Arbuscular mycorrhizal fungi mediate below-ground plant–herbivore interactions: a phylogenetic study

Rachel L. Vannette[1] and Sergio Rasmann*[,2]

[1]*Biology Department, Stanford University, 371 Serra Mall, Stanford, CA, 94305 USA; and* [2]*Department of Ecology and Evolution, University of Lausanne, UNIL Sorge, Le Biophore, CH – 1015 Lausanne, Switzerland*

Summary

1. Ecological interactions are complex networks, but have typically been studied in a pairwise fashion. Examining how third-party species can modify the outcome of pairwise interactions may allow us to better predict their outcomes in realistic systems. For instance, arbuscular mycorrhizal fungi (AMF) can affect plant interactions with other organisms, including below-ground herbivores, but the mechanisms underlying these effects remain unclear.

2. Here, we use a comparative, phylogenetically controlled approach to test the relative importance of mycorrhizal colonization and plant chemical defences (cardenolides) in predicting plant survival and the abundance of a generalist below-ground herbivore across 14 species of milkweeds (*Asclepias* spp.). Plants were inoculated with a mixture of four generalist AMF species or left uninoculated. After 1 month, larvae of *Bradysia* sp. (Diptera: Sciaridae), a generalist below-ground herbivore, colonized plant roots.

3. We performed phylogenetically controlled analyses to assess the influence of AMF colonization and toxic cardenolides on plant growth, mortality and infestation by fungus gnats. Overall, plants inoculated with AMF exhibited greater survival than did uninoculated plants. Additionally, surviving inoculated plants had lower numbers of larvae in their roots and fewer non-AM fungi than surviving uninoculated plants. In phylogenetic controlled regressions, gnat density in roots was better predicted by the extent of root colonized by AMF than by root cardenolide concentration. Taken as a whole, AMF modify the effect of below-ground herbivores on plants in a species-specific manner, independent of changes in chemical defence.

4. This study adds to the growing body of literature demonstrating that mycorrhizal fungi may improve plant fitness by conferring protection against antagonists, rather than growth benefits. In addition, we advocate using comparative analyses to disentangle the roles of shared history and ecology in shaping trait expression and to better predict the outcomes of complex multitrophic interactions.

Key-words: *Asclepias*, *Bradysia*, cardenolides, chemical ecology, *Glomus*, phylogenetic ecology, plant defence, plant–herbivore interaction

Introduction

An increasing number of studies demonstrate that plant associations with mycorrhizal fungi not only alter the performance of individual plants, plant community structure, plant productivity and nutrient cycling (Smith & Read 2008), but can also change the outcome of interactions between plants and their herbivores (reviewed in Borowicz 2001; Gange & Brown 2002b; Gehring & Whitham 2002; Bennett, Alers-Garcia & Bever 2006; Gange 2007; Gehring & Bennett 2009). Indeed, given that more than half of the

total described macro-biodiversity is represented by plants and insect herbivores (Strong, Lawton & Southwood 1984) and that more than 80% of plant species associate with mycorrhizal fungi (Wang & Qiu 2006), it is very likely that many of the often studied pairwise interactions between plants and herbivores are also influenced by mycorrhizal fungi (Bennett, Alers-Garcia & Bever 2006; Hartley & Gange 2009). To date, syntheses of the effect of mycorrhizae on herbivores have shown idiosyncratic effects of both ecto-mycorrhizal (EM) and arbuscular mycorrhizal fungi (AMF) on insect performance (Gange 2007; Gehring & Bennett 2009; Koricheva, Gange & Jones 2009). For instance, Gehring & Whitham (2002) and later Gehring &

*Correspondence author. E-mail: sergio.rasmann@unil.ch

Bennett (2009) observed that mycorrhizal colonization significantly affected the performance of *c.* 2/3 of insect species examined in 17 and later 30 studies reviewed. However, the direction of these effects was highly variable and depended on the identity of fungi, herbivore and plant. Among herbivore feeding guilds, only sucking herbivore performance was higher in the presence of EM than in the presence of AMF. Interestingly, generalist herbivores seem to be more affected by the presence of mycorrhizae than specialist herbivores (Gehring & Whitham 2002; Koricheva, Gange & Jones 2009), suggesting that plant secondary metabolites may mediate these interactions (Bennett, Alers-Garcia & Bever 2006).

Our understanding of plant-insect interactions is largely based on above-ground systems (Hunter 2001; Van der Putten *et al.* 2001), and similarly, most of the work involving the above-mentioned tripartite interactions has focused on the effect of mycorrhizae on above-ground herbivores. Indeed, few studies have looked at the effect of mycorrhizae on soil-dwelling insect herbivores (Gange 2007), despite the ecological importance of below-ground herbivores (Hunter 2001). In one of the few examples, colonization of *Taraxacum officinale* by the AMF *Glomus mosseae* resulted in nearly 50% reduction in growth rate and survival of the root-feeding larvae of the black vine weevil (*Otiorhynchus sulcatus*) (Gange, Brown & Sinclair 1994). Similarly, colonization of strawberry plants (*Fragaria × ananassa*), by either of two AMF fungi (*G. mosseae* and *G. fasciculatum*) decreased the performance of the same herbivore, and the effect of single species inoculations was attenuated when both fungi were co-inoculated (Gange 2001).

Mycorrhizal fungi may alter plant interactions with herbivores through several hypothetical mechanisms (Gehring, Cobb & Whitman 1997; Bennett, Alers-Garcia & Bever 2006). Mycorrhizal colonization can increase plant nutritional quality, in turn increasing the performance of herbivores (White 1984; Price 1991; Goverde *et al.* 2000; Bennett, Alers-Garcia & Bever 2006; Vannette & Hunter 2009). Mycorrhizae can also affect plant vigour, which in turn can increase (Kula, Hartnett & Wilson 2005) or decrease (Bennett & Bever 2007; Garrido *et al.* 2010) plant tolerance to herbivory in a species and genotype-specific way. Mycorrhizal fungi may also alter toxic plant secondary metabolite production, thus altering resistance to herbivores (Jones & Last 1991; Vannette & Hunter 2011a). In addition to these indirect and plant-mediated effects, mycorrhizae may also directly deter root herbivores through physical or chemical interactions in the root or rhizosphere (Schulz *et al.* 2002; Bennett, Alers-Garcia & Bever 2006; de la Peña *et al.* 2006; Rasmann *et al.* 2011a).

The relative importance of each of these mechanisms remains unknown. There is indeed evidence that mycorrhizae can increase the levels of toxic secondary metabolites in roots (Gange & West 1994; Peipp *et al.* 1997; Schliemann, Ammer & Strack 2008), but disentangling the effects of mycorrhizal colonization, the presence of fungi within roots and plant-mediated effects on below-ground herbivores remains a challenge. With this study, we aimed to address this question using a comparative phylogenetic approach. First, we used a multi-species approach to overcome the fact that the effect of mycorrhizal fungi on plant chemistry – and subsequently on herbivores – is conditional on the species chosen (Gange & Brown 2002a). Second, strong evidence indicates phylogenetic trait conservatism in plant functional traits related to growth and defence (Futuyma & Agrawal 2009). Therefore, comparative phylogenetic analyses of anti-herbivore traits need to account for shared ancestry. Finally, recent analyses suggest that host plant phylogeny can affect plant response to mycorrhizal colonization among plants that share the same habitat (Reinhart, Wilson & Rinella 2012). However, we are not aware of studies that examine AMF associations with closely related plant species that share defence compounds and subsequent plant–herbivore interactions. Our phylogenetically controlled approach may allow us to disentangle the role of plant defence expression, mycorrhizal colonization and shared ancestry on the outcome of multitrophic interactions.

Milkweeds, in the Pan-American genus *Asclepias*, are optimal candidates to investigate plant defence theories in a comparative framework. They have a well-characterized defensive arsenal (including toxic cardenolides) (Zalucki, Brower & Alonso 2001), tremendous variation among species in the expression of these traits (Agrawal & Fishbein 2006; Rasmann & Agrawal 2011b) and known phylogenetic relationships (Fishbein *et al.* 2011). Almost all of the studied milkweed species contain cardenolides (Abe & Yamauchi 1994; Rasmann & Agrawal 2011b), molecules which can disrupt the sodium and potassium flux in animal cells when ingested (Malcolm 1991). Cardenolides occur in all milkweed tissues, including roots (Rasmann *et al.* 2009). Despite insect behavioural and physiological adaptations to reduce cardenolide exposure and toxicity (Dussourd & Eisner 1987; Holzinger & Wink 1996), empirical evidence suggests that cardenolides continue to be detrimental to both above-ground (Zalucki, Brower & Alonso 2001; Agrawal 2005) and below-ground herbivores (Rasmann *et al.* 2011b). Milkweeds are also colonized by mycorrhizal fungi that affect plant growth, latex exudation, specific leaf mass and cardenolide production (Vannette & Hunter 2011b).

In this study, we assessed the role of arbuscular mycorrhizal symbiosis as a mediator of plant–herbivore interactions for 14 milkweed species. Specifically, we asked: (i) how does AMF colonization affect plant performance and response to below-ground herbivory? (ii) How does AMF colonization affect the performance of a generalist below-ground herbivore? (iii) What is the relative importance of cardenolides and mycorrhizal colonization in determining below-ground herbivore abundance and plant–herbivore interactions? We hypothesized that (i) AMF improve the plant performance, (ii) AMF protect plants against

below-ground herbivory by increasing plant resistance and (iii) AMF decrease the performance of below-ground herbivores by both physically shielding the roots and by enhancing root secondary metabolism.

Material and methods

To disentangle the role of plant defence from direct mycorrhizal protection of roots, we experimentally manipulated the presence and absence of mycorrhizal fungi available to 14 milkweed (*Asclepias*) species. We then quantified mycorrhizal colonization and chemical defence within roots and assessed their effects on root infestation by a generalist below-ground herbivore and subsequent plant growth and survival (see Table S1, Supporting information).

PLANTS AND MYCORRHIZAL INOCULUM

We chose 14 species of milkweeds based on high variation in cardenolide concentration in the roots (Rasmann & Agrawal 2011b). All seeds were germinated at room temperature after stratification at 4 °C on moist filter paper for 2 weeks. Individual seedlings (n = 10–26 replicates per plant species depending on germination success) were then transplanted into 10-cm diameter plastic pots in a mixture of low nutrient, autoclaved potting soil (Metro-Mix 360; Metro-Mix Sun Gro Horticulture Canada CM Ltd., Vancouver, BC, Canada) and perlite (3 : 1 parts potting soil: perlite), and grown in a single growth chamber (10 h daylight, 26 °C day : 17 °C night). Metro-Mix 360 is a formulated Canadian *Sphagnum* peat moss, coarse perlite, bark ash, starter nutrient charge (with Gypsum) and slow release nitrogen and dolomitic limestone (specifications include NO_3 [50–100 ppm], NH_4 [10–14 ppm], P [12–40 ppm], K [90–175 ppm] www.sungro.com). The soil therefore becomes relatively poor in nutrients only after plant establishment, and perlite promotes soil aeration. As a result, this soil composition should promote optimal mycorrhizal development. The two mycorrhizal treatments were prepared by combining the soil mixture with live or autoclaved mycorrhizal inoculum consisting of a combination of root fragments and spores. We obtained inoculum of cosmopolitan fungal species known to associate with *Asclepias syriaca* (Vannette & Hunter 2011b) including *Glomus intraradices*, *G. mosseae*, *G. aggregatum* and *G. etunicatum* from Mycorrhizal Applications (Grants Pass, OR, USA). These fungal species have been found in forests, grassland, wetlands and arable fields (Öpik *et al.* 2006), habitats in which all *Asclepias* species used here are also found. Plastic pots were filled with 300 mL of sterilized soil mixture containing 20 mL of live or autoclaved mycorrhizal inoculum, and the top of all pots were capped with another 100 mL of soil mixture to prevent transfer of mycorrhizal spores among treatments. Therefore, half of the plants were inoculated with mycorrhizal fungi, and the other half were considered control, non-mycorrhizal plants. Replicates were randomized within the growth chamber. Plants were watered *ad libitum* and received no fertilizer.

BELOW-GROUND HERBIVORE COLONIZATION

Through regular watering, we prevented soil desiccation in the greenhouse, thereby allowing the natural colonization of roots by fungus gnat flies (Diptera: Sciaridae). This ubiquitous group of herbivorous, fungivorous and saprophytic larvae that dwell below-ground has been used to test classic hypotheses of plant resistance against herbivores (McConn *et al.* 1997). This system provides a 'semi-natural' framework for testing effect of mycorrhizae on below-ground herbivore–plant interactions. By allowing colonization within the growth chamber, our measurements of lar-

val abundance in roots account for both oviposition choice by females and survivorship of larvae as a function of plant toxins and mycorrhizal interactions. In addition, we hypothesized that the effect of mycorrhizal fungi and cardenolides on generalist herbivores such as gnat larvae may be more pronounced than their effects on a very specialized herbivore (Van der Meijden 1996). We suspect that fly eggs were present in the growth chamber or adults migrated to the growth chamber during watering.

After 2 months of plant growth, soil colonization by fungus gnats was measured following Cabrera, Cloyd & Zaborski (2003). Fresh potato discs (1-cm diameter, 2-cm long) were placed in the soil around the roots of each plant at about 1 cm from the stem. After 3 days, nearly all larvae migrated from the roots to the highly palatable potato discs (this was confirmed by visual observation of roots and soil after potato disk removal), and were counted. Identification of adults that emerged from the potato discs placed the collected gnats in the family Sciaridae, all belonging to the genus *Bradysia* (Borror, Triplehorn & Johnson 1989). All dead plants were recorded to measure the effect of larval infestation on plant mortality. A wilted plant was considered dead when, after watering, it did not recuperate. Subsequent observation showed signs of scarring and tunnelling throughout the dead plants' root systems done by the sciarid maggots, suggesting that sciarids were the cause of mortality.

HARVEST AND ASSESSMENT OF MYCORRHIZAL COLONIZATION

After larval colonization and plant mortality measurements, soil around plants was carefully washed away with tap water. Roots and leaves were dried at 40 °C for 1 week and weighed. Roots of each plant were then divided into two equal parts, one for measuring fungal colonization and the other for measuring cardenolide concentrations. For fungal colonization, a subset of fine root tissue (about 200 mg) was cleared with 10% KOH for 10 min, acidified using 2% HCl and stained in 0·05% trypan blue in 1 : 1 : 1, water/glycerine/lactic acid. Roots were mounted on slides and scored at × 20 magnification with a Nikon E600 (Melville, NY, USA) microscope using the magnified gridline intersect method (McGonigle *et al.* 1990), with at least 100 intersections scored per sample except for species with low root biomass, where we scored at least 50 intersections. An intersection was considered colonized if AM hyphae, arbuscules or vesicles were present. The proportion of root intersections occupied by arbuscules was also recorded. Stained roots were also analysed for colonization by non-AM fungi, which are distinguishable from mycorrhizal hyphae by their linear hyphae and regular septa. Because of their limited root mass, roots of *Asclepias hirtella* and *Asclepias humistrata* were completely devoted to cardenolide measurements as described later.

ANALYSIS OF ROOT CARDENOLIDES

Root cardenolides were assessed following established protocols (Zehnder & Hunter 2007). Briefly, about 25 mg of root tissue was weighed and ground in a ball mill, then extracted in 1 mL of methanol using a sonicating water bath at 50 °C for 60 min. Samples were dried under vacuum and resuspended in methanol with digitoxin (Acros Organics) as an internal standard and analysed by UPLC (Waters Inc, Milford, MA, USA) using an Acquity BEH C18 column (1·7 μm, 2·1 × 50 mm, Waters). Each 2 μL of injection was eluted at a constant flow of 0·7 mL min^{-1} with a gradient of acetonitrile (ACN) and water, maintained at 20% ACN for 3 min, increasing to 45% ACN through the nine-minute run. Peaks were detected by a diode array detector at 218 nm, and absorbance spectra were recorded from 200 to 400 nm. Peaks with symmetrical absorbance between 218 and 222 nm were quantified

as cardenolides (Malcolm & Zalucki 1996). Cardenolide concentrations were calculated using digitoxin as an internal standard and initial root mass, and total cardenolides were calculated as the sum of individual peaks.

STATISTICAL ANALYSIS

To examine the effect of plant species identity and AMF treatment on plant response variables, we used a series of ANOVAs. Because of non-random mortality due to fungus gnats, sample sizes for each species × treatment combination ranged from 10 to 16 and were not balanced among treatments, so we used restricted maximum likelihood to estimate *F* values using function gls in the nlme package (Pinheiro 2012) in R v. 2.15.0 (R Development Core Team 2008). Plant biomass, AMF, non-AM fungi and cardenolide concentrations were log transformed to conform to the assumptions of normality and homoscedasticity. We used a one-way ANOVA to examine whether plant species differed in AMF colonization. Two-way ANOVAs were used to discriminate the presence of AMF and plant species on fungus gnat total plant biomass (above- and below-ground biomass were highly correlated across species; $n = 14$, $r = 0.97$, $P < 0.0001$), root-to-shoot ratio, root cardenolide concentration and larval abundance per plant. *Post hoc* Student's *t*-tests were performed to assess the treatment effect for individual species, and Bonferroni corrections were used to account for multiple tests. The effect of mycorrhizae on non-AM fungi was analysed separately with one-way ANOVAs because of high levels of plant mortality and the subsequent loss of degrees of freedom. We used a t-test to examine how the presence of AMF affected plant mortality, using the proportion of plants surviving for each species by treatment combination as replicates.

In addition to examining the global means, we tested for correlations among traits across individual species means ($n = 14$) by taking in account the effect of phylogenetic non-independence in a maximum likelihood phylogenetic generalized least squares (PGLS) framework (Pagel 1999). A comprehensive phylogeny was pruned with the retention of branch lengths to create a phylogram for the 14 *Asclepias* species used in the experiment (Fishbein *et al.* 2011). Using PGLS, models of trait evolution differing in complexity can be compared using a likelihood ratio test (LR), in which LR = −2[log-likelihood of the better-fitting model−log-likelihood of the worse-fitting model]. Under the assumption of model equivalence, the LR statistic should be chi-square distributed with one degree of freedom (when a single parameter is altered between the models compared). For the phylogenetic independent correlation analyses, in Continuous (Pagel 1999), the LR parameter was estimated from a random-walk model using Pagel's estimated lambda (λ) with and without an estimated covariance.

Specifically, we analysed the effect of larval abundance per plant species on plant mortality. We then examined the effect of AMF colonization, non-AM fungal infestation and root cardenolides on larval density in PGLS framework. We here used larval density (number of larvae per root biomass) to account for effects of root size on fungus gnat abundance (see Results). Finally, we used a phylogenetically corrected multiple regression to account for trait correlations among plant traits to predict larval performance. Using the regression module of Continuous (Pagel 2007), we compared the likelihood values of a full model (with both arbuscular colonization and cardenolides) with likelihood values of models with each predictor trait removed separately. All regressions were performed only on plants that received the mycorrhizal treatment. Number of mycorrhizal arbuscules and hyphae were highly correlated in both raw ($n = 12$, $r = 0.97$, $P < 0.0001$) and a maximum likelihood generalized least squares (PGLS, $n = 12$, $\lambda = 0$, LR = 32·61, $P < 0.0001$) analyses. Therefore, we used arbuscular colonization in all regression analyses involving mycorrhizae.

Results

EFFECTS OF THE MYCORRHIZAL TREATMENT

Mycorrhizal treatment was effective for most *Asclepias* species included in the experiment, with an average of 14% of roots colonized by all AMF structures and 9·5% of the root colonized by arbuscules across all milkweed species in the mycorrhizal treatment, compared to 0·4% and 0·1% colonization by total AMF and arbuscules, respectively, in the control plants. Plant species varied in the proportion of root that was colonized by AMF (Fig. 1a, species effect; $F_{9,77} = 2.79$, $P = 0.006$). Only *Asclepias tuberosa* and *Asclepias verticillata* were scored as not having any visible AMF or pathogenic colonization in the roots, so plants from these species were removed from analyses that examined how the presence of AMF affected plant traits. Across all species, all but five inoculated plants contained mycorrhizal structures, and only six plants in the non-mycorrhizal treatment were colonized by mycorrhizal structures; these plants were also removed from further analysis.

Plant species varied widely in biomass production (Fig. 1b, species: $F_{10,130} = 16.9$ $P < 0.0001$), and the presence of mycorrhizal fungi tended to decrease slightly plant biomass overall (AMF: $F_{1,130} = 5.66$, $P = 0.02$). Plant species did not differ significantly in their growth response to AMF (species × AMF: $F_{10,130} = 1.51$, $P = 0.12$). In contrast, mycorrhizal colonization changed the distribution of plant biomass, sharply decreasing root-to-shoot ratio by 20% overall (Fig. 1c, AMF: $F_{1,130} = 4.53$, $P = 0.01$). Plant species also differed in root-to-shoot ratio (species: $F_{10,130} = 9.79$, $P < 0.0001$) and mycorrhizal fungi decreased root-to-shoot ratio to a greater extent in some species than in others (AMF × species: $F_{10,130} = 2.07$, $P = 0.03$).

The presence of mycorrhizal fungi did not substantially affect root cardenolide concentration overall (Fig. 1d, AMF: $F_{1,134} = 2.47$, $P = 0.06$), but cardenolide concentration was highly dependent on species identity of *Asclepias* (species: $F_{10,134} = 10.22$, $P < 0.0001$), and the presence of mycorrhizal fungi increased total cardenolide concentration in some species but not in others (AMF × species $F_{10,134} = 5.04$, $P < 0.0001$).

Species of *Asclepias* varied in larval density (Fig. 1e, species effect: $F_{10,123} = 19.86$, $P < 0.0001$), and AMF decreased fungus gnat infestation to a greater degree in some species than others (AMF × species $F_{10,123} = 2.33$, $P < 0.02$). On average, the presence of mycorrhizal fungi decreased the density of root-feeding fungus gnat in plant roots by 28% overall, although the main effect was not statistically significant (AMF: $F_{1,123} = 0.026$, $P = 0.87$). In addition, roots in the mycorrhizal treatment were colonized 70% less by non-AM fungi than were their non-mycorrhizal counterparts (treatment effect: $F_{1,67} = 7.17$, $P < 0.01$, and species effect: $F_{9,67} = 1.34$, $P = 0.23$). Among plant species that hosted AMF, the mycorrhizal treatment decreased plant mortality by an average of 27% (Fig. 2, $t = 2.29$, $P = 0.01$).
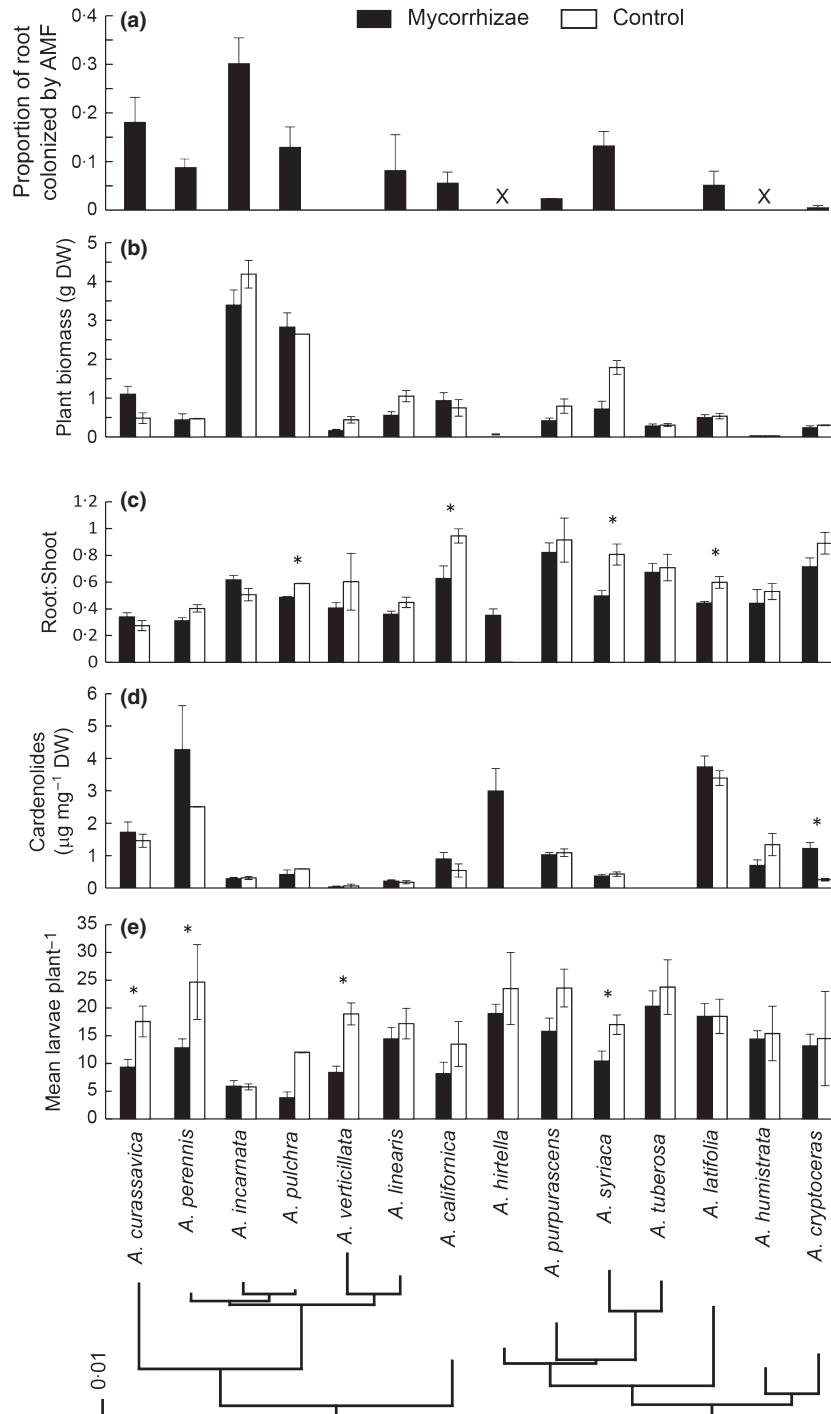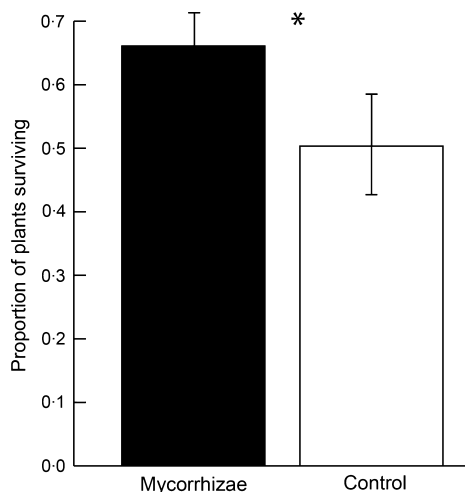
Fig. 1. Effect of mycorrhizal treatment on (a) proportion of roots colonized by arbuscular mycorrhizal fungi (AMF) (X represents missing data and not null mycorrhization), (b) total plant biomass, (c) root-to-shoot ratio, (d) total cardenolides and (e) fungus gnats larvae per plant for all 14 species of milkweeds. Shown are means (±1SD). Black bars represent mycorrhizal treatment, and open bars represent control, non-mycorrhizal plants. (*<0·05, *t*-test). Bottom of figure shows the phylogenetic relationship of all 14 species of *Asclepias*. Graph pruned after Fishbein *et al.* (2011).

PHYLOGENETIC ANALYSIS OF PLANT DEFENCE TRAITS AND LARVAL PERFORMANCE

To examine how mycorrhizal colonization affected plant traits and larval abundance in roots, all subsequent analyses were performed only on plants inoculated with mycorrhizal fungi. Overall, we detected little or no phylogenetic signal for most traits we measured (λ values for root biomass = 0·11, for AMF in roots = 0·08, for cardenolides = 0, and for non-AM fungi = 0). Interestingly, however, plant mortality had a λ value = 1 and abundance of larvae in roots had a λ = 0·44. We used the estimated

Fig. 2. Effect of mycorrhizal treatment on the proportion of *Asclepias* plants surviving to the end of the experiment. Bars represent the average proportion of plants surviving among all species for mycorrhizal treatment (black bars) and control (open bars) plants ± 1SE.

phylogenetic signal for all subsequent analyses. In mycorrhizal plants, we observed a positive correlation between plant mortality and larval infestation (Fig. 3, PGLS, $n = 14$, $\lambda = 0.89$, $r = 0.52$, LR = 4.76, $P = 0.03$). Plant species with larger root biomass hosted fewer larvae overall (PGLS, $n = 14$, $\lambda = 0.21$, $r = -0.66$, LR = 8.06, $P = 0.01$), so we controlled for AMF-induced changes in plant biomass by dividing the number of larvae by total root biomass and used this larval density for subsequent analyses. Overall, plant species that hosted higher larval densities were significantly more prone to mortality than those with low larval density, across 14 *Asclepias* species (PGLS, $n = 14$, $\lambda = 0.89$, $r = 0.81$, LR = 14.21, $P < 0.001$).

We next asked which plant traits might confer protection against this generalist below-ground herbivore. Specifically, we examined the effect of mycorrhizal colonization, root cardenolide concentration and non-AM fungi in pre-
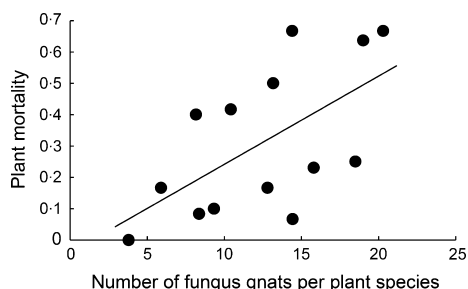
Fig. 3. Correlation between average plant mortality and fungus gnat colonization of plant roots. Points represent the average plant mortality and number of fungus gnats per plant for each of the 14 species of milkweed studied. The relationship is significant even when taking in account phylogenetic relationship between species.

dicting fungus gnat abundance. Because AMF treatment also affected plant root biomass allocation (Fig. 1c) in a species-specific matter, we used larval density in roots as mentioned earlier as a response variable. Arbuscular colonization negatively predicted larval density (Fig. 4a, PGLS, $n = 12$, $\lambda = 0$, $r = -0.69$, LR = 4.49, $P = 0.03$). However, neither the proportion of root colonized by non-AM fungi nor increasing levels of cardenolides were associated with larval density (non-AM: Fig. 4b, PGLS, $n = 12$, $\lambda = 0$, $r = 0.43$, LR = 2.46, $P = 0.12$, cardenolides: Fig. 4c, PGLS, $n = 14$, $\lambda = 0$, $r = 0.22$, LR = 0.50, $P = 0.48$). In addition, average cardenolide concentration per plant species did not affect AMF colonization (PGLS, $n = 12$, $\lambda = 0$, $r = -0.21$, LR = 0.55, $P = 0.46$), nor did the presence of non-AM fungi (PGLS, $n = 12$, $\lambda = 0$, $r = -0.29$, LR = 1.14, $P = 0.29$).

We then attempted to disentangle the effects of AMF, non-AM fungi and cardenolides on larval density in a
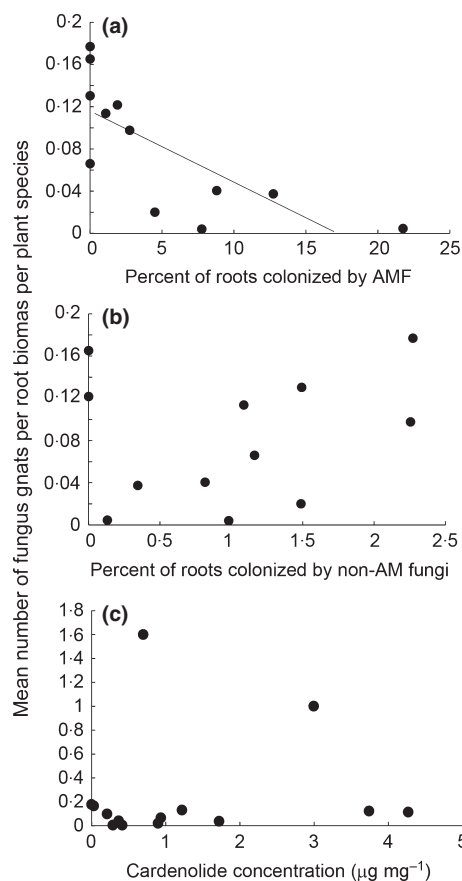
Fig. 4. Larval abundance per unit of root biomass as a function of (a) arbuscular mycorrhizal fungi (AMF) root colonization by arbuscules, (b) non-AM fungi in roots and (c) cardenolide across 12 species of *Asceplias*. *Asclepias hirtella* and *Asclepias hirsuta* are only included in the root cardenolide analysis ($n = 14$). High larval density of these two species (two outliers in panel c) results from low root biomass compared to the other milkweed species. Only the number of mycorrhizal fungi negatively predicted the abundance of the herbivore larvae in phylogenetically corrected analyses.

multivariate phylogenetic framework (Table 1). Removal of the total arbuscular colonization from the full model significantly decreased the ability to predict larval abundance, but the removal of cardenolide concentration or non-AM fungi did not affect model fit (Table 1).

Discussion

We investigated how AMF mediate interactions between a generalist soil-dwelling herbivore and plants in a comparative phylogenetic framework. Our results reveal the potential of AMF symbionts to modify plant–herbivore interactions, even when fungal colonization has weak effects on plant biomass. Mycorrhizal plants exhibited reduced larval density of the herbivorous fly, leading to higher survivorship. In contrast to our predictions, this protective effect was not associated with among-species variation in root cardenolide concentration or mycorrhizal-induced changes in cardenolides. This was confirmed in a multivariate phylogenetic analysis, where the removal of AMF, but not cardenolides, from the model significantly decreased its ability to predict colonization by fungus gnats across 14 species of milkweeds. Instead of increased expression of resistance in roots, the level of colonization by mycorrhizal fungi most closely predicted the protective effect of mycorrhizal fungi on plants exposed to below-ground herbivores.

EFFECT OF AMF ON MILKWEED PERFORMANCE AND CARDENOLIDES

Our inoculation treatment was largely effective, but *A. verticillata* and *A. tuberosa* remained free of mycorrhizae (Fig. 1a). Additionally, plants colonized by mycorrhizal fungi also tended to have slightly lower biomass than non-mycorrhizal plants (Fig. 1b), although some plant species (e.g. *A. curassavica* and *A. californica*) tended to be larger in the mycorrhizal treatment. The fungal species in our inoculum could represent a novel AMF community for some *Asclepias* species in our experiment, which may con-

Table 1. Phylogenetically independent multiple regression analysis of total root cardenolides, non-AM infection and AMF colonization as predictors of fungus gnats' larval abundance across 14 species of *Asclepias*

	Full	−Cardenolides	−Non-AM	−AMF (arbuscles)
LH	21·10	21·09	21·06	17·80
LR		0·02	0·08	6·60
P		0·90	0·78	**0·01**

AMF, arbuscular mycorrhizal fungi.
Probability values are derived from likelihood ratio (LR), calculated as two times the difference between the likelihood (LH) of the full model minus the LH of the model lacking each of the plant traits measured. A *P* value < 0·05 (in bold) indicates a significant effect of the trait on larval survival when all other traits and phylogenetic non-independence are taken in account.

tribute to the observed effects of mycorrhizal inoculum on plant biomass. A constant AMF inoculum allowed us to compare the response of many *Asclepias* species to a single fungal community, although future experiments should attempt to include native AMF communities if possible. In addition, most of the fungi used in this experiment are cosmopolitan species found in many habitats (Öpik *et al.* 2006), including those where milkweeds also occur and are likely to form associations with *Asclepias* species in natural systems. We believe that the growth conditions imposed in this experiment, although somewhat artificial, might represent those in disturbed habitat where many *Asclepias* species commonly grow. In addition, plants in our experiment shifted allocation of biomass to shoots in the mycorrhizal treatment, a common response to mycorrhizal colonization (Marler, Zabinski & Callaway 1999; Smith & Read 2008; De Deyn *et al.* 2009). Furthermore, the slight plant growth depression in response to the AMF inoculum is not uncommon: similar results are frequently documented, and some can be explained by high level of P in the soil (Graham & Abbott 2000; Sena, Labate & Cardoso 2004), variation in mycorrhizal abundance, species or genotype identity (De Deyn *et al.* 2009; Vannette & Hunter 2011b), or a short time period since establishment of the mycorrhizal association (e.g. Ronsheim 2011). In short, we believe that plant responses to AMF in our experiment are ecologically relevant.

In contrast to our predictions, plant species responded to the mycorrhizal treatment differentially in the expression of total root cardenolides. The direction and extent of this change was not directly related to the degree of AMF colonization, or AMF-induced changes in plant biomass or root-to-shoot ratio among species (Fig. 1). Tao & Hunter (2012) recently demonstrated that fertilization with nitrogen, but not phosphorus, increases cardenolide concentration in *A. syriaca*. The degree of change in root cardenolides in *Asclepias* species may thus depend on N benefit gained from AMF among plant species.

EFFECTS OF MYCORRHIZAL FUNGI ON BELOW-GROUND HERBIVORES

In our experiment, mycorrhizal fungi altered plant–herbivore interactions, increasing plant survival in the presence of below-ground herbivores. The degree of plant associations with AMF, rather than root chemistry, determined the effect of mycorrhizae on plant–herbivore interactions. In addition, mycorrhizal colonization of plant species decreased the density of below-ground herbivores and plant mortality independently of root biomass and shared ancestry between plants. This result is consistent with previous work that also documents the protective effects of AMF against generalist root herbivores, assessed in single species of plants. Similarly, mycorrhizal colonization decreases the performance of the generalist root feeder, *O. sulcatus*, on *Fragaria × ananassa* and *T. officinale* (Gange, Brown & Sinclair 1994; Gange 2001). Other root

herbivores such as nematodes are known to avoid roots already colonized by micro-organisms (Piskiewicz, Duyts & van der Putten 2008, 2009). However, results of such experiments are system-specific and the opposite effect has also been demonstrated. For example, the clover root weevil *Sitonalepidus,* a specialist root feeder, displayed higher survival when fed on mycorrhizal host plants than on non-mycorrhizal plants (Currie, Murray & Gange 2011).

The mechanisms underlying the effect of AMF on root herbivores remain unclear – besides a possible oviposition choice by *Bradysia* females, which has not been previously documented, changes in root structure, nutrition or chemistry may have mediated this effect (Gange 2001; Rasmann *et al.* 2011a). Among *Asclepias* species, we documented a minor increase in root chemical defence with mycorrhizal colonization, but plants with higher cardenolide expression did not have lower gnat densities. This is in contrast with a previous study using an *Asclepias* specialist root herbivore. Rasmann & Agrawal (2011a) found that the survival of root-feeding *Tetraopes tetraophthalmus* larvae on 18 species of milkweeds was predicted by phylogenetic distance from the true host plant (*A. syriaca*). When dissecting putative plant-related and ecological traits, both cardenolides and the habitat of the plant were shown to contribute to the observed pattern (i.e. more cardenolides in plant species reduced larval survival, and plant species from extreme habitats were also poor hosts). While AMF-mediated increases in root defence may be effective against some herbivores or pathogens (Gange, Brown & Sinclair 1994; De Deyn *et al.* 2009), this was not the case in our system. Here, we show that cardenolides have little to no effect on this generalist soil herbivore. Recent reviews have highlighted a series of possible mechanisms insects have adopted to cope with toxic cardenolides (Dobler, Petschenka & Pankoke 2011; Agrawal *et al.* 2012). Our study would suggest that *Bradysia* flies are insensitive to these molecules as well, but future physiological studies are needed to confirm this.

Rather, the proportion of the root containing mycorrhizal fungi was correlated with reduced fungus gnat infestation and decreased plant mortality. We suggest that local interactions between fungi, roots and larvae mediate the protective role of AMF: increased structural defence, improved plant tolerance to the effects of root feeders, suppression the colonization of roots by non-mycorrhizal fungi or fungi serving as an alternative food source may have improved plant survival (Kennedy 1974). Indeed, previous studies have also documented protective effects of AMF against below-ground herbivores mediated through local interactions. For example, in a split-root experiment, mycorrhizal colonization of dune grass *Ammophila arenaria* reduced infestation and reproduction of root-feeding nematode *Pratylenchuspenetrans*, but only when these organisms co-occurred in the same root compartment (de la Peña *et al.* 2006). In addition, previous studies have demonstrated that AMF colonization increases root life span (Atkinson *et al.* 2003), likely mediated through the changes in root structure and morphology, which may also affect root herbivory (Rasmann *et al.* 2011a).

While the effects of AM fungi on below-ground herbivores are likely mediated by changes in root morphology or another indirect mechanism, we cannot rule out the role of mycorrhizal fungi in the production of mycotoxins (Schulz *et al.* 2002), allelochemicals that deter or are toxic to the larvae entering in contact with the fungus. In addition, AMF can also affect plant tolerance to or induction of defences against herbivores (Bennett, Bever & Bowers 2009; Garrido *et al.* 2010; Kempel *et al.* 2010), but we were unable to examine these effects as all plants were infested by larvae to some extent. Positive feedbacks among plants, AMF and herbivores may also benefit plants (Hoffmann, Vierheilig & Schausberger 2011). Root grazing by insect herbivores can increase AMF colonization (Currie, Murray & Gange 2006). This in turn would provide plant with higher tolerance to grazing via increased nutrient acquisition as well as enhancement of the direct protective benefits described earlier.

In addition to the protective benefits conferred by mycorrhizal fungi against below-ground herbivores, we also identified a negative correlation between mycorrhizal colonization and non-AM fungi colonization of roots. Indeed, mycorrhizal colonization often protects plants against pathogenic fungi (Borowicz 2001; Pozo & Azcon-Aguilar 2007), particularly when the AMF colonist is a member of the Glomeraceae (Sikes, Cottenie & Klironomos 2009). When insect herbivores are also vectors for pathogenic fungi, as are *Bradysia* spp. (Gillespie & Menzies 1993) mycorrhizal fungi may provide dual benefits to plant hosts. Finally, our soil treatment did not include a microbial wash, which may contribute to the effects documented here. We speculate this to be unlikely. AMF have been shown to protect plants from pathogenic microbial attack (Newsham, Fitter & Watkinson 1995).

Conclusions

Our results suggest that the local effects of mycorrhizal colonization, rather than AMF-mediated changes in fine root chemistry, protect milkweeds against a generalist below-ground herbivore. This effect was robustly documented by using a comparative phylogenetic approach, which demonstrated a consistent effect of mycorrhizal colonization across many *Asclepias* species. Colonization by AMF protects *Asclepias* against below-ground herbivores, and this effect may be mediated by multiple protective factors including increased vigour, tolerance and resistance to secondary infection by pathogens. Although AMF did not increase the growth of most plant species, increased protection against herbivory and survival in the presence of root herbivory might represent a mechanism for mycorrhizal fungi to increase plant fitness and contribute to the evolutionary stability of the mycorrhizal mutualism. Future studies should further examine the potential direct

mechanisms by which plant symbionts mediate multi-trophic interactions, particularly below-ground.

Acknowledgements

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Plant traits including plant biomass, plant mortality and cardenolide levels, fungus gnat larvae abundance in roots, number of hyphae, arbuscules, and non-AM fungi in roots of 14 mycorrhized species of milkweeds (*Asclepias* spp).

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