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Plant and insect genetic variation mediate the impact of arbuscular mycorrhizal fungi on a natural plant–herbivore interaction

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Abstract. 1. While both arbuscular mycorrhizal (AM) fungi and plant and insect genotype are well known to influence plant and herbivore growth and performance, information is lacking on how these factors jointly influence the relationship between plants and their natural herbivores.

2. The aim of the present study was to investigate how a natural community of arbuscular mycorrhizal fungi affects the growth of the perennial herb *Plantago lanceolata* L. (Plantaginaceae), as well as its interaction with the Glanville fritillary butterfly [*Melitaea cinxia* L. (Nymphalidae)]. For this, a multifactorial experiment was conducted using plant lines originating from multiple plant populations in the Åland Islands, Finland, grown either with or without mycorrhizal fungi. For a subset of plant lines, the impact of mycorrhizal inoculation, plant line, and larval family on the performance of *M. cinxia* larvae were tested.

3. Arbuscular mycorrhizal inoculation did not have a consistently positive or negative impact on plant growth or herbivore performance. Instead, plant genetic variation mediated the impact of arbuscular mycorrhizal fungi on plant growth, and both plant genetic variation and herbivore genetic variation mediated the response of the herbivore. For both the plant and insect, the impact of the arbuscular mycorrhizal community ranged from mutualistic to antagonistic. Overall, the present findings illustrate that genetic variation in response to mycorrhizal fungi may play a key role in the ecology and evolution of plant–insect interactions.

Key words. Arbuscular mycorrhizal fungi, genetic variation, *Glomeromycota*, *Melitaea cinxia*, multitrophic interaction, *Plantago lanceolata*, plant–herbivore interaction.

Introduction

Studies of belowground–aboveground interactions have shown that soil biota can play an important role in plant growth and plant defence against natural enemies (Bezemer & van Dam, 2005; Tack *et al.*, 2015; Biere & Goverse, 2016). However, we lack insights into how genetic variation in the aboveground community influences the nature of interactions among the aboveground and belowground communities. Importantly, such interactions between the soil biota and genetic variation within the aboveground community may influence the ecology and evolution of aboveground species interactions (Tack *et al.*, 2015; Mursinoff & Tack, 2017). To explore this within a multitrophic context, we may first assess how soil biota and plant genetic

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variation jointly affect plant growth. Next, we can pinpoint how the response of the plant, in combination with genetic variation in the natural enemy, affects the performance of organisms at higher trophic levels.

One particularly prominent group of soil organisms, the arbuscular mycorrhizal (AM) fungi, are important root symbionts that associate with more than 80% of all terrestrial plant species (Smith & Read, 2008). AM fungi share hard-to-acquire nutrients in exchange for carbon (Smith & Smith, 2011), and thereby play a key role in aboveground community dynamics and ecosystem functioning owing to the effects that the fungi can have on plant growth, community structure, and nutrient cycling (Hartnett & Wilson, 1999; Klironomos *et al.*, 2000; van der Heijden *et al.*, 2008, 2015). In addition, the fungi may provide other benefits to the plant, such as protection against drought, pathogens, and herbivores (Gange & West, 1994; Newsham *et al.*, 1995; Augé, 2001). One of the many ways AM fungi may influence herbivores is through, often positive, changes in plant growth and nutritional quality, as well as changes in defence-related pathways and plant priming for defence (Bennett *et al.*, 2006; Gehring & Bennett, 2009; Jung *et al.*, 2012). The role of AM fungi is not the same for all plant–insect species interactions. Notably, specialised herbivore species more often perform better on AM fungal plants than generalists (Koricheva *et al.*, 2009). In addition, differences have been found in how herbivores respond to AM fungi depending on their feeding guild (e.g. sucker, chewer) leading to either positive (Gange & West, 1994; Goverde *et al.*, 2000; Koricheva *et al.*, 2009) or negative (Gange & West, 1994; Koricheva *et al.*, 2009) effects of AM fungi on herbivore performance.

A review by Gehring and Bennett (2009) made the convincing case that we need to use natural AM fungal communities to further advance our understanding of the role of AM fungi in natural systems. Indeed, the interaction of plants with a diverse natural AM fungal community may explain the strong variation in the impact of AM fungal inoculation on plant resistance: interactions between multiple AM fungal species may either reduce the impact (by cancelling out positive and negative effects) or have a consistently positive or negative effect on plant defences (Bennett *et al.*, 2009; De Deyn *et al.*, 2009). However, the majority of studies on AM fungi and their effect on plants and their natural enemies conducted thus far have used inoculations by only a single AM fungal species or commercial inocula, often from the genus *Glomus* (Gehring & Bennett, 2009). These studies have provided elegant demonstrations of the importance of AM fungal identity on both plants (Koch *et al.*, 2006; Vogelsang *et al.*, 2006; Scheublin *et al.*, 2007) and their natural enemies (Gange, 2001; Gange *et al.*, 2005; Bennett & Bever, 2007; Wooley & Paine, 2007). However, some recent studies have compared the effects of using inoculations with single species and isolates with that of multiple species (Gange *et al.*, 2005; Bennett *et al.*, 2009; Currie *et al.*, 2011; Roger *et al.*, 2013; Ortas & Ustuner, 2014). Emam (2016) found that natural communities of AM fungi and not commercial inocula increased plant growth.

Genetic variation in the plant and herbivore may affect the outcome of direct and indirect interactions with AM fungi. In an experiment where researchers mimicked natural herbivore damage by manually removing leaf area, Garrido *et al.* (2010) demonstrated that the response of plants to simulated herbivory differed between plant families when inoculated with commercial AM fungal inoculum (*Glomu*s sp.). In another study, Kant *et al.* (2008) showed that the response of spider mites to plant defences was determined by the spider mite genotype. Lastly, Bennett *et al.* (2016) used a natural community of AM fungi to investigate the multitrophic interactions between AM fungi, potatoes, aphids, and parasitoids and the role of plant genetic variation. They found that the effect of AM fungi and the potato genotype did not have a direct, measurable impact on aphid performance, but that the effect of AM fungi and potato genotypes was found to influence the performance of the parasitoids. These studies show that AM fungi and plant genetic variation have the potential to impact multiple trophic levels. However, we lack studies that investigate the impact of both plant and herbivore genetic variation, especially in natural systems and with natural communities of AM fungi.

In the present, we set out to investigate how a natural community of AM fungi, as well as plant and insect genetic identity, affect the interaction between the perennial herb *Plantago lanceolata* (Plantaginaceae) and its specialist herbivore *Melitaea cinxia* (Lepidoptera: Nymphalidae). More specifically, we investigated (i) the impact of mycorrhizal fungi and plant line on plant growth, and (ii) the impact of mycorrhizal fungi, plant line, and insect family on larval growth. As a result of the positive effect that AM fungi can have on plant nutrition, we expected that AM fungi would positively affect the growth of *P. lanceolata*. Nutritional effects may cascade up to higher trophic levels, and thereby also positively affect herbivore performance. However, as based on the variable responses of genetic lineages reported in the literature, we expected that both plants and larvae would be influenced differently by AM fungal colonisation as depending on their genetic background.

Materials and methods

Study organisms

Plantago lanceolata is a rosette-forming, perennial herb that has a cosmopolitan distribution. It has wind dispersed pollen and is an obligate outcrosser as a result of several outcrossing mechanisms (Cavers *et al.*, 1980; Krohne *et al.*, 1980). It contains high amounts of iridoid glycosides, a group of defensive terpenoids (El-Naggar & Beal, 1980) which are sequestered by specialised herbivores such as *M. cinxia* (Suomi *et al.*, 2003), perhaps as a protection against generalist predators (Harvey *et al.*, 2005; Lampert *et al.*, 2014).

The butterfly *M. cinxia* is widely distributed in Europe, but in Finland, it is only found on the Åland Islands (Marttila *et al.*, 1990). In June, females lay eggs in clusters of around 150–200 on the underside of leaves of one of its two host plants, *P. lanceolata* and *Veronica spicata* (Plantaginaceae). Eggs hatch in July and larvae live gregariously in groups of full siblings in silken webs. Larvae stay in groups as they diapause for the winter, often in their fifth instar and feed gregariously in the spring until the seventh, or in some cases eighth, larval

Fig. 1. The location of the Åland Islands, where the inset shows the three *Plantago lanceolata* populations from which seeds (A, B, C) and soil used for inocula (A) were collected.

instar, when they disperse to pupate (Kuussaari *et al.*, 2004; Saastamoinen *et al.*, 2013).

Study site and field sampling

The seeds of *P. lanceolata*, soil for extraction of the AM fungal spores, and adult butterflies were all collected from the Åland Islands, Finland (Fig. 1). Within this study area, *P. lanceolata* plants are found in *c*. 4000 small meadows, some of which are occupied by the butterfly *M. cinxia* (Ojanen *et al.*, 2013).

Seeds of *P. lanceolata* were collected from a total of 10 mother plants (henceforth '*plant lines*') from each of three *P. lanceolata* populations within the Åland Islands, with three plant lines collected from population A, four from population B, and three from population C. In spring 2013, wild butterflies were collected from multiple sites on the Åland Islands and interbred. One egg clutch from each of 10 wild-caught females were used in the experiment. These egg clutches consisted of full siblings, and are henceforth referred to as '*larval family*'. None of the butterflies were collected from the same sites as plants and soil were collected. To minimise environmental variation, the larvae were kept in growth chambers (LD 16:8 h at 28:10 °C) and fed with mixed wild-collected *P. lanceolata* plants before the start of the experiment.

Soil was collected from a single *P. lanceolata* population (population A), based on a preliminary survey that showed a high diversity of AM fungal spores within this population. The litter layer was removed and soil was collected from a depth of 0–15 cm, corresponding to the root depth. The AM fungal community was extracted using the wet sieving and sucrose density gradient centrifugation method (Daniels & Skipper, 1982). Three replicate subsamples of the inoculum had 31.33 ± 64.35 spores ml^{-1} consisting of an average of eight morphospecies, matching inoculum size and diversity of that found by Bennett *et al.* (2016). This method has been used extensively and consistently results in mycorrhizal colonisation of plant roots (e.g. Bennett *et al.*, 2016; Karley *et al.*, 2017). Notably, soil sterilisation removes most soil organisms, and the addition of AM fungal inocula adds both AM fungi and microbes associated with or co-extracted with the AM fungi. To control for this microbial community, we extracted the community of soil microbes from the mycorrhizal inoculum (a microbial wash) by vacuum filtering a portion of the inoculum through a Grade 1 11 -μm Whatman filter paper (125 mm, Buckinghamshire, England), and autoclaved half of the collected microbial inoculum (Bennett *et al.*, 2016). We then added live microbial wash to plants in the sterile treatment and sterile microbial wash to plants in the live treatment, resulting in equal volumes of live microbes added to each treatment. This approach incorporates both AM fungi and their associated microbial communities but allows us to compare the effect of AM fungi only (Koide & Li, 1989). One-millilitre liquid AM fungal inoculum (either live or sterilised) and 1 ml liquid microbial wash (either live or sterilised) was pipetted into the root zone.

The impact of arbuscular mycorrhizal fungi on plant performance

In this experiment, we assessed the impact of mycorrhizal fungi and plant line on plant performance. Before implementing the experimental AM fungal treatment, the seeds of *P. lanceolata* were grown for 3 weeks in 700-ml pots filled with autoclaved

soil (121 °C for 2 h, repeated twice). We then divided the plants into two treatments: half of the plants were inoculated with AM fungal inoculum ($n = 50$ plants), and the other half was inoculated with sterilised inoculum (autoclaved at 121 °C), thereby serving as a control ($n = 50$ plants). Plant measurements (leaf width of the longest leaf, leaf length of the longest leaf, and a total number of leaves) were taken 10 and 50 days after inoculation. Plants were grown under greenhouse conditions and watered regularly. The positions of the plants were randomised several times over the course of the experiment.

The impact of arbuscular mycorrhizal fungi on insect performance

We used a subset of plant lines ($n = 6$) from two populations (populations A and B) to investigate the impact of mycorrhizal inoculation on larval performance (18 mycorrhizal plants; 18 control plants; see Table S1 in File S1 for details on replication). To determine how larval performance was affected by both mycorrhizal treatment and its interaction with the plant and herbivore genetic variation, the plants were subjected to herbivory by adding 15 third instar *M. cinxia* larvae to each plant 22–24 days after AM fungal inoculation. Placement date varied due to small differences in moulting time. To test for differences among larval families, we split each larval family into subsets of 15 larvae. The larval groups were assigned to the experimental plants and treatments at random. This was due to a lack of replication of some plant lines and larval families in both mycorrhizal treatments. Larval weight per group of 15 larvae ($n = 36$) was measured before the groups were placed on the plants and after 6 and 12 days. After 12 days of feeding on the experimental plants, the larvae were removed and placed in Petri dishes in growth chambers (LD 16:8 h at 28:10 °C). The larvae were fed either leaves from mycorrhizal or control plants (depending on their treatment) until they stopped eating in preparation for diapause (on average 10 days). As a result of a lack of plant material from some plant lines, we fed the larvae a mixture of leaves irrespective of the plant line. For a subset of larval groups ($n = 25$), we recorded three additional performance measures at the level of the individual larva: developmental time (i.e. days to diapause), fresh weight at diapause (with an accuracy of 0.0001 g), and the percentage fat per dry weight at diapause. To avoid pseudoreplication, we calculated averages per larval group for use in subsequent analyses. To determine the total amount of fat, we followed a modified procedure of Knapp and Knappová (2013). First, larvae were dried at 60 °C for 24 h and weighed. Then a mix of 1:1 diethyl ether and chloroform was added to each larva, and after 48 h this solvent was removed, and the larvae were dried for an additional 72 h at 60 °C. The total fat content was then calculated by subtracting the weight at 72 h from the weight at 24 h of drying, and the fat percentage of each individual larva was calculated by dividing the total weight of fat for each larvae by their initial dry weight after 24 h.

Statistical analyses

We analysed the data using a two-step approach. First, we assessed the impact of mycorrhizal inoculation on plant performance and explored whether there was variation between plant lines within and among plant populations in the response of plants to mycorrhizal inoculation. Second, we analysed larval performance on a subset of plant lines to explore how mycorrhizal inoculation, plant line, and larval family was related to larval performance. The analyses were conducted in R v. 3.1.2 (R Core Team, 2014).

The impact of arbuscular mycorrhizal fungi on plant performance. To determine the effect of mycorrhizal inoculation on plant performance, we modelled plant growth traits as a function of the fixed effect '*AMF treatment*' (inoculated or control). To account for variation in plant growth among plant populations, as well as among plant lines within plant populations, we included the variables '*Plant population*' and '*Plant line*' (as nested within '*Plant population*') as random effects. Moreover, we included two interaction terms to assess the spatial scale (within or among populations) at which plants are genetically differentiated in their response to arbuscular mycorrhizal fungi: (i) to compare populations in their response to inoculation by arbuscular mycorrhizal fungi, we included the interaction between '*AMF treatment*' and '*Plant population*'; and (ii) to assess the response of plant individuals within populations to inoculation by arbuscular mycorrhizal fungi, we included the interaction between '*AMF treatment*' and '*Plant line*' (as nested within '*Plant population*'). According to previous studies on the ecology and evolution of *P. lanceolata*, we focused on the plant traits leaf length, leaf width, number of leaves, leaf allometry, leaf size, and leaf area (Bowers & Stamp, 1993; Case *et al.*, 1996; van Hinsberg & Tienderen, 1997; Reudler Talsma *et al.*, 2008). Models were implemented using the package *lme4* (Bates *et al.*, 2014). The function ANOVA in the *car* package (Fox & Weisberg, 2011) was used to determine the significance of the fixed effects, and the function *rand* in the package *lmerTest* (Kuznetsova *et al.*, 2015) was used to assess the significance of the random effects. The function *glht* in the package *multcomp* was used to run pairwise comparisons (Hothorn *et al.*, 2008). We refer to Table S2 in File S1 for details on the calculation of the response variables, the number of replicates, and transformations.

The impact of arbuscular mycorrhizal fungi on insect performance. To assess the impact of mycorrhizal inoculation, plant line, and larval family on larval performance, we modelled larval growth as a function of the fixed effects '*AMF treatment*' (inoculated or control), '*Plant line,*' and '*Larval family*'. We here added '*Plant line*' as a fixed effect as we only used a limited subset of the original set of plant lines. We focused on plant line *per se* (rather than on genetic variation among plant populations), as (i) we were only looking at a subset of plant lines from two populations and (ii) the results on plant performance (see Results) showed that the main variation in plant performance was within plant populations. To assess differences among plant lines and larval families in response to AM fungal inoculation, we included the two-way interactions '*AMF treatment* × *Plant line*' and '*AMF treatment* × *Larval*

Table 1. The impact of mycorrhizal (AMF) treatment and plant genetic variation on plant growth traits at 10 and 50 days post inoculation (DPI).

	AMF treatment			Population			Plant line (nested in population)			AMF treatment × population			AMF treatment × plant line (nested in population)		
Plant growth traits	Df	χ^2	P	Df	χ^2	P	Df	χ^2	P	Df	χ^2	P	Df	χ^2	P
At 10 DPI															
Leaf length ($n = 100$)	1	0.03	0.86	1	0.00	1	1	7.06	**0.008**	1	0.00	1.00	1	0.00	1.00
Leaf width ($n = 100$)	1	1.11	0.74	1	0.00	1	1	5.03	**0.02**	1	0.00	1.00	1	0.15	0.70
Number of leaves ($n = 100$)	1	0.13	0.72	1	0.00	1	1	2.38	0.10	1	0.00	1.00	1	0.00	1.00
Leaf allometry ($n = 100$)	1	1.11	0.37	1	0.00	1	1	7.13	**0.008**	1	0.04	0.85	1	0.00	1.00
Leaf size ($n = 100$)	1	0.02	0.89	1	0.00	1	1	5.81	**0.02**	1	0.00	1.00	1	0.00	0.98
Leaf area ($n = 100$)	1	0.23	0.64	1	0.00	1	1	6.07	1.00	1	0.00	1.00	1	0.00	1.00
At 50 DPI															
Leaf length ($n = 95$)	1	0.09	0.77	1	0.12	0.73	1	0.84	0.36	1	0.00	1.00	1	5.43	**0.002**
Leaf width ($n = 95$)	1	0.90	0.34	1	0.00	1.00	1	5.03	**0.02**	1	0.00	1.00	1	0.15	0.70
Number of leaves ($n = 95$)	1	0.99	0.32	1	1.59	0.21	1	0.00	1.00	1	0.00	1.00	1	4.82	**0.03**
Leaf allometry ($n = 95$)	1	0.57	0.45	1	0.00	1.00	1	7.13	**0.008**	1	0.04	0.85	1	0.00	1.00
Leaf size ($n = 95$)	1	0.41	0.52	1	0.81	0.40	1	1.09	0.30	1	0.00	1.00	1	2.58	0.10
Leaf area ($n = 95$)	1	0.26	0.61	1	0.82	0.37	1	0.00	1.00	1	0.00	1.00	1	6.39	**0.01**

Significant *P*-values are in bold. Number of replicates (n) are listed between brackets.
Shown are the results of a generalised linear mixed model.

family', respectively. Given the low level of replication, we did not include the three-way interaction.

To assess the effect of mycorrhizal inoculation on development time, weight at diapause, and fat percentage, we modelled larval performance as a function of the fixed effect '*AMF treatment*' (inoculated or control), and to determine the effect of variation among larval families we added the fixed effect '*Larval family*'. In addition, to determine how differences among larval families might lead to different responses to mycorrhizal inoculation we included the interaction '*AMF treatment*' and '*Larval family*'. However, unlike the model for larval growth (above), we did not include the factor '*Plant line*', as larvae were fed with a mixed-plant line diet from the last weight measurement until diapause (see Materials and methods for more details). The function *glht* in the package *multcomp* was used to run pairwise comparisons (Hothorn *et al.*, 2008). We refer to Table S2 in File S1 for details on the calculation of the response variables, the number of replicates, and transformations.

Results

The effect of AM fungi on plant performance

At 10 days after mycorrhizal inoculation, the plant lines differed in the majority of plant traits (Table 1). This variation was present among plant lines within populations. At this early stage, we detected no differentiation among plant populations or plant lines within populations in their response to AM fungal inoculation (Table 1). In contrast, 50 days after inoculation there was a strong effect of AM fungal treatment on leaf length, the number of leaves, and leaf area, but both the strength and direction of the effect differed between plant lines (Fig. 2a,b, Table 1). In contrast, traits describing leaf shape (leaf width and leaf allometry) differed among plant lines but were not affected by mycorrhizal inoculation (Table 1).

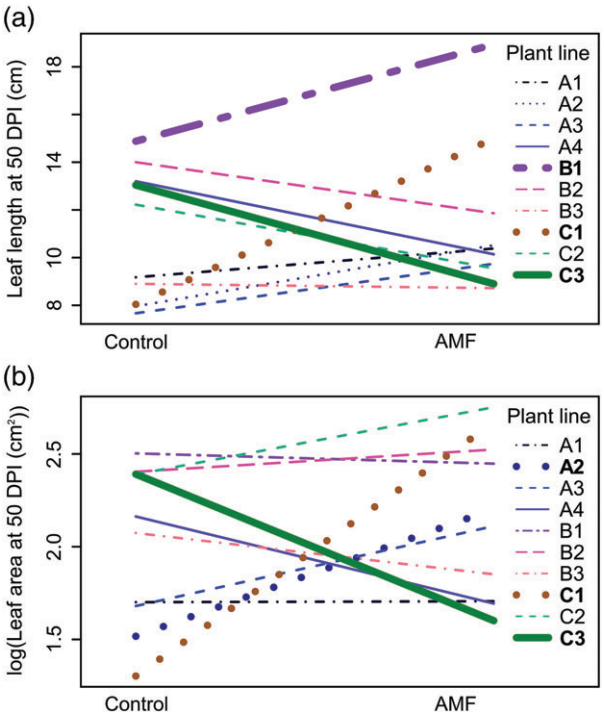

Fig. 2. Interaction plot showing the impact of mycorrhizal treatment and plant genetic variation on growth of *Plantago lanceolata* at 50 days post inoculation (DPI). The lines connect, for each plant line, the mean values for the control and mycorrhizal treatment. Panel (a) shows the interaction between treatment and plant line on leaf length, and panel (b) shows the interaction between treatment and plant line on leaf area. Bold lines indicate a significant difference between the control and mycorrhizal treatment for a given plant line.

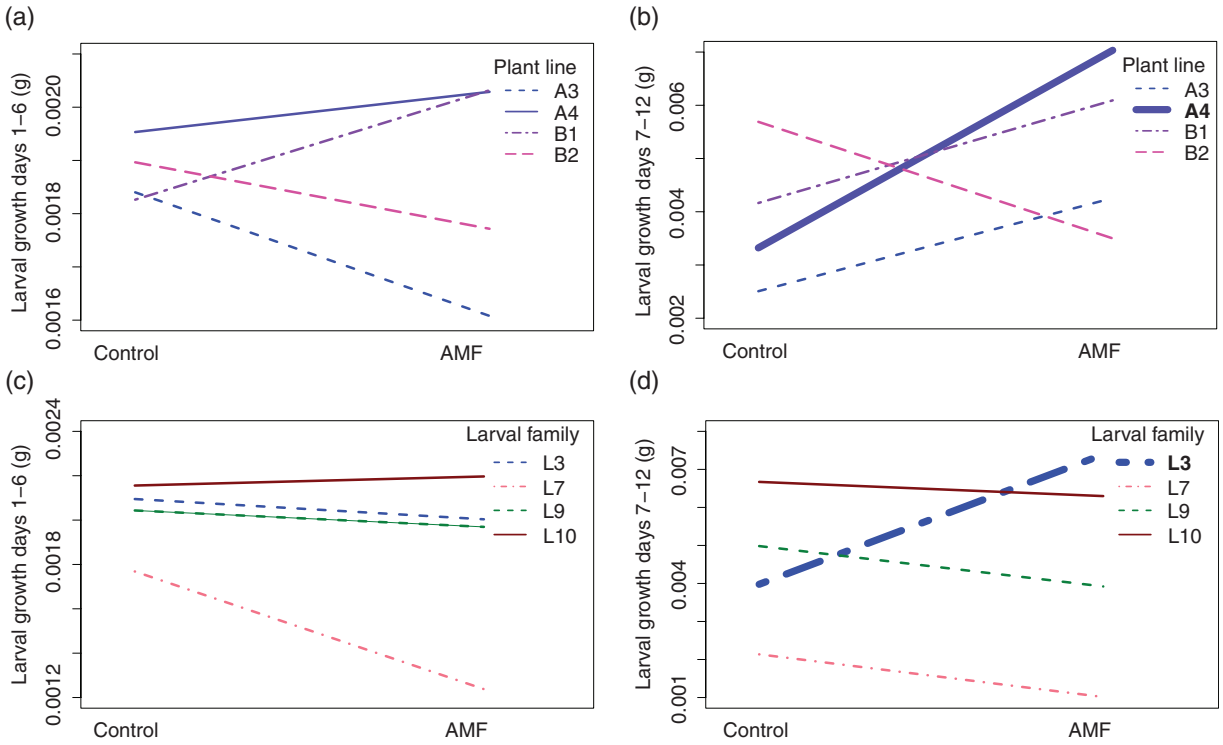

Fig. 3. Interaction plot showing the impact of mycorrhizal treatment and larval (*Melitaea cinxia*) and plant (*Plantago lanceolata*) genetic variation on larval performance. The lines connect, for each plant line (panels a and b) or larval family (panels c and d) the mean values for the control and mycorrhizal treatment. Panels a and b show the interaction between treatment and plant line on larval growth at days 1–6 and days 7–12, respectively. Panels (c) and (d) show the interaction between treatment and larval family on larval growth at days 1–6 and 7–12, respectively. Bold lines indicate a significant difference between the control and mycorrhizal treatment for a given plant line (panels a and b) or larval family (panels c and d). Shown are only those plant lines ($n = 4$) and larval families ($n = 4$) that are replicated within each treatment.

The effect of AM fungi on larval performance

Larval growth between days 1–6 was not affected by either AM fungal treatment, plant line, or larval family; however, larval growth from days 7–12 was affected by both AM fungal treatment and plant line (a significant interaction effect; Fig. 3a,b, Table 2). For days 7–12 the significant effect of the interaction between AM fungal treatment and plant line on larval growth showed that most larvae responded positively to most plant lines inoculated with AM fungi (average increase of 75% on AM fungal plants compared to control plants) while larvae responded negatively to AM fungal treatment on one plant line (38% decrease compared to control plants). Interestingly, the impact of mycorrhizal inoculation on larval growth rate differed by larval family (significant interaction effect; Fig. 3c,d, Table 2). At days 7–12 half of the larval families responded positively to the mycorrhizal treatment (average growth increase of 133% compared to control), whereas the other half responded negatively to AM fungal inoculation (average growth decrease of 25% compared to control). Larval growth was not affected by plant line or by the interaction between mycorrhizal treatment and plant line when taking into account the full 12-day feeding period. However, across the full feeding period, there was a strong effect of the larval family (Table 2). We did not detect an effect of treatment or larval family on the development time, weight at diapause, and larval fat percentage (Table 2).

Discussion

In this study, we set out to investigate the effects of a natural community of arbuscular mycorrhizal fungi, as well as plant and larval genetic variation, on the performance of both a plant and its specialised herbivore. There was no consistently positive or negative effect of AM fungal inoculation on plant performance; instead, our findings illustrate that plant genetic variation mediated the effect of AM fungal inoculation on plant growth. Furthermore, we found that both plant line and larval family mediated the effect of AM fungal inoculation on larval growth. The interactions between AM fungi and plant and larval genetic variation may have pronounced consequences for the ecology and evolution of plant–herbivore interactions.

The effect of AM fungi on plant performance

In this study, we showed that plant line and AM fungi jointly affected plant performance and the suitability of the plant to a herbivore. Similar to this, other studies have shown that

Table 2. The impact of mycorrhizal treatment and plant and larval genetic variation on larval performance.

	AMF treatment			Plant line			Larval family			AMF treatment × plant line			AMF treatment × larval family		
Plant growth traits	d.f.	*F* value	*P*	d.f.	*F* value	*P*	d.f.	*F* value	*P*	d.f.f	*F* value	*P*	d.f.f	*F* value	*P*
Larval growth (days 1–12, $n = 36$)	1	0.99	0.35	5	3.16	0.07	9	8.07	**0.004**	3	2.82	0.11	8	3.41	0.05
Larval growth (days 1–6, $n = 36$)	1	0.01	0.92	5	0.39	0.85	9	1.60	0.25	3	0.46	0.72	8	0.63	0.74
Larval growth (days 7–12, $n = 36$)	1	1.56	0.25	5	3.75	**0.048**	9	10.37	**0.002**	3	4.96	**0.031**	8	3.99	**0.034**
Development time ($n = 25$)	1	0.16	0.70	–	–	–	9	3.02	0.07	–	–	–	6	1.83	0.21
Weight at diapause ($n = 25$)	1	0.88	0.38	–	–	–	9	1.67	0.24	–	–	–	6	1.74	0.23
Fat percentage ($n = 25$)	1	0.21	0.66	–	–	–	9	0.87	0.58	–	–	–	6	0.99	0.49

Significant *P*-values are in bold. Number of replicates (n) are listed between brackets.
Shown are the results of a linear model of larval performance.

AM fungi influence plant genotypes differently, although the most focus has been on cultivated species such as wheat or maize (Al-Karaki & Al-Raddad, 1997; Kaeppler *et al.*, 2000). In a natural system, Ramos-Zapata *et al.* (2010) found that genotypes of the weed *Ruellia nudiflora* (Acanthaceae) differed in both survival and number of leaves produced in response to a natural community of AM fungi. Overall, these results suggest that both in natural and agricultural communities there is genetic variation in the response of plants to AM fungi.

We showed that at 10 days after inoculation there was a strong difference among plant lines in growth traits, but not yet any imprint of AM fungal inoculation. Most likely, it was too early for the AM fungi to have fully colonised the roots and influenced aboveground plant growth (Moorman & Reeves, 1979). At 50 days after inoculation, plant lines differed in the strength and direction of their response to AM fungal inoculation, where the response ranged from negative (indicating an antagonistic interaction) to positive (indicating a mutualistic interaction). AM fungi are generally considered plant mutualists, but other studies have shown that in some instances AM fungi can act as antagonists (Johnson *et al.*, 1997, 2015). This suggests extensive functional diversity in AM symbiosis (Johnson *et al.*, 1997, 2005; Klironomos, 2003). Hence, AM fungi may shape not only the structure of the plant community (Klironomos, 2003), but also the genetic composition and the evolutionary trajectory of plants in natural systems. Such a variable response by plant lines match those obtained by Ramos-Zapata *et al.* (2010) and Sylvia *et al.* (2003). A differential response of the plant lines to AM fungal colonisation may be related to differences between plant lines in their ability to acquire nutrients, especially phosphorus, or related to differences between plant lines in their interactions and responsiveness to AM fungi (Al-Karaki & Al-Raddad, 1997; Bryla & Koide, 1998; Kaeppler *et al.*, 2000). Here we found that not all plant traits were affected by AM fungal inoculation, indicating that some plant traits, like leaf shape, may be genetically based but unaffected by AM fungi, whereas other traits, representing plant growth, are affected by the interaction between AM fungi and plant line.

The use of natural communities of AM fungi is important to make realistic predictions on the outcome of interactions with AM fungi and other organisms. We note however that, given a diverse inoculum, the differences between plant lines in their response to AM fungi can be due to variation in which AM fungal species are colonising the roots, and at what abundances. Between plant lines, there may be differences in the identity of the dominant strains, which could be caused by plant lines favouring different AM fungal species, or AM fungi favouring different plant lines. Notably, as we did not directly assess root colonisation by AM fungi, we cannot unequivocally conclude whether the effect of inoculation is as a result of AM fungal colonisation alone or if it may be because of other factors associated with the AM fungal inoculum. An interesting future avenue would be to use molecular methods (e.g. Öpik *et al.*, 2013) to determine the species colonising the plants, as well as their relative abundances, to determine how plant genotypes differ in their AM fungal communities when growing in the same soil environment.

The effect of AM fungi on herbivore performance

Our results showed that *M. cinxia* larval growth was either positively or negatively affected by mycorrhizal inoculation depending on which family the larvae originated from, as well as the mycorrhizal treatment of the plant line they were fed. This pattern was found only for the last 6 days of larval feeding and not the first 6 days, which could be caused by delayed herbivore-induced responses in the plant (Biere & Goverse, 2016). A previous study found that the effect of plant quality (iridoid glycoside content) was most pronounced in the fourth instar of *M. cinxia* larvae when compared to the third instar (Saastamoinen *et al.*, 2007). This corresponds to the findings reported here, where larval growth was more affected at a later larval stage. While previous work has suggested that specialist herbivores consistently perform better on AM fungal inoculated plants, whereas generalists perform worse (Gehring & Whitham, 2002; Koricheva *et al.*, 2009), we here demonstrate that both plant and larval variation mediated the impact of AM fungal inoculation on a specialist herbivore. Supporting our findings that both plant line and larval family are important for plant–insect interactions, Saastamoinen *et al.* (2007) previously found an impact of both plant and larval family on larval performance in this system. We found no impact of AM fungi, or an interaction between AM fungi and larval family, on the development time, weight at diapause or fat content of larvae. However, the lack of an effect on these response variables may

need further scrutiny, as the effect may have been obscured for two reasons: (i) the effect could have weakened due to the fact that the larvae were – because of a lack of plant material – fed with a mixed-plant line diet during the final days before diapause, or (ii) the detached leaves may not represent the live leaves, owing to differences in induced responses.

A possible mechanism for the variable effects of AM fungi on larval growth could be differences in plant chemical composition and production of chemical defences. The production of secondary metabolites, iridoid glycosides, in *P. lanceolata* differs between plant genotypes (Bowers *et al.*, 1992; Marak *et al.*, 2002). Moreover, AM fungi have been shown to influence iridoid glycoside content in *P. lanceolata* (Gange & West, 1994), which has an important impact on the performance of *M. cinxia* butterflies: high iridoid glycoside content has been found to increase larval weight and increase development time (Nieminen *et al.*, 2003; Harvey *et al.*, 2005; Saastamoinen *et al.*, 2007). Diet quality has also been shown to affect the defence of *M. cinxia* larvae against parasitoids and pathogens (Laurentz *et al.*, 2012). Future studies may, therefore, focus on the variable response of plant lines to AM fungal colonisation, especially with regards to the chemical composition of plants, such as nutrient content and investment in plant defences.

While our study showed the effect of AM fungi and genetic variation on pre-diapause growth of *M. cinxia* larvae under controlled conditions, our findings do not unequivocally demonstrate their ecological relevance. Nonetheless, we note that increased larval growth may deplete hosts plants more quickly than they can grow new leaves, increasing host-plant switching (and thus movement). Increased movements may increase mortality, due to the risk of movement and the difficulty of finding an alternative host plant. Moreover, a meta-analysis by Chen and Chen (2016) showed that increased larval growth rate might affect parasitism rate, which in turn may affect the survival and extinction dynamics of butterfly populations (van Nouhuys & Laine, 2008). Overall, we hope that future studies will assess the relevance of interactions between AM fungi and genetic variation in the plant and insect in a field context, ideally throughout the butterfly's entire life-cycle, and thereby pinpoint the relative importance of plant and insect genetic variation as compared to other ecological factors in driving the plant and butterfly dynamics.

Intriguingly, our findings illustrate that, depending on plant line and larval family, the impact of AM fungal inoculation can be beneficial for the plant, herbivore, or both. Hence, in the absence of natural enemies, natural selection may favour plants responding positively to AM fungal inoculation; however, in the presence of its specialist herbivore, there may be selection for increased plant resistance. From the larval perspective, resource quality will be heterogeneous, with some plants within the population being more resistant than others. This may result in local adaptation of either the plant or the herbivore (Biere & Tack, 2013). Notably, the responses of plant lines to AM fungal inoculation were highly variable among plant individuals, but not among plant populations, suggesting that plant populations have not differentiated their response to AM fungi in response to different selection pressures; moreover, it indicates that the herbivore may face small-scale heterogeneity (i.e. among plants), which may prevent local adaptation due to high rates of gene flow between butterflies within plant populations. Assessing whether – and at what spatial scale – plants and herbivores are locally adapted to AM fungi would be an interesting research avenue.

Conclusion

Our findings demonstrate that plant and insect genetic variation are important for determining the impact of AM fungi in a natural plant–herbivore interaction. The outcome of both plant–AM fungi and herbivore–AM fungi interactions ranged, depending on the identity of the plant and insect, from mutualistic to antagonistic. Taken together, our findings indicate that plant and insect variation, in combination with below-ground biotic heterogeneity, may be a major driver of the ecology and evolution of plant–herbivore interactions in natural systems. We hope that future studies will validate the generality, and ecological and agricultural relevance, of these findings across a range of plant–herbivore systems.

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Supporting Information

Additional Supporting Information may be found in the online version of this article under the DOI reference: 10.1111/een.12453

File S1. Supporting information files.

Table S1. Sample sizes for plant lines and larval families for the mycorrhizal treatments.

Table S2. Details on the calculation of response variables, number of replicates and transformations.

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