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Kevin Hoang April 15, 2016

Effects of milkweed species on monarch butterflies and their parasites

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An abstract of
a thesis submitted to the Faculty of Emory College of Arts and Sciences
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Abstract

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Parasites contribute to much of the species diversity and cover a wide range of hosts and environments. By definition, parasites induce fitness reductions in hosts, whether it is through reduction in host size, immune defense, lifespan, or fecundity. The intricate host-parasite relationship is affected by a number of factors, such as host diet, nutrition, and immune system. For example, the monarch butterfly (*Danaus plexippus*), which can be infected with the protozoan parasite *Ophryocystis elektroscirrha*, exhibits oviposition preferences when infected. Infected females, which spread the parasite during oviposition, seek out medicinal milkweeds (*Asclepias spp.*) to lay their eggs upon in order to alleviate the effects of *O. elektroscirrha* in the next generation. These medicinal milkweeds differ in their toxic chemical contents and reduce parasite loads as well as increase lifespans of infected monarchs compared to those reared on non-medicinal milkweeds. Here, we explore how different milkweed species affect the host-parasite interaction between the monarch butterfly and its parasite. Specifically, we compare *Morrenia odorata*, a member of the milkweed family, as a host plant for monarchs with more common milkweed species (*Asclepias spp.*). We found that *M. odorata* can be used to rear monarch larvae to adulthood but that the lifespans of these monarchs are generally lower than those reared on *Asclepias* milkweeds. Additionally, *M. odorata* does not appear to have any medicinal effects on infected monarchs. On top of this, female monarchs significantly preferred to oviposit on *Asclepias* milkweeds over *M. odorata* when given a choice. A separate experiment we conducted was to study the effects of different monarch larval diets on parasite morphology. Here, we found a significant relationship between larval diet and parasite size. The toxic chemicals within milkweeds significantly reduced parasite size, a hidden benefit of medicinal milkweeds on *O. elektroscirrha*. Not only do these milkweeds reduce parasite loads, but they also reduce parasite sizes, another possible mechanism to lower parasite fitness. In the future, additional studies on the effects of *M. odorata* and monarch larvae diets on monarch-parasite interactions need to be conducted to further expand our knowledge on this intricate and alluring host-parasite relationship.

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ABSTRACT

Parasites normally have significant fitness costs on hosts. In order to reduce parasite loads or to clear parasites, hosts have evolved several behavioral changes, ranging from passive parasite avoidance or to dietary changes in response to infection. Interestingly, some infected insects alter oviposition behaviors in response to infection. The monarch butterfly is an example, where infected females preferentially oviposit on milkweeds with medicinal properties in order to alleviate parasite loads in the next generation. Recent reports suggest that *Morrenia odorata*, a member of the milkweed family, completely clears infected monarchs of infection. Here, we evaluate *M. odorata* as a host plant for monarchs and its effects on parasite infection. We show that monarchs reared on *M. odorata* have lower survival rates compared to monarchs reared on other milkweed species, and importantly, *M. odorata* does not reduce infection probability or clear parasite infection in monarchs. This experiment, due to small sample sizes, should be repeated in order to further evaluate *M. odorata*'s medicinal properties and qualities as a host plant.

INTRODUCTION

Parasites are one of the most diverse and ubiquitous life forms on earth and, by definition, require host resources for replication and transmission (Windsor 1998). Parasites can decrease both host fitness and longevity, thus shaping host ecology and evolution (Dobson and Hudson 1986). As a result, hosts have evolved many mechanisms of combating parasites (Hart 1988, Beckage 1997), including canonical immune defenses through phagocytosis or by encapsulation of the parasites (Hoffmann et al. 1999, Tzou et al. 2002) and non-immunological defenses (Parker et al. 2011). For example, behavioral fever, which increases the body temperature above

typical set points, is a defense mechanism set to inhibit the ability of pathogens or parasites to develop (Kluger et al. 1998, Moore 2002, Kluger 2015). Another form of defense against parasites is avoidance of infection (Hart 1994, Moore 2002), which has been seen in many systems and is the most effective means of preventing infection (Hausfater and Meade 1982, Karvonen et al. 2004, Reckardt and Kerth 2007). For example, yellow baboons (*Papio cynocephalus*) alternate sleeping locations every few days, a behavioral trait that may partially have been developed to increase avoidance of intestinal nematodes that spread through fecal samples and reside in the soil (Hausfater and Meade 1982). In addition to behavioral changes, hosts can also undergo dietary changes in response to infections. For example, chimpanzees willingly ingest the bitter pith of *Vernonia amygdalina* to treat intestinal nematode infections (Huffman 2003). Wooly bear caterpillars (*Grammia incorrupta*) also exhibit dietary changes in response to infection by their lethal endoparasites, tachinid flies. Infected wooly bear caterpillars preferentially choose diets with high amounts of pyrrolizidine alkaloids. These infected caterpillars exhibit higher survival rates than those on diets without pyrrolizidine alkaloids (Singer et al. 2009). Collectively, therapeutic behavioral responses against parasites or infections are known as self-medication (Janzen 1978, Rodriguez and Wrangham 1993, Huffman 2003).

One interesting form of medication behavior is trans-generational medication (Lefèvre et al. 2010). Trans-generational medication leads to indirect benefits: while the individual receives no benefit, their offspring do. For example, female fruit flies (*Drosophila melanogaster*) have been shown to preferentially oviposit on Petri dishes with food sources high in ethanol content in the presence of the wasp parasite *Leptopilina heterotoma*, thus protecting future larvae from infection (Kacsoh et al. 2013). Similarly, female monarch butterflies (*Danaus plexippus*) infected with their protozoan parasite *Ophryocystis elektroscirrha* preferentially oviposit on milkweed

host plants (*Asclepias spp.*) with higher toxic chemicals in order to reduce parasite loads in the next generation (de Roode et al. 2008, Lefèvre et al. 2010, Sternberg et al. 2012).

Milkweeds are the host plants of monarch butterflies and produce cardenolides, secondary plant chemicals that are toxic to most animals. Cardenolides are steroid chemicals that dirsupt animal Na^+/K^+-ATPase. They are 23-carbon structures consisting of three main components: a steroid backbone made of four fused carbon rings, a five-membered lactone group, and a carbohydrate or sugar moiety on the first carbon ring (Figure 1) (Agrawal et al. 2012). Milkweeds produce a wide variety of cardenolides and in various concentrations; the differences in milkweed cardenolides can be both in polarity (functional groups attached to the cardenolide) and in concentration (amount of cardenolides present) (Rasmann and Agrawal 2011, Agrawal et al. 2012, Tao et al. 2015).

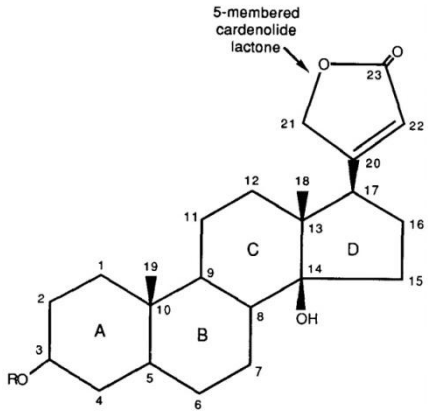

Figure 1. A general structure of cardenolides. Image adapted from Malcolm 1991.

Monarchs are specialized herbivores that can sequester milkweed cardenolides for defense against predation (Brower 1969). As mentioned previously, cardenolides act as medication for monarchs infected with *O. elektroscirrha*. *O. elektroscirrha* is a protozoan parasite that is transmitted vertically during oviposition and horizontally during mating (Altizer

et al. 2004). This parasite undergoes two main life stages: an actively reproducing phase within the monarch larval gut and a dormant, transmissible spore phase on the abdomen of adult butterflies (McLaughlin and Myers 1970, Leong et al. 1992). Monarchs infected by *O. elektroscirrha* suffer fitness costs such as reduced lifespans, body mass, flight ability, and mating success (Altizer and Oberhauser 1999, Bradley and Altizer 2005). Infected monarchs reared on milkweeds with high cardenolide concentrations have shown reduced parasite loads as well as increased longevities compared to infected monarchs raised on low cardenolide milkweeds (de Roode et al. 2008, Sternberg et al. 2012). On top of this, infected female monarchs have been shown to practice trans-generational medication by preferentially ovipositing on milkweeds of higher cardenolide concentrations, which can alleviate parasite loads in the next generation (de Roode et al. 2008, Lefèvre et al. 2010, Sternberg et al. 2012). As such, larval host plant species can have serious effects on infected monarch fitness as well as drive behavioral adaptations in monarch butterflies.

While monarchs typically consume milkweeds (*Asclepias spp.*) during growth, they can be reared on alternative food sources. For example, monarchs can be reared on an artificial diet containing proper nutrients (Glass and Pan 1983) or on other plant species. For example, monarchs on the Caribbean island of Barbados have been observed to survive on the giant milkweed *Calotropis procera* (Blakley and Dingle 1978). Although not in the genus *Asclepias* as other milkweed species, *C. procera* is in the same family as *Asclepias* milkweeds (family: *Apocynaceae*). Another interesting milkweed of the *Apocynaceae* family is the milkweed vine *Morrenia odorata*. The southern monarch (*D. erippus*) is a non-migratory, close relative of the eastern monarch (*D. plexippus*) and has been shown to successfully feed on *M. odorata* (Silveira-Guido et al. 1977). Unpublished reports circulating in the monarch community have shown the

successful rearing of eastern monarchs on *M. odorata*. In addition, these reports suggest that *M. odorata* completely clears infected monarchs of *Ophryocystis elektroscirrha*. As such, tests to confirm the medicinal effects of *M. odorata* can have serious implications in future monarch butterfly research. Here, we evaluate the milkweed vine *Morrenia odorata* both in its medicinal effects against *O. elektroscirrha* and in its ability to act as a host plant for monarch butterflies.

In this study, we compared the medicinal properties of the milkweed vine *M. odorata* with three known milkweeds, specifically *Asclepias incarnata*, *A. curassavica*, and *A. syriaca*. We first assess the viability of *M. odorata* as a monarch host plant and comparing monarch survival and lifespan on different milkweeds and on *M. odorata*. We also test whether *M. odorata* can reduce the probability of infection or reduce spore load in monarchs.

MATERIALS AND METHODS

Host and Parasite sources

Monarchs for this experiment were lab-reared progeny of wild-caught migrating monarchs obtained in St. Marks, Florida during Oct 2014 and are representative of the eastern United States migrating monarch population. Monarchs were of three different lineages, and lineages were randomly distributed between the milkweed groups.

Parasite clones of *Ophryocystis elektroscirrha* were obtained from infected monarchs previously caught by lab members. These clones were propagated by inoculating monarch larvae with a single parasite spore. Spores taken from adults of the resulting butterflies were used in this experiment and should be genetically identical to one another in order to reduce differences due to parasite genetics (Sander et al. 2013).

Plant sources

In this experiment, we used three milkweed species of the *Asclepias* genus: *A. incarnata*, *A. curassavica*, and *A. syriaca* and compared their effects on monarch-parasite interactions with the milkweed *M. odorata*. Milkweed seeds were obtained from Butterfly Encounters Inc. (San Ramon, CA, USA) and *M. odorata* seeds were obtained from Georgia Vines (Claxton, GA, USA). Seeds were sowed on sterilized seedling soil from Fafard (Agawam, MA, USA). When seedlings were roughly 3 cm tall, they were transferred to individual pots with sterilized, 3B soil from Fafard. Plants were grown in a greenhouse where the temperature was between 24°C and 33°C and the humidity was between 30% and 60%. Plants were watered twice daily and were approximately three months old when used for experiments.

Experiment 1:Effects of M. odorata on monarch growth, fitness and parasite infection

Monarchs were separated into five different plant groups for rearing to adulthood: *A. curassavica*, *A. incarnata*, *A. syriaca*, *M. odorata*, and *M. odorata* + *A. incarnata*. For the *M. odorata* + *A. incarnata* group, monarch larvae were fed *M. odorata* prior to inoculation and *A. incarnata* after inoculation. Previously, studies have shown that the effects of milkweeds on monarchs occur mostly at early larval stages (Zalucki et al. 2001, De Roode et al. 2011, Tao and Hunter 2012). In this design, the timing of the effects of the possible medicinal qualities of *M. odorata* could be shown: either they act before inoculation, in which no differences would be seen between the *M. odorata* and *M. odorata* + *A. incarnata* groups would be seen; or they act after inoculation, in which differences would be seen between the two groups.

Forty monarch eggs were randomly assigned into each plant group and specific plants were assigned to each monarch, yielding a total of 200 individuals. For each group, 15 were to be

uninfected and 25 were to be infected. A single leaf from the fourth leaf pair from the top of each plant was placed on a moist filter paper in a 10 cm Petri dish and was used to rear individual monarch eggs to the second instar stage.

Monarch larvae were inoculated using methods adapted from de Roode *et al.* 2007. To do this, a leaf disk of 0.64 cm diameter was taken from a leaf of the third leaf pair from each assigned milkweed plant. Leaf disks were placed on moist filter papers in individual, 10 cm Petri dishes, and ten parasite spores were placed onto each leaf disk using a drawn-out glass capillary tube. These larvae were kept until complete consumption of their leaf disks, and transferred to individual, clear plastic tubes (7.62 cm in diameter, 30.48 cm in length; Visipak, MO, USA) that contained their assigned milkweed plant. Uninfected individuals were treated similarly, although without placing parasite spores on leaf disks.

Larvae were kept in their individual containers until pupation or until complete consumption of their plant, upon which they were fed cuttings of the same milkweed species grown in a separate lab room. Survival was recorded only for eggs that reached the adult stage. Pupae were transferred to individual, 473 mL solo cups and placed in a separate lab room until emergence. Upon emergence, monarchs were placed into individual, 8.9 cm x 8.9 cm glassine envelopes and stored in a 12°C incubator. Monarchs were left unfed and checked daily for death, upon which the lifespan was recorded.

Parasite spore load of infected monarchs was determined using methods adapted from those described in de Roode *et al*. 2007. Thirty days after monarchs died, their wings were removed and the bodies were vortexed (Vortex-Genie 2; Scientific Industries, Inc., Bohemia, NY, USA) in plastic, 20 mL vials (Fisher Scientific, Pittsburgh, PA, USA) with 5 mL of H_2O at high

speed for 5 minutes. Bodies were removed from vortex vials and the solution remaining contained the parasite spores to be quantified.

Parasite load was measured by counting the number of parasite spores in four, 10 μL samples of the vortexed solutions containing spores. The vortex solution was vortexed for 5 seconds before pipetting out the four samples to be analyzed in order to resuspend the spores. Parasite spores were counted by pipetting each 10 μL sample into different wells of a microscope slide with wells. Each well consisted of a 3 x 3 grid, and parasite spores were counted in the four corners of the grid. Spores on the bottom and right edges of the cells were counted if over 50% of the spore was within the cell. If a cell contained monarch scales or materials other than parasite spores, a different cell was used to count spores. The number of the parasite spores from all sixteen cells (four cells from the 3 x 3 grid in each well and four wells per slide) was averaged, and original parasite load was quantified using a logarithmic scale with the equation $\log_{10}(\text{average}*50000+1)$.

This experiment was conducted twice, once in the summer of 2015 and once in the fall of 2015. Both were conducted using the same methods; the only difference between the two trials, however, due to logistic reasons, is that in the fall 2015 trial, only three milkweed groups were used: *A. curassavica*, *A. incarnata*, and *M. odorata*. Monarchs in the fall 2015 trial were outbred monarchs derived from crosses between de Roode lab monarchs and monarchs from the Alitzer lab at the University of Georgia. All other methods were kept consistent between the two trials. For the fall 2015 trial, several monarchs were used as parasite sources for a previous lab member. While sporeload data was not collected for these monarchs, longevity and other data were used in subsequent analyses.

Experiment 2: Oviposition choices of female monarchs between M. odorata and A. incarnata

Females used in this experiment were of different lineages than those used in *Experiment 1* and were randomly reared on milkweed species other than *A. incarnata* and *M. odorata*. This design prevents any female predisposition choice due to larval food choices, as females never encountered *A. incarnata* or *M. odorata* prior to choice tests. Previous studies on monarch oviposition show that uninfected monarchs show no milkweed preference and distribute eggs fairly even when given two milkweed choices (Lefèvre et al. 2010). In this choice test experiment, we placed one fertile female monarch in a mesh cage with *A. incarnata* and *M. odorata* on either side of the mesh cage (39 cm in diameter, 60 cm in length; Carolina Biological Supply Co., Burlington, NC, USA). Females were placed on the tops of both plants for five seconds, which introduces them to the plants available and were allowed one hour to oviposit. Females were removed after one hour, and eggs were counted on both plant species. In order to minimize differences due to plant size, plants used for each trial were roughly identical in size. Individual monarchs were allowed to undergo choice tests only twice for data collection. This limit was to prevent future preferences for oviposition.

Statistical tests

All data analyses were conducted using R v. 3.2.2 (R Development Core Team 2015) and RStudio v. 0.97.551 (© 2009-2012 RStudio, Inc.). We performed logistic regressions to test whether milkweed plant species had effects on monarch survival and infection probability. Subsequently, we performed linear models to see whether plant species and infection status had significant effects on monarch pupation time, lifespan, and sporeload. This was done separately

for the two datasets from summer 2015 and fall 2015. To test whether female monarchs prefer *M. odorata* or *A. incarnata* to oviposit on, we performed a chi-squared test.

RESULTS

Experiment 1

Summer 2015

Milkweed species had a significant effect on monarch survival (χ^2_4 = 10.7, p = 0.03, Figure 2). Specifically, monarchs in the *A. curassavica* and *A. incarnata* groups had the greatest survival (67.5%) while those in the *A. syriaca* and the *M. odorata* + *A. incarnata* groups had the lowest (40% and 45%, respectively). Monarchs reared solely on *M. odorata* had 47.5% survival. The effect of milkweed species on pupation time was nonsignificant ($F_{4, 101}$ = 1.94, p = 0.11). We found that milkweed species had significant effects on monarch lifespan ($F_{4, 96}$ = 5.11, p < 0.001, Figure 3). Average monarch lifespans were highest on *A. syriaca* and *M. odorata* while lowest on *A. incarnata*, a finding that may be due to small sample sizes. Infection status did not have significant effects on monarch lifespans ($F_{1, 96}$ = 2.14, p = 0.15); nor was milkweed species on parasite virulence, as there was no significant interaction between milkweed species and parasite infection on monarch lifespan ($F_{4, 96}$ = 1.22, p = 0.31). Milkweed species also had no significant effects on infection probability (χ^2_4 = 1.3, p = 0.86, Figure 4) or spore loads of infected butterflies ($F_{4, 6}$ = 0.93, p = 0.51, Figure 5). Again, these results may be due to small sample sizes, as only 10 of the 106 surviving monarchs were successfully infected. Also, low infection probabilities may have been due to poor spore quality.

Fall 2015

Milkweed species during the Fall 2015 trial also had significant effects on monarch survival ($\chi^2_2 = 9.9$, $p = 0.007$, Figure 6). Survival was highest when monarchs were reared on *A. curassavica* (37.5%), second for monarchs reared on *A. incarnata* (17.5%), and lowest for monarchs reared on *M. odorata* (7.5%). The effect of milkweed species on pupation times was not significant ($F_{1, 17} = 0.056$, $p = 0.82$). The effect of milkweed species on monarch lifespans was significant ($F_{2, 19} = 5.54$, $p = 0.013$, Figure 7). Specifically, average monarch lifespans were highest when reared on *A. curassavica*, second when reared on *A. incarnata*, and lowest when reared on *M. odorata*. Infection by parasites marginally reduced monarch lifespan ($F_{1, 19} = 3.39$, $p = 0.08$), and milkweed species did not affect parasite virulence ($F_{2, 12} = 0.51$, $p = 0.61$). Similar to the Summer 2015 trial, milkweed species did not affect infection probability or spore loads ($\chi^2_2 = 1.0 \times 10^{-5}$, $p = 1.00$ and $F_{1,3} = 0.005$, $p = 0.95$, respectively) (Figures 8 and 9, respectively).

Experiment 2

Oviposition choice tests for five unique female monarchs were conducted. Eggs on each plant species were counted from each trial and totaled for comparison. We found a large difference in the number of eggs laid by female monarchs on *A. incarnata* versus *M. odorata* ($\chi^2 = 146.57$, $p < 0.001$). Specifically, females highly preferred to oviposit on *A. incarnata* over *M. odorata*; 177 out of 188 (94%) of eggs were laid on *A. incarnata* over *M. odorata*.

DISCUSSION

We performed this study to explore the viability of *M. odorata* as a host plant to monarch butterflies and its effects on monarch-parasite interactions. Overall, we found that monarch survival is generally lower when reared on *M. odorata* compared to other milkweed species. We

also found that monarchs reared on *M. odorata* had significantly lower lifespans compared to monarchs reared on *A. incarnata* and *A. curassavica* during the Fall 2015 trial. Additionally, female monarchs actively avoid oviposition on *M. odorata* in the presence of *A. incarnata*. As such, we have shown that although *M. odorata* can be used to rear eastern, migratory monarchs from egg to adulthood, the performance of monarchs is low compared to other *Asclepias* species. More importantly, in contradiction to other reports, our study suggests that *M. odorata* does not reduce infection probabilities when monarchs are exposed to *O. elektroscirrha*, or reduce infection loads of *O. elektroscirrha*; follow-up experiments, however, are needed to confirm this. These last two findings are especially important in monarch-parasite research and should elucidate that *M. odorata* is not beneficial to monarchs, as previous reports have suggested.

As mentioned previously, our results need to be interpreted with caution as we have experienced high mortality in monarchs. This might be due to viral and bacterial infection from the soil. Also, plant qualities and quantities in these experiments were low due to logistic reasons and pest infections. Specifically, plants of *A. syriaca* were of poor quality and small quantities during the Summer 2015 trial, which could explain the low monarch survival on the species. Reduced food supplies may have also induced stress in monarch larvae. Additionally, human errors such as unsuccessful inoculation procedures reduced the infection probability and the sample sizes for infected groups, which may explain the fact that we did not find significant effects of milkweed species on infected monarch lifespans or on parasite loads (de Roode et al. 2008, Lefèvre et al. 2010, Sternberg et al. 2012).

Future work on *M. odorata* is needed to fully assess the plant's medicinal properties. Although we showed that *M. odorata* does not clear infected monarchs of parasites, our sample sizes were small and data had several inconsistencies with previous studies. Characterizing

cardenolide data on *M. odorata* is also an important future experiment, as cardenolide types differ between milkweeds and may contribute to parasite defenses (Lefèvre et al. 2010, Sternberg et al. 2012). Overall, repeating this experiment successfully is one of the first steps to understanding the effects of *M. odorata* on monarch-parasite interactions.

FIGURES

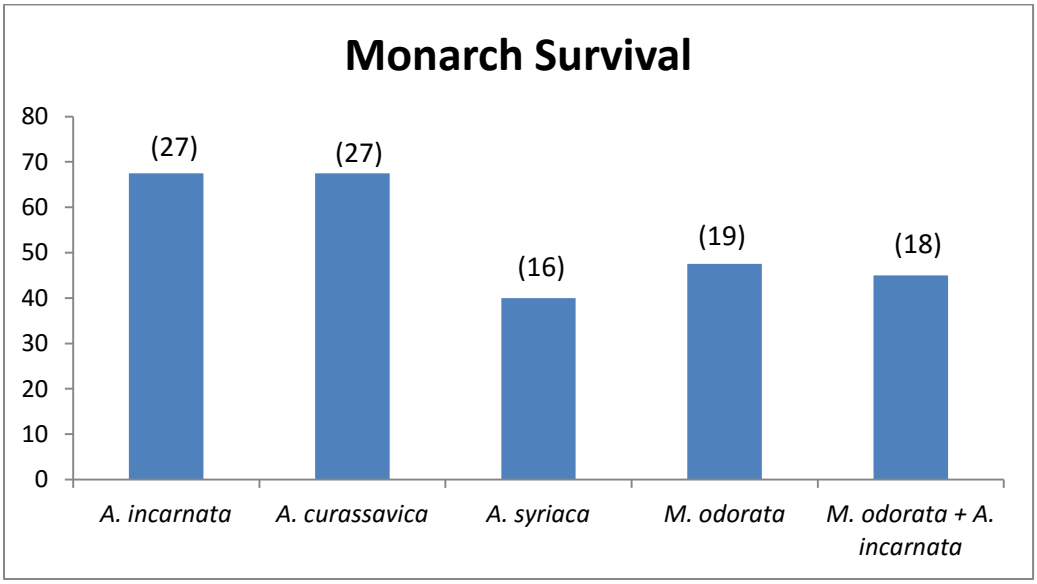

Figure 2. Percent of monarch survival to adulthood, separated by plant species (Summer 2015). Numbers above bars represent the total number of monarchs that survived to adulthood.

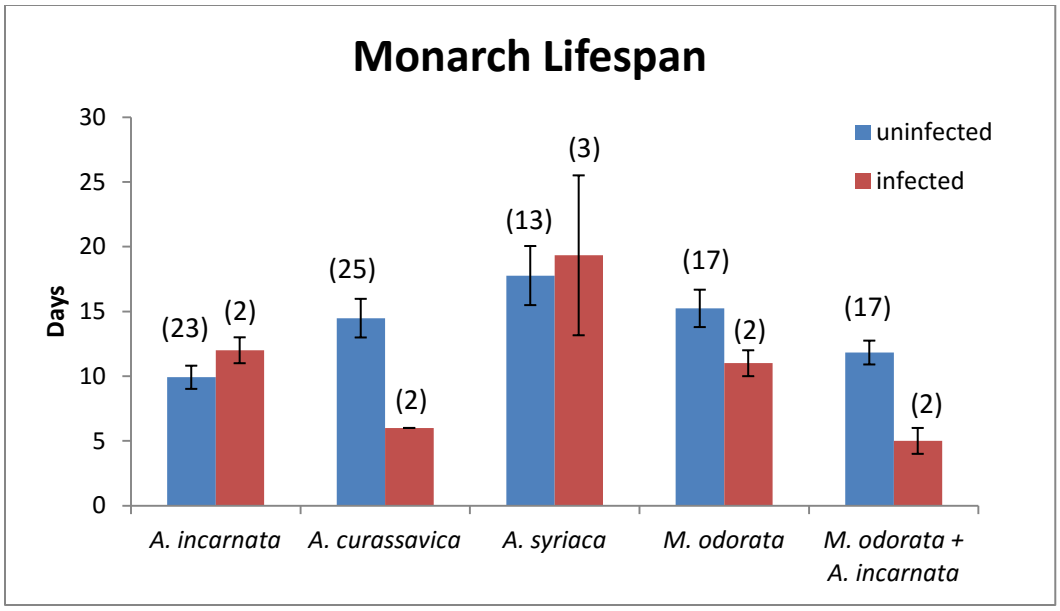

Figure 3. Monarch lifespans by plant species (Summer 2015). Blue bars represent uninfected monarchs while red bars represent infected monarchs. Error bars are ± 1 SEM. Numbers above bars represent sample size. Note: two monarchs from the uninfected, *A. incarnata* group were excluded from longevity analyses, as they were infected (they cannot be included in the infected group, as they were not inoculated with the standard ten parasite spores). Also, one monarch was labeled incorrectly early in the experiment and was in the *M. odorata* + *A. incarnata* group. This monarch was ignored during survival analyses but was included in longevity analyses as being part of the *M. odorata* + *A. incarnata* group.

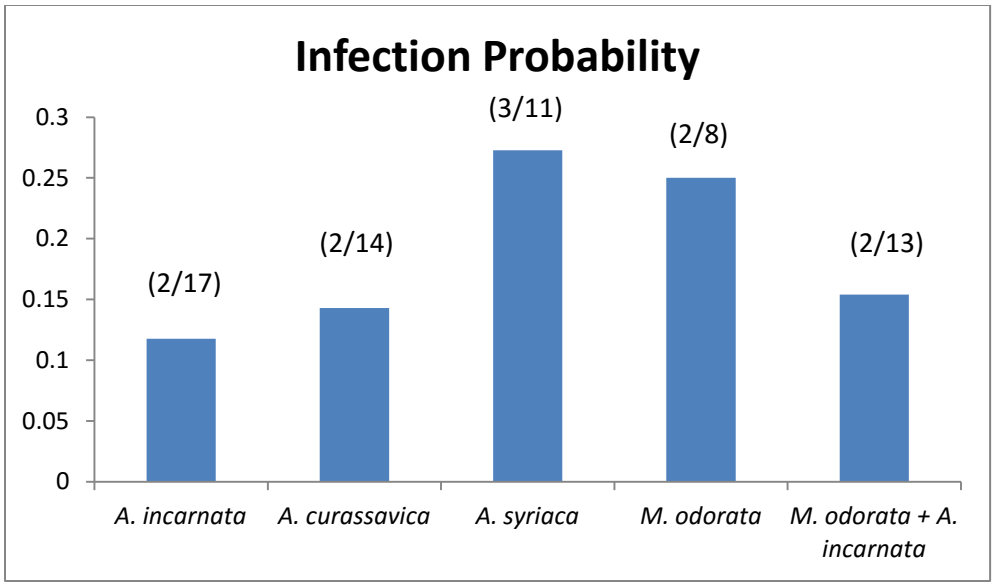

Figure 4. The infection probability of monarchs reared on different milkweed species (Summer 2015). Fractions above bars represent the number of individuals infected over the number of individuals from the infected group that survived to adulthood.

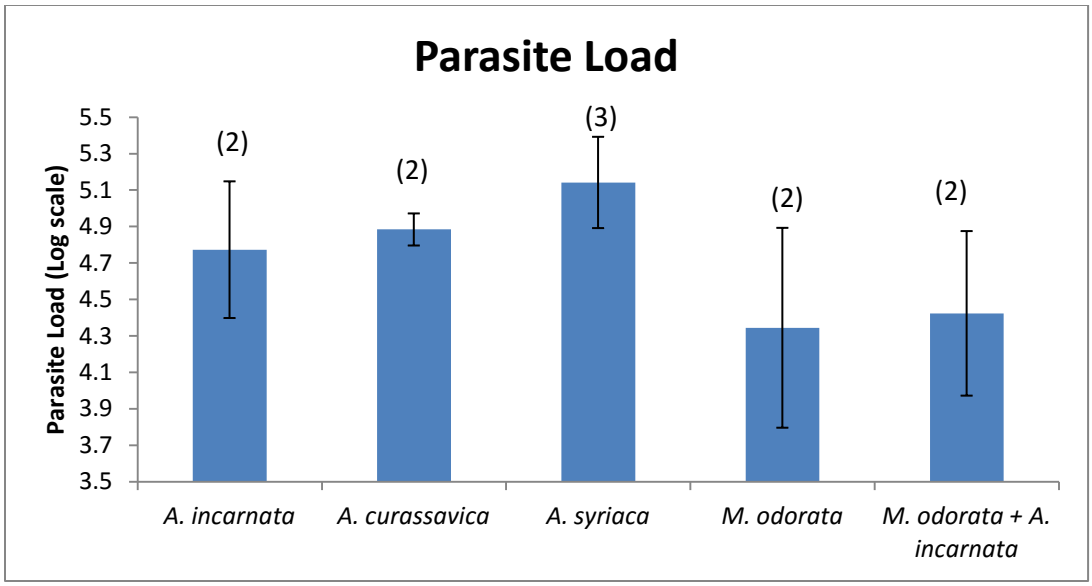

Figure 5. Parasite loads for infected monarchs (Summer 2015). Numbers above bars represent sample sizes. Error bars are ± 1 SEM.

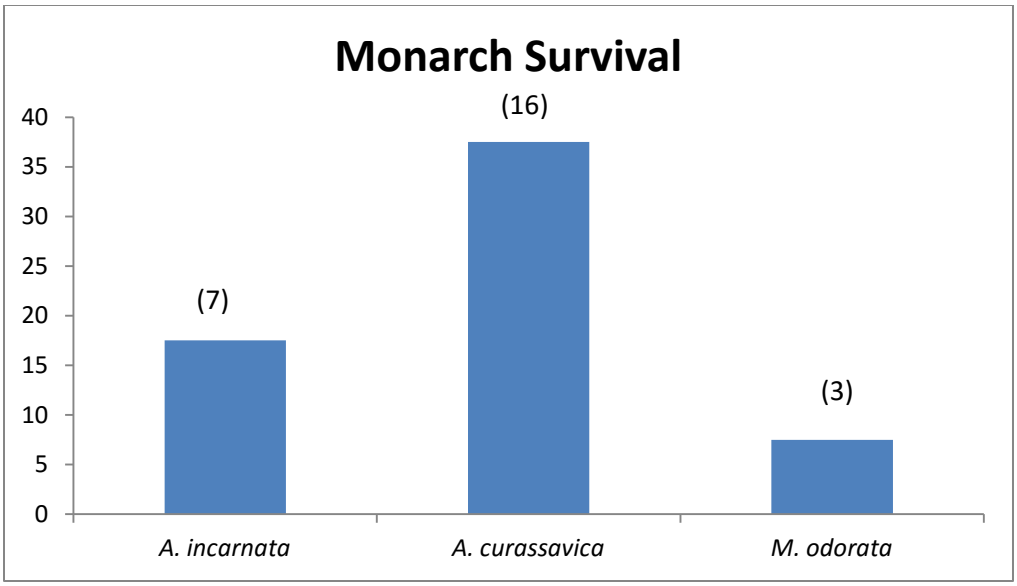

Figure 6. Percent of monarch survival to adulthood, separated by plant species (Fall 2015). Numbers above bars represent the total number of monarchs that survived to adulthood.

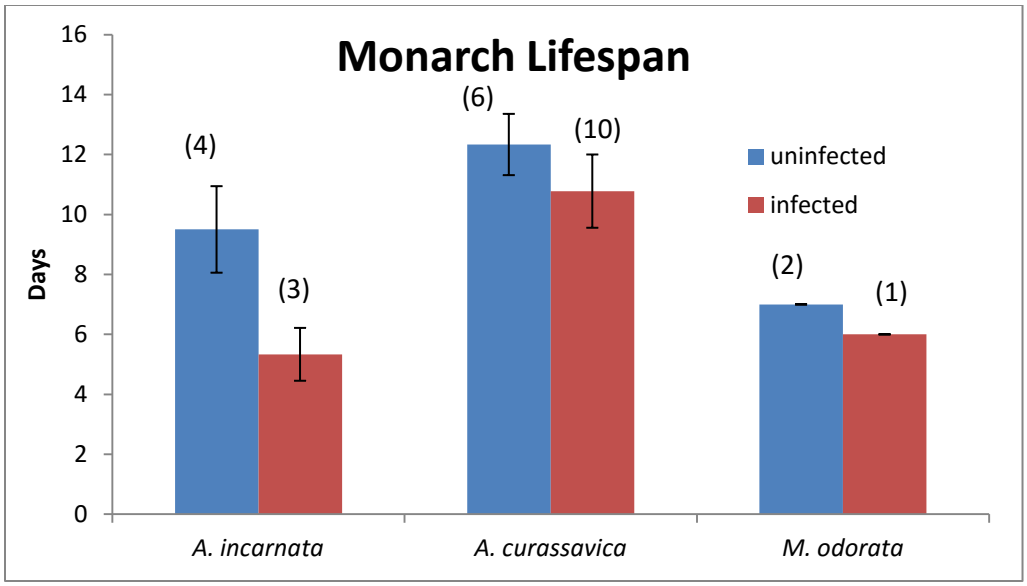

Figure 7. Monarch lifespans by plant species (Fall 2015). Blue bars represent uninfected monarchs while red bars represent infected monarchs. Error bars are ± 1 SEM. Numbers above bars represent sample size.

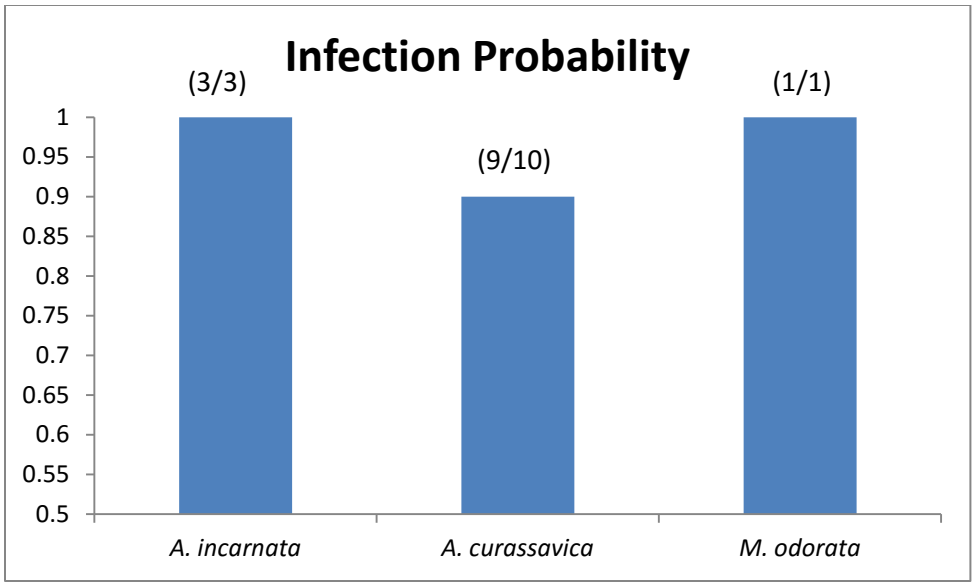

Figure 8. The infection probability of monarchs reared on different milkweed species (Fall 2015).

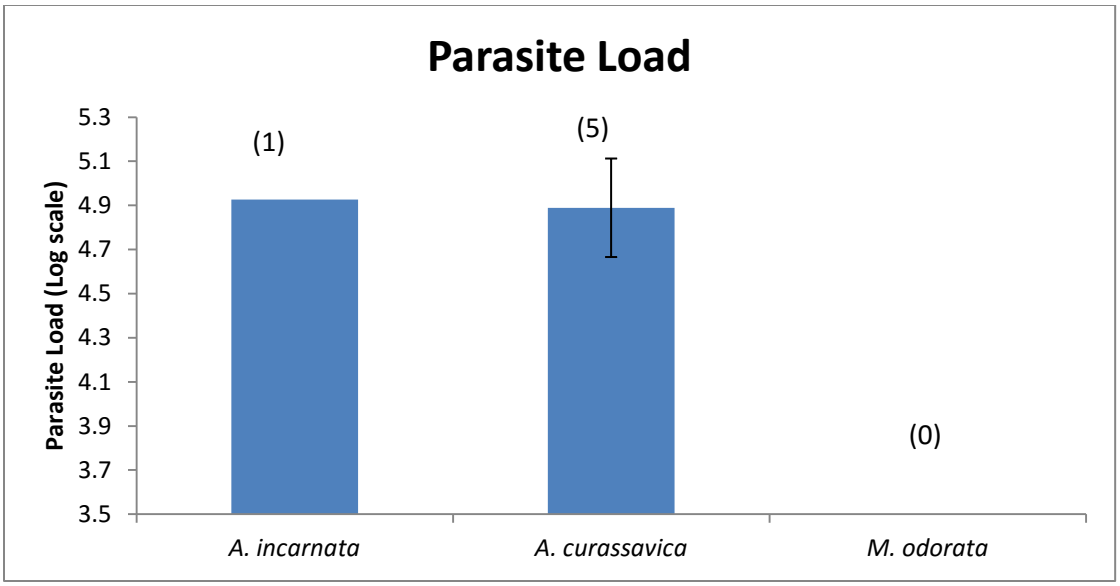

Figure 9. Parasite loads for infected monarchs (Fall 2015). Numbers above bars represent sample sizes. Error bars are ± 1 SEM. Note: As mentioned above, several infected monarchs were used as parasite sources for a lab alumnus. Two were from the *A. incarnata* group, five were from the *A. curassavica* group, and one was from the *M. odorata* group.

Chapter 2: Host diet affects the morphology of a butterfly pathogen

ABSTRACT

Understanding the effects of parasites on hosts has been a major field of study and has many implications regarding human health. Host-parasite interactions, therefore, have been studied and documented for many systems, where many infected hosts undergo changes in response to infection. While many studies focus on quantitative traits of these interactions such as changes in parasite loads due to host dietary changes, less focus is placed on qualitative traits such as the effects of host diet on parasite size and shape (parasite morphology). Parasite morphology has significant effects on parasite fitness such as initial colonization of hosts, avoidance of host immune defenses, and starter resources for parasite replication. As such, understanding the factors of host-parasite interactions on parasite morphology can help expand knowledge on the consequences of such interactions on parasite fitness. Here, we measure how host diet affects parasite morphology in the relationship between the monarch butterfly and its protozoan parasite. Previous studies have found that infected monarchs reared on milkweeds of high cardenolide content have reduced parasite loads. Adding to this benefit of high cardenolide milkweeds, we have found that infected monarchs reared on milkweeds of higher cardenolide concentrations yielded smaller parasites, a potentially hidden characteristic of these milkweeds that can possibly have serious consequences on parasite fitness.

INTRODUCTION

Parasites are one of the most diverse and common life forms on earth (Windsor 1998). As such, understanding their effects on wildlife, agriculture, and humans is especially important (Smith et al. 1995, Aramini et al. 1998, Liberti et al. 2003, King et al. 2007, Wargo et al. 2007).

Parasites depend on hosts for growth, replication, and transmission; and these three processes are deeply connected to host conditions such as the immune system and nutrition (Bundy and Golden 1987, Coop and Holmes 1996). While all parasites depend on the host for resources, parasites differ in their responses to the host immune system. For example, some parasites actively evade host immune responses by altering surface antigens, releasing signaling molecules, or by residing within host cells (Sacks and Sher 2002, Olivier et al. 2005). Other parasites, however, are only able to establish and propagate in immunocompromised hosts (Scott and Koski 2000, Keiser and Nutman 2004, Snydman et al. 2005). In order to complete lifecycles, most parasites undergo physiological changes in order to transfer between hosts, either through an intermediate host or through a dormant stage (Decaestecker et al. 2004, Roberts et al. 2009, Cox 2010). In some parasites, the stages of dormancy are called spores, which can typically survive both harsh environmental conditions and time (Roberts et al. 2009).

While extensive research has been conducted to study active parasites, less focus has been directed towards understanding the dormant (spore) stage. Spore morphology is especially important in considering the success of parasites, as it has been shown to contribute to parasite transmission (Salt 1940, Wenner and Windsor 1979, Poulin 1995, Coop and Kyriazakis 1999, Leonardos and Trilles 2003, Tsotetsi et al. 2004, Kropf et al. 2005). For example, three different spore types of the microsporidium *Octosporea bayeri*, a parasite of the water flea *Daphnia magna*, have been observed, each with different spore shapes and sizes; while the exact roles of these different spore types are unknown, they may contribute to transmission or to protection against environmental stress (Vizoso et al. 2005). More generally, similar to other organisms, larger parasite sizes typically imply higher fitness (Blueweiss et al. 1978, Moore 1981, Peters 1986). For example, the parasitic isopod *Ichthyoxenus fushanensis* consists of heterosexual pairs

that infect the freshwater fish *Varicorhinus bacbatulus*. Due to host constraints, male counterparts of the isopod parasite normally have reduced body sizes in order to allow females to grow larger and to increase clutch sizes (Tsai et al. 2001). Larger parasite sizes can also provide more resources for host colonization as well as for protection from the host immune system (Poulin 1995). For example, the cells of the pathogenic fungus *Cryptococcus neoformans*, which infect human lungs, can grow up to twenty times normal sizes, which reduces phagocytosis from host cells as well as oxidative and nitrosative damage. This morphological change in *C. neogormans* greatly increases survival and host colonization during initial stages of infection (Okagaki et al. 2010, Zaragoza et al. 2010). Aside from mass, spore shape is important in determining parasite fitness (Sander et al. 2013). Overall, variations in spore size and shape can change both transmission and specificity to hosts (Monis et al. 2003, Roper et al. 2008, Wang and Lin 2012).

Spore morphology is affected by many factors, among which host conditions are the most important (Bundy and Golden 1987, Coop and Holmes 1996). The size of *Mothocya epimerica*, an isopod parasite of the sand smelt fish, has been shown to increase with the size of its host (Leonardos and Trilles 2003). Similarly, the size of *Lamproglena clariae*, an ectoparasite that infects gills of sharptooth catfish, has also been shown to correlate with host size (Tsotetsi et al. 2004). However, the role of host diet on spore morphology remains unknown. Host diet has been shown to affect many aspects of parasite-host interactions, including immune systems, parasite virulence, and host vigor, all of which may subsequently affect parasite morphology (Poulin 1995, Coop and Holmes 1996, de Roode et al. 2008, Tao et al. 2015).

In this study, we explored how host diet affects parasite morphology using the monarch butterfly (*Danaus plexippus*) and its protozoan parasite *Ophryocystis elektroscirrha*. *O.*

elektroscirrha displays two main life stages: an actively reproducing cycle while in the monarch larvae gut and a dormant, transmissible, spore cycle on the abdomen of the monarch adult (McLaughlin and Myers 1970, Leong et al. 1992). The dormant spores are typically observed as elliptical shapes (McLaughlin and Myers 1970, Vickerman et al. 1999, Sternberg et al. 2012). Monarchs infected by *O. elektroscirrha* exhibit decreases in fitness, which can be measured by decreases in body mass, lifespan, and mating success (Altizer and Oberhauser 1999, Bradley and Altizer 2005). Typically, *O. elektroscirrha* is spread vertically from parent to offspring during oviposition, where infected monarch females scatter parasite spores from their abdomens onto eggs and plant material that the larvae subsequently consumes; however, horizontal transmission between adults can occur during mating as well as when infected monarchs spread spores onto plants (Altizer et al. 2004). These tritrophic interactions (host plant-host-parasite interactions) have been extensively studied, which suggest that host plant species have important consequences for host-parasite interactions (Cory and Hoover 2006, de Roode et al. 2008, Sternberg et al. 2012).

Specifically, monarch caterpillars feed on milkweeds (*Asclepias spp.*), which produce cardenolides, toxic steroid chemicals that milkweeds produce to decrease herbivory and that disrupt animal Na^+/K^+-ATPase (Agrawal et al. 2012). Monarchs are specialized to milkweeds and can sequester cardenolides for their defense against predators (Brower 1969). Previously, it has been shown that infected monarchs reared on milkweeds with high cardenolide concentrations exhibit both reduced parasite load as well as longer lifespans compared to infected monarchs reared on milkweeds with low cardenolide concentrations (de Roode et al. 2008, Sternberg et al. 2012). While both genetic and environmental effects on parasite morphology have been studied in the monarch-parasite system (Sander et al. 2013), the direct

effects of host diet and nutrition on parasite morphology have not. In addition to cardenolide concentrations, milkweed species also differ in nutritional (carbon, nitrogen, and phosphorous) contents (Tao et al. 2015). Therefore, we hypothesize that milkweed species have direct effects on *O. elektroscirrha* morphology through their differences in both nutritional and cardenolide profiles.

MATERIALS AND METHODS

Host and Parasite sources

Monarchs used in this experiment were lab-reared progeny of wild-caught migrating monarchs obtained in St. Marks, Florida during Oct 2013 and are representative of the eastern United States migrating monarch population. Monarchs used were from five different lab-reared lineages, and lineages were randomly distributed between the three milkweed species.

Parasite clones of *O. elektroscirrha* were obtained from infected monarchs caught previously by lab members. These clones were propagated by inoculating lab-reared monarch larvae with one parasite spore. Spores were taken from successful inoculations at the adult stage. For this experiment, one parasite clone was used to minimize differences due to parasite genetics (Sander et al. 2013).

Plant sources

Milkweeds used in this experiment were *A. verticillata*, *A. syriaca*, and *A. latifolia*, which have been shown previously to range from low to high cardenolide concentrations, respectively (Tao et al. 2015). These three milkweeds are indigenous to North America and are found as follows: *A. verticillata* and *A. syriaca* in the east and mid United States and *A. latifolia* in the mid

and west United States (Woodson 1954). Seeds were obtained from Butterfly Encounters Inc. (San Ramon, CA, USA) and were sowed on autoclaved seedling soil from Fafard (Agawam, MA, USA). When seedlings were roughly 3 cm tall, they were transferred to individual 3.6 cm pots. Plants were grown in a greenhouse where the temperature was between 24°C and 33°C and the humidity was between 30% and 60%. Plants were watered twice daily and were approximately three months old when used for experiments.

Experimental design

Milkweed chemical and nutritional (nitrogen and phosphorous) analyses were conducted prior to feeding monarch larvae. To do this, six leaf disks (each of 0.64 cm diameter) were collected from one leaf of the fourth leaf pair (counting down) on each milkweed using a paper hole puncher. Another six leaf disks were taken from the other side of the same leaf for a total of twelve leaf disks. The first six leaf disks were placed in 1 mL of methanol and stored at -20°C, and the second six were placed into a glassine envelope to estimate dry mass. The leaf was then removed, dried, and ground into powder to analyze nitrogen (N) and phosphorous (P) contents. For *A. verticillata*, which has thin leaves that are inadequate for hole punches, two whole leaves were stored in methanol for chemical analyses and two opposite leaves for dry mass estimations as well as nitrogen and phosphorous analyses. One monarch egg was randomly assigned to each milkweed plant and was fed with one leaf from the third leaf pair upon hatching to the caterpillar's second instar stage. Chemical and nutritional analyses of the larval food were conducted during this time due to previous findings that showed milkweed chemical and nutritional effects caterpillars are most significant during earlier instar stages (Zalucki et al. 2001, De Roode et al. 2011, Tao and Hunter 2012).

Monarch larvae were inoculated at the second instar stage with ten parasite spores. To do this, a leaf disk (from the third leaf of each caterpillar's assigned plant) was placed on a moist filter paper in a 10 cm Petri-dish, and the ten parasite spores were placed on the leaf disk using a drawn-out glass capillary tube. These larvae were kept in the Petri-dishes with the leaf disks until complete consumption. Larvae were then moved to assigned plants and were confined to the plant in an 18.9 liter mesh (Trimaco, Morrisville, NC, USA).

If a caterpillar finished its assigned milkweed plant before pupation, it was fed cuttings of *A. incarnata* until pupation. *A. incarnata* was chosen because it has been shown to contain very low cardenolide concentrations (Agrawal et al. 2012, Sternberg et al. 2012, Tao et al. 2015). This switch in larval food, if it occurred, was during the final days of the larval stage, and milkweed chemical and nutritional effects on larval growth at this time were most likely minimal (Zalucki et al. 2001, Tao and Hunter 2012). Previous work has also shown that milkweed chemical and nutritional effects on parasites were greatest during early stages of monarch development and infection (De Roode et al. 2011).

Pupae were allowed to harden for one day and were transferred to individual, 473 mL solo cups from Solo Cup Company (Urbana, Illinois) in a separate laboratory room. When adult monarchs emerged, they were transferred to individual, 8.9 x 8.9 cm glassine envelopes and stored in a 14ºC incubator. Sex and emergence data were recorded for each monarch, and monarchs were left unfed. Three weeks after death, dried monarchs were weighed to the nearest 0.1 mg using a Mettler Toledo microbalance (Columbus, OH, USA).

Parasite morphology analysis

Shortly after the butterflies emerged, we pressed individual sticky mailing seals (Avery Inc, Pasadena, CA, USA) against the abdomen of each butterfly firmly for 2 seconds, which removed butterfly scales and parasite spores. Then the seals were placed on white index cards (Pendaflex Inc, Melville, NY, USA) and inspected under the light microscope (Olympus BX51, Tokyo, Japan) under 400 × dimension attached with a digital camera (Olympus DP71, Tokyo, Japan). For each seal, we took five photos with an internal 10 μm scale from the Olympus DPcontroller software (Olympus Inc, Tokyo, Japan). Typically, each photo included 20~100 parasite spores; we randomly selected ten spores for morphology analysis. This resulted in 10 × 5 = 50 spores analyzed for each butterfly.

To measure morphological data of selected spores, we used Adobe Photoshop CS5 (Adobe systems, Mountain View, CA, USA) to measure the length of the long axis and the breadth of the perpendicular axis in μm. Then we calculated the area and aspect ratio (ratio between the long and perpendicular axes) for each spore.

Statistical tests

To explore if the three milkweed species differed in their foliar cardenolide, N and P concentrations, we performed one-way ANOVAs using species identity as fixed factor and each foliar traits as dependent variables. To explore if plant species affected spore morphology (long axis, short axis, area and aspect ratio), we used these traits as dependent variables, and used species identity as the fixed factor, individual butterfly as the random factor and the weight of each butterfly as a covariate in four mixed linear models. Subsequently, to test how plant traits affect spore morphology, we repeated the above analysis while replacing plant species with each foliar trait as fixed factors. Lastly, we incorporated both plant chemistry and plant species

identity as fixed factors. Butterfly weight was included as a co-variate to eliminate indirect effects of host plant on parasite morphology (Sander et al. 2013).

Mixed linear model analysis was performed using the nlme package (Pinheiro et al. 2007) in R 3.2.3 (R Development Core Team 2012). For all regression models, homogeneity of variance of dependent variables was confirmed by the Levene's test from the CAR package in R (Fox and Weisberg 2010), and normality of errors was confirmed by the Shapiro–Wilk normality test.

RESULTS

The three milkweed species differed significantly in their cardenolide concentrations (Fig. 1a; $F_{2, 31} = 15.60$, $p < 0.001$) and N concentrations (Fig. 1b; $F_{2, 37} = 10.50$, $p < 0.001$). Specifically, cardenolide concentrations were highest in *A. latifolia* (2.77 ± 0.49 mg/g), followed by *A. syriaca* (0.66 ± 0.31 mg/g) and *A. verticillata* (0.08 ± 0.04 mg/g). On the other hand, *A. vercillata* had the highest N concentration (2.88 ± 0.16%), followed by *A. latifolia* (2.33 ± 0.08%) and *A. syriaca* (1.97 ± 0.10%). However, they did not differ significantly in their P concentration (Fig. 1c; $F_{2, 23} = 1.58$, $p = 0.23$).

To account for potential effects of host diet on spore morphology simply from changes in monarch sizes, we incorporate monarch weight as a co-variate in all subsequent analyses (Tables 1-3). Host plant species significantly affected the size of parasite spores by affecting the length of the long axes, but not the short axes (Fig. 2a-c; $F_{2, 37} = 3.97$, $p = 0.03$; $F_{2, 37} = 1.59$, $p = 0.22$, respectively). As a result, plant species marginally affected the spore area ($F_{2, 37} = 2.65$, $p = 0.08$). However, they did not affect the shape of the spores (Fig. 2d; $F_{2, 37} = 2.23$, $p = 0.12$).

Foliar cardenolide concentrations, but not N or P concentrations had significant effects on the length of short axis and area (Fig. 3; effects of cardenolides: long axis: $F_{1, 32} = 3.62$, $p = 0.07$; short axis: $F_{1, 32} = 3.59$, $p = 0.04$; area: $F_{1, 32} = 4.47$, $p = 0.04$; effects of N: long axis: $F_{1, 38} = 0.27$, $p = 0.61$; short axis: $F_{1, 38} = 0.04$, $p = 0.85$; area: $F_{1, 38} = 0.02$, $p = 0.88$; effects of P: long axis: $F_{1, 24} = 0.03$, $p = 0.87$; short axis: $F_{1, 24} = 0.57$, $p = 0.29$; area: $F_{1, 24} = 0.85$, $p = 0.33$). Additionally, cardenolide concentration had a marginal effect on long axis ($F_{1, 32} = 3.62$, $p = 0.07$). In general, higher foliar cardenolide concentration led to reduced spore sizes. None of the three traits affected spore shape (Fig. 3; $F_{1, 32} = 0.24$, $p = 0.63$; $F_{1, 38} = 0.87$, $p = 0.36$; $F_{1, 24} = 0.33$, $p = 0.63$).

Subsequently, we included both foliar chemical traits and plant species identity in above models. After incorporating foliar cardenolide concentration, plant species no longer had any significant effects on spore long axis or spore area (Table 1). By contrast, incorporating foliar N or P concentration did not remove the significant effect of plant species (Table 2, 3). This again suggests that effects of plant species on spore morphology were mainly driven by foliar cardenolides.

DISCUSSION

As we have shown, host diet can have significant effects on parasite morphology. This is important, as parasite morphology can affect its replication and transmission. Here, we found that cardenolide concentrations in monarch host plants have significant negative effects on its protozoan parasite's size, reducing overall parasite sizes with increases in cardenolide concentrations. Plant nutritional concentrations (nitrogen and phosphorous), however, do not significantly affect parasites. To our knowledge, these results are one of the first to show that host diet has significant effects on parasite morphology, which may translate into parasite

virulence and transmission (Salt 1940, Poulin 1995). The effects of host diets on parasites can be broadened to human applications. For example, some spices and vegetables in human diets have been shown to possess antimicrobial, antiviral, and anti-parasitic qualities as well as anti-cancer chemicals (Ohigashi et al. 1992, Murakami et al. 1994, Murakami et al. 1996, Billing and Sherman 1998, Sherman and Billing 1999), although we do not know how they affect parasite morphology and subsequently, their pathogenicity and transmission (Okagaki et al. 2010, Zaragoza et al. 2010).

Generally, understanding factors involved in spore morphology is essential to understanding parasite growth, transmission, and fitness. Parasites that undergo dormant stages typically incur some benefits as a spore, whether it is increased environmental tolerances or prolonged infectivity (Gest and Mandelstam 1987, Kennedy et al. 1994, Potts 1994, Nicholson et al. 2000, Roberts et al. 2009). For example, infective spores of microparasites to *Daphnia magna* in different pond sediment depths can remain infective for many years (Decaestecker et al. 2004). Similarly, bacteria *Bacillus spp.*, which are abundant in soil, form spores in times of nutritional deficits. These spores are resistant to extreme environmental stresses such as heat and cold, protecting the bacteria until favorable conditions arise (Nicholson et al. 2000, Nicholson 2002, Driks 2004). Dormant spores are normally also able to withstand long periods of dryness and UV damage . For example, blastospores of *Paecilomyces fumosoroseus* were shown to withstand months of desiccation while retaining infectivity and virulence (Jackson et al. 1997).

In addition to qualitative traits mentioned above that increase dormant parasites' tolerance to stressors, spores are key to the fitness of some parasites through morphological traits such as spore size and shape. Larger spores imply greater resources for parasite replication and establishment into hosts, typically by evading host immune defenses (Blueweiss et al. 1978,

Sacks and Sher 2002, Olivier et al. 2005). Larger parasite sizes have been correlated with larger host sizes (Bundy and Golden 1987, Coop and Holmes 1996, Leonardos and Trilles 2003, Tsotetsi et al. 2004). Here, we did not find significant effects of monarch butterfly host mass with *O. elektroscirrha* spore size. Previously, the sizes of *O. elektroscirrha* have been shown to positively correlate with monarch size, although the effects are quite weak (Altizer and Davis 2010, Sander et al. 2013). This is contrary to our findings, where we did not detect any significant effects of host sizes on parasite sizes. The differences in results can be explained simply and can further show how host diet affects parasite morphology. In the study by Sander *et al.*, monarch larvae were reared on a single milkweed species, specifically, *A. incarnata*, which has been shown to have low cardenolide concentrations (Agrawal et al. 2012, Sternberg et al. 2012, Tao et al. 2015). Our study, which uses three milkweed species with varying cardenolide concentrations, shows that effects of cardenolide concentrations on parasite size are more important than host size, and that cardenolides most likely play a larger role in monarch-parasite interactions.

To our knowledge, our study is one of the first to show how host diet affects parasite spore morphology. Previously, studies have shown that infected monarchs reared on milkweeds of high cardenolide concentrations reduced parasite load as well as increased tolerance to parasites (longer lifespans under the same parasite load) (de Roode et al. 2008, Lefèvre et al. 2010, Sternberg et al. 2012). This reduction may be due to boosts in the immune system, which has been seen in other systems (Lee et al. 2008, Povey et al. 2009, Simpson et al. 2015), or direct interference with the parasites (Cory and Hoover 2006). Here we pointed out another hidden effect of cardenolides on *O. elektroscirrha* through reducing their sizes, which may render them a lower probability to successfully infect new hosts and a disadvantage in competitiveness

against other larger parasites. Future studies can be performed to test these predictions by comparing monarch-parasite interactions with parasites of reduced size and normal size. Such reduction in sizes may also help explain increases in butterfly tolerance when feeding on plants with high cardenolides: since the parasites are smaller, their per capita damage is smaller. Combining previous studies, our results suggest that cardenolides can confer monarchs with many advantages through reducing parasite loads, increasing butterfly tolerance, and reducing parasite sizes.

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TABLES

Table 1. Results (F and p values) of linear mixed models testing effects of foliar cardenolide concentration, plant species and monarch weight on spore morphology.

Spore morphology	Independent variables	F and p values
Long axis	Cardenolides	$F_{1,30} = 3.82$; $p = 0.06^{+}$
	Species	$F_{2,30} = 1.85$; $p = 0.18$
	Weight	$F_{1,30} = 0.12$; $p = 0.73$
Short axis	Cardenolides	$F_{1,30} = 4.42$; $p = 0.04^{*}$
	Species	$F_{2,30} = 0.44$; $p = 0.65$
	Weight	$F_{1,30} = 0.22$; $p = 0.64$
Spore Area	Cardenolides	$F_{1,30} = 4.48$; $p = 0.04^{*}$
	Species	$F_{2,30} = 1.08$; $p = 0.35$
	Weight	$F_{1,30} = 0.17$; $p = 0.68$
Aspect Ratio	Cardenolides	$F_{1,30} = 0.23$; $p = 0.63$
	Species	$F_{2,30} = 0.63$; $p = 0.54$
	Weight	$F_{1,30} = 0.03$; $p = 0.86$

Note: +, $p < 0.1$; *, $p < 0.05$

Table 2. Results (F and p values) of linear mixed models testing effects of foliar N concentration, plant species and monarch weight on spore morphology.

Spore morphology	Independent variables	F and p values
Long axis	Nitrogen	$F_{1,36} = 0.31$; $p = 0.58$
	Species	$F_{2,36} = 3.92$; $p = 0.03$*
	Weight	$F_{1,36} = 0.65$; $p = 0.42$
Short axis	Nitrogen	$F_{1,36} = 0.04$; $p = 0.85$
	Species	$F_{2,36} = 2.11$; $p = 0.14$
	Weight	$F_{1,36} = 1.72$; $p = 0.20$
Spore Area	Nitrogen	$F_{1,36} = 0.03$; $p = 0.88$
	Species	$F_{2,36} = 3.00$; $p = 0.06^{+}$
	Weight	$F_{1,36} = 1.26$; $p = 0.27$
Aspect Ratio	Nitrogen	$F_{1,36} = 0.91$; $p = 0.35$
	Species	$F_{2,36} = 1.94$; $p = 0.16$
	Weight	$F_{1,36} = 0.56$; $p = 0.46$

Note: +, $p < 0.1$; *, $p < 0.05$

Table 3. Results (F and p values) of linear mixed models testing effects of foliar P concentration, plant species and monarch weight on spore morphology.

Spore morphology	Independent variables	F and p values
Long axis	Phosphorous	$F_{1,22} = 0.31$; $p = 0.86$
	Species	$F_{2,22} = 3.74$; $p = 0.04$*
	Weight	$F_{1,22} = 2.12$; $p = 0.16$
Short axis	Phosphorous	$F_{1,22} = 0.34$; $p = 0.56$
	Species	$F_{2,22} = 1.54$; $p = 0.24$
	Weight	$F_{1,22} = 0.73$; $p = 0.40$
Spore Area	Phosphorous	$F_{1,22} = 0.04$; $p = 0.84$
	Species	$F_{2,22} = 2.56$; $p = 0.10^{+}$
	Weight	$F_{1,22} = 1.44$; $p = 0.24$
Aspect Ratio	Phosphorous	$F_{1,22} = 1.00$; $p = 0.33$
	Species	$F_{2,22} = 1.43$; $p = 0.26$
	Weight	$F_{1,22} = 0.28$; $p = 0.60$

Note: +, $p < 0.1$; *, $p < 0.05$

FIGURES

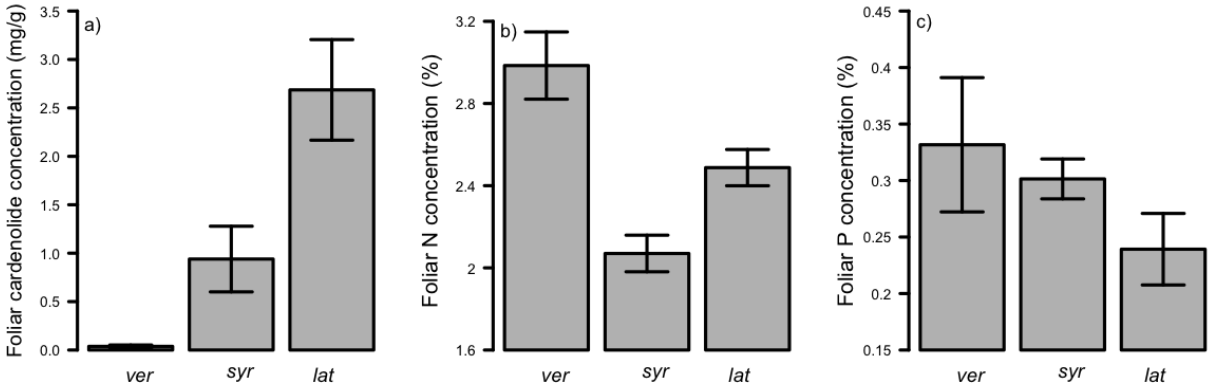

Figure 1. Foliar cardenolide (a), nitrogen (b), and phosphorous (c) concentrations in *Asclepias verticillata* (*ver*), *A. syriaca* (*syr*) and *A. latifolia* (*lat*). Each bar represents the mean value ± 1 SEM.

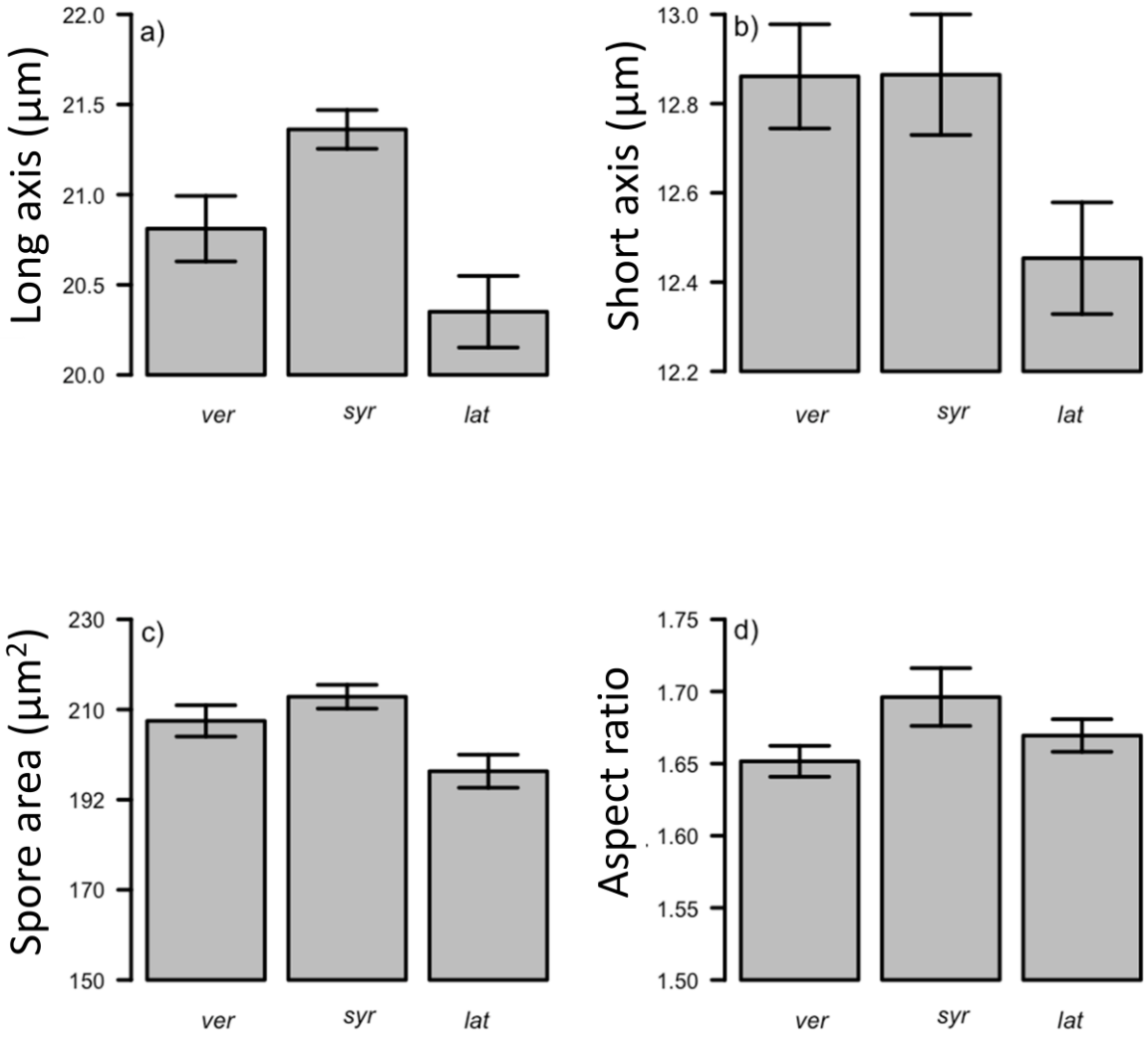

Figure 2. The effects of *A. verticillata*, *A. syriaca*, and *A. latifolia* on parasite long axis (a), short axis (b), area (c), and aspect ratio (d). Each bar represents the mean value ± 1 SEM.

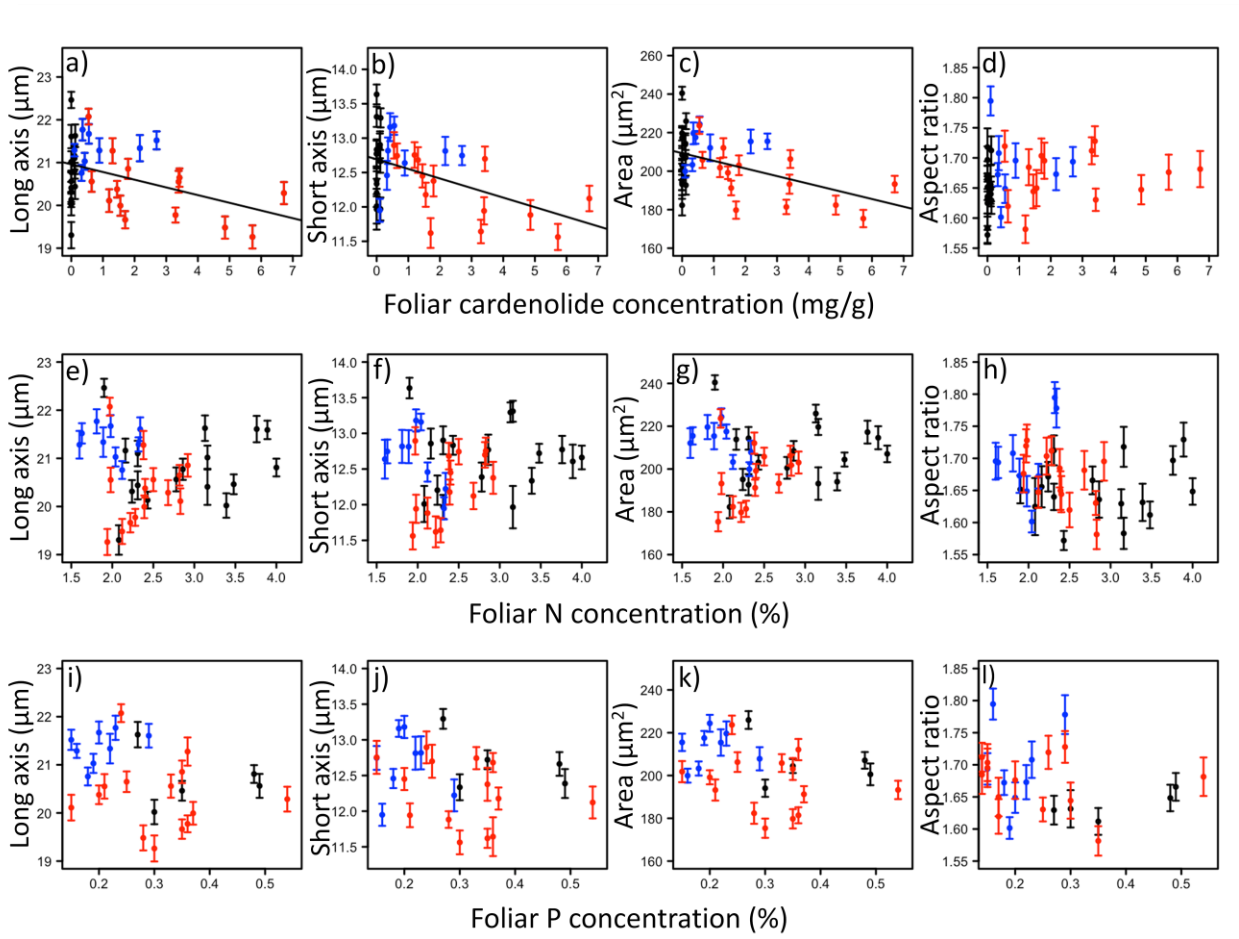

Figure 3. The effects of foliar cardenolide (a-d), nitrogen (e-h), and phosphorous (i-l) concentrations on parasite long axis (a, e, i), short axis (b, f, j), area (c, g, k), and aspect ratio (d, h, l). Individual data points are color-coded as follows: *A. latifolia*, red; *A. syriaca*, blue; and *A. verticillata*, black. Each point represents the mean value ± 1 SEM from fifty spores on individual butterflies.