beall, G. 1948. The fat content of a butterfly,* Danaus plexippus Linn.*, as affected


The Center for Biological Diversity, The Center for Food Safety, The Xerces Society, and L. P. Brower. 2014. Petition to protect the monarch butterfly (Danaus plexippus) under the endangered species act.


Flockhart, D.T., J. B. Pichancourt, D. R. Norris, and T. G. Martin. 2015. Unravelling the annual cycle in a migratory animal: breeding season


CHAPTER 4: MONARCH BUTTERFLIES DO NOT PLACE ALL OF THEIR EGGS IN ONE BASKET: OVIPOSITION ON NINE MIDWESTERN MILKWEED SPECIES

A manuscript published in Ecosphere.

Abstract

Over the past two decades, the population of monarch butterflies east of the Rocky Mountains has experienced a significant decline in overwintering numbers. Habitat restoration that includes planting milkweeds is essential to boost monarch numbers within the breeding range. Milkweeds are the only host plants for larval monarch butterflies, but female oviposition preference for different milkweed species, especially those with overlapping ranges, is not well documented. We examined the relative inclination to lay eggs on nine milkweed species native to Iowa (no choice), and oviposition preference (choice) among the four most commonly occurring Iowa species (A. incarnata, A. syriaca, A. tuberosa, and A. verticillata). In both experiments, eggs were counted daily for four days. The milkweeds tested were Asclepias exaltata (poke milkweed), A. hirtella (tall green milkweed), A. incarnata (swamp milkweed), A. speciosa (showy milkweed), A. sullivantii (prairie milkweed), A. syriaca (common milkweed), A. tuberosa (butterfly milkweed), A. verticillata (whorled milkweed), and Cynanchum laeve (honeyvine milkweed).

When females were given only a single species on which to lay eggs there were significant differences among milkweed species in the average number of eggs laid; A. incarnata had the highest average egg count. When females were given a choice among A. incarnata, A. syriaca, A. tuberosa, and A. verticillata,
there were also differences among milkweed species in the number of eggs laid; again, *A. incarnata* had the highest average number of eggs laid. Additionally, females laid more total eggs when four plants of different milkweed species were available than when there were four plants of a single milkweed species. Our results show that monarch butterflies will lay eggs on all nine milkweeds, but that there are clear preferences for some milkweed species over others.

**Introduction**

The monarch butterfly (*Danaus plexippus* L.) population east of the Rocky Mountains has experienced a significant decline in overwintering numbers over the past two decades (Brower et al. 2012, Stenoien et al. 2016). This decline has been attributed to multiple factors including the loss of milkweed, the only host plants of monarch larvae, (Oberhauser et al. 2001, Pleasants and Oberhauser 2013, Pleasants 2017, Pleasants et al. 2017, Zaya et al. 2017). Recent models have implicated the loss of habitat, including milkweeds, within the breeding range as the largest threat to the monarch population (Zalucki and Lammers 2010, Flockhart et al. 2015, Zalucki et al. 2016). Because the majority of monarchs that overwinter in Mexico originate from the Midwest (Seiber et al. 1996, Wassenaar and Hobson 1998, Flockhart et al. 2017), restoration of monarch habitat in this region, especially on marginal agricultural lands (Thogmartin et al. 2017), is essential to increase population numbers (Oberhauser et al. 2016). Federal, state, and non-profit groups have undertaken efforts to reestablish monarch habitat. These projects have focused on adding milkweed plants to the landscape.
In the past, milkweeds that grew in crop fields in the Midwest (A. syriaca) were among the most heavily used monarch host plants in the North American breeding range (Malcolm, et al. 1993, Oberhauser 2001, Pleasants and Oberhauser 2013). Increased use of glyphosate herbicide in corn and soybean fields in conjunction with glyphosate-tolerant crops has all but eliminated A. syriaca from crop fields (Pleasants and Oberhauser 2013). Although historic Midwestern grassland and wetland habitats contained multiple milkweed species (Hayden 1919, Pleasants 2015), virtually all restoration recommendations to date are based on A. syriaca (Landis 2014, Pleasants and Oberhauser 2013, Pleasants 2017). Monarchs could potentially use multiple milkweed species for oviposition, but more information is needed about monarch oviposition preference and behavior on these milkweeds to ensure that these plants could contribute to population growth.

We examined monarch oviposition on nine milkweed species native to Iowa because it is a high priority area for Midwestern conservation efforts (The Center for Biological Diversity 2014) and because most milkweeds native to the Midwest, especially those with narrow ranges, have not been included in prior oviposition studies. The species we examined were: Asclepias exaltata (poke milkweed), A. hirtella (tall green milkweed), A. incarnata (swamp milkweed), A. speciosa (showy milkweed), A. sullivantii (prairie milkweed), A. syriaca (common milkweed), A. tuberosa (butterfly milkweed), A. verticillata (whorled milkweed), and Cynanchum laeve (honeyvine milkweed). These species look very different (Figure 4.1), have overlapping ranges (Woodson 1954), and different habitat
Figure 4.1  Pictures of each of the nine milkweed species used in the no-choice experiment representing differences in plant architecture. Milkweed species are pictured in the field during the summer of 2017 at eight weeks old. Names in gray were also used in the oviposition preference experiment.

Few prior studies have focused explicitly on monarch oviposition preference across multiple native milkweed species. Those studies that did contribute to this knowledge examined milkweed chemical composition, both cardenolides (Zalucki et al. 1990) and quercetin glycosides (Haribal and Renwick 1996, 1998a,b), in relation to monarch oviposition and post-alightment behavior. These studies laid the foundation for later preference experiments considering plant chemicals as a factor in oviposition behavior. Other work focused on monarch use and preference across regional milkweed species in North America (Cohen and Brower 1982, Calvert 1999, Bartholomew and Yeargan 2002, Casagrande and Dacy 2007) and established that monarchs use some milkweed species over others within localized areas, specifically in Texas (Calvert 1999), Florida (Cohen and Brower 1982, Zalucki et al. 1990) and Kentucky (Bartholomew and Yeargan 2002). Prior work also identified that swallowworts (Vincetoxicum nigrum and Vincetoxicum rossicum), milkweed relatives, did not act as a monarch population sink because few eggs were laid on these species.
(Ditomasso and Lacy 2003) and that monarchs with different natal origins (California and Michigan) did not display oviposition preferences for the milkweed species from their natal region (Ladner and Altizer 2005). To better identify how monarchs choose to distribute their eggs when multiple milkweed species are present on the landscape, we used both choice and no-choice experiments with young plants of different milkweed species.

We examined the inclination to lay eggs on young plants of the nine milkweed species listed above in a no-choice experiment to determine the egg-laying baseline for each. This was done for several reasons: previous work did not provide a baseline egg laying rate on different milkweed species, no work has been done on the egg laying rate (eggs laid by a female per day per plant) on any of these nine milkweed species, monarchs are adaptable and use multiple host milkweed species throughout their annual cycle (Agrawal 2016), and many previous studies did not compare the same milkweed species. Following the no-choice experiment, we conducted an oviposition preference test using four broadly distributed native milkweeds: *A. incarnata*, *A. syriaca*, *A. tuberosa*, and *A. verticillata* because these species are most common across Iowa (Woodson 1954, Eilers and Roosa 1994).
Materials and methods

Female Monarchs Used in Experiments

Females used in the experiments were obtained from a monarch butterfly colony that was started by collecting 312 monarch eggs and young larvae on A. *syriaca* plants from June 6 to July 28, 2015 from Boone, Hamilton, and Story Counties in Iowa. Larvae were reared on *A. syriaca* through the summer growing season and *A. curassavica*, a tropical milkweed, from greenhouse-grown plants through the fall and winter. Each generation of adults was tested for *Ophryocystis elektroscirrha* (O.E.) before entering the colony; individuals that tested positive for this parasite (under 5) were frozen. Adults were allowed to mate and eggs were collected for propagation of the colony on a weekly basis. Twelve generations of colony breeding preceded the beginning of this experiment. Individuals from generations 13-15 were used in these experiments. Inbreeding should not affect monarch preferences, as colony breeding of multiple generations of monarchs did not influence monarch oviposition in prior experiments; there is no evidence that inbreeding influences oviposition preference, even when colonies are formed through continuous matings of monarchs collected from different locations (Ladner and Altizer 2005).

Females were allowed to eclose and dry; all females were tested for O.E. before they were placed into a breeding cage; no females that tested positive for O.E. were used in these experiments. Females were allowed to mate and feed, but had never encountered a milkweed plant prior to the beginning of the experiment. Females used in both experiments were between 7 and 11 days old.
Milkweed Plants Used in Experiments

Milkweeds of all nine species were grown from seed (Prairie Moon Nursery, Winona MN, USA) without the use of chemical pesticides in a greenhouse (21.1-35 °C, 16h photophase, and 56%rh) at Iowa State University. Seeds were sown in 128-cell plug trays (Landmark Plastics, Akron OH) and then at approximately 6 weeks from germination were transplanted into 3.5 inch square deep perennial pots (Kord, Ontario Canada). Plants ranged from 10-30cm in height depending on milkweed species; milkweeds were 8-12 weeks old when used in each trial; all plants used within one trial were the same age. Groups of 48 plants were transported to the laboratory (19.5-34.5°C, 16h photophase, and 50%rh) 24 hours before the beginning of each oviposition trial. Plant height and leaf number were recorded for each milkweed plant; leaf dimensions for the two largest leaves were recorded on each plant used in the no-choice experiment. Each set of four plants was used for one four-day trial to keep plants in good condition; only one female used each group of four plants in both oviposition experiments.

Relative Inclination to Lay Eggs

Plants were placed into the four corners of 4'x4'x4.5' breathable plastic cages (PlantHouse 4, Flowerhouse, Clio, MI); plants were watered daily. Each trial consisted of 12 cages with four plants of the same milkweed species in each cage (ex. 4 plants of A. syriaca in each cage). There was one full trial for each of the nine milkweed species resulting in 8-12 replicates per milkweed species (there was some loss of monarchs during the trials); light, temperature and relative humidity fell within the same ranges for all trials. A dish lined with a
circular sponge and filled with artificial nectar (Gatorade, Pryor, Oklahoma, USA) was placed in the center of each cage; sponges were included to aid butterfly feeding. One mated female monarch never exposed to a milkweed plant was introduced to each cage, and allowed to lay eggs for 4 days. At the end of each day, the total number of eggs on each plant was counted. Contrary to Drury and Dwyer (2005), we did not observe females avoiding plants on which eggs were already present, although no eggs were present at the beginning of this experiment. All eggs were removed daily from the plants to prevent larval feeding/injury to the milkweeds as plant damage can result in chemical defense induction in some milkweed species (Agrawal 2017), which could influence monarch oviposition preference. Only females that survived all four days of each trial were included in the analysis.

**Oviposition Preference**

Plants and female monarchs were reared and treated as described above except that in this case each of the four plants in a cage was a different species. Only four of the most common Iowa milkweed species were tested in this experiment: *A. incarnata, A. syriaca, A. tuberosa, and A. verticillata* (Woodson 1954, Eilers and Roosa 1994, USDA-NRCS 2017). Plants were placed into each cage in a randomized order to reduce issues of plant adjacency. Trials lasted four days; all monarch eggs were removed from the milkweed plants daily. This experiment contained 14 replicates with 12 cages included in each replicate for a total of 168 females and 672 milkweed plants, 168 of each milkweed species. Only females that survived all four days of each trial and laid at least 50 eggs were included in the analysis.
Statistical Analysis

Data were analyzed using R version 3.1.2 (R Core Team 2014). Data were combined across replicates within each experiment, as replicates were not significantly different from one another. Daily egg counts from each female were combined across each four-day trial, as there was no significant difference in the number of eggs a female laid on day one vs. days two, three, or four when all milkweed species were combined or analyzed individually. Differences in egg counts were determined using a Poisson regression (Kaitala 1996, Mery and Kawecki 2002) with individual butterfly as a random effect and milkweed species as a fixed effect. Pairwise differences were determined by comparing least square means for each milkweed species; p values were adjusted using Tukey’s range test for multiple comparisons. Leaf widths were averaged for each plant, and eggs were totaled for each plant over the course of the four-day trial. Egg totals were square root transformed for normality, and a Pearson correlation was used to determine the correlation between total number of eggs per plant and the average leaf width per plant. The square root of the number of eggs per centimeter of plant height was analyzed using a mixed effect ANOVA for the oviposition preference study (Ladner and Altizer 2005). Pairwise differences in eggs per cm of plant height were tested using a t-test with a Bonferroni correction. Proportions of egg counts from both studies were arcsine square root transformed and analyzed using one-way ANOVA (Ladner and Altizer, 2005). Pairwise differences in transformed proportions were assessed using a Tukey’s test. The total number of eggs laid per female per plant was compared across experiments for milkweed species included in both experiments (A. incarnata, A.
syriaca, A. tuberosa, and A. verticillata) using a Poisson regression as described above.

**Results**

**Oviposition Inclination**

Female monarchs laid eggs on all nine milkweed species, but laid more eggs on plants of some milkweed species than others (Figure 4.2). When milkweed species was included as a fixed effect and individual butterfly was included as a random effect, milkweed species had a significant effect on the number of eggs laid per female. Females laid 26.8 times more eggs on *A. incarnata* than on *A. tuberosa* (z=4.27, p<0.01; Figure 4.2) and 22.6 times more eggs on *A. incarnata* than on *A. verticillata* (z=4.4, p<0.01; Figure 4.2). Females laid more eggs on *A. sullivantii* than on *A. verticillata* (z=3.35, p<0.05; Figure 4.2) and *A. tuberosa* (z=3.19, p<0.05; Figure 4.2). There were no significant differences in the number of eggs laid based on female age in days (z= -1.44-1.53; p=0.998-1.00). There was no significant relationship between the total number of eggs laid per plant and the average leaf width or length (r=0.056, d.f.=517, p>0.2). Plant height and leaf number were not significant predictors for the number of eggs laid per species.

**Oviposition Preference**

When given four different milkweed species at the same time, female monarchs laid eggs on all four but the number of eggs laid on each milkweed species was significantly different when individual variation in fecundity was included as a random effect and milkweed species was a fixed effect in a Poisson regression (Figure 4.3). Females laid 1.7 times more eggs on *A.
*incarnata* than on *A. syriaca* ($z=25.49$, $p<0.01$), 14.9 times more eggs than on *A. tuberosa* ($z=61.90$, $p<0.01$), and 4.5 times more eggs than on *A. verticillata* ($z=54.24$, $p<0.01$, Figure 4.3a). Females laid nine times more eggs on *A. syriaca* than on *A. tuberosa* ($z=54.14$, $p<0.01$) and 2.7 times more eggs than on *A. verticillata* ($z=40.63$, $p<0.01$, Figure 4.3a). Females laid 0.31 times fewer eggs on *A. tuberosa* than on *A. verticillata* ($z=-27.52$, $p<0.01$, Figure 4.3a). When the data were examined as eggs per centimeter of plant height, *A. tuberosa* and *A. verticillata* were different from *A. incarnata* and *A. syriaca* ($F=34.7$, d.f.=3, 9874, $p<0.01$); *A. incarnata* had the highest number of eggs per cm of plant height (Figure 4.3b; $p<0.01$). Females laid 53.7%, 31.3%, 11.5%, and 3.5% of their eggs on *A. incarnata*, *A. syriaca*, *A. tuberosa*, and *A. verticillata*. When proportions of eggs laid on each milkweed species were compared, all egg proportions were significantly different from one another (Figure 4.3c, $F=68.92$, d.f.=3, 336, $p<0.001$). Egg numbers did not increase with the number of leaves per plant on any milkweed species.

When the number of eggs laid per female on each plant in the inclination to lay trials and preference trials were compared, females laid 2.5 times more eggs when there were multiple species of milkweeds present compared to only one. Egg counts were significantly higher in the preference trials compared to the no-choice trials when the four milkweed species included in both experiments (*A. incarnata*, *A. syriaca*, *A. tuberosa*, and *A. verticillata*) were combined (Figure 4.4, $z=4.34$, $p<0.001$).
Figure 4.1  Average total eggs counted per female over the course of four days when relative inclination to lay eggs was examined. Each bar represents one milkweed species: EXA= A. exaltata (N=8 females), HIR= A. hirtella (N=11 females), INC= A. incarnata (N=11 females), LAE= C. laeve (N=10 females), SPE= A. speciosa (N=12 females), SUL= A. sullivantii (N=12 females), SYR= A. syriaca (N=12 females), TUB= A. tuberosa (N=11 females), and VER= A. verticillata (N=12 females); error bars represent 95% confidence intervals. Females laid more eggs on A. incarnata than on A. tuberosa (p<0.01) and A. verticillata (p<0.01); females laid more eggs on A. sullivantii than on A. tuberosa (p<0.05) or A. verticillata (p<0.05) in a pairwise comparison of least mean squares. P-values were adjusted using the Tukey method for multiple comparisons.
Figure 4.2  Average total eggs laid per female on each plant (A), the untransformed average number of eggs per centimeter of plant height (B), and the average percentage of eggs laid on each milkweed species (C) from the oviposition choice study. Each graph represents eggs counted from 85 females and 340 milkweed plants. Each bar represents one milkweed species; error bars represent 95% confidence intervals. In A and C, all four milkweed species are significantly different from each other (pairwise t-test (a) Tukey HSD (c), p<0.01). In B, A. syriaca and A. incarnata are significantly different from A. tuberosa and A. verticillata (p<0.01).
Figure 4.3  Average total eggs laid per female on each plant when all eggs from *A. incarnata*, *A. syriaca*, *A. verticillata*, and *A. tuberosa* were combined. This graph represents eggs counted from 130 females and 520 milkweed plants. Each bar represents the average total egg count from one experiment (C=oviposition preference or choice trials, NC= inclination to lay eggs or no-choice trials); error bars represent 95% confidence intervals. The total number of eggs per female per plant was significantly different among the experiments; more eggs were present on average in the oviposition preference tests (p<0.01). Each female had four milkweed plants on which to lay eggs (C= one plant each of four different species, NC= four plants of the same milkweed species).

Discussion

Our findings suggest that monarch butterflies will lay eggs on all milkweed species tested in no-choice experiments although they are more inclined to lay on some species than others (Figure 4.2). In choice experiments females still lay eggs on all four species available but prefer some milkweed species over others with preference generally mirroring the pattern exhibited in the inclination to lay experiment (Figure 4.3). Interestingly, monarchs females laid more total eggs during the choice experiment when a diversity of milkweeds were present in each cage than would be expected based on the no-choice experiments (Figure 4.4). In no-choice tests, we saw the highest egg counts on *A. incarnata* followed by *A.*
*sullivantii* and *A. syriaca* (Figure 4.2). In preference tests, over half of all eggs laid were on *A. incarnata* (Figure 4.3). Females laid fewer eggs on *A. tuberosa* and *A. verticillata* in both preference and no-choice tests (Figures 4.2 and 4.3), even though larval survival was high on both of these species in prior experiments (Pocius et al. 2017a,b).

It is important to note that monarchs use multiple different milkweed hosts each year throughout their annual cycle (Agrawal 2017). Although these milkweed species appear on the landscape in different proportions, monarchs do not specialize on one milkweed species even when both have co-evolved within a smaller region (ex. Eastern vs. Western North America). Monarchs from both the eastern and western populations exhibited the same oviposition preferences when given access to milkweed species from both eastern and western North America (Ladner and Altizer 2005). Our results support the adaptability of monarchs even when milkweed species were closer in proximity than usually seen in the field. Females used all four milkweed species in each preference trial and females laid more eggs overall when a mix of milkweeds were present in each cage than when a single species was present (Figure 4.4). Because of monarchs’ broad use of host species, other explanations for oviposition preference must be explored.

One possible explanation is the difference in secondary plant compounds across milkweed species. Across the monarch breeding range, monarchs encounter a variety of milkweed hosts with different plant architecture and chemical concentrations (Zalucki 1986, Malcolm et al. 1989, Agrawal 2016).
Cardenolide and quercetin glycoside concentrations are plant characteristics thought to influence both monarch oviposition and larval performance in prior studies (Zalucki et al. 1990, Malcolm 1991, Haribal and Renwick 1998a, Ladner and Altizer 2005, Agrawal et al. 2015). Adult females have been shown to reject high cardenolide hosts even though monarch larvae sequester cardenolides for their own defense as they feed on milkweed plants (Oyeyele & Zalucki 1990, Zalucki et al., 1990; Haribal and Renwick, 1998a). Females may reject these high cardenolide hosts in response to chemical cues. High cardenolide levels have been linked with low larval survival and slower development rates (Erickson, 1973, Zalucki et al., 2001, Zalucki et al. 2012). As such, there may be chemical cues that affect oviposition choice. Alternatively, high quercetin glycoside level located on the leaf surface (Agrawal 2017) stimulate oviposition; monarchs respond to these chemicals as part of host plant recognition and females have laid eggs in response to the presence of these chemicals without a plant (Haribal and Renwick 1996).

In our study, the least preferred milkweed species A. tuberosa (no-choice, Figure 4.2), and A. verticillata (choice, Figure 4.3a) both have low cardenolide levels recorded in the literature (Roeske et al. 1976, Agrawal et al. 2009, Rasmann and Agrawal 2011, Agrawal et al. 2015), but A. verticillata has a higher level of quercetin glycosides than A. tuberosa (Agrawal et al. 2009). Females may be able to sense these chemical differences by “dabbing” their ovipositor on the underside of a leaf prior to oviposition (Zalucki et al. 1990, Arikawa 2001). Additionally, A. tuberosa has a layer of trichomes, which may inhibit oviposition
or decrease a female’s ability to sense leaf chemicals. *A. incarnata*, the most preferred milkweed in both experiments (Figures 2 and 3ab), also has a relatively low level of cardenolides compared to some of the other species tested (*A. speciosa* and *A. hirtella*), but has a higher level of quercetin glycosides than *A. tuberosa* as reported in the literature (Woodson 1954, Roeske et al. 1976, Agrawal et al. 2009, Rasmann and Agrawal 2011, Agrawal et al. 2015) and lacks leaf hairs. *A. syriaca* also has similar cardenolide levels to *A. incarnata*, but slightly lower levels of quercetin glycosides as reported in the literature (Woodson 1954, Roeske et al. 1976, Agrawal et al. 2009, Rasmann and Agrawal 2011, Agrawal et al. 2015), which could contribute to the differences we observed in egg totals between these two milkweed species. Interestingly, *A. incarnata* and *A. verticillata* have very similar levels of quercetin glycosides (Agrawal et al. 2009). Although these plant chemicals play a role in oviposition preference, additional plant traits undoubtedly contribute to egg laying preference.

Other plant characteristics that may play a role in female oviposition preference include leaf trichomes, leaf morphology (overall dimensions), and overall plant architecture (height, number of leaves, etc.) We counted fewer eggs on the narrow leafed milkweeds (*A. tuberosa* and *A. verticillata*) in both the no-choice and oviposition preference tests although the total number of eggs laid on each plant is not correlated with leaf width. Observations of ovipositing females showed that *A. verticillata* plants bent under the weight of female monarchs, and that the strength of the stems and size of the leaves may present
a physical challenge to oviposition (Pocius, personal observation). In the wild, females may encounter more robust stalks of *A. verticillata*. Thus, presenting females with young plants in the lab may have artificially reduced the number of eggs laid on this species.

Females laid a moderate number of eggs on *C. laeve*, the only vine included in this study. This species was not significantly different from the highly preferred or highly unpreferred milkweed species. It is difficult to explain why females didn’t utilize this milkweed species more often in the oviposition inclination experiment because the cardenolide and quercetin glycoside concentrations for this plant are currently unknown. The structure of each individual vine also may have been difficult for females as the wider leaves are often tangled in the stem in young plants (Pocius, personal observation).

Although egg counts were highest on *A. incarnata*, restoration efforts should focus on planting a variety of milkweeds, not just the milkweed species with the highest egg counts reported here because plant quality is important for both monarch larvae and adults. Specialization on one milkweed species is not the optimal strategy for female monarchs; weather conditions, like temperature and precipitation, can have massive impacts on the quality of milkweed plants. For example, *A. incarnata* thrives in wet years, but plants deteriorate in drought conditions (V. Pocius, personal observation). Conversely, *A. hirtella* thrives in drier conditions. Females need to have multiple milkweed species to place their eggs on the most viable milkweed hosts during each breeding season.
These milkweed species will perform best in sites that match their habitat requirements. All nine milkweeds tested in our experiments favor different habitats. For example, *A. syriaca*, *A. incarnata*, *A. tuberosa*, and *A. verticillata* are found across the entirety of Iowa, but *A. syriaca* and *A. verticillata* are found in drier locations than *A. incarnata* (Woodson 1954, Eilers and Roosa 1994, USDA-NRCS 2017). See Pocius et al. (2017b) for a summary of milkweed distributions. Given the differences in flowering time and plant maturation phenology, a suite of different milkweed species may provide a broader set of resources across the flight season compared to only one milkweed species. Additionally, females laid more eggs when a diversity of milkweed species were present in their environment. Because our conservation goals include increasing the number of eggs laid per female to boost monarch numbers, adding a diverse array of milkweeds to restorations is likely to increase the number of eggs laid in these locations.

Future research should investigate adult female egg load (number of mature eggs contained in the ovaries daily) and potential fecundity for individuals that have fed on different milkweed species in order to assess the value of different milkweeds on the landscape. Future trials should use mature milkweed plants so that monarchs encounter buds, blooms, and differing leaf quality (young and mature leaves) of various milkweed species. We used young milkweed plants in this study as females will more readily lay eggs on young plants compared to mature plants (Zalucki and Kitching, 1982), but young plants do not resemble mature plants in the field, as they often have fewer stems, and
no buds or flowers. In the field, differing chemical concentrations among clones, differing modularity, and differing phenology among milkweed species also contribute to oviposition preference. We acknowledge that females used in this experiment encountered a simplified array of milkweeds compared to nature. Understanding how females respond to mature plants in the context of oviposition will allow scientists and managers to even more specifically gauge their potential value in habitat restoration.

**Acknowledgements**

This work was partially funded by the USDA National Institute of Food and Agriculture, Hatch project number 1009926 (IOW05478) and by the USDA, Natural Resources Conservation Service’s Conservation Innovation Grant program under Agreement Number 69-3A75-16-006. Additional support was provided by Prairie Biotics Inc., the Iowa Native Plant Society and the Iowa Monarch Conservation Consortium. Mention of a proprietary product does not constitute an endorsement or a recommendation by Iowa State University or USDA for its use. The authors would like to thank Ali Ford, Nicholas Oppedal, Jaclyn Appelhans, Cory Haggard, and Nancy Shryock for their help with experimental set up and collecting measurements. We thank the staff of the ISU Forestry Greenhouse for their help with plant care as well as Lincoln Brower, Amy Toth, Sue Blodgett, Dean Adams, and three anonymous reviewers for their insightful comments that improved this manuscript.
Literature Cited


The Center for Biological Diversity, The Center for Food Safety, The Xerces Society, and L. P. Brower. 2014. Petition to protect the monarch butterfly (Danaus plexippus) under the endangered species act.


Manson, J. S., S. Rasmann, R. Halitschke, J. D. Thomson, and A. A. Agrawal. 2012. Cardenolides in nectar may be more than a consequence of allocation to other plant parts: a phylogenetic study of *Asclepias*. Functional Ecology 26:1100-1110.


CHAPTER 5: COMMON GARDEN STUDIES SUGGEST MONARCH BUTTERFLIES SHOW OVIPOSITION PREFERENCES AMONG NINE MILKWEED SPECIES NATIVE TO IOWA

A manuscript in preparation for submission.

Abstract

Over the past two decades, the population of monarch butterflies east of the Rocky Mountains has experienced a significant decline in overwintering numbers. Habitat restoration that includes planting milkweeds is essential to boost monarch numbers within the breeding range. Milkweeds are the only host plants for larval monarch butterflies, but female oviposition preference for different milkweed species, especially those with overlapping ranges, is not well documented. We examined oviposition preference by comparing the number of eggs laid by free flying wild monarchs on each of nine native milkweed species occurring in Iowa (A. exaltata, A. hirtella, A. incarnata, A. speciosa, A. sullivantii, A. syriaca, A. tuberosa, A. verticillata, and C. laeve). Eggs were counted weekly at 14 sites across the state of Iowa in June, July and August 2015-2017. Each site had one 1m² plot planted with each of the nine species.

When egg totals were compared for each milkweed species across all sites in each year, there were significant differences among eggs deposited among the nine milkweed species examined. Females preferred A. incarnata and A. syriaca in all years. Fewer eggs were counted on A. exaltata, A. hirtella, A. tuberosa, A. verticillata, and C. laeve. Our results show that monarch butterflies will lay eggs on all nine milkweeds, but that there are clear preferences for some milkweed species over others.
Introduction

Habitat loss is one of the leading causes of species decline and extinction worldwide (Wilcove et al. 1998; Pimm and Raven 2000; Ceballos and Ehrlich 2002; Kerr and Cihlar 2004; Venter et al. 2006). One way to mitigate these losses is through carefully executed habitat restoration. However, an intimate knowledge of species preferences is necessary to effectively restore habitat targeted for one species. Although monarch butterflies are dependent upon milkweeds \( (Asclepias \text{ spp.}) \) as larvae, there are over 100 species of milkweeds in the U.S. (Woodson, 1954); and scientists are only beginning to understand monarch preference for different milkweed species as larvae and adults (e.g., see Pocius et al. 2017a,b, Pocius et al. 2018). Regardless of preference, disturbance in modern agricultural landscape favors \( Asclepias syriaca \), but this species may not have been as prevalent in historic landscapes (Pleasants 2015).

Monarch butterfly \( (Danaus plexippus) \) populations both east and west of the Rocky Mountains have experienced a significant decline in overwintering numbers over the past two decades (Brower et al. 2012, Espeset et al. 2016, Inamine et al. 2016). Although this decline may not be representative of the monarch population size during other times of the year, such as migration and breeding (Davis 2012, Davis and Dyer 2015), this decline has been attributed to multiple factors including the loss of milkweed (Oberhauser et al. 2001, Pleasants and Oberhauser 2013, Pleasants 2017, Zaya et al. 2017) and nectar sources (Inamine et al. 2016) within the breeding range. Recent models have implicated loss of habitat, including milkweeds, within the breeding range as the main threat to the monarch population (Flockhart et al. 2015). Because a large...
proportion of monarchs that overwintered in Mexico originate from the Midwest (Wassenaar and Hobson 1998, Flockhart et al. 2017), restoration of monarch habitat in this region is essential to increase population numbers (Oberhauser et al. 2016). Federal, state, and non-profit groups have undertaken efforts to establish monarch habitat. These projects have focused on adding milkweed plants to the landscape (Thogmartin et al. 2017).

Historic midwestern grassland and wetland habitats contained multiple milkweed species (Hayden 1919). Agriculture led to a loss of Midwest grassland habitat while increased disturbance resulted in an increase of Asclepias syriaca (Martin and Burnside, 1980). Before the advent of glyphosate-tolerant crops, common milkweeds that grew in-between corn and soybean rows (Martin and Burnside 1980) were among the most heavily used monarch host plants in the U.S. North Central breeding range (Oberhauser 2001, Pleasants and Oberhauser 2013). After the advent of glyphosate use, the number of milkweed plants in corn and soybean fields virtually disappeared from 1999 to 2010 leading to a 58% decline in milkweed abundance on the Midwestern landscape (Hartzler, 2010, Pleasants and Oberhauser 2013). Virtually all current restoration recommendations are based on A. syriaca (Landis 2014, Pleasants and Oberhauser 2013, Pleasants 2017). Monarchs could potentially use multiple milkweed species for oviposition, but more information is needed about monarch oviposition on these milkweeds to ensure that these plants could contribute to population growth and add resilience to this system through increased plant diversity.
We examined monarch oviposition and larval presence on nine milkweed species native to Iowa, which is a high priority area for Midwestern conservation efforts (Flockhart et al. 2015, Thogmartin et al. 2017). Most milkweeds native to the Midwest, especially those with narrow ranges, have not been tested in field experiments. The species we examined were: *A. syriaca* (common milkweed), *A. incarnata* (swamp milkweed), *A. tuberosa* (butterfly milkweed), *A. verticillata* (whorled milkweed), *A. speciosa* (showy milkweed), *A. exaltata* (poke milkweed), *A. sullivantii* (prairie milkweed), *A. hirtella* (tall green milkweed), and *C. laeve* (honeyvine milkweed). These species have varying concentrations of cardenolides (Woodson 1954, Roeske et al. 1976, Malcolm 1991, Agrawal et al. 2009, Rasmann and Agrawal 2011) quercetin glycosides, (Haribal and Renwick 1996, Agrawal et al. 2009), different architecture, and overlapping ranges (Woodson 1954), but different habitat requirements (Woodson 1954, Kaul et al. 1991, Eilers and Roosa 1994).

Prior work has contributed to our understanding of monarch oviposition preference on different milkweed species. Haribal and Renwick (1996, 1998a,b) examined milkweed chemical composition in relation to monarch oviposition and post alightment behavior. Calvert (1999) compared oviposition preference among monarchs migrating northward on seven endemic species in Texas; Cohen and Brower (1982) examined monarch oviposition and subsequent larval survival in Florida on *A. humistrata* in the field. Bartholomew and Yeargan (2002) compared oviposition behavior of monarchs laying on *C. laeve* and *A. syriaca* in Kentucky; and both DiTommaso and Losey (2003) and Casagrande and Dacey
(2007) examined female oviposition on non-milkweed species in the laboratory. Malcolm and Brower (1986) studied the difference in oviposition preference between *A. curassavica* and *A. incarnata* in mixed stands in southern Florida, while Zalucki et al. (1990) assessed female oviposition preference based on cardenoid content in the field and lab in the southeastern U.S.; and Ladner and Altizer (2005) evaluated both larval survival and oviposition in the laboratory on leaf cuttings, and trimmed milkweed stems, respectively. Pocius et al. (2018) examined oviposition preference on nine native milkweed species in both no choice and oviposition preference tests. Few studies have focused on monarch oviposition preference across multiple native milkweed species; none have replicated a design on a large scale in the field.

We established 14 milkweed demonstration sites across Iowa, each site containing plots of nine native milkweed species. We counted the number of monarch eggs and larvae on each block of five milkweed plants and noted the number of surviving plants and blooming period at each location between June and August 2015-2017. We predicted that monarchs will use all nine species, and that the oviposition patterns that we observed in the field would follow those observed in prior laboratory studies (Pocius et al. 2018); we expected higher egg totals on *A. incarnata* than on *A. tuberosa* and *A. verticillata* (Pocius et al. 2018). Information about monarch oviposition in the field with wild females is necessary to develop and execute effective, economical habitat restoration in the Midwest.
Materials and methods

Experimental Milkweed Plot Plants and Demonstration Site Establishment

Midwestern ecotype milkweed seeds (A. exaltata, A. hirtella, A. incarnata, A. speciosa, A. sullivantii, A. syriaca, A. tuberosa, A. verticillata, and C. laeve, Prairie Moon Nursery, MN, USA) were stratified in wet sand for 6 weeks. After stratification, seeds were sown into 128-cell plug trays (Landmark Plastics, Akron OH, USA) and transplanted into 8.9 cm square, deep perennial pots (Kord, Ontario Canada) at approximately 6 weeks following germination. When milkweed plants were 12 weeks old, 5 young plants of each species were transported to each location. Demonstration sites were established at ten Iowa State Research and Demonstration Farms, Luther College, Pella High School, and Adel Conservation (Figure 5.1). At least one site was located in each quadrant of the state. Plants were distributed to each site and planted by the second week of June, 2015.

Each of nine milkweed species was randomly assigned to a 1m$^2$ plot within one row. Each block was separated by a 1 m wide grass or stone path. Five milkweeds of the same species were placed within each 1m$^2$ plot (Figures 5.2, 5.3). During May 2016 and 2017, each plot was monitored for plant emergence; any plants that did not survive were replaced with young plants (6-8 weeks old). A. hirtella plants were not replaced due to a lack of seed in 2016 and 2017.
Figure 5.1  Map of milkweed demonstration plot locations modified from ISU Research and Demonstration Farms (farms.ag.iastate.edu). Colored regions represent varying soil types across the state; red circles denote milkweed demonstration plot locations.
Figure 5.2  Milkweed plot arrangement. Each blue square denotes a 1m$^2$ plot of an individual species. Species were randomly assigned to plots at each farm. The arrow denotes the plant arrangement within each milkweed species plot. Each green circle represents one milkweed plant.
Figure 5.3  Milkweed demonstration plot at the Allee Research Farm in Newell, Iowa in mid-June 2016.
Demonstration Site Monitoring

Each site was monitored weekly from the first week of June through the end of August from June 2015-August 2017. In 2017, the sites in central Iowa were monitored from April-October 2017 to obtain additional information on milkweed plant phenology. Plant emergence was recorded at each site in late May-early June in 2017. Temperature was recorded immediately before weekly monitoring began at each site; monitoring was conducted in all but severe weather (e.g. thunderstorms, hail). Each week, the number of live milkweed plants, bloom presence, the number of blooms, the height of the tallest plant, the presence of seed pods, and the presence of mature seed pods was recorded for each milkweed species. Each plant was examined for the presence of monarch eggs, larvae, or other insects using a modified protocol from the Monarch Larva Monitoring Project (Oberhauser 2013).

Statistical Analysis

Egg counts on each block of five plants were summed across June, July, and August in each year, and the results were analyzed separately. Only sites where observers recorded egg numbers for at least eight weeks were included in the analysis of each year; sites without any eggs during the summer were removed from the analysis (N=12 sites in 2015, N=13 sites in 2016, and N=10 sites in 2017). Egg counts were only reported for milkweed species with live plants at each site over the observation period. Differences in total egg counts in single years were determined using a Poisson regression with milkweed species and site as fixed effects (Kaitala 1996, Mery and Kawecki 2002); plant height and number of blooms were not significant predictors of the number of eggs laid per
species and thus were excluded from the final model. Pairwise differences in egg counts were determined by comparing least square means for each milkweed species; p values were adjusted using Tukey’s range test for multiple comparisons. Concordance was determined using a Kendall coefficient of concordance. R version 3.3.3 (R Core Team 2014) was used for statistical analyses.

Results
During each of the three years, female monarchs laid eggs on all nine milkweed species, but laid more eggs on plants of some milkweed species than others (Figure 5.4). Monarchs laid more eggs in 2015 than in 2016 or 2017 (Figure 5.4) and they laid more eggs later in the season on most species in 2015, 2016, and 2017. Monarchs laid fewer eggs on A. hirtella in August than in June or July, but these differences are not statistically significant. Monarchs’ species preference was highly concordant across years (W=0.94). There was no discernable effect of the number of blooms per plant or plant height on the number of eggs laid on each milkweed species; monarchs laid many eggs on milkweeds that were not blooming. Additionally, monarchs did not lay eggs at every site.
Figure 5.4  Average eggs counted on each milkweed species over the course of the summer breeding season in 2015 (A), 2016 (B), and 2017 (C). Each bar represents one milkweed species. EXA=A. exaltata, HIR=A. hirtella, INC=A. incarnata, LAE=C. laeve, SPE=A. speciosa, SUL=A. sullivantii, SYR=A. syriaca, TUB=A. tuberosa, and VER=A. verticillata; error bars represent 95% confidence intervals. N= 12 sites in 2015, 12 sites in 2016, and 10 sites in 2017. Bars that do not share a letter within each panel are significantly different from each other. Females laid more eggs on A. incarnata and A. syriaca than on A. exaltata, A. hirtella, C. laeve, A. tuberosa, and A. verticillata in all years (p<0.05). P values were adjusted using the Tukey method for multiple comparisons.
2015

Peak egg laying was observed from August 3-10, 2015. When milkweed species and site were included as fixed effects, milkweed species had a significant effect on the number of total eggs laid per milkweed species. *A. incarnata* and *A. syriaca* had the highest egg totals over the first summer of plot observation, when counts from all sites were combined (Figure 5.4). Females laid about 1.3 times more eggs on *A. incarnata* than *A. syriaca*, although this difference was not significant. *A. incarnata* and *A. syriaca* were followed by *A. sullivantii*, *A. speciosa*, *A. hirtella*, *A. tuberosa*, *A. verticillata*, *C. laeve*, *A. verticillata*, and *A. exaltata* in number of eggs counted. One of the largest differences in total egg counts was between *A. incarnata*/*A. syriaca* and *A. exaltata*. Females laid 6.8 times more eggs on *A. incarnata* and 5.4 times more eggs on *A. syriaca* than on *A. exaltata* (Figure 5.4). For all pairwise comparisons, see Table 5.1.

2016

Peak egg laying was observed from August 22-28 2016. When milkweed species and site were included as fixed effects, milkweed species had a significant effect on the number of total eggs laid per milkweed species. *A. syriaca* had the highest egg totals followed by *A. incarnata*, when counts from all sites were combined (Figure 5.4). Females laid 1.4 times more eggs on *A. syriaca* than on *A. incarnata* although this difference was not significant. *A. incarnata* and *A. syriaca* were followed by *A. sullivantii*, *A. speciosa*, *A. hirtella*, *A. verticillata*, *C. laeve*, *A. tuberosa* and *A. exaltata* in number of eggs counted. The largest difference in egg counts was observed between *A. syriaca*, *A. incarnata*...
Table 5.1  Pairwise Comparisons for total number of eggs laid on each milkweed species during June-August 2015 when all demonstration sites were combined. A Tukey's range test was used to adjust p-values for multiple comparisons. EXA= *A. exaltata* (poke milkweed), HIR= *A. hirtella* (tall green milkweed), INC= *A. incarnata* (swamp milkweed), LAE= *C. laeve* (honeyvine milkweed), SPE= *A. speciosa* (showy milkweed), SUL= *A. sullivantii* (prairie milkweed), SYR= *A. syriaca* (common milkweed), TUB= *A. tuberosa* (butterfly milkweed), and VER= *A. verticillata* (whorled milkweed). Bold text signifies a significant difference in which p<0.05.

<table>
<thead>
<tr>
<th>Milkweed Species Comparison</th>
<th>Total Egg Estimate</th>
<th>S.E.</th>
<th>z-ratio</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>EXA-HIR</td>
<td>-1.098</td>
<td>0.27</td>
<td>-4.04</td>
<td>0.0018</td>
</tr>
<tr>
<td>EXA-INC</td>
<td>-0.511</td>
<td>0.25</td>
<td>-7.65</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>EXA-LAE</td>
<td>-0.511</td>
<td>0.29</td>
<td>-1.71</td>
<td>0.74</td>
</tr>
<tr>
<td>EXA-SPE</td>
<td>-1.27</td>
<td>0.27</td>
<td>-4.75</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>EXA-SUL</td>
<td>-1.40</td>
<td>0.26</td>
<td>-5.32</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>EXA-SYR</td>
<td>-1.68</td>
<td>0.26</td>
<td>-6.56</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>EXA-TUB</td>
<td>-0.916</td>
<td>0.28</td>
<td>-3.29</td>
<td>0.28</td>
</tr>
<tr>
<td>EXA-VER</td>
<td>-0.721</td>
<td>0.29</td>
<td>-2.51</td>
<td>0.23</td>
</tr>
<tr>
<td>HIR-INC</td>
<td>-0.831</td>
<td>0.16</td>
<td>-5.10</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>HIR-LAE</td>
<td>0.588</td>
<td>0.23</td>
<td>2.58</td>
<td>0.19</td>
</tr>
<tr>
<td>HIR-SPE</td>
<td>-0.17</td>
<td>0.18</td>
<td>-0.92</td>
<td>0.99</td>
</tr>
<tr>
<td>HIR-SUL</td>
<td>-0.30</td>
<td>0.18</td>
<td>-1.68</td>
<td>0.75</td>
</tr>
<tr>
<td>HIR-SYR</td>
<td>-0.586</td>
<td>0.17</td>
<td>-3.45</td>
<td>0.016</td>
</tr>
<tr>
<td>HIR-TUB</td>
<td>0.18</td>
<td>0.20</td>
<td>0.903</td>
<td>0.99</td>
</tr>
<tr>
<td>HIR-VER</td>
<td>0.378</td>
<td>0.21</td>
<td>1.77</td>
<td>0.701</td>
</tr>
<tr>
<td>INC-LAE</td>
<td>1.42</td>
<td>0.20</td>
<td>6.975</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>INC-SPE</td>
<td>0.661</td>
<td>0.15</td>
<td>4.30</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>INC-SUL</td>
<td>0.530</td>
<td>0.15</td>
<td>3.59</td>
<td>0.01</td>
</tr>
<tr>
<td>INC-SYR</td>
<td>0.245</td>
<td>0.13</td>
<td>1.81</td>
<td>0.674</td>
</tr>
<tr>
<td>INC-TUB</td>
<td>1.01</td>
<td>0.17</td>
<td>5.82</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>INC-VER</td>
<td>1.21</td>
<td>0.18</td>
<td>6.46</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>LAE-SPE</td>
<td>-0.760</td>
<td>0.22</td>
<td>-3.42</td>
<td>0.018</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>----------</td>
<td>-----</td>
<td>-----</td>
<td>------</td>
<td>-----</td>
</tr>
<tr>
<td>LAE-SUL</td>
<td>-0.890</td>
<td>0.22</td>
<td>-4.10</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>LAE-SYR</td>
<td>-1.17</td>
<td>0.21</td>
<td>-5.62</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>LAE-TUB</td>
<td>-0.410</td>
<td>0.23</td>
<td>-1.72</td>
<td>0.73</td>
</tr>
<tr>
<td>LAE-VER</td>
<td>-0.210</td>
<td>0.24</td>
<td>-0.85</td>
<td>0.99</td>
</tr>
<tr>
<td>SPE-SUL</td>
<td>-0.130</td>
<td>0.17</td>
<td>-0.77</td>
<td>0.99</td>
</tr>
<tr>
<td>SPE-SYR</td>
<td>-0.415</td>
<td>0.16</td>
<td>-2.58</td>
<td>0.19</td>
</tr>
<tr>
<td>SPE-TUB</td>
<td>0.352</td>
<td>0.19</td>
<td>1.81</td>
<td>0.67</td>
</tr>
<tr>
<td>SPE-VER</td>
<td>0.550</td>
<td>0.21</td>
<td>2.65</td>
<td>0.16</td>
</tr>
<tr>
<td>SUL-SYR</td>
<td>-0.284</td>
<td>0.15</td>
<td>-1.83</td>
<td>0.66</td>
</tr>
<tr>
<td>SUL-TUB</td>
<td>0.484</td>
<td>0.19</td>
<td>2.55</td>
<td>0.21</td>
</tr>
<tr>
<td>SUL-VER</td>
<td>0.680</td>
<td>0.20</td>
<td>3.37</td>
<td>0.02</td>
</tr>
<tr>
<td>SYR-TUB</td>
<td>0.768</td>
<td>0.18</td>
<td>4.26</td>
<td>&lt;0.0007</td>
</tr>
<tr>
<td>SYR-VER</td>
<td>0.964</td>
<td>0.19</td>
<td>4.98</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>TUB-VER</td>
<td>0.196</td>
<td>0.22</td>
<td>0.882</td>
<td>0.99</td>
</tr>
</tbody>
</table>
Table 5.2 Pairwise Comparisons for total number of eggs laid on each milkweed species during June-August 2016 when all demonstration sites were combined. A Tukey’s range test was used to adjust p-values for multiple comparisons. EXA= A. exaltata (poke milkweed), HIR= A. hirtella (tall green milkweed), INC=A. incarnata (swamp milkweed), LAE= C. laeve (honeyvine milkweed), SPE= A. speciosa (showy milkweed), SUL= A. sullivantii (prairie milkweed), SYR= A. syriaca (common milkweed), TUB= A. tuberosa (butterfly milkweed), and VER= A. verticillata (whorled milkweed). Bold text signifies a significant difference in which p<0.05.

<table>
<thead>
<tr>
<th>Milkweed Species Comparison</th>
<th>Total Egg Estimate</th>
<th>S.E.</th>
<th>z-ratio</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>EXA-HIR</td>
<td>-2.40</td>
<td>1.04</td>
<td>-2.30</td>
<td>0.344</td>
</tr>
<tr>
<td>EXA-INC</td>
<td>-3.87</td>
<td>1.01</td>
<td>-3.83</td>
<td>0.004</td>
</tr>
<tr>
<td>EXA-LAE</td>
<td>-2.15</td>
<td>1.07</td>
<td>-2.01</td>
<td>0.534</td>
</tr>
<tr>
<td>EXA-SPE</td>
<td>-3.61</td>
<td>1.01</td>
<td>-3.56</td>
<td>0.011</td>
</tr>
<tr>
<td>EXA-SUL</td>
<td>-3.61</td>
<td>1.01</td>
<td>-3.56</td>
<td>0.011</td>
</tr>
<tr>
<td>EXA-SYR</td>
<td>-4.19</td>
<td>1.01</td>
<td>-4.16</td>
<td>0.0011</td>
</tr>
<tr>
<td>EXA-TUB</td>
<td>-1.61</td>
<td>1.10</td>
<td>-1.45</td>
<td>0.870</td>
</tr>
<tr>
<td>EXA-VER</td>
<td>-2.20</td>
<td>1.05</td>
<td>-2.08</td>
<td>0.480</td>
</tr>
<tr>
<td>HIR-INC</td>
<td>-1.47</td>
<td>0.334</td>
<td>-4.41</td>
<td>0.0004</td>
</tr>
<tr>
<td>HIR-LAE</td>
<td>0.245</td>
<td>0.484</td>
<td>.506</td>
<td>0.99</td>
</tr>
<tr>
<td>HIR-SPE</td>
<td>-1.21</td>
<td>0.343</td>
<td>-3.53</td>
<td>0.012</td>
</tr>
<tr>
<td>HIR-SUL</td>
<td>-1.21</td>
<td>0.343</td>
<td>-3.53</td>
<td>0.012</td>
</tr>
<tr>
<td>HIR-SYR</td>
<td>-1.79</td>
<td>0.325</td>
<td>-5.50</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>HIR-TUB</td>
<td>0.788</td>
<td>0.539</td>
<td>1.46</td>
<td>0.873</td>
</tr>
<tr>
<td>HIR-VER</td>
<td>0.201</td>
<td>0.449</td>
<td>0.446</td>
<td>1.00</td>
</tr>
<tr>
<td>INC-LAE</td>
<td>1.72</td>
<td>0.410</td>
<td>4.23</td>
<td>0.0008</td>
</tr>
<tr>
<td>INC-SPE</td>
<td>0.260</td>
<td>0.219</td>
<td>1.19</td>
<td>0.959</td>
</tr>
<tr>
<td>INC-SUL</td>
<td>0.260</td>
<td>0.219</td>
<td>1.19</td>
<td>0.959</td>
</tr>
<tr>
<td>INC-SYR</td>
<td>-0.318</td>
<td>0.190</td>
<td>-1.68</td>
<td>0.759</td>
</tr>
<tr>
<td>INC-TUB</td>
<td>2.26</td>
<td>0.470</td>
<td>4.81</td>
<td>0.0001</td>
</tr>
<tr>
<td>INC-VER</td>
<td>1.67</td>
<td>0.363</td>
<td>4.61</td>
<td>0.0001</td>
</tr>
<tr>
<td>LAE-SPE</td>
<td>-1.46</td>
<td>0.413</td>
<td>-3.53</td>
<td>0.0126</td>
</tr>
<tr>
<td>LAE-SUL</td>
<td>-1.46</td>
<td>0.413</td>
<td>-3.53</td>
<td>0.0126</td>
</tr>
</tbody>
</table>
Table 5.2 Continued

<p>| | | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>LAE-SYR</td>
<td>-2.04</td>
<td>0.400</td>
<td>-5.10</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>LAE-TUB</td>
<td>5.43</td>
<td>0.586</td>
<td>0.927</td>
<td>0.990</td>
</tr>
<tr>
<td>LAE-VER</td>
<td>-4.44</td>
<td>0.505</td>
<td>-0.088</td>
<td>1.00</td>
</tr>
<tr>
<td>SPE-SUL</td>
<td>-1.27x10^{-13}</td>
<td>0.232</td>
<td>0.00</td>
<td>1.00</td>
</tr>
<tr>
<td>SPE-SYR</td>
<td>-0.578</td>
<td>0.205</td>
<td>-2.81</td>
<td>0.110</td>
</tr>
<tr>
<td>SPE-TUB</td>
<td>2.00</td>
<td>0.476</td>
<td>4.20</td>
<td>0.0009</td>
</tr>
<tr>
<td>SPE-VER</td>
<td>1.41</td>
<td>0.372</td>
<td>3.80</td>
<td>0.0045</td>
</tr>
<tr>
<td>SUL-SYR</td>
<td>-0.578</td>
<td>0.205</td>
<td>-2.81</td>
<td>0.110</td>
</tr>
<tr>
<td>SUL-TUB</td>
<td>2.00</td>
<td>0.476</td>
<td>4.20</td>
<td>0.0009</td>
</tr>
<tr>
<td>SUL-VER</td>
<td>1.41</td>
<td>0.371</td>
<td>3.80</td>
<td>0.0045</td>
</tr>
<tr>
<td>SYR-TUB</td>
<td>2.58</td>
<td>0.463</td>
<td>5.56</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>SYR-VER</td>
<td>1.99</td>
<td>0.355</td>
<td>5.61</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>TUB-VER</td>
<td>0.587</td>
<td>0.558</td>
<td>-1.05</td>
<td>0.980</td>
</tr>
</tbody>
</table>
Table 5.3  Pairwise Comparisons for total number of eggs laid on each milkweed species during June-August 2017 when all demonstration sites were combined. A Tukey’s range test was used to adjust p-values for multiple comparisons. EXA= *A. exaltata* (poke milkweed), HIR= *A. hirtella* (tall green milkweed), INC= *A. incarnata* (swamp milkweed), LAE= *C. laeve* (honeyvine milkweed), SPE= *A. speciosa* (showy milkweed), SUL= *A. sullivantii* (prairie milkweed), SYR= *A. syriaca* (common milkweed), TUB= *A. tuberosa* (butterfly milkweed), and VER= *A. verticillata* (whorled milkweed). Bold text signifies a significant difference in which p<0.05.

<table>
<thead>
<tr>
<th>Milkweed Species Comparison</th>
<th>Total Egg Estimate</th>
<th>S.E.</th>
<th>z-ratio</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>EXA-HIR</td>
<td>1.12x10^{-15}</td>
<td>0.535</td>
<td>0.00</td>
<td>1</td>
</tr>
<tr>
<td>EXA-INC</td>
<td>-1.79</td>
<td>0.408</td>
<td>-4.39</td>
<td>0.0004</td>
</tr>
<tr>
<td>EXA-LAE</td>
<td>-0.245</td>
<td>0.536</td>
<td>-0.46</td>
<td>1</td>
</tr>
<tr>
<td>EXA-SPE</td>
<td>-0.619</td>
<td>0.469</td>
<td>-1.32</td>
<td>0.925</td>
</tr>
<tr>
<td>EXA-SUL</td>
<td>-1.15</td>
<td>0.434</td>
<td>-2.64</td>
<td>0.170</td>
</tr>
<tr>
<td>EXA-SYR</td>
<td>-1.49</td>
<td>0.418</td>
<td>-3.56</td>
<td>0.011</td>
</tr>
<tr>
<td>EXA-TUB</td>
<td>-2.36x10^{-15}</td>
<td>0.535</td>
<td>0.00</td>
<td>1</td>
</tr>
<tr>
<td>EXA-VER</td>
<td>-0.251</td>
<td>0.504</td>
<td>-0.50</td>
<td>0.999</td>
</tr>
<tr>
<td>HIR-INC</td>
<td>-1.79</td>
<td>0.408</td>
<td>-4.39</td>
<td>0.0004</td>
</tr>
<tr>
<td>HIR-LAE</td>
<td>-0.251</td>
<td>0.536</td>
<td>-0.46</td>
<td>1</td>
</tr>
<tr>
<td>HIR-SPE</td>
<td>-0.619</td>
<td>0.469</td>
<td>-1.32</td>
<td>0.925</td>
</tr>
<tr>
<td>HIR-SUL</td>
<td>-1.15</td>
<td>0.434</td>
<td>-2.64</td>
<td>0.170</td>
</tr>
<tr>
<td>HIR-SYR</td>
<td>-1.49</td>
<td>0.418</td>
<td>-3.56</td>
<td>0.011</td>
</tr>
<tr>
<td>HIR-TUB</td>
<td>-3.48x10^{-15}</td>
<td>0.535</td>
<td>0.00</td>
<td>1.00</td>
</tr>
<tr>
<td>HIR-VER</td>
<td>-0.251</td>
<td>0.504</td>
<td>-0.50</td>
<td>0.999</td>
</tr>
<tr>
<td>INC-LAE</td>
<td>1.55</td>
<td>0.411</td>
<td>3.77</td>
<td>0.0052</td>
</tr>
<tr>
<td>INC-SPE</td>
<td>1.17</td>
<td>0.317</td>
<td>3.70</td>
<td>0.0068</td>
</tr>
<tr>
<td>INC-SUL</td>
<td>0.647</td>
<td>0.263</td>
<td>2.46</td>
<td>0.253</td>
</tr>
<tr>
<td>INC-SYR</td>
<td>0.304</td>
<td>0.237</td>
<td>1.28</td>
<td>0.937</td>
</tr>
<tr>
<td>INC-TUB</td>
<td>1.79</td>
<td>0.408</td>
<td>4.39</td>
<td>0.0004</td>
</tr>
<tr>
<td>INC-VER</td>
<td>1.54</td>
<td>0.367</td>
<td>4.19</td>
<td>0.0009</td>
</tr>
<tr>
<td></td>
<td>LAE-SPE</td>
<td>0.374</td>
<td>0.471</td>
<td>-0.79</td>
</tr>
<tr>
<td>-------</td>
<td>----------</td>
<td>-------</td>
<td>-------</td>
<td>-------</td>
</tr>
<tr>
<td>LAE-SUL</td>
<td>-0.900</td>
<td>0.436</td>
<td>-2.06</td>
<td>0.499</td>
</tr>
<tr>
<td>LAE-SYR</td>
<td>-1.24</td>
<td>0.421</td>
<td>-2.95</td>
<td>0.076</td>
</tr>
<tr>
<td>LAE-TUB</td>
<td>0.245</td>
<td>0.536</td>
<td>0.46</td>
<td>1.00</td>
</tr>
<tr>
<td>LAE-VER</td>
<td>-6.19x10^{-3}</td>
<td>0.506</td>
<td>-0.01</td>
<td>1.00</td>
</tr>
<tr>
<td>SPE-SUL</td>
<td>-0.526</td>
<td>0.350</td>
<td>-1.50</td>
<td>0.854</td>
</tr>
<tr>
<td>SPE-SYR</td>
<td>-0.869</td>
<td>0.330</td>
<td>-2.63</td>
<td>0.174</td>
</tr>
<tr>
<td>SPE-TUB</td>
<td>0.619</td>
<td>0.469</td>
<td>1.32</td>
<td>0.926</td>
</tr>
<tr>
<td>SPE-VER</td>
<td>0.368</td>
<td>0.434</td>
<td>0.85</td>
<td>0.995</td>
</tr>
<tr>
<td>SUL-SYR</td>
<td>-0.343</td>
<td>0.279</td>
<td>-1.23</td>
<td>0.950</td>
</tr>
<tr>
<td>SUL-TUB</td>
<td>1.15</td>
<td>0.434</td>
<td>2.64</td>
<td>0.170</td>
</tr>
<tr>
<td>SUL-VER</td>
<td>0.894</td>
<td>0.396</td>
<td>2.26</td>
<td>0.367</td>
</tr>
<tr>
<td>SYR-TUB</td>
<td>1.49</td>
<td>0.418</td>
<td>3.56</td>
<td>0.011</td>
</tr>
<tr>
<td>SYR-VER</td>
<td>1.24</td>
<td>0.379</td>
<td>3.27</td>
<td>0.030</td>
</tr>
<tr>
<td>TUB-VER</td>
<td>-0.251</td>
<td>0.504</td>
<td>-0.50</td>
<td>0.999</td>
</tr>
</tbody>
</table>
and *A. exaltata*. Females laid over twenty times more eggs on *A. syriaca* and *A. incarnata* than on *A. exaltata* in 2016. For all pairwise comparisons, see Table 5.2.

2017

Peak egg laying was observed from July 23-29 2017. When milkweed species and site were included as fixed effects, milkweed species had a significant effect on the number of total eggs laid per milkweed species. *A. incarnata* had the highest egg totals while *A. syriaca* had the second highest egg counts when eggs from all sites were combined (Figure 5.4). Females laid about 1.3 times more eggs on *A. incarnata* than *A. syriaca* although this difference was not significant. *A. incarnata* and *A. syriaca* were followed by *A. sullivantii*, *A. speciosa*, *A. verticillata*, *C. laeve*, *A. hirtella*, *A. tuberosa*, and *A. exaltata* in number of eggs counted. Females laid eight times more eggs on *A. incarnata* than on *A. exaltata* and six times more eggs on *A. incarnata* than on *A. hirtella* in 2017. For all pairwise comparisons, see Table 5.3

Discussion

The findings of our field-based oviposition preference experiment suggest that monarch butterflies will lay eggs on all milkweed species tested (Figure 5.4), although they prefer to lay more eggs on some species compared to others. Importantly, wild monarch females were consistent in their milkweed species preference across three breeding seasons (2015-2017) even when these females had encountered other milkweed species before reaching a milkweed plot; species ranks were highly concordant across years. *A. incarnata* and *A. syriaca* were consistently preferred for oviposition when egg counts were
combined across sites within each year. This observed preference for *A. incarnata* and *A. syriaca* in the field matches that of a prior laboratory study in which females were naïve to milkweed before the start of the study (Pocius et al. 2018). We saw more eggs on all species in 2015, which could be due to the young plant age (Zalucki and Kitching 1982) throughout the first season but more likely due to higher level of egg laying in the Midwest in 2015 than 2016 and 2017 (J. Pleasants *pers comm.*). Contrary to Zalucki and Kitching (1982), we did not see an increase in egg counts with plant height in any year; we saw no relationship between the number of eggs laid and plant height (Pocius, unpublished data). Eggs were not present at all sites each year, but no site had zero eggs in two consecutive years, demonstrating the variability of egg distribution across Iowa during these three years. Such differences could be related to how monarchs populate the full extent of the breeding range in the spring or wind patterns across the state each year.

Across years, *A. exaltata*, *A. hirtella*, *A. tuberosa* and *C. laeve* had low egg counts when compared to *A. incarnata* and *A. syriaca*. *A. exaltata* and *A. hirtella* were difficult to establish in sites across Iowa. Only four demonstration sites had five live plants of both species by August 2017. *A. exaltata* also senesced by early July in all years, well before peak oviposition occurred. The few eggs that we did observe on *A. tuberosa* were located on flower buds; however, we observed 4th and 5th instars feeding on this species in August. Older larvae may have moved to this milkweed from other milkweed species within the demonstration plot. Because *A. tuberosa* was in better condition (ex.
greener leaves, no visible senescence) compared to *A. incarnata*, *A. speciosa*,
and *A. syriaca* late in the growing season, *A. tuberosa* may be more valuable as
a larvae food source than for oviposition in August. The utility of *C. laeve* may be
underestimated in our analysis; we observed more eggs on this species in
September in central Iowa after plot monitoring across the state ended and data
from September were not included here. However, it is unlikely that eggs laid
that late will successfully produce adults that migrate to Mexico.

Monarchs use multiple milkweed hosts each year throughout their annual
cycle (Agrawal 2017). Although these milkweed species appear on the
landscape in different proportions, monarchs do not specialize on one milkweed
species even when both have co-evolved within a geographic region (e.g.,
Eastern vs. Western North America) due to their migratory life history (Zhan et al.
2014, Agrawal 2017). Monarchs from both the eastern and western populations
exhibited the same oviposition preferences when given access to milkweed
species from both eastern and western North America (Ladner and Altizer 2005).
Our results support the adaptability of monarchs even when milkweed species
were closer in proximity to each other than usually seen in the field. Females
used all nine milkweed species during each breeding season, but demonstrated
oviposition preference (Figure 5.4).

Differences in secondary plant compounds across milkweed species could
play a role in explaining differences in egg counts. Across the monarch breeding
range, monarchs encounter a variety of milkweed hosts with different plant
architecture and chemical concentrations (Zalucki 1986, Malcolm et al. 1989,
Agrawal 2017). Cardenolide and quercetin glycoside concentrations are plant characteristics thought to influence both monarch oviposition and larval performance (Zalucki et al. 1990, Malcolm 1991, Haribal and Renwick 1998a, Ladner and Altizer 2005, Agrawal et al. 2015). Adult females have been shown to reject high cardenolide hosts even though monarch larvae sequester cardenolides for their own defense as they feed on milkweed plants (Oyeyele & Zalucki 1990, Zalucki et al., 1990; Haribal and Renwick, 1998a). High cardenolide levels have been linked with low larval survival and slower development rates (Erickson, 1973, Zalucki et al., 2001, Zalucki et al. 2012). As such, there may be chemical cues that affect oviposition choice. Alternatively, high quercetin glycoside levels on the leaf surface (Agrawal 2017) stimulate oviposition; monarchs respond to these chemicals as part of host plant recognition and females have laid eggs in response to the presence of these chemicals without a plant (Haribal and Renwick 1996).

In our study, some of the least preferred milkweed species, A. tuberosa, and A. verticillata (Figure 4), both have low cardenolide levels recorded in the literature (Roeske et al. 1976, Agrawal et al. 2009, Rasmann and Agrawal 2011, Agrawal et al. 2015), but A. verticillata has a higher level of quercetin glycosides than A. tuberosa (Agrawal et al. 2009). Females may be able to sense these chemical differences by “dabbing” their ovipositor on the underside of a leaf prior to oviposition (Zalucki et al. 1990, Arikawa 2001). Additionally, A. tuberosa has a layer of trichomes, which may inhibit oviposition or decrease a female’s ability to sense leaf chemicals. A. incarnata, the most preferred milkweed in both 2015
and 2017 (Figure 5.4), also has a relatively low level of cardenolides compared to some of the other species tested (A. speciosa and A. hirtella), but has a higher level of quercetin glycosides than A. tuberosa (Woodson 1954, Roeske et al. 1976, Agrawal et al. 2009, Rasmann and Agrawal 2011, Agrawal et al. 2015). Interestingly, A. incarnata and A. verticillata have very similar levels of quercetin glycosides (Agrawal et al. 2009). We used values reported in the literature to make broad comparisons among these milkweed species; chemical levels can vary widely within milkweed species and even among parts of the same milkweed plant (Agrawal et al. 2012).

Although these plant chemicals play a role in oviposition preference, additional plant traits contribute to egg laying preference. Other plant characteristics that may play a role in female oviposition preference include leaf trichomes, leaf morphology (dimensions), and overall plant architecture (height, number of leaves, etc.). We counted fewer eggs on the narrow leafed milkweeds (A. hirtella, A. tuberosa and A. verticillata) across all three breeding seasons, but prior work showed no correlation between the number of eggs laid and leaf width (Pocius et al. 2018). Observations of ovipositing females in the laboratory on young plants showed that A. verticillata plants bent under the weight of female monarchs, and that the strength of the stems and size of the leaves may present a physical challenge to oviposition (Pocius, personal observation). In the wild, females may encounter better lignified stems than in the greenhouse or larger patches of A. verticillata that allow for wind protection and easier oviposition. The number of open flowers and the overall size of each plant (biomass, height)
also could impact oviposition preference, but plant metrics and egg counts would need to be recorded more often than once per week to elucidate this relationship. To understand the effects of blooming flowers on monarch oviposition, more information is needed regarding monarch behavior to determine if feeding and oviposition behaviors occur together in the wild.

Milkweed species relatedness could also explain oviposition preference if preferred species are members of the same clade. *A. exaltata*, *A. speciosa*, *A. sullivantii*, *A. syriaca*, *A. tuberosa*, and *A. verticillata* appear in the same clade in a phylogeny built on plant defense traits, while *A. incarnata* was in a different clade (Agrawal et al. 2008). All species tested, with the exception of *C. laeve* that was not included in this phylogeny, were included in the North American clade of milkweeds when species relatedness was investigated using non-coding chloroplast DNA (Fishbein et al. 2011). *A. hirtella*, *A. speciosa*, *A. sullivantii*, *A. syriaca*, and *A. tuberosa* clustered within the same clade with species that were distributed in northern, temperate regions. *A. incarnata* and *A. verticillata* appeared in a subclade that contained species with distributions in the southwestern U. S. A. and the montane regions of Mexico (Fishbein et al. 2011). Most importantly for this study, the most preferred species for oviposition did not all fall within the same clade or subclades, although relationships within the North American clade need to be resolved further (Fishbein et al. 2011). Other differences among these milkweed species must affect oviposition preferences.

Nutritional differences among milkweed species may also influence oviposition preference. Nitrogen content of individual plants may vary based on
their proximity to agricultural fields (J. Pleasants, person. comm.); these differences could impact monarch oviposition as well as larval survival. Although nitrogen content did not impact monarch oviposition on *A. fruticosa* in Australia (Oyeyele and Zalucki 1990), it may affect egg-laying preference across milkweed species when nitrogen content can vary greatly across the landscape.

The modularity of these milkweed species differs greatly, and the number of available stems on each plant within a milkweed species plot may have contributed to the differences in egg totals that we observed. *A. syriaca*, *A. speciosa* and *A. sullivantii*, and *C. laeve* plants went from having one stem per plant in 2015 to an average of 8 stems per plant by July 2017 (Pocius, personal observation). In contrast, *A. exaltata* plants regrew only one stem per plant each summer (Pocius, personal observation). This difference in the number of undamaged, available stems may have contributed to oviposition preference, especially when monarch larvae, milkweed tussock moth larvae (*Euchaetes egle*), or adult beetles had already fed on leaves of plants with few stems (*A. exaltata, A. verticillata, A. hirtella*).

Plant quality is important for both monarch larvae and adults throughout the growing season, and the presence of nectar resources also may contribute to the plant’s value. Inter-annual variation in temperature and precipitation can also have significant impacts on the quality of milkweed plants. For example, *A. incarnata* thrives in wet years, but even mature plants deteriorate in drought conditions as observed in late summer 2017 (V. Pocius, personal observation). Conversely, *A. hirtella* and *A. tuberosa* thrive in drier conditions. Having access
to multiple milkweed species during a breeding season provides females the opportunity to place their eggs on the most viable milkweed hosts during each breeding season. As such, specialization on one milkweed species may not be the optimal strategy for female monarchs.

Milkweed species will perform best in sites that match their habitat requirements. All nine milkweeds tested in our experiments favor different habitats. For example, *A. syriaca*, *A. incarnata*, *A. tuberosa*, and *A. verticillata* are found across the entirety of Iowa, but *A. syriaca* and *A. verticillata* are found in drier locations than *A. incarnata* (Woodson 1954, Eilers and Roosa 1994, USDA-NRCS 2017). See Pocius et al. (2017b) for a summary of milkweed distributions.

Future research should investigate milkweed phenology and use across the monarch breeding range because timing for peak oviposition likely differs by location. Future trials should use mature, naturally occurring milkweed patches so that monarchs encounter buds, blooms, and differing leaf quality (young and mature leaves) of various milkweed species in a natural setting. Understanding how females respond to mature milkweed patches in the context of oviposition will allow scientists and managers to further gauge the value of different milkweed species in habitat restoration.
Acknowledgements

This work was partially funded by the USDA National Institute of Food and Agriculture, Hatch project number 1009926 (IOW05478), Prairie Biotics, Inc., The Center for Global and Regional Climate Research (CGRER), and by the USDA, Natural Resources Conservation Service’s Conservation Innovation Grant program under Agreement Number 69-3A75-16-006. Additional support was provided by the Iowa Monarch Conservation Consortium. Mention of a proprietary product does not constitute an endorsement or a recommendation by Iowa State University or USDA for its use. The authors would like to thank Ali Ford, Nancy Shryock, Cory Haggard, Jacqueline Appelhans, Royce Bitzer, Kristen Siewert, Kirk Larsen, Lyle Rossiter, Dallas Maxwell, Randy Breach, Steve Jonas, Nick Howell, Logan Wallace, Myron Rees, Brandyn Chapman, Ken Pecinovsky, Matt Schnabel, Terry Tuttle, and Chris Beedle for their help with demonstration site establishment, plant replacement, and data collection. The authors would like to thank Nick Oppedal, Kelsey Fisher, Teresa Blader, and Niranjana Krishnan for their help planting, transplanting, and watering milkweed plants grown at Iowa State for this project.
Literature Cited


Oberhauser, K. S. 2013. Monarch Larva Monitoring Project


Restoring monarch butterfly habitat in the Midwestern US: ‘all hands on deck’.
Environmental Research Letters, 12: 074005.


Zalucki, M. P., L. P. Brower, and A. Alonso. 2001. Detrimental effects of latex and cardiac glycosides on survival and growth of first-instar monarch butterfly larvae \textit{Danaus plexippus} feeding on the sandhill milkweed \textit{Asclepias humistrata}.

Ecological Entomology 26:212-224.


CHAPTER 6: GENERAL CONCLUSION

This dissertation summarizes the growth, development, and oviposition preferences of monarch butterflies on nine milkweed species native to the Midwest (A. exaltata, A. hirtella, A. incarnata, A. speciosa, A. sullivantii, A. syriaca, A. tuberosa, A. verticillata, and C. laeve). In chapter two, we investigated larval survival over the first five days of life. Larvae were reared on fresh leaves and young milkweed plants of the nine native milkweed species. Larvae ranged from 1-3rd instar by the end of this experiment. We found that there were differences in mass and the instar larvae reached based upon the milkweed species that the larvae consumed; there were also differences in larval lipid stores based on the milkweed species consumed. As expected, larvae that fed on excised leaves were more likely to survive and weighed more than larvae that fed on whole plants because they did not have to contend with milkweed defenses. All larvae reached the third instar during the study with the exception of those that fed on C. laeve. Survivorship did not differ among milkweed species, but larval mass was significantly different among species. Larvae that fed on A. verticillata weighed more than larvae that fed on any other species and were significantly different from C. laeve, A. incarnata, A. speciosa, A. sullivantii, or A. tuberosa. Larvae that fed on C. laeve weighed the least. Larvae that fed on A. incarnata had a higher percentage of lipids than larvae that fed on A. exaltata, A. hirtella, A. sullivantii, A. syriaca, A. tuberosa, or A. verticillata. These experiments contribute to our understanding of early instar survival on a variety of milkweed species and how these species can influence larval energy stores.
The results of chapter two were the impetus for further investigation of larval survival and nutrition on different milkweed species. In chapter three, we examined monarch survival from first instar through adult eclosion on the same nine native milkweed species. The purpose of this study was to gain a baseline estimate of monarch survival on each milkweed species in the absence of predation and to see if there were any differences in survival and developmental rates among larvae that fed on different milkweed species. Furthermore, we analyzed the lipid content of the resulting adults that fed on each of the nine species to compare the lipid content patterns of early instar larvae to those in adults. Survivorship from first instar to adult varied from 30-70% across milkweed species, averaging 58% across all milkweeds species. Fewer monarchs that fed on *A. hirtella* survived than those that fed on *A. tuberosa*, or *A. exaltata*. When adults were dried before lipid analysis, adults that fed on *A. hirtella* weighed less than adults that fed on *A. incarnata*. The total amount of lipid (mg) was significantly different among adults that fed on the nine different milkweed species. Adults that fed on *A. exaltata*, *A. incarnata*, and *A. syriaca* had higher lipid content than those that fed on *A. hirtella* as larvae. This experiment contributes to our understanding of monarch survival on different milkweed species and how these differing food sources can contribute to varying lipid levels in freshly eclosed adults.

With an understanding of both survival and use of these nine milkweeds species by monarch larvae, we wondered if there were differences in oviposition preference using the same nine milkweed species and if larval survival and
Oviposition species preferences would be similar. The findings from chapter four illustrate oviposition preference in the lab, which provides a baseline for egg laying in the absence of differing temperature, site, plant age, and egg predation. Using four of the nine milkweed species we found that monarch butterflies will lay eggs on all four tested in choice experiments although they are more inclined to lay on some species than others, with preference generally mirroring the pattern exhibited in the no-choice experiment. Interestingly, monarch females laid more total eggs during the choice experiment when a diversity of milkweeds were present in each cage than would be expected based on the no-choice experiments. In no-choice tests, we saw the highest egg counts on *A. incarnata* followed by *A. sullivantii* and *A. syriaca*. In preference tests, over half of all eggs laid were on *A. incarnata*. Females laid fewer eggs on *A. tuberosa* and *A. verticillata* in both preference and no-choice tests, even though larval survival was high on both of these species in prior experiments (Pocius et al. 2017a,b). These results indicate that milkweed species that support high oviposition rates are not necessarily those that provide the best food resources for monarch larvae. These results did not include data from large arrays of mature milkweed stands or blooming milkweed plants; more information is necessary to understand oviposition preference in the wild and how milkweed species could be used to boost monarch numbers within restored habitat.

In Chapter five, we investigated similar oviposition questions in a three-year field trial using the same nine milkweed species planted at 15 locations across Iowa. This experiment allowed us to compare the distribution of eggs on
different milkweed species and across several breeding seasons (2015, 2016, and 2017). The findings of our field-based oviposition preference experiment suggest that monarch butterflies will lay eggs on all milkweed species tested, although they prefer to lay more eggs on some species compared to others. Importantly, monarch females were consistent in their milkweed species preference across three breeding seasons (2015-2017); species ranks were highly concordant across years. *A. incarnata* and *A. syriaca* were consistently preferred for oviposition when egg counts were combined across sites within each year. This observed preference for *A. incarnata* and *A. syriaca* in the field matches that of a prior laboratory study (Pocius et al. 2018). We saw more eggs on all species in 2015, which could be due to the young plant age (Zalucki and Kitching 1982) throughout the first season but more likely due to higher egg production throughout the Midwest in that year. Eggs were not present at all sites in each year, but no site had zero eggs in two consecutive years, demonstrating the variability of egg distribution across Iowa during these three years. Such differences could be related to variability in how monarchs populate the full extent of the breeding range in the spring perhaps due to wind patterns across the state each year.

The findings from chapter five have great potential as a resource for selecting milkweed species to include in pollinator habitat restorations, especially in Iowa. The study did not investigate the reasons why certain milkweed species are preferred over others, but it did allow us to test milkweed preference in field conditions. The highest egg totals were found on *A. incarnata* and *A. syriaca*
during the summers of 2015-2017 when egg counts from all locations were combined. Given our results, we recommend that future research focus on how plant chemicals and additional plant traits (e.g. trichomes, latex, leaf toughness, plant age, plant modularity, nectar availability, plant biomass) contribute to observed oviposition preferences. Future research should also address larval usage of different milkweeds in the field and take larval movement into account for future habitat restoration efforts.

Conclusions

This work highlights the value of having multiple milkweed species available throughout the core monarch breeding range for larval feeding and oviposition. Because milkweeds that support high oviposition are not the same milkweed species that encourage the highest larval survival, multiple milkweed species should be included in habitat restorations, especially *A. incarnata* and *A. syriaca*.

*A. incarnata* had the highest egg totals on average in the laboratory and field experiments and performed well in the larval survival studies; this species is easy to establish and thrives in wet conditions. *A. syriaca* performed well, within the top third, in both larval survival and oviposition experiments, supporting the utility of this broadly distributed milkweed in restorations. Surprisingly, *A. sullivantii*, which has been thought to be an egg sink because of strong latex pressure, performed well in oviposition trials; this species had the third highest egg total in 2015-2017, but only about 36% of larvae reached adulthood while feeding on this milkweed. Both *A. tuberosa*, and *A. verticillata* were good larval food sources, but few eggs are laid on these species. Although *A. speciosa*, and
A. *exaltata* performed relatively well as larval food sources, I would not recommend these species for broad restorations; *A. exaltata* is difficult to establish and both species senesce early in the season well before peak oviposition. *C. laeve* was very easy to establish at field locations and served as a good larval food source, but received very few eggs, and was difficult to control in a garden setting; this species may be more useful south of Iowa when other milkweeds senesce earlier in the summer. Since it appears to be used mostly in September, its utility may be limited because late egg production is rarely successful. *A. hirtella* should not be included in broad habitat establishment. It was difficult to establish and did not perform well in larval survival or oviposition experiments. A summary of each milkweed species’ utility for larval survival, oviposition, and establishment is above (Table 6.1).

Monarchs do prefer some milkweed species over others for oviposition, but laid more eggs when several milkweed species were present. Thus, milkweed diversity in a habitat may boost egg numbers; this needs to be examined in a filed setting. Different milkweeds also provide differing levels of nutrition to larvae, resulting in differing lipid levels in freshly eclosed adults. These lipid differences could influence foraging and mating decisions by adult monarchs.
Table 6.1  Summary of the utility of nine milkweed species examined in this dissertation. Habitat information is summarized from Kaul et al. 1991 and Eilers and Roosa 1994. Larval survivorship designated as high if over 60% of larvae reached adulthood; under 60% survival is designated as low. Oviposition use is designated as high if species were in the top third for both laboratory and field oviposition experiments, medium if species were in the second third for both experiments, and low if the species were in the bottom third of egg totals for both experiments. Species are designated as easy to establish if over 60% survived within the demonstration plots from 2015-2017.

<table>
<thead>
<tr>
<th>Milkweed Species</th>
<th>Common Name</th>
<th>Habitat Requirements</th>
<th>Larval Survivorship</th>
<th>Oviposition Use</th>
<th>Ease of Establishment from Plugs</th>
<th>Recommended for Restoration</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. exaltata</td>
<td>Poke Milkweed</td>
<td>Partial shade, woodland edges, upland woods</td>
<td>High</td>
<td>Medium</td>
<td>Difficult</td>
<td>No</td>
</tr>
<tr>
<td>A. hirtella</td>
<td>Tall Green Milkweed</td>
<td>Full sun, prairie remnants, fields</td>
<td>Low</td>
<td>Medium</td>
<td>Difficult</td>
<td>No</td>
</tr>
<tr>
<td>A. incarnata</td>
<td>Swamp Milkweed</td>
<td>Partial to full sun, wetlands, floodplains, marshes</td>
<td>High</td>
<td>High</td>
<td>Easy</td>
<td>Yes Wet Locations Only</td>
</tr>
<tr>
<td>A. speciosa</td>
<td>Showy Milkweed</td>
<td>Full sun, roadsides, untilled fields, forest clearings</td>
<td>High</td>
<td>Medium</td>
<td>Easy</td>
<td>Yes, outside the range of A. syriaca</td>
</tr>
<tr>
<td>A. sullivantii</td>
<td>Prairie Milkweed</td>
<td>Full sun, prairies, roadsides, field edges</td>
<td>High</td>
<td>Medium</td>
<td>Medium</td>
<td>Yes, but only within the Midwest</td>
</tr>
<tr>
<td>A. syriaca</td>
<td>Common Milkweed</td>
<td>Full sun, any disturbed areas</td>
<td>High</td>
<td>High</td>
<td>Easy</td>
<td>Yes</td>
</tr>
<tr>
<td>A. tuberosa</td>
<td>Butterfly Milkweed</td>
<td>Full sun, prairies, open woodlands</td>
<td>High</td>
<td>Low</td>
<td>Easy</td>
<td>No</td>
</tr>
<tr>
<td>A. verticillata</td>
<td>Whorled Milkweed</td>
<td>Partial to full sun, disturbed areas, roadsides, prairies</td>
<td>High</td>
<td>Low</td>
<td>Easy</td>
<td>No</td>
</tr>
<tr>
<td>C. laeve</td>
<td>Honeyvine Milkweed</td>
<td>Full sun, disturbed areas, prairies, cities</td>
<td>Low</td>
<td>Medium</td>
<td>Easy</td>
<td>No</td>
</tr>
</tbody>
</table>
This dissertation provides baseline information on monarch utilization of different milkweed species. Future research must investigate how larvae move in the field, and if they can use multiple species of milkweeds as hosts. We also need to understand whether there is a link between the milkweed that a monarch’s mother ingested as a larva, the species on which she lays her eggs, and species that her offspring prefer to eat and oviposit upon. Finally, more work is necessary to elucidate how plant chemicals (cardenolides and quercetin glycosides) vary within individual milkweed stems and how the distribution of these chemicals impacts larval survival and oviposition.

**Literature Cited**


APPENDIX: MILKWEED TRAIT TABLE

Table A.1  Summary of milkweed species traits. Information compiled from Woodson (1954), Eilers and Roosa (1994), and USDA PLANTS (2018).

<table>
<thead>
<tr>
<th>Milkweed Species</th>
<th>Mature Height (m)</th>
<th>Trichomes</th>
<th>Milky Latex</th>
<th>Bloom Period</th>
<th>Vining Ability</th>
<th>Brief Leaf Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. exaltata</td>
<td>0.5-1.5</td>
<td>Absent</td>
<td>Present</td>
<td>April-June</td>
<td>Low</td>
<td>Opposite Leaves</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Broadly ovate blades</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(10-17cm long)</td>
</tr>
<tr>
<td>A. hirtella</td>
<td>0.4-1</td>
<td>Absent</td>
<td>Present</td>
<td>July-August</td>
<td>Low</td>
<td>Alternate Leaves</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Oblong to narrow lance</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>olate blades(7.62 cm</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>long; 1.3 cm wide)</td>
</tr>
<tr>
<td>A. incarnata</td>
<td>0.6-1.8</td>
<td>Absent</td>
<td>Absent</td>
<td>June-August</td>
<td>Low</td>
<td>Opposite Leaves</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Linear to linear-lance</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>olate blades (5-15 cm</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>long; 0.3-1.2 cm wide)</td>
</tr>
<tr>
<td>A. speciosa</td>
<td>0.4-1.5</td>
<td>Present</td>
<td>Present</td>
<td>May-September</td>
<td>Low</td>
<td>Opposite Leaves</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Broadly ovate blades</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(10-17cm long)</td>
</tr>
<tr>
<td>A. sullivantii</td>
<td>0.6-1</td>
<td>Absent</td>
<td>Present</td>
<td>June-August</td>
<td>Low</td>
<td>Opposite Leaves</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Oblong to ovate-oblong</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>blades (7.15cm long;</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>3.3-7.6 cm wide)</td>
</tr>
<tr>
<td>A. syriaca</td>
<td>0.5-2.5</td>
<td>Present</td>
<td>Present</td>
<td>May-August</td>
<td>Low</td>
<td>Opposite Leaves</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Broadly ovate to ellipt</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>ic blades (10-20 cm</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>long; 5-11 cm wide)</td>
</tr>
<tr>
<td>A. tuberosa</td>
<td>0.3-0.7</td>
<td>Present</td>
<td>Absent</td>
<td>June-August</td>
<td>Low</td>
<td>Alternate Leaves</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Linear-oblong to lance</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>olate-oblong blades</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(6.3-9cm long; 1.3-1.9 cm wide)</td>
</tr>
<tr>
<td>A. verticillata</td>
<td>0.15-0.6</td>
<td>Absent</td>
<td>Present</td>
<td>June-August</td>
<td>Low</td>
<td>Opposite Leaves</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Filiform to linear</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>blades (5-7.5 cm long;</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2-3mm wide)</td>
</tr>
<tr>
<td>C. laeve</td>
<td>0.5-3</td>
<td>Variable</td>
<td>Present</td>
<td>July-August</td>
<td>High</td>
<td>Opposite Leaves, cordate,</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>ovate, acute to acuminate</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>blades (10cm long; 8cm wide)</td>
</tr>
</tbody>
</table>