Disease ecology across soil boundaries: effects of below-ground fungi on above-ground host–parasite interactions

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Host–parasite interactions are subject to strong trait-mediated indirect effects from other species. However, it remains unexplored whether such indirect effects may occur across soil boundaries and connect spatially isolated organisms. Here, we demonstrate that, by changing plant (milkweed Asclepias sp.) traits, arbuscular mycorrhizal fungi (AMF) significantly affect interactions between a herbivore (the monarch butterfly Danaus plexippus) and its protozoan parasite (Ophryocystis elektroscirrha), which represents an interaction across four biological kingdoms. In our experiment, AMF affected parasite virulence, host resistance and host tolerance to the parasite. These effects were dependent on both the density of AMF and the identity of milkweed species: AMF indirectly increased disease in monarchs reared on some species, while alleviating disease in monarchs reared on other species. The species-specificity was driven largely by the effects of AMF on both plant primary (phosphorus) and secondary (cardenolides; toxins in milkweeds) traits. Our study demonstrates that trait-mediated indirect effects in disease ecology are extensive, such that below-ground interactions between AMF and plant roots can alter host–parasite interactions above ground. In general, soil biota may play an underappreciated role in the ecology of many terrestrial host–parasite systems.

1. Introduction

Interactions between hosts and their parasites depend on more than just two partners. Community composition, including the presence of competitors, predators and reservoir species, mediates strong indirect effects that influence parasite virulence and transmission, which subsequently affect host fitness [1–3]. Some indirect effects are density-mediated. For example, decreases in the density of parasite-competent hosts in the presence of incompetent competitors can reduce parasite prevalence [4]. Alternatively, indirect effects can be trait-mediated, wherein changes in host quality and behaviour influence parasite replication, virulence and transmission [5,6]. For example, when feeding on low-quality phytoplankton, Daphnia reduce their foraging activity, leading to lower disease transmission [7,8]. Identifying how indirect effects alter host–parasite interactions is key to understanding host–parasite ecology and the management of disease.

An open question is how extensive trait-mediated effects are in disease ecology, and whether they can occur across ecosystem boundaries to connect spatially isolated organisms. Herbivorous insects and their parasites provide excellent opportunities to study trait-mediated indirect effects across the soil surface boundary. A rapid growing literature suggests that below-ground and above-ground biota can be intimately connected through the traits of plants [9,10]. Below-ground organisms, such as root symbiotic fungi and decomposers, can change plant quality and, therefore, have a profound impact on above-ground herbivore and predator performance [11–15], biodiversity [10] and ecosystem...
processes [16]. Additionally, food plants can change herbivore quality and immune function, and/or interfere directly with parasite infection or growth [5,17,18]. The important yet missing link is the connection between below-ground organisms and above-ground host–parasite interactions through changes in plant traits. Given the vast diversity of herbivorous insects and their parasites, understanding such a linkage is important for the advancement of community ecology in general and disease ecology in particular.

Parasite virulence, defined as the reduction in the fitness of hosts due to parasite infection, is a crucial driver of parasite transmission and host fitness and, therefore, the ecology and evolution of host–parasite interactions [19]. Virulence is co-determined by host resistance and tolerance to parasites. Host resistance is the ability of the host to prevent infection or reduce parasite replication, whereas tolerance is the ability to maintain fitness in the presence of parasite infection [20,21]. Both host resistance and tolerance are affected by host diet [22], and in the case of herbivorous insects, the quality of their food plants. For example, plant defence chemicals ingested by hosts from their food can inhibit parasites, and/or alter host immune functions [17,23–25]. Additionally, plant nutrient levels also influence parasite virulence. Higher nutrient levels can improve host vigour and increase allocation to immune defence [26–28], which may both reduce parasite replication [29] and increase host tolerance.

Plant secondary chemical profiles and nutrient levels are affected strongly by below-ground organisms including arbuscular mycorrhizal fungi (AMF). AMF are important root symbiotic fungi for more than 85% of terrestrial plant species. In return for sugars, AMF provide plants with nutrients and water, stimulate growth, and may relieve abiotic and pathogen stress [30]. In addition, interactions between AMF and their host plants stimulate signal transduction pathways, which mediate the synthesis of secondary chemical compounds [31]. Consequently, we hypothesized that AMF are indirect mediators of parasite virulence and the performance of infected herbivores through changes in plant nutrient and secondary chemical traits.

In this study, we tested the above predictions by exploring interactions among the monarch butterfly (Danaus plexippus), its protozoan parasite (Ophryocystis elektroscirrha) and its milkweed host plants. Milkweeds (mostly in the genus Asclepias) contain cardenolides, toxic steroids that disrupt animal Na⁺/K⁺-ATPase [32]. Previous studies have shown that milkweeds with high foliar concentrations of cardenolides can reduce O. elektroscirrha growth and virulence in monarchs [17,18]. Additionally, monarch performance is affected strongly by the nutrient status of its host plants [33]. By measuring and correlating the effects of AMF on host resistance and tolerance with their effects on plant nutrient and cardenolide traits, we show here that AMF have variable and significant effects on host performance across six milkweed species. Importantly, we illustrate that the combination of plant nutrient and chemical traits appears to explain the specific effects of AMF on infected butterfly hosts.

2. Material and methods

(a) Study system

Milkweeds readily form symbiotic relationships with AMF in their natural environments. AMF colonization changes the cardenolide and nutrient concentrations of milkweed foliage, with the magnitude and direction of the effects varying with milkweed species and AMF abundance [34,35]. We used six milkweed species in our experiments: A. curassavica, A. latifolia, A. syriaca, A. purpurascens, A. verticillata and A. incarnata. These species are native to North and Central America, and vary widely in their cardenolide concentrations. Out of the six species that we used, four occur naturally in Georgia (where the experiments were conducted), and A. curassavica occurs naturally in Florida with similar climates. A. latifolia occurs naturally in the western USA under drier conditions. Notably, all milkweed species used prefer sunny and hot climates, similar to our greenhouse conditions. We obtained seeds from Butterfly Encounters Inc. (CA, USA). We used a commercially available mixture of AMF (Plant Success Inc., CA, USA), which has equal proportions of Rhizopogon intraradices, Funneliformis mosseae, Glomus aggregatum and Claroideoglomus etunicatum. Asclepias species can form associations with these cosmopolitan AMF species in natural and experimental populations [35], although, as with most systems, the relative frequency of such relationships in natural populations remains unknown.

(b) Experimental set-up

We performed two experiments. In the pilot experiment, we used three milkweed species (A. latifolia, A. syriaca, A. verticillata); in the main experiment, we used all six species listed above. Experimental procedures were the same for both experiments.

We used a 3 : 1 soil (MetroMix 380, SunGro Inc., MA, USA) to sand (Quikcrete, GA, USA) mixture, which was evenly mixed and autoclaved (KIQ Inc., GA, USA; 120 °C/15 psi/30 min) before the experiment. Three levels (control, low and high) of AMF were used, where the high level was achieved by mixing 7 g of AMF mixture with 11 of soil/sand mixture, the low level by using 2 g of live and 5 g of autoclaved AMF mixture and the control by mixing 7 g of autoclaved AMF mixture with the same amount of soil/sand. Milkweed seeds were sterilized with 20% bleach before germination on damp filter paper in Petri dishes. Seedlings were randomly assigned to treatments and transferred to 4-inch pots of their respective treatment. Plants were grown in a greenhouse with natural sunlight and watered daily. The temperature was maintained between 24 and 33 °C, and the humidity between 30 and 60%. In the pilot experiment, plants were germinated in a growth chamber at 26 °C and 90% humidity with an LD 16 : 8 h (Philips F32T8/ ADV835/ALTO 32 Watt T8 high lumen fluorescent bulbs) at the University of Michigan before transferring to the greenhouse at Emory University. Each treatment had between 6 and 10 replicates in the pilot experiment. In the main experiment, each treatment had between 20 and 30 replicates.

When the plants were approximately two months old, we measured plant chemical traits. Briefly, one leaf from the fourth leaf pair on each plant was chosen, and six leaf discs (total 424 mm²) were taken with a paper hole punch from one side of the leaf, placed immediately into 1 ml of cold methanol and stored at −20 °C for subsequent cardenolide analysis. Another six identical discs were taken from the opposite side of the same leaf to estimate sample dry mass. The remaining leaf was then removed from the plant, dried and ground into fine powder for nitrogen (N) and phosphorus (P) analysis. Because A. verticillata have thin leaves with small leaf area, unsuitable for taking hole punches, we stored two whole leaves in methanol for cardenolides and two opposite leaves to estimate sample dry mass and nutrient content.

Immediately after chemical sampling, each plant was randomly assigned to one of two caterpillar treatments: infected or control. We collected caterpillar eggs from five outcrossed monarch lineages in a laboratory stock and randomly assigned them
to treatments. We reared the newly hatched caterpillars on the remaining 4th leaf from their individual plant in a Petri dish for 2 days, upon which the caterpillars became second instar. On the 3rd day, we took a hole punch from the third leaf pair of each plant. For the parasite treatment, 10 parasite spores were deposited onto the leaf disc, which was then fed to its pre-assigned caterpillar; control caterpillars received leaf discs without spores. After each caterpillar had fully consumed its leaf disc (usually within 48 h), both the caterpillar and its host plant were transferred into clear plastic tubes with 20 venting holes in the lid, where they were allowed to completely consume their plant. Because plants were generally not big enough to support complete larval development, larvae were then supplied with a separately grown batch of *A. incarnata* cuttings grown in sterilized soil until pupation. The procedure is justified because previous studies have shown that the effects of milkweed chemistry on parasite infection, growth and virulence are conferred during the time of infection, but not during the larval development stage following infection [36]. Similarly, the effects of nutrients and secondary chemicals on uninfected caterpillars are most prominent when they are in early instars [33,37]. We specifically chose *A. incarnata* as supplementary food because of its low cardenolide concentration. Importantly, by the time they had finished their experimental host plant, the caterpillars were mostly fifth instars and had spent an average of 8.5 days on their individual plants, leaving an average of only 1.5 days of pre-pupal development on these new supplementary cuttings.

After emerging from their pupae, butterflies were placed in 8.9 x 8.9 cm glassine envelopes, stored in a 12°C incubator, and inspected daily until they died, upon which the lifespan of each butterfly was recorded. This measurement, used routinely in this host–parasite system [17,18,21,38] combines longevity and starvation resistance, both of which are highly correlated with the lifespan and life-time fitness of monarchs under more natural conditions [17]. After death, the spore load of each butterfly was measured following described methods [17]. Spore load estimates the total number of spores on a butterfly, which is positively correlated with parasite transmission potential and negatively correlated with butterfly resistance and fitness.

After each plant was completely consumed by its assigned caterpillar, we harvested the root tissues by gently washing the soil from the roots. The roots were stored in formalin–ethanol–acetic acid for AMF staining. For root staining, eight samples from each treatment were randomly chosen, cleared with KOH, and acidified in trypan blue in 1 : 1: 1 water : glycerine : lactic acid. Roots were then mounted on slides and AMF were scored with eyepiece crosshairs. An intersection site was considered colonized if AM hyphae, arbuscules or vesicles were present. Percentage of colonization was then calculated as the number of colonized sites divided by the total inspected sites. Analysis of foliar cardenolide, N and P concentrations followed [33].

### (c) Statistical analysis

Analyses were carried out separately for the pilot and main experiment, using the following procedures. To test for effects of AMF on plant traits, we used plant species (*A. syriaca*, *A. verticillata* and *A. latiofolia* in pilot experiment; *A. cinnassavica*, *A. verticillata*, *A. purpureascens*, *A. syriaca*, *A. latifolia* and *A. incarnata* in the main experiment), AMF presence (absence/presence) or AMF abundance (control, low and high) and their interactions as independent variables, and plant cardenolide, N or P concentration as dependent variables in three separate linear models.

To analyse effects on parasite virulence, we used a linear model with adult monarch lifespan as the dependent variable, parasite treatment (uninfected versus infected), plant species (three species in the pilot experiment; six species in the main experiment), AMF presence (absence/presence) or abundance (control, low and high) and their interactions as independent variables. As parasite virulence is determined as reduction in fitness of infected individuals relative to uninfected individuals, significant interactions between parasite treatment and other variables on lifespan indicate significant effects on virulence. To estimate host resistance, we used parasite spore load, which was log transformed to achieve homogeneity of variance and normality of errors. Lower spore load indicates higher host resistance. To estimate host tolerance, we measured the negative slope between log transformed spore load and lifespan of both uninfected and infected butterflies [21]. More negative slopes indicate lower tolerance to infection.

To test whether AMF affect host resistance, we used log-transformed spore load of infected hosts as the dependent variable, plant species identity (three in the pilot experiment; six in the main experiment), AMF presence (absence/presence) or abundance (control, low and high) and their interactions as independent variables. Finally, to determine if AMF affect host tolerance to parasites, we used adult lifespan of butterflies as the dependent variable, AMF presence (absence/presence) or abundance (control, low and high), plant species (three in the pilot experiment; six in the main experiment), log transformed spore load and their interactions as independent variables.

Next, we explored any independent effects of plants traits (N, P, cardenolide) on the lifespan of uninfected and infected butterflies. This analysis allows us to consider whether the potential benefits or costs of particular plant traits (N, P, cardenolide) for butterfly fitness differ between infected and uninfected monarchs [18]. Models were run separately for each plant trait and experiment, with adult lifespan as the dependent variable, and parasite treatment (uninfected versus infected), the given plant trait, and the interaction between plant trait and infection status as independent variables.

Because we were particularly interested in how plant traits might interact to influence the consequences of infection, we performed additional analyses on infected butterflies only. Separately for each experiment, we adopted a forward selection process using AICc (corrected Akaike information criterion) scores to explore any additive and interactive effects of plant traits on butterfly lifespan. The plant trait that yielded the lowest AICc score entered the model first. Any additional variable (and its interactions) was incorporated only if the new model reduced AICc by more than two points and if the significance level of the new variable was below 0.05 [40]. The above procedure was repeated until the minimal model was determined.

For all regression models, homogeneity of variance of dependent variables was confirmed by Levene’s test from the car package in R [41], and normality of errors was confirmed by the Shapiro–Wilk normality test. All statistical tests were performed using R v. 2.15.3 [42].

### 3. Results

In the pilot experiment with fewer milkweed species and replicates, we found qualitatively similar results to those found in the main experiment. We report the results of the pilot experiment in the electronic supplementary material; only results from the main experiment are reported in the following text.

The AMF treatment resulted in successful root infections across milkweed species (*t* = 32.96, *p* < 0.001). Specifically, control plants had negligible AMF infection (0.03 ± 0.03%; mean ± s.e.m.), while AMF infection rates for plants from low and high treatment groups were 20.70 ± 3.77%
Table 1. AMF colonization rates (± s.e.m.) for each milkweed species.

| species         | A. curassavica | A. verticillata | A. purpurascens | A. syriaca | A. latifolia | A. incarnata |
|-----------------|----------------|-----------------|-----------------|------------|--------------|--------------|
| control         | 0.18 ± 0.18%   | 0.00 ± 0.00%    | 0.00 ± 0.00%    | 0.00 ± 0.00% | 0.00 ± 0.00% | 0.03 ± 0.03% |
| low             | 36.57 ± 9.90%  | 15.20 ± 7.04%   | 5.02 ± 2.63%    | 33.93 ± 11.02% | 27.35 ± 10.94% | 6.36 ± 3.26% |
| high            | 41.28 ± 9.54%  | 26.34 ± 8.39%   | 13.21 ± 4.29%   | 32.42 ± 5.80% | 28.10 ± 11.17% | 42.64 ± 6.53% |

and 30.67 ± 3.43%, respectively. Tukey’s post hoc comparisons confirmed that across all six species, all three groups differed significantly in their AMF infection rates (low versus control, p < 0.001; high versus control, p < 0.001; low versus high, p = 0.04; table 1). However, in A. syriaca and A. latifolia, AMF colonization rates between low and high AMF treatment groups were very similar (table 1).

(a) Effects of arbuscular mycorrhizal fungi on parasite virulence

Infection with *O. elektroscirrha* greatly reduced butterfly lifespan (a measure of parasite virulence; *F*<sub>1,274</sub> = 481.88, p < 0.001; electronic supplementary material, table S1), and the magnitude of lifespan reduction varied among plant species (infection × plant species: *F*<sub>5,274</sub> = 3.35, p = 0.006; electronic supplementary material, table S1; figure 1), illustrating that plant species affect parasite virulence. For example, reductions in lifespan by parasites were smallest when caterpillars fed on *A. verticillata* and *A. curassavica*, and were greatest when caterpillars fed on *A. incarnata*.

Importantly, the presence and the abundance of AMF affected the extent to which parasite infection reduced monarch lifespan (figure 1; infection × AMF presence × plant species: *F*<sub>5,528</sub> = 2.78, p = 0.02; infection × AMF abundance × plant species: *F*<sub>10,274</sub> = 1.75, p = 0.07; electronic supplementary material, table S2). For *A. verticillata* and *A. latifolia*, AMF decreased parasite virulence. By contrast, AMF increased parasite virulence in monarchs reared on *A. purpurascens*. On *A. curassavica*, *A. incarnata* and *A. syriaca*, AMF had no effects on the virulence of the parasite.

(b) Effects of arbuscular mycorrhizal fungi on host resistance and tolerance

The spore load of infected butterflies is a measure of host resistance to the parasite (high spore load = low resistance). Host resistance varied among milkweed species (*F*<sub>5,138</sub> = 20.10, p < 0.001; electronic supplementary material, table S2; figure 2a). However, neither AMF presence nor abundance affected host resistance. By contrast, the presence of AMF influenced host tolerance to parasites differentially among plant species (*F*<sub>5,290</sub> = 2.43, p = 0.04; electronic supplementary material, table S4; figure 2b). Specifically, AMF effects on tolerance were qualitatively similar to those on lifespan of infected individuals, whereby AMF increased tolerance of caterpillars reared on *A. verticillata*, *A. syriaca* and *A. latifolia* but decreased tolerance of caterpillars reared on *A. purpurascens* (electronic supplementary material, table S4; figure 2b).

(c) Effects of arbuscular mycorrhizal fungi on host–parasite interactions explained by plant traits

Foliar P concentration was correlated positively with the lifespan of both uninfected and infected butterflies (*F*<sub>1,39</sub> = 8.11, p = 0.006; figure 3a). There was also a positive relationship between foliar cardenolide concentration and lifespan of infected butterflies, but not of uninfected ones (cardenolide × infection: *F*<sub>1,302</sub> = 5.17, p = 0.02; figure 3b). Foliar nitrogen (N) concentration, on the other hand, did not affect the lifespan of uninfected or infected butterflies (*F*<sub>1,99</sub> = 0.69, p = 0.41). The minimal statistical model exploring the effects of plant traits on the lifespan of infected butterflies included

![Figure 1](http://rspb.royalsocietypublishing.org/Downloaded from http://rspb.royalsocietypublishing.org/...
the positive effects of both foliar P and cardenolide concentrations (table 2).

Parasite spore load on monarch butterflies declined with foliar cardenolide concentrations ($F_{1,153} = 79.332, p < 0.001$). Foliar cardenolide concentration, however, did not affect host tolerance to the parasites (cardenolide $\times$ spore load parameter estimation: $0.01 \pm 0.03; F_{1,306} = 0.31, p = 0.58$).

There were no relationships between foliar N and P concentrations and parasite spore load. Foliar P concentration was correlated marginally and positively with host tolerance to parasites (P $\times$ spore load parameter estimation: $2.22 \pm 1.28; F_{1,57} = 3.05, p = 0.08$).

The effects of AMF on foliar P and cardenolide concentrations varied among milkweed species (electronic supplementary material, table S5; figure 4). For example, high AMF treatment increased foliar P concentrations in *A. curassavica*, but decreased foliar P concentrations in *A. syriaca*. In *A. purpurascens* and *A. incarnata*, low, but not high AMF treatment increased foliar P concentration. There was also a marginally non-significant interaction between plant species and AMF treatment on cardenolide concentration ($F_{10,320} = 1.59, p = 0.10$); foliar cardenolide concentrations tended to decrease with increasing AMF density in *A. curassavica*, but increase in *A. purpurascens*, and *A. syriaca* (figure 4).

4. Discussion
In this study, we illustrate that interactions among members of four biological kingdoms link the fast growing fields of below-ground–above-ground species interactions and disease community ecology by showing complex interactions among plant symbiotic fungi, plants, herbivores and parasites. Specifically, the relationship between a protozoan parasite and its herbivore host above ground depends upon the presence and the abundance of soil fungi that influence plant nutritional and defensive traits. Our study also adds to the growing body of literature showing that community composition has profound effects on host–parasite interactions [1–3], and that trait-mediated indirect effects can connect below- and above-ground ecosystems [9,10]. As with other studies of AMF, we also found that these effects were dependent on both the abundance of mycorrhizal fungi and the identity of plant species that they colonize [34,35].

Through changes in plant traits, below-ground biota can have large effects on above-ground tri-tritrophic interactions [12,43–45]. Sometimes the indirect effects of below-ground biota on above-ground interactions are density-mediated; for example, AMF-induced changes in the numbers of thrips feeding on soy beans translate directly into higher population densities of thrip predators [46]. In other cases, such effects are largely trait-mediated. For instance, although soil organisms reduce the population sizes of the aphid *Rhopalosiphum padi*, parasitoid numbers are unaffected: increases in aphid body size caused by below-ground organisms reduce the mortality rate of the parasitoids [43]. While the larger body size of aphids may result from changes in concentrations of primary nutrients (amino acids) in the phloem [43], plant secondary metabolites appear to play a bigger role in other systems [11]. Here we illustrate that plant nutritional (P) and defensive chemicals (cardenolides) can mediate simultaneously the indirect effects of soil biota on species interactions above ground.
Effects of community composition on disease ecology are also characterized by the presence of density-mediated and trait-mediated indirect effects. For example, dilution effects on disease transmission are essentially density-mediated effects in which numerical changes in the relative abundance of competent hosts affect disease transmission [4]. Likewise, trait-mediated indirect effects on host quality, size and behaviour can be equally important [3,5–8,45]. Among the factors that influence host–parasite interactions, host diet quality can play a particularly significant role because it can affect host growth rate, immune function and foraging behaviour [7,24,26,27]. Importantly, we demonstrate that AMF can affect parasite virulence by affecting plant primary and secondary chemical traits simultaneously.

Consistent with previous experiments [17,18], we found positive effects of milkweed cardenolides on monarch butterfly resistance to their parasites. Dietary secondary metabolites can affect host resistance to parasites through many mechanisms [5]. First, they can directly reduce parasite numbers. Furthermore, secondary metabolites can affect the host’s immune system. Detoxifying secondary metabolites may reduce resources available for allocation to immune systems [25]. However, the opposite effect has also been observed, whereby secondary chemicals boost immune function [24]. Which mechanism operates in the monarch–parasite system is as yet unclear. Although protozoans lack the complete machinery of Na\(^+/\)K\(^+\)-ATPase, they still possess the α subunit of modern Na\(^+/\)K\(^+\)-ATPase [47], which is the direct target site of cardenolides [32]. Moreover, cardenolides may interact with monarchs through other biochemical pathways that influence immune function and overall resistance to O. elektroscirrha. In addition to effects on resistance, previous work has found that cardenolides increase the tolerance of monarchs to O. elektroscirrha [18]. In this study, we found positive effects of cardenolides on tolerance in the pilot experiment, but not in the main experiment, suggesting that milkweed secondary chemicals may have more consistent effects on host resistance than on tolerance.

Dietary nutrient quality is another important factor influencing host–parasite interactions. Immune defences are nutrient-costly and can improve under high nutrient availability [26,27]. In this study, we found no obvious effects of foliar N or P concentrations on host resistance, as they did not affect spore production. However, foliar P concentration was positively associated with monarch tolerance to disease, possibly because higher P concentrations improve lipid metabolism, which helps to resist starvation [48] and general vigour. Similarly, in Daphnia magna, higher P concentrations in food sources also increase the fitness of hosts that are infected by bacterial pathogens. However, spore production in Daphnia is also higher when it consumes P rich food, resulting in higher virulence [29].

We estimated the fitness of uninfected and infected butterflies by measuring the lifespan of adults under constant temperature (12 °C), a standard procedure in this system [17,18,21,38]. Although we would prefer to measure fitness under more natural conditions, logistical constraints simply prohibit measuring individual butterfly fitness under natural conditions with the sample sizes required for our experiments. However, previous studies have demonstrated that monarch lifespan is correlated positively with their mating probability and life fecundity under more natural rearing conditions, and is therefore a good proxy for fitness [49]. Moreover, using lifespan as an estimate of virulence has proven repeatedly to correlate well with estimates of parasite reproduction, monarch resistance and monarch tolerance to infection (e.g. [17,18,21]).

Importantly, our results illustrate that the lifespan of infected monarch butterflies is best explained by the combined contributions of foliar P and cardenolide concentrations on lifespan. Effects of AMF on plant traits are very species-specific and sometimes nonlinear [34,50,51], therefore, they display large variation in their effects on host–parasite interactions. By changing both foliar P and cardenolide concentrations simultaneously, AMF had significant effects on the lifespan of infected butterflies. For instance, in A. curassavica, AMF increased foliar P concentration while decreasing foliar cardenolide concentration, yielding overall neutral effects on the lifespan of infected butterflies. Likewise, although AMF increased cardenolide concentration in the leaves of
A. purpurascens, high AMF levels also decreased foliar P concentration, associated with greater adverse effects on lifespan. More generally, this suggests that it may be necessary to include the effects of both primary and secondary metabolites in studies of host–parasite interactions.

Notably, the fitness of uninfected butterflies was associated positively with foliar P and slightly negatively with foliar cardenolide concentrations. For instance, reductions in cardenolide concentration but increases in plant P caused by high levels of AMF led to longer lifespans of uninfected butterflies that used A. purpurascens as host plants. As parasite virulence is determined by the differences in fitness between uninfected and infected individuals, AMF have the potential to influence overall virulence estimates by their effects on both infected and uninfected butterflies. This is what we observed in our experiment, in which AMF influenced the lifespan of both infected and uninfected butterflies. This is what we observed in our experiment, in which AMF influenced the lifespan of both infected and uninfected monarchs (figure 1), associated with the effects of AMF on foliar P and cardenolide concentrations.

The fungal community that we used in our experiments was from a commercially available mixture of AMF species that undergo symbioses with milkweeds in natural populations. However, we have not matched fungal isolates with their local milkweed hosts, nor determined the degree of root colonization by each of the AMF species derived from the mix. Much of the species-specific nature of our results may depend upon differential colonization of AMF species with our milkweed species [52]. In nature, fungal community composition exhibits substantial geographical variation and fungal species vary in their effects on plant traits [50]. In addition, our milkweed species differed in their AMF colonization rates, which could also have driven the variable effects of AMF on host–parasite interactions that we observed in our experiments. However, there were no clear patterns linking milkweed-specific variation in AMF colonization with milkweed-specific effects on the parasite. Understanding spatial and temporal variation in milkweed–AMF interactions in natural populations will demand detailed future work with local fungal communities and their plant hosts [53]. Nonetheless, under natural conditions, milkweeds readily form associations with the cosmopolitan AMF species that we used. By using a commercial mixture of AMF, we have demonstrated that root fungi can influence parasite–host interactions above ground. Given the ubiquity of plant–AMF associations, and their substantial influence on plant nutrition and defence, we suggest that soil biota may play an underappreciated role in the ecology of many terrestrial host–parasite systems.

Figure 4. Effects of AMF on natural log transformed foliar cardenolide (black) and phosphorus concentrations (grey). Data represent mean ± 1 s.e.m. Treatment annotations and species abbreviations are as in figure 1.

Data accessibility. Data from the main experiment are deposited in Dryad at: http://dx.doi.org/10.5061/dryad.164t6.

Competing interests. We declare we have no competing interests.

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References

1. Keesing F et al. 2010 Impacts of biodiversity on the emergence and transmission of infectious diseases. Nature 468, 647–652. (doi:10.1038/nature09575)
2. Tompkins DM, Dunn AM, Smith MJ, Telfer S. 2011 Wildlife diseases: from individuals to ecosystems. J. Anim. Ecol. 80, 19–38. (doi:10.1111/j.1365-2656.2010.01742.x)
3. Rohr JR, Civitello DJ, Crumrine PW, Halstead NT, Miller AD, Schotthoefer AM, Stenoien C, Johnson LB, Beasley VR. 2015 Predator diversity, intraguild predation, and indirect effects drive parasite transmission. Proc. Natl Acad. Sci. USA 112, 3008–3013. (doi:10.1073/pnas.1415971112)
4. Johnson PT, Preston DL, Hoverman JT, Richgels KLD. 2013 Biodiversity decreases disease through predictable changes in host community competence. Nature 494, 230–233. (doi:10.1038/nature11883)
5. Cory JS, Hoover K. 2006 Plant-mediated effects in insect–pathogen interactions. Trends Ecol. Evol. 21, 278–286. (doi:10.1016/j.tree.2006.02.005)
6. de Roode JC, Ranick RM, Mongue AJ, Gerardo NM, Hunter MD. 2011 Aphids indirectly increase
47. Sáez A, Lozano E, Zaldívar-Riverón A. 2009 Evolutionary history of Na,K-ATPases and their osmoregulatory role. Genetica 136, 479 – 490. (doi:10.1007/s10709-009-9356-0)

48. Alonso-Mejía A, Rendon-Salinas E, Montesinos-Patino E, Brower LP. 1997 Use of lipid reserves by monarch butterflies overwintering in Mexico: implications for conservation. Ecol. Appl. 7, 934 – 947. (doi:10.1890/1051-0761(1997)007[934:UOLRBM]2.0.CO;2)

49. de Roode JC, Yates AJ, Altizer S. 2008 Virulence-transmission trade-offs and population divergence in virulence in a naturally occurring butterfly parasite. Proc. Natl Acad. Sci. USA 105, 7489 – 7494. (doi:10.1073/pnas.0710909105)

50. Bennett AE, Bever JD. 2007 Mycorrhizal species differentially alter plant growth and response to herbivory. Ecology 88, 210 – 218. (doi:10.1890/0012-9658(2007)88[210:MSDAPG]2.0.CO;2)

51. Gange AC, Ayres RL. 1999 On the relation between arbuscular mycorrhizal colonization and plant ‘benefit’. Oikos 87, 615 – 621. (doi:10.2307/2546829)

52. Klironomos J et al. 2011 Forces that structure plant communities: quantifying the importance of the mycorrhizal symbiosis. New Phytol. 189, 366 – 370. (doi:10.1111/j.1469-8137.2010.03550.x)

53. Maherali H, Klironomos JN. 2007 Influence of phylogeny on fungal community assembly and ecosystem functioning. Science 316, 1746 – 1748. (doi:10.1126/science.1143082)