

Simulated folivory increases vertical transmission of fungal endophytes that deter herbivores and alter tolerance to herbivory in *Poa autumnalis*

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• **Background and Aims:** The processes that maintain variation in the prevalence of symbioses within host populations are not well understood. While the fitness benefits of symbiosis have clearly been shown to drive changes in symbiont prevalence, the rate of transmission has been less well studied. Many grasses host symbiotic fungi (*Epichloë* spp.), which can be transmitted vertically to seeds or horizontally via spores. These symbionts may protect plants against herbivores by producing alkaloids or by increasing tolerance to damage. Therefore, herbivory may be a key ecological factor that alters symbiont prevalence within host populations by affecting either symbiont benefits to host fitness or the symbiont transmission rate. Here, we addressed the following questions: Does symbiont presence modulate plant tolerance to herbivory? Does folivory increase symbiont vertical transmission to seeds or hyphal density in seedlings? Do plants with symbiont horizontal transmission have lower rates of vertical transmission than plants lacking horizontal transmission?

• **Methods:** We studied the grass *Poa autumnalis* and its symbiotic fungi in the genus *Epichloë*. We measured plant fitness (survival, growth, reproduction) and symbiont transmission to seeds following simulated folivory in a 3-year common garden experiment and surveyed natural populations that varied in mode of symbiont transmission.

• **Key Results:** *Poa autumnalis* hosted two *Epichloë* taxa, an undescribed vertically transmitted *Epichloë* sp. PauTG-1 and *E. typhina* subsp. *poae* with both vertical and horizontal transmission. Simulated folivory reduced plant survival, but endophyte presence increased tolerance to damage and boosted fitness. Folivory increased vertical transmission and hyphal density within seedlings, suggesting induced protection for progeny of damaged plants. Across natural populations, the prevalence of vertical transmission did not correlate with symbiont prevalence or differ with mode of transmission.

• **Conclusions:** Herbivory not only mediated the reproductive fitness benefits of symbiosis, but also promoted symbiosis prevalence by increasing vertical transmission of the fungus to the next generation. Our results reveal a new mechanism by which herbivores could influence the prevalence of microbial symbionts in host populations.

Key words: Defensive mutualism, symbiosis, *Epichloë*, induced response, resistance, tolerance, transgenerational effects

INTRODUCTION

Identifying the factors that promote the persistence of symbioses (or cause their extinction) can yield new insights into the underlying ecological and evolutionary processes that lead to integrated partnerships (Kiers and West, 2015). Through symbioses, both plants and animals have acquired new functions and metabolic capabilities (e.g. White and Torres, 2009; Douglas, 2010). Plants can acquire nitrogen through symbiosis with bacteria, arthropods gain amino acids synthesized by gut symbionts, ruminants house cellulolytic bacteria to use plant cellulose as an energy source, and plants obtain anti-herbivore resistance from microbial endophytes (Zilber-Rosenberg and Rosenberg, 2008; Rodriguez *et al.*, 2009; Kandel *et al.*, 2017). Despite the apparent benefits of these interactions, such symbioses often involve mutual exploitation, with cryptic costs that can vary with the ecological context (Jones *et al.*, 2015).

Most studies on symbiosis emphasize reciprocal fitness effects as the key driver of interaction dynamics (e.g. Sachs and Simms, 2006). However, while fitness effects are clearly important to the prevalence of symbiosis, the mode and rate of symbiont transmission can also play major roles (Rudgers *et al.*, 2010; Gundel *et al.*, 2011; Bibian *et al.*, 2016). Symbiont transmission can be vertical, in which microorganisms pass exclusively from parents to offspring; horizontal, in which hosts contagiously acquire symbionts from the environment (Bright and Bulgheresi, 2010); or mixed, with a combination of vertical and horizontal transmission (Brem and Leuchtman, 2003). Unlike horizontal transmission, vertical transmission tightly couples the fitness of the host and symbiont, selecting for mutualism via partner fidelity feedback (Ewald, 1987). Despite the central role of transmission dynamics in models of host–symbiont interactions (Lipsitch *et al.*, 1995; Genkai-Kato and

Yamamura, 1999; Gundel *et al.*, 2008), relatively few empirical studies have investigated ecological controls on the rate and mode of transmission (Douglas, 2010; Gundel *et al.*, 2011; Sneek *et al.*, 2019). However, observations suggest that abiotic environmental context, such as climatic variability and type of soil, could underlie variable transmission (Gundel *et al.*, 2009; Gibert and Hazard, 2013; Sneek *et al.*, 2017). Ecological interactions with other species could also influence transmission dynamics, but have received little study.

In plants, the prevalence of foliar endophytes within and among natural host populations is highly variable (Semmartin *et al.*, 2015), suggesting that fitness outcomes are context-dependent (Davitt *et al.*, 2011; Rho *et al.*, 2018) or that variable transmission rates create frequent opportunities for symbiont loss from individual hosts (e.g. Gundel *et al.*, 2011, 2012; Gibert and Hazard, 2013). Symbioses between plants and foliar fungal endophytes are ubiquitous (Rodriguez *et al.*, 2009), and the best-studied of these occur between cool-season grasses (subfamily Pooideae) and systemic fungal endophytes (genus *Epichloë*; Ascomycota, Clavicipitaceae). As in other symbioses, empirical studies of *Epichloë* species have focused primarily on fitness benefits as drivers of symbiont prevalence, rather than on the transmission process (Gundel *et al.*, 2008, 2011; Rudgers *et al.*, 2010). *Epichloë* can increase plant resistance to herbivores and pathogens as well as improve plant performance under abiotic stresses (see reviews by: Clay and Schardl, 2002; Malinowski and Belesky, 2019). Alternatively, endophytes may reduce plant tolerance to herbivory by reducing regrowth after defoliation (Qin *et al.*, 2016), particularly if plants have less carbon for regrowth because of allocation to the symbiosis. Whether the fungal endophytes that confer herbivore resistance constrain or enhance the ability of host plants to regrow after damage (tolerance to herbivory) remains unresolved because few experiments have been conducted (Partida-Martínez and Heil, 2011).

In addition to this high potential for variable fitness benefits, endophyte transmission modes can also be highly variable, ranging from exclusively vertical to mixed to exclusively horizontal (Brem and Leuchtman, 2003; Rodriguez *et al.*, 2009). Vertical transmission occurs with asexual hyphal growth into developing seeds (Liu *et al.*, 2017), whereas horizontal transmission occurs when the fungal fruiting bodies (stromata) arrest development of grass inflorescences, which may reduce opportunities for vertical transmission if fewer seeds are produced (Leuchtman *et al.*, 2000; Schardl *et al.*, 2004). If the ecological context alters these transmission processes, there is strong potential for changes in the prevalence of symbiosis in host populations to arise from transmission dynamics rather than context-dependency in the fitness benefits of symbiosis (Gundel *et al.*, 2011; Cavazos *et al.*, 2018).

The ecological factors that generate variation in vertical transmission rates or influence the relative amounts of vertical vs. horizontal transmission remain unclear. Exogenous ecological contexts, such as climate or species interactions, could affect symbiont transmission, particularly if symbionts contribute to transgenerational (or maternal) effects (Gundel *et al.*, 2017). Maternal provisioning is one of the most common mechanisms through which progeny acquire traits associated with the environment experienced by their parents (Herman and Sultan, 2011; Zas and Sampedro, 2015). Induced mechanisms of maternal (or

transgenerational) effects can increase progeny fitness via protection against abiotic or biotic stressors (Pieterse, 2012). For example, plants challenged with herbivores can produce more resistant offspring than plants that have not been exposed to herbivory (Agrawal *et al.*, 1999; Kellenberger *et al.*, 2018). Transgenerational effects mediated by microorganisms could occur if maternal plants provision offspring with protective symbionts through the process of vertical transmission (Gundel *et al.*, 2017). Thus, the dynamics of grass–*Epichloë* symbioses, in which fungal-derived alkaloids can protect against herbivores (Clay and Schardl, 2002; White and Torres, 2009), could be altered if herbivory or other stressors alter the process of vertical transmission from maternal plants to seed. Indeed, the accumulation of fungal alkaloids in the seeds of grasses (Gundel *et al.*, 2018) suggests potential for a transgenerational effect mediated by endophyte symbiosis. However, to our knowledge, no previous studies have manipulated herbivory to test whether it alters the amount of vertical transmission of the grass–endophyte symbiosis.

Using the native woodland plant autumn bluegrass (*Poa autumnalis*) and its endophytic *Epichloë* species as a model system, we investigated the potential for herbivory to increase symbiont transmission and compared the magnitude of that effect to the more commonly studied fitness benefits of symbiosis. We used a common garden experiment with clipping to simulate folivory in order to test the following questions: (1) Does symbiont presence increase or decrease plant tolerance to herbivory? (2) Does folivory increase symbiont vertical transmission to seeds or hyphal density in seedlings? To contextualize experimental work within natural host–symbiont dynamics, we used observations of endophyte presence across populations to test a hypothesized trade-off between vertical and horizontal transmission, and investigated whether vertical transmission rate is associated with symbiont prevalence. Specifically, we asked: (3) Do plants with symbiont horizontal transmission have lower rates of vertical transmission than plants lacking horizontal transmission?

MATERIALS AND METHODS

Study system

Autumn bluegrass (*P. autumnalis*), is distributed from the North American Atlantic coast to eastern Texas, which represents the western edge of its distribution (USDA/NRCS, 2012). This caespitose grass is common in mesic, hardwood forest understoreys in eastern Texas. A preliminary field survey showed a high incidence of *Epichloë* species (~96 % of individuals; Rudgers *et al.*, 2009) with some individuals producing stromata – the mechanism of horizontal transmission for sexually reproductive *Epichloë* species. A stroma is the fruiting body of the fungus that sterilizes the reproductive tissues of the plant (the pathogenic manifestation is known as ‘choke disease’), a process that ends in the production of sexually formed ascospores that can colonize new plants (Tadych *et al.*, 2012; Leuchtman *et al.*, 2014). Previous field observations revealed 50 % reduced folivory when the endophyte was present than in plants from which the endophyte was experimentally removed, and laboratory assays showed an increased preference of

endophyte-disinfected plants over endophyte-symbiotic plants by aphid, grasshopper and caterpillar herbivores, as well as reduced caterpillar performance in no-choice assays (Crawford *et al.*, 2010). In the present study, we surveyed the diversity of endophyte mating types and alkaloid genes of natural populations, including one population in which individuals produced the stromata that enable horizontal transmission.

Does symbiont presence increase or decrease plant tolerance to herbivory? Does folivory increase symbiont vertical transmission to seeds or hyphal density in seedlings?

Experimental design. In 2009, a 3-year common garden experiment was conducted at Stephen F. Austin Experimental Forest near Nacogdoches, Texas, USA (31°29'44"N, 94°45'39"W). Endophyte status (endophyte present: E+, or absent: E-) and simulated herbivory (clipped or control) were manipulated in a 2 × 2 factorial design, with 52 replicates per treatment combination (*N* = 208 plants). Individuals were planted into the common garden in a rectangular grid (21 × 10 m) at 1-m spacing with no watering following planting. Plant position in the grid was assigned at random.

Endophyte treatment and propagation. We grew plants from seeds collected from 25 individual plants at the Stephen F. Austin Experimental Forest on 2 May 2007 (SFA-3 population containing *Epichloë* sp. PauTG-1, Table 1). We experimentally removed the endophyte by heating seeds at 60 °C in a drying oven for 7 d (further details in Supplementary Data Appendix S1). Plants were grown in the Rice University glasshouse facility. Four weeks before field planting, each plant (genet) was divided into equally sized ramets (about five tillers each) then replanted into separate 115-mL pots. One of the ramets from each genetically independent plant was assigned to the simulated herbivory treatment while the other one was the control (see below). Only ramets for which the endophyte was present as intended (E+) or effectively eliminated (E-) were used in the experiment. The endophyte status of plants was verified by the detection of endophyte hyphae in thin sections of the inner leaf sheath stained with aniline blue lactic acid and examined at ×200 magnification (Bacon & White, 1994). The common garden was planted on 16 December 2009, adjacent to the site of seed collection (SFA-3, Table 1). Ramets were removed from pots and planted into the natural matrix of vegetation using a hand trowel, with minimal disturbance.

Herbivory treatment. We used a paired design to reduce error associated with genetic variation among plant individuals that were subjected to herbivory treatments. One ramet from each of 52 genetically unique endophyte symbiotic plants (E+) and from each of 52 genetically unique endophyte-free (E-) plants was randomly assigned to a simulated herbivory (clip) treatment; the other ramet of each genotype served as a control. Clipped plants had ~25 % less leaf biomass via removal of the tips of leaves with scissors. The clipping treatment replicated damage by lepidopteran larvae and orthopterans, for which we observed an average damage at the site of 8.4 % tissue removed per leaf (maximum of 50 % of leaf tissue removed) (Crawford *et al.*, 2010). Because plants go dormant during the summer

months, after flowering, clipping occurred once per month from December 2009 to May 2010 and from October 2010 to April 2011. Control plants were visited and leaves were touched, but not clipped. All clipped material was removed from the site.

Plant fitness. From 2010 to 2012, we scored each plant for survival and counted the number of vegetative tillers and inflorescences during 11–22 May, when plants were reproductive. As seeds matured, we collected them into coin envelopes. In 2011, we also counted the number of spikelets per inflorescence for a subset of four inflorescences per individual. We estimated total seed production per plant by multiplying the number of inflorescences × mean number of spikelets per inflorescence × mean number of seeds per spikelet. *Poa autumnalis* produced, on average, 2.8 ± 0.4 s.d. seeds per spikelet, regardless of endophyte status.

Fungal endophyte fitness. In 2011, we evaluated three variables associated with fitness of the symbiont: vertical transmission to the seed and to the seedlings, and hyphal density in the seedlings. Vertical transmission was evaluated by manually dissecting ten seeds per plant at ×10 magnification following soaking overnight in 5 % sodium hydroxide. We removed the endosperm, added one or two drops of aniline blue lactic acid stain following Bacon & White (1994), and squashed each seed under a cover-slip. Seeds were scored for endophyte presence at ×200 magnification. For determination of endophyte presence, we only scored seeds from E+ plants (10 seeds × 104 plants), ~1040 seeds in total.

We examined 20 seedlings per individual plant in the common garden experiment. We removed the lemma and palea then placed ten seeds into a Petri dish containing 1 % water agar, then sealed this with parafilm. After cold stratification at 4 °C for ~4 weeks, the sealed plates were placed in the glasshouse (~23 °C) during 24–30 June 2010. After seedlings had produced at least one true leaf, we stained them with rose Bengal and evaluated them for endophyte presence at ×200–400 magnification following Belanger (1996). For each maternal plant, we determined the proportion of seeds that germinated and the proportion of seedlings with an endophyte. We scored a total of 1935 seedlings for endophyte presence.

To assess the density of *Epichloë* hyphae in the seedlings, we measured seedling height for a subset of randomly chosen seedlings from 11 maternal plants in each clipping treatment. We then stained the seedlings as before, and determined hyphal density per linear millimetres of leaf sheath tissue. We also corrected for the possibility that plants differed in cell size by measuring plant cell size (pixels) at ×200 magnification using ImageJ analysis software (see Schneider *et al.*, 2012). We scored a total of 22 endophyte-symbiotic seedlings for both hyphal density and plant cell size.

Common garden experiment: data analysis

Does symbiont presence increase or decrease plant tolerance to herbivory? If endophytes increase plant tolerance to herbivory, then when plants are subjected to a fixed amount of damage, plants with the endophyte should have greater fitness than endophyte-free plants. This result would be confirmed by a statistical interaction

TABLE 1. Summary of data on symbiosis and germination in 18 populations of autumn bluegrass, *Poa autumnalis*, with latitude and longitude for each population. Populations in bold were additionally surveyed for prevalence in seedlings produced by field-collected seeds from the surveyed adult plants.

Site	Endophyte taxa	Collection dates	Latitude	Longitude	Adult prevalence (%)	N (plants)	Stromata adults (%)	N (seeds)	Seed prevalence (%)	N (germ.)	Seed germination (%)	N (seedlings)	Seedling prevalence (%)
ANF-1	1 & 2	2009-04-11	31°02'52"	-94°23'16"	100.0	30	0.0			400	23.2	73	70.9
ANF-2	1	2009-05-08	31°05'01"	-94°19'21"	97.1	35	0.0			438	47.0	192	96.3
ANF-3	1 & 2	2009-04-11	31°05'13"	-94°19'15"	96.7	43	20.0			1351	33.9	480	96.9
		2009-05-08											
ANF-5	1 & 2	2006-04-12	31°05'23"	-94°19'21"	100.0	14	0.0	21	90.5				
ANF-6	1	2006-04-12	31°02'52"	-94°23'14"	100.0	12	0.0	22	100.0				
BNP-1	1 & 2	2006-04-16	30°34'24"	-94°38'32"	93.3	15	0.0	9	100.0				
BNP-2	1 & 2	2006-04-17	30°29'15"	-94°50'27"	100.0	14	0.0	20	100.0				
BNP-3	1	2006-04-17	30°23'00"	-94°51'00"	100.0	22	0.0	20	100.0				
DNF-1	1	2009-05-04	31°27'56"	-95°07'44"	93.3	30	0.0	19	78.9	705	56.0	259	99.4
DNF-2	1	2006-05-16	31°27'46"	-95°07'44"	100.0	14	0.0	19	78.9				
LOC-1	1 & 2	2006-04-26	31°38'57"	-94°46'37"	100.0	20	0.0	23	100.0				
SFA-1	1	2009-04-30	31°29'44"	-94°45'38"	100.0	30	0.0	71	94.4	970	89.0	593	98.8
SFA-2	1	2006-04-30	31°30'07"	-94°47'18"	90.0	20	0.0	71	94.4				
SFA-3*	1	2007-05-02	31°29'44"	-94°45'39"	100.0	25	0.0	71	94.4	<i>n.t.</i>	<i>n.t.</i>	98	95.9
SNF-1	1 & 2	2009-04-23	30°30'30"	-95°05'22"	100.0	30	0.0	16	68.8	780	35.3	151	81.6
SNF-2	1 & 2	2006-04-12	30°30'30"	-95°05'22"	94.1	17	0.0	18	61.1				
SNF-3	1	2006-04-12	30°30'48"	-95°07'23"	91.7	12	0.0	18	61.1				
TWR-1	2	2006-04-17	30°21'58"	-94°51'43"	91.7	12	0.0	21	100.0				

*The SFA-3 population was used in the field experiment; the other populations were used to survey natural endophyte prevalence. Collection dates are given in ISO 8601 format. Prevalence (in adults, seeds or seedlings) is the mean percentage of individuals with the endophyte. N = sample size. *n.t.* = not tested.

Endophyte taxa were distinguished by PCR; taxon 1 = PauTG-1 (hybrid), taxon 2 (*E. typhina* subsp. *poae*, non-hybrid), 1 & 2 = a mixture of both PauTG-1 and *E. typhina* subsp. *poae*.

between the endophyte treatment and simulated herbivory (clip) treatment in analyses of plant fitness metrics. We used repeated-measures general linear mixed effects models with clip treatment, endophyte treatment, clip \times endophyte interaction, and the year effect as well as interactions with year. Models included the random effect of genetic pair, and the random effect of ramet to account for the non-independence of observations on the same plant in different years (restricted maximum likelihood, Proc GLIMMIX, SAS Institute v. 9.3, Cary, NC, USA). In all models, we evaluated the *a priori* hypothesis that fungal endophytes modulate plant tolerance to herbivory by testing the contrast between endophyte-symbiotic (E+) vs. endophyte-free (E-) plants within the clip treatment and the same contrast within the herbivory control treatment. To meet assumptions of normality of residuals and homogeneity of variances, we log-transformed inflorescence counts, tiller counts and seed counts. For survival (0/1 data), we used a log-linear mixed effects model with a binomial distribution and the same independent factors as for plant growth and reproduction. Plant survival was high in 2010 at 98 %, but declined through time (2011: 89 %; 2012: 50 %). Including 2010 survival data resulted in models that did not converge due to extremely high survival; thus we only examined survival responses to treatments for 2011 and 2012.

Does folivory increase symbiont vertical transmission to seeds or hyphal density in seedlings? For the subset of common garden plants with endophytes, we tested the independent factors of clip treatment (fixed) and plant genetic pair (random) on five fungal response variables: the proportion of seeds with the endophyte, the proportion of seedlings with the endophyte, the proportion of seedlings that germinated, mean hyphal density/cm of plant tissue, and mean plant cell size (mm²) (restricted maximum likelihood, Proc MIXED, SAS Institute v.9.3). All proportion data were logit-transformed following [Warton et al. \(2011\)](#).

Field survey: do plants with symbiont horizontal transmission have lower rates of vertical transmission than plants lacking horizontal transmission?

Field collection and microscopy. To characterize the natural prevalence and genetic diversity of endophytes in *P. autumnalis*, we collected leaves and seeds from ~12–30 adult plants in each of 18 populations during the reproductive phase (April–May) ([Table 1](#)). Leaves were stored at 4 °C for up to 7 d, and seeds were stored at –20 °C. We used microscopy to detect the endophyte in leaves for all populations. Then, for a subset of seven populations (**bold in [Table 1](#)**), we also germinated and scored seedlings for endophyte presence, following methods for the Common Garden Experiment. Sample sizes are presented in [Table 1](#). We used a Spearman rank correlation to relate the proportion of symbiotic adult plants in each population to the proportion of symbiotic offspring either using seeds ($n = 11$ populations) or seedlings ($n = 7$ populations). We observed stomata formation in only one of the 18 surveyed populations (ANF-3, [Table 1](#); [Supplementary Data Fig. S1](#)). For this population, we compared the efficiency of vertical transmission for seeds of stomata-bearing plants ($N = 11$) against that of asymptomatic plants ($N = 32$) using one-way ANOVA.

Endophyte genotyping. To determine possible endophyte diversity within and between the *P. autumnalis* populations, we

used a PCR approach to identify endophyte infection and characterize endophyte diversity. To characterize endophyte genotypes, we used PCR on seeds from endophyte-symbiotic plants with primers specific for alkaloid genes and fungal mating types ([Charlton et al., 2012](#)). In total, 19 primer sets were used to test for endophyte presence and diversity ([Table S1](#)). Each total DNA sample was extracted from up to eight individual seeds per individual plant or from up to four seeds from a bulk collection with at least four independent samples ([Table S1](#)). Each gene primer set was evaluated in a single PCR. In 2016, a subset of seed samples (21 populations and individual plants from population ANF-3) were further tested by PCR using the protocol of [Charlton et al. \(2014\)](#), which used the primers in five multiplex primer combinations. These primer combinations are used to provide information on endophyte diversity within the populations examined and can highlight specific classes of bioactive alkaloids that might be produced by the endophyte, such as those known to increase plant resistance to herbivores.

The populations were split into three distinct groups containing taxon 1 only, taxon 2 only, or a mixed population with taxon 1 and taxon 2 present ([Table 1](#)). Endophytes were identified to individual taxon based on phylogenetic trees of the mating-type genes. Due to the quality of the DNA isolated from the seed in our collections, we could only confirm the phylogenetic placement using sequence data from the mating-type genes *mtAC* and *mtBC*. The phylogenetic placement of each sequenced gene was performed with phylogeny.fr ([Dereeper et al., 2008, 2010](#)). Accession numbers for mating-type genes are MN311480–MN311482.

RESULTS

Does symbiont presence increase or decrease plant tolerance to herbivory?

Endophyte presence increased host survival under the controlled folivory (25 % leaf tissue removed) imposed in our experiment. Over both endophyte treatments, simulated herbivory by clipping leaves with scissors reduced plant survival by 29 % ($F_{1,78} = 12.64$, $P < 0.001$), despite significant interannual variability in survival (Year effect: $F_{1,101} = 57.64$, $P < 0.001$). In the clipped treatment, plants with endophytes (E+) showed 33 % greater survival than endophyte-free (E-) plants (contrast E+ vs. E- under clipping: $F_{1,204} = 5.27$, $P = 0.023$), indicating improved tolerance. However, survival of E+ and E- plants was similar in the non-clipped, control treatment ($F_{1,204} = 0.83$, $P = 0.363$; [Fig. 1A](#); main effect of endophyte symbiosis $F_{1,204} = 4.33$, $P = 0.038$). When clipped, E- plants showed 30 % lower survival than non-clipped controls, whereas E+ plants showed only a 15 % reduction in survival.

Endophyte-symbiotic plants did not have better tolerance to clipping than endophyte-free plants in their growth response, as indicated by a non-significant clipping \times endophyte interaction for tiller production ($F_{1,78} = 0.52$, $P = 0.472$). Simulated herbivory caused a larger decline in plant growth compared with removal of the endophyte ([Fig. 1B](#)). Across years, E+ plants made 26 % more tillers than E- plants ($F_{1,78} = 4.48$, $P = 0.037$), but non-clipped plants grew 45 % larger than clipped plants ($F_{1,78} = 7.90$, $P < 0.001$).

The reproductive fitness effects of both simulated herbivory and endophyte presence varied among years, as evidenced by the three-way interaction among endophyte, clipping treatment and year for inflorescence production ($F_{2,383} = 3.97$, $P = 0.019$). First, in year 2010, E+ plants produced 40–50 % more inflorescences than E– plants, regardless of the clipping treatment. Second, in 2011, the endophyte did not increase reproduction (Fig. 1C). Total seed production per plant was recorded only in 2011 and was reduced by 88 % under clipping ($F_{1,37} = 12.37$, $P < 0.001$) but was not significantly increased by endophyte presence ($F_{1,90} = 1.72$, $P = 0.193$), mirroring the lack of an endophyte effect on inflorescence number during 2011. However, there was a trend for clipped plants to show a stronger benefit of the endophyte than controls during 2011 (contrast E+ vs. E–, clipped: $F_{1,37} = 2.89$, $P = 0.097$; control: $F_{1,37} = 0.00$, $P = 0.953$) (Fig. 1D), a closer match to the endophyte-enhanced tolerance that we observed for the plant survival response. After 3 years (by 2012), E+ plants in the control treatment made 126 % more inflorescences than E– plants (contrast, $P = 0.047$), but in the clipped treatment, E+ plants made a statistically equivalent number of inflorescences to E– plants ($P = 0.425$, Fig. 1C), demonstrating that symbiosis was beneficial only in the non-clipped treatment.

Does folivory increase symbiont vertical transmission to seeds or hyphal density in seedlings?

Simulated herbivory increased vertical transmission of the endophyte from maternal plants to seeds ($F_{1,48} = 5.39$,

$P = 0.025$) and from maternal plants to seedlings ($F_{1,48} = 10.13$, $P = 0.003$). The mean difference between clipped and control plants was ~2 % for plant-to-seeds and ~5 % for plant-to-seedlings (Fig. 2A, B). In addition, endophyte hyphae were significantly denser in seedlings of maternal plants that had been clipped ($F_{1,20} = 6.23$, $P = 0.021$; Fig. 2C). This effect was not a consequence of plant cell size because there was no significant difference in plant cell size between seedlings from clipped vs. control maternal plants ($F_{1,20} = 1.3$, $P = 0.268$). Although simulated folivory increased the prevalence of vertical transmission of the endophyte, folivory had no significant effect on the proportion of seeds that germinated ($F_{1,48} = 0.00$, $P = 0.982$) (data not shown).

Do plants with symbiont horizontal transmission have lower rates of vertical transmission than plants lacking horizontal transmission?

Vertical transmission did not correlate positively with endophyte prevalence. Across 18 populations, there was no significant correlation between the prevalence of symbiosis (percentage of adult plants in the population with *Epichloë*) and vertical transmission (percentage of seeds or seedlings produced by endophyte-bearing plants with *Epichloë*) (Fig. 3, Spearman's $r = 0.09$, $P = 0.730$, $N = 18$). The lack of pattern did not change when we removed the one stromata-forming population (ANF-3) from the analysis. However, both variables, and

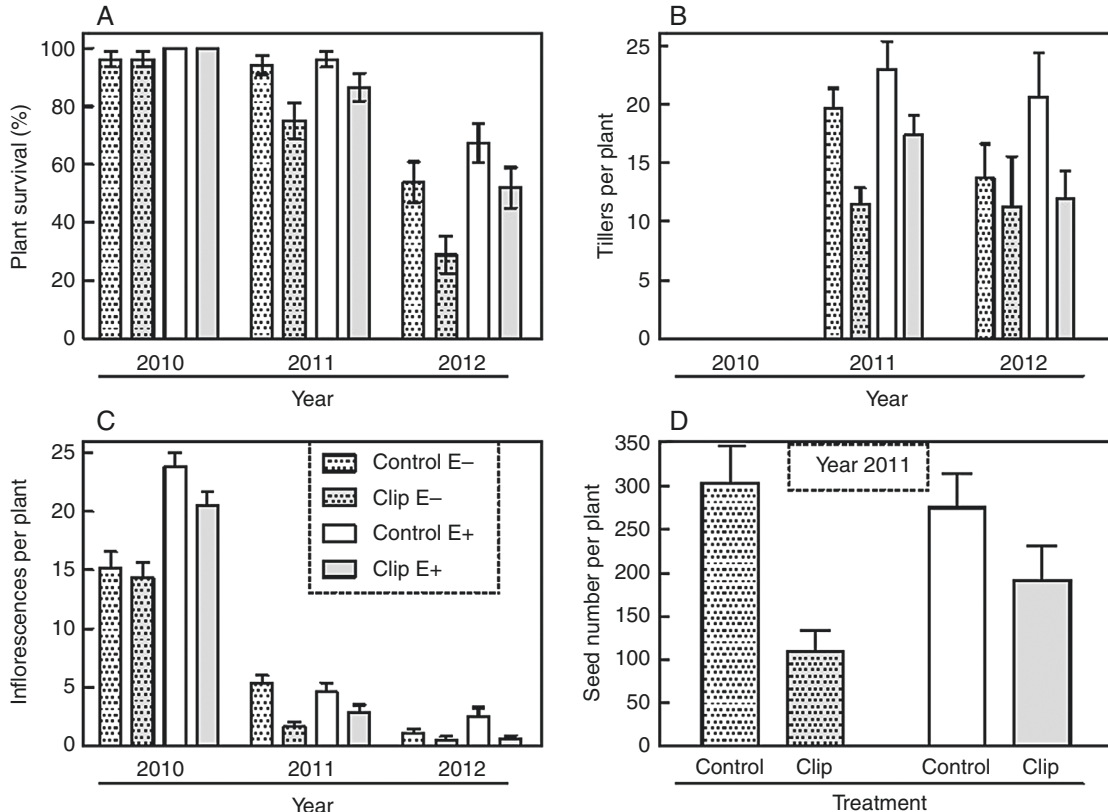


FIG. 1. Effects of clipping to simulate folivory and the presence of *Epichloë* on autumn bluegrass (*Poa autumnalis*): (A) plant survival over the period 2010–2012, (B) number of tillers per plant recorded in 2011 and 2012, (C) number of inflorescences per plant recorded in 2010, 2011 and 2012, and (D) number of seeds per plant recorded in 2011. Symbols show means \pm s.e. Sample size was $n = 52$ plants per treatment combination at the beginning of the experiment and declined yearly as plants died.

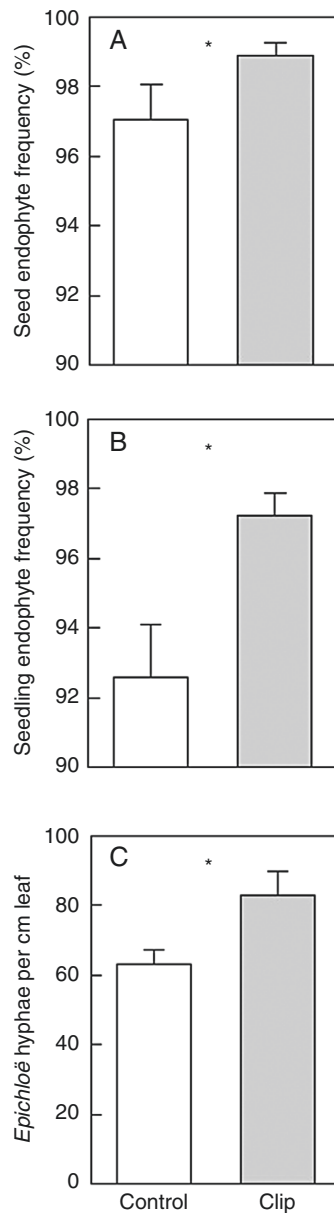


FIG. 2. Effects of clipping to simulate folivory on autumn bluegrass (*Poa autumnalis*): (A) seed endophyte frequency per plant (percentage of ten seeds that had *Epichloë*), (B) seedling endophyte frequency per plant (percentage of 20 seedlings that had *Epichloë*), and (C) *Epichloë* hyphal density in seedlings (per centimetre of leaf tissue). Bars show means \pm s.e. Sample size was 49 endophyte-bearing plant genotypes; each genotype was split into two plants, one was clipped, the other served as an unmanipulated control. Asterisks indicate significant differences ($P < 0.05$).

particularly vertical transmission, had low variation, which constrained the ability to detect a relationship. Populations differed significantly in both the level of vertical transmission to seedlings (Fig. 4A; $F_{5,175} = 8.47$, $P < 0.001$) and the proportion of seeds that germinated (Fig. 4B; $F_{5,173} = 56.36$, $P < 0.001$). With a greater range of variation in germination than in endophyte vertical transmission, the former was positively associated with the latter across the six populations with both types of data (Fig. 4). Endophyte transmission to seedlings was highly

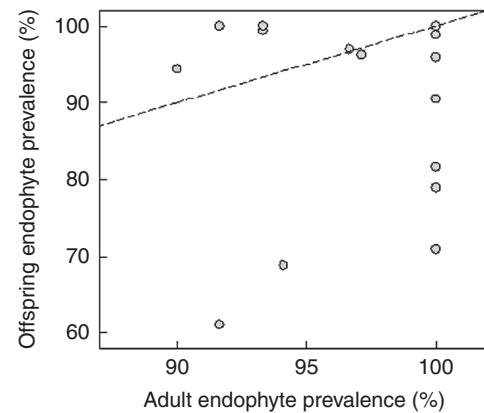


FIG. 3. Relationship between prevalence of *Epichloë* in *Poa autumnalis* (percentage of field-collected adult plants) and the prevalence of vertical transmission (percentage of seedlings with *Epichloë*) across 18 populations in Texas, USA. The dashed line shows the expectation for a 1 : 1 relationship between adult and offspring endophyte prevalence.

effective ($\approx 100\%$) in five populations (Table 1), while it was ≈ 70 – 80% in two others (Table 1).

Populations varied in endophyte. We detected two endophyte genotypes among the *P. autumnalis* populations surveyed that equated to two different *Epichloë* taxa (Supplementary Data Fig. S2). In total, ten populations contained only the interspecific hybrid endophyte *E. sp. PauTG-1* (*Poa autumnalis* Taxonomic Group 1, taxon 1), and as yet to be named *Epichloë* species but previously reported endophyte (Kutil *et al.*, 2007; Schardl *et al.*, 2012). Four populations contained only the non-hybrid *E. typhina* subsp. *poae* endophyte (taxon 2), and eight populations contained a mixture of both endophyte taxa (Table 1; Table S2). The *E. sp. PauTG-1* endophyte is considered asexual and would only transmit vertically, whereas *E. typhina* subsp. *poae* is considered a sexual endophyte and could transmit both vertically and horizontally.

Vertical transmission rates did not decline with horizontal transmission. Only the ANF-3 population had plants with stromata (Table 1), and thus had potential for horizontal transmission. Evaluation of the endophytes in 20 of the 60 individual plants from ANF-3 revealed that both endophyte taxa were present. Plants with stromata all had taxon 2 (*E. typhina* subsp. *poae*), and the asymptomatic plants had either taxon 1 (asexual PauTG-1) or taxon 2 (*E. typhina* subsp. *poae*). Of the 20 ANF-3 plants tested, five contained the hybrid PauTG-1 endophyte, whereas the remaining 15 had the non-hybrid *E. typhina* subsp. *poae*. Maternal plants bearing stromata had a similar prevalence of vertical transmission to asymptomatic maternal plants ($F_{1,41} = 0.61$, $P = 0.438$; Fig. 5A). However, germination was $\approx 15\%$ lower in seeds produced by stromata-forming plants ($F_{1,39} = 4.83$, $P = 0.034$; Fig. 5B) compared to plants lacking stromata, suggesting a potential fitness cost of stromata production in the ANF-3 population.

DISCUSSION

Here, we show that exogenous ecological interactions with simulated consumers altered the fitness benefits as well as

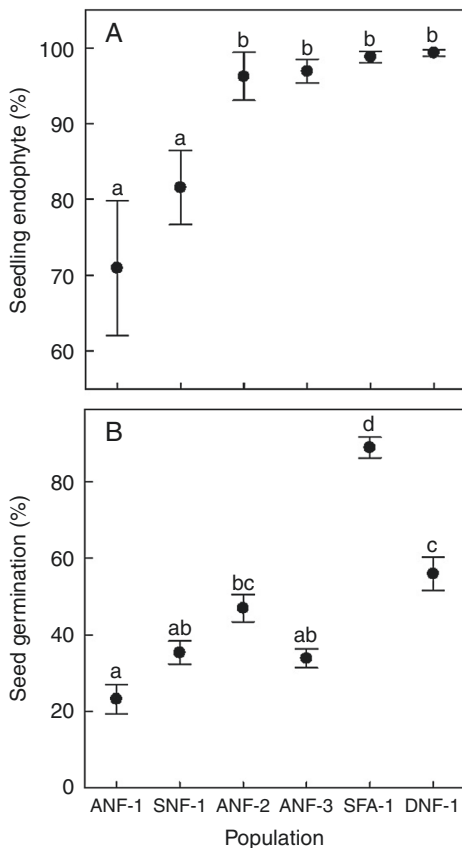


FIG. 4. Variation among six populations of autumn bluegrass (*Poa autumnalis*) in which we assessed both (A) vertical transmission (percentage of laboratory-grown seedlings bearing *Epichloë*; $F_{5,175} = 8.47$, $P < 0.0001$, $r^2 = 0.19$), and (B) germination (percentage of seeds that germinated in the laboratory; $F_{5,173} = 56.36$, $P < 0.001$, $r^2 = 0.62$). Populations were ordered by increasing seedling endophyte prevalence. Symbols show means \pm s.e. Sample sizes are given in Table 1. Different letters indicate significant differences in means ($P < 0.05$).

transmission dynamics of symbioses. However, the symbiosis had variable effects on plant fitness at different life stages. Clipped plants with the endophyte survived better than endophyte-free plants, suggesting that improved tolerance to herbivory can be an additional fitness benefit of symbiosis beyond the herbivore resistance reported in our previous study (Crawford et al., 2010). However, under clipping, reproduction was equivalent between symbiotic and endophyte-free plants, and benefits of the endophyte to seed production were significant only in the absence of clipping. Altogether, several plant fitness correlates were either improved or unaffected by endophyte symbiosis in *P. autumnalis* under simulated folivory. For example, even when clipped, symbiotic plants made similar or greater numbers of tillers and inflorescences than non-clipped, non-symbiotic plants. Previous studies testing endophyte effects on tolerance of perennial ryegrass (*Lolium perenne*) or tall fescue (*Schedonorus phoenix*, ex. *Festuca arundinacea*) in response to damage yielded variable outcomes on tolerance that highlighted interactions among symbiotic status, plant species, genotype identity and timing of damage (Cheplick, 1998; Belesky and Fedders, 1996). For example, simulated grazing imposed late in the growing cycle of annual ryegrass

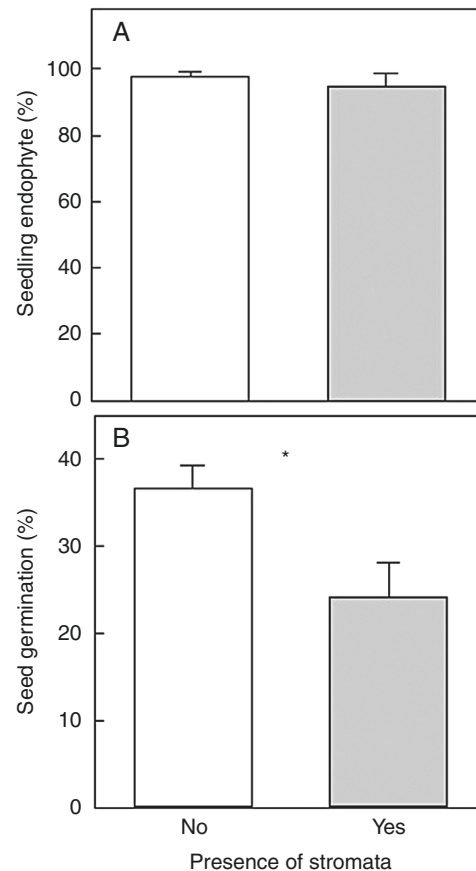


FIG. 5. Association between presence of stromata on maternal plants of autumn bluegrass (*Poa autumnalis*) from population ANF-3 and two attributes of offspring (A) vertical transmission [seedling endophyte frequency (percentage of laboratory-grown seedlings with *Epichloë*)], and (B) germination (percentage of seeds that germinated in the laboratory). Bars show means \pm s.e. Sample sizes, No: $n = 9-11$, Yes: $n = 32$. Asterisk indicate a significant difference ($P < 0.05$).

(*L. multiflorum*) reduced seed production in endophyte-bearing plants (Garcia Parisi et al., 2012). The impact of a symbiont on plant recovery from herbivore attack is likely to depend also on the severity of the damage (Gundel et al., 2011), herbivore feeding guild (grazer mammals, chewing and sap-sucking arthropods) and nutritional status. It is also possible that plant and symbiont responses to actual folivory by mammals or chewing insects are stronger (or weaker) than responses to simulated clipping. Integrating the opposing effects of symbiosis during different life stages and years into a net benefit of symbiosis to population growth would require a demographic model (e.g. Rudgers et al., 2012; Bibian et al., 2016).

In addition to modulating the fitness benefits of symbiosis, simulated folivory also increased the amount of vertical transmission of the endophyte to both seeds and seedlings. Together with the increase in the density of endophyte hyphae in the seeds, these results suggest, for the first time, that symbiont vertical transmission can function as an induced response to herbivory. However, we evaluated endophyte mycelium concentration at only two stages: seeds and seedlings with one or, at most, two tillers. Therefore, although we are confident our measurements of hyphae concentration are reliable, they represent only two

snapshots during host ontogeny. Fungal endophyte hyphae in adult plants are heterogeneously distributed among different parts and are variable among tillers (e.g. Philipson and Christey, 1986, Gagic et al., 2018). Further studies examining the phenology of hyphal density, in parallel with changes in fungal metabolites (e.g. Fuchs et al., 2017), could provide finer resolution on the underlying physiological mechanisms of responses to herbivory, and more precise estimates of potential effects on seedling herbivory, an aspect of plant life history that is understudied (Barton and Hanley, 2013).

Our results complement previous studies in other systems that showed herbivory-induced increases in fungal-derived alkaloids or gene expression in damaged, adult plants (e.g. Bultman and Bell, 2003; Sullivan et al., 2007; Zhang et al., 2009; Fuchs et al., 2017). Although we did not directly measure fungal-derived alkaloids in this study, we would expect higher levels of alkaloids in seeds with greater hyphal densities, based on previous studies (Spiering et al., 2005; Rasmussen et al., 2007). The *E. sp.* PauTG-1 endophyte identified within the SFA-3 population has been previously reported to reduce herbivory (Crawford et al., 2010) and is known to produce insect-deterrent lolines (Kutil et al., 2007; Schardl et al., 2012). In accordance with previous observations that endophyte growth can be affected by exogenous factors that influence plant growth (e.g. seasonality, temperature, resources, stress; reviewed by Gundel et al., 2011), our results support the existence of transgenerational effects of consumers on symbiont dynamics.

Transgenerational effects in plants, particularly effects that are induced by herbivores, have received considerable attention in recent years, with a particular focus on understanding the underlying mechanisms and evolutionary consequences (Agrawal et al., 1999; Gundel et al., 2017). Our results suggest the new hypothesis that symbiosis with vertically transmitted microbes can be an efficient mechanism of transgenerational information transfer in response to herbivory. Given the ubiquity of both fungal and bacterial endophytes in plant seeds (Hodgson et al., 2014; Truyens et al., 2015), microbially mediated transgenerational effects may warrant additional study. Vertical transmission to seeds has been documented not only for *Epichloe* species in grasses, but also for other fungal endophytes including: *Curvularia* species that enhance heat resistance in the grass *Dichanthelium lanuginosum* (Redman et al., 2002); *Undifilum oxytropis* that produce the herbivore-deterrent toxin swainsonine in *Astragalus* and *Oxytropis* species (locoweeds) (Ralphs et al., 2011); and other Clavicipitaceae ('*Periglandula*' spp.) responsible for toxic ergot alkaloid production in the morning glory family (Steiner et al., 2006). In addition to fungi, plants can convey bacteria to offspring (reviewed by Truyens et al., 2015). However, because the mode of transmission has not been characterized for the majority of above-ground endophytes in plants, it remains unclear how widespread this possible mechanism of transgenerational effects may be.

Many *Poa* species have been reported with accompanying *Epichloë* endophytes that vary in transmission, with vertical, horizontal and mixed transmission modes (Schardl et al., 1997, 2012; Moon et al., 2004; Rudgers et al., 2009; Tadych et al., 2012; Shymanovich et al., 2017; Leuchtmann et al., 2019). Interestingly, single populations of *P. autumnalis* were observed to host more than one *Epichloë* taxon, including the non-hybrid *E. typhina* subsp. *poae*, which has been observed in other *Poa* species (Tadych et al., 2012; Leuchtmann et al., 2014), and the

interspecific hybrid PauTG-1, a new undescribed species. Some populations of *P. autumnalis* appear to be sympatric with both taxa, but our study was not designed to provide information on the frequency of each *Epichloë* taxon or compare their fitness benefits to plants. Other studies have also resolved endophyte taxonomic diversity for multiple populations of a single host, with some host species able to associate with multiple *Epichloë* taxa (Charlton et al., 2012, 2014; Shymanovich et al., 2017, 2019a). For example, a latitudinal transect collection of *Poa alsodes* populations identified two endophyte taxa and high endophyte frequency, but one *Epichloë* taxon was more prevalent than the other. The two *Epichloë* taxa of *P. alsodes* differed in insect defence mechanisms (Shymanovich et al., 2019b), which could also differ between the *Epichloë* taxa we identified in *P. autumnalis*.

In addition to herbivory, other environmental and genetic factors probably regulate the prevalence of endophyte symbiosis in plant populations (Rudgers et al., 2009; Gundel et al., 2011; Semmartin et al., 2015; Sneek et al., 2017, 2019) through their effects on symbiont transmission. In comparison with other studies (e.g. Afkhami and Rudgers, 2008; Gundel et al., 2009; Rudgers et al., 2009; Gibert and Hazard, 2013), our populations of *P. autumnalis* showed, overall, high endophyte prevalence ($\geq 90\%$), similar to that previously reported for *P. alsodes* (Shymanovich et al., 2019a). Although we found variation among populations in the prevalence of vertical transmission (from ~60 to 100%, Table 1), transmission was not positively correlated with the high standing prevalence of symbiosis in populations (Fig. 3), perhaps because there was little variation in adult plant prevalence. At present, we cannot parse out how much the differences among populations were caused by plant genotype vs. endophyte genotype because we lack data on plant genotype. For example, Gibert and Hazard (2013) demonstrated that the variation in endophyte frequency among populations of *Lolium perenne* was largely caused by plant genotype. By contrast, in a recent study of *P. alsodes*, frequency of the more common *E. alsodes* symbiont (interspecific hybrid endophyte) was higher than that of *E. schardlii* var. *pennsylvanica* (intra-specific hybrid endophyte) and was probably due to greater compatibility with its host (Shymanovich et al., 2017, 2019a). Further investigation into the roles of plant genotype vs. fungal genotype or taxon in the *P. autumnalis* system, where *Epichloë* genotypes are diverse, could aid in determining the relative importance of genotype vs. environment in symbiont prevalence.

We expected to find a lower vertical transmission rate and greater fitness cost associated with horizontal transmission of the endophyte (stromata formation) in our mixed transmission population (ANF-3). However, there was no evident penalty in terms of reduced vertical transmission for plants associated with stromata formation, in contrast to the expectation of a trade-off between these transmission modes (e.g. Tintjer et al., 2008). We did detect a potential fitness cost via reduced seed germination from maternal plants with stromata than in those without. The predominance of vertical transmission among the populations of *P. autumnalis* we surveyed (Table 1) may suggest a history of selection toward mutualistic associations, for which vertical transmission creates a partner fidelity feedback (Ewald, 1987; Sachs and Simms, 2006). However, the persistence of stromata-forming variants indicates potential maintenance of some pathogenicity in this symbiosis. Our field experiment

demonstrated superior net fitness of endophyte-symbiotic plants over non-symbiotic plants, consistent with previous results in this system and in other *Poa* species (Crawford *et al.*, 2010; Yule *et al.*, 2011; Shymanovich *et al.*, 2019b). However, integration of results on both fitness effects and transmission into demographic models would further resolve the question of long-term persistence of fungal endophytes (e.g. Gundel *et al.*, 2008; Cavazos *et al.*, 2018).

In summary, besides the fungal alkaloid-conferred resistance to herbivores, our study provides evidence for an endophyte-mediated enhanced tolerance to herbivory. Also, our results demonstrate that simulated folivory not only mediates the relative fitness benefits of symbiosis in host plant populations, but also promotes the persistence of symbiosis by increasing endophyte vertical transmission to the next generation. The synergism between fitness and transmission mechanisms of symbiont persistence should maintain long-term symbiosis under frequent herbivory and probably explains the high prevalence of the grass–*Epichloë* symbiosis and low frequency of pathogenic variants in populations of *P. autumnalis*.

SUPPLEMENTARY DATA

Supplementary data are available online at <https://academic.oup.com/aob> and consist of the following. Appendix 1: Detailed methods. Figure S1: Detection of *Epichloë* sp. in *Poa autumnalis*. Figure S2: Phylogenetic tree of partial *mtAC* and *mtBA* gene sequences of representative *Epichloë* species. Table S1: PCR primers used in this study. Table S2: Overview of PCR-based genotyping of endophytes in natural populations of *Poa autumnalis*.

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