

Monarch (*Danaus plexippus*) Growth and Survival in a Changing World

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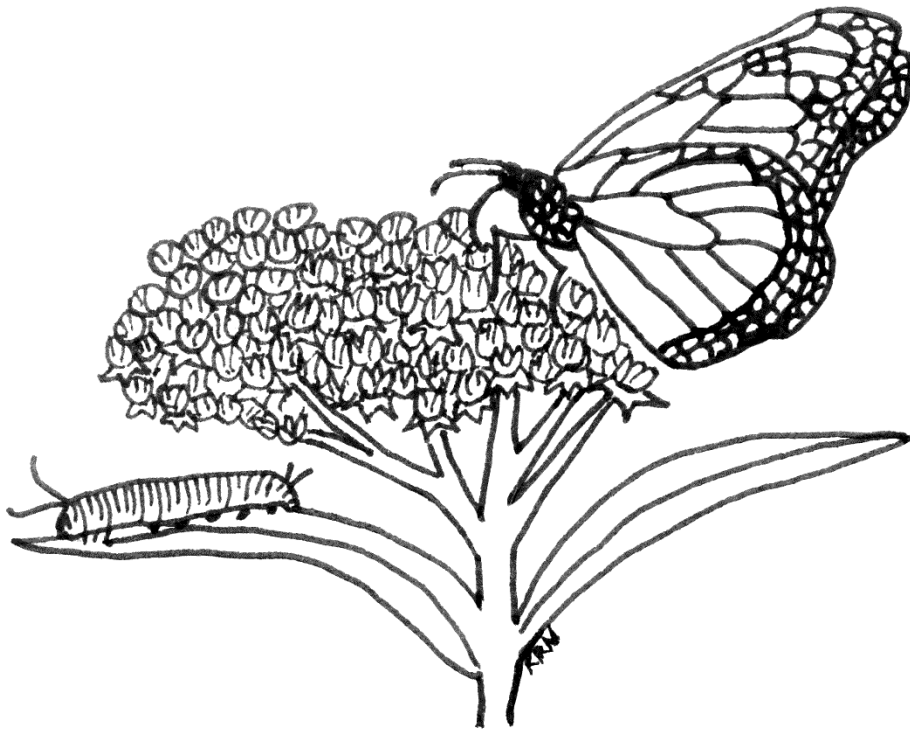
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Dedication

This dissertation is dedicated to all endangered pollinators. My hope is that the work in this dissertation plays a small part in conserving the natural beauty, fascinating life history, and awe-inspiring migration of monarchs, but that it also brings awareness to other insect species in peril. My ultimate desire is that people reading this dissertation in the future still have the chance to see, know, and continue to conserve not only monarchs, but also Poweshiek skipperlings, rusty patched bumble bees, and Karner blue butterflies.

In the name of the Bee -
And of the Butterfly -
And of the Breeze - Amen!
-Emily Dickinson



Abstract

Eastern North American monarch butterflies (*Danaus plexippus*) are well known for being charismatic insects that undergo a yearly long-distance migration. With a world changing in both habitat and climate, and a monarch population in decline, my work attempts to better understand the impacts of these changes and to help better inform conservation efforts. Effective conservation of a migratory species requires an understanding of an organism at all areas throughout its migratory path. My dissertation seeks to understand both the thermal requirements of monarchs at novel overwintering sites along the Gulf Coast and the habitat requirements of monarchs throughout their breeding grounds.

My work on cold tolerance enhances our understanding of the thermal impacts on immature monarchs that overwinter along the Gulf Coast, rather than the more utilized strategy of overwintering as adults in central Mexico. In my first chapter, I investigate the extreme temperature limits of immature monarchs by determining the supercooling points and lower lethal temperatures at different stages (including larval stadia). I then examine how cool temperatures influence immature monarchs, both directly and indirectly, as mediated through the impacts of cool temperatures on the monarch host plant, milkweed (Chapter 2).

In addition to laboratory studies, my dissertation also uses citizen science data to examine the eastern North American monarch population. In Chapter 3, I use a novel combination

of stage-specific citizen science sightings of immature monarchs along the Gulf Coast, along with a growing degree day model, to determine how long monarchs are predicted to remain in the area and to quantify the coldest temperatures that these monarchs are exposed to as immatures. Chapter 4 also uses citizen science data to estimate monarch survival and the habitat site characteristics that are associated with increased survival. Additionally, this work produced an estimate of the number of milkweeds needed to produce a migratory monarch.

The insights that are gained from my dissertation are important to better understand the eastern North American population of monarchs, and combined with other research, can be used to help better inform conservation measures. This dissertation takes multiple approaches across two areas of habitat of monarchs, their northern breeding grounds and the Gulf Coast, through which they migrate through in the spring and fall, and in which they sometimes spend the winter. In a changing and increasingly fragmented world, this unifying approach looking at all aspects of a migratory species will be increasingly useful in conservation.

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Preface

My work with monarchs has always been in the context of conservation biology. Over the course of my time in graduate school, the size of the Eastern North American population of monarchs went from what may have been a few low years to a statistically significant population size decline (Brower et al. 2012, Rendón-Salinas and Tavera-Alonso 2014, Vidal et al. 2014), with the lowest three years ever recorded happening during my tenure at the University of Minnesota. An urgency to understand and conserve monarchs informed and shaped the direction of my research.

I began by investigating the cold tolerance of immature monarchs (Ch. 1) to better understand the thermal effects on monarchs as they employed an alternative strategy of overwintering and breeding along the U.S. Gulf Coast (Satterfield et al. 2015). I combined and published my work on supercooling points and lower lethal temperatures alongside the complementary heat tolerance work of former graduate student Reba Batalden (published in “Monarchs in a Changing World: Biology and Conservation of an Iconic Butterfly” in 2015). This work in turn led to my study of the direct and indirect effects of cooler temperatures on immature monarchs as mediated through milkweed (Ch. 2). This full factorial laboratory study of temperature on both immature monarchs and milkweed provided a better understanding of the *ex situ* physiological effects of cool temperatures. I then used citizen science data to study *in situ* effects of cold temperature on monarchs. By compiling several sources of citizen science winter sightings of immature monarchs along the U.S. Gulf Coast and applying a growing degree day model,

I was able to determine how long observed monarchs would be expected to live and to what temperatures they would be exposed (Ch. 3). Finally, my work with citizen science data sets led my co-authors and I to study immature monarch survival in the wild and factors associated with increased survival (Ch. 4; published in 2015 in *Annals of the Entomological Society of America*). This work also elucidated the number of milkweeds in the North Central region needed to produce one adult monarch in the migratory generation, a measure that is important for conservation, particularly as monarchs are currently being considered for listing as threatened under the Endangered Species Act (Center for Biological Diversity et al. 2014).

My work forms a coherent picture of the effects of cool temperatures on immature monarchs, as well as factors associated with immature survival. My research centers on monarch conservation, whether through better understanding their ability to withstand different thermal conditions or by predicting the number of milkweeds needed in the wild to sustain a healthy population of migratory adults. All chapters are either published or are being prepared for publication in different peer-reviewed publications, which means there are slight differences in formatting between chapters in this dissertation.

CHAPTER 1

WHAT'S TOO HOT AND WHAT'S TOO COLD?

LETHAL AND SUBLETHAL EFFECTS OF EXTREME TEMPERATURES ON
DEVELOPING MONARCHS

SUMMARY

We exposed immature monarchs to extreme cold and hot temperatures for varying lengths of time and quantified both sublethal (adult size and development time) and lethal effects. Larvae have upper and lower thermal limits of 42 and -20 °C. Although most larvae survived short exposure during the daytime to temperatures up to 42 °C, they suffered sublethal effects from temperatures 38 °C and higher, including smaller adult mass and slower development. Nighttime temperatures of 34 °C during periods with daytime temperatures of 38 °C resulted in lower survival, showing that respites from elevated temperatures are important in allowing monarchs to survive temperature stress. Median supercooling points (SCPs) for immature monarchs ranged from -26.1 to -9.6 °C, with eggs having the coldest SCP and third instars having the warmest SCP. Larvae appear to be freeze-intolerant, with 50% mortality not occurring until temperatures fall below each stage's respective SCP; however, eggs seem to be chill-intolerant, with mortality occurring before their SCP. Interestingly, third instars were most susceptible to both cold and heat stress. These findings can help inform future modeling and conservation efforts for monarchs throughout their life cycle.

INTRODUCTION

Monarchs are affected by climate in their wintering sites (Oberhauser and Peterson 2003, Zalucki and Rochester 2004, Ramírez et al. 2015, Williams and Brower 2015) and during the breeding season (Zalucki and Rochester 2004, Batalden et al. 2007), when extensive rain or prolonged cool and cloudy conditions can reduce egg laying and

increase development time, and prolonged hot or dry spells can reduce adult life span and fecundity (Zalucki 1981, Masters et al. 1988, Masters 1993). Zalucki and Rochester (2004) predicted fluctuations in monarch abundance in the eastern North American population due to the effects of climate on their phenology and fecundity (but see Zalucki et al. 2015). Climate can also influence milkweed abundance and quality (Zalucki and Kitching 1982; Zalucki and Rochester 2004).

Work on upper thermal limits of monarchs has included both modeling and empirical studies. Ecological niche models based on occurrence data from the Monarch Larva Monitoring Project (MLMP; see Oberhauser et al. 2015a) predict a marked northward summer range shift for eastern North American monarchs under climate change scenarios (Batalden et al. 2007), driven largely by increasing temperatures. MLMP data show no monarch presence above a monthly mean temperature of 30 °C (all temperatures in this paper refer to degrees Celsius). Continuous exposure to temperatures of 36° causes significant larval mortality (Zalucki 1982), while single or repeated 12-hour pulses of 36° increase development time without increasing mortality (York and Oberhauser 2002). This finding suggests that low nighttime temperatures are important, yet observed climate change and future predictions indicate increased frequency of higher nighttime lows (IPCC 2007).

To our knowledge, no studies have examined the lethal and sublethal impacts of temperatures above 36° or explored the effects of elevated nighttime temperatures on immature monarchs. It is possible they can survive warmer or cooler temperatures than those used to build the models. If monarchs can withstand conditions different from those

they currently inhabit, it is possible that published models (Oberhauser and Peterson 2003; Zalucki and Rochester 2004; Batalden et. al. 2007) do not reflect their actual thermal tolerance and that they will be able to survive the severe changes predicted by climate models. Additionally, none of the niche models incorporate selection on character traits such as thermal tolerance and resulting evolution.

Contrary to the typical strategy of overwintering in Mexico, some monarchs breed in the southern United States throughout the winter, although the proportion of breeding vs. diapausing individuals is not known (Howard et al. 2010; Batalden and Oberhauser, 2015). However, breeding during the winter poses risks; winter temperatures in the southern United States can fall below freezing. While adult monarchs can leave areas with unsuitable temperatures, immature monarchs may be exposed to cold and possibly lethal temperatures.

The lower development threshold for monarch larvae is 11–12° (Zalucki 1982), but lower lethal thresholds for immature stages are poorly understood. Although little is known about immature monarch cold tolerance, much work has been done on insect cold tolerance in general. Insects are classified into three categories of cold temperature-dependent mortality: chill-intolerant, freeze-intolerant, or freeze-tolerant (Lee 2010). Chill-intolerant insects experience mortality before freezing. Freeze-intolerant, or freeze-avoidant, insects survive until their body freezes (i.e., ice nucleation occurs). Finally, freeze-tolerant insects can survive ice formation within a limited temperature range. The classification of the cold-tolerance strategy of monarchs is not known for any life stage. Adult monarchs do not survive internal ice formation (Larsen and Lee 1994), so they may

be freeze-intolerant. Although the supercooling points of adult monarchs is known (-8° if they are dry and -4° if wet; Anderson and Brower 1996), no previous work has assessed freezing points of immature monarchs or cold-mediated lethal and sublethal effects.

METHODS, RESULTS, AND DISCUSSION

In a series of four experiments, we investigated lethal and sublethal impacts of elevated and lowered temperatures on immature monarch survival and development. Experiment 1 determined the upper physiological limits of larval development. Experiment 2 tested the effect of high nighttime temperatures combined with a daytime temperature that caused sublethal impacts (increased development time and decreased size), but no lethal impacts. Experiment 3 measured the supercooling points (SCPs, subzero temperature at which intracellular fluid freezes) of immature monarchs. Finally, experiment 4 determined the lower lethal temperatures of immature monarchs.

In all four experiments, wild-caught monarchs laid eggs on *Asclepias curassavica* in a greenhouse. For experiments 1 and 2, immature monarchs were kept in Percival growth chambers (LD 12:12 h photoperiod; 30° : 25°), except when they were exposed to treatment conditions. In experiments 3 and 4, they were kept in the same growth chambers (LD 15.5:8.5 h photoperiod; 22° : 20°) for at least 12 hours for eggs and 48 hours for all other stages, before being exposed to cold temperatures during a specific stage. After cold exposure, these monarchs were returned to the same growth chamber conditions for the duration of their development. The pre-exposure photoperiod and temperatures in experiments 3 and 4 simulated conditions experienced during winters in

the southern United States. In experiments 1, 3, and 4, individuals were kept throughout their development in separate 500 ml plastic deli containers with ventilation holes in the lids. In experiment 2, they were kept in petri dishes until they became fifth instars and then moved to the deli containers to pupate. In all experiments, rearing containers were cleaned and larvae given fresh milkweed (*A. syriaca*) daily.

Experiment 1: Upper temperature limits of larval development

Experiment 1 was completed in two sequential rounds; round 1 treatment temperatures were 38° and 40°, and round 2, 42° and 44°. All experimental larvae were offspring of wild-caught individuals collected from first-generation monarchs in St. Paul, MN, in June 2007.

Within 12 hours of hatching, individuals were placed in rearing containers and randomly assigned to an experimental group (stage, temperature, and duration of heat exposure). We exposed larvae within 24 hours of hatching or molting into first, third, or fifth instars to 12-hour pulses of 38°, 40°, 42°, or 44° over periods of 1, 2, 4, or 6 days. They were always returned to nighttime temperatures of 25°. Sample sizes ranged from 17 to 20 for each treatment group, including the control, which was kept at 30° during the day and 25° at night.

We observed the larvae daily, tracking mortality and development. We measured development time in days and degree days using 12° as the lower threshold temperature (Zalucki 1982). We assessed two additional effects of heat exposure: the ability to pupate without falling and adult size. If individuals fell from the lids of their containers when they attempted to pupate, we recorded the event and used thread and tape to affix the

cremaster back to the lid. Adult size was measured as mass and right forewing length (i.e., distance from wing base to apex). We measured each individual's mass after its wings dried but before it had fed.

All monarchs exposed to 44° for any length of time died before adulthood; of 234 individuals exposed to 42°, only 16 survived to be adults. Therefore, we could not compare sublethal effects across treatments at these temperatures. Mortality did not differ between control groups in rounds 1 and 2 (Figure 1; 0.125 mortality for both groups, Fisher's exact test, $P = 1.00$), so we compared mortality across all treatments.

Survival varied with temperature, treatment timing, and treatment duration (Figure 1), with higher survival at lower temperatures and shorter treatment duration. Individuals exposed as third instars were less likely to survive to adulthood, particularly after 6 days of exposure, with 70% and 20% surviving at 38° and 40°, respectively. Only 53% of fifth instars survived to adulthood after 6 days exposure to 40°. Overall mortality increased with exposure to higher temperatures; 18% died at 38°, 28% at 40°, and 93.3% at 42°.

Of 451 individuals that survived to pupate in all treatments, 143 dropped from the lids of their containers during or just after pupation. Under natural conditions, falling is likely to result in death, either directly from the fall or from another source, such as predation (although container lids may not provide as good of a substrate to pupate on compared to what a monarch might use in nature). Here, the distance to the floor of the container was small, and when we taped pupae back onto the container lid, subsequent survival was 94%. Consequently, this potentially lethal effect of exposure to heat stress was not represented in our mortality estimates. The proportion of individuals that fell did

not depend on temperature, but individuals exposed as fifth instars were more likely to fall at durations of 4 or 6 days, with 58% and 72% falling, respectively (Figure 2). Individuals exposed as third instars also showed elevated risk of falling, with 33%, 38%, and 46% falling if exposed for 1, 2, or 4 days, respectively. The lack of effect for third instars in the 6-day treatment may be due to the high mortality (and consequent low sample size) of this group. There was no difference in pupation ability whether the individual pupated while still in the treatment or after returning to control conditions ($\chi^2 = 0.218$, $df = 1$, 63 , $P = 0.64$).

Temperature, but not the duration or timing of treatment, affected adult mass. Mass decreased with increasing temperature, and monarchs exposed to temperatures above 38° were lighter than controls. Wing length in the treatment groups did not differ from controls (Table 1).

When we assumed that development rates continue to increase with increasing temperature, development time to adult, measured in degree days, was longer in nearly all treatments compared to controls (Figure 3a, b). This effect was not as strong for fifth instars as it was for first and third instars. However, insects have developmental maxima as well as minima, so we know that our assumption of continually increasing development rates with increasing temperature is inaccurate. Thus, we recalculated degree days to exclude time exposed to elevated daytime temperatures, assuming that development ceased while monarchs were in the heat treatments. The adjusted degree-day totals (Figure 3c, d) reflect all the time spent in control conditions but only the 12 nighttime hours each day (at 25°) spent under treatment conditions. This assumption

worked fairly well for some treatments, resulting in degree-day development times similar to the controls.

It is possible that some individuals acclimated to the hot temperatures, and started to develop under these conditions. With the assumption that development ceases in hot conditions, acclimation could explain the fact that some individuals with longer exposure times spent fewer degree days to develop than control individuals. At 40°, fifth instars exposed for 4 or 6 days used significantly fewer adjusted degree days to develop than controls. The fact that this was not true for first and third instars suggests that younger larvae were less able to acclimate to high temperatures (Figure 3d). Many insects produce heat-shock proteins (HSPs) in response to exposure to high temperatures, including other Lepidoptera (Fittinghoff and Riddiford 1990; Sakano et al. 2006). These HSPs may then in turn increase thermotolerance at temperatures previously unsuitable for development (Neven 2000).

We determined when the developmental lags occurred by detailed tracking of individuals (Table 2; note that only the 40° treatment is shown in this table for illustrative purposes). All individuals exposed to elevated temperatures as first instars took longer to become second instars than control larvae. At 38°, their average time as second instars was not significantly different from control, even though individuals in the 4 or 6 day exposure groups were still exposed to elevated temperatures as second instars. At 40°, their average development times as second and third instars were longer than the control only for larvae exposed for 6 days.

Third instars exposed to 38° did not slow development, but those exposed to 40°

took longer than control individuals to become fourth instars (Table 2). Their development times as fourth instars were not significantly different from control, except for those exposed to 40° for 4 and possibly 6 days.

For individuals exposed to elevated temperatures as fifth instars, 38° did not slow their development, but all those exposed to 40° spent longer as fifth instars than the control group (Table 2). Fifth instars exposed to 40° for 6 days spent more time as pupae than the controls.

Experiment 2: Effects of high nighttime temperatures

Experiment 2 was completed in three sequential rounds. Each round tested a different treatment temperature, which differed from the control during the day and night, rather than only during the day as in experiment 1. Daytime treatment temperature was 38° for all replications and 30°, 32°, or 34° at night in rounds 1, 2, and 3, respectively. We used the same factorial design for treatment timing and duration as in experiment 1, and measured the same lethal and sublethal indicators of heat stress. Larvae for round 1 were F1 offspring of wild-caught individuals collected in St. Paul, MN, and western Wisconsin in June 2008. Rounds 2 and 3 individuals were F2 offspring of these individuals.

Control mortality did not differ among rounds ($\chi^2 = 1.875$, $df = 2$, 57 , $P = 0.39$), so our analysis of mortality includes comparisons across all rounds. Control development time, however, was different across rounds, so we cannot compare this response variable among rounds (ANOVA $F = 3.61$, $df = 2$, 45 , $P = 0.04$). Round 1 was conducted in

midsummer, while rounds 2 and 3 occurred into late fall; thus, milkweed quality may have contributed to the observed changes in development time. Alternatively, differences between F1 and F2 generations could have affected development time.

Across all treatments, mortality increased with length of exposure to high nighttime temperatures longer than 2 days, with 72% and 68% survival after 4 and 6 days, respectively, compared with 81% survival for the control group (Figure 4a). At nighttime temperatures of 34°, only 3.8% of larvae exposed as first instars survived to adulthood (Figure 4b), but exposure at other ages or temperatures did not affect survival.

Experiment 3: Supercooling points of eggs and larvae

We determined SCPs of individual eggs; first, third, and fifth instars; and pupae in a -80° freezer. We used contact thermocouple telemetry and lowered the temperature by approximately 1°/min using a standardized foam-insulated box (Carrillo et al. 2004). Fifth instars were held in filter paper capsules to ensure constant contact with the thermocouple, whereas firsts and thirds were attached to the probe using high vacuum grease. Temperatures were recorded 10 times per second by a multichannel data logger. SCP was recorded as the lowest temperature reached before the observed increase in temperature (latent heat of fusion) indicating a state change of the intercellular fluid from liquid to solid. Sample sizes ranged from 10 to 14 individuals for each developmental stage tested. All experimental individuals were F1 offspring of wild-caught larvae collected in Minnesota and western Wisconsin in summer 2011.

All life stages had median SCPs well below 0°, and no individuals within any of the treatment groups froze at temperatures warmer than -4° (Figure 5). Eggs had the lowest

median SCP of -26.1° , followed by pupae at -17.5° . First instars had a median SCP of -12.4° , and third and fifth instars, -9.6° and -10.3° , respectively. All individuals were lowered to temperatures well below their SCP (below -70°) and there was no survival after removal from the freezer.

Experiment 4: Lower lethal temperatures of eggs and larvae

We measured lower lethal temperatures (LT_{50} , the temperature at which 50% of individuals die from the effects of cold temperature) for eggs and first- and third-instar larvae, using F1 offspring of wild-caught individuals collected in Minnesota and western Wisconsin in summer 2011. We cooled monarchs in groups of 10 (eggs and first instars) or 5 (third instars) at a rate of approximately $0.3^{\circ}/\text{min}$ using a calibrated polystyrene box placed in a -80°C freezer, and then immediately removed them when the desired temperature was reached (Carrillo et al. 2004). We tested three (eggs and first instars) or five (third instars) groups at each temperature; thus total sample sizes at each temperature were 30 (eggs and first instars) and 25 (third instars). We assessed their response to several temperatures, beginning at 0° and decreasing temperatures at intervals of 5° , with the minimum temperature for each age class at least 10° below its SCP. We warmed monarchs gradually and assessed survival by recording the number hatched (eggs) or alive after one day (larvae); larvae were considered alive if they were observed moving or if they moved in response to a tactile stimulus. We also raised monarchs to eclosion to assess post-exposure survival. Our response variable was the proportion of each group that survived; we then used logistic regression on these proportions to determine the LT_{50} .

Some survival was recorded below the median SCP for each life stage, but no survival occurred below the lowest recorded SCP for the respective life stage (Figure 6). The temperature predicted to be lethal for half the population (with standard error) is $-15.6^{\circ} \pm 1.2$ for eggs, $-14.0^{\circ} \pm 0.76$ for first instars, and $-12.7^{\circ} \pm 0.56$ for third instars (Figure 6). Of the initial 359 immature monarchs that hatched from eggs or survived the initial freezing as larvae or pupae, only 85 survived to eclosion, including only 23% of the control group, so we were unable to compare sublethal effects of cold treatments.

CONCLUSION

These experiments provide additional information on immature monarch tolerance of extremely high and low temperatures. Larval mortality increased substantially between 40 and 42°, regardless of the timing or duration of exposure. This suggests a physiological limit between 40 and 42°. No larvae survived any exposure to 44°. Third instars showed higher mortality than either first or fifth instars in the heat stress experiments, particularly when exposed to 40° for 6 days, suggesting that third instars are more susceptible to heat stress. Similarly, York and Oberhauser (2002) found that mortality was higher for third than for first or fifth instars when exposed to 12-hour pulses of 36°. Third instars that survived the initial heat exposure were less able to pupate successfully after being removed to control conditions, indicating a long-term effect of exposure to high temperatures. We cannot explain the increased susceptibility of third instars but suspect that development during this stage is particularly vulnerable to thermal stresses.

Fifth instars exposed for 6 days to 40° also had higher mortality than controls,

possibly because individuals attempted to pupate while exposed to treatment conditions, or because of increased physiological stress as a result of preparing to pupate coupled with heat stress. Fifth instars exposed to elevated temperatures for 4 or 6 days were also more likely to fall as they pupated.

There were also sublethal effects of heat stress. Individuals exposed to higher temperatures weighed less, which could result in decreased male mating success (Solensky and Oberhauser 2009), fecundity (Oberhauser 1997), and survival during migration (Masters et al. 1988; Arango 1996; Van Hook 1996; Alonso-Mejía et al. 1997). There may also be direct effects of exposure to high temperatures on these fitness correlates; it would be interesting, for example, to measure effects on fecundity. Additionally, overall development time increased with exposure to elevated temperatures, which could lead to increased risk of predation during this vulnerable stage (Zalucki and Kitching 1982; Oberhauser et al. 2015b).

High temperatures affected development time in ways that are not easily explained by degree-day calculations; this is expected when individuals are exposed to supraoptimal temperatures. When total degree days were recalculated to exclude daytime hours at temperatures above 38°, the total degree days needed for development did not differ from control individuals for most treatment combinations, suggesting that development stopped during exposure to elevated temperatures. This assumption was less accurate for longer exposure times, suggesting that after a certain amount of time under unfavorable conditions, monarchs no longer confined development to more favorable nighttime hours. This appeared to be true for all larvae exposed to 38° for 6 days, but

only for fifth instars when exposed to 40°. Larvae may be acclimating to higher temperatures, or the rate of development may be determined by a balance between the risks of delayed development and the risks of development at elevated temperatures.

Developmental delays largely occurred while individuals were exposed to heat stress; when they returned to control conditions, their development returned to a normal pace. Larvae appeared to try to escape the hot temperatures; we observed first instars apparently seeking shade under the container labels and older larvae under leaves or the filter paper at the bottom of their container. Individuals also ate less, if at all, under high temperatures. These behaviors were recorded only anecdotally but suggest behavioral changes in response to elevated temperatures, as observed by Serratore et al. (2012) in a field study.

Increased nighttime temperatures also pose a risk. Monarchs exposed constantly to 36° die before adulthood, while repeated 12-hour pulses of 36° do not increase mortality (York and Oberhauser 2002). These findings, coupled with our data showing some survivorship up to 42° when nighttime temperatures were 25°, indicate that a decrease in temperature at night is necessary to cope with extreme temperatures. When the temperature dropped only to 34° at night, first instars suffered substantial mortality, possibly caused by desiccation, since first instars have the highest ratio of surface area to body mass. Dead first instars exhibited signs of dehydration, but because observations were made only once a day, it is unknown whether they desiccated before dying or postmortem. Nighttime temperatures of 34° or lower did not lead to increased mortality for third or fifth instars, suggesting that any decrease in temperature from daytime to

nighttime is beneficial in the short term, as long as temperatures drop below the 36° threshold (Zalucki 1982; York and Oberhauser 2002).

While monarchs can survive short exposures to temperatures above the previously assessed limit of 36°, individuals exposed to higher temperatures were more likely to fall during pupation, weighed less, and developed more slowly. Ecological niche models do not predict monarchs present in areas with a mean monthly temperature above 30° (Batalden et al. 2007), although other models predict monarchs present, but doing poorly, at this temperature (Zalucki and Rochester 2004). Even though monarchs can survive temperatures above 30°, potentially lethal temperatures (40° or higher) as well as temperatures that cause sublethal impacts (38° or higher) are possible within a month with a mean temperature of 30°.

In the cold tolerance experiment, no larvae survived exposure to -20°, but many survived at -10°. For eggs, no hatching was recorded after exposure to -30°, but many eggs hatched after exposure to temperatures as low as -20°. Monarch eggs appear to be chill-intolerant, as their LT_{50} (-15.6°) is warmer than the median SCP (-26.1°). Conversely, first- and third-instar larvae appear to be freeze-intolerant, with half the monarchs dying at or near their respective SCPs. These findings show that monarchs can survive brief periods of cold, as much as 22° to 37° below the stage-specific developmental zeroes reported by Zalucki (1982). However, the high mortality rates in the control group (reared in cooler, late-fall Texas temperatures) suggest that extended periods at cool temperatures (warmer than the SCP and LT_{50} s) may also be lethal; hence, mortality of both mature and immature monarchs resulting from extended exposure to

nonlethal cold conditions should be tested with future experiments (see Chapter 2- this volume).

Species can respond to climate change in three general ways: movement, adaptation, or extirpation (Holt 1990). Mobile species may be able to track their ecological niches as the climate changes; there is evidence that some European and North American butterflies have done so (Parmesan et al. 1999; Parmesan and Yohe 2003; Crozier 2004; Breed et al. 2012). According to ecological niche models, in the future, monarchs will need to move northward from their current range in June and July, and then return southward in August to track the conditions they currently use for reproduction (Batalden et. al. 2007). Currently, only the spring generation appears to move northward before laying eggs (Journey North 2013); during most of the summer, monarchs remain in approximately the same geographic range (MLMP 2013).

It is unclear whether monarch summer generations will respond to suboptimal (i.e., too hot) conditions with movement. If, under a changed climate, an individual monarch survives a stressful temperature regime as a larva, it might attain greater fitness by relocating as an adult, perhaps moving north before laying eggs to promote offspring fitness. Such movement occurs in the spring generation, but the mechanisms that prompt migration out of the southern United States are unclear. If monarchs are to track their moving thermal niche (the area with a range of temperatures in which they can survive) throughout the summer, they will probably need to respond to different cues from those that currently trigger spring or fall migration.

Climate models predict that the overwintering grounds in Mexico may soon be

unsuitable for monarchs (Oberhauser and Peterson 2003) and oyamel fir trees (Ramírez et al. 2015), indicating that the eastern North American monarch population may require different overwintering habitat. Whether monarchs can successfully overwinter in other areas depends in part on their being able to survive the colder temperatures and different habitats present in areas such as the southern United States. The absolute minimum temperatures present in the southern United States are warmer than the LT_{50s} for all stages tested, indicating that extreme cold temperatures are unlikely to be a limiting factor for monarchs overwintering there, although we have measured cold tolerance only of monarchs and not their host plants. There may be time for a frozen host plant to regenerate leaves in time to provide food for an egg or possibly a first instar; however, this is unlikely for a later instar. On the other hand, pupae exposed to freezing temperatures do not depend on fast regeneration of milkweed leaves. Thus, the risks imposed by freezing temperatures are likely to vary with different monarch stages, and understanding the effects of freezing on milkweed plants, including leaf regeneration times, will help us interpret the results reported here.

Table 1. Adult mass and right wing length by treatment temperature in Experiment 1.

Temp (°C)	Male mass (g) (SE)	Female mass (g) (SE)	Male RWL (mm) (SE)	Female RWL (mm) (SE)
38	0.59 (0.006) ^a	0.55 (0.007) ^a	52.4 (0.22) ^a	52.0 (0.23) ^a
40	0.57 (0.007) ^b	0.52 (0.007) ^b	51.7 (0.24) ^b	51.2 (0.24) ^b
42	0.52 (0.025) ^c	0.49 (0.023) ^b	50.0 (0.86) ^b	50.8 (0.83) ^{ab}
Control	0.61 (0.02) ^a	0.57 (0.02) ^a	51.2 (0.64) ^{ab}	51.3 (0.55) ^{ab}

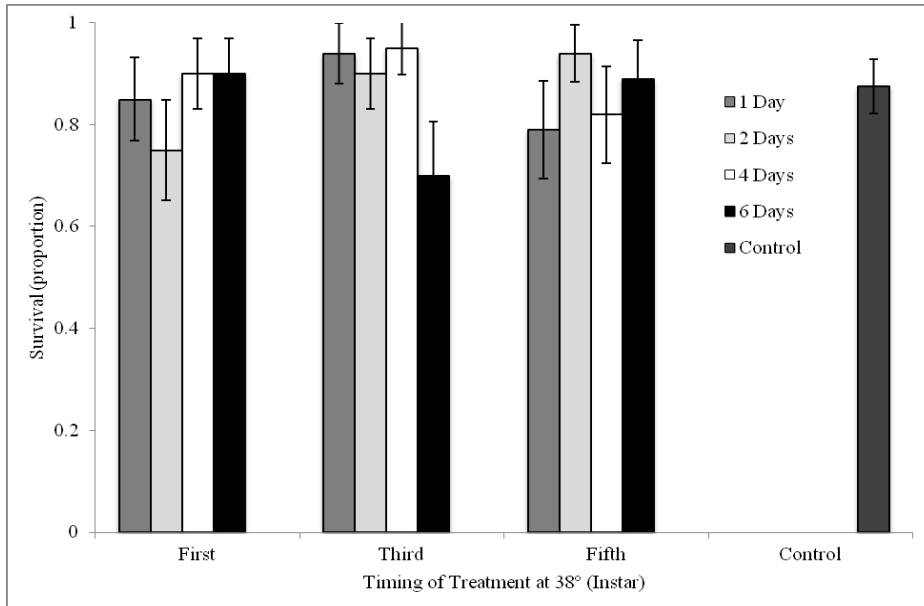
Note: Different letters indicate treatments that are significantly different within columns (ANOVA, Tukey LSD tests, $P < 0.05$).

Table 2. Development time (followed by sample size) for each larval instar by each factorial combination of treatment, timing, and duration.

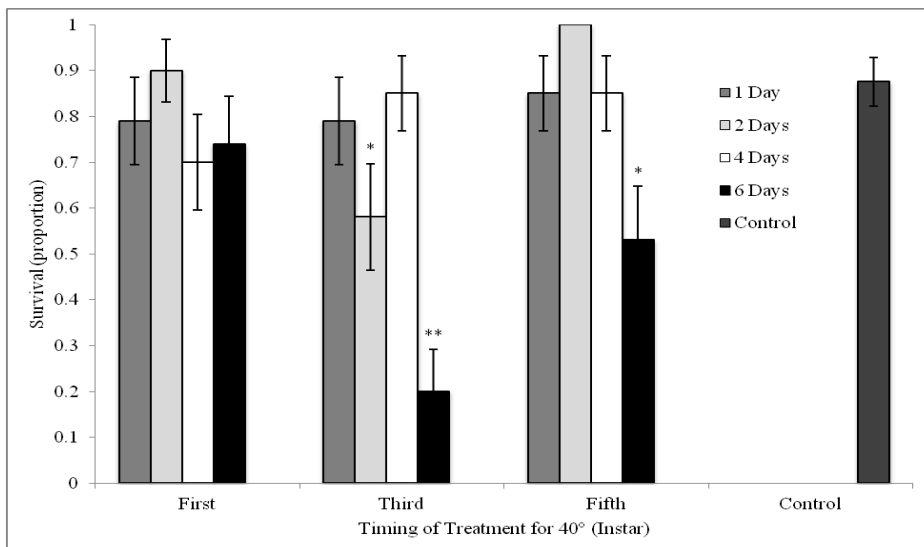
Timing (instar)	Duration (days)	1st	2nd	3rd	4th	5th	Pupa	Total
Control	Control	2.1 (40) ^a	1.72 (39) ^a	1.87 (39) ^a	2.42 (38) ^{abcde}	3.74 (39) ^a	8.10 (36) ^{ab}	19.94 (36) ^a
1	1	2.71 (17) ^b	1.71 (17) ^a	2.00 (16) ^{ab}	2.19 (16) ^{abc}	3.75 (16) ^a	8.07 (15) ^{abc}	20.47 (15) ^{abcd}
	2	2.83 (18) ^b	2.12 (17) ^a	1.95 (19) ^{ab}	2.26 (19) ^{abcd}	4.00 (19) ^{abcd}	8.00 (18) ^{abcd}	21.00 (18) ^{cdefg}
	4	2.80 (15) ^b	2.29 (14) ^{ab}	2.00 (14) ^{ab}	2.29 (14) ^{abcdef}	4.00 (14) ^{abcde}	8.00 (14) ^{abcd}	21.43 (14) ^{defg}
	6	2.84 (19) ^b	2.89 (18) ^b	2.69 (15) ^b	2.67 (15) ^{abcdef}	4.13 (15) ^{abcde}	8.00 (15) ^{abcd}	23.40 (15) ^h
3	1	–	–	2.61 (17) ^b	1.82 (17) ^a	4.12 (17) ^{abcde}	8.20 (15) ^a	19.93 (15) ^{ab}
	2	–	–	2.53 (13) ^{ab}	2.77 (13) ^{abcdef}	4.00 (12) ^{abcde}	8.18 (11) ^a	21.00 (11) ^{bcdefg}
	4	–	–	2.61 (19) ^b	3.21 (19) ^f	4.22 (18) ^{abcde}	7.88 (17) ^{bcd}	21.82 (17) ^g
	6	–	–	2.59 (13) ^b	3.23 (13) ^{ef}	4.67 (6) ^{abcdef}	7.75 (4) ^{bcd}	22.25 (4) ^{efgh}
5	1	–	–	–	–	4.56 (18) ^{bcdef}	8.18 (17) ^a	20.24 (17) ^{abc}
	2	–	–	–	–	5.05 (20) ^f	7.85 (20) ^{cd}	20.65 (20) ^{abcde}
	4	–	–	–	–	5.21 (19) ^f	8.00 (17) ^{abcd}	20.59 (17) ^{abcde}
	6	–	–	–	–	5.31 (13) ^f	8.67 (12) ^e	21.42 (12) ^{defg}

Notes: Only the 40° treatment is shown, for illustrative purposes. Shaded values represent time larvae spent in the heat treatment. Times are shown as mean number of days for each instar. Treatment combinations followed by different letters are significantly different within columns (ANOVA, Tukey LSD tests, $P < 0.05$).

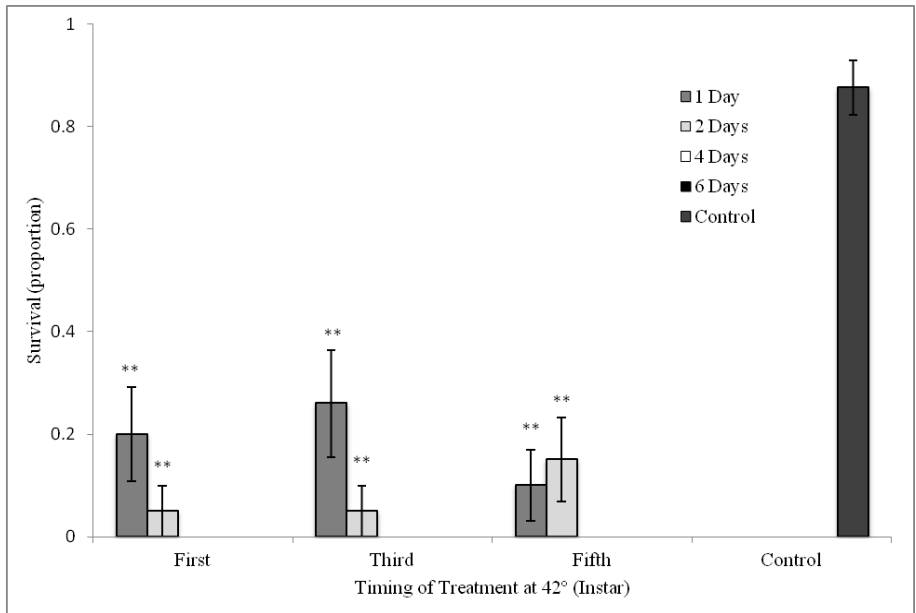
Figure 1. Probability of survival by treatment timing and duration for 38° (a), 40° (b) and 42° (c). * and ** indicate treatment combination is significantly different from control with $p < 0.05$ and $p < 0.01$, respectively. Error bars represent standard error. There was no survival of monarchs exposed to 44° for any length of time.



1a.



1b.



1c.

Figure 2. Probability that pupae fell by treatment timing and duration. * indicates treatment combination is significantly different from control with $p < 0.05$. Error bars represent standard error.

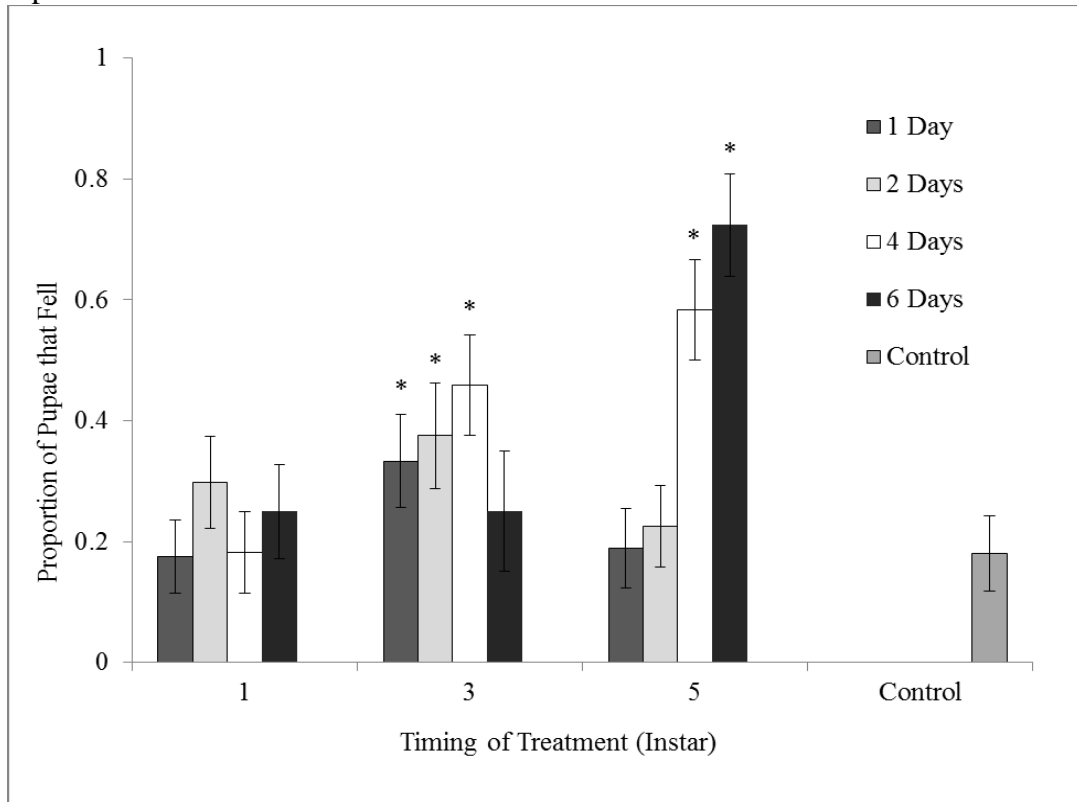
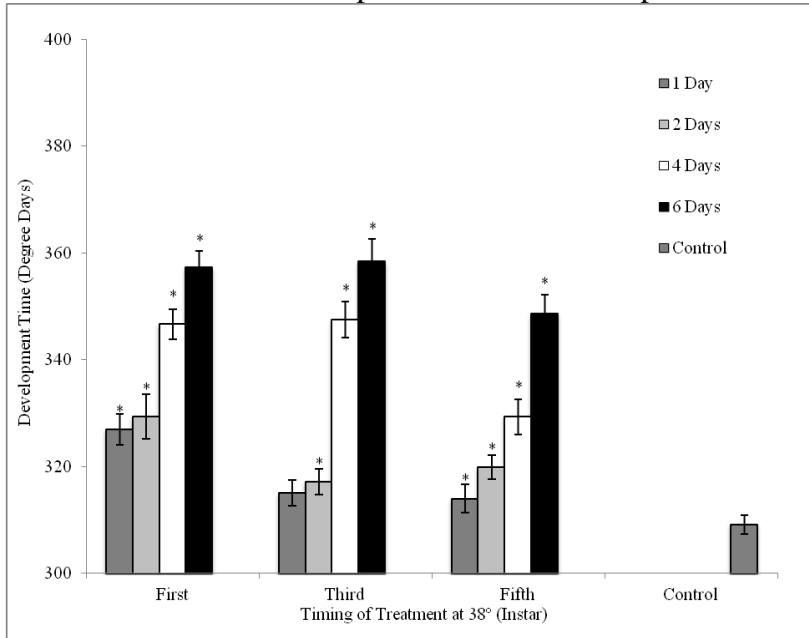
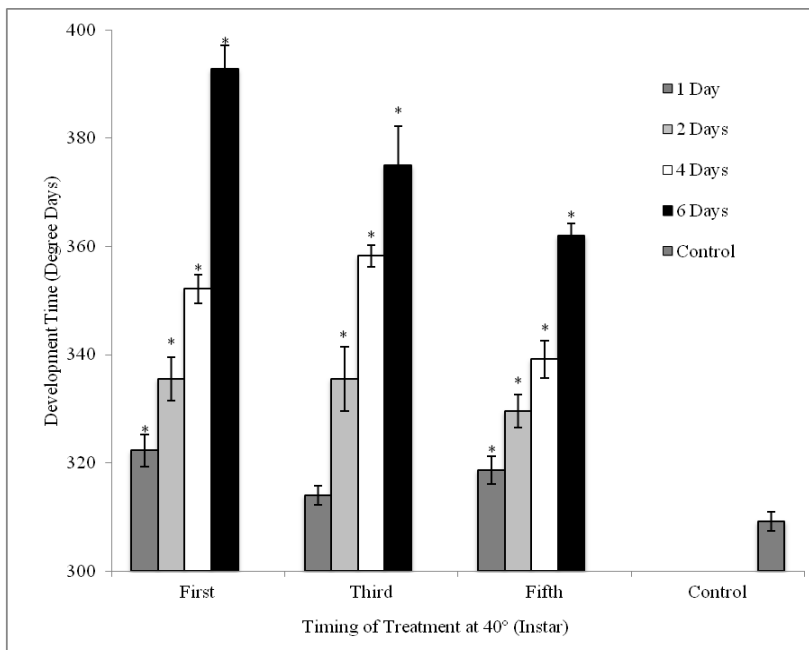


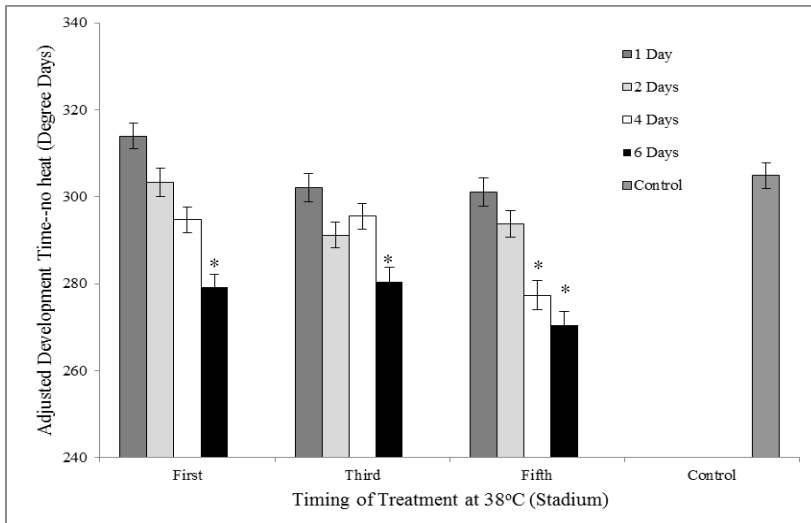
Figure 3. Development time, measured in degree days, by treatment timing (first, third, or fifth instar) and duration at 38° C (a) and 40° C (b). Adjusted development time, measured by not including time spent at elevated temperatures, by treatment timing and duration at 38° C (c) and 40° C (d). * indicates treatment combination is significantly different from control with $p < 0.05$. Error bars represent standard error.



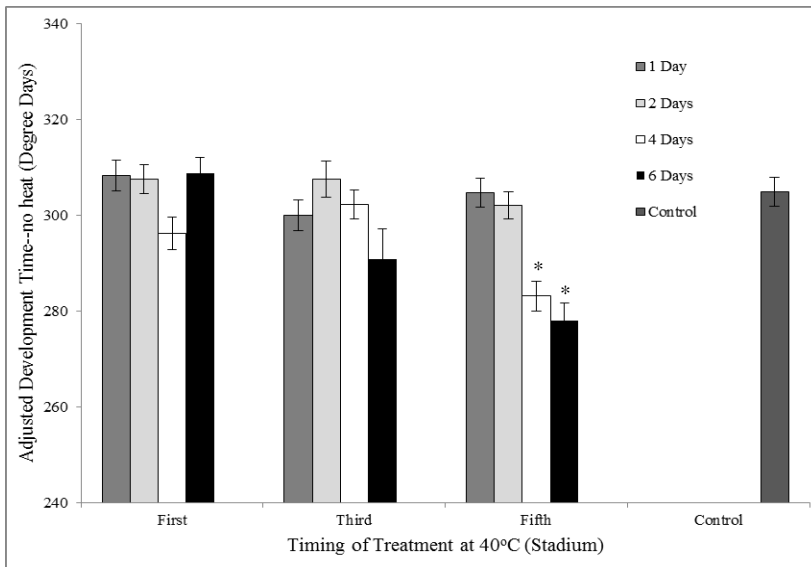
3a.



3b.

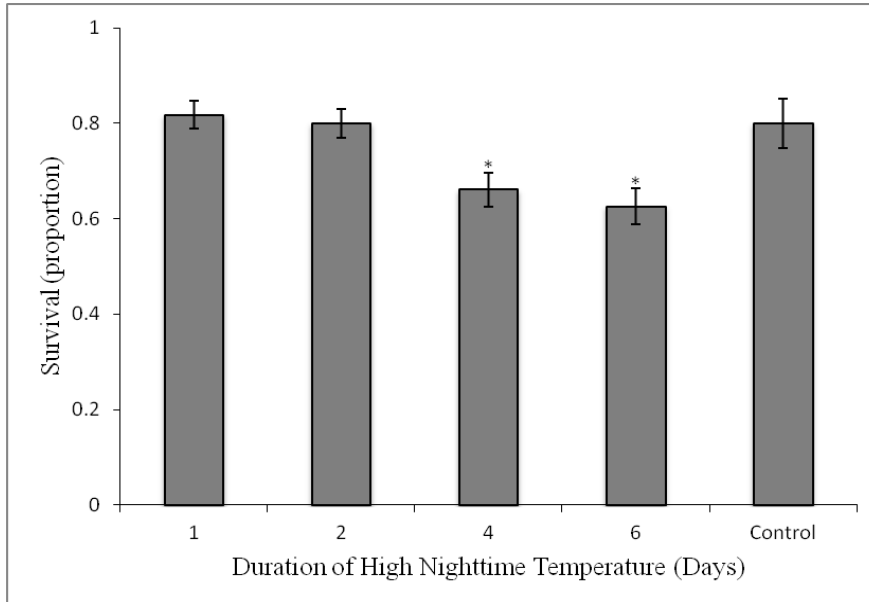


3c.

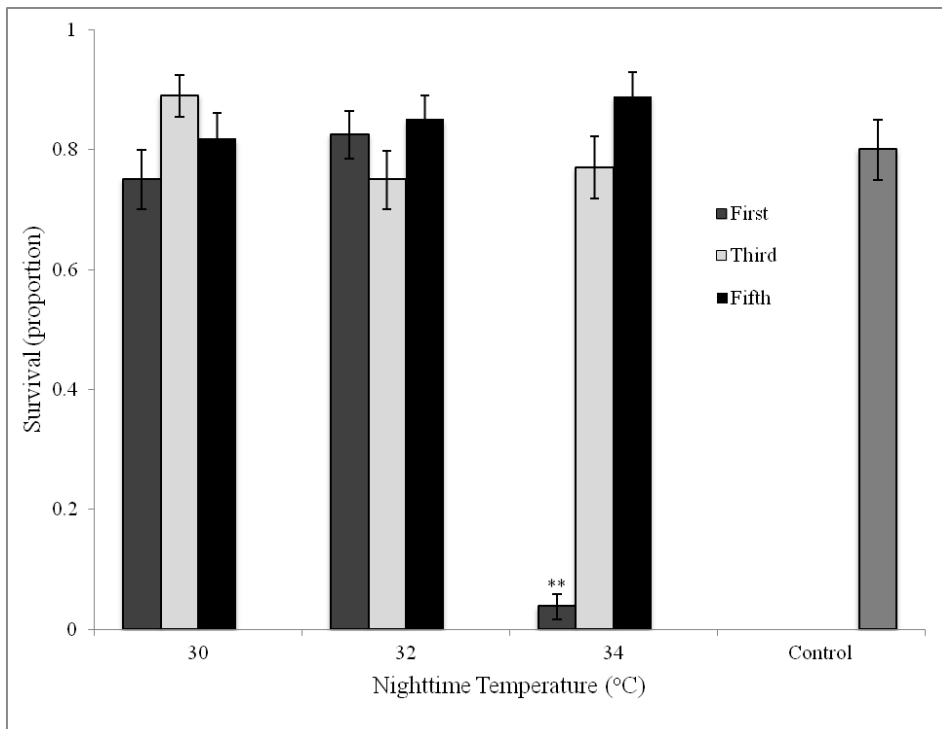


3d.

Figure 4. Probability of surviving elevated nighttime temperatures when daytime temperature was 38° by treatment duration (a) and temperature and timing (b). * and ** indicate treatment combination is significantly different from control with $p < 0.05$ and $p < 0.01$, respectively. Error bars represent standard error.



4a.



4b.

Figure 5. Box plot of observed supercooling points along with LT_{50} s for respective age classes. Line inside box represents the median SCP, with the box representing the first to third quartile of SCP data. Dashed lines outside the box represent the range of data, with outliers represented by open circles (outliers are data points more than $1.5 \times$ Interquartile Range). Sample sizes are listed in parentheses. LT_{50} s for eggs, and first and second instars shown for comparison.

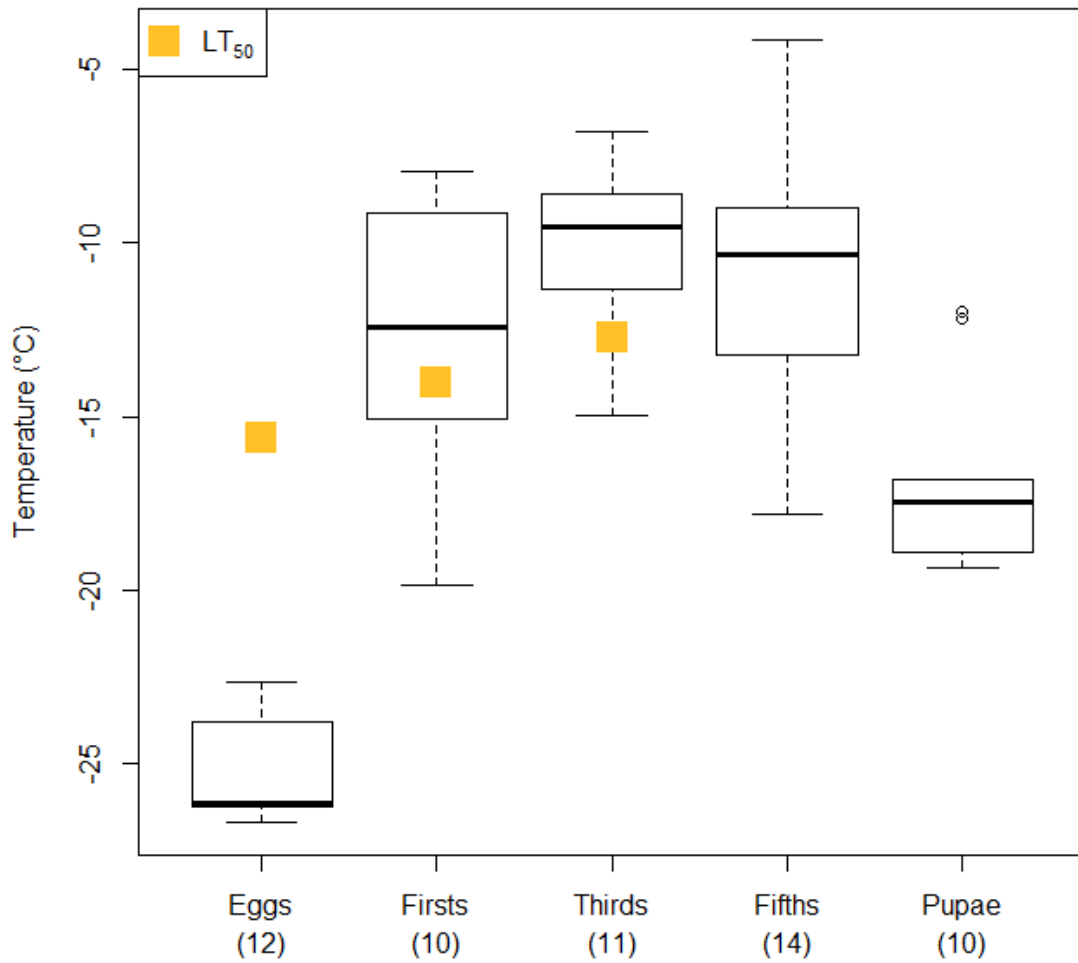
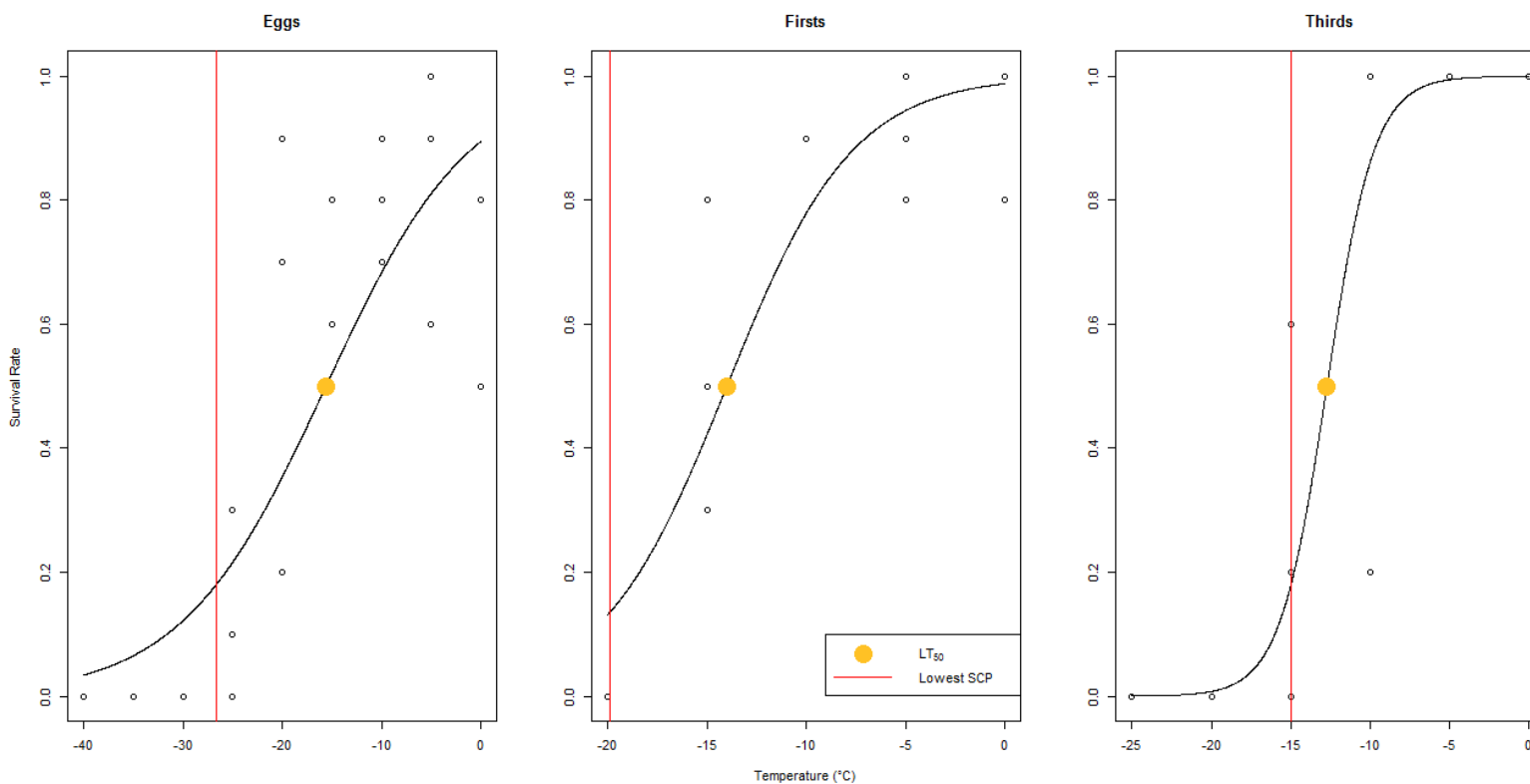


Figure 6. Lower lethal temperatures for monarch eggs, first and third instar larvae. Logistic regression fitted lines used to calculate LT_{50} for each stage: Eggs= $1/(1+e^{-(2.136 + 0.137 * \text{temperature})})$; Firsts= $1/(1+e^{-(4.406 + 0.314 * \text{temperature})})$; Thirds= $1/(1+e^{-(8.509 + 0.669 * \text{temperature})})$. Vertical lines on each graph represent the lowest recorded SCP for that life stage, and closed dots represent the predicted temperature at which half of the larvae would die (LT_{50}).



Supplementary Text S1.1: While this dissertation is written by the dissertation author, the manuscript version of this chapter has the following authorships and affiliations listed below. Rebecca Batalden conducted the first two experiments on heat tolerance, while Kelly Nail conducted the last two experiments on cold tolerance.

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CHAPTER 2

MONARCHS IN A CHANGING CLIMATE: DIRECT AND INDIRECT EFFECTS OF COOLER TEMPERATURES

SUMMARY

An increasing proportion of the population of eastern North American monarchs (*Danaus plexippus*) have been overwintering and breeding along the Gulf Coast throughout the winter, rather than migrating to and overwintering in Mexico in reproductive diapause. These Gulf Coast monarchs most commonly utilize tropical milkweed (*Asclepias curassavica*), a non-native host plant that is typically found in human-maintained landscapes. We investigated the extent to which monarch growth and development are affected by the low temperatures experienced along the Gulf Coast in the winter and compared these to the effects of moderate summer breeding ground temperatures. In addition to the direct effects of temperature, we also examined whether monarchs are affected indirectly by milkweed grown under different temperature regimes, and whether cardenolide concentration is affected by temperature. Monarch temperature treatment was the only consistently significant factor correlated with immature monarch development. While warm temperature monarch growth aligns well with growing degree day calculations, the effects of cool temperature monarch development were variable, particularly between experimental blocks. Adult and pupal mass were both higher in the cool monarch treatment, but forewing length was not affected by monarch temperature treatment in a consistent way. Cool temperatures may be associated with lower cardenolide concentration, but the variability between experimental blocks suggests that other factors influence cardenolide concentrations. These results show that the average temperatures of winter sites along the Gulf Coast are likely to directly increase

development time, but not indirectly mediated through milkweed. We discuss the implications of this study for winter breeding Gulf Coast monarchs.

KEY WORDS

Danaus plexippus, *monarch*, *Lepidoptera*, *Asclepias curassavica*, *milkweed*, *cardenolides*, *climate change*, *cold tolerance*

INTRODUCTION

Eastern North American monarch butterflies (*Danaus plexippus*) are one of the most well-known insects, both for their conspicuous bright coloration and their incredible annual migration. This migration takes monarch butterflies up to 4,500 km from their summer breeding grounds in the northern U.S. and southern Canada south to overwintering sites in central Mexico (Solensky 2004). While fall migratory monarchs are typically in diapause (a non-reproductive state), a subset of this population winters along the Gulf Coast of the U.S. and continues to reproduce (Howard et al. 2010). When this phenomenon began is unknown, but sightings of winter breeding monarchs appear to be increasing in recent years (Satterfield et al. 2015). More winter breeding monarchs could be due to the increased use and maintenance of tropical milkweed (*Asclepias curassavica*) and warmer temperatures that have made the habitat more suitable (Batalden and Oberhauser 2015).

Monarch reproduction throughout the winter in Texas and other Gulf Coast states means that the immature stages (eggs, larvae, and pupae) are potentially exposed to cooler temperatures than they would encounter in their summer breeding grounds (Nail et al. 2015b). As ectotherms, immature monarchs develop more slowly in cooler temperatures until reaching a developmental zero, or low temperature at which development is halted (approximately 12.2°C for eggs, 11.5°C for larvae, and 13.5°C for pupae; Zalucki 1982, Chapter 3- this volume). The lower extreme temperature limits that monarchs can survive have been quantified, and show that monarchs can survive temperatures below 0°C, but

freeze and die between -0.5 and -26.1°C , depending on their developmental stage, whether they are wet or dry, and their surrounding environment (Larsen and Lee 1994, Anderson and Brower 1996, Nail et al. 2015b). Temperature is a primary driver of where adult monarchs can survive in their wintering grounds (Ramírez et al. 2015, Williams and Brower 2015) and throughout their breeding grounds (Zalucki and Rochester 2004, Batalden et al. 2007). In addition to affecting survival, temperature can affect many other aspects of monarch biology, from coloration of larvae and adults (Solensky and Larkin 2003, Davis et al. 2005) to oviposition and lifespan (Zalucki 1981, Oberhauser 1997) to diapause induction and migration (Goehring and Oberhauser 2002, Guerra and Reppert 2013). While the direct effects of hot and cold temperatures on adults and hot temperatures on immature monarchs are well documented, there has been little research on the direct and indirect effects of cold temperature on the growth and survival of immature monarchs (but see Nail et al. 2015b).

In addition to suitable temperatures, monarchs also require milkweed (*Asclepias* spp. and a few other closely related genera) as their host plant (Malcolm and Brower 1986). Gulf Coast winter breeding monarchs almost exclusively use the non-native tropical milkweed, *A. curassavica*, since native host plants are not consistently available during the winter (Batalden and Oberhauser 2015). Throughout much of the Gulf Coast, tropical milkweed is primarily available in cultivated gardens, where it is weeded and watered, but it has naturalized in Florida (tropical milkweed is native to South and Central America; Woodson 1954). In addition to being newly available to monarchs north of

Mexico, *A. curassavica* has interesting effects on monarch fitness. Monarchs sequester cardiac glycoside toxins (cardenolides) from milkweed to become less palatable to predators. High toxicity milkweeds, such as *A. curassavica*, can also cause lowered survival and growth in monarchs (Zalucki et al. 2001a,b), but they might also provide some resistance against the debilitating protozoan parasite, *Ophryocystis elektroscirrha* (*OE*) (Lefèvre et al. 2010, 2012; Sternberg et al. 2014). Conversely, the continual availability of tropical milkweed and thus year-round monarch populations may promote *OE* infections, as the novel interaction between the plant and insect does not provide migratory escape or migratory culling to prevent disease spread (Altizer et al. 2011, Satterfield et al. 2015).

In addition to affecting immature monarch growth and survival directly, temperature may affect the milkweed that monarchs consume. Low temperatures, among other factors, have been shown to reduce the production of toxins in some plants (Parker and Williams 1974). Additionally, recent studies have suggested that plants raised in cooler conditions can provide more nutrient-rich food, hence promoting more lepidopteran growth (including increased body mass and decreased development time), regardless of the temperature regime in which the larva itself was grown (Bauerfeind and Fischer 2013). Previous work on a monarch congener (*D. chrysippus*) has shown that cooler temperatures may cause larvae to consume more milkweed and grow larger (Mathavan and Pandian 1975). The direct and indirect effects of temperature on milkweed composition, and in turn, monarch growth and survival, remain to be seen.

With the eastern North American monarch population is currently experiencing a statistically significant decline (Brower et al. 2012, Vidal et al. 2014, WWF 2016), it is imperative to understand the potential effects of changing temperature regimes on monarch development and survival. Monarchs breeding throughout the Gulf Coast are likely to continue inhabiting the area, so understanding how temperature affects immature monarchs both in this region and elsewhere is important for future conservation efforts, particularly if the portion of the population overwintering in Mexico continues to decrease. Using a full factorial design, our study addresses two key questions about the ways in which immature monarchs might cope with different temperature regimes: 1) How does temperature affect the growth and survival of immature monarchs raised on tropical milkweed? and 2) How does the temperature at which milkweed was grown affect the cardenolide concentration of milkweed and, also, monarch growth and survival (separate from the direct effects of temperature)? Based on previous research and our experiments on thermal limits, we hypothesize that: (1) monarch growth will be slowed by cooler temperatures at a rate greater than what would be expected due to degree day calculations and that, similar to past studies, monarchs reared in cooler conditions will be more likely to die, but that monarchs in this treatment that survive will be larger (Wensler 1977, Nail et al. 2015b). Additionally, (2) based on studies of other plants, cool temperatures may reduce toxicity and make the milkweed more nutrient-rich, possibly promoting monarch growth and decreased development time.

METHODS

Experimental monarchs were F1 and F2 progeny of wild caught Minnesota adult monarchs. These monarchs were lab reared from mid-summer through the fall and winter, approximately when monarchs from the migratory generation are seen breeding in and along the Gulf Coast. Monarchs were reared in Percival incubators at two different temperature regimes, with the cooler regime approximating winter temperatures in Houston, a major Gulf Coast city with many documented winter monarch sightings (cool: L:D 16:8; 18:13°C). The warm temperature regime was approximately what monarchs experience in central Minnesota in June, when monarchs have returned to this region (warm: L:D 16:8; 26:21°C). We kept the photoperiods the same between both treatments to separate the effects of temperature from those of light. Humidity was kept high by placing a bowl of water with a large sponge at the bottom of each incubator. Tropical milkweed was reared from seed (from Outsidepride.com, Inc.) in two Conviron E15 growth chambers with the same temperature regimes as the monarchs. Plants were watered *ad libitum*, fertilized bi-weekly, and rotated randomly within the growth chamber to ensure they received equal lighting. Over the course of this entire experiment, we used predatory mites [*Amblyseius cucumeris*] to control thrips, soil dwelling mites [*Stratiolaelaps scimitus*] to control fungus gnats and thrips, and lady beetles [*Hippodamia convergens*] to control aphids. On the day an individual monarch larva hatched, it was randomly assigned to be reared in either warm or cool conditions, and then further assigned to be fed milkweed from cool or warm conditions. Monarchs were fed fresh milkweed as needed and containers were cleaned daily (containers were Petri dishes

through the third stadium, pint size plastic deli containers for the fourth stadium through pupation, and quart plastic deli containers through eclosion). Monarch developmental stage, including larval stadium, was recorded daily. Additionally, pupal mass (~24 hours after pupation), monarch sex, forewing length (FWL), and adult mass before nectaring were recorded (~24 hours after eclosion). Pupal mass was only recorded starting at the third experimental block, resulting in a smaller sample size (Table 1). Monarchs were also non-destructively checked for *OE* infection (Altizer et al. 2000). Due to space and time constraints, we conducted five rounds of experiments (hereafter referred to as experimental blocks).

To determine the effect of temperature on the composition of milkweed, leaves from both temperature conditions were collected. Milkweed samples were taken two different times: at the beginning of experimental block 1 and during the middle of trial 5. Multiple leaves were taken from various parts of different plants in order to capture cardenolide concentration variability that may have been present. These leaves were freeze dried (FreeZone Cascade Benchtop Freeze Dry System; Labconco Corp.), and the cardenolides extracted. The cardenolides from each sample were run through high-performance liquid chromatography (HPLC) at facilities at Western Michigan University, using digitoxin and *Calotropis procera* extracts as internal standards. Cardenolide concentration in the plant tissue from both treatments was determined by summing all peaks from HPLC output (Rasmann et al. 2009). Peaks were considered cardenolides if they had a

symmetrical absorbance maximum detected between approximately 207 and 222nm (Malcolm and Zalucki 1996).

We used analysis of variance (ANOVA) to examine the effects of temperature treatment, host plant treatment, experimental block, and all interactions on development time, and on pupal and adult morphometric measurements. Logistic regression was used to examine these same effects on survival. We further examined significant relationships between temperature treatments and measured traits using Least Squares Means (LSM). T-tests were used to examine the effects of temperature on milkweed cardenolide concentration. Residuals were examined to verify that all model assumptions were met. Unless otherwise specified, all error measurements are standard error. All analyses conducted in R 3.2.3 (R Core Team 2015).

RESULTS

Between late 2014 and early 2016, a total of 383 monarchs were reared; only 60 survived to the adult stage (Fig. 1). Mortality was spread relatively evenly between the four treatment combinations (Fig. 1), suggesting that it was not related to either monarch or milkweed temperature (see also *Survival, Sex, and OE* sub-section).

Immature development time

Time in each stage/stadium was significantly affected by monarch temperature treatment, and experiment block (Table 1). The first instar was completed 3.2 days slower in the

cool monarch temperature treatment than in the warm monarch treatment (cool monarch treatment = 5.7 days \pm 0.11; warm monarch treatment = 2.5 days \pm 0.10). This difference was similar in other stadia and in the pupal stage (Table 2). In total, average monarch development time in the cool monarch temperature treatment was 58.6 days (from hatching to adult eclosion) and the total development time in the warm treatment was 24.0 days, compared to the predicted 82.2 days and 25.9 days based on growing degree day models for cool and warm temperatures, respectively (Table 2; Zalucki 1982).

Milkweed temperature treatment did not significantly affect development time of any stadium or stage. Experimental block and the interaction with monarch temperature treatment were also significant predicting factors of developmental time for most stadia. These relationships were complicated and varied, but in general, the cool monarch treatment tended to have more variation in larval development times between experimental blocks than the warm monarch treatment (Figure 2).

Pupal and adult measurements

Pupal mass: Monarch temperature treatment was the only factor significantly correlated with pupal mass. Monarchs had a greater average pupal mass in the cool treatment (1.10 g \pm 0.03) than those in the warm treatment (0.92 g \pm 0.04).

Forewing length and adult mass: Milkweed temperature treatment, block, and the interaction between block and monarch temperature treatment were all significant

predictors of forewing (FWL). Monarchs fed milkweed from the warm treatment had slightly longer wing lengths ($47.68\text{mm} \pm 0.69$) than did those fed milkweed from the cool treatment ($45.25\text{mm} \pm 0.71$). Monarch temperature treatment, block, and the interaction between block and monarch temperature treatment were all significant predictors of adult mass. Adult monarchs grown in warm conditions were smaller before nectaring ($0.44\text{ g} \pm 0.03$) than those grown in cool conditions (0.53 ± 0.03).

Survival, Sex, and OE:

A total of 60 monarchs survived to adulthood. Survival was significantly correlated with experimental block, with blocks 1 and 3 having higher survival rates. Survival was not significantly correlated with either monarch or milkweed temperature treatment (Fig. 1). Out of the surviving adults, 53% (31) were females and 47% (28) were males (one individual did not fully eclose and was not sexed). Sex was added as a predictor to the full model for all traits measured, but was not significant for any trait and thus removed. No adults were infected with *OE*.

Milkweed cardenolide concentration:

In the first sample (experimental block 1), some thrips were present, but by experiment 5, thrips had been controlled using biocontrol agents. Additionally, experimental blocks 3, 4, and 5 used a different set of growth chambers due to facility availability. In the first samples (block 1), there was a statistically significant difference ($p = 0.028$; $t = 3.05$, $df = 5.01$) between the average cardenolide concentration for the warm ($10.25\text{ }\mu\text{g}/0.1\text{g dry}$

plant weight ± 3.63 , $N=6$) and cool ($0.90 \mu\text{g}/0.1\text{g}$ dry plant weight ± 0.11 , $N=7$) treatments. For the second sample (experimental block 5), the warm treatment milkweed also had a higher cardenolide concentration ($92.78 \mu\text{g}/0.1\text{g} \pm 25.09$, $N=4$) than the cool treatment milkweed ($21.92 \mu\text{g}/0.1\text{g} \pm 6.37$, $N=4$), but the difference was only marginally significant ($p = 0.068$, $t = 2.74$, $df = 3.39$). In both blocks, milkweed plants in the cool condition were shorter and had smaller leaves (based on visual observations only; data on plant size were not collected).

DISCUSSION

Immature monarch development time appears to be driven primarily by the temperatures that the monarch experienced directly, and not significantly affected by temperature indirectly mediated via milkweed. This direct effect of temperature was expected based on previous studies (Zalucki 1982, Solensky and Larkin 2003). However, unlike previous work (Bauerfeind and Fischer 2013), there appears to be no indirect effect of the host plant (milkweed) on monarch growth and development, even with large differences in milkweed cardenolide concentration.

The average development times for the cool and warm monarch temperature treatments were 58.6 and 24.0 days, respectively. These times were similar to those seen in previous studies where there was no difference in milkweed [monarchs reared at 17°C and 25°C took 56 days and 24 days to develop, respectively (Wensler 1977)]. As predicted, however, the average development time of the monarchs in the cool temperatures (58.6

days) was 28.7% less than what would be predicted by degree day calculations (82.2 days), but the warm temperature treatment was close to the predicted days (actual: 24.0 days, predicted: 25.9 days; 7.3% less than expected). Monarch degree day calculations are based on a linear model (Zalucki 1982), which works for many temperatures monarchs experience, but doesn't capture the "ends" of the sigmoidal curve at extreme temperatures (Zalucki 1982, Chapter 3- this volume).

Adult and pupae has less mass in the warm monarch temperature treatment than in the cool monarch temperature treatment. Adult forewing length was the only measured variable that was significantly correlated with milkweed temperature treatment, although the difference (2.43mm) may not be ecologically significant. These results align with previous research (York and Oberhauser 2002, Nail et al. 2015b, Mathavan and Pandian 1975), where extreme hot temperatures produced individuals with lower mass [although Lemoine and colleagues (2015) found no difference in mass between temperature treatments when looking at temperatures below 30° C). Although monarch and milkweed temperature treatments had significant effects on adult measurements, our work shows that neither of these treatments had any effect on monarch survival (see also Lemoine et al. 2015).

Milkweed cardenolide concentrations were quite low compared to many published reports (Zalucki et al. 1990, Malcolm 1990), but *A. curassavica* has a very wide reported range of cardenolide concentrations (Malcolm 1990). There was a significant difference

between the warm and cool treatment from our first experimental block, and a marginally significant difference between the cool and warm treatment from the final experimental block, with both cool treatment milkweeds having lower cardenolide concentrations. However, even if temperature influenced cardenolide concentration, the large difference between the two blocks indicates that other issues (e.g., aphids and other pests [Martel and Malcolm 2004, Zehnder & Hunter 2007, de Roode et al. 2011] or undetected variation in growth chamber conditions) are likely more of a factor in the variation. While we did not analyze the cardenolide concentration of monarchs, previous adults reared at different temperatures showed no difference in cardenolide concentrations (Dixon et al. 1978). While the warmer temperatures led to increased consumption of milkweed by larvae, monarchs reared at cooler temperatures were more efficient at sequestering cardenolides from the milkweed they consumed. Additionally, while temperature may have limited effects on milkweed cardenolide concentration, other aspects of climate change, including increased CO₂ and water stress, are known to affect cardenolide concentration in milkweed (Vannette and Hunter 2011, Couture et al. 2015). Environmental variation may also affect other aspects of milkweed biology, including increased nitrogen, lignin, and fiber content (Lemoine et al. 2015, Couture et al. 2015). Monarchs can compensate for changes in plant quality with increased consumption rates (Lavoie and Oberhauser 2004), but may also grow faster in response host plants with increased nitrogen levels (Lemoine et al. 2013).

Our research indicates that while temperature affects monarch development times directly, it does not influence monarch development or growth indirectly through milkweed (with the possible exception of FWL). Monarch temperature treatment affects mass in ways similar to previous work, with warmer temperatures producing smaller monarchs. The ecological impact of these differences in mass is unclear and should be investigated further. Neither monarch nor milkweed temperature significantly affected survival, indicating that average cool temperatures are unlikely to be a large source of mortality for Gulf Coast wintering monarchs. However, these monarchs must contend with a multitude of other threats, including extreme temperatures, natural enemies, and density dependent competition for food (Chapter 3- this volume). Future research should work to elucidate the impacts of these threats on this novel winter breeding system, as this will allow us to not only better understand this behavior, but also determine if (and what) conservation measures are needed.

Table 1. Measured variables and significant predicting factors from linear models with monarch temperature treatment (“monarch”), milkweed temperature treatment (“milkweed”), experimental block (“experiment”), and all interactions, followed by p-values in parentheses. N indicates sample size for each measured variable. Note that *OE* was not included as a measured variable since no adults were found to be infected.

Measured variable	N	Significant predicting factors*
First stadium development time	360	monarch (p < 0.001); experiment (p < 0.001); experiment×monarch (p = 0.001)
Second stadium development time	331	monarch (p < 0.001); experiment (p = 0.001); experiment×monarch (p = 0.004)
Third stadium development time	269	monarch (p < 0.001); experiment×monarch (p < 0.001); experiment×milkweed (p = 0.008); monarch×milkweed×experiment (p = 0.001)
Fourth stadium development time	167	monarch (p < 0.001); milkweed (p = 0.029), experiment×monarch (p < 0.001)
Fifth stadium development time	94	monarch (p < 0.001); experiment (p < 0.001)
Pupal development time	62	monarch (p < 0.001)
Pupal Mass	49**	monarch (p = 0.002)
Adult Mass	60	monarch (p = 0.002); experiment (p < 0.001); experiment×monarch (p = 0.011)
FWL	57	milkweed (p = 0.049), experiment (p < 0.001), experiment×monarch (p = 0.033)
Survival	382	experiment (p < 0.001)

*at the p=0.05 level

**Pupae were not weighed for the first two experimental blocks, resulting in a smaller sample size.

Table 2. a) Immature monarch development times in days based on monarch treatment temperature (warm or cool condition) \pm standard error, with sample sizes in parentheses. Treatment times are calculated based on least squares means (averaged over milkweed treatment) and expected treatment times are based on the actual temperatures and degree day calculations (see Zalucki 1982 and chapter 3 of this volume). **b)** Immature monarch development times in days based on both monarch treatment temperature and milkweed temperature treatment (warm or cool) \pm standard error, with sample sizes in parentheses.

a.

Stadium/Stage	Cool treatment time \pm SE (<i>N</i>)	Expected cool treatment time*	Warm treatment time \pm SE (<i>N</i>)	Expected warm treatment time*
First Stadium	5.7 \pm 0.11 (169)	7.8	2.5 \pm 0.10 (191)	2.7
Second Stadium	5.4 \pm 0.14 (142)	5.8	2.2 \pm 0.12 (189)	2.2
Third Stadium	5.6 \pm 0.13 (117)	5.1	2.3 \pm 0.11 (152)	1.9
Fourth Stadium	6.8 \pm 0.23 (81)	7.4	2.9 \pm 0.22 (86)	2.8
Fifth Stadium	12.2 \pm 0.55 (48)	13.8	5.7 \pm 0.57 (46)	5.2
Pupa	22.9 \pm 0.35 (29)	42.3	8.4 \pm 0.33 (33)	11.1

*Expected treatment time calculated using degree day calculations and known temperatures (Zalucki 1982, chapter 3 of this volume).

b.

	Treatment Combination			
	Warm Milkweed, Warm Monarch	Cool Milkweed, Warm Monarch	Warm Milkweed, Cool Monarch	Cool Milkweed, Cool Monarch
First Stadium	2.5 \pm 0.14 (93)	2.4 \pm 0.14 (98)	5.8 \pm 0.15 (87)	5.6 \pm 0.15 (82)
Second Stadium	2.2 \pm 0.17 (97)	2.2 \pm 0.17 (92)	5.1 \pm 0.20 (72)	5.7 \pm 0.20 (70)
Third Stadium	2.3 \pm 0.16 (78)	2.2 \pm 0.16 (74)	5.6 \pm 0.18 (61)	5.7 \pm 0.18 (56)
Fourth Stadium	3.0 \pm 0.30 (46)	2.8 \pm 0.32 (40)	7.2 \pm 0.31 (42)	6.4 \pm 0.33 (39)
Fifth Stadium	5.2 \pm 0.73 (27)	6.2 \pm 0.87 (19)	12.2 \pm 0.78 (24)	12.2 \pm 0.78 (24)
Pupa	8.6 \pm 0.41 (19)	8.4 \pm 0.50 (13)	23.0 \pm 0.50 (13)	22.8 \pm 0.45 (16)

Figure 1. Number of individuals that survived each larval stadium and pupal stage, and total number of monarchs that made it to be adults, grouped by both milkweed and monarch treatment. Total numbers of surviving individuals are above each bar.

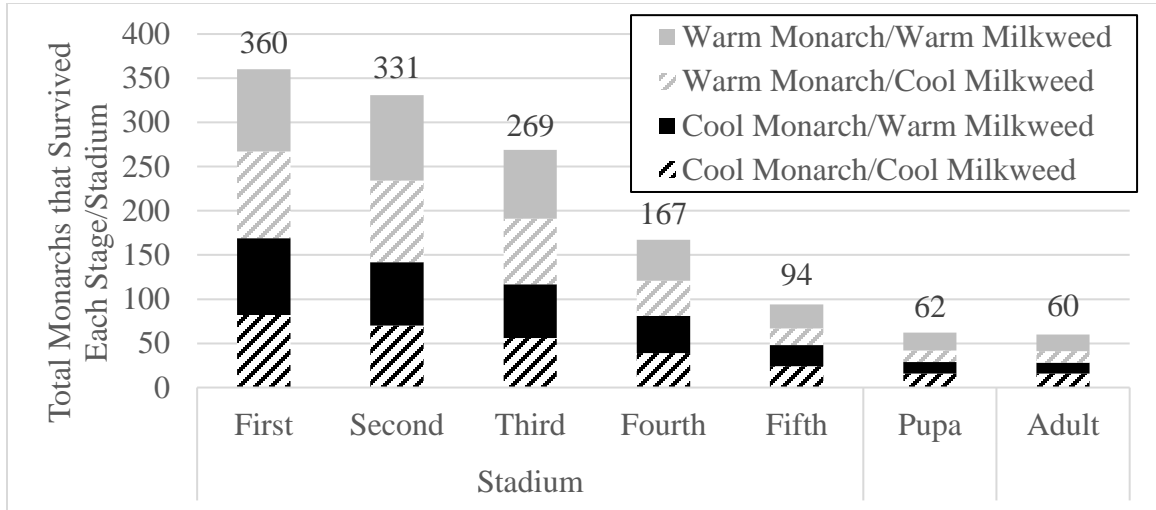
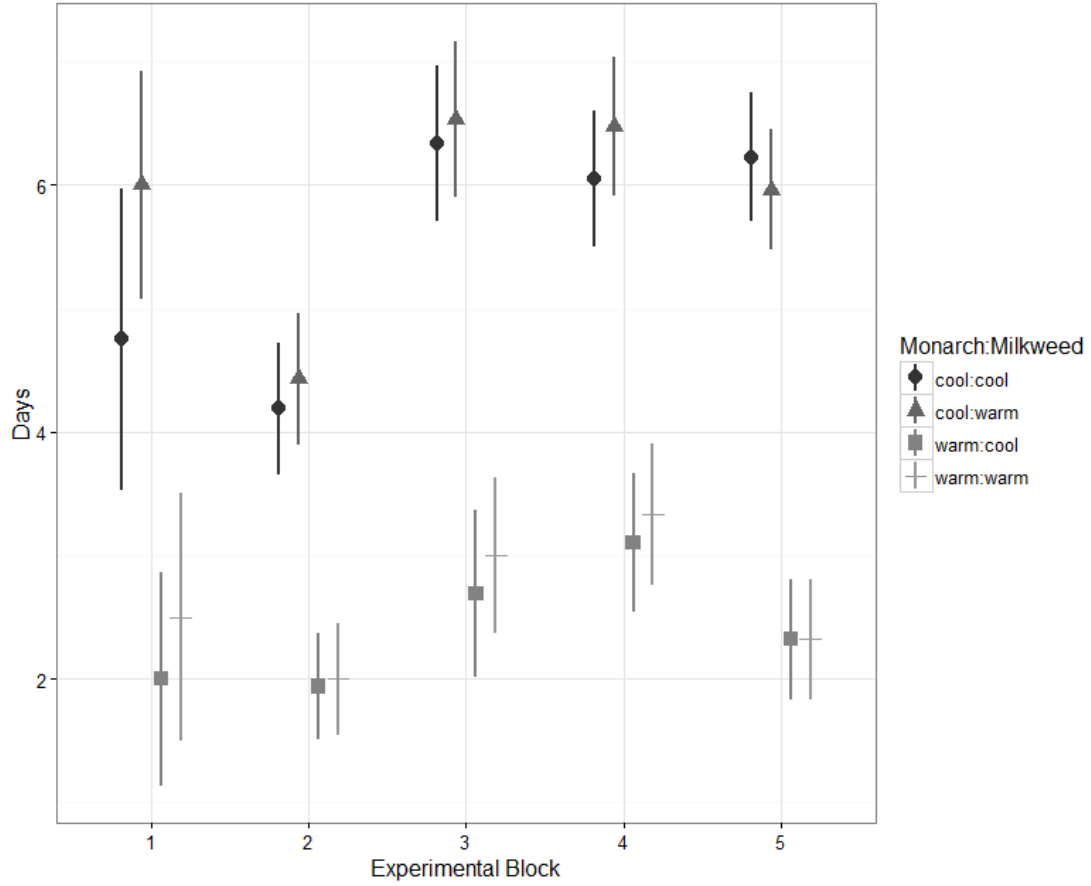
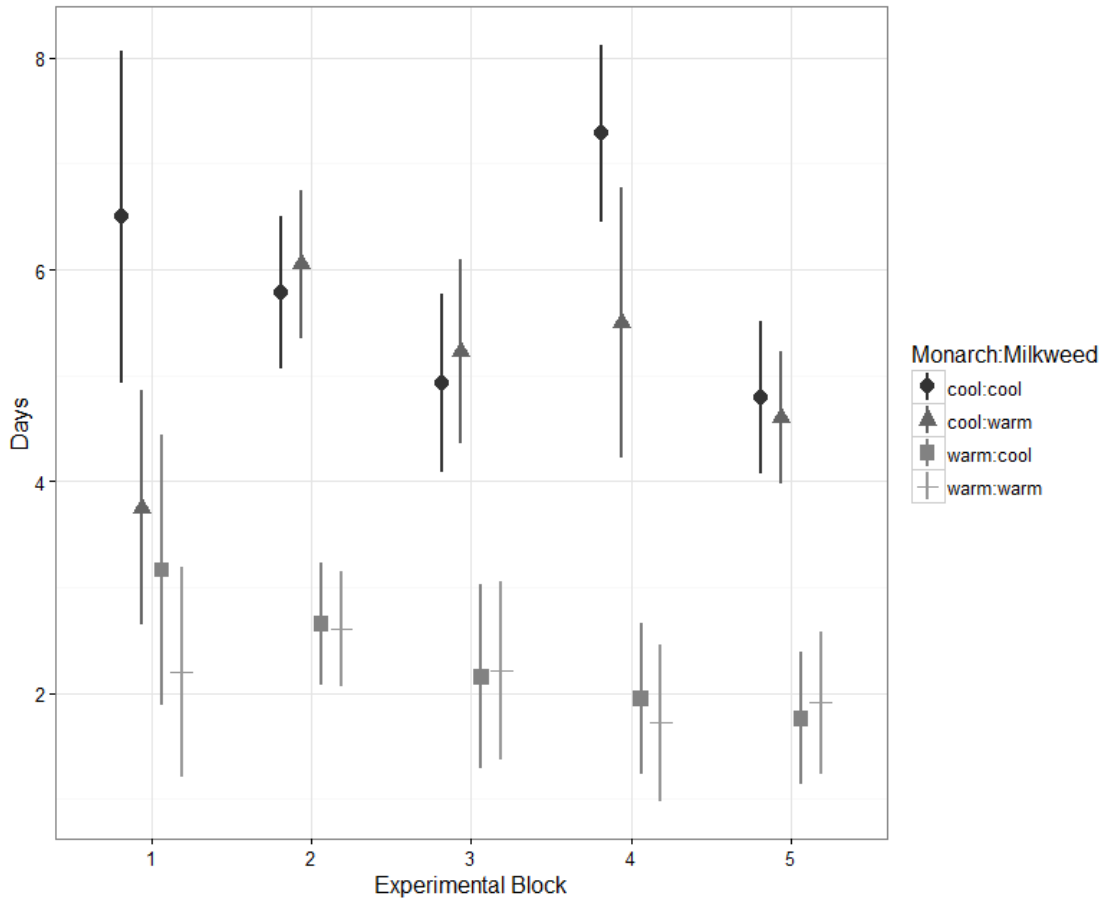


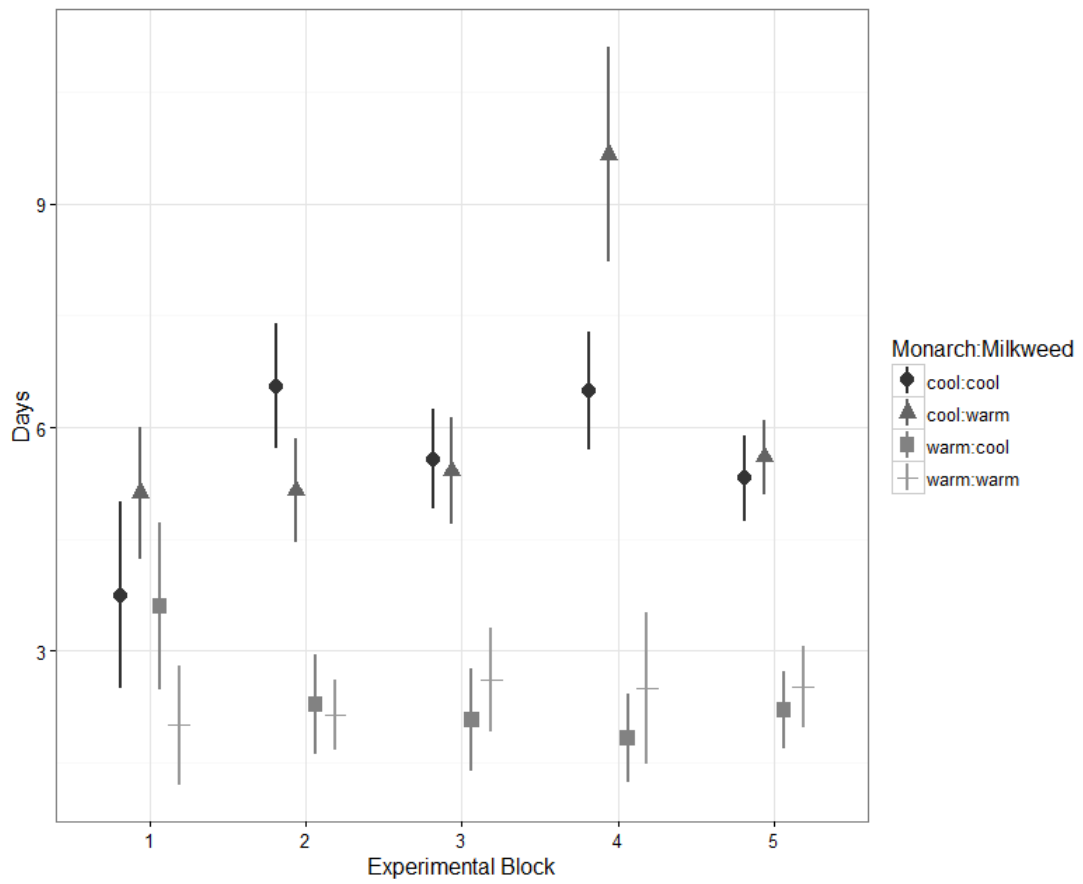
Figure 2. Mean development time and 95% confidence intervals days of development based on a linear model including monarch temperature, milkweed temperature, experimental block, and all interactions for **a)** first stadium, **b)** second stadium, **c)** third stadium, **d)** fourth stadium, **e)** fifth stadium, and **f)** the pupal stage of monarchs.



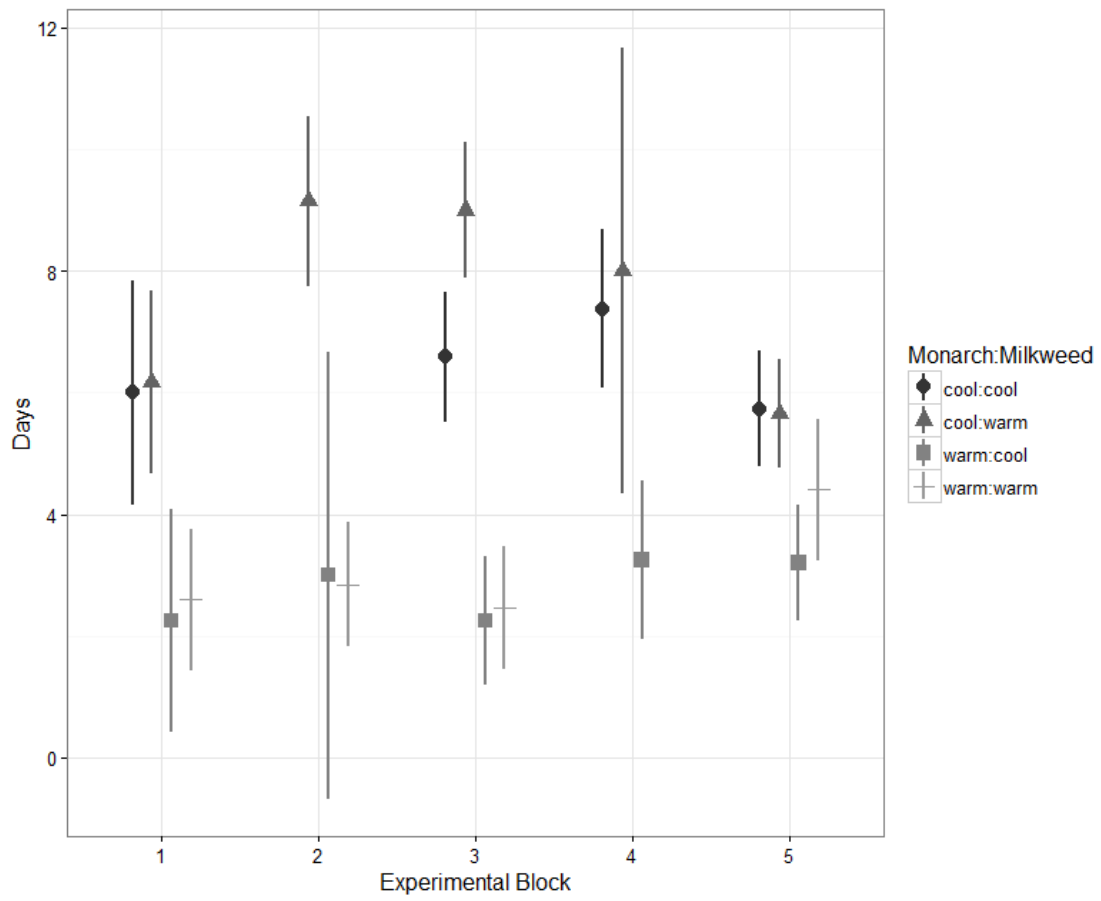
a. First Stadium



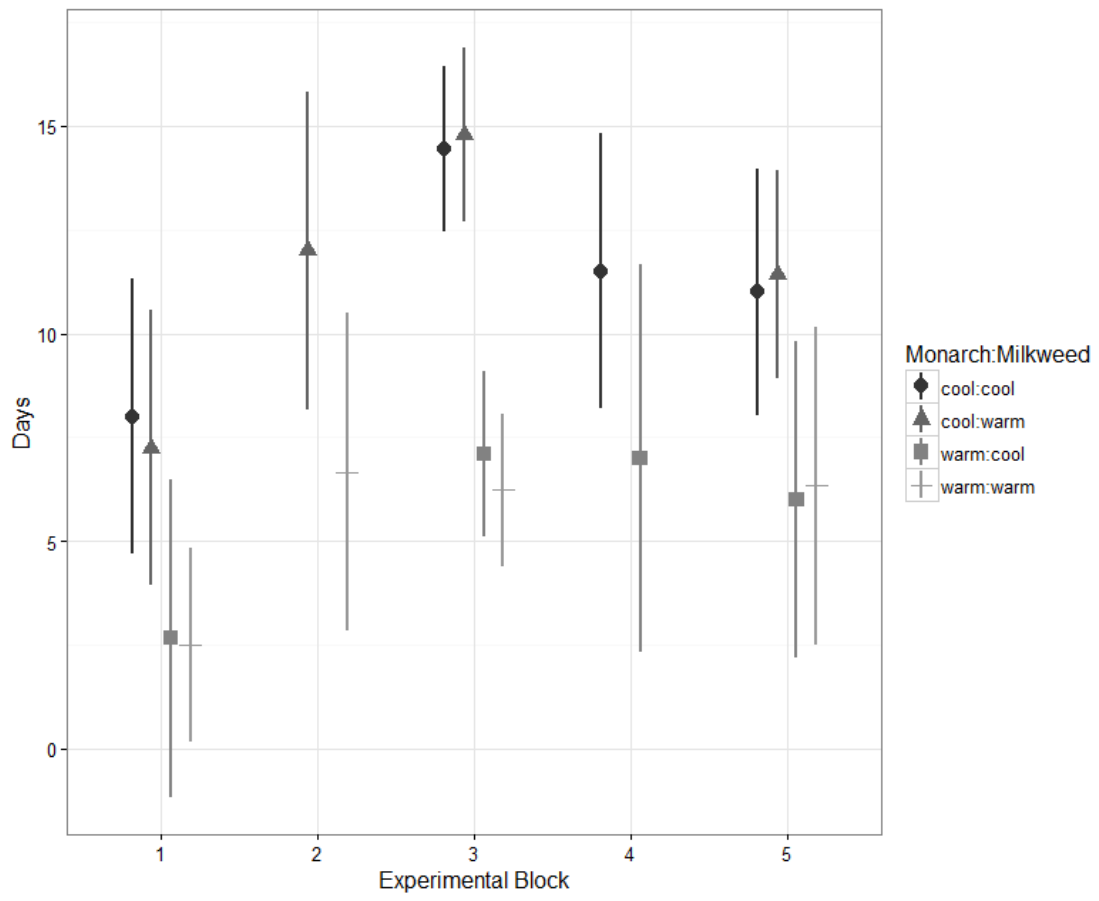
b. Second Stadium



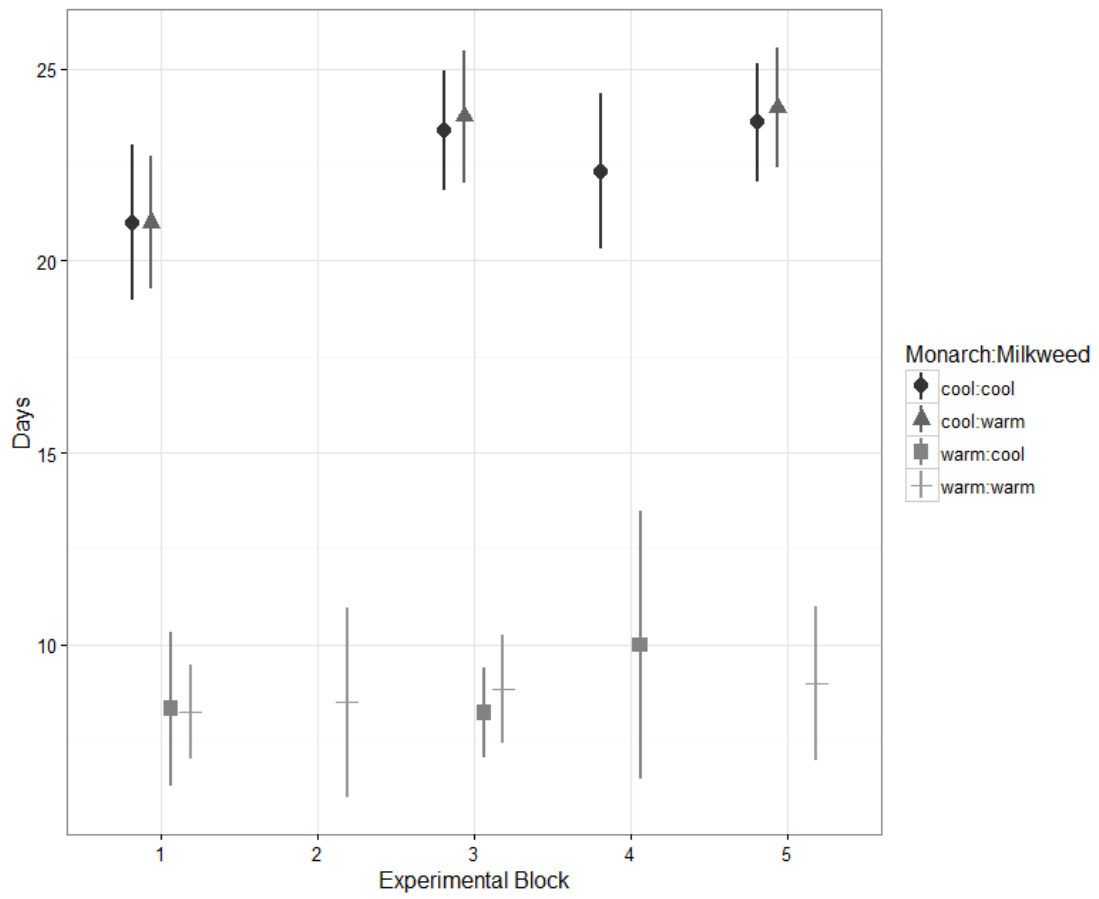
c. Third Stadium



d. Fourth Stadium

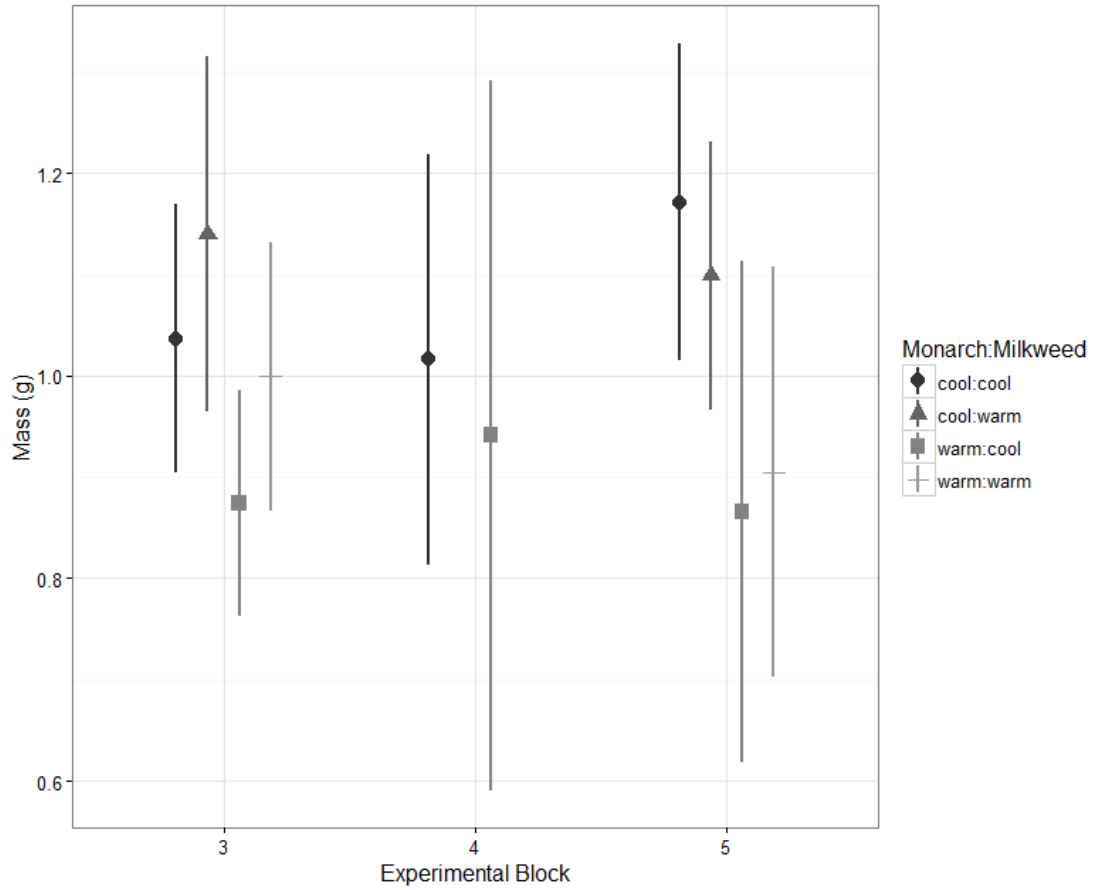


e. Fifth Stadium

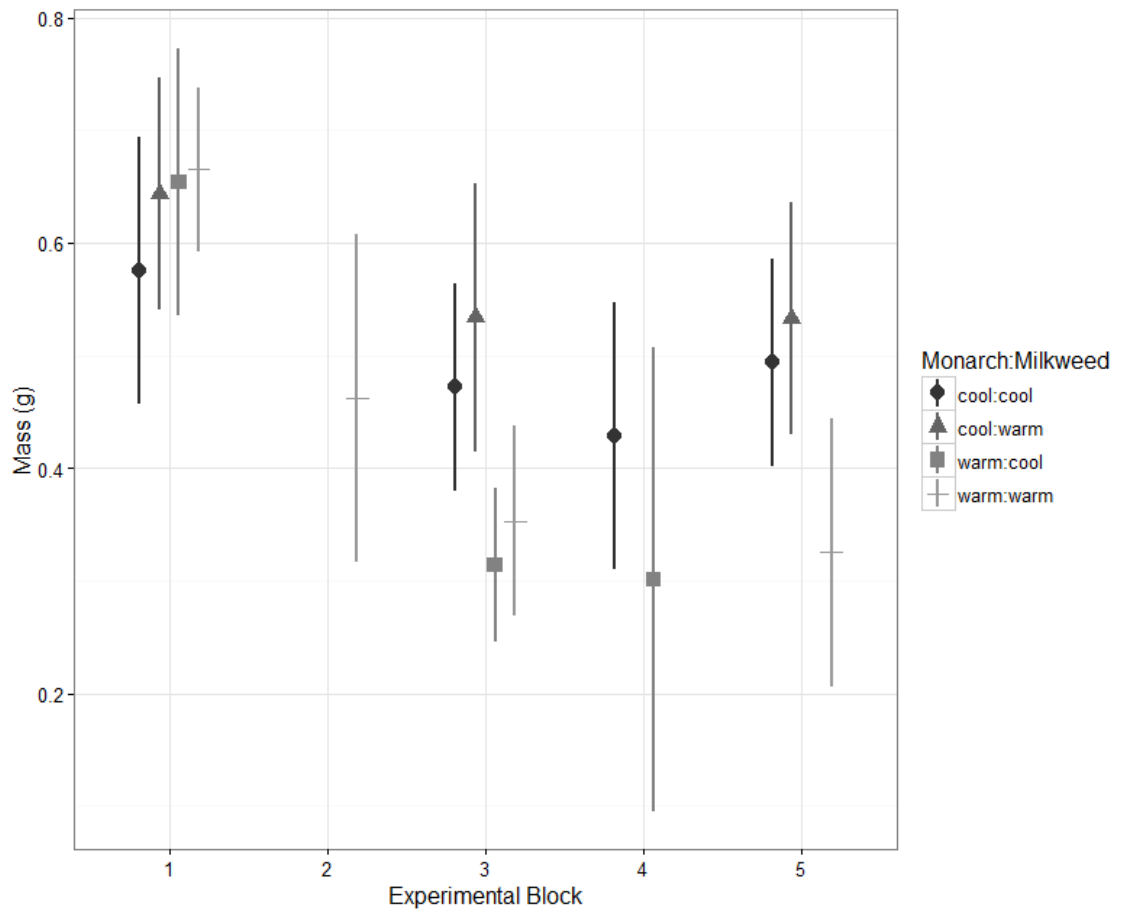


f. Pupal Stage

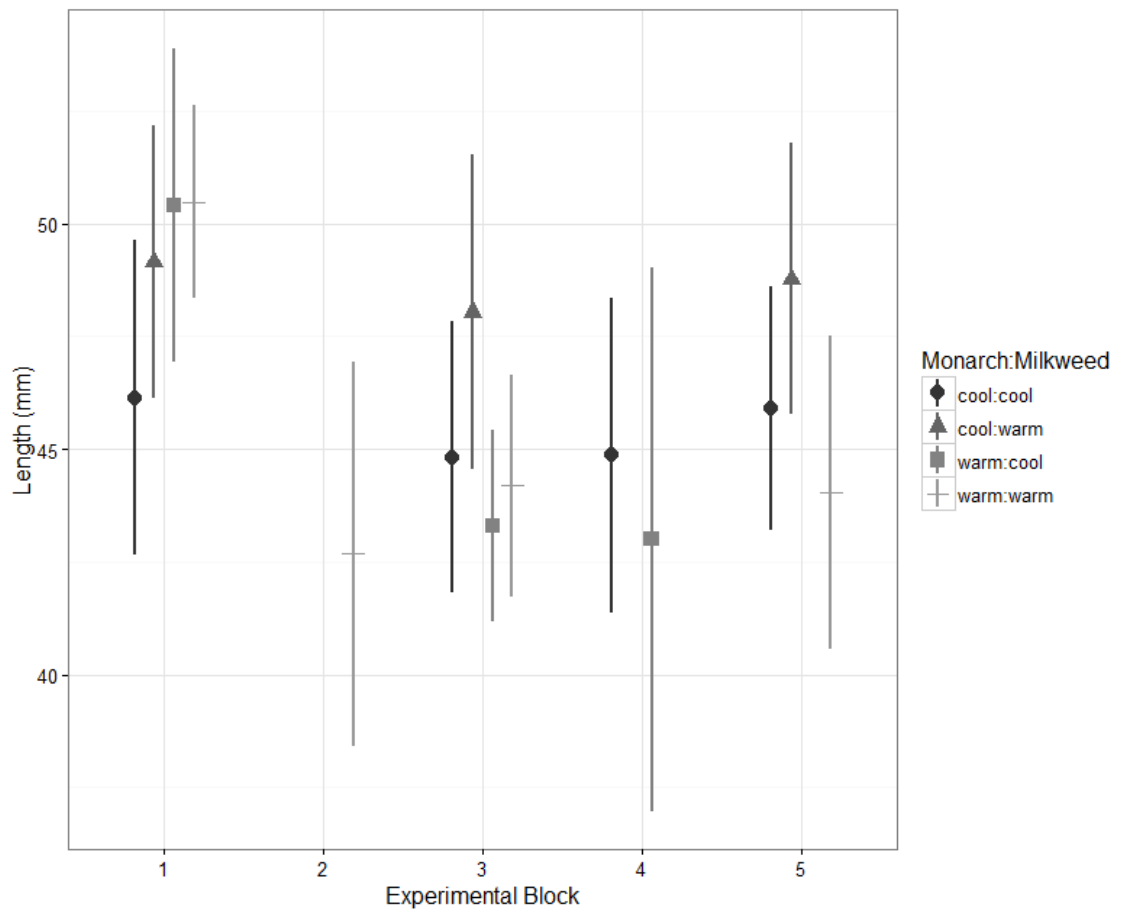
Figure 3. a) Mean pupal mass, **b)** mean adult mass, and **c)** mean forewing length based on linear model including monarch temperature, milkweed temperature, experimental block, and all interactions. Bars indicate 95% confidence intervals.



a. Pupal mass

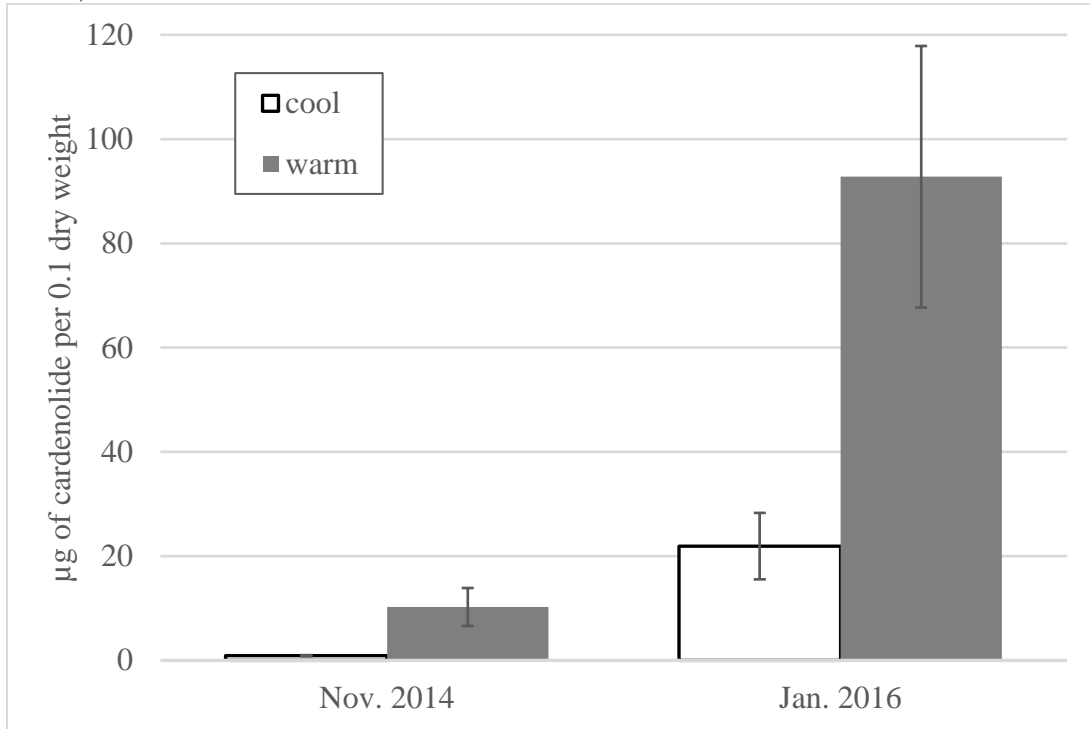


b. Adult mass



c. Forewing length

Figure 4. Total μg of cardenolide per 0.1 dry weight sorted by milkweed temperature treatment with SE bars. Samples were taken once in November 2014 and once in January 2016. The difference between the Nov. 2014 samples is statistically significant ($p = 0.028$), but the difference between the Jan. 2016 values is only marginally significant ($p = 0.063$).



Supplementary Text S2.1: While this dissertation is written by the dissertation author, the manuscript version of this chapter has the following authorships and affiliations:

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CHAPTER 3

**PREDICTING IMMATURE MONARCH DEVELOPMENT TIME AND EXPOSURE TO
EXTREME TEMPERATURES USING GROWING DEGREE DAYS AND CITIZEN
SCIENCE DATA**

SUMMARY

An increasing proportion of the population of eastern North American monarchs (*Danaus plexippus*) breed along the Gulf Coast throughout the winter, rather than migrating to and overwintering in Mexico in reproductive diapause. This partial migration means the resulting immature monarchs (eggs, larvae, and pupae) are exposed to cooler temperatures along the Gulf Coast in the winter than they would likely encounter in their summer breeding grounds. In order to further investigate the potential effects of this phenomenon, we used location- and stage-specific immature monarch observations from citizen scientists and temperature data for each location at which monarchs were found. We employed a growing degree day (GDD) model to estimate when observed individuals had been laid as eggs, how long it would take them to develop to maturity, and the lowest temperatures to which they had been and would be exposed during their development. Immature monarchs were often exposed to temperatures below 12° C (approximately their developmental zero), and the GDD model predicted an average egg to adult development time more than double than summer development times. Extended lifespan in the vulnerable and relatively immobile immature stages could put monarchs at higher risk of predation, disease, and potentially lethally cold temperatures, and also limits the number of generations that can be produced by winter breeding monarchs. We discuss the implications of these results for both monarchs and monarch migration.

KEY WORDS *Danaus plexippus*, monarch, Lepidoptera, degree days, climate change, cold tolerance, migration

INTRODUCTION

Animal migrations are highly visible phenomena which occur in a wide variety of organisms, from butterflies to whales. Many factors can affect or interrupt migration, including barriers that may be created, habitat destroyed or altered along the migratory route, disruption of demographic connectivity, and alteration of migratory cues from stressors such as climate change (Wilcove 2008, Wilcove and Wilkelski 2008). Ensuring sustained and continued migration often requires coordination over state and country boundaries, and protection of migratory animals while they are still abundant (Wilcove 2008).

Complicating conservation is the wide and changing array of behaviors of species and populations classified as migratory. Many species have changed their migratory patterns (Sutherland 1998), including a number of species that have altered their migration likely in response to the changing temperatures regimes of climate change (Visser et al. 2009). These changes may arise from selection on a species that exhibits partial migration (where individuals within a species exhibit both migratory and sedentary behavior; Chapman et al. 2011). We look at one species with a potentially recent change in migration and focus on the effects of temperature in this novel overwintering system.

The eastern North American monarch (*Danaus plexippus*) is a charismatic insect species well known for its incredible yearly migration. This autumnal migration takes monarch butterflies up to 4,500 km from their summer breeding grounds in southern Canada and

the northern U.S., south to overwintering sites in mountainous regions of central Mexico (Solensky 2004). While most eastern North American monarchs migrate to Mexico in diapause (a non-reproductive state), a small portion of the population reproduces throughout the winter along the Gulf Coast of the U.S. (Howard et al. 2010). While sightings of adult monarchs wintering along the Gulf Coast have been reported previously (Urquhart 1960, Brower 1995), there appears to be an increase in sightings in recent years, particularly of immature monarchs. This is possibly due to increased use and maintenance of a non-native host plant, tropical milkweed (*Asclepias curassavica*) or temperatures that make the Gulf Coast more suitable for monarchs year-round (Batalden and Oberhauser 2015, Satterfield et al. 2015). This behavior, along with monarchs being non-migratory year-round in Florida and other countries where the monarch has been introduced, indicates that monarchs are more accurately described as partial migrants. We investigate the effects of temperature on monarchs wintering along the Gulf Coast and discuss the potential consequences of temperature on monarchs in this region.

While average temperatures in the Gulf Coast during the winter may be warming and making it increasingly suitable for adult monarchs, the minimum temperatures reached there are cooler than those experienced by immature monarchs in their breeding range (Nail et al. 2015b). Cool temperatures may affect monarchs in at least two non-exclusive ways. First, monarchs may experience direct effects of the cold, including sublethal effects and cold-mediated mortality (Nail et al. 2015b, KRN unpublished data, Chapter 2- this volume). Second, cool temperatures may indirectly compound immature monarch

mortality by extending their time in the vulnerable immature stages (Kitching 1977), thus exposing them to increased mortality from other causes. Since monarchs already suffer high levels of mortality in immature stages, extra time spent in these stages may increase the likelihood of mortality due to predators, parasitoids, and disease (Zalucki 1982, de Anda and Oberhauser 2015). Monarchs suffer upwards of 90% mortality before becoming adults, with eggs and the earliest stadia suffering the highest mortality (Zalucki et al. 2002, de Anda and Oberhauser 2015, Nail et al. 2015a).

The goal of this study is to begin to assess the risks (due to increased mortality as a result of exposure to cool temperatures) and benefits (due to additional generations) of the monarch strategy of overwintering and breeding along the Gulf Coast. Monarchs are intensely monitored by both professional scientists and volunteer citizen scientists throughout the world, but especially in North America (Ries and Oberhauser 2015). Citizen science observations have been particularly helpful in illuminating the phenomenon of breeding monarchs along the Gulf Coast (Howard et al. 2010), and allow us to determine when monarchs are present and at what stage. Additionally, monarchs require a known number of growing degree days to develop (Zalucki 1982). Using a growing degree day (GDD) model and measured temperatures, we estimated the development time of monarchs observed along the Gulf Coast in the winter, and assessed the daily maximum and minimum temperatures to which observed monarchs had been and would be (assuming they survived to adulthood) exposed. This novel use of citizen science data with degree day calculations allows us to determine the degree to which

observed monarchs had been or were likely to be exposed to lethal or sublethal temperatures, and additionally, predict how many generations could be contributed to the eastern migratory monarch population through winter breeding.

METHODS

Citizen science observations of immature monarchs (eggs, larvae, and pupae) were compiled from 1997-2014 from four sources: Journey North, Monarch Larva Monitoring Project (MLMP) sightings at established sites, anecdotal observations submitted to MLMP website, and emails received by MLMP or by the authors. Observations were only used if the citizen scientist provided a specific location and date, and identified the monarch as an egg, pupa, or specific larval stadium. Observations also had to occur between November 1st and March 19th, as this period occurs after the primary fall migration and before the spring remigration. All observations were within the eastern North American migratory population range (monarchs located in most of Florida were excluded as these may be part of the southern Florida non-migratory population [anything west of the 85° longitude and south of 30° latitude]). Finally, observations that were unlikely to be wild monarchs were removed (e.g., the one egg reported in Minnesota in the winter was either a mistake or from a captive reared female, and observations with notes indicating that a monarch had been reared indoors were also removed).

We used the DAYMET daily surface temperature dataset (compiled by Oak Ridge National Laboratory; <http://daymet.ornl.gov>; Thornton et al. 2014) to determine the

temperature profile at each site where an immature monarch had been observed, both for the day the monarch was sighted and for the days before and after the sighting. Temperatures from the DAYMET dataset are produced throughout North America at a 1km resolution and are interpolated from nearby weather stations using models that account for complex terrain (Thornton et al. 2014). Since monarch development is temperature dependent, we used growing degree days (GDD) to calculate total development time. GDD are an estimate of the heat accumulated during a single day, and can be used to measure the amount of growth possible each day based on daily temperatures (Baskerville and Emin 1969). The number of GDD available at any given site on a particular day was calculated in R 3.2.3 (R Core Team 2015) based on the daily minimum and maximum temperatures, using a single sine wave approximation (Baskerville and Emin 1969, Allen 1976). The total number of GDDs required for monarch development during the egg stage, each larval stadium, and the pupal stage were previously calculated by Zalucki (1982; Table 1), and thus, the stage a monarch is in after a given number of days can be predicted given the available GDD. We assumed that each monarch observed was in the middle of its particular stage or stadium, and then worked backwards and forward through time to develop temperature profiles (daily minimum and maximum temperature) preceding and following the day of observation using the DAYMET data layers. We used that profile to calculate both the date on which the monarch had been laid as an egg (backcasting) and the date on which the monarch would be expected (if it survived) to eclose as an adult (forecasting).

After estimating the approximate number of days an individual monarch was likely to have been present at a particular site, we used the same temperature profile to determine both the warmest and coolest temperatures to which an individual had been previously exposed, and those to which they would be exposed after they were observed (assuming that they survived to adulthood). We used t-tests in R 3.2.3 to examine if both low and high temperatures varied between backcasted and forecasted days. Additionally, we used paired t-tests to determine if the most extreme temperatures an individual was exposed to differed between backcasted and forecasted days.

RESULTS

A total of 451 unique citizen science observations of immature monarchs met our criteria (Fig 1): 121 eggs, 39 first instars, 50 second instars, 58 third instars, 81 fourth instars, 65 fifth instars, and 37 pupae. Initial monarch observations were spread out over the entire monitoring period (Fig. 2), and primarily along the Gulf Coast, with 93% of immature winter observations located in Texas (Fig. 1a). Based on our GDD model, the mean number of days a Gulf Coast wintering immature monarch required to develop from egg to adult, assuming they survived, was 97 days \pm 1.2 (SE), with development times for 68% of all observed monarchs predicted to fall between 80 and 119 days (Fig. 3). We can use the correction factor of actual development time being 28.7% less than GDD predicted development times to produce an estimate of 69.2 days (Chapter 2- this volume)

The coldest temperature to which monarchs would have been exposed before being observed was -6°C . Forecasted days had colder minimum temperatures, going as low as -12°C (however, only 13 out of 29,565 forecasted days reached below -10.3°C , which is the warmest larval supercooling point, or point at which the monarch body freezes; Nail et al. 2015b). A perhaps more relevant metric is the number of days with minimum temperatures below 12°C , which is approximately the developmental zero for all stages of immature monarchs (the temperature at which immature monarchs would have ceased growth for at least part of the day; Table 1). Backcasted days had a minimum temperature under 12°C for 9,935 days (72% of total days) and forecasted days for 20,571 days (69.6% of total forecasted days). Additionally, the maximum temperature was below 12°C on 1,071 backcasted days (7.8%) and 3,093 forecasted days (10.5%), meaning that no development was likely to occur at all on these days (Table 2).

The differences between the average minimum daily temperature between forecasted and backcasted monarchs was highly significant ($p < 0.001$), but small and perhaps not ecologically significant (8.4°C for average minimum backcasted daily temperature and 8.1°C for forecasted temperatures; Fig. 4). Similar results were found for the average maximum daily temperature (19.7°C for average maximum backcasted daily temperature and 19.5°C for forecasted temperatures, $p = 0.045$). Paired t-tests for individual monarchs showed that there was also a significant difference between the most extreme low temperature that a monarch would have experienced and would be likely to experience ($p < 0.001$), with a colder extreme low temperature predicted for the future (forecasted)

versus the warmer backcasted extreme low temperatures (mean extreme low temperature = 1.8 °C for backcasted and -0.7°C for forecasted; Fig. 4).

DISCUSSION

Currently, the size of the eastern North American monarch population is declining, as evidenced by the size of the area they occupy on their wintering sites in Mexico (Brower et al. 2012, Rendón-Salinas and Tavera-Alonso 2014, Vidal et al. 2014), and egg counts in their summer breeding grounds (Stenoien et al. 2015a). They are being considered for listing under the Endangered Species Act (Center for Biological Diversity et al. 2014), and a recent population viability analysis (Semmens et al. 2016) suggests that they face an unacceptable risk of extinction unless the population reaches a number that occupies 6 ha in Mexico, substantially more than the 2015-2016 value of 4.01 ha (WWF 2016), a year noted to be a “rebound” year. As the monarch-occupied area at the Mexican overwintering sites has been decreasing, the number of non-migrating monarchs along the Gulf Coast (although probably small in comparison to the number that winter in Mexico) appears to be increasing (Satterfield et al. 2015). It is important to understand the ecological consequences of continuous winter breeding in all areas of monarch habitat (James 1981). By coupling a GDD model with citizen science observations of monarchs, we were able to examine the possible temperature-related risks posed by winter breeding, and the possible benefits that result from multiple generations that would not otherwise be produced.

The predicted development time of 97 days (or 69 days corrected) is much longer than that predicted under typical summer conditions. At constant room temperature (23°C), a monarch would develop from egg to adult in 33 days. With typical temperatures in the summer breeding grounds well above 23°C, immature monarchs develop even faster up to an upper thermal threshold (approximately 38°C before experiencing any sublethal effects; Nail et al. 2015b). Thus, the cool temperatures experienced by monarchs wintering and breeding in the southern U.S. mean that they only produce slightly more than one generation while their migratory counterparts are in Mexico (from November through mid-March). While this additional generation could provide a fitness advantage, there are two important caveats.

First, mortality and disease rates could rise with increasing development time. Monarchs suffer upwards of 90% mortality in their immature stages (Zalucki et al. 2002, de Anda and Oberhauser 2015, Nail et al. 2015a). Our predicted 97-day (or ~69-day corrected) duration before reaching maturity means that monarchs are exposed to risks specific to eggs, larvae and pupae for longer, including disease, parasitism, and predation. Monarchs breeding along the Gulf Coast of the U.S. throughout the winter are already likely to have extremely high rates of the debilitating protozoan parasite, *Ophryocystis elektroscirrha* (*OE*), which can reduce the fitness of the adult monarch, and sometimes lead to death (Satterfield et al. 2015, Altizer and Oberhauser 1999). While no studies have documented an interaction between increased development time leading to increased exposure to *OE*

spores, it is reasonable to assume that the longer a monarch larva is on a plant, the greater the likelihood that an infected adult will visit the plant and spread spores.

Extended development times also render immature monarchs more vulnerable to parasitoids present along the Gulf Coast, including tachinid flies (*Lespesia archippivora*) and the specialist parasitoid wasp, *Pteromalus cassotis* (Oberhauser 2012, Stenoien et al. 2015b), as well as a multitude of predators (Oberhauser et al. 2015b). Finally, while we do not yet understand the mechanisms for these observations, we have noted that, even in predator-free lab conditions, larval survival tends to be low when reared in cool conditions, even when those conditions do not reach developmental minima. Larvae sometimes remain in one stadium without eating for much longer than predicted by degree day calculations, before eventually dying (KRN and KSO, personal observations).

Secondly, the benefits of an extra generation may be further negated by density dependent factors. Monarchs overwintering along the Gulf Coast occupy a much smaller area than the summer breeding grounds (Fig. 1a). This area also has fewer milkweed plants in the winter (primarily *A. curassavica* in gardens and other maintained landscapes), which leads to extreme larval crowding. There are many anecdotal reports and KRN has observed monarchs stripping milkweed when larvae are present in large numbers (Fig. 1b). Density dependent effects will only worsen if more monarchs employ the strategy of overwintering along the Gulf Coast.

The lowest recorded temperatures (-6°C for backcasted and -12°C for forecasted) rarely occurred and were not below lower lethal temperature calculations (Nail et al. 2015b). However, extended exposure to these cold temperatures is likely to cause sublethal effects (Nail et al. 2015b). There were statistically significant differences between the average and extreme minimum backcasted and forecasted temperatures (Table 2), with forecasted days having cooler temperatures. The differences between forecasted and backcasted average minimum and maximum temperatures (0.3 and 0.2°C , respectively) was small enough that it is not likely to be ecologically significant. The extreme minimum temperature that an individual monarch was likely to experience was significantly lower for forecasted days than for backcasted days. The difference (2.5°C) may be enough to cause harm to the monarchs and milkweed exposed to sub-zero temperatures. The fact that minimum forecasted temperatures are lower in the future may indicate that similar cold temperatures were already lethal to immature monarchs in the past, as these monarchs would not be present for observation and are hence absent from our dataset.

Our fore- and backcasted GDD model was based on ambient temperatures. However, Rawlins and Lederhouse (1981) found that monarch larvae behavior can increase larval temperature 3 to 8°C above ambient temperatures. These warming behaviors include basking and moving to different parts of the milkweed plant. There are large temperature variations on different parts of the milkweed plant (KRN, unpublished data), so by altering which part of the plant it is on, a larva can effectively raise or lower its body

temperature. Any rise in larval temperature above the developmental zero (approximately 12.2°C for eggs, 11.5°C for larvae, and 13.5°C for pupae) will mean a shortened development time relative to what our model predicts. Additionally, while GDD needed for monarch development can be predicted using a linear model for moderate temperatures, the relationship between temperature and growth rate is actually slightly sigmoidal, meaning that there is more growth at extreme low temperatures than a linear model predicts (Zalucki 1982, Chapter 2- this volume; Fig. 5). Additionally, going outside the range of temperatures used to create this GDD linear model is likely to lead to spurious results, but is right now the best (and only) model we have available (Allen 1976). More research is needed on the degree to which monarch larvae can modulate their temperatures under cool conditions, particularly with daily temperature fluctuations.

Another area for future study is the cold tolerance of milkweed along the Gulf Coast. Even though immature monarchs may be able to withstand temporary periods of exposure to both below zero and below developmental zero temperatures, their survival depends on milkweed withstanding these temperatures as well. Monarchs overwintering along the U.S. Gulf Coast use primarily *A. curassavica*, a tropical species. Cold events are often the limiting factor for tropical plant species, and low temperatures have been predicted to be the biggest limiting factor for *A. curassavica* (Cavanaugh et al. 2014, Lemoine 2015). Even though the U.S. Gulf Coast is predicted to continue to be suitable for tropical milkweed in the future, it is on the edge of the species' suitability range and short term exposure to extreme temperatures may kill the aboveground plant (Cavanaugh

et al. 2014, Lemoine 2015). Most native milkweed species die back in the winter, and dependence on an introduced tropical species that cannot survive freezing temperatures could pose risks in addition to those associated with increased *OE* levels (Satterfield et al. 2015).

While we focus here on the risks associated with monarch overwintering and breeding along the U.S. Gulf Coast, there may also be benefits. With monarch summer breeding habitat predicted to move northward with climate change (Batalden et al. 2007), the offspring of individuals that stayed in the U.S. will have a shorter northward migration than their Mexico overwintering counterparts. Additionally, the current overwintering sites in Mexico are predicted to be climatically unsuitable both for monarchs (Oberhauser and Peterson 2003) and their most common roosting trees, oyamel firs (*Abies religiosa*) (Ramírez et al. 2015) within this century. Thus, foregoing the migration to the Mexican wintering sites may allow the eastern migratory monarch population to survive a changing climate, albeit with a very different migratory behavior and with a much smaller area than the eastern North American breeding grounds. Cooler temperatures, while perhaps directly detrimental to monarchs, may provide indirect benefits such as decreased abundance of predators and lowered competition for resources (Öhlund et al. 2015). The relative costs and benefits of winter breeding merit further attention.

When implementing monarch conservation in eastern North America, it is important to consider all parts of the migratory cycle. While monarchs breeding along the Gulf Coast

may shorten their southward migration in the fall and their northward journey in the spring, they face exposure to low temperatures that could have both direct and indirect negative effects on their growth and survival. Additionally, while winter breeding could potentially add individuals to the eastern population, thermal limits suggest that only slightly over one generation could be produced in the winter, and this additional generation may suffer due to both natural enemies and density dependent competition for food.

Monarchs display an impressive array of migratory and non-migratory behaviors throughout their worldwide range (James 1981, Brower 1995, Solensky 2004). With continued monitoring of these Gulf Coast wintering monarchs, we can better understand what is happening with this segment of the eastern North American population and why. Our work suggests that cold temperatures in this area play a role in extended monarch development and perhaps in direct mortality. Determining what happens to the offspring of this winter generation, especially where the generation(s) proceeding these winter breeders goes (Batalden and Oberhauser 2015) will be important in more clearly comprehending the implications of winter breeding for the population as a whole, and in turn, what informed actions we should take to better conserve this iconic insect.

Table 1.

Developmental zeros (DZ) and growing degree days (GDD) for *Danaus plexippus*. Standard errors are in parentheses (table adapted from Zalucki 1982). These values were calculated using a linear model based on monarchs grown ex situ in constant temperature treatments of 10, 15, 17, 19, 22, 25, 29, 31, 33, and 36° C.

	DZ (°C)	GDD
Egg	12.2 (0.85)	45.0 (0.53)
First Instar	11.6 (1.91)*	32.3 (1.07)
Second Instar	10.4 (2.27)*	27.8 (1.20)
Third Instar	12.0 (1.49)*	24.5 (0.79)
Fourth Instar	11.7 (1.73)*	35.7 (1.50)
Fifth Instar	12.0 (1.62)*	66.6 (4.43)
Pupa	13.5 (1.58)	119.9 (7.58)

*The larval developmental zero used for all of our calculations was 11.5°C, based on personal communications with Zalucki.

Table 2.

Extreme and average minimum temperatures, average maximum temperatures, and percentage of temperatures below 0°C and 12°C for both backcasted and forecasted days. All temperatures are statistically different ($p < 0.05$) between backcasted and forecasted days.

	Backcasted Days	Forecasted Days
Extreme minimum temperature*	1.8°C	-0.7°C
Average minimum temperature	8.4°C	8.1°C
Average maximum temperature	19.7°C	19.5°C
% of total days with minimum temperature below 0°C	7.8%	10.5%
% of total days with maximum temperature below 12°C	72%	69.6%

*The extreme minimum temperature is the coldest temperature an individual monarch would have experienced. The numbers here are the averages of these extreme temperatures (not all minimum temperature) across the 451 individual monarchs.

Figure 1. a.) Map of winter monarch observation sites throughout the southern U.S. from 1997 – 2014. **b.)** Tropical milkweed plant with leaves completely eaten by monarch caterpillars in November, 2013 (Galveston, Texas).

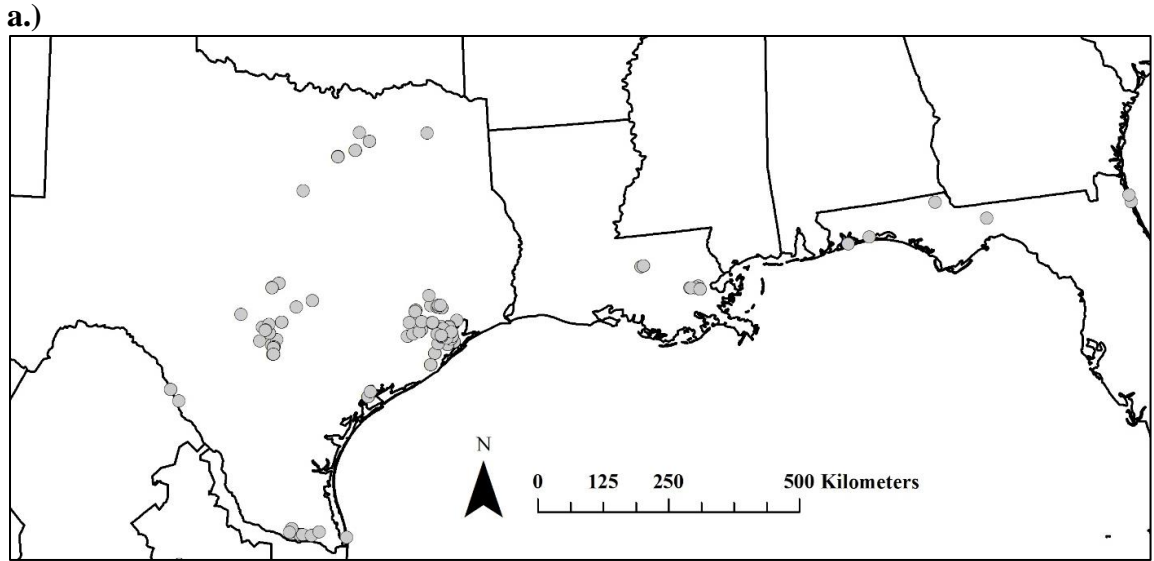


Figure 2. Histogram of monarch observations by ordinal day over all 17 years of the study, grouped into 5-day bins, sorted by eggs (black bars), larvae (light gray bars), and pupae (dark gray bars). Average low (solid line) and high (dotted line) daily temperatures from Houston, TX (data obtained from National Oceanic and Atmospheric Administration; accessed January 2016; available at <http://www.noaa.gov>).

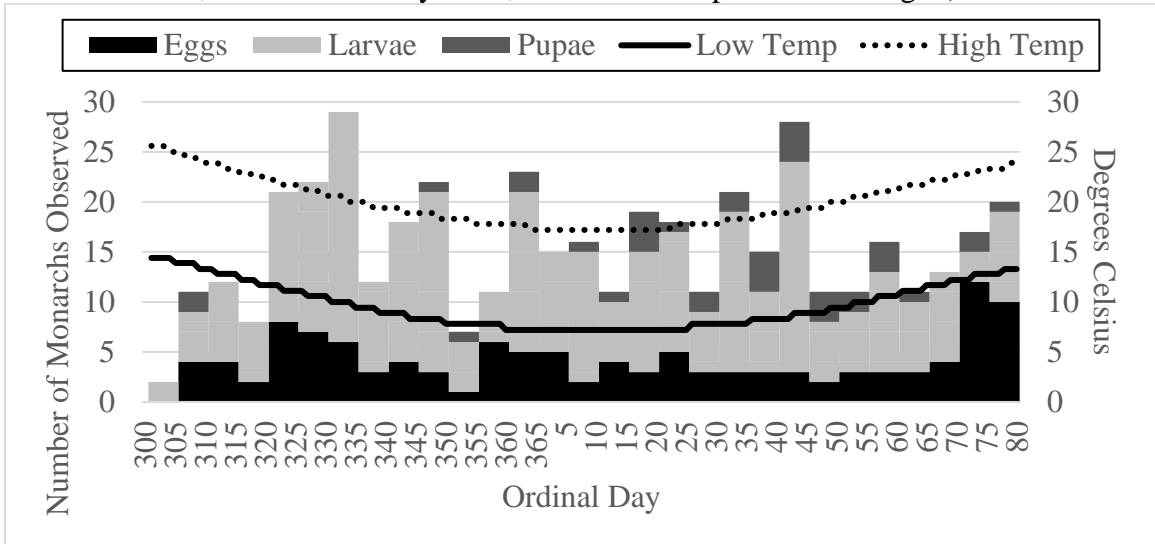


Figure 3. Histogram of total number of days required for development from egg to adult for each observed monarch, split into percentage of each bin made up of forecasted days and backcasted days. Percentage of each bin that is made up of forecasted days is above each bar.

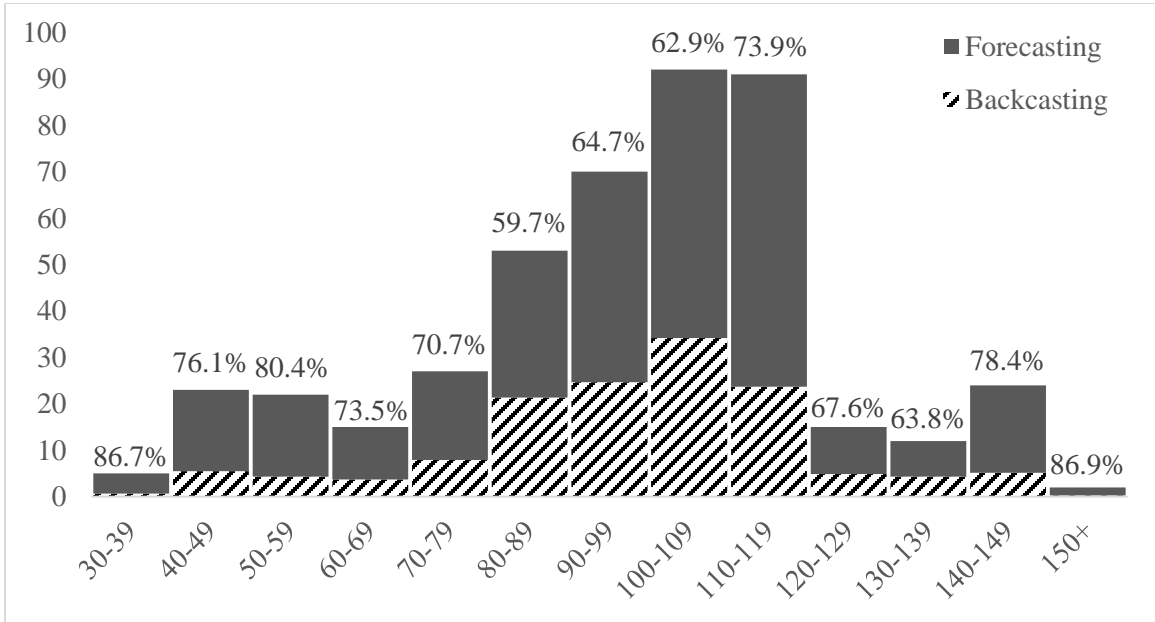


Figure 4. Histogram of minimum daily temperatures at sites where immature monarchs were observed and expected to be present. Black bars show minimum temperatures for backcasted days and bars with diagonal lines show minimum temperatures for forecasted days.

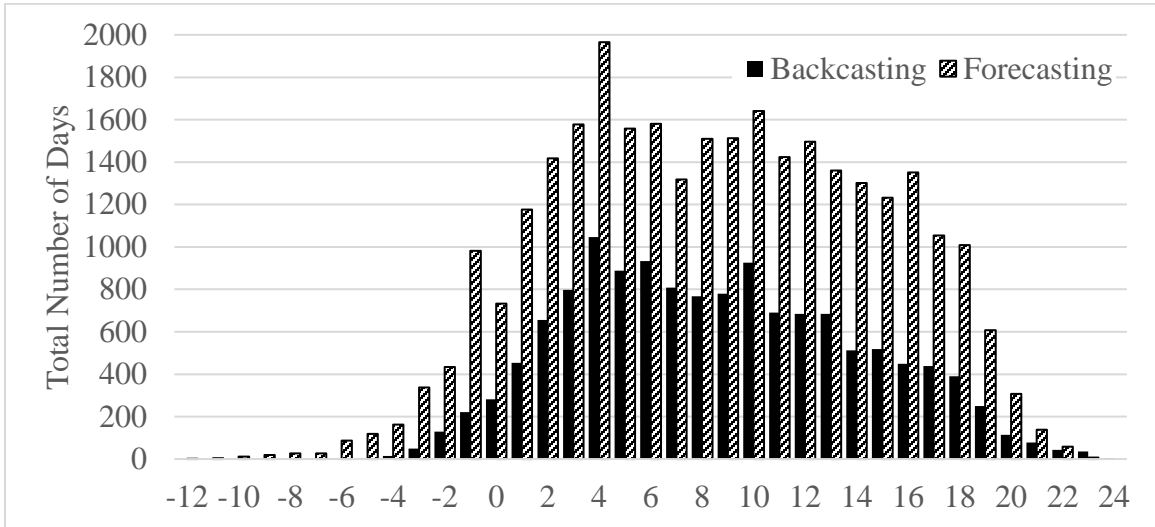
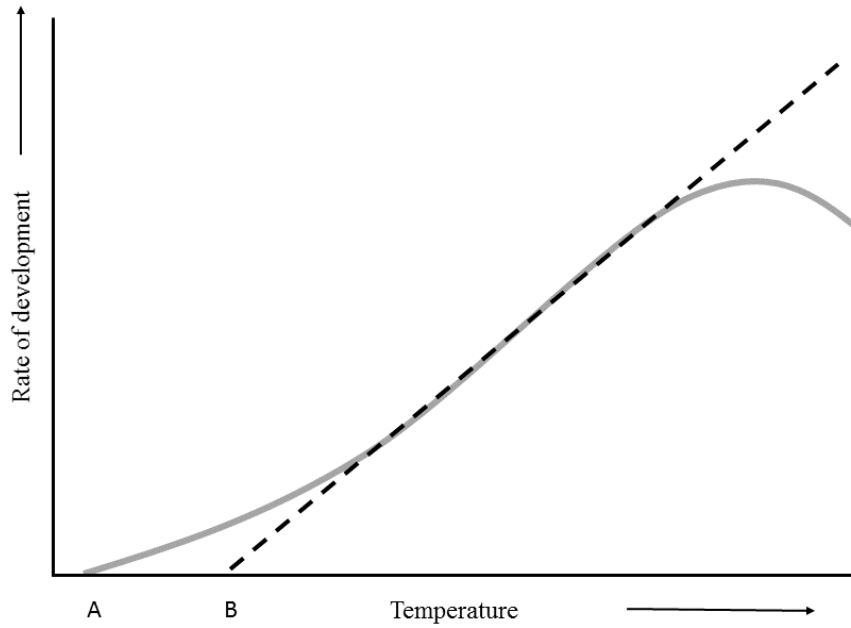


Figure 5. Visual representation of actual developmental rate (gray solid line) compared to modeled rate of development based on degree days (black dashed line). The linear degree day model adequately models actual rate of development for most temperature that monarchs experience, but overestimates growth at extreme high temperatures and underestimates growth at extreme low temperatures. This leads to the actual developmental zero (A) being a lower temperature than the estimated developmental zero (B). Figure adapted from of Zalucki 1982.



Supplementary Text S3.1: While this dissertation is written by the dissertation author, the manuscript version of this chapter has the following authorships and affiliations:

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CHAPTER 4

IMMATURE MONARCH SURVIVAL: EFFECTS OF SITE CHARACTERISTICS, DENSITY, AND TIME

SUMMARY

The eastern North American monarch population is declining, as evidenced by the area occupied by overwintering adults. Recently, decreasing availability of breeding habitat has been most strongly implicated in this decline. An alternative, nonexclusive explanation for the recent population decline is decreasing survival. We used 18 yr of data from the Monarch Larva Monitoring Project, a citizen science program, to determine immature monarch mortality rates over time as well as factors associated with increased mortality. Our data included field measures of mortality from egg to the final larval instar, and mortality due to parasitoids and other causes, assessed by rearing field-collected monarchs. Average egg to fifth-instar survival ranged from ~7 to 10% across all regions. Survival from fifth instar to adult ranged from ~60 to 90%, although this overestimates survival because monarchs are not exposed to many mortality factors when reared indoors. Both survival rates showed a great deal of temporal and spatial variation. Survival tended to be higher in sites that were planted and had more milkweed plants. There was a negative effect of per plant egg density on survival, suggesting density dependence. Survival rates appear to be declining from 1997 to 2014, and we discuss possible reasons for this pattern. Finally, we estimate that across all years in the north-central United States, where we have the most data, a minimum number of ~29 milkweed plants are required to produce an adult monarch that will be part of the fall migratory generation.

KEY WORDS: *Danaus plexippus*, monarch, Lepidoptera, habitat, survival

INTRODUCTION

Eastern North American monarchs [*Danaus plexippus* L. (Lepidoptera: Nymphalidae)] are well-known for their annual fall migration, which takes them up to 4,000 km from their summer breeding grounds throughout the northern United States and southern Canada to mountainous overwintering sites in central Mexico (Solensky 2004). The generation that migrates south in the fall also begins the spring migration, laying eggs as they head northward. It is these descendants that finish the journey back to the summer breeding grounds, where an additional two to three summer generations are produced.

Recent analyses show that monarch abundance, measured at the Mexican overwintering sites, has significantly declined (Brower et al. 2012, Rendón-Salinas and Tavera-Alonso 2014, Vidal et al. 2014). It is likely that a decrease in milkweed host plant (*Asclepias* spp.) availability throughout the monarchs' breeding grounds is contributing to the decline in the overwintering monarch population. While monarchs use many *Asclepias* species as host plants, most of the monarchs that migrate to Mexico in the fall have consumed *Asclepias syriaca* L., common milkweed (Malcolm et al. 1993). *A. syriaca* growing in agricultural fields was a key source of monarchs before the widespread use of genetically modified crops (Oberhauser et al. 2001), though it has since all but disappeared (Pleasants and Oberhauser 2013, Pleasants 2015) as adoption rates of herbicide tolerant crops have reached ~90% (Stenoien et al. 2015a). Another nonexclusive hypothesis is that overwintering abundance is declining because of increasing monarch mortality rates during the egg and larval stages. Changes in immature

mortality could be due to several top-down and bottom-up forces, including 1) increased consumption of monarchs by predators or parasitoids, 2) increased larval competition (resource limitation) as females are forced to lay eggs in smaller and more dispersed habitats, or 3) increased infection rates of diseases.

The relative importance of bottom-up (resources) versus top-down (natural enemies) forces in structuring insect herbivore populations has long been debated (Hairston et al. 1960, White 1978, Oksanen et al. 1981, Hunter and Price 1992). Price et al. (1980) contended that to fully understand plant–herbivore interactions, a tri-trophic approach is required, that includes interactions between plants, herbivores, and natural enemies. For lepidopterans, bottom-up forces are likely to include the availability of larval host plants and adult nectar sources, as well as the physical and chemical defenses of larval host plants. Top-down forces are likely to include predators, parasitoids, and disease. It is also essential to acknowledge the importance of abiotic factors, especially weather, in determining insect–herbivore population dynamics (Birch 1957).

Several factors hypothesized to influence monarch survival or fecundity have been previously investigated. Zalucki and Lammers (2010) modeled the potential bottom-up impacts of the loss of agricultural milkweed on individual monarch fecundity, and suggested that fecundity is likely to decline with a decreasing density of host plants. Additionally, milkweed physical and chemical characteristics have been shown to influence oviposition behaviors and larval survival (Zalucki et al. 1990, 2001a,b; Malcolm 1995; Malcolm and Zalucki 1996; Zalucki and Malcolm 1999). While site characteristics, such as the availability of flowering plants and site size, have been

correlated with pollinator abundance (Westphal et al. 2003, Belfrage et al. 2005), little is known about the degree to which site characteristics affect insect survival. Monarchs are also known to be affected by many top-down forces, including a large suite of egg, larval, and pupal predators and parasitoids (reviewed by Oberhauser et al. 2015b). Mortality can also be caused by disease, such as that inflicted by the protozoan parasite *Ophryocystis elektroscirrha* (Altizer and Oberhauser 1999).

Survival rates of eggs and larvae have been previously documented by Borkin (1982), Oberhauser et al. (2001), Prysby (2004), Calvert (2004), and De Anda and Oberhauser (2015). All of these studies reported survival rates of $\leq 10\%$ from the egg to late-instar larva stage, but they all involved limited temporal and spatial scales. All but Oberhauser et al. (2001) covered only one or a few locations, and all but De Anda and Oberhauser (2015), which was a 2-yr study, covered only a single year. Additionally, because these studies all tracked only eggs and larvae, they did not document most of the mortality caused by parasitoids, which usually occurs after pupation. In addition to resource availability and natural enemies, other factors, including temperature (Zalucki 1982, York and Oberhauser 2002, Nail et al. 2015b) and insecticides (Oberhauser et al. 2006, 2009), are known to affect immature monarch survival. Because most monarch mortality occurs in the egg and larval stages, immature mortality has the potential to be an important population driver.

While monarch fecundity and survival can be measured in laboratory studies (Svärd and Wiklund 1988, Oberhauser 1989, Oberhauser 1997), these rates are difficult to measure in the field. One source of monarch field occurrence data is a citizen science

venture called the Monarch Larva Monitoring Project (MLMP). MLMP volunteers from 37 U.S. states, the District of Columbia, three Canadian provinces, and one Mexican state collected data on monarch egg and larval density at sites with milkweed (Fig. 1). These data, with records beginning in 1997, include habitat characteristics of the monitoring site and surrounding area (Prysby and Oberhauser 1999, MLMP 2015), and can thus be used to identify large- and small-scale habitat features that are associated with monarch survival.

The first goal of this study was to estimate immature survival over broad spatial and temporal scales, and to determine what local- and landscape-level site characteristics, as well as spatial and temporal factors, are correlated with egg and larval survival. Monarch survival could be influenced by several factors that vary between habitats: 1) heavily managed sites (gardens and other planted sites) might have higher survival, if weed control and watering increase host plant quality. Alternatively, 2) these planted sites might be habitat islands that attract ovipositing females that do not leave, leading to increased immature monarch densities and possible density-dependent effects (see number 7 below). 3) Survival could decrease with proximity to agricultural lands, if monarchs are affected by pesticide applications or other practices in these habitats, but 4) agricultural habitats could contain fewer predators and thus support higher survival, as suggested by Oberhauser et al. (2001) and Pleasants and Oberhauser (2013). 5) More diverse habitats could also support a more diverse and abundant suite of predators (Oberhauser et al. 2001). 6) Monarch survival might be negatively affected by egg density owing to higher disease rates (Lindsey et al. 2009, Satterfield et al. 2015) and

scramble competition, defined as a situation in which the finite amount of food available for each individual decreases with a larger population size. Alternatively, 7) egg density might be an indicator of female preference for high-quality host plants and, hence, might be associated with higher survival. A second, related goal was to then determine if there are temporal trends in monarch survival, and thus if immature survival rates could be drivers of the observed decline in monarch numbers.

Finally, using monarch egg density, larval survival, and MLMP parasitism data, we calculate the approximate number of adult monarchs that come from a single milkweed plant during the summer in the northcentral region of North America, the most important source of overwintering monarchs in Mexico (Wassenaar and Hobson 1998). This is particularly important for monarch conservation, as having an estimate of number of milkweeds is necessary to produce an adult monarch in the migratory generation will inform the magnitude of conservation actions required to achieve population targets.

MATERIALS AND METHODS

MLMP volunteers record weekly counts of monarchs identified to instar at sites with milkweed throughout North America (Fig. 1). For a more detailed description of collection procedures and data recorded, see Prysby and Oberhauser (2004) and MLMP (2015). Briefly, data are collected as densities of eggs and each larval instar per milkweed plant observed, and several characteristics of monitoring sites are recorded each year. We used data from 1997 to 2014 for this analysis, divided into six different regions (Fig. 1) and analyzed over four different time periods defined to encompass relevant generations

in regions used by monarchs throughout the year (Table 1). These time periods were determined using visualizations of the monitoring data from the 18 project years. We chose time periods that were long enough to encompass year-to-year variation in monarch phenology and include all of the eggs and larvae that were produced during a single generation in most years (with the exception of the northern summer period, which covers two overlapping generations). For each time period, the monitoring events used to obtain the number of fifth instars were shifted 2 weeks later than those used to obtain eggs numbers. This allows us to account for monarch development time and the fact that MLMP volunteers monitor weekly. While generations sometimes overlap between these time periods, our visualizations of region-wide data suggested that this overlap is usually not relevant within in any given region, as the vast majority of the population moves in a predictable way from one region to another during the year (Batalden et al. 2007). An alternative would have been to measure survival over the entire year, but we wanted to capture differences from one generation to the next.

Our units of analysis for egg to fifth-instar larva survival were individual monitoring sites in a given time period and year. Data were excluded for site–time period combinations if there were ≤ 10 milkweeds monitored per monitoring event, or if the site was monitored fewer than five times over most multimonth time periods. However, we included sites that were monitored four or more times for the northern spring season in the north-east and north-central regions, as volunteers often did not monitor five times in April–June owing to the late appearance of milkweed (truncating the data on the early side of the time period), and because monarchs stay in these regions to produce

succeeding generations (so if we extended the interval to later, data would overlap with the next generation). These monitoring frequency requirements needed to be met during both the egg and fifth-instar time periods (see Table 1). Data were also removed if volunteers recorded more larvae of a single instar than eggs, as this indicates an inability to distinguish monarch eggs or selective observation of plants with caterpillars. Finally, because we were evaluating monarch survival through the fifth instar, we also removed data from observers who collected most or all of the eggs or first through third-instar larvae that they observed, as collecting these would lead to a lack of late-instar larvae in the following weeks (collecting later instars, however, does not affect our survival estimates because of the weekly monitoring intervals).

Once the data were cleaned, we determined survival from egg through the larval stage by dividing the number of fifth instars observed at a site by the number of eggs seen at the same site in the same time period during the same year, but shifted 2 wk earlier:

Equation 1.

$$Larval\ Survival_{i,t,y} = \frac{Number\ of\ fifth\ instars_{i,t,y}}{Number\ of\ eggs\ from\ two\ weeks\ earlier_{i,t,y}}$$

where i = site, t = time period, and y = year

We then compared mean survival between different regions and time periods and across years. To understand survival drivers, we used a mixed-effects model with binomial error structure in R 3.2.3 (R Core Team 2015, Vienna, Austria) with package lme4 (Bates et al. 2015). As many sites are monitored for multiple years, site ID was used

as a random effect (Table 2). Year was also treated as a random effect to account for stochastic variation over time. Fixed effects included time period (season), egg density (measured as total number eggs/total number of plants observed during the time period at a given site), mean number of milkweeds monitored per week, whether a site was planted, and site type (garden, natural area, crop-based agricultural area, noncrop-based agricultural area, roadside, or other [Stenoien et al. 2015 includes details on the assignment to these categories based on volunteer descriptions]). The average number of milkweeds per monitoring event was a proxy for the number of plants at a site, as these are correlated (see Stenoien et al. 2015a). We used stepwise backward selection and AIC scores to choose the top supported model (Sakamoto et al. 1986). To determine whether immature survival rates are changing over time, we conducted a second analysis with the same predictors and site ID as a random effect, but with year as a fixed effect.

Since 1999, a subset of MLMP volunteers have collected monarch eggs and larvae to rear in their homes, and we used the outcomes of these rearings to estimate larva to adult survival. While many of these monarchs are collected from regular monitoring sites, volunteers also record data on monarchs that they rear from other locations. Thus, we do not have site characteristics for these nonsite locations, and only assigned them to region for analysis (see Fig. 1). Most volunteers collect and rear fourth and fifth instars, but our database also includes records from monarchs collected as eggs and younger larvae. Volunteers record the date, location, and larval instar at collection, as well as the outcome of each rearing (adult monarch, died of unknown cause, died accidental death, parasitized by fly, or parasitized by wasp). A notes data field allows volunteers to record additional

information that they consider relevant. Volunteers only identify parasitoids to order, but the vast majority are flies, and all of the flies that we have identified to species (several dozen from throughout the United States) have been *Lespesia archippivora* (Oberhauser et al. 2007).

For this analysis, we omitted cases in which monarch death was accidental (e.g., the specimen was dropped, or crushed between the lid and rearing container), and from volunteers whose reared larvae consistently suffered rates of mortality from unknown causes at rates $>40\%$, as our rearing experience suggests that this high mortality is likely to be due to diseases transmitted as a result of mass rearing or poor rearing techniques, and thus may not be an accurate reflection of natural causes of mortality. We also omitted data if the monarch stage at collection was not recorded, and if 100% of the monarchs reared by the volunteer were parasitized, as it is possible that these volunteers only reported parasitized monarchs. However, because 100% parasitism could be accurate with small sample sizes, deleting only the small sample size cases in which mortality was 100% could lead to an under-representation of parasitized monarchs from small sample sizes. We thus omitted all of the cases in which the total proportion of parasitized monarchs in a given sample size category was significantly different from the overall parasitism rate (as determined by a chi-square association test). Using this criterion, we omitted all cases in which a volunteer reared fewer than four monarchs from a given site in a given year. We combined data across the entire year because previous analyses showed no consistent effect of season (Oberhauser et al. 2007).

We used MLMP egg density and survival data to estimate the number of milkweed

plants needed to produce an adult monarch that will migrate to Mexico. The estimates used to calculate this value will vary across seasons, milkweed plant species, years, and regions, but the information has conservation importance that is broadly useful. We were limited in survival data (both egg to fifth instar as estimated by field observations, and fifth instar to adult as estimated by the rearing study) for all but the north-central region, but because this region produces a large portion of the monarchs that overwinter in Mexico (Wassenaar and Hobson 1998), it provides a valid starting point for estimating restoration targets. We estimated the value across all years (using our cleaned data set and only data from the late summer, which produces the migratory generation) as follows:

Equation 2.

$$\frac{\text{monarchs}}{\text{milkweed}} = \frac{\frac{\text{eggs}}{\text{plant}}}{\text{week}} \times \frac{\text{5th instars}}{\text{egg}} \times \frac{\text{adults}}{\text{5th instar}} \times \text{weeks}$$

where eggs per plant per week = the average of the total number of eggs/total plants observed in the northcentral region per week, starting with 7–13 July (chosen because it is likely that the adults resulting from eggs laid on or after 7 July will join the migratory population); fifth instars over eggs = all of the fifth instars observed from 21 July or later/all of the eggs observed from 7 July or later; adults/fifth instar = all of adults that were reared from larvae collected as fifth instars/ the number of fifth instars collected; and weeks = the total number of weeks, starting with 7–13 July, for which we had data from the north-central region across all years of the study.

RESULTS

Field Survival. Our analyses included 1,462 site–season–year combinations that met our inclusion criteria, representing a total of 424 sites. These sites were spread across the monarch range in approximately the same proportion, as illustrated in Fig. 1 (north-central: 1,057 site–season–year combinations from 289 sites; north-east: 86 from 40 sites; mid-central: 76 from 22 sites; mid-east: 118 from 36 sites; south-central: 144 from 36 sites; south-east: 1 from 1 site). Figure 2 illustrates mean egg to fifth instar survival rates across all years in regions for which we had >30 site–season–year observations that met our inclusion criteria. Summer survival in the north-central region was higher than spring survival in the north-central region, but there were no other significant differences between region–season combinations across all years.

Figure 3 illustrates yearly survival in the southcentral region in the spring, and the north-central region in the spring and summer (where sample sizes were >75 site–season–year observations). While no long-term trends are readily apparent in Fig. 3, it illustrates large year-to-year variation in survival, spanning at least an order of magnitude in each region.

In our best supported mixed-effects model with site and year as random effects (Table 2a), survival tended to be higher in sites that were planted and that had more milkweeds (e.g., larger sites), and lower in sites that had higher egg densities. Sites that were classified as noncrop agricultural (such as Conservation Reserve Program land, old pastures, and fields) or natural (such as nature reserves and state parks) had higher survival. There was lower survival during the spring season in the north (note the positive

effect of all other seasons, Table 2). Surrounding area was not included in either final model. When we included year as a fixed effect, while accounting for site by keeping it as a random effect, the same terms as above were significant in the same direction. Year was also significant, with a negative effect on immature survival (Table 2b).

Reared Monarch Survival. MLMP volunteers reared 18,157 monarchs for the survival study. Of these, 16,075 met all of our inclusion criteria. The vast majority (13,931) were from the north-central region, with most of the rest (1,830) from the mid-east region. Thus, we present some summary data across all regions, but only present comparisons between the north-central and mid-east regions.

The rate of parasitism increased (and, concomitantly, adult survival decreased) with the stage at collection (Oberhauser 2012), so we only used data from monarchs collected as fifth instars to investigate year-to-year variation in survival rates of larvae to adults. In the north-central region, parasitism rates varied from 3.2 (in 2004) to 38.4% (in 2012), and in the mid-east region, from 0 (in 2011, 2013, and 2014) to 55.6% (in 2002). Fifth instar to adult survival of reared monarchs in the north-central region varied from 56 (in 2012) to 90% (in 2003), and in the mid-east region, from 38 (in 2002) to 100% (in 2014; Fig. 4). There are no significant trends over time in parasitism or fifth instar to adult survival in either region (Pearson correlation tests, all $P > 0.24$), nor are there differences between the two regions. However, because we could not control for site (as a random effect) or site characteristics, these comparisons are less robust than comparisons using egg to fifth-instar survival. Note that data from the north-central region across all years are from 99 different monitoring sites (total n from sites = 3,495 plus $n = 1,689$ from

non-site locations, and the most from a single site = 458), while the data from the mid-east region are from 13 different sites, and 970 of the 1,335 are from a single site. Thus, our data from the north-central region are more likely to represent the region as a whole.

Monarchs per Milkweed Plant and Survival to Adult. Egg density in the north-central region, calculated from the total numbers of eggs and plants observed in a given week across all years, varied a great deal from July 7 to the end of the summer (Fig. 5). The mean weekly egg density, across the 14 wk illustrated in Fig. 5, was 0.043. Survival from egg to the fifth instar, calculated from survival from the northern summer period as defined in Table 1, was 0.077 (from a total of 3,912 fifth instars and 51,059 eggs). Survival from the fifth instar to adult, calculated from reared monarchs across all years in the north-central region, was 0.76 (from a total of 4,731 monarchs collected as fifth instars). The product of these three values and the number of weeks during which data were collected (14) is 0.035 monarchs per milkweed. Thus, in the region for which we have all of the data required to calculate this value, MLMP data suggest that one monarch that is likely to migrate to Mexico is produced per ~28.5 (the inverse of 0.035) milkweed plants, across all years of this study.

Across all years and weeks, summer survival in the north-central region from egg to adult is $\sim 0.077 \times 0.76 = 0.058$ (5.8%). In the spring, egg to fifth-instar survival (from a total of 1,986 fifth instars and 34,876 eggs) in this region across all years of the study is 0.057. Assuming that fifth instar to adult survival rates are the same in the spring and summer (Oberhauser et al. 2007), egg to adult survival in the spring is $0.057 \times 0.76 = 0.0431$ (4.3%). Note that both of these estimates depend on reared monarchs for fifth

instar to adult survival estimate.

DISCUSSION

Here, we discuss several factors that were associated with monarch immature survival, including variation between regions and seasons, planted versus nonplanted sites, and site types. We also discuss evidence for density-dependent mortality, and a decline in survival rates over the time of our study. Finally, we discuss how our findings can inform planting targets for milkweed, by estimating the number of plants needed to produce a monarch that will migrate to Mexico.

While we documented a great deal of variation in immature survival rates from year-to-year field survival rates (egg to fifth instar) across all regions and seasons, when combined across all years of the study, are similar to rates found in many other studies (Borkin 1982, Oberhauser et al. 2001, Prysby 2004), ranging from ~7 to 10%. Our rates are higher than those reported by Calvert (2004) and De Anda and Oberhauser (2015). Both of the latter studies were conducted in one or a few sites and over only two years, and survival in our study varied a great deal across years, so this difference is not surprising.

Across all years, survival rates were lower in the spring in the north-central region than they were in the summer in the north-central region (Fig. 2). The difference between north-central spring and summer is counterintuitive, as predator abundance might be expected to increase over the course of the summer. However, slower development rates in May and June could increase exposure to predators. Other hypotheses for this

difference are that cooler temperatures have direct effects on survival (Nail et al. 2015b), or that higher per plant densities in the spring (Stenoien et al. 2015a) lead to scramble competition. Our full model also showed lower survival during the spring season in the north (Table 2). When both egg to fifth instar and fifth instar to adult survival (the latter is overestimated because of indoor rearing) are taken into account in the north-central region, where we have enough data to estimate both parameters, our results suggest ~6% survival from egg to adult in the summer, and ~4% survival in the spring.

Several site-level factors affected survival. In our best supported model, planted sites had higher survival, suggesting that site management practices could influence survival or that planted sites might be selected in areas with attributes that promote survival (e.g., sites might be chosen in areas that are ideal for milkweed growth). Two site types (natural and noncrop agricultural) were also associated with higher survival. These site types include areas such as Conservation Reserve Program land (noncrop agricultural) and nature reserves (natural) that are often intended to increase habitat for wildlife, including pollinators, but more work is needed to investigate the mechanisms for the correlation between these site types and immature monarch survival. None of the other site characteristics that we hypothesized could be important (agricultural sites or garden sites) significantly affected survival in our final model.

Egg density was negatively correlated with survival, suggesting that density-dependent factors may affect survival, although it should be noted that overall density throughout this study period has not increased (Stenoien et al. 2015a). As further support for density-dependent survival, sites in which more milkweed plants were monitored had

higher survival rates, and there is a negative correlation between the number of plants monitored and per plant egg densities (Stenoien et al. 2015a). Density dependence could be driven by predation, parasitism, disease (Lindsey et al. 2009), or competition amongst immature monarchs for resources. Many studies suggest that immature monarch survival is largely driven by the top-down effects of predators and parasitoids (Borkin 1982, Prysby 2004, De Anda and Oberhauser 2015), and there is evidence that natural enemies drive some species' population dynamics (e.g., larch budmoths [Turchin et al. 2003] and the southern pine beetle [Turchin et al. 1999]), although other work points to the importance of bottom-up forces on populations (e.g., White 1978). Future research should attempt to ascertain the interacting influences of natural enemies, milkweed characteristics, the potentially cascading effects of sequestered milkweed toxins on natural enemies, and abiotic factors (including temperature and precipitation) on these year-to-year and density-dependent variations in immature monarch survival.

There was a significant negative effect of year on monarch survival from egg to fifth instar, when we controlled for site, suggesting that immature survival rates declined over the time period of this study. We are not sure what is causing this decline over time; one possible explanation is that the rate of *O. elektroscirra* infection is increasing (Satterfield et al. 2015), and monarch larvae infected with *O. elektroscirra* show decreased survival (Altizer and Oberhauser 1999). Another possibility that cannot be tested using the data from this study is that predators are killing larval monarchs at higher rates, although we did not detect an increase in tachinid fly parasitism over the course of the study (Fig. 4). The decline does not appear to be driven by increased larval

competition as females are forced to lay eggs in smaller and more dispersed habitats because overall density did not increase over the period of this study (Stenoien et al. 2015a). While there was a negative trend over time when we accounted for site, neither of our survival metrics (egg to fifth instar or fifth instar to adult) improved a regression model against the area occupied by monarchs overwintering in Mexico (Stenoien et al. 2015a). Thus, our calculated survival rates do not appear to be driving the fluctuations observed in monarch numbers at the overwintering sites, at least not in a simple way. However, it is possible that this analysis did not uncover complex interactions between abiotic conditions, egg densities, and the large suite of predators that attack monarchs (reviewed in Oberhauser et al. 2015b).

We could not conduct the analysis of fifth instar to adult survival at the site level because few individual sites had enough data to do robust analyses of survival, and many volunteers collect larvae at a variety of sites. Thus, our analyses of fifth instar to adult survival are done at the regional level, and we did not detect any differences in this survival metric when comparing regions (we could only compare the north-central and mid-east regions) or over time. There was a fairly constant rate of pupal death from other causes across the years of the study, at least in the north-central region for which we have the most data, suggesting that tachinid fly parasitism was an important driver of survival from the fifth instar to adult, at least for reared monarchs. However, our assessment of fifth instar to adult survival is calculated based on indoor rearing, and thus does not include pupal mortality from predators such as *Polistes* wasps (Oberhauser et al. 2015b) or pupal parasitoids (Oberhauser et al. 2015b), or from extreme weather conditions.

Addressing this data gap will be difficult because finding pupae in the wild is rare (our complete database only includes 31 monarchs that were collected as pupae). We do know that parasitism by *Pteromalus cassotis* Walker, a gregarious parasitic wasp of lepidopteran pupae, can cause high mortality (up to 100% at some sites; Oberhauser et al. 2015b) that is not taken into account in our analysis.

A key finding of this study is the large variation in immature monarch survival from year to year, both from the egg to the fifth instar, as measured by field sampling, and from fifth instars to adults, as measured by reared larvae collected from the wild. While we are not accounting for mortality from pupal parasitoids or predators, or abiotic factors that could affect pupal survival, this finding demonstrates that it will be important to consider immature mortality in population models and conservation actions. Another key finding is the positive association between survival and the number of plants monitored, suggesting that conservation actions should encourage plantings with large numbers of milkweed plants, not only because more plants will support more monarchs but also because survival is likely to be higher.

The amount of milkweed required to produce an adult monarch has, to our knowledge, never been calculated using long-term field data. While ~29 milkweeds for the production of one adult monarch is a good starting point for conservation efforts, this is a conservative estimate because pupae were not exposed to many sources of mortality that are likely to be important in the wild. Additionally, this estimate is likely to only apply to the north-central region and the milkweed species common to sites in this region (*A. syriaca*). However, depending on the region and species of milkweed available for

planting, this measure can be used as a minimum in monarch conservation planning.

Finally, this study documents the value of the intensive monitoring done in a program like the MLMP; none of the parameters estimated here would have been obtainable with less intense monitoring. MLMP volunteers have provided an incredible amount of data across a wide geographic range. However, there remain significant data gaps that document the need for recruitment in regions that are not currently well-represented by MLMP volunteers. Of the 16 possible region–time combinations for this analysis, only 7 had enough data for robust analyses (see Fig. 2). While we have fairly good coverage in the northern and south-central regions, more data from the mid-latitudes and the south-east would provide a more complete picture of monarch survival and population dynamics.

Table 1. Date ranges for different seasons and the monarch stage and regions analyzed (NC = north-central, NE = north-east, MC = mid-central, ME = mid-east, SC =south-central, SE = south-east).

Season	Monarch stage	Dates	Regions* analyzed
Southern Spring (1st generation)	Eggs	March 1- May 31	ME, MC, SE, SC
	Fifth Instars	March 15- June 14	
Northern Spring (2nd generation)	Eggs	April 1- June 30	NE, NC, ME, MC
	Fifth Instars	April 15- July 14	
Northern Summer (3rd and 4th generations)	Eggs	July 1st- September 30	NE, NC, ME, MC
	Fifth Instars	July 15th- October 14	
Southern Summer (final generation produced by a portion of the population)	Eggs	August 1- October 31	ME, MC, SE, SC
	Fifth Instars	August 15- November 14	

See text for explanation of criteria used to select date ranges.

*See Fig. 1 for delineation of regions.

Table 2. Fixed effect variables in the best supported binomial mixed-effects models for monarch survival. a) Site ID and year as random variables. b) Only site ID as a random variable. See text for explanation of models.

	Estimate	SE	Test statistic*	P-value
a. Final model with year and site as random effects				
Intercept	-4.60	0.154	-29.8	<0.0001
Planted	0.748	0.196	3.82	<0.0001
Non Crop Agricultural	0.626	0.208	3.01	0.00014
Natural	0.483	0.198	2.44	0.0147
Average milkweeds	0.00143	0.000133	10.7	<0.0001
Northern Summer	1.16	0.0434	26.7	<0.0001
Southern Fall	1.19	0.0770	15.5	<0.0001
Southern Spring	1.26	0.121	10.3	<0.0001
Egg Density	-1.19	0.0844	-14.1	<0.0001
b. Final model with site as random effect				
Intercept	-4.12	0.140	-29.4	<0.0001
Planted	0.794	0.196	4.04	<0.0001
Non Crop Agricultural	0.670	0.210	3.19	0.00014
Natural	0.560	0.199	2.82	0.00488
Average milkweeds	0.000981	0.000117	8.39	<0.0001
Northern Summer	1.22	0.0432	28.2	<0.0001
Southern Fall	1.26	0.0765	16.5	<0.0001
Southern Spring	1.22	0.122	10.1	<0.0001
Egg Density	-1.23	0.0844	-14.61	<0.0001
Year	-0.0494	0.00469	-10.51	<0.0001

*test statistic = z-value

Figure 1. Analysis regions and MLMP sites (before data cleaning). While the vertical lines dividing the east and central regions from each other are slightly different than those used in other regional analyses (e.g., Ries et al. 2015), very few sites with usable data were affected by the difference, and the data sorting was more feasible using longitude lines. (See Table 1 for region abbreviations).

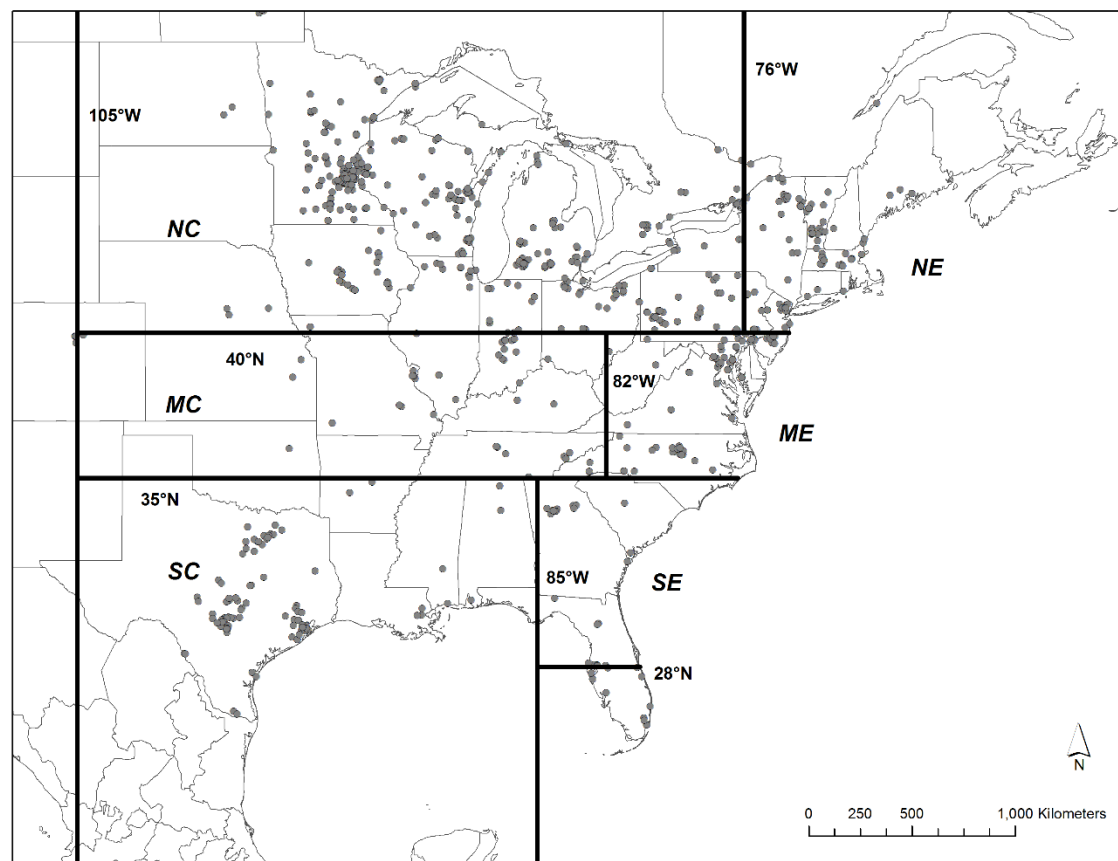


Figure 2. Mean proportion of monarchs surviving from egg to fifth instar (as defined by equation $1 \pm SE$) across all years of the study for regions and time periods with a minimum sample size of 30 (sample size [number of site/time period combinations] shown above error bars). Means with different letters are significantly different (Tukey's honestly significant difference, $P < 0.05$).

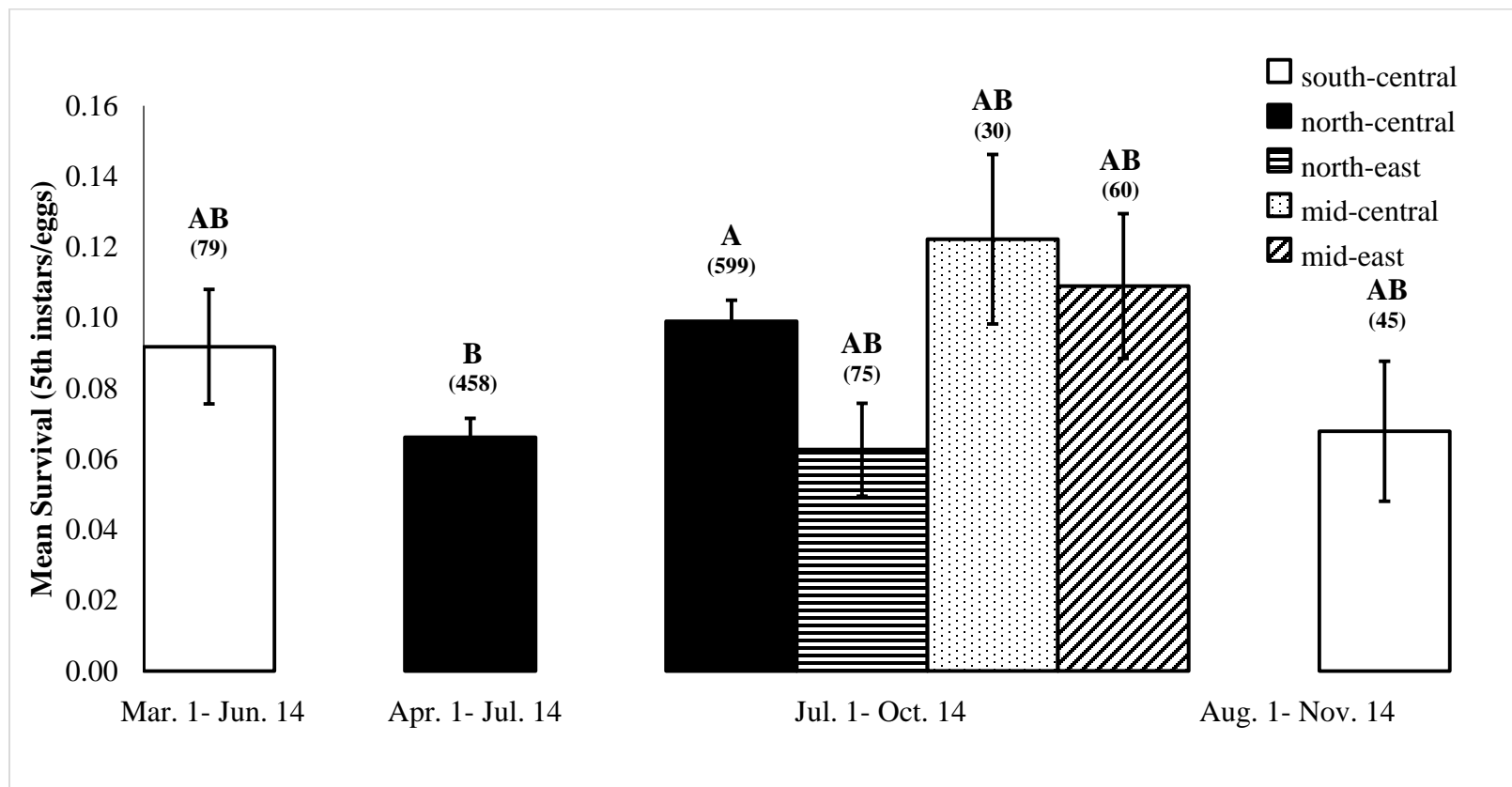


Figure 3. Mean egg to fifth instar survival (across all sites that met our inclusion criteria) over time. The mean yearly sample sizes for each region are 4.9 (\pm 0.7 SE) for spring in the south-central region, 25.4 (\pm 3.1 SE) for spring in the north-central region, and 33.3 (\pm 3.8 SE) for summer in the north-central region.

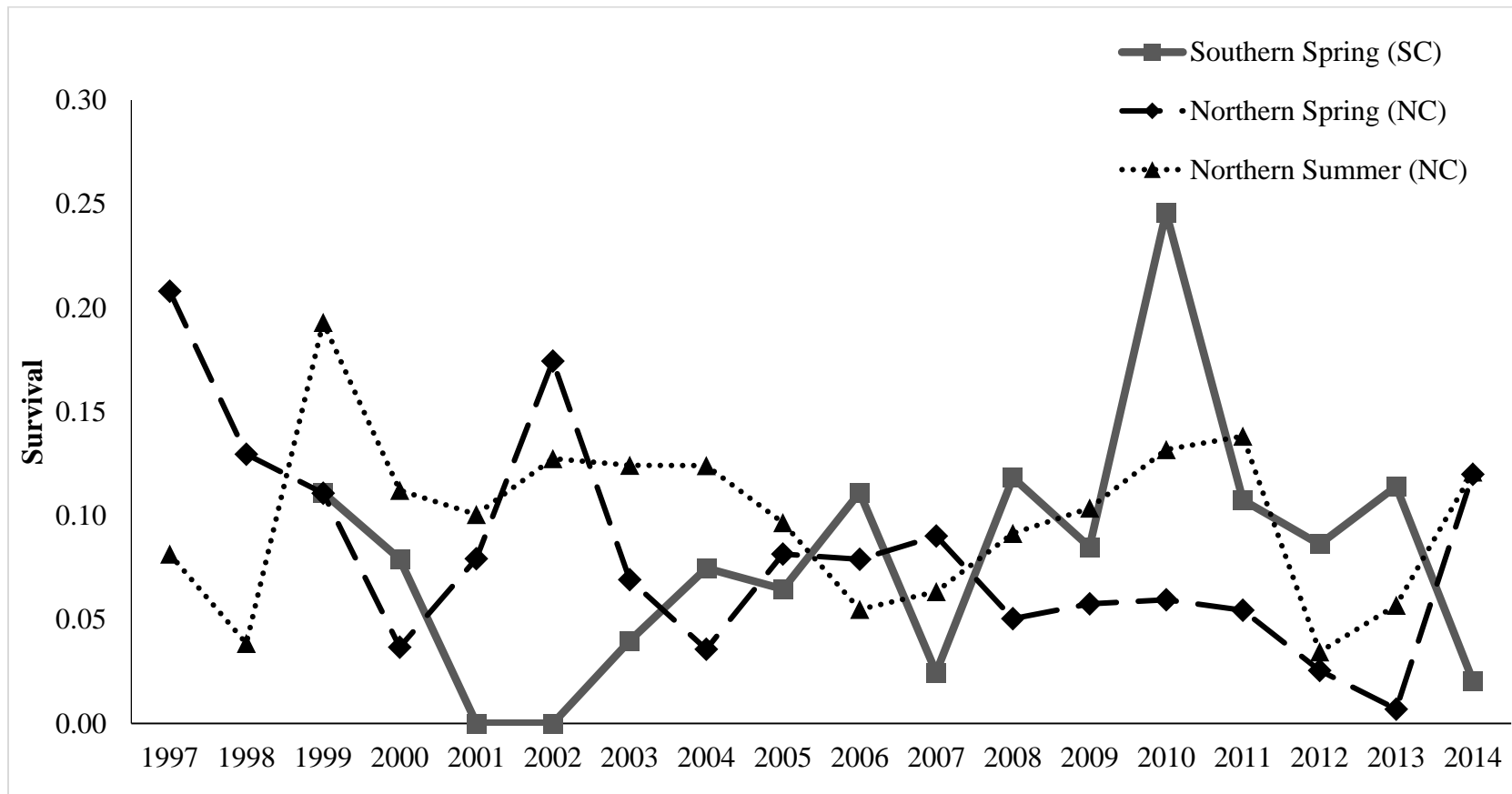
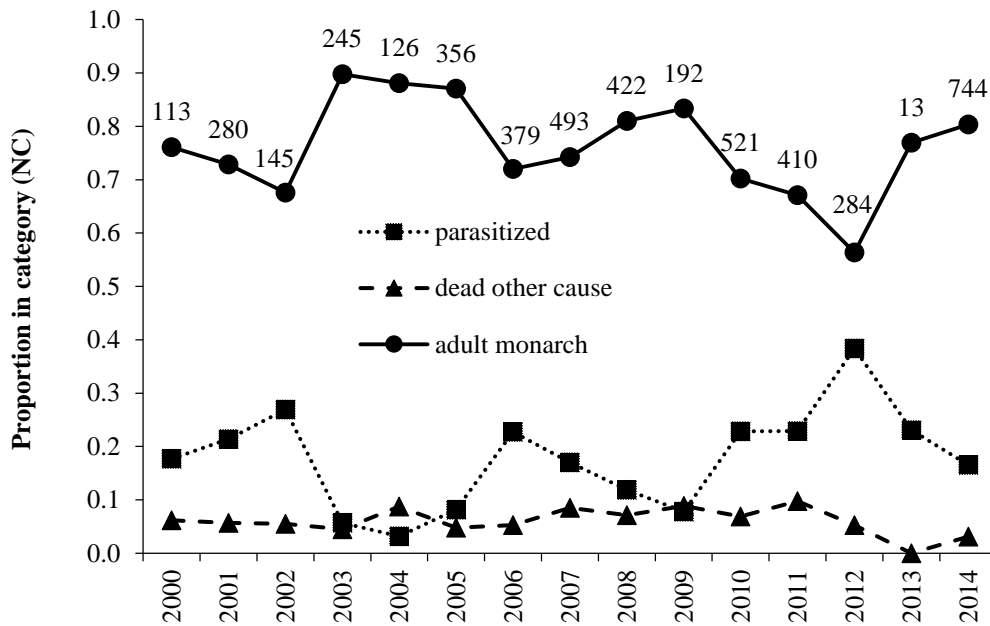


Figure 4. Proportion of monarchs collected as fifth-instar caterpillars that were parasitized by tachinid flies, died of other causes, and survived to the adult stage in the (a) north-central and (b) mid-east regions. Years in which < 10 fifth instars were reared are not illustrated. Sample sizes (total number of fifth instars that were collected) for each year are indicated.

a)



b)

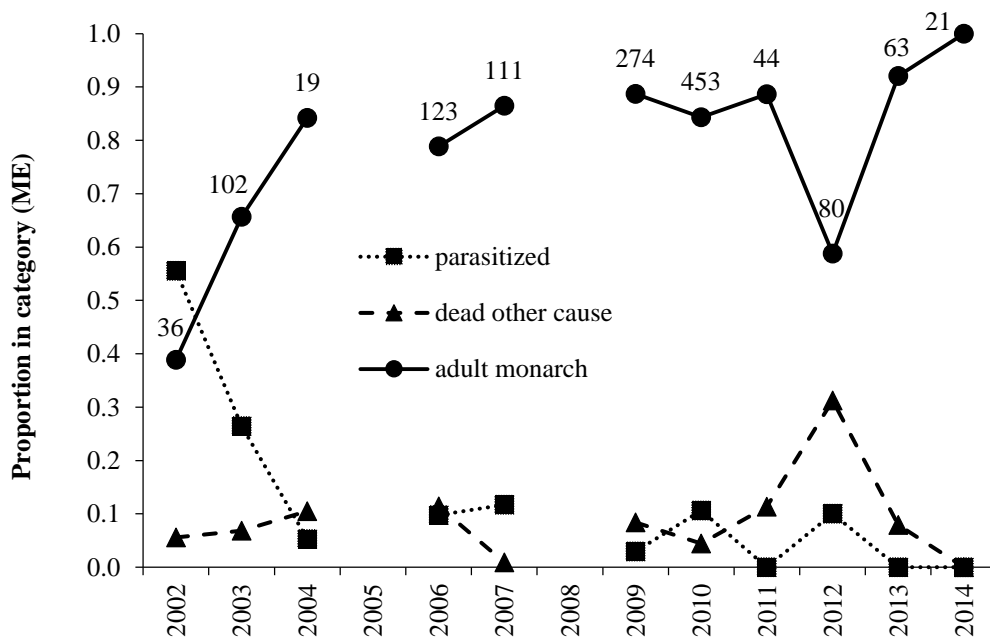
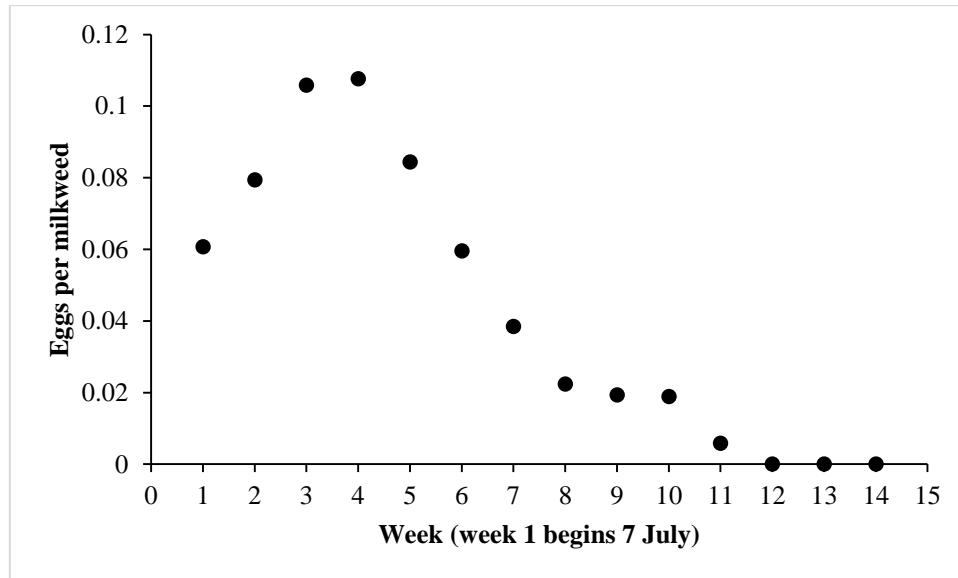


Figure 5. Weekly per milkweed plant egg density in the north-central region for the monarchs that are likely to migrate to Mexico. Values calculated by summing all eggs and all plants observed in a given week across all years of the study (egg $n = 1,046$ and milkweed $n = 885,188$).



Supplementary Text S4.1: While this dissertation is written by the dissertation author, the manuscript version of this chapter has the following authorships and affiliations:

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