Final Report to Texas Comptroller of Public Accounts
Economic Growth and Endangered Species Management

Pilot study on the potential role of Red Imported Fire Ants (*Solenopsis invicta*) on Monarch Butterfly (*Danaus plexippus*) reproductive recruitment in northeast Texas.

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Freshly emerged first generation adult photographed at Cooper Lake WMA, 7 May 2016

Acknowledgements

Abstract

This pilot study was conducted to determine if sufficient numbers of ovipositing Monarch Butterflies (*Danaus plexippus*) could be located in northeast Texas to warrant future, more detailed studies on the role of Red Imported Fire Ants (RIFA) (*Solenopsis invicta*) and other sources of mortality on monarch egg and larval survivorship. Other objectives were to modify the methods of previous studies and develop recommendations for future research.

Monarch survivorship on control plants and on experimental plants surrounded by an exclosure were much higher than most other estimates in the literature. Survivorship of experimental eggs and larvae was higher than that of control eggs and larvae. Emigration from host plants made accurate analyses of the survivorship of fourth and fifth instars impossible. Data from sticky traps showed that RIFA were three times more abundant than any other arthropod, predatory or otherwise. Around experimental plants, where survival to the third instar was highest, RIFA were more abundant. RIFA mounds were closer to control plants where eggs survived to third instar than they were to control plants where eggs did not survive to third instar. Over 20% of plants that had RIFA on them had instars survive to third instar and RIFA accounted for only 7.7% of observed mortalities. Monarch eggs and larvae lived longer on plants with more spiders and ants. Monarch survival may be dependent on predator-mediated indirect effects in which RIFA are an important component. However, problems with the arthropod data make these conclusions tenuous.

Three studies are proposed to generate more accurate and precise evaluation of monarch survival. The first is to census arthropod communities on plants using two sets of controls, plants in areas with suppressed RIFA populations, and plants in areas with enhanced RIFA foraging densities. The second is to conduct choice experiments to determine RIFA prey preferences among arthropods found on milkweed plants. The third is to use harmonic radar to follow fourth and fifth instars in order to document dispersal behavior and the mortality rates of older instars.

Introduction

In 2014 the Monarch Butterfly (*Danaus plexippus plexippus*) was petitioned for listing under the Endangered Species Act (ESA) (Monarch ESA Petition 2014). This came in response to 90% declines in populations of monarch butterflies east of the Rocky Mountains in the previous decade (Monarch ESA Petition 2014). Listing of the monarch butterfly under the ESA has enormous economic ramifications across this species' near continental distribution because protections provided under the ESA have major influences on land use, land management, and development. Critically important to the listing of a species under the ESA, and the protections delineated by that listing, is the quality and extent of scientific information regarding that species. The purpose of this project was to investigate oviposition and brood success among spring (generation 1) monarchs in northeast Texas. Particular attention was paid to the role that the Red Imported Fire Ant (RIFA) (*Solenopsis invicta*) might play in reducing egg and larval survivorship in monarch butterflies.

The eastern population of the monarch butterfly in North America has a near continental distribution that covers the area east of the Rocky Mountains to the Atlantic Ocean in Canada and south into central Mexico (Scott 1986). The species is migratory, spending the winter in mountain refuges in central Mexico and migrating north in spring. Spring migration is accomplished through successive reproductive events; a first generation that occurs in the southern tier of the U.S., a second generation that occurs across the central U.S., and a third and fourth generation that occurs in the northern tier of the U.S. and southern Canada. This breeding distribution encompasses more than 12 million km^2, though only a portion of this breeding distribution may be active at any given time (Flockhart et al. 2013). Population size is lowest during the winter due to an extended period of predation and mortality without reproductive recruitment (Malcolm et al. 1993). Because of this, first generation recruitment in spring is extremely important for establishing the size of subsequent generations. Recent isotopic analyses have shown that the most important portion of North America for the production of first generation adults is in Texas and Oklahoma (Flockhart et al. 2013).

Despite the important role that north Texas plays in reproductive recruitment of the monarch butterfly, there is little information on what factors affect spring reproduction in this region. Studies in other areas report that monarch survival rates from egg to fifth instar are extremely low, as low as 4% in Louisiana but more generally ranging from 5% to 20% across the species' breeding distribution (Prysby and Oberhauser 2004). Survivorship curves of monarchs in Wisconsin demonstrate that most mortalities occur within seven days of the eggs being laid and, in some cases, there was 50% mortality within the first 24 hours (Prysby 2004). In Minnesota, it was found that only 20% of eggs survived long enough to hatch into 1st instar larvae (De Anda and Oberhauser 2015). Mortality rates among larvae beyond the first instar tends to be lower.

One study in central Texas showed complete reproductive failure (0% survival), a result that was attributed to depredation by RIFA (Calvert 1996). None of the 33 eggs survived past the first instar. A follow-up study using exclosures to exclude fire ants and other terrestrial predators found survivorship rates of 1.6% to 27% inside the exclosures and 0 to 1.4% outside the exclosures (Calvert 2004). These results strongly suggest that terrestrial predation, and in particular RIFA, has an important impact on monarch reproductive success in Texas.

RIFA are known to have negative impacts on at least some vertebrates (Kopachena et al. 2000, Allen et al. 2004) and are well known to have negative community-wide impacts on arthropod populations (Porter and Savignano 1990, Morrison 2002). However, there is also evidence that some arthropods may benefit from the presence of RIFA (King and Tschinkel 2006) and, in some cases, there is a positive relationship between RIFA density and arthropod diversity (Morrison and Porter 2003). This can occur if RIFA influence trophic cascades as found in one species of tropical ant (Dyer and Letourneau 1999) and could also occur if RIFA had negative impacts on other predators of monarch eggs and larvae.

The studies conducted in Texas (Calvert 1996, 2004) suggest that RIFA have important negative impacts on monarch reproduction. However, the 1996 study was based on a crude correlation between

high RIFA mound density at the study site, a single observation of a RIFA attacking a first instar larva, and complete reproductive failure based on only 33 eggs. The follow-up study, which used exclosures, provides stronger support for the idea that RIFA are important predators on monarch eggs and larvae (Calvert 2004). That study, based on over 700 eggs, found monarch survivorship was 26 times higher inside the exclosures than outside the exclosures and RIFA densities were 3.4 times higher outside the exclosures than they were inside the exclosures. However, the study still did not isolate RIFA as the cause of higher mortalities outside the exclosures because the effect of the exclosures on other predators was not measured. There are myriads of other arthropods that prey on monarchs, including wasps, spiders, stink bug nymphs, syrphid fly larvae, ladybird beetles, assassin bugs, lacewings, and variety of other dipterans (De Anda and Oberhauser 2015, Oberhauser et al. 2015). Lastly, ants other than RIFA, are known as important predators of monarch eggs and larvae (Prysby 2004) and the study by Calvert (2004) did not indicate whether predation rates were higher than would be expected from native ants. To understand the role RIFA play in the reproduction of monarchs in Texas, a more refined approach is necessary.

The purposes of this pilot study were to:

1. Determine if sufficient numbers of ovipositing monarch butterflies can be observed in the area around Commerce, Texas, to warrant more detailed research on the survivorship of monarch eggs and larvae. The concern was that monarch populations in northeast Texas are so dramatically depressed that it would no longer be possible to collect sufficient samples for valid statistical comparisons.

2. Refine the protocols used by Calvert (2004), Prysby and Oberhauser (2004), and Prysby (2004) to document survivorship of monarch eggs and larvae and the potential role of RIFA in the survivorship of monarch eggs and larvae in the area around Commerce, Texas, and to compare these rates to published studies in other geographic locations.

3. Use the results of the current pilot study to develop more detailed study of monarch reproduction in northeast Texas with the goal of producing a key factor analysis that identifies those factors with the greatest impact on survivorship. These factors would then be used to inform future management decisions.

Methods

Two contiguous study areas in Hopkin's County in northeast Texas were used (Figure 1). These sites were chosen because of the abundance of milkweed plants, the presence of RIFA, and ease of access. The only milkweed species at both sites was Green-flowered Milkweed (*Asclepias viridis*), though a small patch of Butterfly Weed (*Asclepias tuberosa*) was found adjacent to one site. The first site was in the Cooper Lake Wildlife Management Area adjacent to the Tira Boat Ramp and the TPWD office at Lake Jim Chapman (Figure 1 and 2). This site, the Tira site, was about 17 ha in size and was burned in the spring or winter of 2004, 2007, 2011, and 2013. It was most recently burned on 11 February, 2016 (Howard Crenshaw, personal communication). Burning clearly impacted the amount of exposed ground at this site and, early in the season, made locating milkweed plants and eggs much easier (Figure 2).

The second site was in the Cooper Lake State Park South Sulphur Unit in an area formerly known as Cooper Lake Center (CLC) and was about 6 ha (Figure 1 and 3). This site is contiguous with the Tira site (Figure 1) and has not been burned in over 20 years. The ground cover at CLC contained much more thatch (Figure 3B) which made finding milkweed plants in early spring more difficult.

Monarch eggs and larvae were monitored 28 March 2016 - 14 May 2016. Two methods were used to find eggs. First, ovipositing females were followed and observed laying eggs (Figure 4A). Second, milkweed plants were searched for eggs (Figure 4B). It was found that female monarchs laid multiple eggs on the same plant and that multiple females laid eggs on the same plant. To keep track of these eggs, leaves were marked with a permanent felt-tipped marker (Figure 5). Individual eggs on

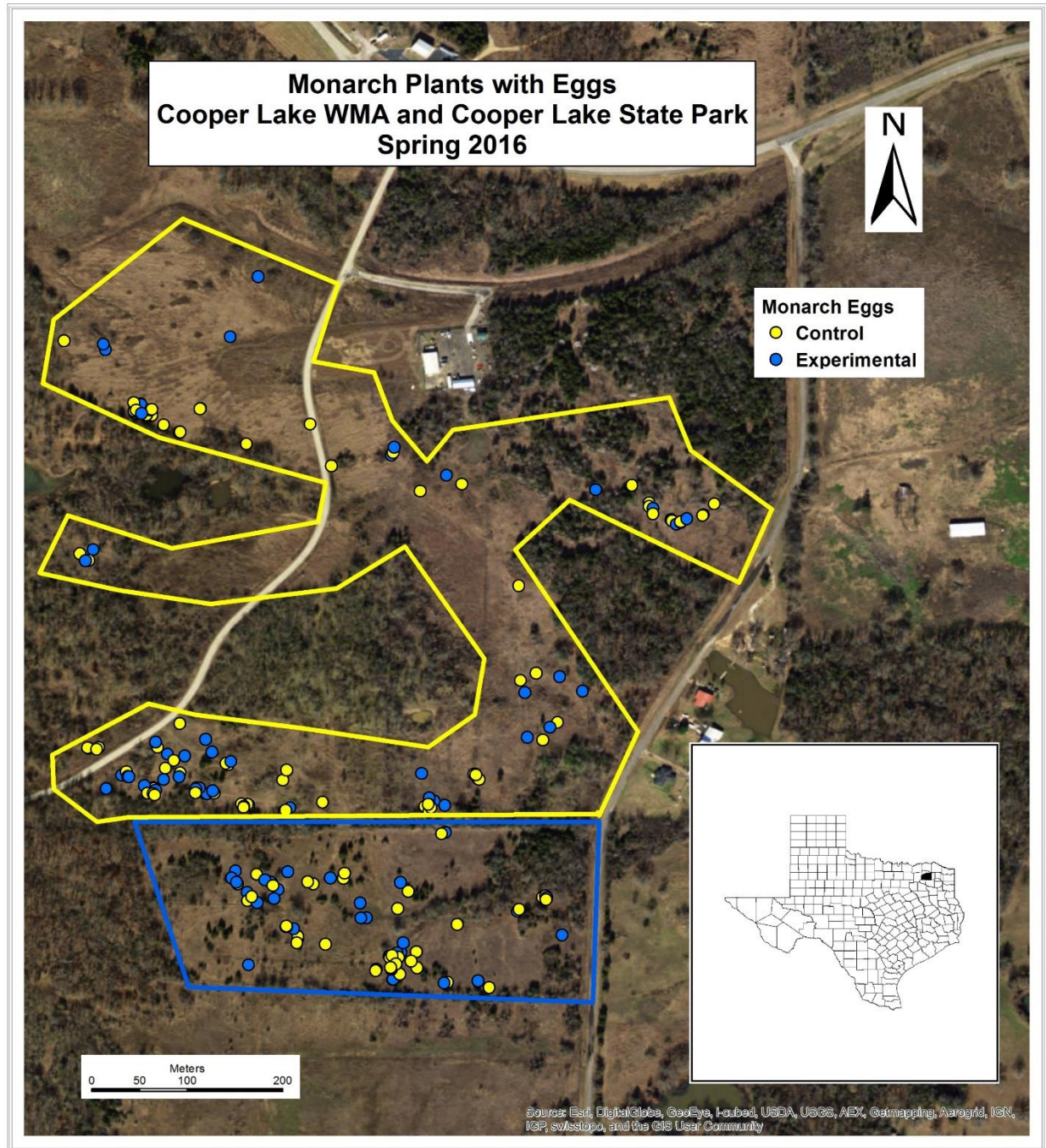Figure 1. Map of study sites used. Inset shows the location of Hopkins Co. in Texas. Area outlined in yellow is the Tira site. Forested areas were excluded. The area outlined in blue is the CLC site. Yellow markers are locations of control plants; blue markers are locations of experimental plants. Owing to the proximity of many plants, many of the markers are overlain on this image and, therefore, not visible.

Figure 2. The Tira study site. A. Overview of vegetation on 31 March, 2016. B. Typical ground cover of Tira site on 31 March, 2016. C. Overview of vegetation at the Tira site on 27 April 2016. D. Typical ground cover at Tira site on 28 April 2016.

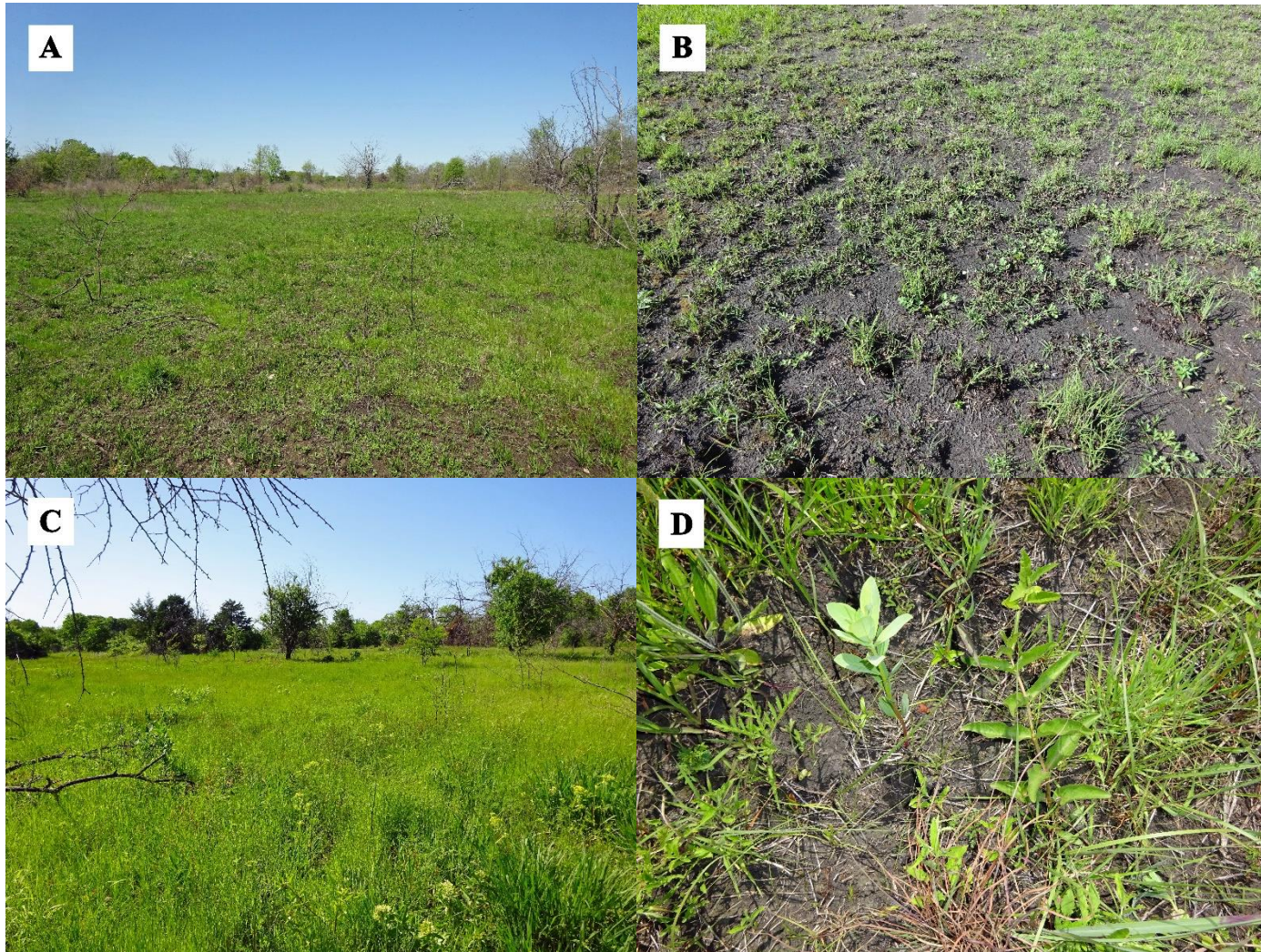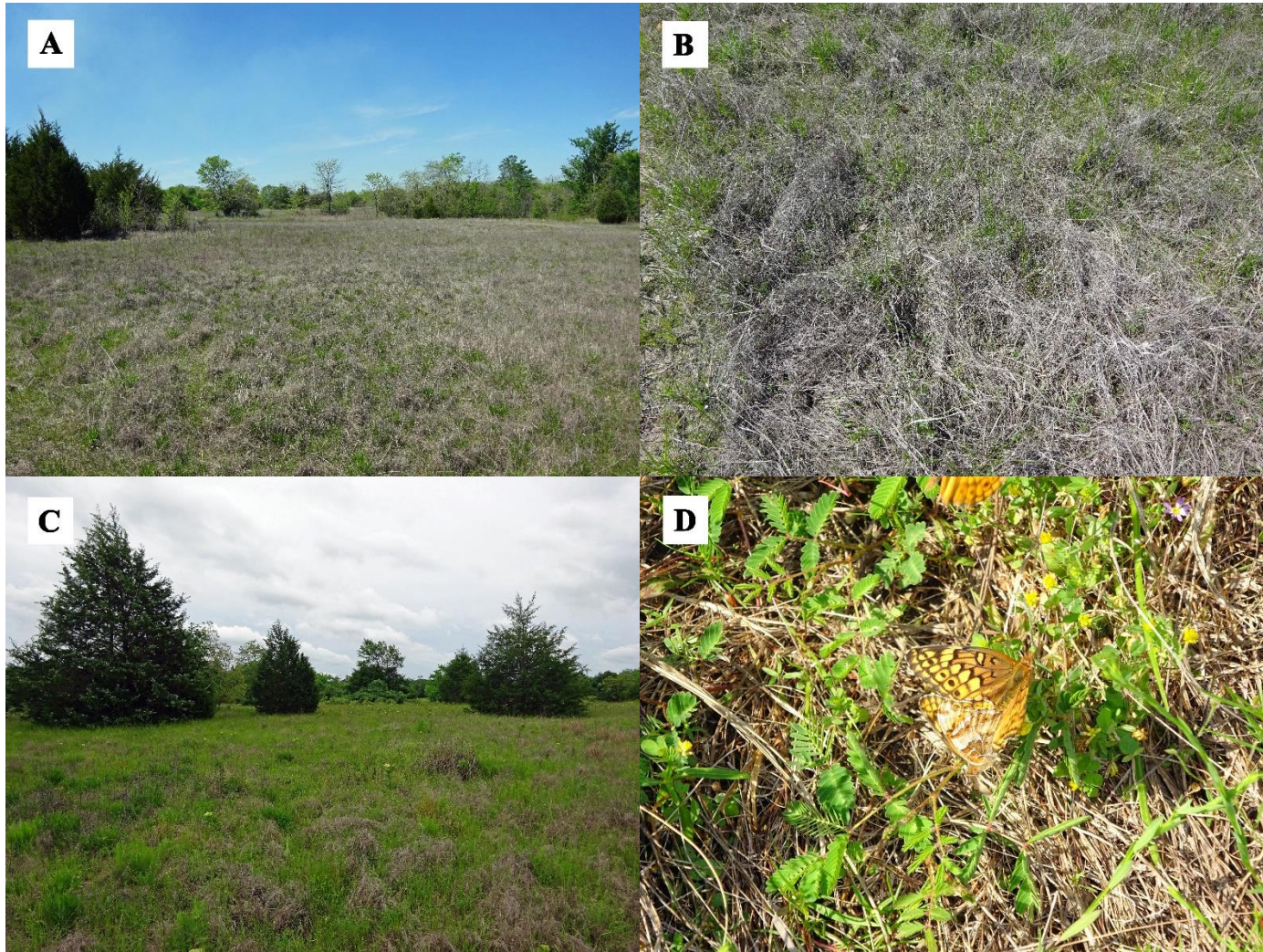Figure 3. The CLC study site. A. Overview of vegetation on 5 April, 2016. B. Typical ground cover of CLC site on 5 April 2016. C. Overview of vegetation at the CLC site on 8 May, 2016. D. Typical ground cover at CLC site on 28 April 2016

Figure 4. A. Monarch ovipositing on milkweed plant at Tira site on 31 March, 2016.
B. Monarch egg found on milkweed plant at Tira site on 31 March, 2016.

Figure 5. Leaves marked to identify eggs. A. Freshly marked leaf with egg. B. Marks on a leaf that were put there 11 days prior. Note the lack of necrotic tissue or discoloration of leaf tissue. This mark was for the third egg found on this plant.

plants were numbered sequentially as they appeared and numbers up to five were indicated with dots. Numbers over five were indicated using alphanumeric characters. These m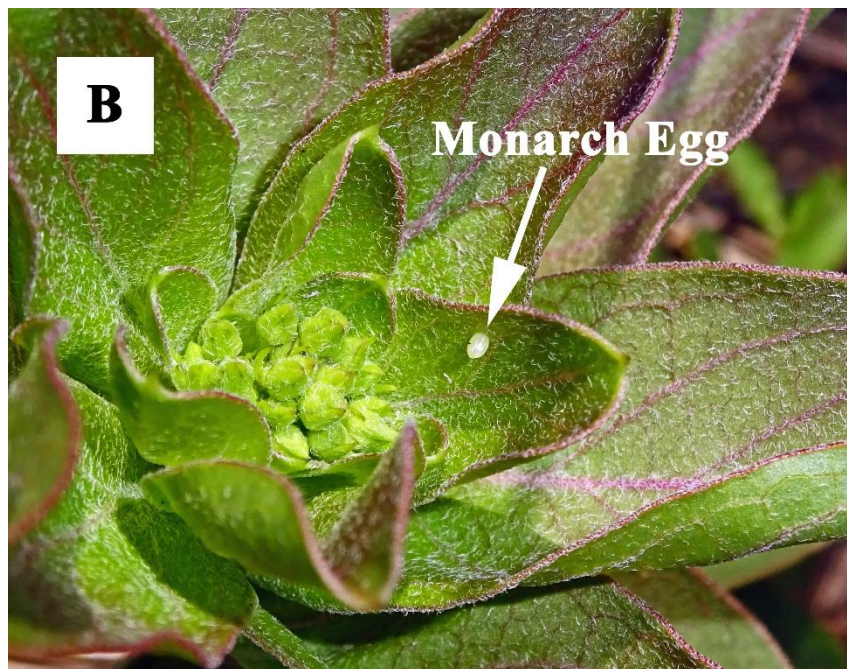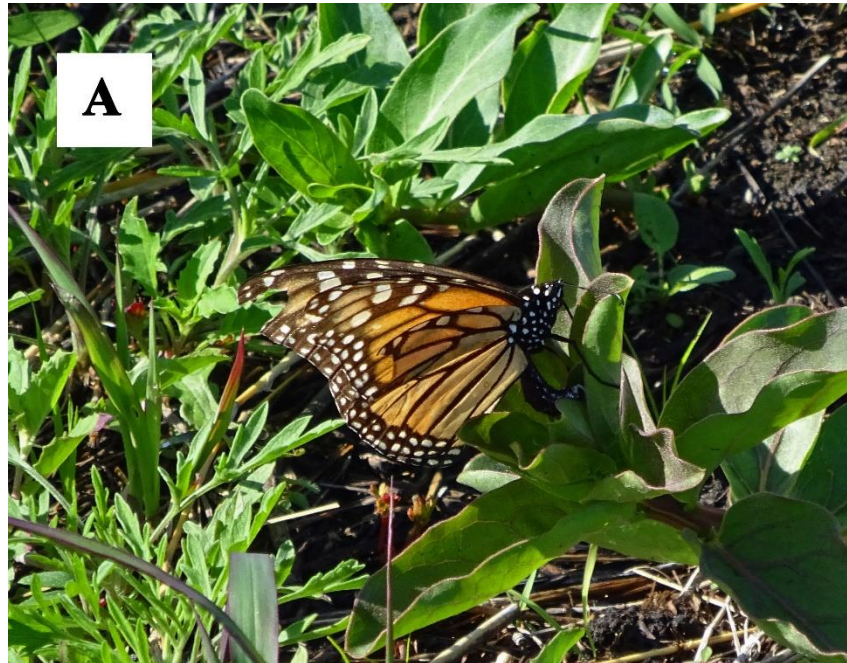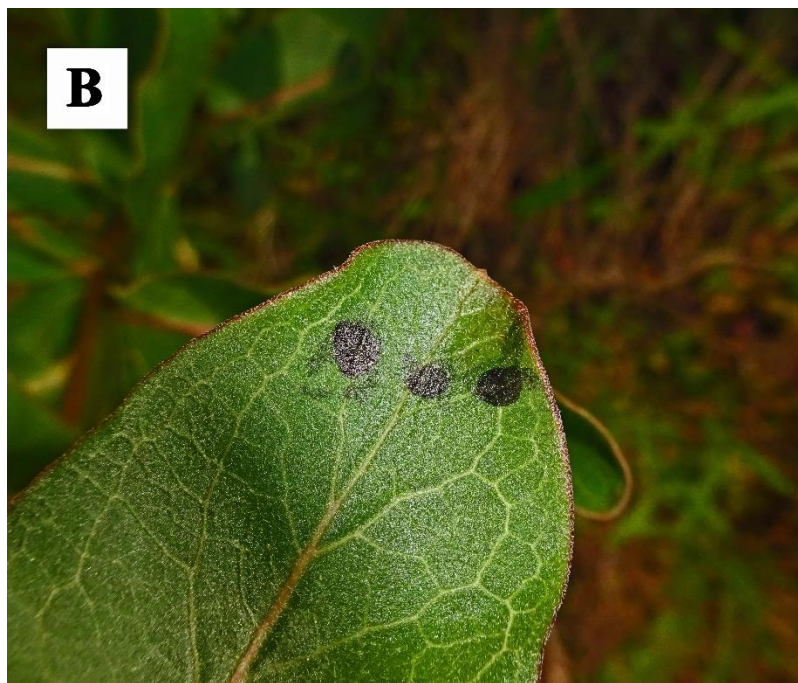arks had no impact, positive or negative, on the leaf, the eggs, or the instars (Figure 5B). After heavy rains the dots tended to fade and sometimes had to be re-marked.

At the time that eggs were located, GPS coordinates were taken and the eggs assigned to one of two treatment groups, control or experimental (Figure 1). An attempt was made to alternately assign eggs to one or the other treatment as they were found. This was not always possible because multiple eggs on a plant necessarily resulted in all of those eggs being assigned to whatever treatment the plant had been assigned. Some plants, by virtue of their size or location adjacent to RIFA mounds or RIFA runways were unsuitable for use as experimental plants. Lastly, time constraints made it difficult to maintain equal numbers of experimental and control plants. Control plants were marked with a white flag that contained the plant's identification number and a blue flag that improved the plant's visibility for ease of relocating the plant (Figure 6A).

Experimental treatments were designed to measure the effect of terrestrial (non-volant) arthropod predators of monarch eggs and larvae. For this purpose, exclosures were built (Figure 6B). All of the vegetation was cleared as near as possible to bare soil in a circular area with a diameter of 60 cm around the plant. Any arthropods observed in the cleared area were removed and if RIFA were observed within the cleared area, the plant was not used for experimental treatment. A circular barrier, 30 cm in diameter, and constructed of 25 cm wide galvanized sheet metal was placed around the plant and sunk 8 cm into the ground to prevent RIFA from tunneling under the exclosure wall. A drywall cutting knife was used to cut through the soil to enable the exclosure to be inserted into the ground. This left an above ground physical barrier of 17 cm. with an outside buffer of bare ground around the exclosure of 15 cm. Tall vegetation in the vicinity was removed to ensure that it did not lean on or over the barrier or would be blown onto the barrier by wind or storm activity. Each exclosure had four 1.6 cm drainage holes at ground level. The drainage holes were covered with stainless steel wire mesh (0.05 cm mesh) affixed to the wall of the
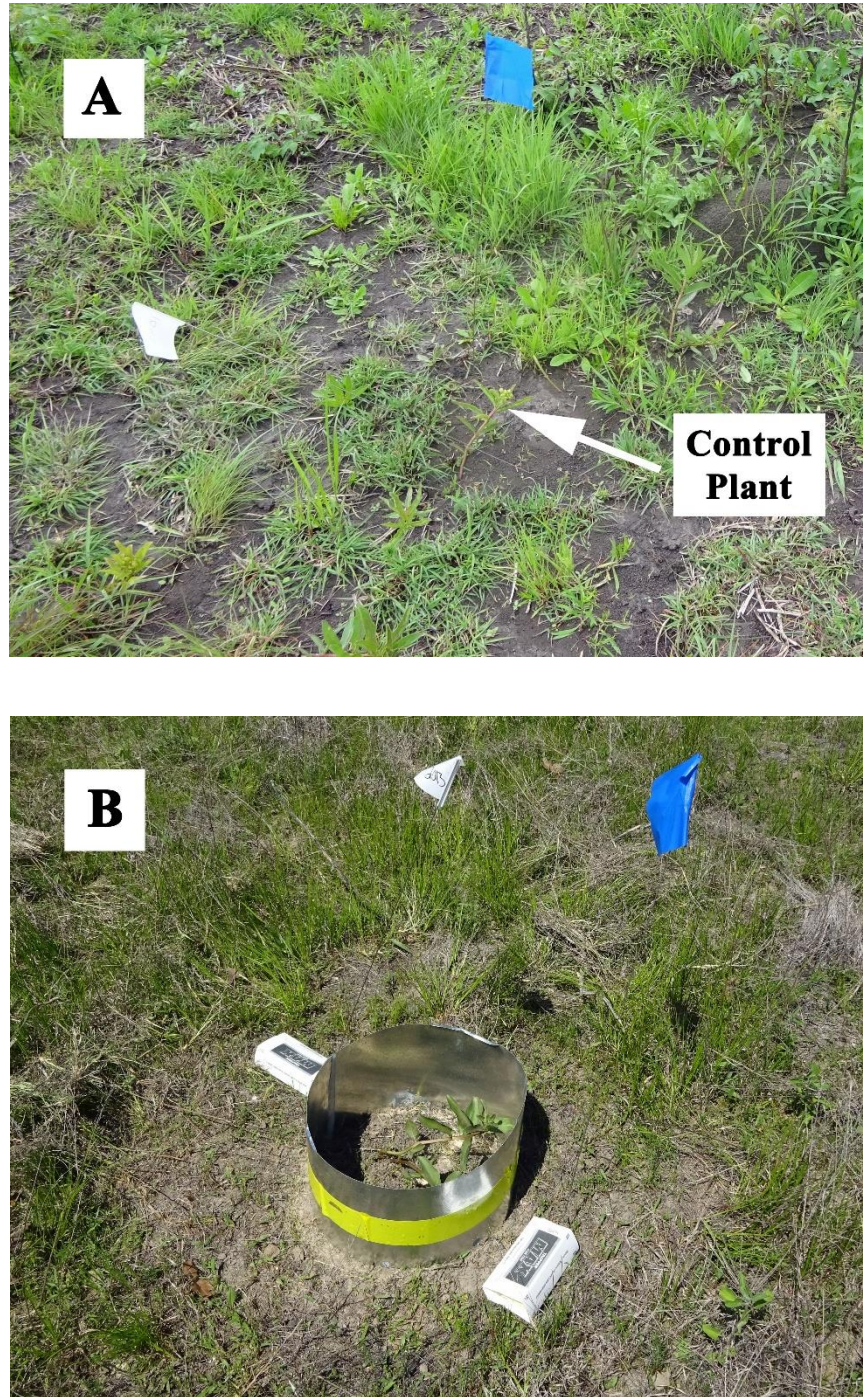
Figure 6. A. Control plant at the Tira site on 12 April, 2016, marked with a white flag and a blue flag. There is a RIFA mound in the upper right of this picture. B. Experimental plant at the CLC site on 5 April, 2016, showing exclosure, marker flags, and sticky traps. Sticky traps were used at both control and experimental plants (see methods).

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exclosure with silicon sealant. Green-flowered milkweed frequently has a decumbent growth form, so to prevent the plant from leaning on or over the barrier, some of the plants needed to be supported with a wire stake. To prevent ants and other arthropods from climbing over the barrier, a 4" strip of sticky tape (Gemplers Turf and Pest Management) was wrapped around the outside of the exclosure (Figure 6B). Seams in the exclosure were sealed with silicon sealant.

For control and exclosure plants, the occupancy of the plant was monitored daily between 09:00 h and 18:00 h and the stage of development of noted. Larval instars were identified according to Oberhauser and Kuda (1997). As noted other studies (Prysby and Oberhauser 2004), very young instars were often extremely difficult to find on the plants. Furthermore, larval monarchs are known to temporarily leave the host plants to seek shelter under cover during hot periods, when they are disturbed, or for a variety of other reasons (Rawlins and Lederhouse 1981, Borkin 1982). This can cause instars that are otherwise present to go undetected. To avoid falsely assuming that an instar was missing, plants were revisited for four days after the instar went missing before concluding that the instar was, in fact, missing from the plant. In this way daily records were kept of which plants were occupied by monarch eggs or larvae, the stage of development of the larvae, and the age at which they went missing. All individuals were monitored until they were either missing for four or more days or until they reached the fifth instar. For each plant the presence of other arthropods on the plant and cases of predation were recorded.

The abundance of potential predators, particularly that of RIFA, was measured by placing pesticide and bait-free Trapper Max™ Glue Traps adjacent to control and experimental plants (Figure 6B). For each trapping episode, two traps were nestled at ground level into the vegetation, one on either side of the plant, 5 cm to 30 cm from the stem. Two traps were placed on the first day that an egg was identified and two traps were set on the last day that the plant was occupied. The traps were left out for 24 hours after which they were taken to the lab. The number and type of arthropods in the traps was recorded.

As an added measure of the potential for RIFA to prey upon monarch eggs and larvae, the distance to the nearest active RIFA mound was measured for each plant. RIFA mounds were found by searching the area around each plant and active mounds were identified by gently disturbing the soil of the mound and looking for ant activity. This also allowed the species of ant to be confirmed as RIFA.

Statistical analyses were conducted using SAS version 9.2. Where it was found that the variables deviated from normality non-parametric tests were used for statistical comparisons.

Results

Though milkweed density was high at both sites, it was higher at Tira at 19,000 plants per ha than at CLC were the density was about 13,000 plants per ha (Kruskal-Wallis Test, Chi-square Approximation, $p = 0.0142$). These density estimates are crude estimates based on nearest neighbor measures (Meuller-Dombois and Ellenberg 1974) adjacent to plants with eggs and may not reflect the true density of the entire study sites.

Both sites had high densities of RIFA. Based on nearest mound distances, the Tira site contained 2005 mounds per ha, whereas the CLC site contained 745 mounds per ha. RIFA mounds were closer to study plants at Tira than at CLC (Kruskal-Wallis Test, Chi-square Approximation, $p = 0.0170$).

At both sites combined, 378 eggs or larvae were found on 210 milkweed plants. Thirty-five eggs were found by observing females lay eggs. Another 323 eggs were found by searching plants. Twenty larvae were first detected on plants as larvae. For this study, only individuals detected as eggs were included. In addition, three eggs were eliminated because they were accidentally damaged or destroyed by observers. Thus, the final analyses are based on 355 eggs; 243 at the Tira site and 112 at the CLC site. The Tira site had 148 control eggs and 95 experimental eggs, the CLC site had 67 control eggs and 45 experimental eggs (Figure 1). The average final instar reached by eggs did not differ between sites for either control (T-test; $t = 0.91$, $df = 213$, $p = 0.3622$) or experimental plants (T-test; $t = 0.93$, $df = 138$, $p = 0.3561$). For this reason, the data from both sites have been combined for all further analyses.

Contrary to what has been emphasized in some of the literature (Prysby and Oberhauser 2004), the number of eggs per milkweed plant did not "rarely exceed" one. In fact, 42% of plants with eggs held more than one egg (Figure 7) and the average number of eggs per plant was 1.78. This is similar to observations in Wisconsin, where 43% of plants held multiple individuals (Borkin 1982). In the current study, the final instar reached did not vary among plants with one individual and those with multiple individuals (T-test; $t = 0.70$, $df = 353$, $p = 0.4835$) and the frequency of individuals reaching each stage did not vary among plants that held single individuals and those with multiple individuals (Chi-square 2x5 Table, Chi-square = 1.6232, $df = 4$, $p = 0.8046$). Similar results are obtained if the data are stratified by treatment. However, the final stage of an individual on a plant that held other individuals was positively correlated to the average final stage of the other individuals on that plant (Pearson's Correlation Coefficient, $r = 0.316$, $p < 0.0001$). An individual did well if the other individuals on that plant also did well. An individual did poorly if the other individuals on the plant did poorly. This is a problem for statistical analyses because individuals on plants that held multiple individuals are not independent. This was not a problem for the survivorship data because those analyses are framed in the context of the population. However, analyses of causes of mortality are based on individuals and pseudoreplication can lead to invalid conclusions. Nonetheless, to be consistent with data reported in the literature (e.g. Prysby 2004), in the current study, these individuals are treated as independent for some analyses.

Age-specific Survival and Survivorship

To enable the current data to be compared to that of other studies, two approaches were used to calculate monarch egg and larval survival: age-specific survival and survivorship. Age-specific survival is the number of individuals entering a specific age class that survive to the next age class.   For monarch eggs and larvae, the age classes are eggs and first through fifth instars. However, because monitoring stopped when the larvae reached the fifth instar, age-specific survival was not calculated for fifth instars. Survivorship is the number of individuals in a cohort surviving to the start of an age class. Age-specific
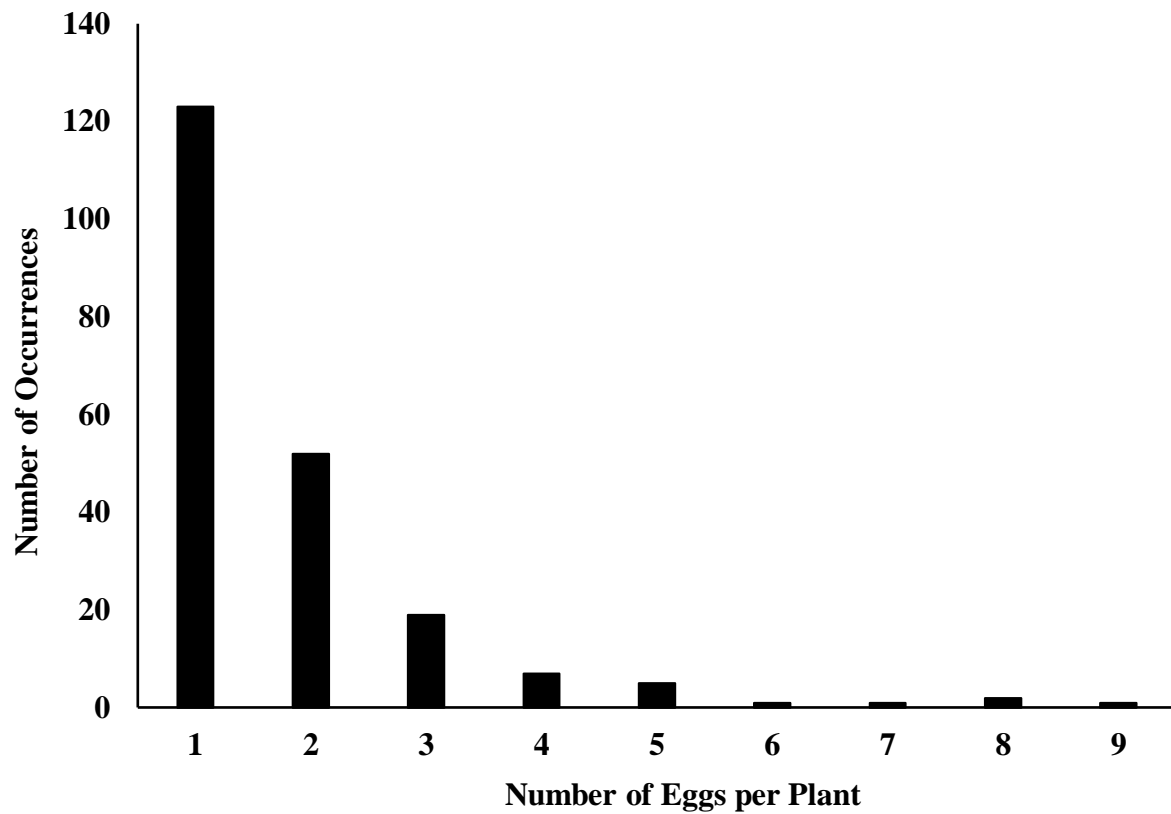
Figure 7. The number of eggs observed per milkweed plant on the 210 plants included in the study. Over 40% of plants held more than one egg; the mean number of eggs was 1.78.

survival and survivorship were calculated separately for control and experimental individuals (Table 1, Figure 8). Age-specific survival was higher for experimental individuals than it was for control individuals for all age classes (Table 1). For control individuals, survival was high, but dropped markedly after the first instar. For experimental individuals, survival varied somewhat among age classes, but was high until the fourth instar.

Age-specific survival was used to construct survivorship curves standardized to a starting cohort of 100 individuals and, therefore, represents percent survivorship of that cohort up to each age class (Figure 8). As was observed in the age-specific survival data, the survivorship curves show that control individuals had lower survivorship than did experimental individuals. None of the controls survived to the fifth instar, whereas about 16% of experimental individuals survived to the fifth instar.

The data presented in Table 1 and Figure 8 contain two sources of bias that must be addressed. First, there is a bias in mortality estimates based on individuals whose age is unknown when they are first identified (Mayfield 1975, Greeney et al. 2010). These mortality rates underestimate mortality because individuals found of unknown age include only those that have survived to that point and not the individuals that have already died. The solution, known as the Mayfield method (Mayfield 1975), is to estimate daily survival rate from the entire sample of each age class and extend this estimate across the average duration of the age class calculated from individuals of known age. In this study the duration of the egg stage can be calculated from the 35 eggs for which the females were observed laying. For consistency, Mayfield estimates of age-specific survival were calculated for eggs and the first four instars (Table 2). The effect was to decrease survival of eggs from 69.3% to 60.8% in control individuals and from 82.9% to 78.3% in experimental individuals. This method also increased the survival of control fourth instars from 0% to 20%.

The second source of bias is emigration. If a larva left a plant and did not return, it was counted as missing and assumed to have died. As documented elsewhere (Rawlins and Lederhouse 1981, Borkin

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Table 1. Age-specific percent survival of monarch eggs and larvae at Cooper Lake WMA and Cooper Lake State Park based on raw data.

	Control			Experimental			Control vs Experimental	
	Lived	Died	Percent Survived	Lived	Died	Percent Survived	Chi-square (2x2 Contingency Table)	p
Egg	149	66	69.3	116	24	82.9	8.23	0.00412
First Instar	79	70	53.0	89	27	76.7	15.79	<0.0001
Second Instar	29	50	36.7	55	34	61.8	10.54	0.00117
Third Instar	6	23	20.7	44	11	80.0	27.72	<0.0001
Fourth Instar	0	6	0	22	21	51.2	-	0.0265[*]

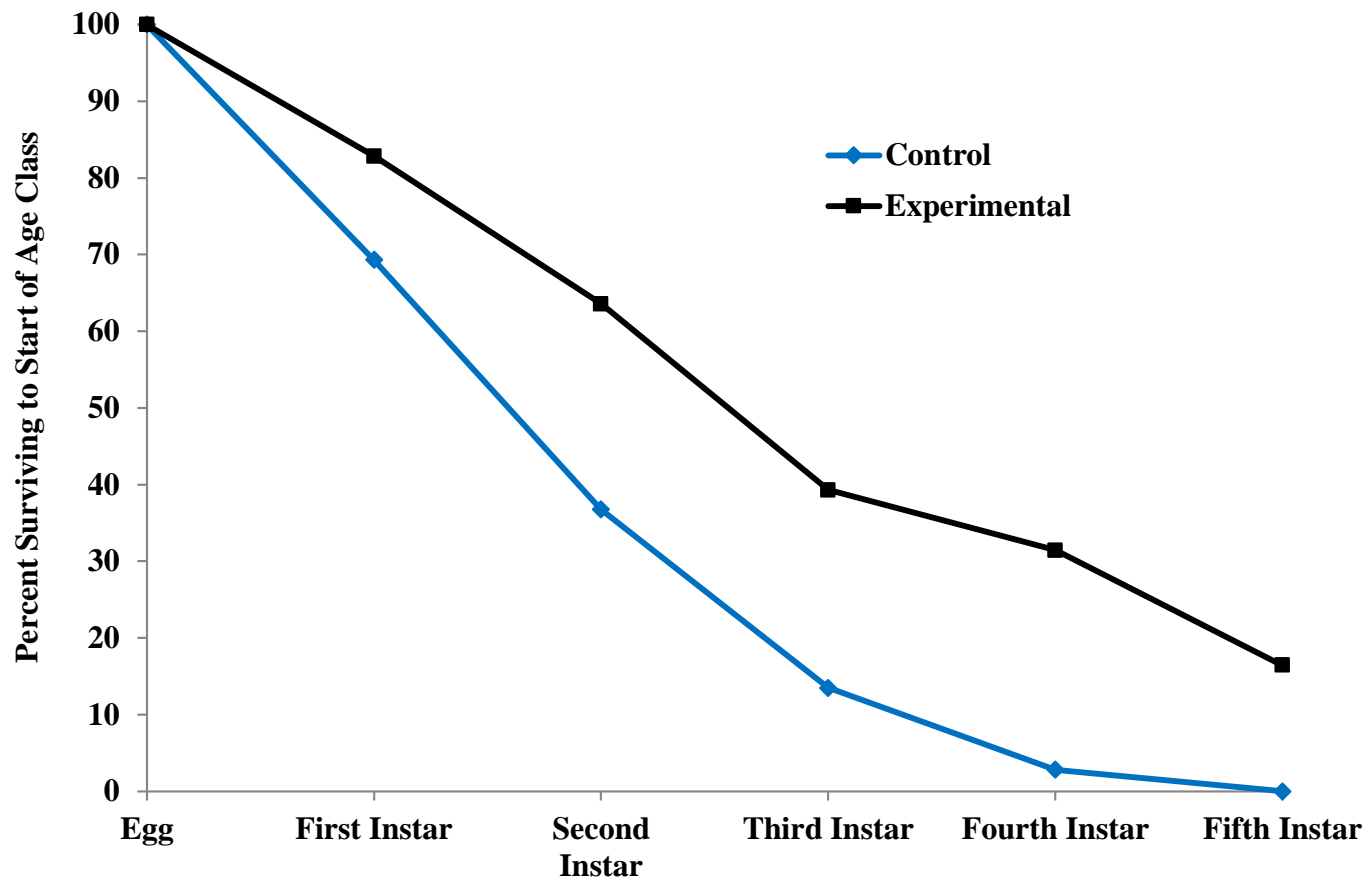[*]Probability based two-sided Fisher's Exact 2x2 Test

Figure 8.   Survivorship curves for control and experimental eggs standardized to a starting cohort of 100 eggs. Data are based on raw values obtained during the study and are not corrected for emigration and errors associated with calculating mortality rates among eggs of unknown age.

Table 2. Mayfield estimates of daily survival probabilities and percent survival for monarch eggs and instars. Means in parentheses represent the mean duration for each stage in days. For eggs, this mean is based on 35 individuals for which females were observed to lay the eggs. For all other instars the mean duration of the stage is based on the entire sample for each age class.

Stage	Measure	Control	Experimental
Egg	Daily survival probability	0.938	0.969
	Percent survival for eggs (mean = 7.85 days)	60.8	78.3
First Instar	Daily survival probability	0.880	0.941
	Percent survival for first instar (mean = 4.71 days)	55.1	75.2
Second Instar	Daily survival probability	0.819	0.911
	Percent survival for second instar (mean = 4.57 days)	40.3	65.5
Third Instar	Daily survival probability	0.646	0.934
	Percent survival for third instar (mean = 3.34 days)	23.7	79.6
Fourth Instar	Daily survival probability	0.625	0.826
	Percent survival for fourth instar (mean = 3.34 days)	20.0	51.7

1982), monarch larvae, particularly older instars, frequently leave their natal plants. The exclosures used in this study also acted as enclosures that inhibited dispersal of the larvae from the natal plant. This was documented through observations of larvae climbing the walls of the exclosure (Figure 9) as well as larvae found on the ground outside the exclosure. Based on the frequency and age of dispersing experimental larvae, it was possible to calculate the proportion of control larvae in each age class that would be expected to be missing due to dispersal. The control data was then adjusted to reflect age-specific dispersal.

Age-specific survival based on the Mayfield method and accounting for emigration shows much more consistent levels of mortality among the first three instars for both treatments (Table 3). These values were used to generate corrected survivorship curves (Figure 10). Based on these data, among control eggs, about 5% are expected to survive to the fifth instar. For experimental eggs, about 16% are expected to reach the fifth instar.

Potential and observed sources of mortality

Three sets of data were used to assess potential sources of mortality: data from sticky traps set adjacent to the plants, observations of other arthropods on the plants themselves, and direct observations of mortality.

Two sticky traps were set the first day an egg was found and two more were set when the plant was determined to be no longer occupied. In some cases, traps went missing or were damaged by heavy rains and could not be used. To account for variations in the number of traps used, the number of arthropods captured was calculated as the mean number per trap. Though the primary interest of this study was in the relationship between mortality and the presence and abundance of RIFA, many other arthropods are known to prey on monarch eggs and larvae (Prysby 2004, De Anda and Oberhauser 2015, Oberhauser et al. 2015). For this pilot study, arthropods other than RIFA were grouped based on their abundance and potential as predators of monarch eggs or larvae (Figure 11). RIFA were more than three times more abundant than aphids, which were the next most common arthropod (Figure 11). Other

Figure 9. A fourth instar monarch larva attempting to escape from an experimental exclosure on 27 April, 2016.

Table 3. Age-specific percent survival calculated using the Mayfield method and adjusted for emigration in control individuals.

	Control before correcting for dispersal	Percent dispersal observed in Experimental larvae[*]	Control after correcting for dispersal	Experimental
Egg	60.8	0	60.8	78.3
First Instar	55.1	0	55.1	75.2
Second Instar	40.3	3.4	43.7	65.5
Third Instar	23.7	21.4	45.1	79.6
Fourth Instar	20	55.6	75.6	51.7

[*]Percent dispersal in experimental larvae was calculated as the percentage of all experimental individuals of each instar that were observed climbing the inside or outside walls of the exclosure or that were observed outside of the exclosure crawling away from it.
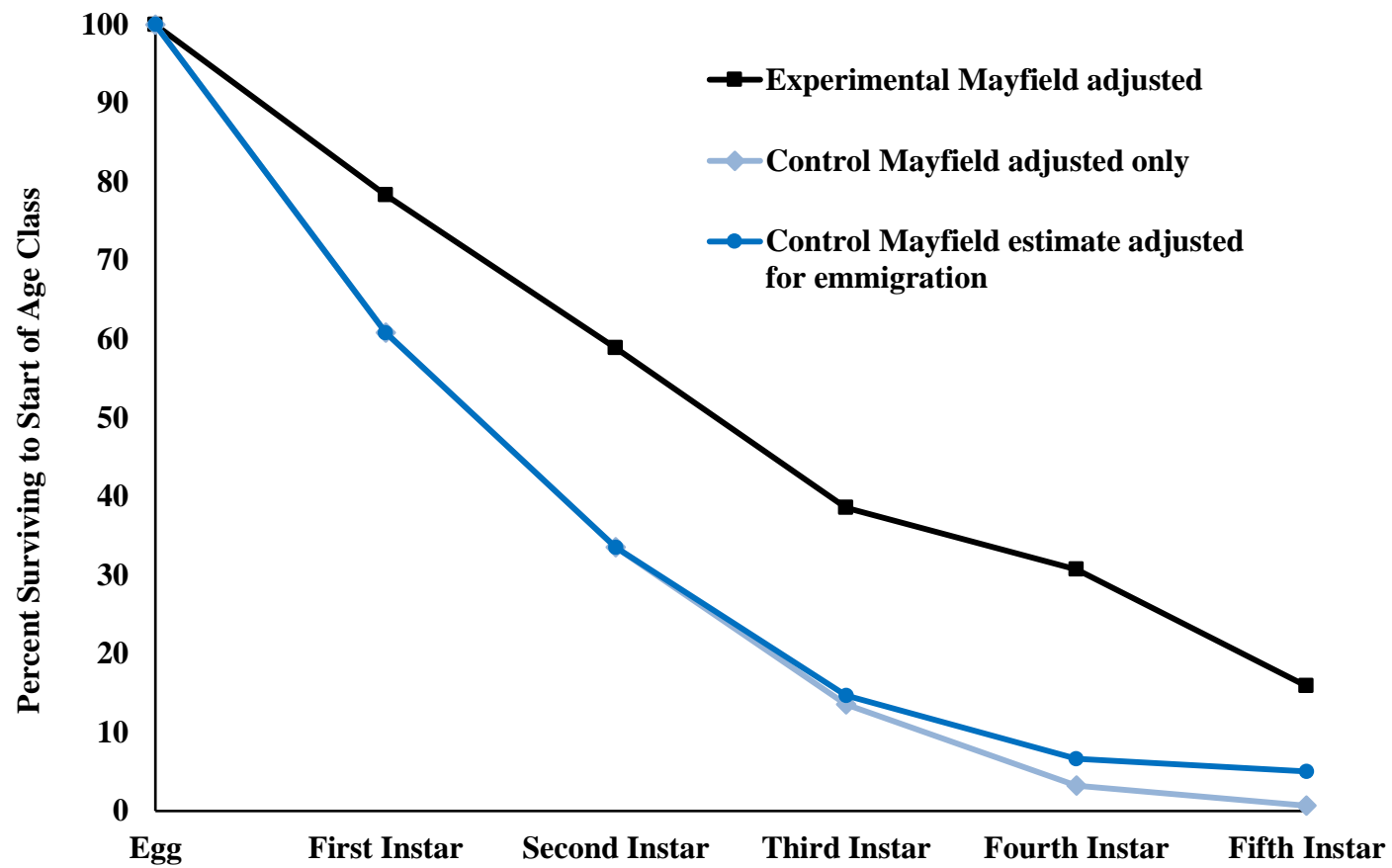
Figure 10. Survivorship curves for control and experimental eggs standardized to a starting cohort of 100 eggs. Data are based on Mayfield estimates and corrected for emigration among third, fourth, and fifth instars.
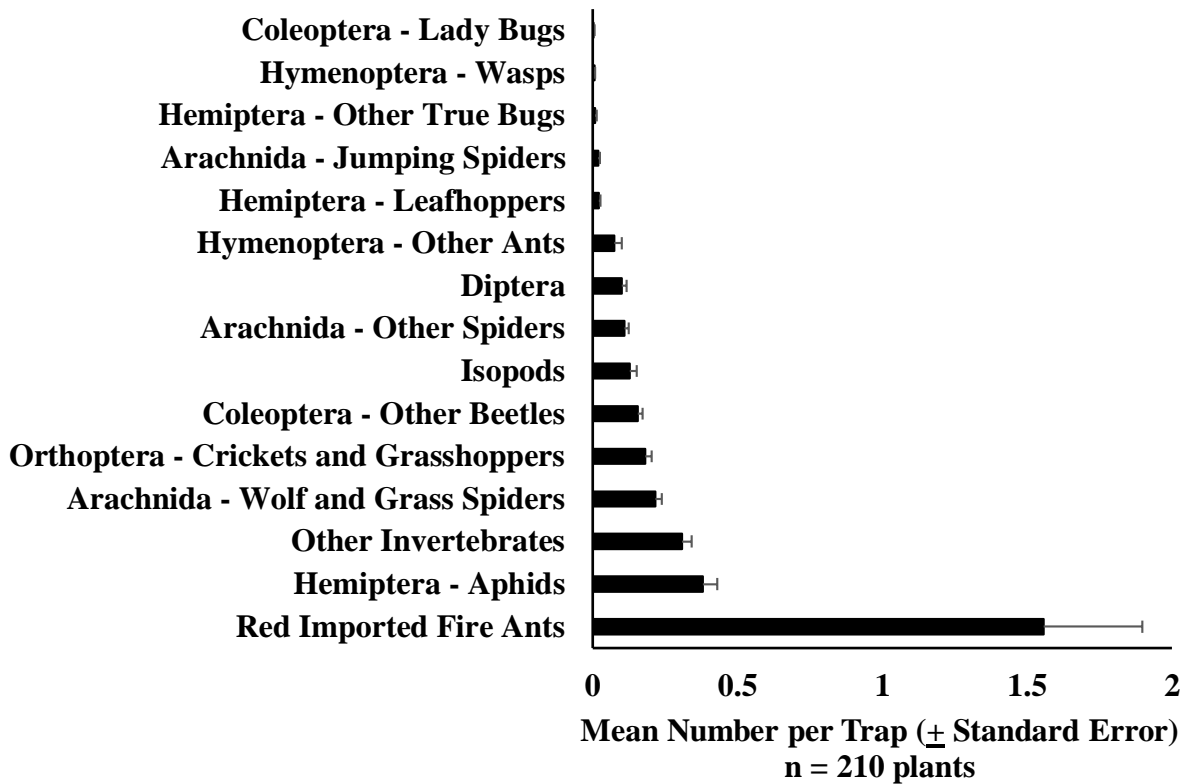
Figure 11. The average number of arthropods captured in sticky traps set adjacent to plants. Data for both control and experimental plants were combined for this figure.

potentially important predators included Wolf and Grass Spiders (Lycosidae and Agelenidae), Coleoptera – Other beetles (which included ground beetles, Carabidae), Arachnida – Other Spiders, Diptera (which included Tachinid flies), Hymenoptera – other ants (which included little black ants (*Monomorium minimum*) and two other species that were not identified), Arachnida – Jumping Spiders (Salticidae), Hemiptera – Other true bugs (which included Pentatomidae and Reduviidae), Hymenoptera – Wasps, and Coleoptera – Lady bugs (Coccinellidae) (Figure 11).

The mortality of monarch eggs and larvae was compared relative to arthropod abundance in the traps. Because of emigration, mortality of third and higher instars could not be used in the analyses so survivorship up to the third instar was used. The hypothesis was that eggs or instars that failed to reach the third instar should be associated with higher abundances of predators and, more specifically, a higher abundance of RIFA. As an added measure of the risk of RIFA predation, the distance of the plant to the nearest RIFA mound was also included in this analysis. For control plants, 29 individuals survived to the third instar whereas 186 individuals failed to reach the third instar. Among these control plants, no statistically significant differences could be found in the abundance of any of the arthropod groups for individuals that failed to reach the third instar and those that survived to the third instar (Table 4). The only group that came close to significance was Wolf and Grass Spiders, but the trend was for Wolf and Grass Spiders to be more abundant at plants where individuals survived. Indeed, RIFA also tended to be more abundant near plants where instars survived to third instar, though this was not statistically significant. Furthermore, the only statistically significant result showed that eggs and instars that died before reaching the third instar were further away from RIFA mounds than were those instars that survived (Table 4). A logistic regression run on these data failed to find a model that predicted survival to the third instar. When these analyses were run on the experimental individuals, no significant trends were found and logistic regression again failed to find a model that predicted survival to the third instar. Consequently, those data are not presented in this report.

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Table 4. Mean number of invertebrates captured in sticky traps adjacent to control plants where instars survived to third instar or died prior to third instar. Also included in this analysis is distance to nearest RIFA mound. Sample sizes: Survived, n = 29, Died, n = 186.

	Survived (Mean + Std Err)	Died (Mean + Std Err)	Wilcoxon's Test p-value
Red Imported Fire Ant	1.32 + 0.51	0.86 + 0.19	0.2640
Hemiptera - Aphids	0.28 + 0.11	0.38 + 0.06	0.7496
Other Invertebrates	0.31 +0.08	0.33 + 0.04	0.9150
Arachnida - Wolf and Grass Spiders	0.24 + 0.06	0.16 + 0.02	0.0838
Orthoptera - Crickets and Grasshoppers	0.09 + 0.03	0.18 + 0.02	0.1080
Coleoptera - Other Beetles	0.14 + 0.04	0.17 + 0.02	0.4679
Isopods	0.07 + 0.03	0.09 + 0.02	0.6415
Arachnida - Other Spiders	0.08 + 0.03	0.09 + 0.01	0.8326
Diptera	0.06 + 0.04	0.07 + 0.01	0.7205
Hymenoptera - Other Ants	0.04 + 0.03	0.06 + 0.01	0.4676
Hemiptera - Leafhoppers	0.03 + 0.02	0.03 + 0.00	0.5143
Arachnida - Jumping Spiders	0.01 + 0.01	0.01 + 0.00	0.8347
Hemiptera - Other True Bugs	0.02 + 0.02	0.01 + 0.01	0.8259
Hymenoptera - Wasps	0.01 + 0.01	0.01 + 0.00	0.5093
Distance to nearest RIFA mound	181.03 $\pm$ 35.28	260.71 $\pm$ 19.44	0.0273

When arthropod captures were compared between treatments it was found that experimental plants had more than twice the abundance of RIFA and 81.3% more Wolf and Grass spiders than did control plants (Table 5). There is no *a priori* reason why these differences occurred based simply on the locations of the plants or any other attributes of the plants themselves. These differences are likely a result of the methodology used to create the experimental treatments. Specifically, they may be a result of clearing the vegetation around the plant and disturbing the soil around the base of the exclosure.

A more refined approach to the trap data is to consider only individuals that failed to reach the third instar and their latency to mortality relative to the abundance of potential predators. The hypothesis is that individuals in areas where predator risk (abundance) is high should have a shorter latency to mortality than those in areas where predator risk is low. Thus, there should be a negative correlation between latency to mortality and predator abundance. The value of this approach is that latency to mortality is a continuous variable that takes advantage of the much larger sample of mortalities. It avoids the pseudoreplication mentioned earlier by using plant as the sample unit and, for plants with multiple instars, using the average latency to mortality for all individuals on a plant. Latency to morality was calculated as the number of days an egg or larva was present from the time it was first identified until it disappeared or was found dead. For this analysis, 109 control plants with individuals that died prior to the third instar were used. For the experimental group, 61 plants with individuals that died prior to the third instar were used.

A Stepwise Regression was run in SAS separately for control and experimental groups using a p-value of 0.05 for either adding or removing a parameter from the regression model. All of the arthropod groups indicated in Table 4 were included in the input data set plus four combined groups: all ant species, all spider species, all predators (which included all species of ants, spiders, wasps, lady bugs, and predatory hemiptera), and all arthropods (which was the grand total of all groups). In addition, the distance of the plant to the nearest RIFA mound was included in the data, putatively as an additional measure of RIFA predation risk. The Stepwise procedure resulted in a significant four-variable model for

Table 5. Mean number of invertebrates captured in sticky traps adjacent to control plants and experimental plants. Sample sizes: Control n= 120, Experimental n = 90.

	Control (Mean + Std Err)	Experimental (Mean + Std Err)	Wilcoxon's Test p-value
Red Imported Fire Ant	1.01 + 0.29	2.28 + 0.69	0.0404
Hemiptera - Aphids	0.46 + 0.08	0.27 + 0.05	0.8811
Other Invertebrates	0.29 + 0.05	0.33 + 0.04	0.1369
Arachnida - Wolf and Grass Spiders	0.16 + 0.02	0.29 + 0.04	0.0054
Orthoptera - Crickets and Grasshoppers	0.17 + 0.03	0.20 + 0.03	0.4293
Coleoptera - Other Beetles	0.15 + 0.02	0.17 + 0.02	0.2538
Isopods	0.10 + 0.03	0.17 + 0.04	0.1382
Arachnida - Other Spiders	0.10 + 0.02	0.13 + 0.02	0.6768
Diptera	0.09 + 0.02	0.12 + 0.03	0.8236
Hymenoptera - Other Ants	0.06 + 0.02	0.09 + 0.06	0.7657
Hemiptera - Leafhoppers	0.02 + 0.01	0.02 + 0.01	0.4512
Arachnida - Jumping Spiders	0.02 + 0.01	0.02 + 0.01	0.4239
Hemiptera - Other True Bugs	0.01 + 0.01	0.01 + 0.01	0.7503
Hymenoptera - Wasps	0.01 + 0.01	0.00 + 0.00	0.4673

control plants which explained about 20% of the variation in latency to mortality ($r^2 = 0.1905$) (Table 6). This model found that latency to mortality was positively related to the abundance of spiders other than Jumping, Wolf, or Grass Spiders, and positively related to the abundance of Orthoptera (primarily crickets (Gryllidae), but also grasshoppers (Caelifera) and katydids (Tettigoniidae)) and the abundance of all ant species. Latency to mortality among control plants was negatively correlated with the overall abundance of all arthropods captured in the sticky traps. The Stepwise regression procedure failed to find any variable or combination of variables that predicted latency to mortality among experimental plants.

Arthropods observed on plants were quantified as presence/absence data. There were 92 observations of other arthropods on the 120 control plants and 57 observations of other arthropods on the 90 experimental plants (Figure 12). There were no statistical differences between control plants and experimental plants for any of the categories of arthropods shown in Figure 12 (Chi-square 2x2 Contingency tables, p all > 0.05). This is somewhat surprising, given that the purpose of the exclosures was to keep terrestrial arthropods such as RIFA off the plants.

It was assumed that once potential predators gained access to the plant they would be a threat to the monarch eggs or larvae regardless of whether it was an experimental or control plant. Therefore, in order to increase sample sizes to a point where statistical comparisons could be made, the data for control and experimental plants were combined. The number of plants with or without each group of arthropods was compared for individuals that survived to the third instar and those that did not survive to the third instar. The hypothesis was that individuals that died before the third instar would more frequently be associated with potential predators than individuals that survived to the third instar. However, there was no relationship between the presence of most of the arthropod groups and the percentage survival of monarch eggs or larvae to the third instar (Table 7). The only statistically significant result was for Wolf and Grass Spiders where monarch survival to the third instar was just over 22% when Wolf and Grass spiders were absent, but was over 60% when Wolf and Grass spiders were present. Interestingly, and contrary to the popular notion that RIFA adopt a "scorched earth" approach to predation, over 20% of

Table 6. Stepwise regression model for latency to mortality for 109 control plants upon which eggs or larvae failed to reach the third instar. For plants containing multiple individuals, the average latency to mortality was used. The overall fit of the model was significant ($F = 6.12$, df = 4, 104, $p = 0.0002$).

Variable	Estimate + Std. Err.	F - Value	p
Intercept	9.58 + 0.65	215.18	< 0.0001
Other Spiders[1]	5.04 + 2.05	6.05	0.0156
Orthoptera[2]	3.51 + 1.42	6.10	0.0152
All Ant Species	0.77 + 0.37	4.33	0.0400
All Arthropods	-1.19 + 0.35	11.54	0.0010

[1] All species of spiders other than Wolf Spiders, Grass Spiders, and Jumping Spiders.

[2] Primarily field crickets, but also includes grasshoppers and katydids.
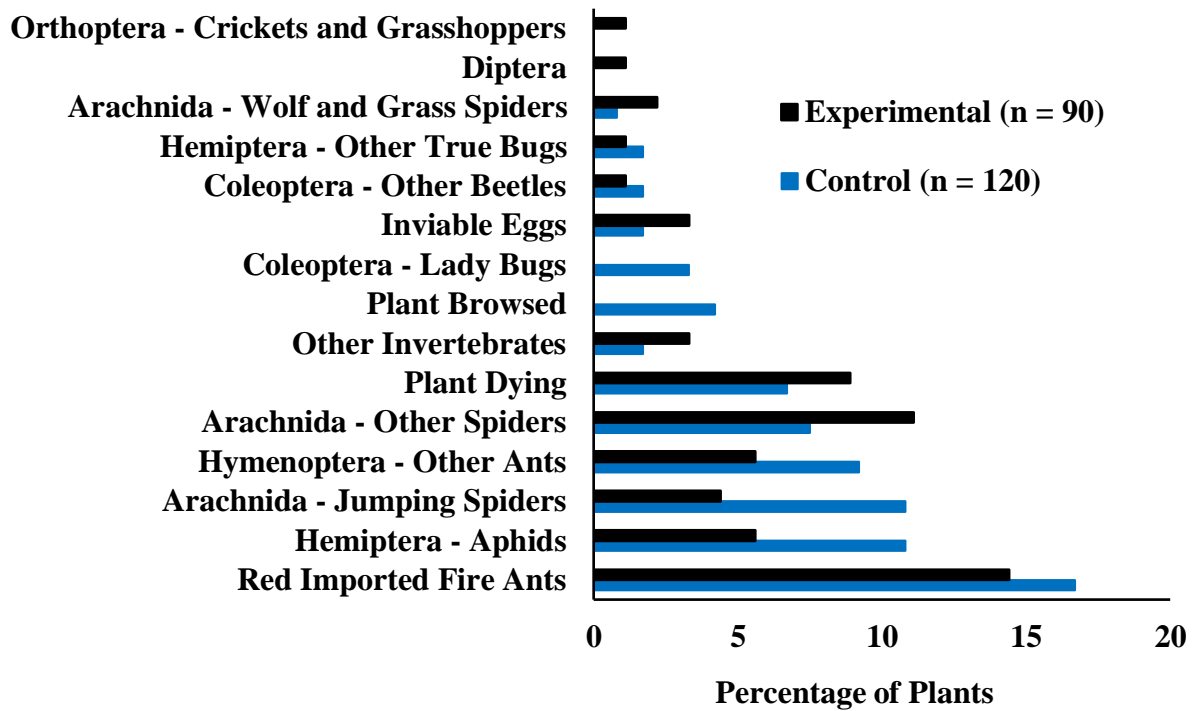
Figure 12. The percentage of control and experimental plants observed to have other arthropods on them, including potential monarch predators. Also included here are plants that were browsed or were dying either from diseases or from heavy aphid or weevil infestations.

Table 7. Percent survivorship to third instar relative to the presence or absence of particular arthropods on the plants. Data is based on 355 individuals first observed as eggs. Numbers in parentheses indicate number of eggs or instars observed with arthropods of each type. Only arthropods with sufficient numbers for statistical analyses are included.

	Present	Absent	Chi-square (df = 1)	p
Red Imported Fire Ant (n = 67)	22.4	24.0	0.010	0.9203
Hemiptera – Aphids (n = 37)	29.7	23.0	0.510	0.4751
Other Invertebrates (n = 18)	38.9	22.8	Fisher's Exact Test	0.1513
Arachnida - Wolf and Grass Spiders (n = 8)	62.5	22.8	Fisher's Exact Test	0.0203
Coleoptera – Lady bugs (n = 12)	0	24.5	Fisher's Exact Test	0.0766
Coleoptera - Other Beetles (n = 11)	18.2	23.8	Fisher's Exact Test	0.7438
Arachnida - Other Spiders (n = 45)	24.4	23.5	0.020	0.8875
Hymenoptera - Other Ants (n = 31)	32.3	22.8	0.920	0.3375
Arachnida - Jumping Spiders (n = 45)	24.4	24.0	0.020	0.8875
Hemiptera - Other True Bugs (n =7)	0	24.1	Fisher's Exact Test	0.2053

eggs or larvae on plants that had RIFA on them survived to the third instar.

Sixty-five mortalities were observed in addition to those that simply went missing. As found in previous studies (Prysby 2004, De Anda and Oberhauser 2015), the majority of "mortalities" were larvae or eggs that simply disappeared. In this study, of 271 eggs or larvae that failed to reach the third instar, 218 (80.4%) simply went missing. The 65 observed mortalities were grouped into several categories (Figure 13) and examples of some of these categories are illustrated in Figures 14 and 15. Sample sizes are too small for statistical comparisons between experimental and control individuals, but there appeared to be a slight trend for more experimental larvae to be killed by RIFA than was observed among control larvae and more experimental larvae were found sucked empty as well. Overall, RIFA accounted for only 5 of 65 mortalities (7.7%). Control individuals appeared to more frequently have inviable eggs and were more likely to be browsed or to be on diseased plants (Figure 13). Browsed stems were cleanly cut (Figure 14B), suggesting that rabbits or hares were the cause. A single control first instar was caught in a sticky trap after a heavy rain event. For both control and experimental individuals, the most common observed source of mortality was individuals that were found dead on the plant. These individuals typically were discolored, often bloated, but otherwise appeared intact (Figure 14D).

Types of mortality seemed to vary with the age of the individual (Figure 16). Eggs often would turn black and fail to hatch. In some cases, eggs became yellowish and withered in appearance. Individuals that were found dead but otherwise intact on the plant occurred most frequently among first and second instars. Those that were sucked empty appeared most frequently among second and third instars. RIFA were most frequently observed killing older larvae. It is possible that these were more easily observed because later instars are too large to be carried off by one or a few individual ants and would have to be broken down by multiple individuals over a longer period of time making it more probable that they would be observed doing so. Similarly, it is possible that many of the eggs or early instars that went missing were the result of RIFA removing the entire egg or larva by single or only a few individuals over a very short time span.
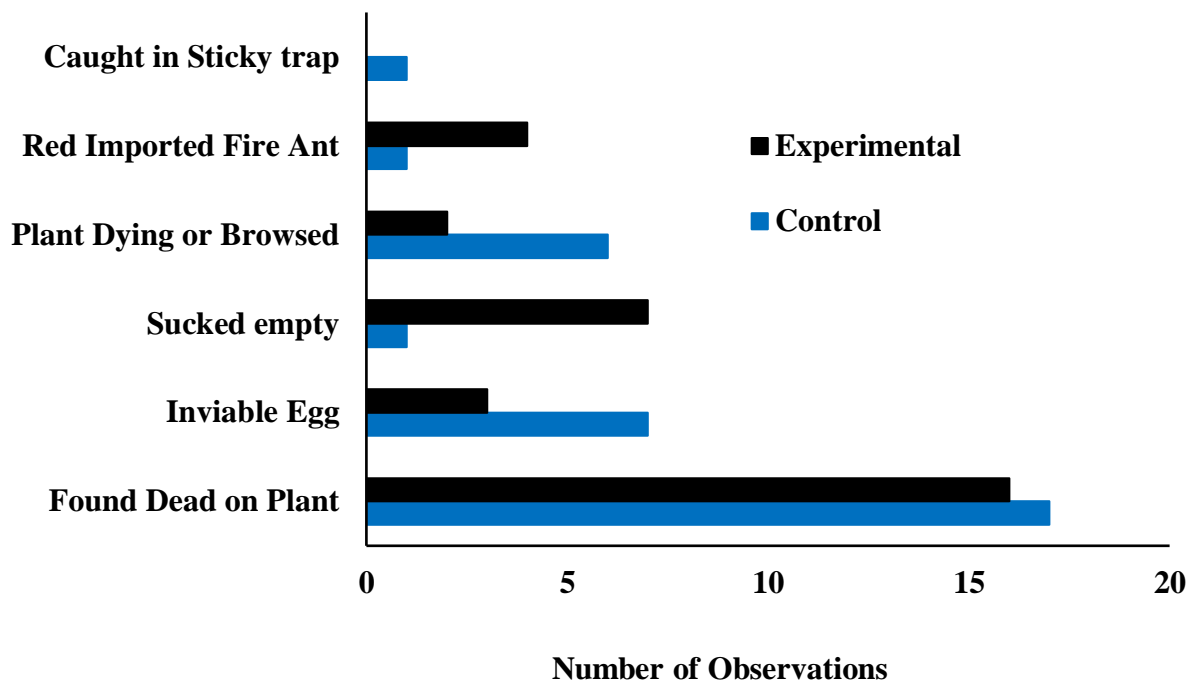
Figure 13. The observed types of mortality for monarch eggs and larvae among control and experimental individuals. These data include all observed mortalities among the 355 eggs or instars in the study.
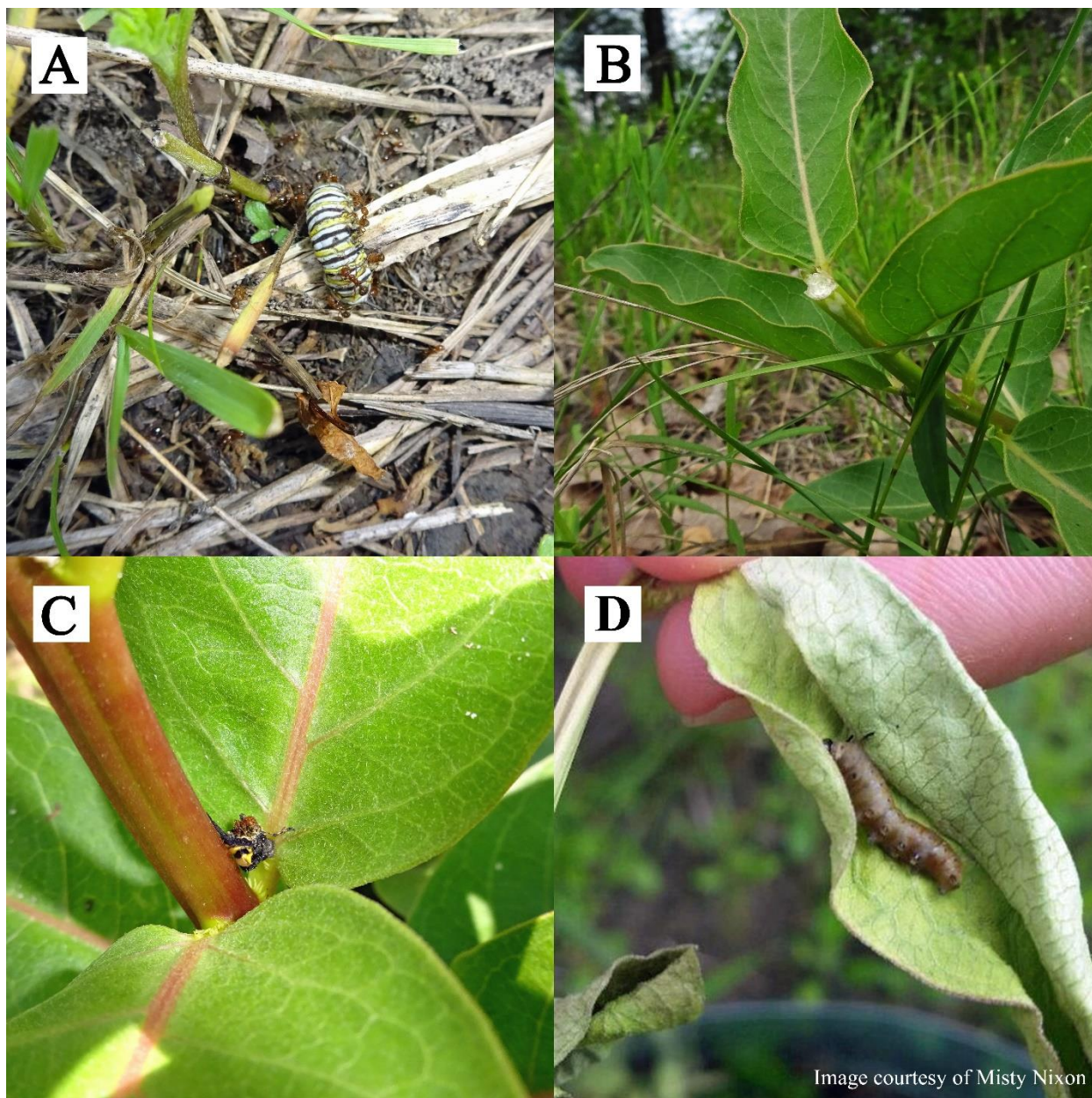
Figure 14. Four examples of mortality observed among monarch eggs and larvae. A. RIFA attacking a fifth instar on 23 April, 2016. B. A stem that contained an egg, but which has been browsed presumably by a rabbit or hare on 16 April, 2016. C. A monarch larva that has been sucked empty found on 23 April, 2016. There is a small brown spider on the top of this otherwise empty carcass. D. A monarch larva that was found dead, discolored, but otherwise undamaged on its natal plant on 25 April, 2016. Most of the observed mortalities fell into this last category.

Figure 15. A jumping spider (Salticidae) feeding on a third instar monarch larva on 23 April, 2016. These observations were grouped in with the "Sucked empty" category for figure 13.

Figure 16. The frequency of the observed causes of mortality stratified by age class. Control and experimental individuals are combined for this figure.

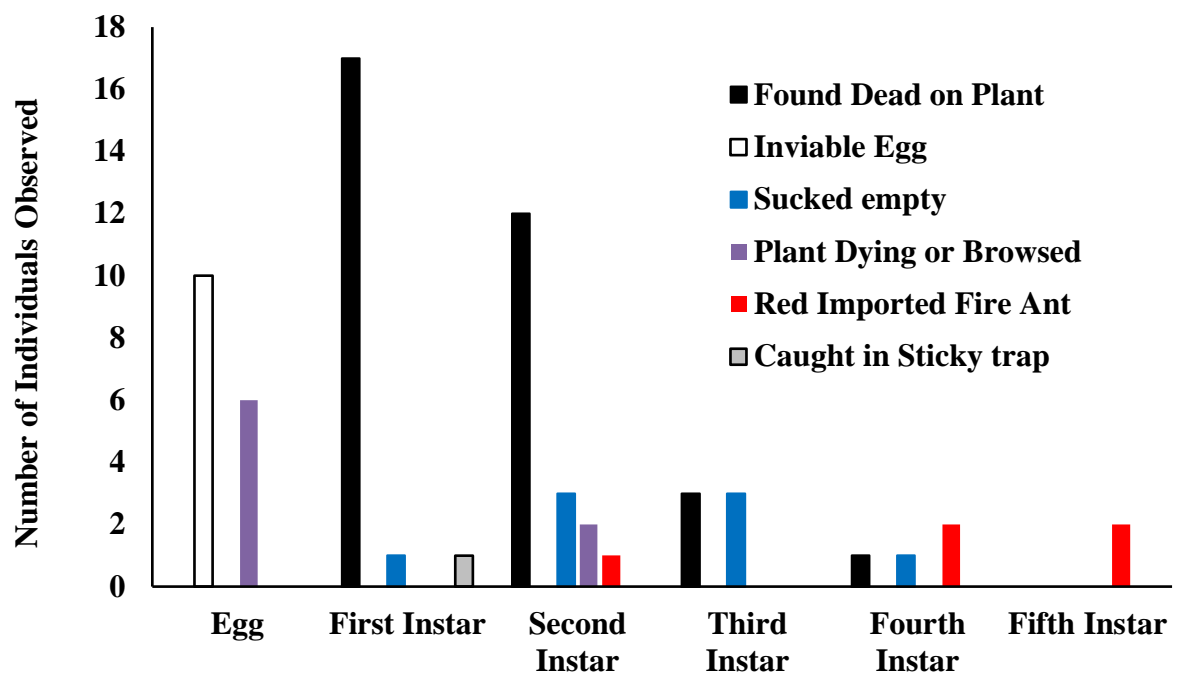<center>**Discussion**</center>

Interpretation of results

      The consensus in the literature is that monarchs suffer high mortality during the period from egg to fifth instar, with survivorship ranging between about 5% and 20% (Oberhauser 2004, Prysby 2004, De Anda and Oberhauser 2015, Nail et al. 2015). In the current study the estimated survivorship of control individuals to the fifth instar was 5%, which is in line with the literature. Due to emigration in third and higher instars, the survival data in this study was most reliable for eggs, first, and second instars and accurate interpretations can only be made using these data. An earlier study in central Texas proposed that the observed 0% survivorship to the second instar found in that study was due to a high density of RIFA mounds, measured at 1011 mounds per ha (Calvert 1996). In that study RIFA were found on 4% of milkweed plants examined (Calvert 1996). In the current study in northeast Texas, where survival was much higher, the RIFA mound density was 1355 mounds per ha and RIFA were found on 14% of control plants and 17% of experimental plants. Furthermore, larvae and eggs on plants that had RIFA were just as likely to survive as larvae and eggs on plants that did not have RIFA.

      A more controlled follow-up study by Calvert (2004) in central Texas used 5 m diameter exclosures to measure the effect of excluding RIFA on monarch survival. In that study, the percent survivorship to the second instar varied from 2% to 9% inside the exclosures and varied from 0% to 1.4% outside the exclosures. However, these data were cited by Calvert (2004) as being unreliable due to the difficulty of detecting early instars when searching unmarked plants over a large area across intervals of several days when plants were not searched. The same study reported survivorship to fifth instars, which are much more easily detected, as varying from 1.6% to 27% inside the exclosures and from 0% to 1.4% outside the exclosures. These values are more comparable to those of the current study where survivorship to fifth instar was estimated to be about 16% inside exclosures and 5% outside the exclosures when the data are corrected for emigration. In the study by Calvert (2004), the higher

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survivorship inside the exclosures was attributed to a lower density of RIFA inside the exclosures measured using baited traps. However, that study did not measure the presence or abundance of other potential predators and, consequently, cannot exclude the possibility that the exclosures had effects on other predators or on other factors such as emigration, that caused the observed differences.

There is one other study conducted close to the geographic location of the current study in northeast Texas. That study (Lynch and Martin 1993) was in southwest Louisiana, parts of southeast Texas, and included one site in Denton Co., Texas, that is about 85 miles from the study site used in the current study. Overall, only 3% of eggs reached the third instar (Lynch and Martin 1993) which is much lower than the 13% recorded among control individuals in the current study. That study (Lynch and Martin 1993) study did not calculate survival separately by site, so the data from Denton Co., Texas, could not be compared directly to the data from the current study.

There are a few other field studies that allow direct comparisons to the current study, all in areas that are a considerable distance from north Texas. Estimates of survival in these studies vary a lot, as do the methodologies used in arriving at these estimates. In Minnesota, daily survival rates comparable to the Mayfield estimates used in the current study were estimated for eggs, first instars, and second instars (De Anda and Oberhauser 2015). They extrapolated their data to calculate the survivorship of eggs to first, second, and third instars to be 16%, 5.6%, and 1.7% respectively. These values are far lower than the 61%, 34%, and 15% observed among control individuals in the current study. In Florida survival from egg to second instar was estimated to be 17.6% (Zalucki and Brower 1992), also below the 34% observed in the current study. In Wisconsin monarch survival in two types of exclosures in addition to controls was measured (Prysby 2004). One type of exclosure excluded terrestrial predators only whereas the second type of exclosure excluded both terrestrial and aerial predators. That study found that fewer than 20% of eggs survived to hatching in control plants and just over 20% of eggs survived to hatching in terrestrial predator exclusion exclosures (equivalent to the ones used in the current study). This is much lower than the Mayfield estimates from the current study which were 61% for controls and 78% in

exclosures. A different, and much earlier, Wisconsin study found survivorship to the first instar to be between 30% and 58% (Borkin 1982), which is lower than the 61% estimated for controls in the current study, but still higher than most other field studies. Similarly, another, earlier study in Florida (Cohen and Brower 1982), found that age specific survival did not vary among age class and was about 57% for all age classes. These values are close to those of the current study except that, in the current study, age-specific survival rates among control individuals declined slightly from 61% in eggs to 40% for second instars. In summary, the current study of age-specific survival in monarchs in northeast Texas, at least to the third instar, showed rates of survival that are very high compared to the values reported for most other field studies. Unfortunately, there are no baseline studies to compare monarch egg and larval survival prior to the arrival of RIFA in north Texas. Consequently, based solely on survival data, it remains unknown whether these values are normal for this area or whether they reflect suppressed survival as a consequence of the presence of RIFA.

Monarch eggs and larvae succumb to numerous types of predators, parasitoids, and diseases (reviewed in De Roode 2015 and Oberhauser et al. 2015). Several predators and potential predators were observed in the current study. These included at least four ant species, several species of spiders, assassin bugs, and an assortment of shield bugs, wasps, and ladybugs. RIFA were much more abundant than any other potential predator caught in sticky traps and were the most frequent potential predator observed on plants that contained eggs or larvae. RIFA accounted for only 7.7% of observed mortalities and high numbers of RIFA were not associated with higher mortality or shorter latency to mortality. Over 20% of eggs or larvae that occurred on plants that contained RIFA survived at least to the third instar, suggesting that the RIFA either did not find them or ignored them. The most commonly observed type of mortality was of individuals who we found dead, but otherwise intact, on their host plant - clearly not the result of RIFA depredation.

In fact, the analyses failed to find any potential predator type that could account for the high levels of mortality observed. On the contrary, five different analyses found positive correlations between the abundance of potential predators and monarch egg or larval survival to the third instar:

1. Wolf or Grass spiders were more abundant near plants where monarch survival was high

2. The survival of monarch eggs and instars was higher on plants that had Wolf or Grass spiders on them

3. The abundance of other spiders and ants was higher near plants where eggs and larvae had longer latencies to mortality

4. RIFA were more abundant near experimental plants where survival was high than they were to control plants

5. Monarch larvae that survived to at least the third instar were closer to RIFA mounds than were those larvae that died prior to the third instar.

There are issues with this data (see below), but these results suggest that the system is more complex than a simple linear predator-prey system (De Roode 2015). Much of this might have to do with the presence of competitors and indirect effects of predators.

Milkweed plants harbor many arthropods that act as competitors, predators, or pollinators. In addition to monarch larvae, at least 10 other species of specialized phytophagous insects are known to feed on milkweeds (Malcolm 1991, Van Zandt and Agrawal 2004). The activity of these phytophagous species have negative effects on the quality of the milkweed plants that, in turn, affect the success of the other species on the plant, particularly monarch larvae (Van Zandt and Agrawal 2004, Agrawal 2005). As well, many pollinators visit milkweed plants (Fishbein and Venable 1996). Pollinators and other phytophagous insects increase the abundance of prey which, in turn, attracts polyphagous predators.

The only factor that decreased the latency to mortality of monarch eggs and larvae was the overall abundance of arthropods. Many of these arthropods, in particular aphids, weevils, leafhoppers, and other

hemipterans, have severe impacts on the health of the plant and its chemical composition (Agrawal 2005). The cardenolides produced by milkweed plants, if present in high concentrations, can be toxic or lethal to monarch larvae (Zalucki and Brower 1992, Zalucki et al. 2001, Zalucki et al. 2012). Like many other plants with well-developed anti-herbivory responses (Ramirez and Eubanks 2016), milkweed plants respond to herbivory by increasing cardenolides to concentrations that may be toxic, or even fatal, to monarchs (Van Zandt and Agrawal 2004, Agrawal 2005). Herbivory on milkweeds also reduces the nitrogen content of the plant tissues (Agrawal 2004) and forces monarch larva to feed more (Lavoie and Oberhauser 2004), which causes them to take up more cardenolides than they would otherwise. This can lead to monarch larval mortality or cause them to leave the plant. It might be significant that most of the larvae that were found dead, but otherwise intact, were first and second instars, stages most vulnerable to the effects of milkweed defenses (Zalucki and Brower 1992, Zalucki et al. 2001, Zalucki et al. 2012). Under this scenario, the observation that monarch larvae died (or disappeared) more quickly on plants with high arthropod populations makes sense.

In ecological communities, indirect effects occur when the impact of a species or group of species on another species or group of species is the result of the presence of a third species or group of species (Wootton 1994, Mittelbach 2012). Indirect effects are important for promoting species richness among trophic levels. For example, top-down regulation by predators has been shown to increase herbivore diversity (Amundrud et al. 2015) and in some cases preferential predation by a predator on one prey species can lead to increases in the population of less preferred prey species (Frago and Godfray 2014, Prado and Frank 2014). In the current study, high densities of predators seemed to improve monarch egg and larval survival. This may be due to an indirect effect in which predators, such as spiders and ants, preferentially fed on other phytophagous insects inhabiting the milkweed plants and, as a result, benefitted the monarchs by reducing competition, improving the plant's quality, and lowering predator pressure. Optimal foraging theory has repeatedly shown that predators ignore less profitable prey when more profitable prey are available and that even slight differences in profitability can cause a prey species

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to be eliminated from the diet of a predator (Giraldeau 2008, Prado and Frank 2014). There is reason to expect other species of insects on milkweed plants to be preferable to predators because not all phytophagous insects on milkweed plants sequester cardenolides or are as efficient as monarchs in sequestering these compounds (Isman et al. 1977) and at least some polyphagous invertebrate predators show an aversion to prey with high levels of cardenolides (Raynor 2004). There is precedent for such indirect effects in monarch predator-prey systems. In both field and laboratory studies of predation on monarch larvae by ladybugs (*Harmonia axyridis*), monarch larvae had increased survival when there were higher densities of an alternate prey in the form of aphids (*Aphis nerii*) (Koch et al. 2005) (but see Prysby 2004 for contrast). There is also precedence for the idea that RIFA may reduce predation on some phytophagus species. In cotton fields, RIFA release cotton aphid (*Aphis gossypii*) from predation by ladybug larvae (Coleoptera: Coccinellidae) and lacewing larvae (Neuroptera: Chrysopidae) (Kaplan and Eubanks 2002). Thus, increased predators on plants infested with other, more profitable, prey could decrease predator pressure on the monarchs and lead to higher survival.

The current study was conducted in an area where invertebrate diversity was high. If monarch survival is dependent on indirect effects of predators, then this system might not operate effectively in a community with low invertebrate diversity such as might be found in managed pastures and hay fields. In those cases, the economics of predation might increase the relative value of monarch eggs and larvae and result in higher mortality rates. Similarly, this study was framed around the survival of monarch eggs through second instars. The much larger, older monarch instars might be more susceptible to predation from predators like RIFA because they are likely to be of higher value as prey items. Unfortunately, based on the study techniques used in most studies of monarch survival it is not possible to separate the loss of older instars due to emigration from those caused by mortality.

In summary, there was no evidence that RIFA had a negative impact on monarch egg and larval survival in the areas used in this study. Indeed, it is possible that RIFA could have a positive effect on monarch survival. There is one other reason why RIFA might not be expected to have a large impact on

monarch larvae: the native distribution of RIFA in South America (*cf* Figure 1 in Mescher et al. 2003)

overlaps that of the Southern Monarch (*Danaus erippus*) (*cf* Figure 20.1 in Malcolm and Slager 2015), a

species that also sequesters cardiac glycosides. Consequently, even though RIFA densities in North

America are much higher than those in South America (Porter et al. 1997), RIFA may have a natural

aversion to preying on the larvae of *Danaus* species, particularly where abundant alternate prey is

available.

Evaluation of methodology and quality of the data

The age-specific survival and survivorship of eggs and first through second instars measured in this study

is likely to be accurate despite the fact that these values are high compared to other studies in Texas and

elsewhere. Much of the accuracy stems from the care used to mark eggs and plants and in checking for

young instars. There were 244 instances where individuals went missing one day, but were relocated in

the following days (69% were found on the next day, 86% within two days). Most of these occurrences

were eggs that had just hatched or were first instars. First instars frequently left the leaf upon which the

egg was laid and burrowed into the terminal leaf buds or flower buds where the instars were very difficult

to observe. In some cases, first instars made a tiny hole at the base of a flower bud, would crawl inside,

and be effectively undetectable. It is possible that some eggs that were laid on the plants were initially

undetected and that larvae that were "found again" were the product of these undetected eggs. There

were a few instances when this was documented; a young instar would be found on a plant that held an

older instar or an unhatched egg (see results). However, as a rule, the plants were closely inspected on a

daily basis and new eggs noted and marked when they appeared. It is unlikely that a large proportion of

the 244 individuals that went missing and were found later, resulted from undetected eggs.

It is also possible, at least for control plants, that some of the instars that went missing and were

later found, were actually individuals that immigrated from other adjacent plants. The literature explicitly

states that first instar larvae never leave their natal plants or, if they do, they die (Zalucki and Kitching

1982, Zalucki and Brower 1992, Prysby 2004). There is, however, no actual data on this statement that to be found in the literature. In the current study it seemed that, in contrast to the citations above, first instars were quite mobile. The leaf they hatched upon was frequently a considerable distance from the terminal buds where they were later found. Some first instars moved from one ramet to another. It is, therefore, quite possible that a first instar could leave its natal plant and climb onto an adjacent plant (mean distance in this study = 77 cm). However, the density of eggs in the study area was too low for immigration to account for a large proportion of the 244 individuals that went missing and were found later. Furthermore, for experimental plants, it would not be possible for first instars to gain access to the interior of the exclosure. Because of these factors, it is likely the that survivorship estimates realistically demonstrate high survivorship, at least to the third instar, for this population at the time the study was conducted.

A clear and significant increase in survivorship was associated with the exclosures. However, the data are ambiguous as to exactly why survivorship was higher in exclosures and what, exactly, the exclosures were keeping out (or in). There are multiple reasons for this, some that relate to the design and method of installing the exclosures and some that relate to how insects were sampled.

Three issues regarding the installation and design of the exclosures affected the results. First, the sticky tape that was wrapped around the outside of the exclosure (Figure 6) was not sticky enough to deter larger insects. This could be corrected by using Tanglefoot ™ instead of the tape. Second, the exclosures were too small to fully contain mature plants. This was not likely to have affected most of the eggs and early instars, because at that time the plants were not as large. However, as the plants and the larvae matured, the plants began to hang over the outside of the exclosure and, because the plants were so robust, staking the plants was ineffectual. It is very likely that many of the late instars that went missing from exclosures simply dropped off the end of the plant and dispersed away from the exclosure and this likely explains the sudden drop in survival estimated for fourth instars in exclosures. In addition, some insects may have gained access to the exclosures by crawling on to the plant from adjacent vegetation.

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This could be solved by making larger exclosures. Exclosures of 1m diameter would be sufficient to contain most plants and prevent unwanted exchange of insects over the walls of the exclosure. The third issue was that clearing the vegetation around the exclosures and setting the exclosures into the soil seems to have attracted the activity of RIFA rather than repelling them. Though considerable effort was made to avoid RIFA foraging tunnels (Markin et al. 1975), these sometimes appeared around the base of exclosures where it appeared that the RIFA took advantage of the disturbed soil for the construction of tunnels. RIFA are known to favor areas with high soil disturbance (Tschinkel 1988). This issue is much more difficult to address, but it may be possible to use a chemical barrier in the soil around the exclosure to prevent incursions by RIFA.

There were problems with the arthropod data. First, the trapping effort, four traps per plant, was too low to provide adequate samples to analyze. The data used to conduct statistical comparisons contained so many zeros that the data did not conform to the standard normal distribution required for more robust statistical analyses. In order to make valid statistical comparisons, many species had to be lumped together, leading to the use of large heterogeneous groupings. For example, one group, Diptera, could include parasitic, predatory, and non-predatory species. Lumping such species together obscures effects of predatory species on the survival of monarch larvae. Similarly, the data for arthropods observed on plants was based on presence/absence only and, therefore, would be more valuable if the actual abundances were quantified. Much of the ambiguity in the results stem from inadequate sampling of arthropods. Increased sampling and more refined quantification of the insects would enable more precise evaluation of the sources of mortality for monarch larvae.

Recommendations for future research

At the outset, it is important to realize that a one-year study does not provide much in the way of conclusive information owing to the vagaries of weather conditions and normal population fluctuations.

Therefore, any future study should, at the very minimum, include at least two years of data. The following suggestions for future research are based on the assumption of at least a two-year study.

The results of this pilot study point toward three areas of research that are essential for understanding monarch egg and larval survival and the potential role of RIFA in on monarch survival in northeast Texas. First, it is clear that monarch survivorship must be analyzed within the context of the arthropod community on and surrounding the host plants. If indirect effects are important, then the interactions between plants, herbivores, and predators needs to be more clearly documented. Secondly, if RIFA are involved in indirect effects by reducing predation and competition for monarchs, then it needs to be demonstrated that monarchs are a lower value prey item than are other species available. Thirdly, understanding the survivorship of monarchs up to the third instar is important, but only represents half the life stages between egg and adult. When a fourth or fifth instar larva leaves a plant, it runs a gauntlet of RIFA mounds (mean distance from host plant to RIFA mound in this study 256 cm). A true understanding of survivorship must take into account the behavior and survival of later instars and pupae. This would require an ability to track the dispersal and the survival of dispersing individuals.

Study 1. Monarch survivorship and arthropod diversity

A conclusive study on factors affecting monarch mortality requires more refined sampling of arthropods. More focus must be placed on direct quantitative observations of communities associated with milkweeds occupied by the monarchs. The current study suggests that monarch mortality is tied to indirect effects mediated by arthropod predators. Since the most abundant of these is the RIFA, the easiest way to manipulate indirect effects is to manipulate the local density of RIFA in the vicinity of milkweed plants containing monarch eggs or larvae.

A series of experiments can be used to identify the local diversity of arthropods on and adjacent to the milkweed plants containing monarch eggs and larvae. These arthropods can be surveyed in two ways. First, daily counts can be made of arthropods directly on the plants.

Second, sticky traps adjacent to the plants can be used. There are, of course, some limitations to these techniques. Daily counts of arthropods on the plants could provide reliable data on arthropods that reside on these plants. However, they would not provide accurate estimates of arthropods that may be important to monarch survival, but are only transient occupants of the plant. Sticky traps are more likely to catch terrestrial arthropods than volant arthropods. Therefore, it is recommended that both methods are used.

To ease identification of arthropods associated with milkweed plants, the arthropods that were captured in the current study will be used to create a reference collection. The reference collection can indicate what species to look for, how best to enumerate them, and what types of groupings represent logical ecologically equivalent species. The primary difficulty encountered with the data on sticky traps was low capture rates. Therefore, it is recommended that eight traps be set on the first and last days that a plant is occupied and every four days in between. This would result in a trapping effort that should provide more reasonable numbers of captures.

High trapping effort around the plant may affect the numbers of arthropods on the plant by temporarily suppressing immigration. Therefore, to control for that effect, it would be important to have some control plants that do not have any traps around them at all. Numbers of arthropods on these control plants would be used to determine the extent to which sticky traps around milkweed plants affect the arthropod populations on those plants.

RIFA density around plants with monarchs can be manipulated using untreated baits and baits with toxins. Eubanks et al. (2002), were able to suppress RIFA without major effects on non-target species in a study on the effects of RIFA on beneficial arthropods in cotton fields. They used a granulated bait containing hydramethylnon (Amdro ^{TM}) that could be broadcast across their study plots. As indicated in that paper, this granulated bait is "composed of an inert, corn "grit" carrier impregnated with soybean oil that is very attractive to foraging fire ant workers

but ignored by other insects" (Eubanks et al. 2002). Fire ant bait could be broadcast over an isolated portion of the study area at the end of February, four weeks prior to the field season and again just prior to the arrival of ovipositing female monarchs at the end of March. Thereafter, individual mounds would be treated within that area on an as needed basis. This would provide data on a relatively intact arthropod community without large populations of RIFA for the duration of monarch season in the study area. The prediction would be that if RIFA benefit monarch eggs and larvae through indirect effects then monarch egg and larval survival should be lower in areas where RIFA populations are suppressed. Alternately, if RIFA have a negative impact on monarch survival, they should do better where RIFA populations are suppressed.

While increasing the population size of RIFA during the course of the study might not be realistic, it is possible to increase the local density of foraging RIFA by attracting them with baits. During some initial trials with sticky traps, when the traps were left in the field for more than one day, it was found that insects captured by the traps during the first 24h attracted large numbers of RIFA to the traps within 48h. Therefore, it should be possible to increase RIFA foraging density around experimental plants. Mealworms (*Tenebrio molitor*) are easily cultured and can also be purchased freeze-dried. Small numbers of dead mealworms spread adjacent to milkweed plants containing monarch eggs or larvae should increase ant activity around these plants. In this case, if RIFA increase monarch survival through indirect effects, then monarch survival should be higher around plants with enhanced RIFA densities. Alternately, if RIFA have a negative impact on monarch survival, they should do worse in areas where the foraging density of RIFA is higher.

This portion of the study, as described above, would consist of 4 treatments:

1. Control plants without traps – provides data on arthropods occupying plants and a baseline for comparisons with plants that have traps. There would need to be 100 replicates for this treatment.

2. Control plants with traps – provides data on arthropods occupying plants which can be compared to control plants without traps to determine the impact of trapping and can be compared with experimental plants to determine the effect of experimental treatment. There would need to be 100 replicates for this treatment.

3. Experimental plants with decreased RIFA densities – provides data on the effect of reduced populations of RIFA on monarch egg and larval survival. There would need to be 100 replicates for this treatment.

4. Experimental plants with increased RIFA densities – provides data on the effect of increased foraging densities of RIFA on monarch egg and larval survival. There would need to be 100 replicates for this treatment.

Study 2. Fire ant prey preferences

Simple field choice trials are proposed to determine the extent to which RIFA actively prefer certain arthropods that normally occur on or near milkweed plants. Randomly chosen RIFA mounds would be chosen in an untreated area and baited sticky traps would be placed approximately 30 cm from the periphery of the mound. A standard mass for each bait would be chosen, possibly 0.25 g and a dead, but non desiccated insect bait would be placed near the center of the sticky trap. Based on their observed abundance and potential direct or indirect impact on monarch larvae, the most relevant bait species would be aphids, weevils, crab spiders, wolf or grass spiders, jumping spiders, monarch second instars, and monarch fourth instars. Mealworms would also be presented as a control. The eight traps would be presented to the ants simultaneously and the latency to detection recorded and the number of ants captured after 1h recorded. Preferred prey items should end up with shorter latencies to detection and higher number of RIFA in the traps. The results of these preference tests would be used to corroborate

evidence that monarch larval survival is due to indirect effects. A minimum of 30 replicates of these preference tests would be needed for statistical analyses.

Study 3. Dispersal and survival of fourth and fifth instars

To study the dispersal and survivorship of fourth and fifth instars, sufficient numbers of these individuals would be needed. The enclosures resulted in 21 individuals reaching the fifth instar among the 92 exclosures set up, but this number could be improved by making the exclosure 1 m wide instead of 30 cm wide. This would help to contain the plant and make accidental early emigration much less likely. Instead of using sticky tape the upper rim of the exclosure would be covered with Tanglefoot™ which would be far more effective for keeping terrestrial predators out. Tanglefoot™ is also soluble in vegetable oil, so if a monarch larva incidentally was caught in the Tanglefoot™ it could be carefully removed, provided it was found soon enough. The exclosures will be carefully monitored on a daily basis and will provide important data on dispersal behavior of different ages of instars.

When the larvae reach the fourth or fifth instar, they will be fitted, using a cyanoacrylate adhesive, with a tiny transponder composed of Schottky diode, an inductor, a conductive strip, and an antenna (Brazee et al. 2005). Once fitted with the transponder they will be released on the natal plant and the exclosure removed. The caterpillars can then be followed using a portable harmonic radar transceiver. The transceiver sends a signal of known wavelength which, when intercepted by the transponder, activates the transponder which sends a signal back to the transceiver. The direction of the signal can then be used to locate the insect carrying the transponder (Brazee et al. 2005). Harmonic radar is an up and coming technology for tracking small invertebrates and has now been used in several studies, including black vine weevils (*Otiorhynchus sulcatus*) (Brazee et al. 2005), honey bees (*Apis mellifera*) (Wolf et al. 2014), potato beetles (*Leptinotarsa decemlineata*), plum curculio (*Conotrachelus nenuphar*), and corn

rootworm (*Diabrotica virgifera virgifer*) (Boiteau et al. 2011). In some cases, detection distances

of 90 m from a 30 mg transponder have been achieved (Tsai et al. 2013). Since a fourth instar

monarch larva weighs around 350 mg, this would be less than 10 of its body mass and could be

easily carried by the caterpillar. The objective would be to track and determine the fates of at

least 20 fourth instars and 20 fifth instars. It is hoped that by tracking fifth instars, some data can

then be collected on natural pupation sites and the survivorship of pupae. There is currently no

data on the survivorship of natural pupae.

Preliminary projected budget

The most limiting factor evident in the current study was the time it took to check and record the

data for each plant. At the height of the study, it took about 8 hours just to check the 272 eggs or

larvae on the study sites at that time. Checking plants, in fact, limited the number of eggs that

could be included in the project. The proposed research would require a total of about 500 eggs

to be found. Furthermore, the protocol of closely censusing all the arthropods on each plant will

be considerably more time consuming. This will require a much larger group of individuals

working on the project. The preliminary budget in Table 8 considers these constraints.

Evaluation of attainment of stated project purposes

The purposes of this pilot study were to:

1. Determine if sufficient numbers of ovipositing monarch butterflies can be observed in the area

   around Commerce, Texas, to warrant more detailed research on the survivorship of monarch eggs

   and larvae.

   a. The data clearly show that sufficient samples can be gathered in the area around

      Commerce, Texas, to warrant more detailed research on the survivorship of monarch

      eggs and larvae.

Table 8. Preliminary projected budget of proposed research project.

Item	Annual Cost	Total
Graduate Assistantships (3 at 2 years each)	$45,000.00	$90,000.00
Undergraduate Assistants (6 at 2 years each)	$13,500.00	$27,000.00
Faculty Time and Effort (10%)	$8000.00	$16,000.00
Glue Traps	$6000.00	$12,000.00
Exclosures	$200.00	$200.00
General supplies	$1000.00	$2,000.00
Harmonic radar system with transponders	$15000.00	$15,000.00
Indirect Costs (10%)		$16,220.00
Total Costs		**$178,420.00**

2. Refine the protocols used by Calvert (2004), Prysby and Oberhauser (2004), and Prysby (2004) to document survivorship of monarch eggs and larvae and the potential role of RIFA in the survivorship of monarch eggs and larvae in the area around Commerce, Texas, and to compare these rates to published studies in other geographic locations.

   a. The protocols used by Calvert (2004), Prysby and Oberhauser (2004), and Prysby (2004) to document survivorship of monarch eggs and larvae were modified by making the exclosures smaller and by careful daily monitoring of marked plants. The revised protocol clearly found a difference in survivorship between control plants and experimental plants.

   b. Further modifications of the protocol are needed to sort out exactly what the exclosures are keeping out (or in). The data collected in this study were ambiguous as to that effect. The data seem to indicate the monarch larvae do better in areas where more potential predators occur. It could be that these polyphagous predators preferentially avoid monarch larvae, but better insect sampling are needed.

   c. Survivorship and age-specific survival was greater than observed in most other studies for both control and experimental individuals.

3. Use the results of the current pilot study to develop more detailed study of monarch reproduction in northeast Texas with the goal of producing a key factor analysis that identifies those factors with the greatest impact on survivorship. These factors would then be used to inform future management decisions.

   a. The methodology used in the current study and the data derived from this methodology were evaluated. A detailed project was proposed with three areas of focus:

      i. Survival and the potential indirect effects of RIFA

ii. Prey preferences of RIFA

iii. Dispersal and survival of fourth and fifth instars

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