Testing the roles of vertical transmission and drought stress in the prevalence of heritable fungal endophytes in annual grass populations

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Summary

- Beneficial inherited symbionts are expected to reach high prevalence in host populations, yet many are observed at intermediate prevalence. Theory predicts that a balance of fitness benefits and efficiency of vertical transmission may interact to stabilize intermediate prevalence.

- We established populations of grass hosts (*Lolium multiflorum*) that varied in prevalence of a heritable fungal endophyte (*Epichloë occultans*), allowing us to infer long-term equilibria by tracking change in prevalence over one generation. We manipulated an environmental stressor (elevated precipitation), which we hypothesized would reduce the fitness benefits of symbiosis, and altered the efficiency of vertical transmission by replacing endophyte-positive seeds with endophyte-free seeds.

- Endophytes and elevated precipitation both increased host fitness, but symbiont effects were not stronger in the drier treatment, suggesting that benefits of symbiosis were unrelated to drought tolerance. Reduced transmission suppressed the inferred equilibrium prevalence from 42.6% to 11.7%. However, elevated precipitation did not modify prevalence, consistent with the result that it did not modify fitness benefits.

- Our results demonstrate that failed transmission can influence the prevalence of heritable microbes and that intermediate prevalence can be a stable equilibrium due to forces that allow symbionts to increase (fitness benefits) but prevent them from reaching fixation (failed transmission).

Introduction

Microbial symbionts of plants and animals are widespread in nature (White *et al.*, 1993; Wilson, 1993; Rudgers *et al.*, 2009) and can affect their hosts in a variety of ways, including growth, competitive ability, stress tolerance and reproduction (Douglas, 1998; Clay & Holah, 1999; Faeth, 2002; Engelstädter & Hurst, 2009). For example, microbial symbionts associated with arthropods can increase host resistance to natural enemies (Haine, 2008), and microbial symbioses between zooxanthellae and corals can modify host responses to climate change and ocean acidification (Berkelmans & Van Oppen, 2006; Hume *et al.*, 2015).

Transmission mode (vertical, horizontal or both) is an important feature of many symbioses. Vertical transmission from parent to offspring generates positive fitness feedbacks between host and symbiont and is therefore expected to select for host–symbiont mutualism (Ewald, 1987; Sachs *et al.*, 2004). Furthermore, there is an expectation that symbionts which are both heritable and beneficial should be highly prevalent in their host

populations because symbiotic hosts regenerate at a greater rate than nonsymbiotic ones (Clay, 1990; Cheplick & Faeth, 2009). However, symbiont prevalence is often quite variable across host populations and species (Gibert & Hazard, 2013; Yule *et al.*, 2013; Semmartin *et al.*, 2015; Sneck *et al.*, 2017). For example, the pea aphid harbors heritable bacterial symbionts that occur at high but variable prevalence between 50 and 80% (Chen & Purcell, 1997; Chen *et al.*, 2000), and other arthropod symbioses show similar patterns (Hilgenboecker *et al.*, 2008). In cool-season grasses, which commonly host heritable fungal endophytes (*Epichloë* spp.), symbiont prevalence is variable to a similar degree (Rudgers *et al.*, 2009; Semmartin *et al.*, 2015).

Theoretical models have suggested that intermediate prevalence of symbionts may reflect the combined effects of fitness benefits and vertical transmission rate (the fraction of offspring of symbiotic parents that fail to inherit the symbiont) (Ravel *et al.*, 1997; Gundel *et al.*, 2008; Hancock *et al.*, 2011; Bibian *et al.*, 2016). Many heritable symbionts, even those thought to be beneficial, are imperfectly transmitted (Afkhami & Rudgers, 2008;

Longdon *et al.*, 2017; Sneck *et al.*, 2017). Imperfect vertical transmission may lead to intermediate prevalence at the population level because it provides a constant source of nonsymbiotic hosts, even when there is a strong fitness advantage to symbiosis (Gundel *et al.*, 2008; Bibian *et al.*, 2016). Theory predicts that intermediate prevalence is stabilized by the combined effects of fitness benefits, which favor an increase in symbiont prevalence, and imperfect vertical transmission, which prevents fixation of symbiotic hosts. All else being equal, stronger fitness benefits should lead to greater symbiont prevalence (Gundel *et al.*, 2008). There also is a prediction from theory that the fitness effects of symbiosis and the vertical transmission rate should interact to determine symbiont prevalence. Specifically, imperfect transmission is predicted to have a greater negative effect on symbiont prevalence when fitness benefits are weaker (Saikkonen *et al.*, 2002; Gundel *et al.*, 2008; Bibian *et al.*, 2016). The effects of symbionts on host fitness are often context-dependent, including positive, neutral or even negative effects depending on environmental conditions (Heath & Tiffin, 2007; Davitt *et al.*, 2011; Chamberlain *et al.*, 2014; Gibert *et al.*, 2015). Context-dependent variability in the fitness effects of symbiosis, combined with imperfect symbiont transmission, may therefore be important sources of variation in symbiont prevalence across host populations and species.

Despite clear theoretical predictions for the influence of fitness benefits and vertical transmission on population-level symbiont prevalence, empirical understanding of these processes lags behind theory. Some observational surveys have supported the hypothesis that symbionts reach higher prevalence in more stressful environments, such as endophyte symbioses that increase in prevalence along gradients related to drought (Lewis *et al.*, 1997) or grazing (Granath *et al.*, 2007), whereas other surveys found more complex patterns of variation with respect to environmental variables (Bazely *et al.*, 2007; Sem-martin *et al.*, 2015; Gundel *et al.*, 2016; Sneck *et al.*, 2017). Data-driven modeling has been used to explore the balance of fitness benefits and transmission in driving symbiont prevalence (Yule *et al.*, 2013; Miller & Rudgers, 2014; Chung *et al.*, 2015; Gibert *et al.*, 2015). Finally, a handful of experimental studies have directly tested for changes in population-level symbiont prevalence in response to manipulations of environmental stressors that are thought to modify the fitness effects of symbiosis (Clay *et al.*, 2005; Oliver *et al.*, 2008; Erickson *et al.*, 2012; Santangelo *et al.*, 2015). For example, in both plant and animal hosts, heritable microbes reached greater prevalence in the presence vs absence of natural enemies, likely due to context-dependent fitness benefits of protective symbiosis (i.e. greater benefit when protection is required; Clay *et al.*, 2005; Oliver *et al.*, 2008). However, most empirical studies of context-dependent symbiosis have focused on fitness benefits without also considering vertical transmission.

Here we report results of a field experiment designed to test predictions for the roles of vertical transmission and environmental stress in the prevalence of heritable fungal symbionts (*Epichloë occultans*) in populations of host grasses (the annual ryegrass *Lolium multiflorum*). Endophyte symbiosis in *L. multiflorum* has

been shown to enhance several aspects of host fitness and increase resistance to herbivores, but these effects vary in magnitude and may depend on environmental context, including water availability and herbivory (Omacini *et al.*, 2001, 2009; Vila-Aiub *et al.*, 2005; Gundel *et al.*, 2011). Prevalence of symbiosis with *E. occultans* varies across natural populations of *L. multiflorum* (White & Cole, 1985; Latch *et al.*, 1987), although the drivers of this variation are poorly resolved. We focused on an annual host because it has the advantages that fitness effects of symbiosis are straightforward to measure (seed production and recruitment provide a complete estimate of fitness) and because changes in population endophyte prevalence should occur rapidly due to annual population turnover.

Our factorial experiment imposed the following treatments on replicated host populations: reductions in effective vertical transmission (by replacing endophyte-positive (E+) seeds with endophyte-free (E−) seeds); elevated precipitation to mimic realistically wet years; both; or neither (control). Water availability is known to modify the fitness effects of fungal endophytes: several studies have shown stronger benefits of endophyte symbiosis under drought stress (reviewed in Cheplick & Faeth, 2009). The biochemical and physiological mechanisms by which endophytes ameliorate drought stress are not well known but drought tolerance-promoting effects of fungal secondary metabolites (especially loline alkaloids) and anti-oxidants have been hypothesized or implicated (reviewed in Malinowski & Belesky, 2006). Previous studies of drought-dependent endophyte benefits in *L. multiflorum* are limited and have generated variable results, including an E+ advantage under water stress for certain life stages in one study (Gundel *et al.*, 2006) but no water-dependent effects in another study, despite their detection of loline alkaloids in E+ hosts (Omacini *et al.*, 2009). Based on trends across the grass–endophyte literature, we hypothesized that elevated precipitation would reduce the fitness benefits of symbiosis, and that reducing the fitness advantage of symbiotic hosts would reduce population-level symbiont prevalence. We further hypothesized that precipitation treatment would interact with vertical transmission. Specifically, if elevated precipitation reduces the benefits of symbiosis, then reduced transmission should cause a greater reduction in symbiont prevalence under the more benign environmental context of elevated precipitation than under ambient precipitation. Our experiment tracked changes in population-level endophyte prevalence from a continuous range of initial prevalence, a space-for-time substitution that allowed us to infer long-term equilibria from short-term observations and to determine whether intermediate prevalence was stabilized by forces that allow symbionts to increase in prevalence (fitness benefits) but prevent them from reaching fixation (imperfect transmission).

Specifically, we addressed the following questions: does endophyte symbiosis confer fitness benefits to hosts and does elevated precipitation reduce these benefits? Are fungal endophytes imperfectly transmitted from maternal plants to seeds and is vertical transmission affected by elevated precipitation? How does the expected equilibrium prevalence of endophyte symbiosis (inferred from our experimental design) respond to factorial
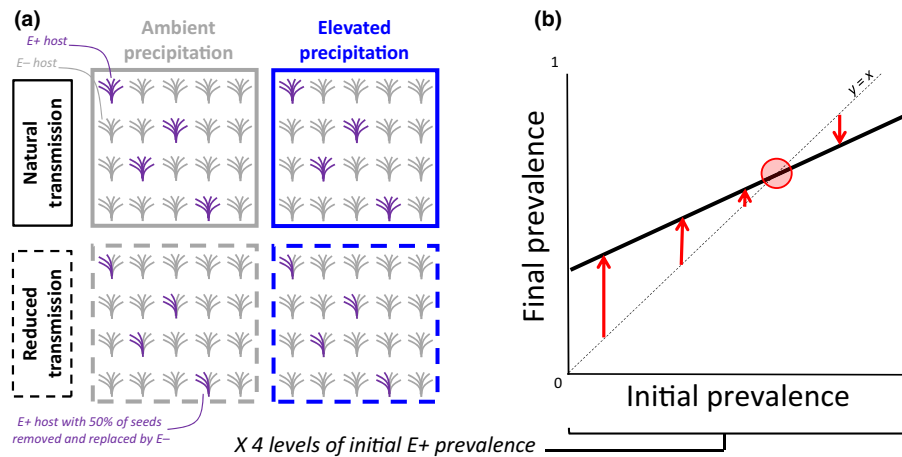
Fig. 1 (a) Overview of experimental design and (b) statistical inference for stable intermediate endophyte prevalence. (a) Experimental populations (boxes) were established with endophyte-positive (E+, purple) and endophyte-free (E−, gray) hosts in varying proportions to meet target initial prevalence of 20 (depicted in the figure), 40, 60 or 80% E+ (realized initial prevalence deviated from these targets; see Fig. 4 and Supporting Information Fig. S3). Populations were assigned to a factorial manipulation of precipitation (ambient/elevated) and vertical transmission (natural/reduced). For reduced-transmission populations, 50% of mature inflorescences (including seeds) were removed from E+ hosts and replaced with the same number of inflorescences from E− hosts. (b) Variation in final endophyte prevalence (2014) was regressed against initial prevalence (2013) to infer long-term equilibrium prevalence (red circle). We hypothesized that fitness benefits would allow endophyte prevalence to increase but that imperfect transmission would prevent endophyte fixation. Arrows illustrate the idea that an increase in prevalence when rare and a decrease when common would result in a stable intermediate equilibrium (red circle), which requires a regression intercept that is positive and a slope less than one.

manipulation of precipitation and vertical transmission? Is intermediate endophyte prevalence an ecologically stable state?

Materials and Methods

Study system and plant material

Lolium multiflorum (Lam.) is a cosmopolitan forage grass native to Eurasia and naturalized throughout temperate regions of South and North America, including east Texas, where our experiment was conducted. In our study region, these annual plants germinate in winter (December–January), flower in spring (May) and senesce in summer (July). Flowering plants can produce over 150 inflorescences (hereafter 'spikes'), each of which includes many seeds. Throughout its range, *L. multiflorum* is symbiotic with the seed-transmitted fungal endophyte *Epichloë* (=*Neotyphodium*) *occultans* (Moon *et al.*, 2004), which may enhance tolerance to biotic and abiotic stresses (Andria *et al.*, 2009; Pérez *et al.*, 2013). Endophytes can be detected via microscopy in seeds and seedlings but are difficult to detect in mature plants (hence the name 'occultans'). We never observed horizontal transmission by *E. occultans* (indicated by externally visible fungal stroma) during this experiment or in our previous studies, so we assume that endophyte transmission was strictly vertical.

Experimental plants were derived from a naturalized population in Argentina with high (*c.* 90%) endophyte prevalence. Seeds were collected from old-field populations in the inland Pampa, 400 km west of Buenos Aires (Carlos Casares County, Buenos Aires province, Argentina; 35°55′S, 61°09′W). Additional information about the source population is provided in Uchitel *et al.* (2011). We eliminated the endophyte from a subset

of these seeds using fungicide treatment, as described in Supporting Information Methods S1. Seedlings from these endophyte-positive (E+) and endophyte-free (E−) seed stocks were transferred to 3.8 cm × 12.7 cm cylindrical containers filled with ProMix potting soil and allowed to grow to one to three tillers in size before transplanting into our field experiment.

Field experimental design

Our field experiment was conducted at the USDA NRCS East Texas Plant Materials Center near Nacogdoches, TX, USA (31°30′N, 94°45′W). The mean total annual precipitation at this site (118 cm) is similar that of the source population in Argentina (102 cm). The soil series is a Woden Fine Sandy Loam with 1–4% slopes. *Lolium multiflorum* did not naturally occur at this site during our study.

In December 2012, we established $N = 48$ 2 × 2 m experimental plots in a newly tilled field, with 3 m of spacing between adjacent plots. Each plot had a stake in each corner and was enclosed by deer netting, which was intended to both protect transplants from vertebrate browsers and to limit seed movement between plots. Seed dispersal occurs as spikes break off senescing plants. Although single seeds could pass through the netting, loose spikes could not. Our goal was to treat each plot as an independent population, where recruitment predominantly reflected seed production inside the plot.

Each plot was randomly assigned to one of four treatments in a factorial cross of ambient/elevated precipitation and natural/reduced vertical transmission (Fig. 1a), with $n = 12$ plots per treatment. Within each treatment, each of the 12 plots was randomly assigned to a target initial endophyte prevalence (20%, 40%, 60% or 80% E+), each replicated three times; plot-to-plot

variation therefore reflects the combined influence of endophyte prevalence and random spatial heterogeneity. During December 2012, we transplanted 20 founder individuals into each plot in a 4 × 5 grid, drawing from E+ and E− glasshouse-raised plants to meet the target endophyte frequency and to equalize genetic diversity across plots, applying the rule that no plot could include two siblings from the same maternal plant. Based on our screening of seedlings, we were confident that individuals designated E− were truly endophyte-free. The same was not true of plants from the E+ seed stock, where endophyte prevalence was 83% (Methods S1). We therefore expected that, on average, planting five and 15 founder individuals from the E+ and E− stocks, respectively, would create a population with *c.* 20% endophyte prevalence, for example. By scoring the seeds produced by these founders (below), we were able to quantify the true starting prevalence once the experiment was underway.

Precipitation treatment Plots assigned to elevated precipitation were equipped with an irrigation system that increased precipitation above ambient amounts to approximate an extremely wet year in our study region. We defined 'extremely wet' by averaging over the three wettest years during the period of 1895–2012 for the three weather stations closest to our experimental site. The elevated precipitation treatment began in April 2013 and ran until the end of the experiment, in June 2014. Control plots received ambient precipitation during that time. Additional details of the precipitation treatment are provided in Methods S1.

Vertical transmission treatment For plots assigned to the vertical transmission reduction treatment, we reduced the population mean transmission rate by 50% by replacing half of the spikes of E+ plants per plot with spikes from E− plants (Fig. 1a). We did not manipulate vertical transmission at the scale of the individual plant; instead, we modified the 'effective' mean transmission rate at the population scale, reducing the relative production of E+ vs E− recruits by E+ hosts without changing absolute fecundity. A 50% transmission reduction is ecologically realistic, because some grass–endophyte taxonomic pairs can exhibit up to 10-fold variation in vertical transmission rate across host individuals or populations (Sneck *et al.*, 2017). Further details of the transmission reduction methods are provided in Methods S1.

Response variable data collection

In June 2013, we surveyed survival and the total number of spikes produced for at least 10 founder individuals, including E+ and E−, in the 24 plots assigned to vertical transmission reduction. We focused demographic data collection in the transmission reduction plots (12 ambient and 12 elevated precipitation) because it was efficient to combine these observations with spike counts needed for the seed exchange. Data from these plots should be representative of the transmission control plots because they had been treated identically up until data collection. For a subset of E+ and E− individuals for which we counted spikes, we

also collected three randomly selected spikes and, in the laboratory, weighed their total seed mass to estimate seeds per spike.

For all founders from the E+ stock, we scored a minimum of four seeds for the presence/absence of endophytes (Methods S1), which allowed us to designate the founder as E+ (if any of the four seeds were E+) or E− (if none were E+). We chose four seeds because, on average, it would allow us to detect E+ maternal plants even with a transmission rate as low as 25%, which is lower than documented transmission rates in this system (Gundel *et al.*, 2011). This information allowed us to know the true endophyte status of the founder individuals, and therefore to estimate the initial endophyte prevalence of each plot. For a subset of 22 individuals verified as E+, we scored 20 additional seeds to gain better resolution of the vertical transmission rate. There were 85 founder plants from the E+ stock (out of 578) that we were unable to score, usually because their seeds were unripe or unfilled.

One year later, in June 2014, we revisited each plot to estimate recruitment and endophyte prevalence. We recorded recruit density in three 0.25 × 0.25 m subplots within each plot. We collected at least four seeds from each of 10 recruits within each subplot. Where subplot densities were lower than 10, we sampled outside the subplots to reach a target of 30 recruits per plot. In the lab, these seeds were scored (Methods S1) to designate each plant as E+ or E−.

Data analysis

Host plant seed production Founder survival was very high (> 97.5%), so we focused analyses on seed production and recruitment. Per-capita seed production reflects a combination of spike number and seeds per spike. We had more observations of spike production (233 individuals from 24 plots) than of seeds per spike (57 individuals from 12 plots) so we analyzed the two reproductive measures separately. In these analyses, individuals were designated E+ only if verified by our seed scores. We fitted a linear mixed model (R package 'LME4'; Bates *et al.*, 2015) to the natural logarithm of spike number as a Gaussian response variable, including fixed effects of endophyte status, precipitation treatment, and their interaction, and the random effect of plot. We fitted a similar model to seed mass per spike, which had normally distributed residuals without requiring any transformation. For both responses, we evaluated statistical significance of endophyte status, water addition treatment, and their interaction using Wald Chi-square tests (R package 'CAR'; Fox & Weisberg, 2011). For models with significant fixed-effect predictors, we quantified marginal and conditional R^2, which approximate the proportion of total variance explained by the fixed effects and fixed + random (plot) effects, respectively (Nakagawa & Schielzeth, 2013), using the R package PIECEWISESEM (Lefcheck, 2016).

Vertical transmission We used a generalized linear mixed model to estimate the vertical transmission rate of E+ hosts and test whether water addition modified transmission at the individual level. The model included precipitation treatment

as a predictor variable, where transmission was a binomial response variable (E+ seeds = 'successes', total seeds scored = 'trials') and plot was included as a random effect. We used a Chi-square test of the likelihood ratio to determine whether precipitation treatment significantly explained variability in transmission.

Recruitment In order to estimate the probability of recruitment from seed, we relied on indirect inference based on the combination of seed production in 2013 and recruitment in 2014. To avoid propagation of errors, we used a bootstrapping approach that accounted for the uncertainty associated with each step of the estimation process, as described in Methods S1. We inferred statistical significance of endophyte symbiosis and water addition by comparing the 95% confidence intervals of seed recruitment probability for each treatment derived from the distributions of bootstrap replicates.

Population prevalence Our seed scoring of the original founders (in 2013) and the new recruits that replaced them (in 2014) provided estimates of initial and final endophyte prevalence of each plot. We fitted a linear model to test for precipitation and vertical transmission treatment effects on the intercept and slope of the relationship between initial and final endophyte prevalence at the plot level (Fig. 1b), assuming that the prevalence values were known without error. For 2013 prevalence, we assigned the subset of unverified E+ plants as true E+, because we knew this seed stock to be majority E+, and we accounted for uncertainty in these assignments below. The model included final prevalence as a Gaussian response variable and precipitation treatment, vertical transmission treatment, initial prevalence, and all interactions as predictors; because prevalence observations were at the plot level, there were no plot random effects (we pooled 2014 data across three subplots plus samples from outside subplots). Although endophyte prevalence is not truly Gaussian (it is bounded by zero and one), we found that the data suitably met model assumptions, including normally distributed residuals with constant variance. We evaluated significance of model terms by ANOVA.

Defining the relationship between initial (y^{initial}) and final (y^{final}) prevalence allowed us to infer a long-term equilibrium (prevalence at which no change is expected; Fig. 1b) – assuming the environment remains constant at the conditions of our study period. Given the relationship $y^{\text{final}} = a + b * y^{\text{initial}}$, equilibrium prevalence is given by $\hat{y} = \frac{a}{(1-b)}$. This equilibrium is ecologically stable if prevalence increases when $y^{\text{initial}} < \hat{y}$ and decreases when $y^{\text{initial}} > \hat{y}$, which requires that $a > 0$ and $b < 1$. Significant effects of precipitation or transmission treatments on regression parameters therefore translate to effects on equilibrium prevalence. As a special case, if final prevalence perfectly tracks initial prevalence ($y^{\text{final}} = y^{\text{initial}}$), then symbiont dynamics are perfectly neutral and there is no equilibrium (i.e. it is undefined; $\hat{y} = \frac{0}{0}$). We used a bootstrapping approach to quantify uncertainty distributions for the intercept and slope, and thus equilibrium prevalence ($\hat{y}$), as described in Methods S1.
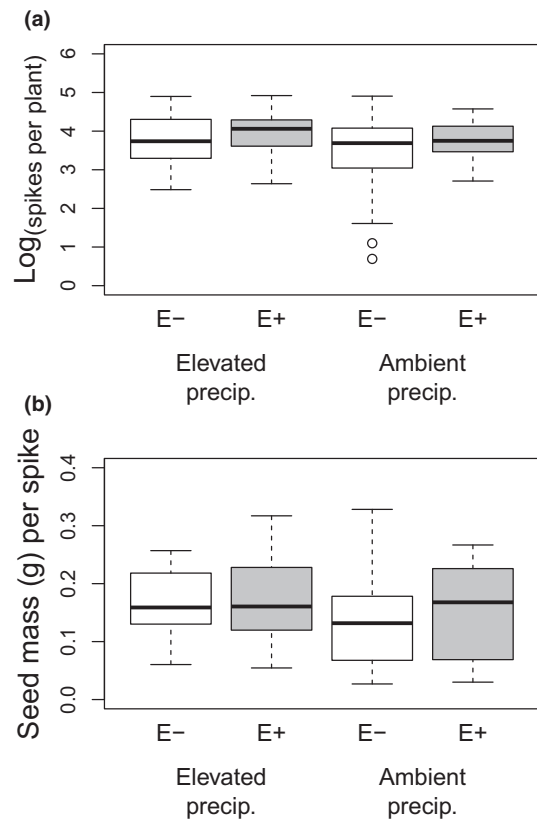
Fig. 2 Reproductive fitness of endophyte-positive (E+) and endophyte-free (E−) *Lolium multiflorum* hosts under ambient and elevated precipitation. (a) Natural logarithm of spike number, and (b) mass (g) of seeds per spike. Boxplots show raw data across individuals and plots (thick lines are medians, box limits show the first and third quartile, whiskers extend beyond box limits 1.5 times the interquartile range, and open points are outliers). In statistical analyses (see Table 1), plot was included as a random effect.

Results

Treatment effectiveness

Manipulations of precipitation, vertical transmission and initial endophyte prevalence had the intended effects (Notes S1). Elevated precipitation increased available soil moisture by 55% over control plots (Fig. S1). The seed replacement method reduced the effective vertical transmission rate at the plot level from 94% for control plots to 55% for transmission reduction plots (Fig. S2). Realized endophyte prevalence at the start of the experiment closely tracked target prevalence (Fig. S3).

Host fitness and symbiont transmission

Host reproduction Endophyte symbiosis and elevated precipitation increased host spike production (Fig. 2a). Both main effects were statistically significant but there was no interaction between them (Table 1). On average, endophyte symbiosis increased spike production by *c.* 7% (mean ± SD spikes per plant, E−: 47.7 ± 29.5, E+: 51.1 ± 29.3), and elevated precipitation increased spike production by *c.* 26% (control:

Table 1 Hypothesis tests for significance of fixed-effect variables in analysis of *Lolium multiflorum* spike and seed production

Fixed-effect predictor variables	Spike production		Seed mass per spike	
	χ^2	P	χ^2	P
Endophyte status	4.14	0.041	0.36	0.55
Precip. treatment	3.65	0.055	2.08	0.15
Endophyte status × Precip. treatment	0.013	0.91	0.0012	0.97

Model included a random effect of plot. Each Wald χ^2 test had 1 df.
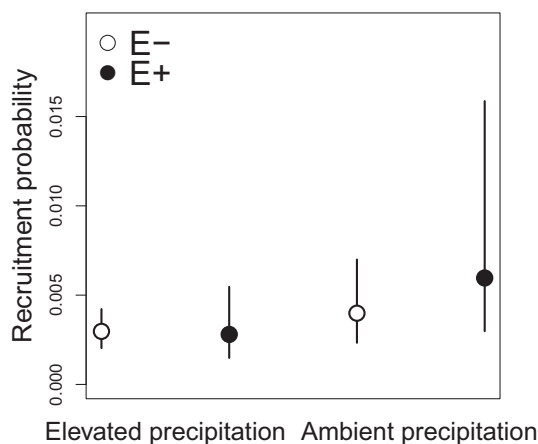
Fig. 3 Bootstrapped estimate of *Lolium multiflorum* recruitment (flowering recruits in 2014 per seed produced in 2013) of endophyte-free (E−) (open points) and endophyte-positive (E+) (closed points) hosts under ambient and elevated precipitation. Points show means and bars represent inner 95% quantiles of bootstrap distributions.

43.9 ± 26.3, addition: 55.6 ± 31.1). The proportion of total variance explained by precipitation treatment and endophyte status (marginal R^2) was 0.055 and the proportion explained by the fixed factors plus random plot effects (conditional R^2) was 0.17, suggesting that there was substantial unexplained variance and that random plot-to-plot variability exceeded the influence of fixed effects. In contrast to spike production, the mass of seeds produced per spike was not significantly influenced by precipitation treatment (mean ± SD mass (g) per spike, control: 14.2 ± 0.801, addition: 16.9 ± 0.067), endophyte symbiosis (E−: 16.2 ± 0.068, E+: 14.9 ± 0.083), nor their interaction (Table 1; Fig. 2b). The fixed and random effects, combined, explained just 4.04% of the total variation in seed mass per spike.

Recruitment Bootstrapped estimates of recruitment to flowering indicated no significant differences between E+ and E− hosts or between ambient and elevated precipitation plots, based on overlap of 95% confidence intervals (Fig. 3). However, there was a trend toward a stronger benefit of endophyte symbiosis under ambient than elevated precipitation. Under ambient conditions, mean establishment probability of E+ seeds was 49% greater than E− seeds, on average (mean ± SD recruits per seed, E− = 0.0039 ± 0.0012, E+ = 0.0054 ± 0.0023). Under elevated

precipitation, endophytes caused a small (5.6%) reduction in mean seed establishment (E− = 0.0029 ± 0.00056, E+ = 0.0028 ± 0.0011).
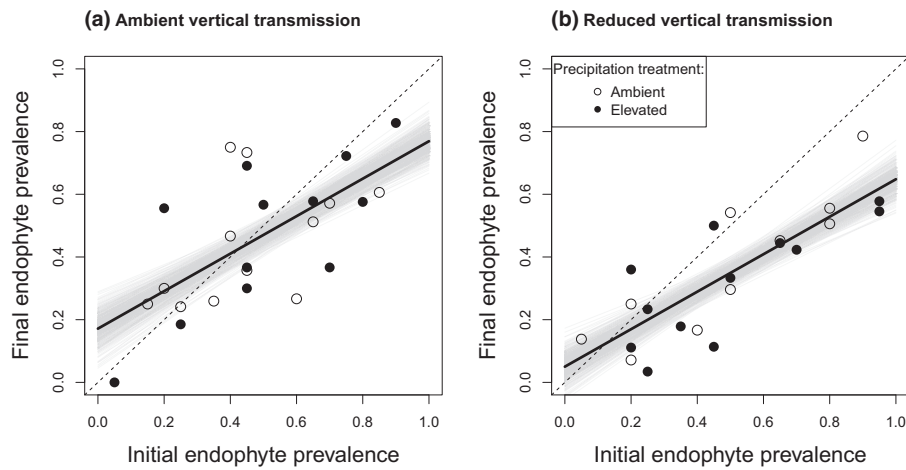
Symbiont transmission Vertical transmission of endophytes was high and not significantly affected by precipitation treatment ($\chi^2 = 1.09$, df = 1, $P < 0.296$). Mean vertical transmission rate was 93.4% under elevated precipitation and 94.6% under ambient precipitation.

Population-level symbiont prevalence

Endophyte prevalence after 1 yr (generation) of population dynamics was positively related to initial endophyte prevalence (Table 2; Fig. 4). Experimental reduction in vertical transmission significantly depressed the intercept of this relationship (significant main effect) but did not modify the slope (nonsignificant initial prevalence × transmission interaction), resulting in overall lower endophyte prevalence in the year following the manipulation (Fig. 4). Precipitation treatment had no significant main or interactive effects on the change in prevalence (Table 2). The statistical model had an R^2 value of 0.58, suggesting that experimental treatments (including initial prevalence) explained more than half of the variation in final prevalence. Bootstrapped parameter estimates showed that, under natural vertical transmission, the intercept was significantly greater than zero (mean (95% CI): 0.172 (0.112–0.24)) and the slope was significantly less than one (0.597 (0.501–0.686)), corresponding to an expected increase in prevalence when endophytes were rare and an expected decrease when they were common (Fig. 4) and resulting in a stable equilibrium prevalence of 42.6% E+ (95% CI: 32.1–51.6% E+) (Fig. 5). Under natural vertical transmission, 53% (eight of 15) of the populations that were initiated at low (< 50%) E+ prevalence increased in prevalence by the end of the experiment, whereas 0% (zero of nine) of the populations initiated at high (> 50%) E+ prevalence increased in prevalence (Fig. 4). By contrast, under reduced vertical transmission, the mean intercept was significantly reduced and its confidence interval included zero (mean (95% CI): 0.049 (−0.0093, 0.107)), indicating that the balance of fitness benefits and experimentally reduced transmission made it difficult for endophytes to spread in populations where they were initially rare. Only five populations in the reduced vertical transmission treatment increased in prevalence

Table 2 Analysis of variance in final (2014) plot-level endophyte prevalence in *Lolium multiflorum* populations

Variable	df	F	P
Initial prevalence	1	43.91	<0.0001
Precip. treatment	1	0.434	0.51
Transmission treatment	1	7.4	<0.01
Initial prevalence × Precip.	1	0.0009	0.97
Initial prevalence × Transmission	1	0.0173	0.89
Precip. × Transmission	1	0.332	0.56
Initial prevalence × Precip. × Transmission	1	1.122	0.29
Residuals	38		

(a) Ambient vertical transmission

(b) Reduced vertical transmission

Fig. 4 Relationships between initial and final endophyte prevalence in *Lolium multiflorum* populations under (a) natural and (b) reduced vertical transmission and under ambient (open points) or elevated (closed points) precipitation. Each point represents one plot. Thick lines are fitted regression models showing a significant difference between vertical transmission treatment (there was no difference between precipitation treatments and the fitted line corresponds to both open and filled points). Each thin gray line represents one bootstrap replicate in which a random 25% of the plots and 25% of the data within plots were excluded and the regression was re-estimated. Thin dashed line shows $y = x$ and corresponds to neutral endophyte dynamics. Intersection of fitted line and $y = x$ indicates equilibrium prevalence (see Fig. 1).
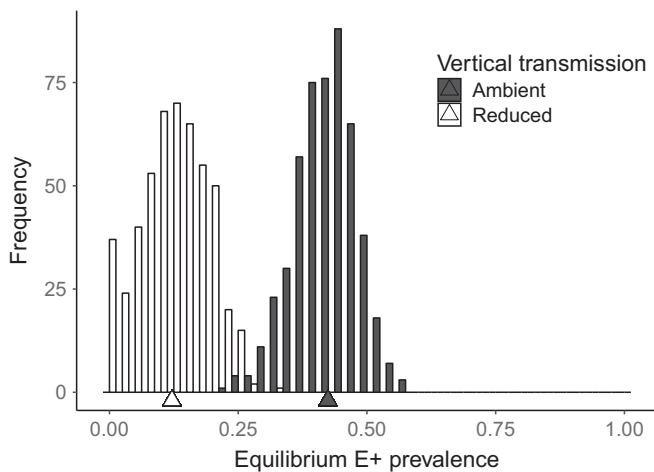
Fig. 5 Distributions of equilibrium endophyte prevalence in *Lolium multiflorum* populations under natural and reduced vertical transmission, derived from the fitted relationships in Fig. 3. Triangles indicate means for each vertical transmission treatment. Uncertainty distributions were generated by bootstrapping 75% of plots and plants within plots 500 times. The 95% confidence interval for prevalence under natural transmission (32.1–51.6% endophyte-positive (E+)) excludes zero (endophyte extinction) and is greater than that of the reduced transmission treatment (0.0–22.7% E+), which includes zero.

and all of these were initiated at low ($< 50\%$ E+) prevalence (Fig. 4). As a consequence, the expected equilibrium endophyte prevalence under reduced vertical transmission was, on average, 11.7% E+ (95% CI: 0.0–22.7% E+), significantly lower than natural transmission (Fig. 5). The distribution of equilibrium prevalence across bootstrap replicates was bimodal for reduced transmission, with zero and nonzero modes. Given the uncertainty in our data, endophyte extinction ($\hat{y} = 0$) was expected with 9.2% probability under reduced vertical transmission and 0.0% under natural transmission.

Discussion

We showed that intermediate prevalence of heritable symbionts can be an ecologically stable outcome, where fitness benefits allow prevalence to increase but failed transmission prevents symbionts from reaching fixation. We also demonstrated experimentally that reduced vertical transmission can reduce equilibrium symbiont prevalence and increase the odds of symbiont extinction from host populations, despite the benefit of increased seed production. The fitness advantage of endophyte symbiosis was not dependent on precipitation context, contrary to expectations from the literature (e.g. Kannadan & Rudgers, 2008; Afkhami *et al.*, 2014), although the opposite has also been found (e.g. Rudgers & Swafford, 2009). As a result, reducing the effective vertical transmission rate in a similar way reduced endophyte prevalence under both ambient and elevated precipitation. Below, we discuss these results in greater detail and their implications for general understanding of host–symbiont dynamics.

There is now growing evidence for variation in the prevalence of heritable symbionts across host populations and species (e.g. Rudgers *et al.*, 2009; Semmartin *et al.*, 2015; Sneck *et al.*, 2017). Nonetheless, observational surveys and even experiments have been unable to rigorously determine whether intermediate symbiont prevalence is a stable state, a neutral state reflecting only historical contingency, or a transitional state on a path toward symbiont fixation or extinction. Our work not only predicted a long-term equilibrium of intermediate symbiont prevalence, but also showed that this equilibrium can be ecologically stable. Previous studies have predicted this result based on theoretical or data-driven modeling (e.g. Ravel *et al.*, 1997; Gundel *et al.*, 2008; Yule *et al.*, 2013; Gibert *et al.*, 2015); our study is, to our knowledge, the first empirical demonstration that intermediate symbiont prevalence exhibits a return tendency. Although we showed that intermediate symbiont prevalence was a stable state,

the equilibrium values that we identified (Figs 4, 5) depended on the assumption that the environment remains constant at the conditions of our 1-yr study. Interannual environmental variability may cause the fitness effects and/or transmission rate to vary through time, and equilibrium prevalence in such a temporally stochastic environment would likely deviate from our findings from a single year. Also, although we focus on ecological stability, we did not demonstrate evolutionary stability of the host and symbiont genotypes involved. Nonetheless, our study provides 'proof of concept' that intermediate prevalence can be stabilized by forces that cause high-prevalence populations to decline and low-prevalence populations to increase.

In all of our treatment combinations, stable intermediate endophyte prevalence arose because, on average, the relative abundance of endophyte-positive (E+) hosts increased when rare and decreased when common (Figs 1, 4). This pattern does not necessarily imply frequency-dependent mechanisms: linear theoretical models of symbiosis that include constant demographic effects and transmission rates (Gundel *et al.*, 2008; Bibian *et al.*, 2016) predict the same qualitative result. However, we were surprised by the low equilibrium prevalence expected under ambient transmission (31–51% E+), especially given high prevalence (*c.* 90%) in naturalized populations from which we collected source material. We expected that the moderate E+ fitness advantage (7% greater spike production) and high vertical transmission rate (94% on average) should give rise to a higher equilibrium prevalence (under any fitness benefit and 100% transmission, equilibrium prevalence should be 100%). We therefore speculate that additional mechanisms caused strong observed declines in prevalence of endophytes when they were common (all populations that began with > 50% E+ declined in prevalence regardless of treatment: Fig. 4). An additional hypothesis is that the fitness effects of symbiosis are negatively frequency-dependent, where rare host 'types' (E+ or endophyte-free (E−)) experience an advantage. This could give rise, for example, to a weaker E+ advantage or perhaps an E− advantage when E+ hosts are initially common. Several previous studies support the possibility of such frequency-dependent dynamics (Omacini *et al.*, 2006; Miller & Rudgers, 2014). However, our *post hoc* analyses do not support this hypothesis and even suggest the opposite: we found that E+ hosts had a slight advantage in spike production at high E+ prevalence, and *vice versa* for E− hosts (Fig. S4). It therefore remains unclear why endophytes declined so strongly in high-prevalence populations. Detailed studies of seed dormancy, germination and seedling survival could reveal additional frequency-dependent processes during early lifecycle transitions.

Given our finding that precipitation treatment did not modify symbiont fitness benefits, we were unable to directly test the hypothesis that vertical transmission and fitness benefits interactively determine symbiont prevalence at the population level. There are several possibilities for why the fitness effects of endophytes did not respond to precipitation. First, the elevated precipitation treatment may have been too conservative to elicit a strong effect relative to ambient conditions. Although the treatment was designed to mimic an extremely wet year in our study region, during three months of our year-long study, ambient precipitation roughly matched our 'wet-year' targets, and little or no precipitation was added (Fig. S1). However, enhanced precipitation elevated soil moisture during the dry periods between rain events (Fig. S1) and significantly increased plant fitness (Fig. 2a; Table 1). These results indicate that host fitness was water-limited, and that elevated precipitation relieved drought stress. A second, more likely explanation is that endophytes confer advantages to hosts that are not related to drought tolerance, such as resistance to herbivory or pathogens. Although our plots were protected from vertebrate browsers, endophytes may have conferred a fitness advantage via protection from insect herbivores (Omacini *et al.*, 2001; Crawford *et al.*, 2010). In addition, previous work documented host protection by *E. occultans* against closely related fungal pathogens (Pérez *et al.*, 2013). We conclude that endophytes did not confer drought tolerance in our study, and so alleviation of drought stress had no influence on endophyte prevalence.

Additional aspects of our experimental design likely affected our results and merit consideration. First, the *L. multiflorum* used in this study were derived from a naturalized population in Argentina (Omacini *et al.*, 2004). We might have seen different effects of climate manipulation had we used a native host–symbiont pair that had evolutionary history in our east Texas climate. Second, we made efforts to reduce the likelihood of between-plot seed movement (plot netting and spacing) but we cannot exclude the possibility. Given the strong dependence of final endophyte prevalence on initial prevalence (Fig. 4), we think that recruitment dynamics at the intended, within-plot scale overwhelmed the effects of any possible seed migration among plots. Third, we know little about the genetic make-up of our host and symbiont populations. Some studies report that endophyte benefits vary among host-plant genotypes in other grass species (Hesse *et al.*, 2004). The genetic match between endophyte and host has also been shown to influence fitness benefits (Sullivan & Faeth, 2004). We intentionally 'averaged over' genetic variation but it is possible that, as in most ecological studies of symbiosis, our study might yield different outcomes with different genetic combinations of host and symbiont.

Variation in the prevalence of heritable microbes that are known or presumed to be beneficial has been widely documented across plant and animal hosts, but the sources of this variation remain unresolved. Our study relied on an annual plant model system for tractability, but the processes that we focused on – symbiont effects on hosts, transmission between generations, and the population-level patterns that result – are general to all host-symbiont systems. We therefore expect our results to provide insight into symbiosis, generally, and not only grass–endophyte interactions. Our study provides novel experimental evidence that mixed populations of symbiotic and nonsymbiotic hosts can achieve a stable state, and that imperfect vertical transmission can suppress symbiont prevalence and increase symbiont extinction risk.

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Author contributions

T.E.X.M. and J.A.R. designed the research; B.R.C., M.E.S., T.F.B., M.L.D., A.S., M.O. and T.E.X.M. propagated plant material, prepared the field site, performed experiments, and conducted fieldwork; and B.R.C. and T.E.X.M. analyzed data and wrote the manuscript, with input from all coauthors.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information tab for this article:

Fig. S1 Pattern and effectiveness of elevated precipitation.

Fig. S2 Effectiveness of vertical transmission reduction.

Fig. S3 Correlation between target and realized endophyte prevalence in the founding generation.

Fig. S4 Frequency dependence in symbiont effects on host fitness.

Methods S1 Detailed description of methods.

Notes S1 Effectiveness of elevated precipitation, vertical transmission reduction treatments and initial prevalence assignment.

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