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SHORT COMMUNICATION

An endophyte-rich diet increases ant predation on a specialist herbivorous insect

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Abstract. 1. All plants form symbioses with microfungi, known as endophytes, which live within plant tissues. Numerous studies have documented endophyte–herbivore antagonism in grass systems, but plant–endophyte–insect interactions are highly variable for forbs and woody plants.

2. The net effect of endophytes on insect herbivory may be modified by their interactions with higher trophic levels, such as predators. Including these multitrophic dynamics may explain some of the variability among endophyte studies of non-grass plants, which are currently based exclusively on bitrophic studies.

3. The abundance of natural foliar endophytes in a Neotropical vine was manipulated and beetles were fed high or low endophyte diets. Experimental assays assessed whether dietary endophyte load affected beetle growth, leaf consumption, and susceptibility to ant predation.

4. Beetles feeding on high- versus low-endophyte plants had almost identical growth and leaf consumption rates.

5. In a field bioassay, however, it was discovered that feeding on an endophyte-rich diet increased a beetle's odds of capture by predatory ants nine-fold.

6. Endophytes could thus provide an indirect, enemy-mediated form of plant defence that operates even against specialist herbivores. We argue that a multitrophic approach is necessary to untangle the potentially diverse types of endophyte defence among plants.

Key words. *Acromis sparsa*, *Azteca lacrymosa*, endophytic fungi, insect herbivory, *Merremia umbellata*, multitrophic interactions.

Introduction

The aboveground tissues of all plants are infected by non-pathogenic, microscopic fungi known as endophytes (sensu Wilson, 1995). Similar to mycorrhizal fungi, foliar endophytes can improve plant nutrient acquisition, confer protection from abiotic stress (Rodriguez *et al.*, 2009), and can also mediate their hosts' interactions with insect herbivores (Hartley & Gange, 2009). Plant–endophyte–insect interactions could be further modulated by feedbacks with higher trophic levels, but the impacts of foliar endophytes on the predators and parasitoids of their host plant's herbivores have not yet been investigated outside of a few model grasses (Hartley & Gange, 2009). While several studies have addressed the potential multitrophic effects of mycorrhizal fungi (e.g. Gange *et al.*, 2003; Laird & Addicott, 2009; Schausberger *et al.*, 2012; Moon *et al.*, 2013), foliar endophytes may present different ecological dynamics because of their potential to interact directly with aboveground herbivores and their natural enemies.

A number of studies have demonstrated that some grasses engage in defensive mutualisms with vertically transmitted endophytes that directly protect their hosts from insect attack by producing toxic secondary metabolites (Schardl *et al.*, 2004; Müller & Krauss, 2005), but these mutualisms may be weakened by negative effects on the herbivores' natural enemies. For example, grass endophyte-produced toxins retained by the herbivore can limit the performance of its predators (de Sassi *et al.*, 2006), pathogens (Richmond *et al.*, 2004), and parasitoids (Bultman *et al.*, 1997; Härri *et al.*, 2008). For forbs

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and woody plants, however, the effects of endophytes and natural enemies on insect herbivory have only been investigated in isolation. In these systems, where endophytes are horizontally transmitted from spore fall in the environment (Herre *et al.*, 2007), direct endophyte effects on insect herbivory are highly variable across studies, and it has been questioned whether the defensive mutualism paradigm is generalisable beyond grasses (Saikkonen *et al.*, 2010). Natural enemies are known to be critical in constraining insect herbivore populations (Strong *et al.*, 1984; Cornell *et al*., 1998), but whether they interact with endophytes to synergistically regulate herbivory on non-grass plants has, to our knowledge, not been addressed.

Endophytes and enemies could interact in a variety of ways, resulting in a diversity of feedbacks on the intensity of herbivore pressure on plants. Endophytes are most likely to influence enemies indirectly through their effects on plants and/or herbivores. For example, an herbivore's body size, developmental rate, nutritional composition, or behaviour, which have been long recognised to modulate predation (Price *et al*., 1980), may also be affected by endophyte-produced toxins or through endophyte alteration of leaf metabolism and physical properties (Clay *et al.*, 1985; Petrini *et al.*, 1992; Mejía *et al.*, 2014). Endophyte-infected plants can have different volatile profiles from endophyte-free plants (Yue *et al*., 2001; Jallow *et al*., 2008); endophytes have also been shown to mediate herbivore-induced plant volatile emission (Li *et al.*, 2014), which in turn may influence enemy attraction (Takabayashi & Dicke, 1996). Furthermore, retention of fungi or sequestration of fungal toxins in or on the herbivore's body could deter enemy attack. Unless these interactions are considered, it will not be known whether the net endophyte effect on plant defence is reinforced or reversed by natural enemies. Studies including such information may help resolve the currently uncertain standing of foliar endophytes as defensive mutualists of non-grass plants.

As a first step in characterising the sign and intensity of links between horizontally transmitted foliar endophytes, plants, herbivores, and their enemies, we experimentally manipulated endophyte abundance in foliage of the tropical vine *Merremia umbellata* L. (Convolvulaceae) and measured herbivore performance, feeding rates, and defence against an ant predator. We first fed high- and low-endophyte leaves to the herbivorous beetle *Acromis sparsa* Boheman (Chrysomelidae: Cassidinae), an obligate specialist and important herbivore on *M. umbellata* (Windsor, 1987), and measured beetle growth and feeding rates. We hypothesised that, in the absence of enemies, endophyte treatment would affect neither herbivore performance nor leaf consumption, as these monophagous beetles may be adapted to tolerate, sequester or detoxify the allelochemicals resulting from endophyte infection of their host plant.

Further, we examined whether dietary endophyte load affected larval survival in a field bioassay with the predatory ant *Azteca lacrymosa* Forel, a natural predator of *A. sparsa* larvae in our study area (Vencl *et al.*, 2013). Cassidine larvae, including *A. sparsa*, construct faecal shields that may chemically protect them from predation (Vencl *et al.*, 1999, 2010), and as faeces may contain ingested endophytes or fungal toxins, we experimentally removed the shields from half of the tested larvae to test whether a possible endophyte effect on survival is mediated by the faecal shield. We hypothesised that high endophyte consumption would lower ant predation on larvae when faecal shields were present. Irrespective of the role of faecal shields, if endophytes do not directly limit beetle herbivory, but release beetles from top-down control by ant predators, the net outcome of endophyte infection could be to increase herbivory on host plants – a finding that would further call into question the role of endophytes as defensive mutualists of forbs and woody plants.

Materials and methods

Our study was conducted at the Smithsonian Tropical Research Institute in Gamboa, Panama, during the summers of 2011 and 2012. We established potted *M. umbellata* plants in a greenhouse by taking cuttings of wild vines growing locally; each cutting was separated into equal numbers of individually potted cuttings or clones. All plants were kept in plastic tents in the greenhouse to reduce endophyte inoculum landing on the plants. We waited until our greenhouse plants flushed all new leaves in this environment, to avoid using leaves that had been exposed in the field. We assigned low- and high-endophyte treatments (hereafter, 'E_{low}' and 'E_{high}', respectively) to equal numbers of each clone, to ensure an equal representation of genotypes in each treatment group. Foliar endophyte abundance in leaves was manipulated according to established protocols (Bittleston *et al.*, 2010). Briefly, we exposed E_{high} plants to naturally occurring communities of airborne fungi at night, while maintaining all plants in plastic tents in the greenhouse during the day to limit colonisation and keep the light environment constant. E_{low} plants were maintained continually inside the plastic tents. All protocols were identical between 2011 and 2012 except that, in 2012, the plants were grown in indoor growth chambers during the day, rather than a greenhouse. To verify the success of endophyte treatment, we plated 16 surface-sterilised 2 mm^2 leaf fragments from E_{low} ($n = 45$) and E_{high} ($n = 27$) plants on 2% malt extract agar (see Van Bael *et al.*, 2009 for details). The proportion of leaf fragments giving rise to fungal colonies after 7 days was used as a measure of foliar endophyte abundance for each plant.

In 2011, 28 wild *A. sparsa* egg masses (containing *c*. 20–40 eggs) were collected from *M. umbellata* plants in the Gamboa area, and separated from their mothers. As soon as the larvae hatched, broods were divided in half and immediately fed either E_{low} or E_{high} cut leaves in plastic containers. We measured the total mass and number of individuals in each of the 56 half-brood groups (containing 14 individuals, on average) on days 4 and 9 of larval development and during the pupal stage. After eclosion, we maintained a randomly selected subset of the adults ($n = 10$ broods per treatment, 3 individuals per brood) in individual containers with E_{low} and E_{high} leaves for 2 weeks and measured their leaf consumption. Feeding rates were taken by photographing leaves pre- and post-feeding daily for 7 days and using ImageJ software to calculate the consumed leaf area. We recorded and analysed growth and consumption variables at the sibling group level, averaging across individuals within each of the groups for each treatment, to avoid pseudoreplication.

In 2012, 36 *A. sparsa* sibling groups were reared up to day 4 of larval development in an identical fashion as the previous

year. We randomly selected two individuals per group and left one faecal shield intact while removing the faecal shield of the other with soft forceps (as in Vencl *et al.*, 1999). We used these larvae for predation bioassays with a wild colony of the ant *A. lacrymosa*, an aggressive generalist species that has served previously as a model predator of *A. sparsa* (Vencl *et al.*, 2005, 2010). As different groups of larvae hatched and reached day 4 of development on different days, and as temperature and rainfall varied considerably from day to day (with major effects on ant foraging activity), all larvae were stored frozen prior to the assays (as in Vencl *et al.*, 2009) enabling us to test larvae of the same age under similar climatic conditions. According to Vencl *et al.* (2005, 2009), we presented thawed, randomly selected beetle larvae to ants one at a time on a wooden platform installed at the base of the colony's tree, and recorded whether or not the larva was 'captured'– defined as being dragged by an ant for at least 1 cm – within 5 min. Excluding five larvae that were misplaced or rolled off the bioassay platform, we tested in total 35 shielded and 34 non-shielded E_{low} individuals, and 36 shielded and 34 non-shielded E_{high} individuals. Trials began when an ant first antennated the larva, and were separated by 3 min to maximise independence by increasing the likelihood that different ants would be tested in each trial. We baited the platform with tuna 1 h prior to the first trial to ensure colony feeding motivation (Vencl *et al.*, 2009); the ants' response to the bait and the thawed larvae was generally similar.

To test whether beetle performance (fresh weight) measured at three developmental stages differed between endophyte treatments, we used a two-way repeated measures ANOVA. Two-sample *t*-tests were used to compare feeding rates between endophyte treatments, after visual confirmation and Shapiro–Wilk tests of normality. Non-parametric Wilcoxon's rank sum tests were used on the foliar endophyte abundance data to evaluate the efficacy of the treatment protocol. To test whether endophyte treatment affected the likelihood of ant capture, we used a generalised linear model with a binomial error distribution (Hosmer & Lemeshow, 2000). The full model included endophyte treatment, shield status (present or absent), and their interaction. We reduced the model by removing non-significant terms in a stepwise fashion, and selected the simplest model that adequately fit the data with analysis of deviance. All analyses were conducted in R v. 3.0.0 (R Core Team, 2013). Raw data and R code used in this study are provided in Tables S1–S5 and File S1.

Results and Discussion

Our experimental manipulation was successful at creating plant groups that significantly differed in foliar endophyte load ($W = 1087.5$, $P < 0.0001$). While E_{low} leaves were not free of endophyte infection, given that the plastic tents they were grown in do not completely block airborne spore deposition, they were approximately one-third as infected as E_{high} leaves (median per-plant endophyte density: 31% vs. 100%, respectively).

In agreement with our first hypothesis that endophytes would not affect beetle performance, growth (measured as fresh weight at days 4 and 9 of larval development, and at the pupal stage) did not differ between endophyte treatments. There was no interaction between developmental stage and treatment ($F_{2,54} = 0.023$, $P = 0.977$), and no significant effect of treatment on overall performance ($F_{1,27} = 0.271$, $P = 0.607$). Thus, in contrast to a number of studies on generalist herbivores (e.g. Vicari *et al.*, 2002; Crawford *et al.*, 2010; Van Bael *et al.*, 2012) endophytes do not appear to directly affect herbivore performance in this system. The discrepancy may be due to the fact that *A. sparsa* is a specialist that feeds exclusively on *M. umbellata*; as there is some evidence for host plant specificity in endophyte communities (Petrini *et al.*, 1992; Arnold *et al.*, 2000), *A. sparsa* may be adapted to deal with *M. umbellata*-associated endophytes. Endophyte studies on insects with restricted diet breadths are limited (Hartley & Gange, 2009), but recent work suggests that foliar endophytes have stronger negative impacts on generalist than on specialist herbivores (Gange *et al.*, 2012).

E_{high} beetles could have reached the same size as E_{low} beetles by eating more leaf material to compensate for higher toxins or lower nutrients present in their diet, which would lower host plant fitness. Yet, again supporting our hypothesis, leaf consumption rates of adult beetles reared in the laboratory were highly similar between endophyte treatments [E_{low} mean (95% CI): 5.182 (4.712–5.652); E_{high} mean (95% CI): 5.081 (4.531–5.630); $t_{18} = 0.295$, $P = 0.771$]. Thus, in the absence of natural enemies, one might conclude that endophytes do not protect *M. umbellata* hosts from herbivory, counter to the commonly held view of endophytes as defensive mutualists.

While endophytes did not directly affect beetle performance or feeding rates, we hypothesised that they may decrease beetle capture by a predator in a fecal-shield-contingent manner. Opposite to these expectations, however, high dietary endophyte load significantly increased the odds of larval capture ($P = 0.041$), and there was no interaction between the two treatments ($P = 0.992$) nor any significant effect of shield presence ($P = 0.322$) (Fig. 1). E_{high} larvae were an estimated nine times more likely to be captured by ants, although the confidence interval surrounding this estimate is fairly wide (OR = 9.049, d.f. = 138, 95% CI: 1.595–170.304). In a natural context, an increase in predation on a herbivore would result in less feeding damage to the plant, and, therefore, foliar endophytic associations could constitute an indirect, multitrophic form of plant defence. The effects of root mycorrhizal fungi on the natural enemies of insect folivores range from positive to neutral to negative (Gange *et al.*, 2003; Guerrieri *et al.*, 2004; Moon *et al.*, 2013). Although it remains to be determined whether there are consistent differences between root- and leaf-associated symbionts in their interactions with aboveground herbivore enemies, foliar endophytes may be under stronger selection for adaptations that increase top-down control on herbivory, as they stand the risk of being consumed along with leaf tissue (see Herre *et al.*, 2007).

Our finding that endophytes increase the odds of ant predation on beetle larvae, without affecting beetle performance or leaf consumption, could be driven by a number of potential mechanisms. First, although freezing itself does not affect ant predation rates on *A. sparsa* (Vencl *et al.,* 2005), we acknowledge that it

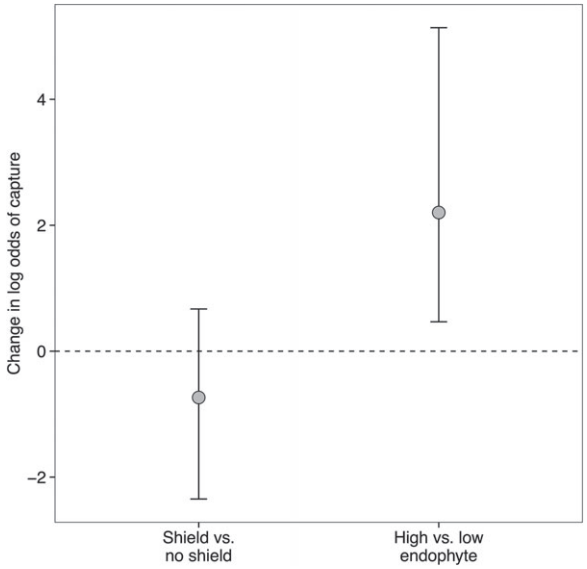

Fig. 1. Larvae fed a high-endophyte diet were significantly more likely to be captured by ants than larvae fed a low-endophyte diet, and presence or absence of the larval faecal shield had no effect on capture odds. The y-axis shows logit coefficients (which correspond to the change in log odds of capture) with their 95% confidence intervals, for the two factors included in the model.

might have had endophyte treatment-specific effects on beetle susceptibility to capture, which future studies comparing live with freeze-killed beetles will examine. As foliar endophytes sometimes increase the nutrient content of leaf tissue (Lyons *et al.*, 1990; Omacini *et al.*, 2001; Zabalgogeazcoa *et al.*, 2006), E_{high} larvae could in turn have constituted a more nutritionally attractive prey item for *A. lacrymosa* ants. Volatiles stored in and emitted from the herbivore could also have served as cues provoking ant attack. Alternatively, endophytes may have made *A. sparsa* larvae less repellent by interfering with their production of defensive chemical compounds. The closely related tortoise beetle *Chelymorpha alternans* (Boheman) metabolises *M. umbellata*-derived chlorophyll in its gut to the catabolite pheophorbide *a*, which has been shown to deter *A. lacrymosa* ants in bioassays similar to those presented here (Vencl *et al.*, 2009). Perhaps secondary metabolites particular to, or elevated in, E_{high} leaves (Petrini *et al.*, 1992) reduced the ability of the beetle larvae to convert chlorophyll to defensive chemical compounds, resulting in higher ant predation without necessarily having an effect on herbivore performance.

In addition to teasing apart the underlying mechanisms, testing for an increase in plant and endophyte fitness in response to elevated ant predation on herbivores would be a worthwhile avenue for future study, as it may indicate whether these endophyte–ant interactions represent adaptations to limit herbivory. Furthermore, our data lead to the prediction that beetle larvae would choose to feed on E_{low} versus E_{high} foliage, because of a reduced predation risk. Given that endophyte density and/or community structure can vary extensively between leaves within a plant (Arnold & Herre, 2003), between conspecific plant individuals (Zimmerman & Vitousek, 2012), and between different host plant species (Arnold & Lutzoni, 2007), herbivores may seek enemy-free space (Jeffries & Lawton, 1984) by incorporating endophyte cues into their foraging decisions.

In conclusion, our study suggests that endophytic fungi may provide indirect, enemy-mediated defensive services to plants in natural, non-grass systems. Effects on herbivore enemies, such as predators and parasitoids, could enable endophytes to defend plants even against specialist insects, which may have evolved counter-defences to the fungi specific to their host plant. Many plants employ predatory ants as a form of indirect defence against herbivorous insects, using morphological adaptations such as nectaries or ant domatia (Rico-Gray & Oliveira, 2007; Heil 2008). Our results suggest that plants may also use mutualistic associations with fungal endophytes to increase ant predation pressure on the insects attacking them. More generally, we argue that endophytes are likely to have critical and complex roles in interactions between insect herbivores and a wide diversity of plants, but that their influence may be masked if higher trophic levels are not considered.

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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.12186

Table S1. Predation assays data.
Table S2. Beetle growth data 1.
Table S3. Beetle growth data 2.
Table S4. Endophyte frequency data.
Table S5. Beetle feeding rate data.
File S1. R code.

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