

Fungal endophytes of *Festuca rubra* increase in frequency following long-term exclusion of rabbits

James S. Santangelo, Nash E. Turley, and Marc T.J. Johnson

Abstract: Plant – fungal endophyte interactions are common in nature and they can shape the ecology of plants. Vertically transmitted endophytes are hypothesized to serve as mutualists, protecting plants from herbivores. If this hypothesis is true, then we expect endophytes to be most abundant in the presence of herbivores and least abundant in their absence, assuming endophytes incur a cost to their host. We tested this prediction by studying the effects of intense rabbit (*Oryctolagus cuniculus* Linnaeus) grazing on grass–endophyte interactions at Silwood Park, UK. We examined seeds of red fescue (*Festuca rubra* L.) collected from 15 natural populations that were protected from rabbits for 0.3–21 years. Contrary to our prediction, the mean proportion of seeds with endophytes increased 1.84×, from 0.45 to 0.83, following 21 years of rabbit exclusion. To better understand the mechanisms driving this increase in frequency, we conducted a fully factorial greenhouse experiment where we manipulated the presence or absence of endophyte infection, intraspecific competition, and simulated grazing on *F. rubra* plants. In both damaged and undamaged treatments, infected plants produced approximately twice as much biomass as uninfected plants, and endophytes did not influence tolerance to herbivory. These results suggest that endophytes directly change plant growth but not compensatory responses to damage. In the absence of competitors, infected plants produced 2.17× more biomass than uninfected plants, whereas in the presence of competitors, infected plants produced only 1.55× more biomass than uninfected plants. This difference suggests that intraspecific competition might lessen the benefits of endophyte infection. Our results do not support the defensive mutualism hypothesis, but instead suggest that endophyte-induced plant growth is important in shaping the costs and benefits of endophytes in our system.

Key words: defensive mutualism hypothesis, herbivory, grassland, plant–fungal, plant–animal, *Epichloë festucae*.

Résumé : Les interactions plante – endophyte fongique sont fréquentes dans la nature et elles peuvent façonner l'écologie des végétaux. Les endophytes transmis de manière verticale sont présumés servir d'organismes mutualistes, protégeant les végétaux des herbivores. Si cette hypothèse s'avère, on peut conséquemment s'attendre à ce que les endophytes soient plus abondants en présence d'herbivores et moins abondants en leur absence, assumant que les endophytes engagent un coût pour leur hôte. Les auteurs ont testé cette prédiction en étudiant les effets d'un broutage intense par le lapin (*Oryctolagus cuniculus* Linnaeus) sur les interactions herbes–endophytes à Silkwood Park, RU. Ils ont examiné les graines de la fétuque rouge (*Festuca rubra* L.) recueillies de 15 populations naturelles protégées des lapins pendant 0,3–21 ans. Contrairement à leur prédiction, la proportion moyenne de graines comprenant des endophytes augmentait de 1,84×, de 0,45 à 0,83, après avoir exclu les lapins pendant 21 ans. Afin de mieux comprendre les mécanismes responsables de cette augmentation de la fréquence, les auteurs ont réalisé des expériences factorielles complètes en serres, où ils ont manipulé la présence ou l'absence d'infection par les endophytes, la compétition intraspécifique et le broutage simulé des plants de *F. rubra*. Dans les cas de traitements dommageables ou non, les plants infectés produisaient approximativement deux fois plus de biomasse que les plants non infectés, et les endophytes n'influençaient pas la tolérance à l'herbivorisme. Ces résultats suggèrent que les endophytes changent directement la croissance des végétaux mais pas les réponses compensatoires au dommage. En absence de compétiteurs, les plants infectés produisaient 2,17× plus de biomasse que les plants non infectés, alors qu'en présence de compétiteurs, les plants infectés produisaient seulement 1,55× plus de biomasse que les plants non infectés. Cette différence suggère que la compétition intraspécifique peut amoindrir les bénéfices conférés par l'infection par des endophytes. Les résultats des auteurs n'appuient par l'hypothèse du mutualisme défensif, mais suggèrent plutôt que la croissance de la plante induite par les endophytes est importante pour façonner les coûts et les bénéfices des endophytes dans leur système. [Traduit par la Rédaction]

Mots-clés : hypothèse du mutualisme défensif, herbivorisme, prairie, plante–champignon, plante–animal, *Epichloë festucae*.

Introduction

Plant–microbe interactions are ubiquitous in nature and play pivotal roles in shaping plant ecology and evolution (Yang et al. 2013). Microbial symbionts, both bacterial and fungal, can shape plant host responses to biotic and abiotic stresses (Schardl et al. 2004; Yang et al. 2013). However, research is increasingly finding widespread variation in the effects of symbionts on host plant fitness (Saikkonen et al. 1998; Klironomos 2003). For example, symbiotic

interactions can change sign (i.e., –, 0, +) and (or) magnitude (strong to weak) across ecological conditions (Davitt et al. 2011; Chamberlain et al. 2014). Understanding the ecological and environmental factors that affect variation in plant responses to microbial symbionts is crucial if we are to understand what drives the prevalence of plant–microbe interactions in natural populations.

One of the best-studied groups of plant microbial symbionts are fungal endophytes, which are ubiquitous among plants (Rodriguez

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et al. 2009). Here we focus on vertically transmitted endophytes in the genus *Epichloë* (Family: Clavicipitaceae, Phylum: Ascomycota), and the cool-season grasses (Subfamily: Pooideae) they infect. These systemic endophytes live asymptotically within host tissues and are transmitted to future generations through seeds (Rodríguez et al. 2009). Fungal endophytes are primarily thought to act as mutualists, protecting plants from herbivores (Clay 1988; Clay and Schardl 2002). This claim is based on observations of endophyte-infected forage plants poisoning cattle and other livestock (Bacon et al. 1977; Fletcher and Harvey 1981; Schmidt et al. 1982), reducing insect growth and survival (Clay et al. 1985; Cheplick and Clay 1988), and the subsequent identification of endophyte-produced toxic alkaloids responsible for these effects (Leuchtman et al. 2000; Schardl et al. 2013). Endophytes can also improve competitive ability (Marks et al. 1991; Clay et al. 1993) and tolerance to abiotic stresses such as drought (Kane 2011; Nagabhyru et al. 2013), low soil nitrogen (Bacon 1993), salt (Reza Sabzalain and Mirlohi 2010; Yin et al. 2014), and heavy metals (Malinowski and Belesky 1999; Ren et al. 2011). However, herbivore resistance remains the most frequently cited benefit of endophytes and some studies find increasing endophyte frequencies in the presence of both invertebrate and vertebrate herbivores (Bazely et al. 1997; Clay et al. 2005; Koh and Hik 2007). For example, endophyte-mediated herbivore resistance is thought to explain the high infection frequencies in agronomic grasses, which often approach 100% (Leuchtman 1992; Clay and Schardl 2002).

While endophytes are typically considered mutualists in non-native, introduced agronomic systems, their effects on native grasses are more variable and less understood (Saikkonen et al. 2006). Endophytes of native grasses can negatively affect host performance by reducing competitive ability (Faeth et al. 2004), growth and reproduction (Faeth and Sullivan 2003). Specifically, they can alter host resource allocation to increase their own transmission, which sometimes reduces allocation to plant reproduction (Faeth 2009; Gorischek et al. 2013; but see Faeth 2010; Rudgers et al. 2010). Conversely, the positive effects of endophytes range from increased competitive ability (Vázquez-de-Aldana et al. 2012) and allelopathy (Vázquez-de-Aldana et al. 2011) to decreased preference and performance of herbivores on infected grasses (Crawford et al. 2010). However, these effects on herbivores can depend on herbivore identity (Afkhami and Rudgers 2009) and endophyte reproductive strategy (i.e., sexual or asexual) (Tintjer and Rudgers 2006). Such variation in native grass performance in response to endophytes may explain the highly variable infection frequencies found in natural populations, which range from 0% to over 50% (Wäli et al. 2007; Iannone et al. 2011). Thus, while endophytes do not appear ubiquitously mutualistic in wild grasses, there have been far fewer studies examining the effects of endophytes in natural systems, and long-term studies testing the defensive mutualism hypothesis are rare (Faeth and Saari 2012; but see Rudgers and Clay 2008; Jani et al. 2010; Faeth and Shochat 2010).

Here we use a long-term (21 year) vertebrate herbivore exclusion experiment in a British grassland to examine the dynamics of the endophyte *Epichloë festucae* Leuchtman, Schardl & M.R.Siegel on its host red fescue (*Festuca rubra* L.). Specifically, we sought to answer the question: Does endophyte frequency decrease following herbivore exclusion, as predicted by the defensive mutualism hypothesis? To identify the ecological mechanisms driving the patterns observed in our long-term field experiment, we conducted a factorial experiment in the greenhouse to understand whether endophytes influence: (1) host plant growth and biomass, (2) tolerance to herbivory, and (3) intraspecific competitive ability of *F. rubra*.

Methods

Study system

Our long-term field experiment was performed at Silwood Park, Imperial College, England (51.409°N, 0.642°W). This site is a species-poor grassland community on sandy, acidic, and low nutrient soils. European rabbits (*Oryctolagus cuniculus* Linnaeus) have long been dominant herbivores at Silwood Park (Crawley 1990), where they are known to influence plant community composition, primary productivity, nutrient dynamics, and seedling recruitment (Crawley 1990; Edwards and Crawley 1999a; Olofsson et al. 2007). Rabbits also alter plant life cycles by limiting plants to vegetative reproduction in the presence of grazing (Crawley 1990; Edwards and Crawley 1999a). Between 1975 and 2009, we created 15 rabbit exclosures that ranged in size from 16 m² to 800 m². These exclosures represent small, local plant communities that vary in the amount of time without rabbit grazing, ranging from 0.3 to 21 years (Supplementary Table S1¹).

Rabbits at Silwood Park are known to feed on many common species including: *Anthoxanthum odoratum* L. (Poaceae), *Holcus lanatus* L. (Poaceae), *F. rubra* (Poaceae), and *Stellaria graminea* L. (Caryophyllaceae). Here, we focus on *F. rubra*, which is a perennial, cool-season grass with a holarctic distribution. At Silwood Park, *F. rubra* is the dominant grass comprising 10%–20% of the total biomass within communities (Edwards and Crawley 1999a), although it tends to decrease in abundance under intense rabbit grazing (Crawley 1990). This grass can spread vegetatively through the production of stolons and rhizomes, resulting in the formation of large clonal patches (Grime et al. 1988). *Festuca rubra* is commonly infected asymptotically with the fungal endophyte *Epichloë festucae* (Zabalgoitia et al. 2006), which lives intercellularly within the sheaths, culms, and rhizomes of plants (Rodríguez et al. 2009). The endophyte is transmitted vertically between generations by infection of the seeds (Clark et al. 1983).

Experiment 1: determining endophyte frequency

Determination of endophyte presence

To test for the effects of rabbit removal on endophyte infection frequency, we collected seeds from all flowering *F. rubra* plants (typically >25 plants per population). We then pooled these seeds within each of the 15 rabbit exclosures. Because intense grazing prevented plants in our youngest plots (i.e., 4 months) from producing seed prior to the establishment of the exclosures, endophyte frequencies within plots should reflect average endophyte frequencies outside of exclosures in the presence of rabbits. Thus, young exclosures represent the shortest point along a gradient of grazing histories, allowing us to test for the effects of rabbit removal on long-term endophyte dynamics. Furthermore, although younger exclosures were also the smallest (Supplementary Table S1¹), they function as a random sample of the larger, continuously grazed, habitat surrounding them. From each exclosure, we randomly sampled 30 seeds and screened them for the presence of endophytic hyphae in the outer layer of the seeds endosperm (i.e., aleurone) by staining them with 0.1% aniline blue–lactic acid and viewing them at 100× under a compound microscope (Clark et al. 1983). We examined seeds longitudinally for 1 min each, which was sufficient to observe the entire surface area. If endophytes were not seen within that time, they were presumed absent. In total, we examined 436 seeds across all plots (one plot only had 16 seeds; see Supplementary Table S1¹). Our estimates of endophyte frequencies represent estimates of the proportion of endophyte-infected flowering ramets within exclosures. Furthermore, using seeds to determine endophyte frequencies provides an accurate and efficient way to quantify the frequency of endophyte infection within populations (Clark et al. 1983).

¹Supplementary data are available with the article through the journal Web site at <http://nrcresearchpress.com/doi/suppl/10.1139/cjb-2014-0187>.

Statistical analysis

To examine changes in endophyte frequency over time, we fit a nested mixed effects logistic regression using enclosure age (i.e., years without rabbit grazing) as a predictor of endophyte presence. The model was fit using the `glmer` function from the `lme4.0` package in R (Bates et al. 2013). The equation of our model using R syntax was as follows:

$$\text{Presence/absence endophytes} \\ \sim \text{Enclosure age*Size} + (1|\text{age/enclosure})$$

This model includes enclosure age and size of enclosures as fixed effect continuous variables and the random effect of enclosure nested within plot age to account for pseudoreplication due to multiple seed samplings per enclosure and age class (Supplementary Table S1^a). We modeled the response variable as a binomial distribution with a logit link function, consisting of either the presence (i.e., 1) or absence (i.e., 0) of endophytes. We determined model parameters and the *p* value of the fixed effect using the Laplace approximation and Wald's *Z*, respectively, which are appropriate for models with fewer than three random effects and lacking overdispersion (Bolker et al. 2009). Using the parameter estimates from the logit model and the `predict` function from the `lme4.0` package, we calculated the marginal probabilities that a randomly selected seed contains endophytes across all enclosure age classes.

Experiment 2: tolerance to herbivory and competition experiment

Germination and growth rate

We randomly selected 258 *F. rubra* seeds from 15 herbivore enclosures and germinated them in 10 cm round pots (382 mL) filled with a soil mixture containing two parts peat moss (Sunshine Mix #1 Professional Growing Mix; Sun Gro Horticulture, Montréal, Canada) and one part sand. We ensured that all enclosures were represented among germinating plants. We placed the seeds individually into pots, ~0.6 cm below the soil surface, and then randomized all pots into a growth chamber set to 20 °C and 10 h light – 14 h dark cycle. Plants were watered as needed.

We checked the seeds daily for germination by gently brushing the soil aside and considered plants germinated once the radicle had emerged from the seed coat. Two weeks following germination we moved all plants into a greenhouse where daily maximum temperatures averaged 30 °C with a photoperiod of 12 h light – 12 h dark. We added approximately 200 mg of slow release fertilizer to each pot (Nutricote total 13-13-13 (N-P-K) controlled release fertilizer with micronutrients, Type 100; Asahi Fertilizer Co., Tokyo, Japan). We recorded the height of the first leaf upon emergence from the soil surface and every week thereafter for the first 4 weeks. The number of leaves on each plant were counted after 3 weeks of growth.

We calculated growth rate over the first 2 weeks as $(LN_{\text{final}} - LN_{\text{initial}})/14$ days where LN_{initial} and LN_{final} represent the height of the first leaf upon emergence and after 2 weeks, respectively. After 4 weeks of growth, we tested all plants for the presence of fungal endophytes by examining a single leaf sheath from the first fully emerged tiller according to the method of Clark et al. (1983). This method of subsampling is sufficient to accurately assess the presence or absence of endophytes because hyphae systemically colonize each emerging leaf blade and are therefore present in all aboveground tissues (Christensen et al. 2008; Rodriguez et al. 2009). Thus, we determined the infection status of all 194 surviving plants and used this to examine differences in growth and germination between infected and uninfected plants. This yielded 123 (63%) endophyte-infected and 71 endophyte-free individuals, which were then used in the establishment of the experiment described below.

Experimental setup

We conducted a factorial experiment to examine how intraspecific competition and grazing affect the influence of endophytes on plant performance. Our design involved a three-way factorial manipulation of focal plant infection status (infected or uninfected), intraspecific competition (infected competitors, uninfected competitors, or no competitors) and simulated grazing (damaged or undamaged). We replicated each treatment 10 times for a total of 120 pots.

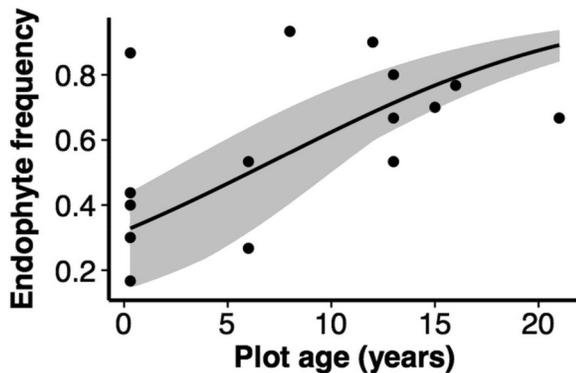
Infected and uninfected ramets were generated from the germinated plants described above by removing them from their pot, gently shaking off all of the soil, and carefully excising a single tiller using a sharp razor blade, which could then be transplanted into the appropriate treatment. Because plants originated from enclosures with different grazing histories, and grazing can shape plant tolerance, competitive ability, and growth, we distributed plants from each enclosure across treatments. We placed focal plants into the center of 15 cm round pots (1 L) and, in competition treatments, we surrounded focal plants by four competitor plants of uniform infection status (i.e., all infected or all uninfected). Prior to transplanting, initial focal plant biomass was estimated by measuring the width and height of the plant, which were used to calculate the volume of a cylinder (Faeth and Fagan 2002). Because this technique has not been applied to *F. rubra*, we destructively harvested 20 plants to test its validity. The cylinder volume was highly correlated with the true biomass measurements for both belowground (Pearson's product moment correlation coefficient, $r = 0.95$, $p < 0.001$, Supplementary Fig. S1a¹) and aboveground ($r = 0.85$, $p < 0.001$, Supplementary Fig. S1b¹) tissues. Following transplant of ramets into experimental treatments, we added approximately 550 mg of the same fertilizer as described above.

We imposed damage treatments 2 and 4 weeks after the start of the experiment by cutting all plants to 1 cm above the soil surface using a sharp razor blade (Didiano et al. 2014). This method is meant to simulate the grazing habits of rabbits in the field and has been successfully used to measure plant tolerance to rabbits in this system (Turley et al. 2013; Didiano et al. 2014). After 6 weeks of growth, we harvested above- and below-ground tissues of all plants and stored them in a -80 °C freezer. We later freeze-dried all tissues in a Labconco freeze drying system prior to weighing them to the nearest 0.1 mg on a Mettler Toledo scale (Model #MS104S).

Statistical analysis

To test for the effects of fungal endophytes on germination time and growth rate, we performed a series of two-sample *t* tests using infection status as a predictor of these response variables. To examine how endophytes influenced the competitive ability of *F. rubra* across the different grazing treatments, we performed an ANCOVA using focal plant infection status, competitor plant infection status, grazing, and all possible interactions as predictors of aboveground biomass. Initial focal plant biomass at the time of transplanting (estimated as described above) and competitor plant biomass were used as covariates. Where significant interactions were found, we performed pairwise *t* tests to assess differences among treatments. To examine tolerance to herbivory, we fit the same linear model as above using log-transformed aboveground biomass values. This transformation was necessary because our damage treatment removed a proportional amount of tissue from all plants and, thus, the effects of grazing on biomass should be assessed on a multiplicative scale (Wise and Carr 2008). A significant focal plant infection status × grazing interaction would provide evidence for differences in tolerance between infected and uninfected plants. Our ANCOVA was fit using the `lm` and `drop1` functions in R, version 2.15.3 (R Core Team 2013). We used type III sums of squares (SS) due to the unbalanced design and presence of significant interactions. R^2 values were calculated as $SS_{\text{factor}}/SS_{\text{total}}$

Fig. 1. Changes in endophyte frequency within seeds as a function of years without rabbit grazing (exclosure age). Points represent mean endophyte frequency at the exclosure level ($n = 15$) and the solid line shows the predicted probability that a randomly selected *Festuca rubra* seed contains endophytes. Probabilities were predicted from the mixed-effects logistic regression model. The grey shaded area corresponds to the 95% confidence interval around the predicted probabilities. There was an 84% increase in mean endophyte frequency over 21 years of rabbit exclusion.



from the ANCOVA models. All data and R scripts are available in Dryad Digital Repository (Santangelo et al. 2015).

Results

Experiment 1: endophyte dynamics in the field

We found that 60% of all *F. rubra* seeds taken from experimental exclosures contained endophytes. There was a significant increase in endophyte frequency with increasing exclosure age (logistic regression, Wald's $Z = 2.68$, $p = 0.007$). Specifically, endophyte frequency increased from 0.45 to 0.83, corresponding to an 84% increase following 21 years of herbivore exclusion (Fig. 1). Neither exclosure size nor the size by age interaction were important predictors of changes in endophyte frequency (Wald's $Z_{\text{Size}} = -1.09$, $p = 0.27$; Wald's $Z_{\text{Age} \times \text{Size}} = 0.55$, $p = 0.59$).

Experiment 2: competition experiment

Infection status

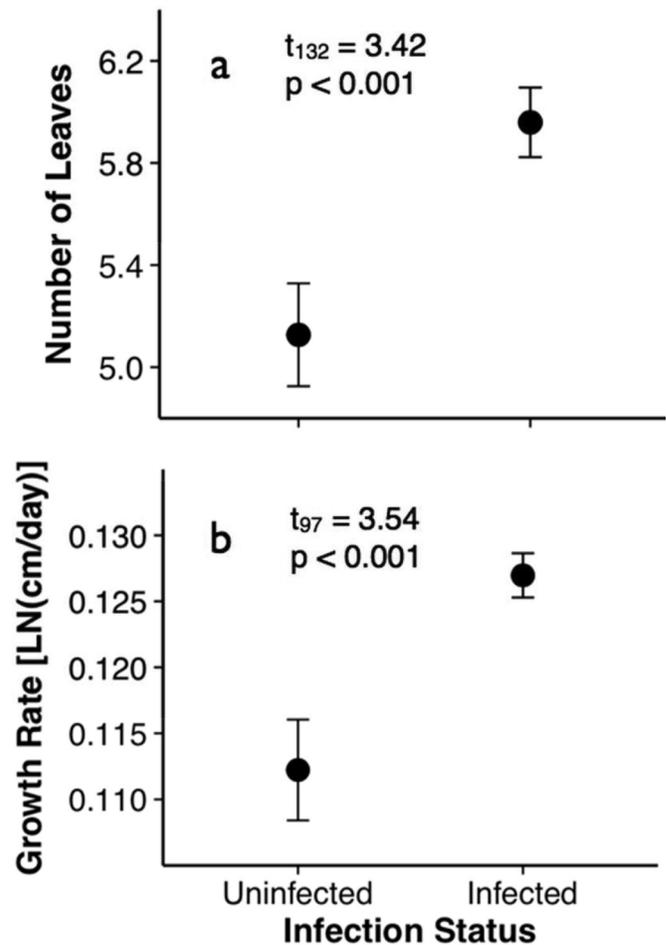
The presence of endophytes influenced most, but not all, of the measured plant traits. Infected and uninfected plants did not differ in time to germination ($t_{142} = 0.46$, $p = 0.64$). However, endophyte infection resulted in the production of 16% more leaves ($t_{132} = 3.42$, $p < 0.001$, Fig. 2a), 13% higher growth rates ($t_{97} = 3.54$, $p < 0.001$, Fig. 2b), and 99% greater total biomass (Table 1).

Competitor plant infection status did not influence the total biomass of focal plants ($F_{[1,65]} < 0.001$, $P = 0.99$), so we ignored competitor infection status in subsequent analyses and pooled the replicates together to examine the effects of competition on focal plant biomass (i.e., competition vs. no competition). For these analyses, above- and below-ground biomass showed similar patterns, so we focus here on total biomass (i.e., aboveground + belowground biomass) for all analyses.

Competition treatment

Although endophyte infection consistently increased focal plant biomass, the magnitude of this effect depended on intraspecific competition (Focal infection \times Competition interaction, Table 1). Infected plants produced 117% more biomass in the absence of competitors (pairwise t test, $p = 0.03$, Fig. 3) and 55% more biomass in their presence (pairwise t test, $p = 0.12$, Fig. 3).

Fig. 2. Plots showing differences in (a) the number of leaves, and (b) growth rate of infected ($n = 123$) and uninfected ($n = 71$) *Festuca rubra* plants. All points represent the means ± 1 SE. Infected plants produced more leaves and had higher growth rates than uninfected plants.



Damage treatment

The effect of the damage treatment on absolute (i.e., untransformed) total biomass differed between infected and uninfected plants (Focal infection \times Damage interaction, Table 1). However, infected plants consistently produced more total biomass than uninfected plants in both the absence (pairwise t test, $p < 0.001$, Fig. 4) and presence (pairwise t test, $p < 0.001$, Fig. 4) of damage. In contrast to the effects on absolute biomass, the relative difference in aboveground biomass between infected and uninfected plants showed no difference across the levels of the damage treatment (Focal \times Damage interaction, log-transformed response variable, $F_{[1,110]} = 0.23$, $p = 0.63$, Supplementary Fig. S2¹). Tolerance to herbivory was also unaffected by the presence of competitors (Focal \times Damage \times Competition interaction, log-transformed response variable $F_{[1,110]} = 1.44$, $p = 0.23$).

Discussion

Contrary to the predictions of the defensive mutualism hypothesis, our long-term field experiment showed that endophyte frequencies increased following rabbit exclusion. Below we discuss a number of alternate hypotheses that may explain our findings. We also found that endophyte-infected plants consistently produced more biomass, yet endophyte infection did not influence tolerance to herbivory. We discuss how the benefits of endophytes on host grasses are likely context-dependent, and suggest

Table 1. Three-way analysis of covariance using type III sums of squares of *Festuca rubra* total focal plant biomass production.

Focal Plant	Total biomass				
	df	F ratio	p	R ²	%*
Initial biomass	1	10.50	0.001	0.28	NA
Competitor plant biomass	1	2.58	0.11	0.007	NA
Focal plant infection status	1	12.06	<0.001	0.033	98.7
Competition	1	0.02	0.88	<0.001	-26.8
Grazing	1	95.01	<0.001	0.257	-96
Focal infection × Competition	1	6.44	0.01	0.017	117 (C), 55 (NC)†
Focal infection × Damage	1	21.52	<0.001	0.069	117.9 (D), 97.9 (U)†
Competition × Damage	1	0.15	0.70	<0.001	-3.1 (D), -27.6 (U)†
Focal infection × Damage × Competition	1	10.20	0.001	0.028	NA
Residuals	101			0.273	NA

Note: C, competition; D, damaged; NA, not applicable; NC, no competition; U, undamaged.

*% column indicates percent change in total biomass for each treatment calculated as [(treatment - control)/control] × 100.

†For two-way interactions, percent change was calculated across the levels of the treatments shown in parentheses and within the levels of the other treatment present in the interaction.

Fig. 3. Mean total biomass (mg) produced by infected ($n = 60$) and uninfected ($n = 60$) *Festuca rubra* plants in the absence ($n = 40$) or presence ($n = 80$) of intraspecific competitors. All points represent the means ± 1 SE. The asterisk denotes a significant difference ($p < 0.05$) between infected and uninfected plants according to a post hoc t test. The presence of competitors differentially impacted infected and uninfected plants.

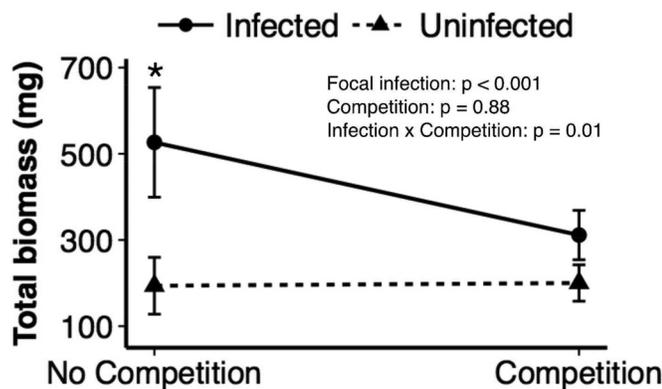
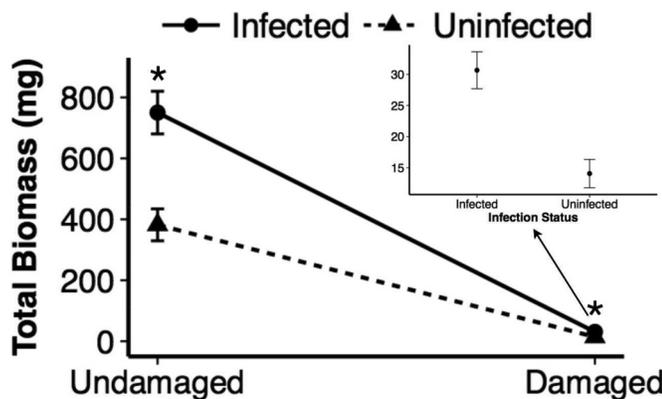


Fig. 4. Mean total biomass (mg) produced by infected ($n = 30$) and uninfected ($n = 30$) *Festuca rubra* plants in the absence ($n = 30$) or presence ($n = 30$) of damage. All points represent the means ± 1 SE. The asterisk denotes significant differences between infected and uninfected plants according to a post hoc t test. Absolute differences in total biomass production between infected and uninfected focal *F. rubra* plants were differentially impacted by the presence of damage.



a mechanism by which such context-dependency may regulate endophyte frequencies in nature, mediated by the effects of endophytes on host plant growth.

Alternate hypotheses explaining endophyte dynamics in the field

The frequency and dynamics of fungal endophytes are highly variable in nature. In our study, 60% of all *F. rubra* plants contained endophytes, which is within the range of other naturally sampled *F. rubra* populations (Bazely et al. 1997; Vázquez-de-Aldana et al. 2007; Wäli et al. 2007). We also found that endophyte frequencies were greater in older rabbit enclosures, which contrasts with previous studies finding increases in endophyte frequencies in the presence of herbivores (Clay et al. 2005), or greater infection rates in naturally grazed populations (Bazely et al. 1997; Koh and Hik 2007). While the possibility of seed dispersal into our plots cannot be ruled out, the fact that we observed changes in endophyte frequency despite this suggests that our estimates of endophyte dynamics are at the very least conservative. Thus, endophytes do not appear to be acting as defensive mutualists against rabbit grazers in this system.

Differences in the role of endophytes in mediating resistance to herbivores may reflect a bias in the literature towards more agricultural systems. A recent meta-analysis found that 64% of studies testing the defensive mutualism hypothesis have focused on two agronomically important crops, perennial ryegrass (*Lolium perenne* L.), and tall fescue (*Schedonorus arundinaceus* (Schreb.) Dumort.) (Saikkonen et al. 2010a). These systems consistently show endophyte-mediated herbivore resistance. For example, studies using tall fescue found increasing endophyte frequencies in the presence of herbivores (Clay et al. 2005), or reduced abundance of arthropod herbivores on endophyte infected plants (Rudgers and Clay 2008). By contrast, studies in native grass systems, although fewer in number, have reported higher herbivore abundances on endophyte-infected plants (Jani et al. 2010; Faeth and Shochat 2010). However, the effects of endophytes on herbivore performance and communities are highly variable and dependent upon many factors, including soil moisture and nutrients (Saikkonen et al. 2006, 2010a; Saona et al. 2010), plant genotype (Yurkonis et al. 2014), types and concentrations of endophyte produced alkaloids (Jani et al. 2010), endophyte reproductive strategy (Tintjer and Rudgers 2006), endophyte hybridization (Saari et al. 2014), and herbivore identity and diet breadth (Hartley and Gange 2009; Afkhami and Rudgers 2009). In our system, for example, *F. rubra* is a preferred food source for rabbits and grazing often results in decreased abundance of this grass (Crawley 1990). It is possible that *F. rubra* in these grasslands are not toxic to rabbits, as *E. festucae* shows spatial and temporal variation in the profiles of herbivore deterrent alkaloids (Vázquez-de-Aldana et al. 2010). Nonetheless, it remains

unclear how often, and under what conditions, endophytes function as defensive mutualists in natural systems.

The defensive mutualism hypothesis would be supported in our system if arthropod herbivory was higher in ungrazed systems and endophytes provided protection against these enemies. In this scenario, increased arthropod herbivory would drive increased endophyte infection in ungrazed plots. We did not study arthropod herbivory, so we cannot rule out this possibility. Previous work at this site using *Holcus lanatus* and *Holcus mollis* documented the importance of insects in regulating endophyte dynamics, although insects had contrasting effects on endophyte frequencies depending on the plant species (Clay and Brown 1997). In North American grasslands, Clay et al. (2005) manipulated both insect herbivores and vertebrate grazers, and only found significant increases in endophyte frequencies when both were present. Invertebrate herbivores may generally be more important than vertebrates in driving infection frequencies due their chronic and persistent herbivory, transmission of plant diseases and pathogens, and consumption of both below- and above-ground tissues (Faeth and Saari 2012). Studies quantifying invertebrate herbivory within rabbit exclosures of different ages are needed to better understand the combined effects of rabbits and invertebrates in affecting endophyte frequencies in our system.

The observed increase in endophyte frequency may be unrelated to the herbivore-deterrent properties of endophytes that are typically reported. Resistance to herbivores in grass–endophyte symbioses occurs via endophyte-produced alkaloids, which can act against both invertebrate and vertebrate herbivores (Leuchtman et al. 2000). However, given that alkaloids are nitrogen-rich compounds that are costly to produce (Faeth and Fagan 2002), they may only be effective in reducing herbivore performance under high nutrient conditions (Lehtonen et al. 2005). While we did not measure alkaloids in this study, soils at our study site are nitrogen limited, and thus it is possible that endophytes do not produce alkaloids at levels required to deter rabbits, resulting in endophyte dynamics being unaffected by the presence or absence of rabbit herbivores.

Imperfect vertical transmission — the production of uninfected seeds from infected mother plants (Ravel et al. 1997; Afkhami and Rudgers 2008) — can result in variable infection frequencies and shape endophyte population dynamics (Gundel et al. 2008, 2011b). Imperfect vertical transmission of endophytes often depends most strongly on plant genetic background (Davitt et al. 2011; Gundel et al. 2011a; Gibert and Hazard 2013), although there is some evidence that biotic interactions (e.g., grazing) can influence endophyte transmission fidelity. For example in a recent study, the presence of grazing reduced the proportion of infected seeds produced by infected *Lolium multiflorum* (García-Parisi et al. 2012). Furthermore, the proportion of endophyte-infected seeds produced by infected parental plants is often positively correlated with endophyte frequencies within populations (Afkhami and Rudgers 2008; Gibert and Hazard 2013). In our system, a combination of reduced seed production and endophyte transmission fidelity may maintain lower infection frequencies in younger, more recently grazed exclosures. As time without grazing increases, increased endophyte transmission and seed production may be the cause of increased endophyte infection following the exclusion of rabbits. In fact, elevated endophyte frequencies may be maintained in natural populations even in cases where their mutualistic effects are minimal, provided there is sufficiently high endophyte transmission (Gundel et al. 2008). While we observed a clear benefit of endophyte infection on vegetative biomass of *F. rubra*, it remains to be tested whether this translates into differences in relative fitness between infected and uninfected plants in the field. Thus, further experiments are needed to accurately assess the influence of biotic and abiotic conditions on the efficiency of vertical transmission within rabbit exclosures in this system

and better assess the relative fitness of symbiotic and nonsymbiotic plants under natural field conditions.

The costs and benefits of endophytes to plants

We found that the presence of endophytes had a net positive effect on plant performance, resulting in increased growth rate, total biomass, and number of leaves. *Epichloë festucae* can have highly variable effects on *F. rubra* growth and biomass production, ranging from no effect (Zabalgogea et al. 2006) to negative (Vázquez-de-Aldana et al. 2012) or positive (Wäli et al. 2009) effects. Variation in these effects has been linked to plant genotype and habitat of origin (e.g., Vázquez-de-Aldana et al. 2012; Wäli et al. 2009). Similar to a previous study (Wäli et al. 2009), we found that endophyte-infected plants invested more in root over shoot tissues than uninfected plants (Supplementary text, Supplementary Fig. S3¹), which may aid in the uptake of nutrients from the nitrogen-limited soils at our study site. The physiological mechanisms by which endophytes promote host plant growth are poorly understood and such effects likely depend on nutrient concentrations (Bacon 1993), the presence of endophyte hybridization (Hamilton et al. 2010), and interactions between endophyte and host genotypes (Cheplick and Cho 2003; Cheplick 2008; Saikkonen et al. 2010b). Endophytes may promote host plant growth via the production of phytohormones such as cytokines and indole-3-acetic acid (IAA) (Tan and Zou 2001). More research on the causes and consequences of endophyte-influenced plant growth rate would help in understanding the mechanisms underlying the costs and benefits of endophytes in this and other systems.

Endophytes can also influence tolerance to herbivory (Faeth and Saari 2012), which is defined as a plant's ability to maintain fitness in the presence of damage by herbivores (Strauss and Agrawal 1999). When endophytes affect tolerance to herbivory, variation in herbivore pressure can affect infection frequency. For example, a recent study of meadow fescue (*Schedonorus pratensis* (Huds.) P.Beauv.) found that endophyte infected plants exhibited reduced tolerance to herbivores and ungrazed pastures exhibited greater endophyte infection frequencies (Saari et al. 2010). Their results imply that endophytes can impose a cost to plants by reducing tolerance. Although we also found greater infection frequencies in older, ungrazed, exclosures, infected and uninfected plants did not differ in tolerance to herbivory (Supplementary Fig. S2¹). Therefore, endophytes do not appear to impose a cost to plants in terms of reduced tolerance that can explain the lower infection frequencies observed in recently grazed exclosures.

The costs and benefits of endophyte infection may also depend on intraspecific competition. Infected plants were affected more by the presence of conspecific competitors than uninfected plants (Fig. 3). This suggests that intraspecific competition may reduce the benefits of endophytes on plant performance. Intraspecific competition should be highest in the absence of rabbit grazing, where *F. rubra* reaches higher densities (Edwards and Crawley 1999b). If greater intraspecific competition drives decreases in endophyte frequency, then infection frequencies should be lower in ungrazed environments, reflecting the reduced benefits of endophytes. By contrast, we found increased endophyte frequencies in the absence of grazing, suggesting that factors other than intraspecific competition regulate endophyte frequencies in this system. The differential impact of competitors on infected and uninfected plants was likely an artifact of the greenhouse experiment having been terminated before treatments with smaller plants obtained a size that allowed for intense competition. In this way, the greater growth rate of infected plants enabled plants to begin competing earlier, while uninfected plants never reached a size where competition was an important factor mediating their growth.

While defense against grazing herbivores does not seem to explain the increase in frequency observed in our study, our results suggest that endophytes confer a benefit to *F. rubra* plants in the

absence of rabbit grazing. Endophytes should only increase in frequency if the benefits they impart on host plant fitness outweigh the costs to the host of harboring them (Rudgers et al. 2012). The increase in endophyte frequency observed in our study may be mediated by the direct effects of endophytes on host plant growth. We showed that endophytes benefit *F. rubra* through increased growth and biomass. However, such benefits may be most prominent in the absence of rabbit grazing, where plants are larger and *F. rubra* typically increases in abundance (Edwards and Crawley 1999a, 1999b). In these populations, infected individuals grow faster and larger, and may become overrepresented in subsequent generations because of greater reproduction via seeds and tillers. In the presence of rabbits, the benefits of endophytes may be diminished because constant grazing reduces absolute differences in biomass between infected and uninfected plants. Thus, small absolute differences in biomass among infected and uninfected plants may not be biologically important in this setting and thus infected plants would not be at an advantage. Long-term reciprocal transplant experiments of infected and uninfected plants across grazed and ungrazed environments are needed to provide a conclusive answer to the relative benefits of endophytes among these environments.

Conclusions

Using a long-term field study coupled with a manipulative factorial experiment, we show that the defensive mutualism hypothesis does not explain interactions between *Epichloë* endophytes and their *Festuca* hosts in a British grassland subject to heavy grazing. Infected plants had greater growth and biomass but did not differ in tolerance to herbivory, suggesting endophytes directly change plant growth but not compensatory responses to damage. Furthermore, endophyte-mediated increases in host plant growth may benefit hosts most in the absence of rabbits, where greater density and seed production of *F. rubra* occur (Edwards and Crawley 1999a, 1999b). Our work demonstrates the need to examine the costs and benefits of native grass – endophyte interactions across a range of ecological conditions using long-term field experiments to better understand the factors regulating their frequencies in natural populations.

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