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## HOST DIET AFFECTS THE MORPHOLOGY OF MONARCH BUTTERFLY PARASITES

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**ABSTRACT:** Understanding host–parasite interactions is essential for ecological research, wildlife conservation, and health management. While most studies focus on numerical traits of parasite groups, such as changes in parasite load, less focus is placed on the traits of individual parasites such as 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 the availability of resources for parasite replication. As such, understanding factors that affect parasite morphology is important in predicting the consequences of host–parasite interactions. Here, we studied how host diet affected the spore morphology of a protozoan parasite (*Ophryocystis elektroscirrha*), a specialist parasite of the monarch butterfly (*Danaus plexippus*). We found that different host plant species (milkweeds; *Asclepias* spp.) significantly affected parasite spore size. Previous studies have found that cardenolides, secondary chemicals in host plants of monarchs, can reduce parasite loads and increase the lifespan of infected butterflies. Adding to this benefit of high cardenolide milkweeds, we found that infected monarchs reared on milkweeds of higher cardenolide concentrations yielded smaller parasites, a potentially hidden characteristic of cardenolides that may have important implications for monarch–parasite interactions.

Parasites are one of the most diverse and common life forms on earth (Price, 1980; Thompson, 1994; Windsor, 1998; Combes, 2001; Poulin and Morand, 2004; Dobson et al., 2008). 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 3 processes are deeply connected to host traits such as the immune system and nutrition (Bundy and Golden, 1987; Coop and Holmes, 1996). To complete their lifecycles, some parasites undergo physiological changes to enable transfer between hosts, either through an intermediate host or through a dormant stage (Decaestecker et al., 2004; Roberts and Janovy, 2008; Cox, 2010). In some parasites, the stages of dormancy are called spores, which can typically survive both harsh environmental conditions and time (Roberts and Janovy, 2008).

While extensive research has been conducted to study active parasites, less focus has been directed toward understanding the dormant (spore) stage. Spore morphology is especially important in considering the success of parasites (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, 3 different spore types of the microsporidian *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, and similar to free-living organisms, larger parasite size typically implies 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 barbatulus*. Due to host constraints, males of this

isopod normally have reduced body size, which allows females to grow larger and to increase clutch size (Tsai et al., 2001). Aside from mass, spore shape is also important in determining parasite fitness (Sander et al., 2013). Overall, variation in spore size and shape is related to 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, including host condition (Bundy and Golden, 1987; Coop and Holmes, 1996). The size of *Mothocya epimerica*, an isopod parasite of the sand smelt fish, increases with the size of its host (Leonardos and Trilles, 2003). Similarly, the size of *Lamproglana clariae*, an ectoparasite that infects gills of sharpnose catfish, also correlates with host size (Tsotetsi et al., 2004). While host diet has been shown to affect many aspects of parasite–host interactions, including immunity, parasite virulence, and host vigor (Poulin, 1995; Coop and Holmes, 1996; de Roode et al., 2008a; Tao et al., 2015), its effects on parasite morphology remain poorly known.

In this study, we explored how host diet affects parasite morphology in the monarch butterfly (*Danaus plexippus*)–protozoan parasite (*Ophryocystis elektroscirrha*) system. *Ophryocystis elektroscirrha* is a neogregarine protozoan parasite specialized on monarch butterflies (Barriga et al., 2016) and was first described in 1970 (McLaughlin and Myers, 1970). Neogregarines are members of the phylum Apicomplexa, a group of obligate unicellular parasites which include the widely-studied parasites *Cryptosporidium* spp., *Toxoplasma* spp., and *Plasmodium* spp. *Ophryocystis elektroscirrha* undergoes 2 main life stages: an actively reproducing cycle within the monarch's tissues and a dormant, transmissible spore stage on the exterior of the monarch adult (McLaughlin and Myers, 1970; Leong et al., 1992). These external spores are dormant and are typically observed as elliptical shapes on the butterfly scales (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 as reduced body mass, lifespan, mating success, and flight ability (Altizer and Oberhauser, 1999; Bradley and Altizer, 2005; de Roode et al., 2007, 2008b; Altizer and de Roode, 2015). Typically, *O. elektroscirrha* is spread vertically from parent to offspring during oviposition, where infected monarch females

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scatter parasite spores from their abdomens onto eggs and plant material, which larvae subsequently consume. Horizontal transmission can also occur when infected monarchs spread spores onto plants and when males spread spores to females during mating, following which they can be consumed by larvae (Altizer et al., 2004; de Roode et al., 2009).

The tritrophic interaction between host plant (milkweeds; *Asclepias* spp.), monarch, and protozoan parasites has been extensively studied. Milkweeds produce cardenolides, toxic steroid chemicals that disrupt animal  $\text{Na}^+/\text{K}^+$ -ATPase and protect milkweeds from herbivory (Agrawal et al., 2012). In addition to varying in cardenolide concentrations, milkweed species also differ in nutritional (carbon, nitrogen [N], and phosphorous [P]) content (Tao et al., 2015). Monarchs are specialized on milkweeds and can sequester cardenolides for their own defense against predators (Brower, 1969). Previously, it has been shown that infected monarchs reared on milkweeds with high cardenolide concentrations exhibit both a reduced parasite load and longer lifespan compared to infected monarchs reared on milkweeds with low cardenolide concentrations (de Roode et al., 2008a; Sternberg et al., 2012; Gowler et al., 2015; Tao et al., 2015). Moreover, infected female butterflies prefer to lay eggs on high-cardenolide milkweed in 2-species choice tests, thereby reducing infection and disease in their offspring (Lefèvre et al., 2010, 2012). These previous studies have shown reductions in parasite number and increases in monarch lifespan, with increasing cardenolide concentrations, but have not investigated effects of cardenolides on spore morphology. In a previous study, Sander et al. (2013) found that parasite genetics significantly affected parasite spore size. This study also found that parasite size and color were positively correlated with monarch wing size and color, demonstrating that both parasite and host traits play a vital role in parasite morphology. Here, we followed up these previous studies to determine whether milkweed species affect *O. elektroscirra* morphology due to variation in nutritional and cardenolide profiles.

## MATERIALS AND METHODS

### Host and parasite sources

Monarchs used in this experiment were lab-reared progeny of wild-caught monarchs obtained in St. Marks, Florida during October 2013. Monarchs used were from 5 different lab-reared lineages, and monarchs were randomly distributed among 3 milkweed species.

For this experiment, a single parasite clone of *O. elektroscirra* was used to minimize any morphological differences due to parasite genetics (Sander et al., 2013). The parasite clone was obtained from a wild-caught infected monarch butterfly and propagated by inoculating a lab-reared monarch larva with a single parasite spore. Spores were taken from adults after successful inoculations of lab-reared monarchs.

### Plant sources

Milkweeds used in this experiment were *Asclepias verticillata*, *Asclepias syriaca*, and *Asclepias latifolia*, which have been shown previously to range from low to high cardenolide concentrations, respectively (Tao et al., 2015). These 3 milkweeds are native to North America and are found as follows: *A. verticillata* and *A.*

*syriaca* in the eastern and mid-United States and *A. latifolia* in the western and mid-United States (Woodson, 1954). Seeds were obtained from Butterfly Encounters Inc. (San Ramon, California) and were sown on autoclaved seedling soil from Fafard (Agawam, Massachusetts). When seedlings were roughly 3 cm tall, they were transferred to individual, 10.16-cm diameter pots. Plants were grown in a greenhouse where the temperature ranged between 24 and 33 °C and the humidity was between 30 and 60%. Controlled environments were necessary for this experiment, as drought stress has been shown to alter milkweed latex production and cardenolide concentrations (Agrawal et al., 2014). Plants were watered twice daily and were approximately 3 mo old when used for experiments.

### Experimental design

Milkweed chemical and nutritional (N and P) analyses were conducted prior to feeding monarch larvae. Specifically, 6 leaf disks (each of 0.64 cm diameter) were collected from 1 leaf of the fourth leaf pair (counting down) on each milkweed using a paper hole puncher. Another 6 leaf disks were taken from the other side of the same leaf for a total of 12 leaf disks. The first 6 leaf disks were placed in 1 ml of methanol and stored at -20 °C for subsequent cardenolide analyses and the second 6 were placed into a glassine envelope to estimate sample dry mass. The leaf was then removed, dried, and ground into powder to analyze N and P contents. Analyses of foliar cardenolide, N, and P concentrations followed the methods described in Tao et al. (2015). For *A. verticillata*, which has narrow leaves that prevent effective hole-punching, 2 whole leaves were stored in methanol for cardenolide analyses and 2 opposite leaves for dry mass estimations as well as for N and P analyses. One monarch egg was randomly assigned to each milkweed plant and neonates were fed from hatching to the second instar stage with 1 leaf from the third leaf pair. Chemical and nutritional analyses of the larval food were conducted just prior to larval hatching because previous findings have established that milkweed chemical and nutritional effects on caterpillars are most significant during the earliest instars (Zalucki et al., 2001; de Roode et al., 2011a; Tao and Hunter, 2012).

On reaching the second instar, monarch larvae were inoculated with 10 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 diameter Petri dish and the 10 parasite spores were placed on the leaf disk using a drawn-out glass capillary tube. Larvae were kept in the Petri dishes until they had completely consumed their leaf disks, and therefore all 10 spores. Larvae were then moved to their assigned plants and confined on them within 18.9-L mesh strainers (Trimaco, Morrisville, North Carolina).

If a caterpillar finished ingesting its assigned milkweed plant before pupation, it was fed cuttings of *A. incarnata* until pupation. *Asclepias incarnata* was chosen because it contains 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 last larval stage, where milkweed chemical and nutritional effects on larval growth have been shown to be minimal (Zalucki et al., 2001; Tao and Hunter, 2012). Previous work has also shown that milkweed chemical and nutritional effects on parasites are greatest during early stages of monarch development and infection (de Roode et al., 2011a).

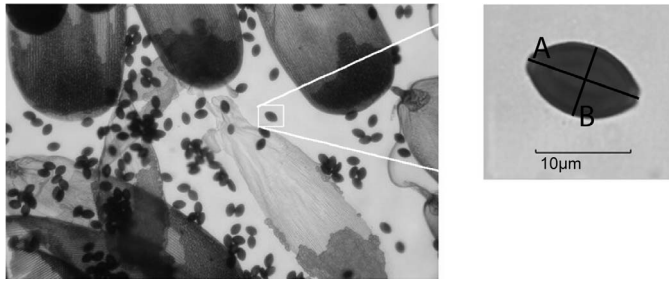


FIGURE 1. A microscope image of abdominal butterfly scales and parasite spores. *Ophryocystis elektroscirrha* spore length (A) and width (B) are indicated in the enlarged image of an individual spore. Images kindly provided by Andrew K. Davis.

Pupae were allowed to harden for a day and were then 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  $8.9 \times 8.9$ -cm glassine envelopes and stored in a 14 C incubator. Sex and emergence date 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, Ohio).

### Parasite morphology analysis

We used a total of 41 butterflies in our analyses (*A. verticillata*, 17; *A. syriaca*, 9; *A. latifolia*, 15). Shortly after the butterflies emerged, we pressed individual sticky mailing seals (Avery Inc., Pasadena, California) against the abdomen of each butterfly firmly for 2 sec, which removed butterfly scales and parasite spores. The seals were then placed on white index cards (Pendaflex Inc., Melville, New York) and inspected under a light microscope (Olympus BX51, Tokyo, Japan) under  $\times 400$  magnification with a digital camera (Olympus DP71, Tokyo, Japan). For each seal, we took 5 photos with an internal, 10- $\mu$ m scale from the Olympus DPcontroller software (Olympus Inc., Tokyo, Japan). Typically, each photo included 20–100 parasite spores; we randomly selected 10 spores for morphology analysis. This resulted in  $10 \times 5 = 50$  spores analyzed for each butterfly.

To collect morphological data of selected spores, we used Adobe Photoshop CS5 (Adobe Systems, Mountain View, California) to measure spore length and width in micrometers (Fig. 1). Then we calculated the area and aspect ratio (ratio between spore length and width) for each spore. Unlike the study by Sander et al. (2013), we used calculations for spore areas because the contrast between parasite spores and the paper backgrounds used made it difficult to accurately outline spores for area measurements. However, because *O. elektroscirrha* spores are typically lemon-shaped (McLaughlin and Myers, 1970), we determined that these calculations are representative of the true spore areas.

### Statistical tests

To determine whether the 3 milkweed species differed in their foliar cardenolide, N, and P concentrations, we performed a one-way analysis of variance using species identity as a fixed factor and each foliar trait as a dependent variable. To explore if plant species affected spore morphology (spore length, width, area, and

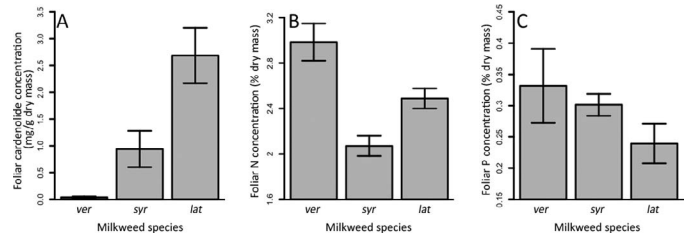


FIGURE 2. Foliar cardenolide (A), nitrogen (B), and phosphorous (C) concentrations in *Asclepias verticillata* (ver;  $n = 17$ ), *Asclepias syriaca* (syr;  $n = 9$ ) and *Asclepias latifolia* (lat;  $n = 15$ ). Each bar represents the mean value  $\pm 1$  SEM.

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 mass of each butterfly as a covariate in 4 separate linear mixed-effects 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 to assess whether significant effects of plant species on spore morphology could be explained entirely by plant chemistry. Butterfly mass was included as a co-variate to eliminate indirect effects of host plant on parasite morphology (Sander et al., 2013).

Linear mixed-effects 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 3 milkweed species differed significantly in their foliar cardenolide concentrations (Fig. 2A;  $F_{2,31} = 15.60$ ,  $P < 0.001$ ) and foliar N concentrations (Fig. 2B;  $F_{2,37} = 10.50$ ,  $P < 0.001$ ). Specifically, cardenolide concentrations were highest in *A. latifolia* ( $2.77 \pm 0.49$  mg/g) followed by *A. syriaca* ( $0.66 \pm 0.31$  mg/g) and *A. verticillata* ( $0.08 \pm 0.04$  mg/g). On the other hand, *A. verticillata* had the highest N concentration ( $2.88 \pm 0.16\%$ ) followed by *A. latifolia* ( $2.33 \pm 0.08\%$ ) and *A. syriaca* ( $1.97 \pm 0.10\%$ ). Milkweed species did not differ significantly in their foliar P concentrations (Fig. 2C;  $F_{2,23} = 1.58$ ,  $P = 0.23$ ).

Spore morphology was unrelated to the mass of monarch butterflies (spore length:  $F_{1,39} = 0.94$ ,  $P = 0.34$ ; spore width:  $F_{1,39} = 2.76$ ,  $P = 0.10$ ; area:  $F_{1,39} = 1.95$ ,  $P = 0.17$ ; aspect ratio:  $F_{1,39} = 0.89$ ,  $P = 0.35$ ). However, to ensure that any observed effects of milkweeds on spore morphology did not stem from milkweed effects on monarch size, we incorporated monarch mass as a covariate in all subsequent analyses (Tables I–III). Host plant species significantly affected the size of parasite spores by affecting spore length but not spore width (Fig. 3A–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 ( $F_{2,37} = 2.65$ ,  $P = 0.08$ ) but did not affect the shape (aspect ratio) of the spores (Fig. 3D;  $F_{2,37} = 2.23$ ,  $P = 0.12$ ).

Foliar cardenolide concentrations, but not N or P concentrations, were associated significantly with spore width and spore

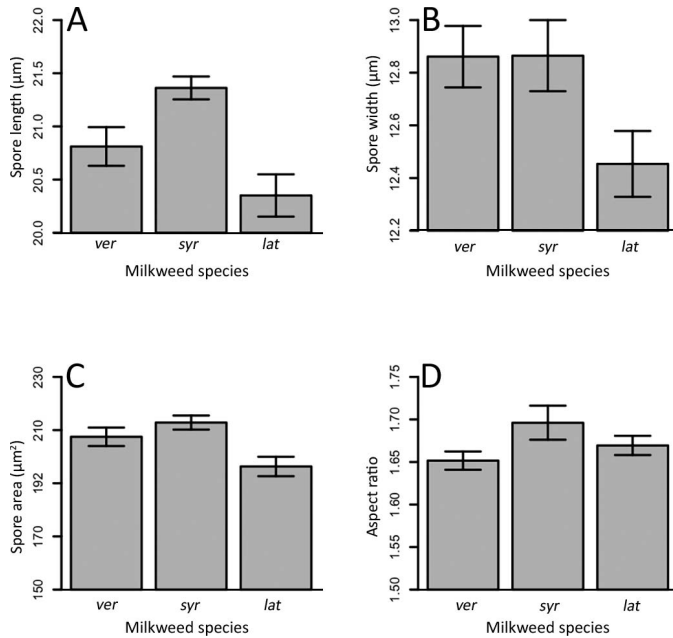


FIGURE 3. The effects of *Asclepias verticillata* ( $n = 17$ ), *Asclepias syriaca* ( $n = 9$ ), and *Asclepias latifolia* ( $n = 15$ ) on parasite spore length (A), spore width (B), area (C), and aspect ratio (D). Each bar represents the mean value  $\pm 1$  SEM.

area (Fig. 4; effects of cardenolides: spore width:  $F_{1,32} = 3.59$ ,  $P = 0.04$ ; area:  $F_{1,32} = 4.47$ ,  $P = 0.04$ ; effects of N: spore length:  $F_{1,38} = 0.27$ ,  $P = 0.61$ ; spore width:  $F_{1,38} = 0.04$ ,  $P = 0.85$ ; area:  $F_{1,38} = 0.02$ ,  $P = 0.88$ ; effects of P: spore length:  $F_{1,24} = 0.03$ ,  $P = 0.87$ ; spore width:  $F_{1,24} = 0.57$ ,  $P = 0.29$ ; area:  $F_{1,24} = 0.85$ ,  $P = 0.33$ ). Additionally, cardenolide concentration was associated marginally with spore length ( $F_{1,32} = 3.62$ ,  $P = 0.07$ ). In general, higher foliar cardenolide concentrations were associated with reduced spore sizes. None of the 3 traits affected spore shape (aspect ratio) (Fig. 4;  $F_{1,32} = 0.24$ ,  $P = 0.63$ ;  $F_{1,38} = 0.87$ ,  $P = 0.36$ ;  $F_{1,24} = 0.33$ ,  $P = 0.63$ ).

TABLE I. Results ( $F$  and  $P$  values) of linear mixed models testing effects of foliar cardenolide concentration (mg/g dry mass), milkweed species, and monarch mass on spore morphology.

| Spore morphology | Independent variables | $F$ values        | $P$ values |
|------------------|-----------------------|-------------------|------------|
| Spore length     | Cardenolides          | $F_{1,30} = 3.82$ | 0.06*      |
|                  | Milkweed species      | $F_{2,30} = 1.85$ | 0.18       |
|                  | Monarch mass          | $F_{1,30} = 0.12$ | 0.73       |
| Spore width      | Cardenolides          | $F_{1,30} = 4.42$ | 0.04†      |
|                  | Milkweed species      | $F_{2,30} = 0.44$ | 0.65       |
|                  | Monarch mass          | $F_{1,30} = 0.22$ | 0.64       |
| Spore area       | Cardenolides          | $F_{1,30} = 4.48$ | 0.04†      |
|                  | Milkweed species      | $F_{2,30} = 1.08$ | 0.35       |
|                  | Monarch mass          | $F_{1,30} = 0.17$ | 0.68       |
| Aspect ratio     | Cardenolides          | $F_{1,30} = 0.23$ | 0.63       |
|                  | Milkweed species      | $F_{2,30} = 0.63$ | 0.54       |
|                  | Monarch mass          | $F_{1,30} = 0.03$ | 0.86       |

\*  $P < 0.1$ .

†  $P < 0.05$ .

TABLE II. Results ( $F$  and  $P$  values) of linear mixed models testing effects of foliar N concentration (% dry mass), milkweed species, and monarch mass on spore morphology.

| Spore morphology | Independent variables | $F$ values        | $P$ values |
|------------------|-----------------------|-------------------|------------|
| Spore length     | Nitrogen              | $F_{1,36} = 0.31$ | 0.58       |
|                  | Milkweed species      | $F_{2,36} = 3.92$ | 0.03*      |
|                  | Monarch mass          | $F_{1,36} = 0.65$ | 0.42       |
| Spore width      | Nitrogen              | $F_{1,36} = 0.04$ | 0.85       |
|                  | Milkweed species      | $F_{2,36} = 2.11$ | 0.14       |
|                  | Monarch mass          | $F_{1,36} = 1.72$ | 0.20       |
| Spore area       | Nitrogen              | $F_{1,36} = 0.03$ | 0.88       |
|                  | Milkweed species      | $F_{2,36} = 3.00$ | 0.06†      |
|                  | Monarch mass          | $F_{1,36} = 1.26$ | 0.27       |
| Aspect ratio     | Nitrogen              | $F_{1,36} = 0.91$ | 0.35       |
|                  | Milkweed species      | $F_{2,36} = 1.94$ | 0.16       |
|                  | Monarch mass          | $F_{1,36} = 0.56$ | 0.46       |

\*  $P < 0.05$ .

†  $P < 0.1$ .

After incorporating foliar cardenolide concentration into the statistical models that tested for effects of plant species, plant species no longer had significant effects on spore length or spore area (Table I). By contrast, incorporating foliar N or P concentrations did not remove the significant effect of plant species (Tables II, III). This suggests that the observed effects of plant species on spore morphology were driven mainly by foliar cardenolides.

## DISCUSSION

As we have shown, host diet can have significant effects on parasite morphology. We found that the spore size of the protozoan *O. elektroscirrha* varied with experimental manipulation of the plant species upon which the host monarchs were feeding. The foliar cardenolide concentrations of those host plants were associated negatively with parasite spore size. In contrast,

TABLE III. Results ( $F$  and  $P$  values) of linear mixed models testing effects of foliar P concentration (% dry mass), milkweed species, and monarch mass on spore morphology.

| Spore morphology | Independent variables | $F$ values        | $P$ values |
|------------------|-----------------------|-------------------|------------|
| Spore length     | Phosphorous           | $F_{1,22} = 0.31$ | 0.86       |
|                  | Milkweed species      | $F_{2,22} = 3.74$ | 0.04*      |
|                  | Monarch mass          | $F_{1,22} = 2.12$ | 0.16       |
| Spore width      | Phosphorous           | $F_{1,22} = 0.34$ | 0.56       |
|                  | Milkweed species      | $F_{2,22} = 1.54$ | 0.24       |
|                  | Monarch mass          | $F_{1,22} = 0.73$ | 0.40       |
| Spore area       | Phosphorous           | $F_{1,22} = 0.04$ | 0.84       |
|                  | Milkweed species      | $F_{2,22} = 2.56$ | 0.10†      |
|                  | Monarch mass          | $F_{1,22} = 1.44$ | 0.24       |
| Aspect ratio     | Phosphorous           | $F_{1,22} = 1.00$ | 0.33       |
|                  | Milkweed species      | $F_{2,22} = 1.43$ | 0.26       |
|                  | Monarch mass          | $F_{1,22} = 0.28$ | 0.60       |

\*  $P < 0.05$ .

†  $P < 0.1$ .

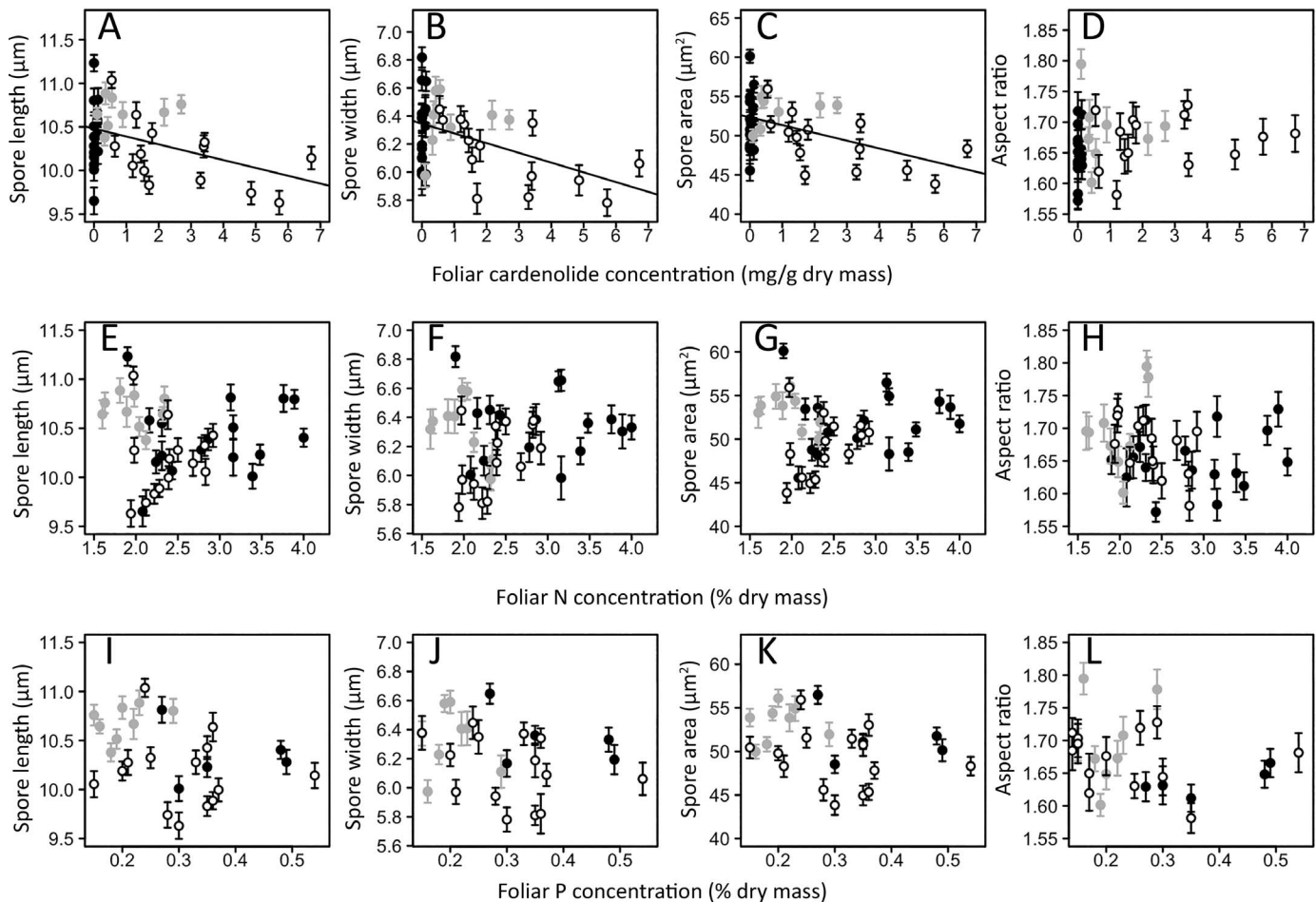


FIGURE 4. Associations among foliar cardenolide (A–D), nitrogen (E–H), and phosphorous (I–L) concentrations and parasite spore length (A, E, I), spore width (B, F, J), area (C, G, K), and aspect ratio (D, H, L). Individual data points are coded as follows: *Asclepias verticillata*, black ( $n = 17$ ); *Asclepias syriaca*, gray ( $n = 9$ ); and *Asclepias latifolia*, white ( $n = 15$ ). Each point represents the mean value  $\pm 1$  SEM from 50 spores on individual butterflies.

foliar concentrations of N and P were unrelated to parasite morphology. These observed effects of host diet on parasite morphology may affect parasite virulence and transmission. Many previous studies have documented the antimicrobial, antiviral, anti-parasitic, and anti-cancer qualities of plant secondary chemicals (Ohgashi et al., 1992; Murakami et al., 1994, 1996; Billing and Sherman, 1998; Sherman and Billing, 1999). In the monarch–parasite system, infected monarch butterflies have shown decreases in parasite loads and increases in tolerance to *O. elektroscirra* when reared on milkweeds with higher cardenolide concentrations (de Roode et al., 2008a, 2011b; Sternberg et al., 2012; Gowler et al., 2015; Tao et al., 2015, 2016). Although the medicinal qualities of numerous plant chemicals are clear (Hunter, 2016), their direct effects on parasite morphology, as well as any related effects on pathogenicity and transmission, are unknown (Okagaki et al., 2010; Zaragoza et al., 2010). Therefore, our results suggest that additional experiments should be conducted to measure the direct effects of different diets on parasite morphology and subsequent effects on host and parasite fitness.

Generally, understanding the factors that generate variation in spore morphology may improve our understanding of parasite

growth, transmission, and fitness. Parasites that produce dormant stages typically incur some benefits as a spore, whether it is increased environmental tolerance or prolonged infectivity (Gest and Mandelstam, 1987; Kennedy et al., 1994; Potts, 1994; Nicholson et al., 2000; Roberts and Janovy, 2008). For example, spores of microparasites of *D. magna* can persist in pond sediments and remain infective for many years (Decaestecker et al., 2004). Similarly, bacteria such as *Bacillus* spp., which are abundant in soil, form spores during times of nutritional deficit. 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 often able to withstand long periods of dryness and UV damage. For example, blastospores of *Paecilomyces fumosoroseus* can withstand months of desiccation while retaining infectivity and virulence (Jackson et al., 1997).

Morphological traits, such as spore size and shape, are key to parasite fitness. Larger spores typically provide greater resources for parasite replication and establishment in hosts, typically by evading host immune defenses (Blueweiss et al., 1978; Sacks and Sher, 2002; Olivier et al., 2005). Greater size can also provide more resources for host colonization and for protection from the

host immune system (Poulin, 1995). For example, the cells of the pathogenic fungus *Cryptococcus neoformans*, which infects human lungs, can grow up to 20 times their normal size, reducing phagocytosis by host cells and oxidative and nitrosative damage. This morphological change in *C. neoformans* greatly increases survival and host colonization during initial stages of infection (Okagaki et al., 2010; Zaragoza et al., 2010). Larger parasite size has been correlated with larger host size in many systems (Bundy and Golden, 1987; Coop and Holmes, 1996; Leonardos and Trilles, 2003; Tsoetetsi et al., 2004). Here, we did not find significant effects of monarch butterfly host mass on *O. elektroscirra* spore size. Previously, spore size of *O. elektroscirra* was shown to correlate positively with monarch size, although this effect was quite weak (Sander et al., 2013). In the present study, which used 3 milkweed species with varying cardenolide concentrations, we found that effects of cardenolide concentration on parasite size were more important than host size, suggesting that the lack of a relationship between host and parasite size may have been caused by the overriding effect of cardenolide concentration on spore size.

In the study by Sander et al. (2013), average spore areas varied from 64 to 70  $\mu\text{m}^2$ . In the current study, average spore areas varied from 48 to 55  $\mu\text{m}^2$ . There are several factors that could contribute to the observed differences in spore area between these studies. First, spore measurements were taken differently, as the study by Sander et al. (2013) measured spore area using an Adobe Photoshop plug-in while the present study calculated spore area using spore length and width only. Additionally, the current study was based on a single parasite genotype, one which may be genetically predisposed to have a smaller spore size (Sander et al., 2013).

Previous studies have shown that infected monarchs reared on milkweeds with high cardenolide concentrations exhibit reduced parasite loads and increased tolerance to parasites (de Roode et al., 2008a, 2011a; Lefèvre et al., 2010; Sternberg et al., 2012; Gowler et al., 2015; Tao et al., 2015, 2016). Cardenolides may cause these reductions in parasite loads by increasing host immunity to the parasite, as has been seen in other systems (Lee et al., 2008; Povey et al., 2009; Simpson et al., 2015), or by direct inhibition of parasites (Cory and Hoover, 2006). Previous studies have shown that the effects of nutrition and milkweed chemicals on monarch larvae are most influential during early instars (Zalucki et al., 2001; de Roode et al., 2011a; Tao and Hunter, 2012). But further studies are necessary to determine whether these effects are mediated through mid-gut-based immunity or anti-parasite toxicity in the mid-gut lumen.

While the exact mechanism by which cardenolides reduce parasite growth and virulence remains unclear, the current study provides a likely explanation for the observed increases in parasite tolerance of monarchs feeding on high-cardenolide milkweed (Sternberg et al., 2012; Tao et al., 2015, 2016). Butterflies fed on high-cardenolide milkweed have higher tolerance, meaning they suffer less fitness loss with increasing parasite spore loads than do monarchs reared on low-cardenolide milkweed. The current study suggests that this increased tolerance could be the result of smaller spore size: because the parasites are smaller, their per capita damage may be smaller as well, resulting in higher butterfly fitness with the same number of parasites. Future studies can be performed to test these predictions by comparing monarch–

parasite interactions with parasites of reduced size and normal size.

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