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PLANT-MICROBE-ANIMAL INTERACTIONS - ORIGINAL RESEARCH

Long-term ungulate exclusion reduces fungal symbiont prevalence in native grasslands

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Abstract When symbionts are inherited by offspring, they can have substantial ecological and evolutionary consequences because they occur in all host life stages. Although natural frequencies of inherited symbionts are commonly <100 %, few studies investigate the ecological drivers of variation in symbiont prevalence. In plants, inherited fungal endophytes can improve resistance to herbivory, growth under drought, and competitive ability. We evaluated whether native ungulate herbivory increased the prevalence of a fungal endophyte in the common, native bunchgrass, *Festuca campestris* (rough fescue, Poaceae). We used large-scale (1 ha) and long-term (7–10 year) fencing treatments to exclude native ungulates and recorded shifts in endophyte prevalence at the scale of plant populations and for individual plants. We characterized the fungal endophyte in *F. campestris*, *Epichloë* species FcaTG-1 (*F. campestris* taxonomic group 1) for the first time. Under ungulate exclusion, endophyte prevalence was 19 % lower in plant populations, 25 % lower within plant individuals, and 39 % lower in offspring (seeds) than in ungulate-exposed controls. Population-level endophyte frequencies were also negatively correlated with soil moisture across geographic sites. Observations of high within-plant variability in symbiont prevalence are novel for the *Epichloë* species, and contribute to a small, but growing, literature that documents phenotypic plasticity in plant-endophyte symbiota. Altogether, we show that native ungulates can be an important driver of symbiont prevalence in native plant populations, even in the absence of evidence for direct mechanisms of mammal deterrence. Understanding the ecological controls on symbiont prevalence could help to predict future shifts in grasslands that are dominated by *Epichloë* host plants.

Keywords *Epichloë* · Epidemiology · *Festuca* · Herbivory · Mutualism

Introduction

Inherited symbionts contribute to major evolutionary transitions and have strong effects on communities and ecosystems. For example, mitochondria and chloroplasts arose from the fixation of inherited symbionts that became integrated components of host genomes—representing a key innovation in the evolution of life (e.g., Margulis 1996). Inherited bacteria can cause major shifts in host sex ratios and alter host demography (Moran et al. 2008; Werren et al. 2008). Inherited fungi can defend plants, such as locoweed and morning glories, from natural enemies by producing toxic secondary metabolites (Kucht et al. 2004; Valdez Barillas et al. 2007). The individual-level effects of inherited symbionts can also extend beyond the host organism.

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For example, by reducing herbivory on a dominant grass, a fungal endophyte slowed the rate of plant succession and reduced arthropod diversity in old fields (Rudgers et al. 2007).

Although host populations are rarely 100 % symbiotic, few studies have investigated the ecological factors that determine the prevalence of inherited symbionts. Such work is important for uncovering the drivers of symbiont fixation or loss from host populations (e.g., Sachs and Simms 2006). When symbionts influence the outcome of interactions between hosts and their natural enemies, changes in enemy abundance can drive symbiont prevalence. For example, in *Drosophila* flies, rapid increases in the population-level prevalence of an inherited *Spiroplasma* bacterium were associated with high levels of attack by a parasitic nematode (Jaenike et al. 2010). Similarly, the presence of parasitoid wasps increased the prevalence of bacterial symbionts that conferred resistance to parasitism in aphid populations (Oliver et al. 2008). And, herbivory promoted higher frequencies of fungal endophytes in populations of a non-native forage grass (Clay et al. 2005).

Ecological drivers of symbiont prevalence can differ in magnitude or even direction among host life history stages. For example, fungal symbionts of some grasses reduce plant survival but improve plant reproduction, with net positive effects on host population growth (Rudgers et al. 2012). Both the percentage of host individuals that are colonized by symbionts and the percentage of host tissue that is colonized can vary among life history stages (e.g., Afkhami and Rudgers 2008; Bibian et al. in review). Although variability in symbiont prevalence within host individuals has received relatively little attention, models from the plant-herbivore literature predict that *within-plant* variability in resistance traits affects herbivore consumption and fitness (reviewed by Karban 2011). Thus, natural enemies could alter within-host variation in symbiont colonization, in addition to their effects on population-level symbiont frequencies.

Most plants support diverse assemblages of fungal endophytes that grow asymptomatically in their leaves (Rodriguez et al. 2009). Some of these fungi are inherited maternally (e.g., in grasses, legumes, morning glories) by growing into seed tissue. Benefits to hosts include improved resistance to herbivores, pathogens, and abiotic stressors, particularly drought (Malinowski and Belesky 2000; Nagabhyru et al. 2013). However, most prior studies focus on a few, agronomic grass species. Less is known about the ecology of native plant-fungal endophyte symbioses (Cheplick and Faeth 2009). For example, it is unclear how much individuals or populations of plants vary in the prevalence of symbiosis, or what factors influence symbiont prevalence either within or across populations (but see Bazely et al. 1997; Semmartin et al. 2015).

We evaluated the prevalence of an endophytic *Epichloë* species in natural populations and individuals of the native, perennial C_3 bunchgrass, *Festuca campestris* Rydb. (rough fescue, Poaceae). Because *Epichloë* species have the potential to deter mammalian herbivores through the production of bioactive alkaloids (Schardl et al. 2013), we asked whether the exclusion of native ungulates reduced the prevalence of *Epichloë* species within a large, long-term field experiment. Herbivores could affect symbiont prevalence at the population scale either by selecting against endophyte-free plants or by altering the percentage of tissue colonized within individual plants. We examined both of these potential effects. To explore the possibility of within-plant response in greater detail, we simulated herbivory on individual plants to determine whether leaf damage alone could induce an increase in the symbiont within a plant. Because drivers of endophyte prevalence may be specific to particular host life history stages, we tested whether ungulate exclusion had similar effects on symbiont frequencies in adult plants versus their offspring (seeds). Finally, to investigate alternative drivers of symbiont prevalence, we examined whether edaphic factors correlated with average endophyte frequencies across geographic sites.

Materials and methods

Study system

Our study was conducted in the Blackfoot Valley in western Montana (47°01′13.11″N, 113°07′59.21″W), where native perennial grasslands are dominated by the long-lived, perennial bunchgrass, *Festuca campestris*. In 2011, an initial screen of *F. campestris* from the region revealed the presence of an *Epichloë* (formerly *Neotyphodium*) species (Leuchtmann et al. 2014) that is inherited by seeds, but had not been previously reported from this plant species. The dominant native ungulates in the Blackfoot Valley are elk (*Cervus elaphus*), white-tailed deer (*Odocoileus virginianus*) and mule deer (*Odocoileus hemionus*). These species winter in high numbers in the Blackfoot Valley and feed on grasses that remain green under snow. Many elk and mule deer move to higher elevations in late spring. Average precipitation is 32 cm year^{-1}.

Ungulate-exclusion treatment

In 2002, we randomly assigned a control or ungulate-exclusion treatment to paired (100–300 m away) 1-ha plots at each of three sites (Bandy, 47°3′55.47″N 113°13′46.87″W; Blackfoot, 46°51′1.73″N 112°57′59.99″W; and Kleinschmidt, 46°58′30.69″N 113°4′2.41″W). A fourth site (Clearwater, 47°1′50.59″N 113°21′32.00 W) was added in

2005 (Maron et al. 2012). The average distance between sites was >23 km; the minimum distance was 7.5 km. At each site, ungulate-exclusion and control plots were paired to ensure similar vegetation, slope, and aspect. To exclude ungulates, we strung 2.4-m-tall, ten-stranded barbed wire to fence posts (strand spacing = 0.24 m). Regular snow tracking in winter and annual fecal pellet counts in spring showed that fencing effectively eliminated ungulates from exclusion plots and that ungulates were active on controls. There was no livestock grazing.

Herbivory treatment: caged, clipped, control

In early May 2012, we randomly selected 20 triads of *F. campestris* that were spread across the 1-ha control and ungulate-exclusion plots at each site. Triads consisted of three nearby (within 5 m of each other) adult *F. campestris* plants that were uniquely marked. One individual of each triad was randomly assigned to one of the following treatments:

1. The caged treatment, in which plants were surrounded by a chicken wire cage (~70 cm wide × ~70 cm high) that was staked to the ground to protect plants from mammalian herbivory.
2. The clipped treatment, which simulated ungulate herbivory: all leaves of focal plants were cut to 3–4 cm above ground level, once in mid-May.
3. The control treatment, in which plants were neither caged nor clipped.

On each ungulate-exclusion plot, 20 diads of *F. campestris* were randomly selected across the entire plot. Diads consisted of two nearby adult *F. campestris* plants. One individual in each diad was randomly assigned to either a caged treatment or clipped treatment (as described above). The caged treatment allowed for us to test for a cage effect independent of herbivory. In May 2013, we re-clipped individuals that had received the clipping treatment in 2012, and we made the experiment factorial by adding control plants to create triads in the ungulate-exclusion plots.

Plant material collection for endophyte detection

We collected plant material for detection of *Epichloë* species in 2012 and 2013. In late May/early June 2012, we collected three individual tillers per plant for 60 plants in each control plot (triads—20 caged, 20 clipped, 20 control plants) and 40 plants in each ungulate-exclusion plot (diads—20 caged, 20 clipped plants) for a total of 1200 tillers examined, spanning 400 plants. In June 2013, we collected six tillers per plant for caged and clipped treatments and up to ten tillers per plant for the control treatment (all triads). Since we used a fully factorial design in 2013, we examined a total of 480 plants and 3486 tillers (some plants had fewer than ten tillers). In 2013, we also collected seeds from all target plants that reproduced (19 individuals). Since relatively few of the target plants flowered, we collected seeds from an additional 15 randomly chosen flowering plants per plot for a total of 106 seed collections, each from a unique plant.

In vegetative tissue, we scored *Epichloë* presence at the scale of individual tillers by examining a minimum of four thin sections of the inner leaf sheath per tiller. Thin sections were stained with aniline blue lactic acid and examined at 200× magnification (following Bacon and White 1994). For each seed collection, we scored ten seeds for *Epichloë* presence, except in cases where fewer than ten seeds were produced (average seeds scored per plant = 5.0 ± 2.5 SD). We first softened and cleared seeds in 5 % NaOH for 22 h. Softened seeds were dissected to expose the seed coat and aleurone layer, then stained with aniline blue lactic acid and examined at 200× and 400× magnification (Bacon and White 1994). Supplementary material Fig. S1 shows examples of detecting *Epichloë* presence in leaf sheaths and seeds of *F. campestris*.

Endophyte prevalence

First, to determine endophyte prevalence at the scale of the host plant *population*, we scored the presence/absence of the endophyte for each individual plant. Plants were scored as symbiotic if at least one tiller showed presence of the endophyte. Second, we determined endophyte prevalence within *individual* plants as the number of symbiotic tillers divided by the total number of tillers examined. Third, we scored endophyte prevalence in *offspring* by dividing the number of symbiotic seeds by the number of seeds examined for each individual plant. For all of these metrics (population, individual, offspring), we calculated the average endophyte prevalence for each treatment combination per site per year for analysis, resulting in 44 observations for population and individual prevalence [2012, 4 sites × 2 ungulate treatments × 2 herbivory treatments (caged, clipped) + 4 additional herbivory controls in control grids = 20 observations; 2013, 4 sites × 2 ungulate treatments × 3 herbivory treatments = 24 observations]. There were 16 observations (averages) for seeds because seeds were examined only in 2013, and few target plants reproduced.

Endophyte species characterization

We determined the phylogenetic placement of the *Epichloë* species in *F. campestris* and characterized its genetic potential to produce alkaloids. Total DNA was extracted from *F. campestris* plant material (tillers or seed) representing endophyte-symbiotic plants from all locations and treatments, using a QIAGEN MagAttract 96 DNA Plant Core Kit (QIAGEN, Valencia, CA). To determine *Epichloë* presence and alkaloid production potential, we used multiplex polymerase chain reaction with 19 established, genus-specific primer pairs representing housekeeping, mating type, and genes encoding key alkaloid biosynthesis steps (Charlton et al. 2014; Takach and Young 2014).

We identified the endophyte species by cloning fragments of the housekeeping genes *tefA* and *tubB* from three endophyte-infected *F. campestris* lines B-C8, B-U16, and K-C3, using the pGEM-T Easy Vector System I (Promega, Madison, WI). The sequences from multiple clones (12 from each gene) were evaluated using Sequencher version 5.0.1 (Gene Codes, Ann Arbor, MI) to identify each gene copy. Sequences representing *Epichloë* species diversity were analyzed using phylogeny.fr with default settings (Dereeper et al. 2008). We aligned sequences using MUSCLE (version 3.7) (Edgar 2004) and inferred phylogenetic trees by maximum likelihood using PHYML (version 3.0) (Guindon and Gascuel 2003) with an approximate likelihood ratio test (Anisimova and Gascuel 2006). Trees were rendered with TREEDYN (version 198.3) (Chevenet et al. 2006).

Statistical analysis

We used general linear mixed models [GLIMMIX (SAS Institute 2012)] to examine how effects of ungulate removal (at the plot scale), herbivory treatment (at the individual plant level—caged, clipped, control), year, interactions between each treatment and year (all fixed factors), and site (random factor) influenced the endophyte prevalence of *populations* (proportion of symbiotic plants in the population), *individuals* (proportion of symbiotic tillers per plant), and *offspring* (proportion of symbiotic seeds per plant). To test whether treatments affected the variability of endophyte prevalence within host individuals, we calculated the coefficient of variation (CV) for the proportion of symbiotic tillers per plant. Response variables met assumptions of normality of residuals and homogeneity of variances, with the exception of CV, which was ln-transformed. Because we did not use a fully factorial design in 2012, we tested for ungulate treatment × herbivory treatment interactions in a separate model that excluded the control herbivory treatment. The ungulate × herbivory treatment interactions were never statistically significant (all $P > 0.75$); thus, final models lacked these interactions.

Alternative correlates of endophyte prevalence

To explore additional correlates of endophyte prevalence, we measured edaphic factors in each plot during 23 and 24 May 2013. We correlated edaphic factors with the *population* prevalence estimate from 2013, using only the control plants from the herbivory treatment in order to represent naturally occurring symbiont prevalence. Edaphic factors included gravimetric water content (%), NH_4–N mg kg^{-1} oven-dry method (ODM) soil, NO_3–N mg kg^{-1} ODM soil, the total inorganic N pool in mg kg^{-1} ODM soil, % N, % C, p.p.m. total N, and p.p.m. total C. Inorganic N was determined by sequential extractions of soil in 2 M KCl (Hart et al. 1994). Fifteen grams of soil was added to 100 mL of 2 M KCl, shaken on an orbital shaker for 1 h, allowed to settle overnight, filtered, and along with an extraction blank, frozen until analysis. We determined concentrations of NO_3^- and NH_4^+ in filtrates colorimetrically using a Synergy 2 Microplate Reader (BioTek, USA). All remaining soil was air-dried and then ground to a fine powder using mortar and pestle. Soil total C and N were determined by combustion (CE Elantech, Lakewood, NT). To evaluate alternative correlates of endophyte prevalence at the population scale, exploratory mixed models included each edaphic covariate individually in addition to the random effect of site, which accounted for the non-independence of ungulate-exclusion and control grids that occurred at the same site ($n = 8$ populations).

Results

Endophyte species characterization

Seeds from individual endophyte-symbiotic plants from each location and in each ungulate treatment were evaluated for the presence of *Epichloë* species-specific markers for fungal mating type and for genes that encode key biosynthesis steps for known classes of fungal alkaloids: the primarily mammalian-deterrent ergot alkaloids (EAS) and indole-diterpenes (IDT/LTM), and the insect-deterrent lolines (LOL), and peramine (PER). Each endophyte-bearing plant sample gave identical banding patterns for each multiplex PCR reaction, indicating low endophyte diversity across the samples we examined (Fig. 1a). All samples were positive for both mating type markers (*MTA* and *MTB*), suggesting that the examined endophytes in *F. campestris* were hybrid as a result from interspecific hybridization between haploid *Epichloë* species. All samples were positive for the *perA* markers (*PER* locus

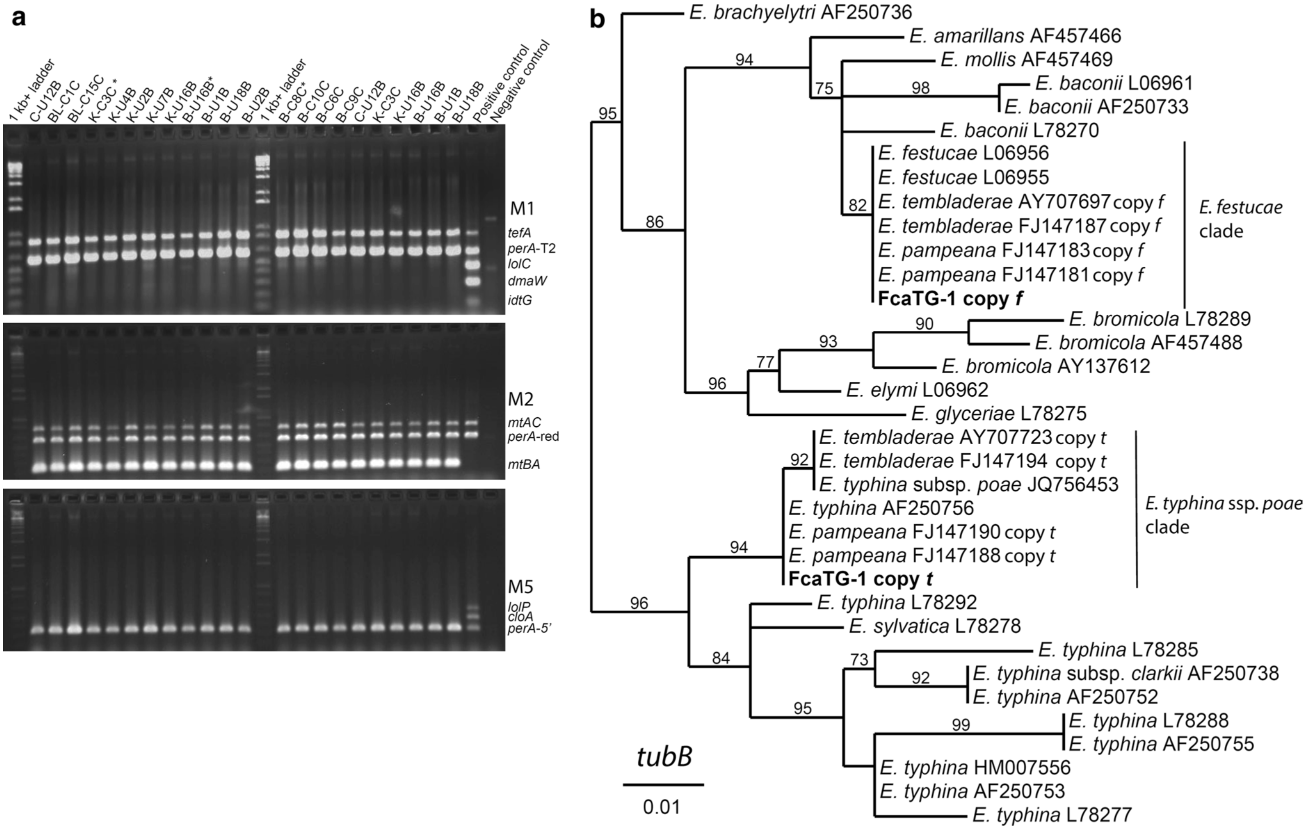

Fig. 1a, b Genotype and phylogenetic inference on the fungal endophyte in *Festuca campestris*. **a** Genotype evaluation of *F. campestris* using multiplex polymerase chain reaction. Only data for multiplexes 1 (*M1*), 2 (*M2*) and 5 (*M5*) are shown. The positive control is *Epichloë coenophiala* (strain AR584) in tall fescue (*Lolium arundinaceum*) and the negative control is endophyte-free *L. arundinaceum*. Samples used to determine phylogenetic placement of the endophyte are indicated by an *asterisk*. Sample names indicate location [Bandy (*B*), Blackfoot (*BL*), Clearwater (*C*), Kleinschmidt (*K*)], treatments [control plots (*C*), ungulate-exclusion plots (*U*)], plant number, and tiller (*last letter*). **b** Phylogram based on alignments from an intron-rich portion of the *tubB* sequences from representatives of *Epichloë* species and FcaTG-1 (*F. campestris* taxonomic group 1). The tree is mid-point rooted and was inferred by maximum likelihood with PhyML (Guindon and Gascuel 2003). The *tubB* and *tefA* (data not shown) trees were congruent so only the *tubB* tree is shown

required for production of peramine), indicating the potential to produce peramine, a known insect herbivore deterrent. Markers for genes within *EAS*, *LOL* and *IDT/LTM* loci, required for the production of ergot alkaloids, lolines and indole-diterpenes, respectively, were not detected in any samples and therefore, the endophyte would be unable to produce these compounds.

The partial endophyte *tefA* and *tubB* sequences were identical among the three evaluated sample lines, Bandy-Control (B-C8), Bandy-Ungulate Exclusion (B-U16), and Kleinschmidt-control (K-C3). Analyses of the consensus of sequences of the *tefA* and *tubB* gene fragments were used for phylogenetic placement of the endophyte from *F. campestris*. In both the *tefA* and *tubB* trees, FcaTG-1 (*F. campestris* taxonomic group 1) allele 1 grouped with *Epichloë festucae* and allele 2 grouped with *Epichloë typhina* ssp. *poae* (Fig. 1b), consistent with a hybrid origin of the *F. campestris* endophyte. FcaTG-1 showed a similar allele grouping as has been identified for the hybrid *Epichloë pampeana* in *Bromus auleticus* from Argentina (Iannone et al. 2009; Leuchtmann et al. 2014). Additional studies would be required to determine if FcaTG-1 and *E. pampeana* are also morphologically similar.

Does ungulate exclusion reduce the percentage of host individuals that are colonized by endosymbionts?

Across plots, the proportion of endophyte-symbiotic grass individuals ranged from 0.2 to 0.9. On average, endophyte prevalence at the *population* scale decreased by 19 % when native ungulates were excluded from 1-ha plots ($X^2 = 11.0$, $P = 0.0009$; Fig. 2a). This effect did not vary between the 2 years sampled (Ungulate exclusion × Year, $X^2 = 0.8$, $P = 0.39$). Population-level endophyte prevalence was consistent across years (Year, $X^2 = 0.5$, $P = 0.47$), as expected given the long lifespan of the host plant.

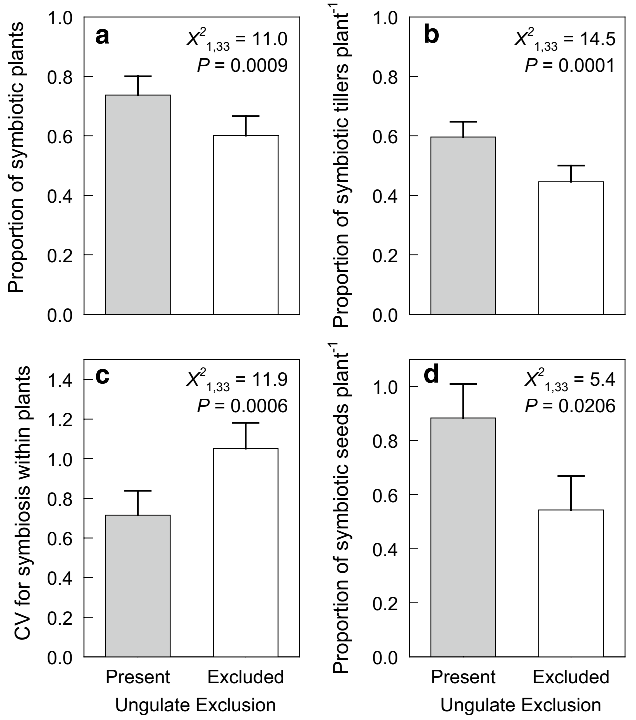

Fig. 2 Effects of ungulate exclusion on **a** symbiont prevalence in *F. campestris* populations (proportion of symbiotic plants), **b** symbiont prevalence in *F. campestris* individuals (proportion of symbiotic tillers per plant), **c** variation within individual plants in symbiont prevalence [coefficient of variation (*CV*) of symbiotic tillers], and **d** symbiont prevalence in *F. campestris* offspring (proportion of symbiotic seeds per plant). *Bars* are means ± 1 SE. Statistics are from general linear mixed models

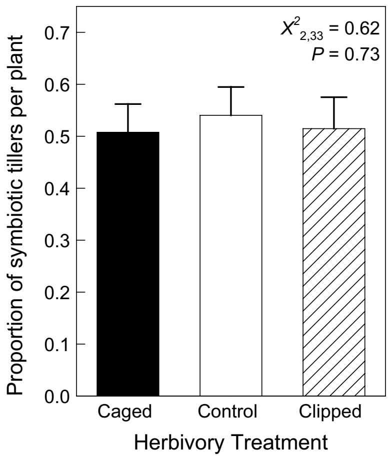

Fig. 3 Mean (±SE) fungal endophyte symbiont prevalence within individuals of *F. campestris* (proportion of symbiotic tillers per plant) that had simulated herbivory (via clipping) or ungulate protection (via caging) to prevent ungulate herbivory. Values averaged over plants treated on larger ungulate-exclusion and control plots due to the lack of a statistically significant Herbivory treatment × Ungulate exclusion effect

Does ungulate exclusion reduce the prevalence of the endosymbiont within individual plants?

Ungulate exclusion reduced endophyte colonization within *individuals* by 25 % relative to control plots with ungulates present ($X^2 = 14.5$, $P = 0.0001$; Fig. 2b). This effect did not vary between years (Ungulate exclusion × Year, $X^2 = 2.5$, $P = 0.11$). However, endophyte prevalence within individuals was, on average, higher in 2013 (mean = 0.59 ± 0.05 SE) than in 2012 (mean = 0.45 ± 0.05 SE; year, $X^2 = 12.2$, $P = 0.0005$), consistent with the hypothesis that endophyte colonization varies plastically within a plant. For symbiotic individuals, the proportion of tillers with an endophyte ranged from 0.1 to 1.0. Across plots, the average proportion of endophyte-symbiotic tillers per plant varied from 0.12 to 0.83. Within-plant *variation* in the proportion of symbiotic tillers (as estimated by the CV) was 47 % greater in grasses with ungulates excluded than in grazed controls ($X^2 = 11.9$, $P = 0.0006$; Fig. 2c). The CV was higher in 2012 (mean = 0.97 ± 0.13 SE) than in 2013 (mean = 0.80 ± 0.12 SE), but the effect size of ungulate exclusion did not vary between years (Ungulate exclusion × Year, $X^2 = 1.9$, $P = 0.16$).

Does simulated herbivory induce an increase in within-plant symbiont prevalence?

Experimental clipping had no significant effect on endophyte prevalence within individual plants (Herbivory treatment, $X^2 = 0.6$, $P = 0.73$; Herbivory treatment × Year, $X^2 = 0.8$, $P = 0.67$; Fig. 3). While caged plants had slightly lower frequencies (mean = 0.51 ± 0.05 SE) than control plants (mean = 0.54 ± 0.05 SE), this difference was not statistically significant (Fig. 3). Clipping and caging treatments also did not affect within-plant variability in endophyte prevalence, as estimated by the CV ($X^2 = 0.6$, $P = 0.76$; Herbivory treatment × Year, $X^2 = 1.3$, $P = 0.54$).

Does ungulate exclusion have similar effects on symbiont frequencies in adult plants versus offspring?

Seeds from ungulate-exclusion plots showed 39 % lower *offspring* endophyte frequencies than seeds from grazed control plots ($X^2 = 5.4$, $P = 0.0206$; Fig. 2d). There were too few reproductive plants in the simulated herbivory treatment to test for effects of caging or clipping on offspring endophyte prevalence.

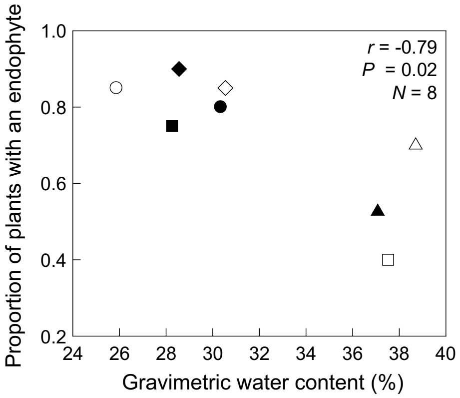

Fig. 4 Correlation between endophyte prevalence at the population level and gravimetric soil moisture. Populations of *F. campestris* at wetter sites had lower endophyte frequencies than at drier sites across the ungulate-exclusion plots (*open symbols*) and control plots (*filled symbols*). *Circles* Bandy, *squares* Blackfoot, *triangles* Clearwater, *diamonds* Kleinschmidt. Mixed-effects model including the random effect of site and the covariate of gravimetric water content (full model, $X^2 = 9.61$, $P = 0.0019$)

What edaphic factors correlate with endophyte prevalence?

The only significant correlate of endophyte prevalence at the geographic scale was soil moisture. Population-level endophyte prevalence was negatively correlated with gravimetric water content across the eight 1-ha plots of the experiment (Fig. 4), and across the four geographic sites ($r^2 = 0.63$). All other examined factors showed no relationship with endophyte prevalence (all $P > 0.43$).

Discussion

Our results show that ungulates can drive increases in the prevalence of endophytic *Epichloë* species in a grass host. This effect occurred even when the symbiont may not have directly deterred ungulate herbivory, as suggested by the lack of fungal genes for ergot alkaloid and indole-diterpene production. Exclusion of ungulates from native grasslands for 7–10 years resulted in declines in endophyte prevalence in *F. campestris* over multiple scales—by 19 % in populations, 25 % within individuals, and 39 % in offspring. To our knowledge, this is the first experimental demonstration that endophyte prevalence in native grass populations responds to ungulate herbivores. Previous experimental work has shown that both vole and insect herbivores can cause increases in endophyte prevalence in a non-native grass (Clay et al. 2005). Observational studies have found strong positive correlations between pika, marmot, or deer herbivory and natural endophyte frequencies (Bazely et al. 1997; Koh and Hik 2007). However, work on native Arizona fescue, also a non-producer of mammalian-deterrent alkaloids, found no effect of experimental ungulate exclusion (Saikkonen et al. 1999).

Evidence for plasticity in endophyte symbiosis

Our work contributes to a small, but growing, body of literature documenting plasticity in fungal endophyte responses to ecological drivers. Prior studies have assumed that symbiotic plants are infected systemically with *Epichloë* species (i.e., the endophyte is present in 100 % of tillers and seeds). Most authors do not record within-plant variability in endophyte colonization, despite evidence to the contrary (e.g., Afkhami and Rudgers 2008).

By what mechanism might differences in endophyte prevalence arise on control versus ungulate-exclusion plots? It is unlikely that our results were due solely to herbivore-driven mortality of endophyte-free host plants on control plots because *F. campestris* is a long-lived, grazing-adapted plant. Populations turn over slowly relative to the timescale of our ungulate exclusion. In addition, the relative magnitude of individual plant response (25 % decline) versus population response (19 % decline) suggests that the majority of change in endophyte prevalence occurred through plastic shifts in the density of fungal hyphae within plants, rather than through births, deaths, or migrations of symbiotic plants into the plant population. First, the natural symbiont prevalence of populations was 75 % in the presence of ungulates. Multiplying this prevalence by the 25 % reduction in symbiotic tillers per plant results in an expected 19 % reduction in individual plants identified as symbiotic under ungulate exclusion—precisely the reduction that we observed at the population level. Second, the plasticity interpretation is also supported by the high variability in symbiont prevalence among symbiotic plants (range 10–100 % of tillers per plant were symbiotic). Similar to our results, Koh and Hik (2007) observed that densities of endophyte hyphae within tillers of an alpine grass were greatest in areas with high herbivore densities (marmots and pikas). Interestingly, *Festuca altaica*, the focus of the Koh and Hik's (2007) study, is a close relative of *Festuca campestris* (Barkworth et al. 2007) and may host a phylogenetically related endophyte, given the broad evidence for co-cladogenesis in *Epichloë* species (Schardl et al. 2008). The endophyte we studied in *F. campestris* lacks the ability to produce the alkaloids most typically associated with mammalian deterrence, based on the absence of genes for biosynthesis of ergot alkaloids and indole-diterpenes, suggesting that alternative fungal metabolites or indirect effects of grazing may be more important mechanisms of governing endophyte dynamics in this host plant (e.g., Schardl et al. 2014).

Conditions that favor plasticity in endophyte symbiosis

Current theory posits that induced responses to herbivory should be favored in environments with high variability (e.g., spatiotemporal variation in herbivore damage) and when costs of constitutive trait expression are high (reviewed by Karban 2011). Although we do not have precise estimates of spatiotemporal variation in herbivore pressure, annual fecal pellet counts across our plots show that ungulate use can be quite variable from year to year. There is also strong temporal variation in the response of grass production to ungulate exclusion. While endophytes can be costly to plants in a number of ways, it is not yet clear how much these costs may be reduced if investment in the endophyte can be adjusted plastically, rather than expressed constitutively. Direct costs of hosting fungal endophytes include the plant carbon provided to the fungus (Thrower and Lewis 1973) and the nitrogen allocated to fungal secondary metabolites, such as the alkaloids that increase herbivore resistance (Schardl et al. 2013). For example, fungal endophytes can reduce host fitness when soil nutrients are in short supply (e.g., Cheplick 2007). Endophytes may also have ecological costs for plants by altering host interactions with other species (Omacini et al. 2012; Strauss et al. 2002). For example, the presence of *Epichloë coenophiala* in tall fescue grass reduced colonization of roots by mycorrhizal fungi (Mack and Rudgers 2008). Documenting the fitness costs of endophyte symbiosis across a number of grass species could help to identify whether higher costs of symbiosis are associated with plant-fungal symbiota that exhibit highly plastic responses to herbivory.

Ecological consequences of plasticity in endophyte symbiosis

High variability in endophyte frequency within individual plants could affect patterns of herbivore damage and herbivore behavior in the same ways proposed for intrinsic, anti-herbivore resistance traits (reviewed by Orians and Jones 2001). While most studies on within-plant heterogeneity in anti-herbivore resistance have focused on insects, it is possible that ungulates also respond to within-plant variability. However, no studies to our knowledge have examined whether within-plant variability affects ungulate bite rates or feeding preferences, even though fungal endophytes are well documented to defend plants against ungulates (reviewed by Belesky and Bacon 2009). We lack data on whether fungal presence or hyphal density correlates with levels of herbivore damage, perhaps indicative of localized allocation by the plant to increase fungal densities within particular leaves or tillers or altered investment by the fungus following damage. In prior work, simulated clipping caused *F. campestris* to reallocate resources to aboveground growth, reducing root production (McInenly et al. 2010). This within-plant reallocation could also influence plant investment in endophytes. An additional next step would be to document how fungal alkaloid production varies with the presence of ungulates and with endophyte hyphal densities at the scale of individual tillers. For example, Rasmussen et al. (2007) showed that fungal alkaloid concentration increased linearly with *Epichloë* hyphal density in perennial ryegrass. Because within-plant variability in induced responses can affect plant-associates, such as arthropods (e.g., Rodriguez-Saona and Thaler 2005), shifts in within-plant endophyte frequencies could influence arthropod and microbial associates of dominant grasses like *F. campestris*, thereby cascading up to the community.

Long-term consequences of ungulate exclusion for endophyte population dynamics

While current evidence suggests that endophytes in adult plants respond plastically to ungulate exclusion, results from our seed data indicate possible demographic effects of ungulate exclusion. Under ungulate exclusion, the reduction in seed endophyte prevalence was substantially larger (39 %) than the reduction in symbiotic tillers per plant (25 %). Thus, shifts in the maternal provisioning of seeds when *F. campestris* were protected from grazers restricted endophyte colonization of offspring to levels below the symbiont prevalence found in maternal plants (see also, Garcia Parisi et al. 2012). Reduced rates of vertical transmission could improve seedling fitness by eliminating the costs of symbiosis in low-herbivory environments. Alternatively, ungulate damage could increase maternal allocation of the endophyte to seeds as an induced mechanism to protect offspring from grazing. For example, in tall fescue pastures, cattle graze on seed heads, which contain high concentrations of the fungal alkaloid ergovaline, to the detriment of these grazers (Goff et al. 2012).

Simulated herbivory did not increase endophyte prevalence

Our direct test of simulated herbivory failed to support the hypothesis that mechanical leaf damage is a mechanism underlying plastic increases in endophyte colonization within plants. There are a number of possible explanations for this result. First, the duration of the treatment was short: 2 years compared to the 7–10 years of our field exclosures. Second, simulated herbivory may differ from ungulate grazing in type, quality, or timing, in ways that affect the plasticity of the plant-fungal symbiotum. For example, foliar application of reindeer and moose saliva inhibited endophyte hyphal growth and alkaloid production in red fescue (Tanentzap et al. 2014), and our simulated clipping lacked saliva. While this particular response does not appear to occur in *F. campestris* because plants exposed to ungulates had higher (not lower) endophyte frequencies than in exclosures, other differences in the mechanics of ungulate herbivory versus clipping with scissors could affect the endophyte response. Third, our plot-level manipulations altered ungulate presence/absence, which affects not only the level of herbivore damage but also levels of disturbance and trampling as well as nutrient inputs from excretion (McNaughton et al. 1997) and other edaphic factors (Hobbs 1996) that could indirectly drive endophyte frequencies. These indirect effects of ungulates warrant further attention.

Alternative correlates of population-level symbiont prevalence

Our results suggest that water availability may be a driver of symbiont prevalence in *F. campestris*, because across sites, a 10 % increment in soil gravimetric water content was associated with a 0.3 drop in the proportion of plants with the endophyte (Fig. 4). No other measured edaphic factor correlated with endophyte prevalence. This result could indicate a benefit of the endophyte under drought, which would select for higher endophyte frequencies in drier soils. Amelioration of drought stress is a common benefit reported for other *Epichloë* species (e.g., Elmi and West 1995; Ren et al. 2014), and past research has shown negative correlations between population-level endophyte prevalence and soil moisture in California, Patagonia, and France (e.g., Afkhami et al. 2014; Iannone et al. 2015; Lewis et al. 1997), similar to the result here. An alternative explanation is that rates of vertical transmission from plants to seeds are higher when soils are drier. However, the latter hypothesis is unlikely given the lack of correlation between the prevalence of offspring (seeds) with the endophyte and plot-level soil moisture ($r = 0.36$, $P = 0.38$, $n = 8$ sites).

A plausible indirect mechanism behind the ungulate effect on endophyte prevalence is reduced soil moisture due to ungulate presence. While soil moisture was not significantly influenced by ungulate presence in our experiment ($P = 0.45$), prior studies have shown that ungulate presence can reduce soil moisture, e.g., by 15 % by elk and deer in one study (Gass and Binkley 2011). Testing for interactive effects of drought and herbivory (e.g., Emery et al. 2010) could resolve the relative importance of these factors in this ecosystem.

Conclusion

Here we show that native ungulates can be an important driver of symbiont prevalence in native plant populations, even when evidence for direct deterrence of ungulates by symbionts is lacking. Observations of high within-plant variability in symbiont prevalence are novel for *Epichloë* species and indicate that these symbiota exhibit phenotypic plasticity. Furthermore, ungulate exclusion reduced the prevalence of endophytes in seeds, conceivably affecting the long-term demography of host and symbiont populations. Given the potential for community and ecosystem-level consequences of grass-endophyte symbioses, understanding the ecological drivers of symbiont prevalence could help to predict future shifts in grasslands that are dominated by hosts of *Epichloë* species.

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Author contribution statement J. L. M. and D. E. P. conceived, designed, and performed the experimental manipulations. R. A. F., E. O., and J. A. R. collected the endophyte data. J. A. R. analyzed the data. C. A. Y. and N. D. C. characterized the endophyte identity. J. A. R. led the writing, with substantial contributions from co-authors.

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