

Does hybridization of endophytic symbionts in a native grass increase fitness in resource-limited environments?

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Abstract:

Hybridization is common among plants, animals and microbes. However, the ecological consequences of hybridization for microbes are far less understood than for plants and animals. For symbiotic *Epichloë* fungi, hybridization is widespread and may augment the well-known benefits of the endophytes to their grass hosts, especially in stressful environments. We tested the hybrid fitness hypothesis (HFH) that hybrid endophytes enhance fitness in stressful environments relative to non-hybrid endophytes. In a long-term field experiment, we monitored growth and reproduction of hybrid-infected (H+), non-hybrid infected (NH+), naturally endophyte free (E-) plants and those plants from which the endophyte had been experimentally removed (H- and NH-) in resource-rich and resource-poor environments. Infection by both endophyte species enhanced growth and reproduction. H+ plants outperformed NH+ plants in terms of growth by the end of the experiment, supporting HFH. However, H+ plants only outperformed NH+ plants in the resource-rich treatment, contrary to HFH. Plant genotypes associated with each endophyte species had strong effects on growth and reproduction. Our results provide some support the HFH hypothesis but not based upon adaptation to stressful environments. Our results reinforce the notion of a complex interplay between endophyte and plant genotype and environmental factors that determine fitness of the symbiont.

Keywords: endophyte | *Epichloë* | *Festuca arizonica* | hybridization | mutualism | symbionts

Article:

Introduction

Heritable variation among individuals within a plant species may have profound consequences at the population, community and ecosystem level (Neuhauser et al. 2003, Whitham et al. 2003, Crutsinger et al. 2006). Genetic variation coupled with the influence of environmental factors

determines phenotypic diversity within plant populations, leading to differences in fitness and relative frequencies among genotypes (e.g., Cheplick 2015). Genetic variation among individuals within plant species or “extended phenotypes” (Whitham et al. 2003) at the primary producer level may cascade upward to alter properties and processes at multiple consumer trophic levels (e.g., Crutsinger et al. 2006, Abdala-Roberts and Mooney 2014). Typically, this genotypic variation at the primary producer level originates from the plant genome.

However, another avenue by which plants acquire the phenotypic and, in some cases, genotypic variation, is via their microbial symbionts. Nearly all, if not all, primary producers in communities harbor microbial symbionts in some form. Symbiotic microbes such as mycorrhizal fungi and nitrogen-fixing bacteria have strong effects on plant fitness and consequently on plant community diversity and some ecosystem functions (e.g., van der Heijden et al. 1998), even though they are largely invisible and usually constitute only a tiny fraction of the biomass of the community.

One group of plant microbial symbionts, the fungal endophytes, has received increasing attention because of their powerful effects on host plant phenotypes (e.g., Hartley and Gange 2009). Fungal endophytes are common, abundant and diverse inhabitants of the above-ground tissues of most plant species (e.g., Cheplick and Faeth 2009). Most of these endophyte infections are localized and horizontally transmitted. However, many cool-season, pooid grasses are systemically infected with clavicipitaceous endophytes in the genus *Epichloë*. Some *Epichloë* species are asexual and are strictly vertically transmitted by hyphae growing into seeds (but see Moy et al. 2000). Thus, the fitness of vertically transmitted endophytes and their host plants are tightly coupled, and these endophytes are generally considered as strong plant mutualists (Clay and Schardl 2002). Infected grasses may show increased resistance and tolerance to biotic (e.g., herbivory) and abiotic (e.g., low soil nutrients and moisture) stresses compared to their uninfected counterparts (e.g., Saikkonen et al. 1998, Cheplick and Faeth 2009). As maternally inherited symbionts, asexual *Epichloë* endophytes may not only increase host plant phenotypic diversity but also plant genotypic richness (sensu Hughes et al. 2008).

However, the realized effects of *Epichloë* endophytes on host fitness depend on plant and endophyte genotypic variation and environmental factors (Saikkonen et al. 1998, 2006, Morse et al. 2007, Cheplick and Faeth 2009, Faeth and Saari 2012). Recent molecular studies show remarkable genetic variation in *Epichloë* endophytes (e.g., Leuchtman et al. 2014) not only across host grass species but also within a given grass species (e.g., Oberhofer and Leuchtman 2014). Host phenotypic variation stemming from different endophyte strains may even be greater than that from infection itself (e.g., Morse et al. 2007). A primary source of genetic variation and speciation events in *Epichloë* endophytes are hybridization events that rapidly infuse genetic variation and result in new, asexual *Epichloë* species (Tsai et al. 1994). About two-thirds of asexual *Epichloë* endophytes across species are of hybrid origin (Schardl and Craven 2003, Leuchtman et al. 2014). Hybridization probably occurs when hyphae of sexual, haploid *Epichloë* endophytes co-occurring in the same plant fuse via parasexual anastomosis to produce asexual, heteroploid (incomplete polyploidy) *Epichloë* endophyte species (Schardl and Craven 2003). Ecologically, host plants infected with hybrids are thought to be fitter in a wider, and hence more stressful, range of biotic and abiotic environments, like some plant hybrids (e.g., Rieseberg 1997), because they express traits from both parental species (Schardl and

Craven 2003). We thus expect the relative fitness of hybrid and nonhybrid infected plants and plants without infections to vary depending on the environmental context. In some wild grasses such as *Festuca arizonica* (Arizona fescue) and *Hordeylmus europaeus*, hybrid and non-hybrid *Epichloë* species can be found within the same population (Oberhofer and Leuchtman 2012, 2014, Saari and Faeth 2012). These systems are ideal to test to how hybridization of endophytic symbionts may change host plant fitness under different resource environments and over multiple growing seasons relative to infection by non-hybrid endophyte symbionts.

However, there have been few tests of the hypothesis that endophyte hybridization increases fitness of host grasses compared to hosts infected with non-hybrid endophytes or hosts that are endophyte free (henceforth, the hybrid fitness hypothesis or HFH). Furthermore, the few studies to date, all conducted in the greenhouse, have yield mixed support for Schardl and Craven's (2003) hypothesis that infection by hybrid endophytes increases growth and performance in a wider range of environmental conditions. In a greenhouse experiment, Oberhofer et al. (2013) found that the wild grass *H. europaeus*, when inoculated with either naturally-occurring hybrid or non-hybrid *Epichloe* endophytes did not differ in growth under drought or drought-free treatments. Non-hybrid endophytes increased seed production whereas hybrid endophytes reduced it, which is not consistent with HFH.

In another wild grass that harbors hybrid and non-hybrid endophytes within the same populations, Hamilton et al. (2009) found in correlational studies that *F. arizonica* hosts infected by the hybrid endophyte are found in habitats where soils have lower nutrients and water availability. In later greenhouse experiments, Saari and Faeth (2012) showed that hybrid infected plants were better competitors than non-hybrid infected plants but only when water and nutrients were limiting, supporting HFH in that infection by hybrid *Epichloë* endophytes may expand ecological niches, especially in marginal habitats. However, in another greenhouse experiment, where hybrid and non-hybrid endophytes were inoculated into hosts, Jia et al. (2016) found that the hybrid endophyte enhanced host growth but these effects were greatest in resource-rich environments, contrary to predictions based on HFH.

All the previously mentioned studies were either correlational or based upon experiments conducted in the greenhouse for short periods (<6 months). To our knowledge, there are no longer term field experiments where the effects of hybrid and non-hybrid endophytes on host growth and reproduction have been tested in different resource environments. To that end, we conducted a long-term field experiment where hybrid (H+) and non-hybrid (NH+) infected, naturally endophyte free (E-) plants and plants from which their endophytes had been experimentally removed (H- and NH-) were grown in a common garden experiment in two resource environments (supplemented or ambient nutrients and water). We then compared plant growth and reproductive parameters over four growing seasons to test HFH using a wild grass that naturally harbors both hybrid and non-hybrid endophyte species.

Methods

Arizona fescue and *Epichloë* species

Arizona fescue (*Festuca arizonica* Vasey), in the subfamily Poöideae, is a dense, perennial bunchgrass that reproduces by seed allogamously and is native and widespread in the southwestern United States and in northern Mexico (Kearney and Peebles 1960). Arizona fescue grows in semiarid ponderosa pine–bunchgrass communities above 2,000 m elevation (Kearney and Peebles 1960), where soils are low in nutrients and seasonal and yearly droughts are common (Faeth and Sullivan 2003). Arizona fescue is frequently infected by either a non-hybrid or hybrid endophyte. Hybrids are readily identified by the presence of multiple alleles at some loci (Schardl et al. 2012). The non-hybrid endophyte is *Epichloë typhina* subsp. *poae* var. *huerfana* (formerly *Neotyphodium huerfanum*, asexual *Epichloë* were formerly placed in the genus *Neotyphodium* but were recently absorbed into the genus *Epichloë*, Leuchtman et al. 2014). Hereafter, we refer to this endophyte taxon as NH (for non-hybrid). The hybrid endophyte (hereafter, H) is *Epichloë tembladera* (formerly *Neotyphodium tembladera*—Leuchtman et al. 2014). The hybrid endophyte in Arizona fescue has resulted from hybridization between co-occurring *E. typhina* and *Epichloë festucae* endophytes (Iannone et al. 2012). *Epichloë tembladera* is found across host grass species and across continents suggesting multiple and independent hybridization events between *E. typhina* and *E. festucae* (Gentile et al. 2005). However, in 30 yr of intense study, we have not encountered either of these parental types in Arizona fescue. Unlike most hybrid endophytes, NH+ plants far outnumber both H+ and E– plants across natural populations of Arizona fescue (Hamilton et al. 2009). Both endophytes are asexual, vertically transmitted and obligate symbionts (no free-living stages) but their hosts remain facultative as endophyte-free (hereafter E–) plants are found in nature.

Common garden experiment

To test the effects of endophyte infection in general and those of hybrid and non-hybrid endophyte species in particular on host plant growth and reproduction, we conducted a long-term common garden field experiment at the Arboretum of Flagstaff, Flagstaff Arizona, USA. A field plot that once harbored Arizona fescue and other native grasses, herbs and forbs was disked in 2009, vegetation removed and then the plot was covered with a weed barrier that is permeable to water and dissolved nutrients (Dalen Products, Inc., Knoxville Tennessee, USA) for 2 yr. The plot was inside an elk fence (elk destroy new plantings in previous experiments). However, all plants in the plot were freely accessible to invertebrate and small vertebrate herbivores.

To separate the effects of endophyte infection and species and plant genotype associated with endophyte species, we first pooled seeds of Arizona fescue from 15 mother plants (to randomize maternal plant genotype within an infection category) of each of the following infection categories: naturally uninfected plants (E–), plants infected with the hybrid endophyte (H+), plants infected with the non-hybrid endophyte (NH+), maternal plants that were originally infected with the H or NH endophyte but were endophyte-free (H– and NH– seeds). The latter two categories of seeds were collected from maternal plants from which the respective endophyte had been experimentally removed (via hydroponic treatment with a fungicide—see Faeth 2009 for details) in the previous generation and thus were several years and one generation removed from the experimental endophyte removal and any associated extraneous effects. These maternal plants were from previous experiments (Faeth and Sullivan 2003, Faeth 2009) that were growing at the Arboretum of Flagstaff. Randomly selected seeds from the pools of maternal plants for these five categories (E–, H+, NH+, H– and NH–) of plants were germinated in the

greenhouse at the Arboretum of Flagstaff in April–May 2010. After germination and growth in the greenhouse for several months, leaf samples from the plants were checked for endophyte status via tissue print immunoblotting (Faeth and Sullivan 2003).

In May 2011, plants in the five categories were transplanted in the field plot into holes cut into the weed barrier at 1 m intervals. To test for any differences within the field plots (e.g., microclimate or soil nutrient differences within the plot), plants were randomly assigned into 10 blocks. Within each block, four plants of each category were randomly assigned a position and planted (a total of 200 plants). All plants were provided supplemental water (0.5 L/d) for the first growing season to facilitate survival and establishment. At the end of the first growing season, 166 plants remain in the experiment. In May of 2012, of the four plants of each category within each block, two were randomly assigned either a low (ambient) or high resource environment. The high resource environment involved continuation of supplemental water (increased to $1 \text{ L} \cdot \text{d}^{-1} \cdot \text{plant}^{-1}$) plus added nutrients (5 g 10N : 10P : 10K fertilizer [Sta-Green (Gro Tec, Inc. Madison, Georgia, USA) every 2 weeks]) during the growing season. Our objective was to test the effect of infection and endophyte species in relatively resource-poor and resource-rich environments. The climate in northern Arizona where the experiment occurred is semi-arid, with an average precipitation of 582 mm/yr, and frequent seasonal and yearly droughts. Precipitation for the years during the years of the experiment were near normal (559, 432, 635 and 544 mm for 2011, 2012, 2013 and 2014, respectively—<http://www.wrh.noaa.gov/fgz/>). In addition, soils are nutrient-poor, especially related to nitrogen (soils average 0.15 mg N/100 g soil (Schulthess and Faeth 1998, Faeth and Sullivan 2003)). This level of supplemented water and nutrients has been used on multiple occasions to achieve large differentials in Arizona fescue growth and reproduction (e.g., Faeth and Sullivan 2003, Faeth 2009).

At the end of each growing season in September 2011–2014, reproductive biomass (culms and seeds) were collected before seeds shattered. All vegetative above-ground plant material was then collected for each plant (above-ground biomass of Arizona fescue naturally senesces in September). Above-ground biomass was dried and weighed and seeds and culms were separated and weighed. Reproductive effort (*sensu* Reekie 1999) was calculated by dividing seed and culm dry biomass by vegetative biomass. At the end of the experiment in fall 2014, we dug up the roots of 50% of the experimental plants and then removed soil with water and dried and weighed them. We then calculated root : shoot biomass ratio by dividing root dry biomass by above-ground dry biomass for 2014. Infection status was confirmed via tissue print immunoblot for all plants at the end of the experiment.

Statistical analyses

The effects of infection. We used repeated measures ANOVA (Systat 12.0, Systat Software, Inc., San Jose, CA, USA) to first test for the effects of non-hybrid and hybrid infection separately on growth, seed production and reproductive effort (arc sine square root transformed) between treatments. For the first of these two analyses, only NH⁺ and NH⁻ plants were included so that we could test for the effects of block, infection, treatment and the interaction between infection and treatment (between subjects factors) over the different years (within subject factors). Because root biomass was only sampled in the last year of the study, we used ANOVA to test for differences in root dry biomass and root : shoot ratio in 2014 between NH⁺ and NH⁻ plants.

Assumptions of normality and homogeneity of variances were tested and met for all RM ANOVA and ANOVA.

To test for the effects of hybrid infection on seed production and reproductive effort between treatments, a similar repeated measures ANOVA was performed as above except only the H⁺ and H⁻ plants were included in the analyses. We also used ANOVA to test the effects of H infection on root biomass and root : shoot ratio in 2014.

The effects of hybrid vs. non-hybrid endophytes. To test for difference between NH and H endophytes, we used repeated measures ANOVA to test for the effects of endophyte species on growth, seed production and reproductive effort between treatments. In this analysis, only NH⁺ and H⁺ plants were included in the analysis since we were interested in the difference in effects on host plants between endophyte species. As noted previously, the effects of block, endophyte species, treatment and the interaction between species and treatment (between subjects factors) on growth, seed production and reproductive effort were compared over years (within subject factors). As earlier, we also tested via ANOVA the effect of species on root dry biomass and root : shoot ratio in 2014.

The effects of plant genotype. Because maternal plant genotypes may be associated with different endophyte species or uninfected plants (e.g., Oberhofer and Leuchtman 2014, Jia et al. 2016), we tested whether plant genotypes that harbor NH or H endophytes or are naturally uninfected (E⁻) differ from each other in growth, seed production and reproductive effort between treatments. For this RM ANOVA, only NH⁻, H⁻ and E⁻ plants were included. We used Tukey HSD tests to compare above-ground and seed biomass and reproductive effort (arcsine square root transformed) means among the NH⁻, H⁻ and E⁻ plant genotypes. As previously, we also tested via ANOVA the effect of plant genotype on root dry biomass and root : shoot ratio in 2014.

Final differences in 2014. Because differences in the effects of infection, endophyte species and plant genotype increased with time for most of the growth and reproductive parameters (Figs. 1-3), we used post hoc ANOVA contrasts within our repeated measures analyses to test for differences in these factors between treatments in the final year of the experiment. In the case of plant genotype, there were multiple pairwise comparisons and *P* values were Bonferroni adjusted.

Results

The effects of infection

Plants infected by the NH endophyte differed from NH⁻ plants in above-ground biomass (Table 1, Fig. 1A, B), seed biomass (Table 1, Fig. 2A, B), and reproductive effort (Table 1, Fig. 3A, B). As expected, growth and reproduction were greater in the high resource treatment than the low resource treatment. Infection status also interacted with treatments to affect above-ground and seed biomass (Table 1). These interactions were clarified by the 2014 analyses. NH⁺ and NH⁻ plants did not differ in above-ground biomass in either the high ($F = 1.41$; 1, 33 df, $P = 0.24$) or low resource treatment ($F = 0.34$; 1, 24 df, $P = 0.56$). However, NH⁺ plants

produced more seeds ($F = 8.09$; 1, 33 df, $P < 0.01$) and had higher reproductive effort ($F = 11.61$; 1, 33 df, $P < 0.01$) than NH⁻ plants, but only in the high resource treatment (Figs. 1B, 2B, 3B). Infection by the NH⁺ endophyte had no effect on root dry biomass or root : shoot ratio (Table 1, Fig. 4A, B).

Table 1. Analysis of variance results for the effect of block, non-hybrid infection (NH⁺ vs. NH⁻) and nutrient and watering treatments (high and low) on growth and reproductive parameters for Arizona fescue plants

	df	Above-ground dry biomass		Seed dry biomass		Reproductive effort		df	Root dry biomass		Root : Shoot	
		<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>		<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>
Between subjects												
Block (B)	1	4.30	0.04	2.35	0.13	5.00	0.03	1	0.85	0.36	0.26	0.61
Infection (I)	1	3.98	0.05	7.65	<0.01	13.40	<0.01	1	0.82	0.38	0.11	0.74
Treatment (T)	1	8.86	<0.01	3.91	0.05	0.58	0.45	1	0.62	0.44	2.96	0.10
I × T	1	0.01	0.96	3.60	0.06	2.52	0.12	1	0.13	0.72	1.51	0.23
Error	58							23				
Within subjects												
Year (Y)	3 (2)	10.34	<0.01	0.90	0.41	3.88	0.02					
Y × B	3 (2)	3.22	0.02	3.06	0.05	6.23	<0.01					
Y × I	3 (2)	1.72	0.16	2.34	0.11	2.55	0.08					
Y × T	3 (2)	4.58	<0.01	0.82	0.44	0.32	0.73					
Y × T × I	3 (2)	0.17	0.92	1.14	0.33	1.77	0.17					
Error	174 (116)											

Notes. Within subjects degrees of freedom vary because above-ground biomass was measured for 4 yr, whereas reproduction did not begin until the second year of monitoring. Root biomass was measured once at the end of the experiment for a subset of the surviving plants. Significant ($P < 0.05$) or marginally significant ($0.10 > P > 0.05$) *P*-values are in bold.

Table 2. Analysis of variance results for the effect of block, hybrid infection (H⁺ vs. H⁻) and nutrient and watering treatments (high vs. low) on growth and reproductive parameters for Arizona fescue plants

	df	Above-ground dry biomass		Seed dry biomass		Reproductive effort		df	Root dry biomass		Root : Shoot	
		<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>		<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>
Between subjects												
Block (B)	1	6.34	0.02	2.50	0.12	4.97	0.03	1	0.31	0.58	0.58	0.45
Infection (I)	1	3.04	0.09	0.88	0.35	0.08	0.78	1	0.07	0.80	2.29	0.14
Treatment (T)	1	1.38	0.24	1.18	0.28	0.39	0.53	1	0.00	0.99	1.95	0.18
I × T	1	8.59	<0.01	5.02	0.03	3.25	0.08	1	0.22	0.65	0.44	0.52
Error	58							23				
Within subjects												
Year (Y)	3 (2)	37.92	<0.001	0.55	0.58	1.97	0.14					
Y × B	3 (2)	8.03	<0.001	2.01	0.14	0.70	0.50					
Y × I	3 (2)	9.30	<0.001	4.45	0.01	0.25	0.25					
Y × T	3 (2)	2.25	0.08	1.52	0.22	0.25	0.25					
Y × T × I	3 (2)	5.10	<0.01	1.92	0.15	0.56	0.54					
Error	174 (116)											

Notes. Within subjects degrees of freedom vary because above-ground biomass was measured for 4 yr, whereas reproduction did not begin until the second year of monitoring. Root biomass was measured once at the end of the experiment for a subset of the surviving plants. Significant ($P < 0.05$) or marginally significant ($0.10 > P > 0.05$) *P*-values are in bold.

Plants infected by the H endophyte differed from H⁻ plants only in above-ground biomass, and then only marginally so (Table 2, Fig. 1A, B) and not in seed dry biomass, reproductive effort, root biomass or root : shoot ratio (Table 2, Figs. 2A, B, 3A, B, 4A, B). However, infection status and treatment interacted to affect above-ground and seed biomass and reproductive effort (Table 2). These interactions were clarified by the 2014 analyses. H⁺ infected plants had greater biomass ($F = 20.63$, $df = 1, 26$, $P < 0.001$) and seed biomass ($F = 5.40$, $df = 1, 26$, $P = 0.03$) than H⁻ plants but only in the high resource treatment (Fig. 1A, B, 2A, B). H⁺ plants did not differ from H⁻ plants in reproductive effort or in root biomass or root : shoot ratio (Table 2).

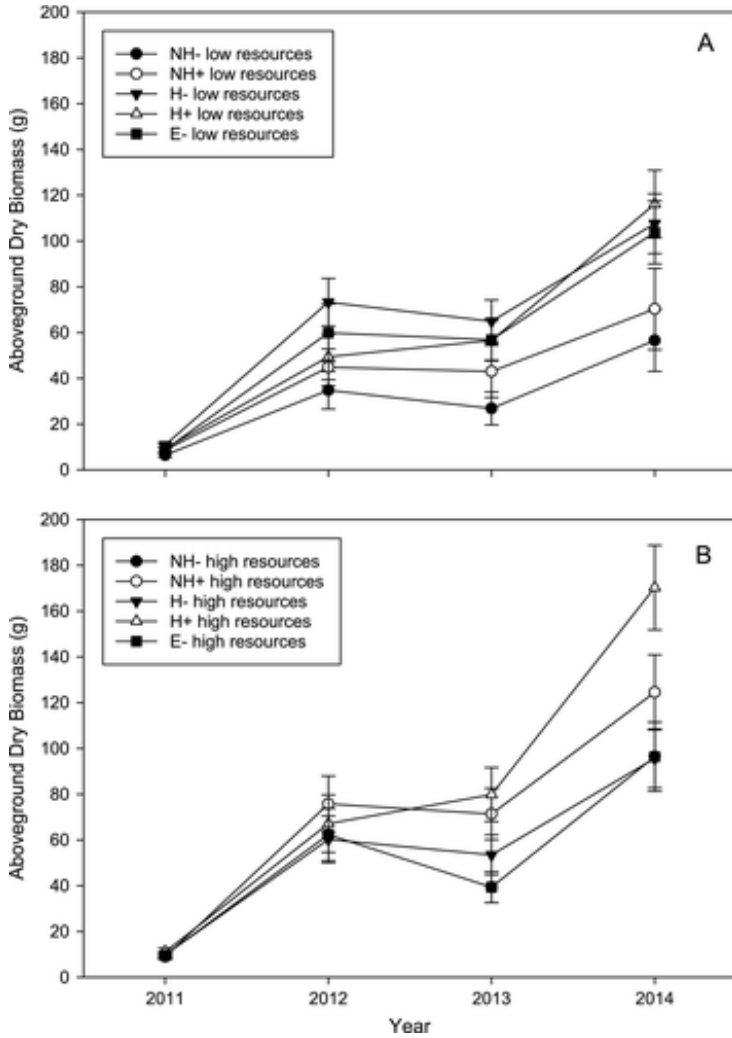


Figure 1. Estimated mean (\pm SE) of above-ground dry biomass of NH⁻, NH⁺, H⁻, H⁺ and E⁻ plants in low (A) and high resource treatments (B).

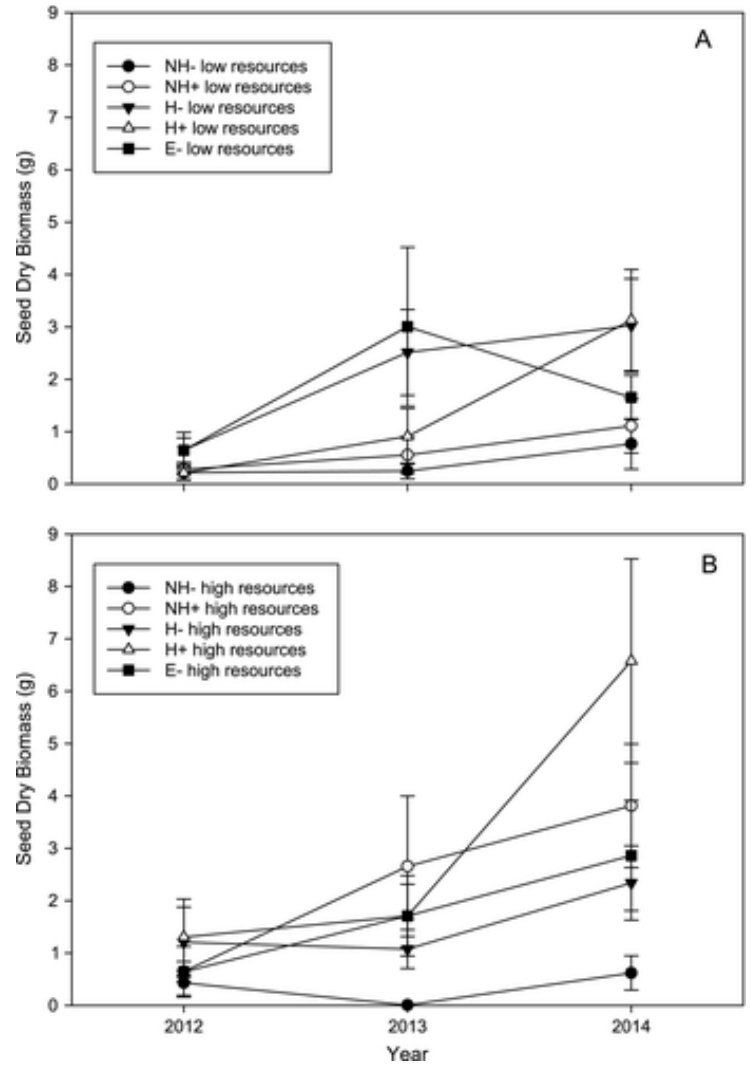


Figure 2. Estimated mean (\pm SE) of seed dry biomass of NH⁻, NH⁺, H⁻, H⁺ and E⁻ plants in low (A) and high resource treatments (B).

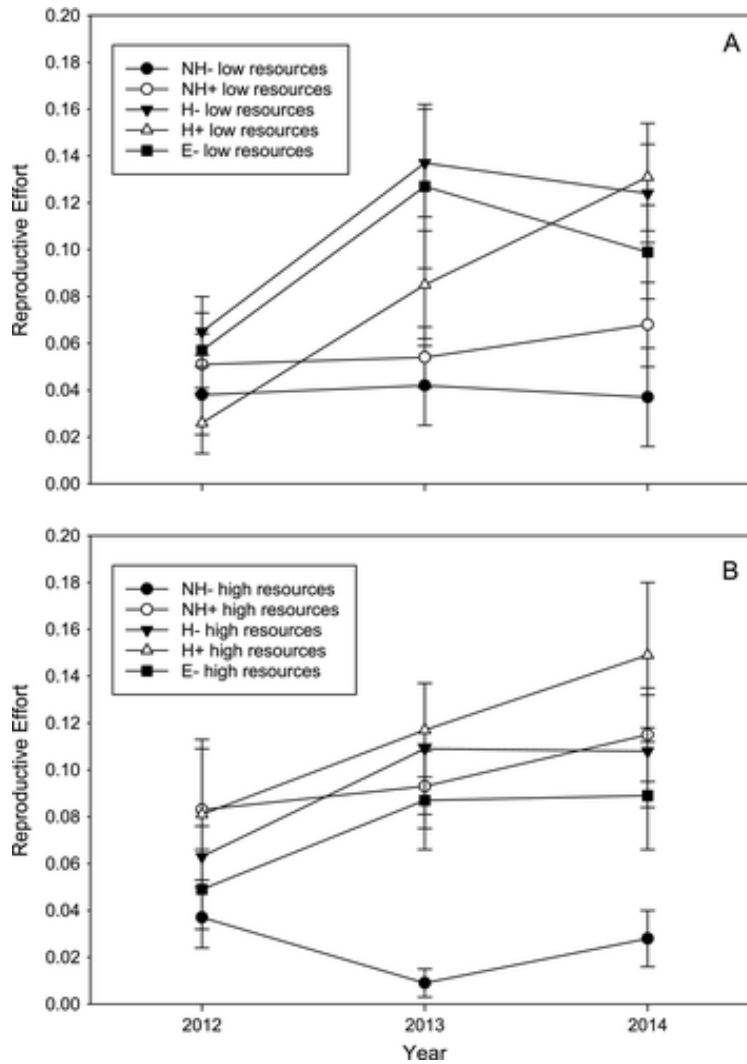


Figure 3. Estimated mean (\pm SE) of reproductive effort of NH⁻, NH⁺, H⁻, H⁺ and E⁻ plants in low (A) and high resource treatments (B).

The effects of endophyte species

Plants infected by the NH and H endophyte differed marginally in above-ground biomass (Table 3, Fig. 1A, B) and did not differ in seed biomass (Table 3, Fig. 2A, B), reproductive effort (Table 3, Fig. 3A, B), root biomass or root : shoot ratio (Table 3, Fig. 4C, D). Analyses of the growth at the end of the experiment in 2014 showed that H⁺ plants had greater above-ground biomass (Fig. 1A, B) than NH⁺ plants but only in the high resource treatment ($F = 5.11$, $df = 1$, 29 , $P = 0.03$). H⁺ plants also differed marginally ($F = 3.59$, $df = 1$, 28 , $P = 0.07$) from NH⁺ plants in reproductive effort but only in the low resource treatment (Fig. 2A, B).

The effects of plant genotype

In this analysis, we compared NH⁻, H⁻ and E⁻ plants to evaluate the effect of plant genotype associated with NH, H and E⁻ plants. Plant genotype had significant effects on above-ground

and seed biomass and reproductive effort (Table 4, Figs. 1A, B, 2A, B, 3A, B) but not on root dry biomass or root : shoot ratio (Table 4, Fig. 4E, F). Plant genotype interacted with treatment to marginally affect above-ground biomass (Table 4). At the end of the experiment, genotypes associated with NH, H and E⁻ plants differed overall in above-ground biomass in the low resource treatment ($F = 4.58$, $df = 2, 41$, $P = 0.02$; Fig. 1A) but not in the high resource treatment ($F = 0.09$, $df = 2, 47$, $P = 0.91$; Fig. 1B). Pairwise comparisons (Tukey HSD) showed that genotypes associated with E⁻ ($P = 0.04$) and H plants ($P = 0.02$) had greater above-ground biomass than NH associated plant genotypes. Above-ground biomass did not differ between E⁻ and H⁻ plants.

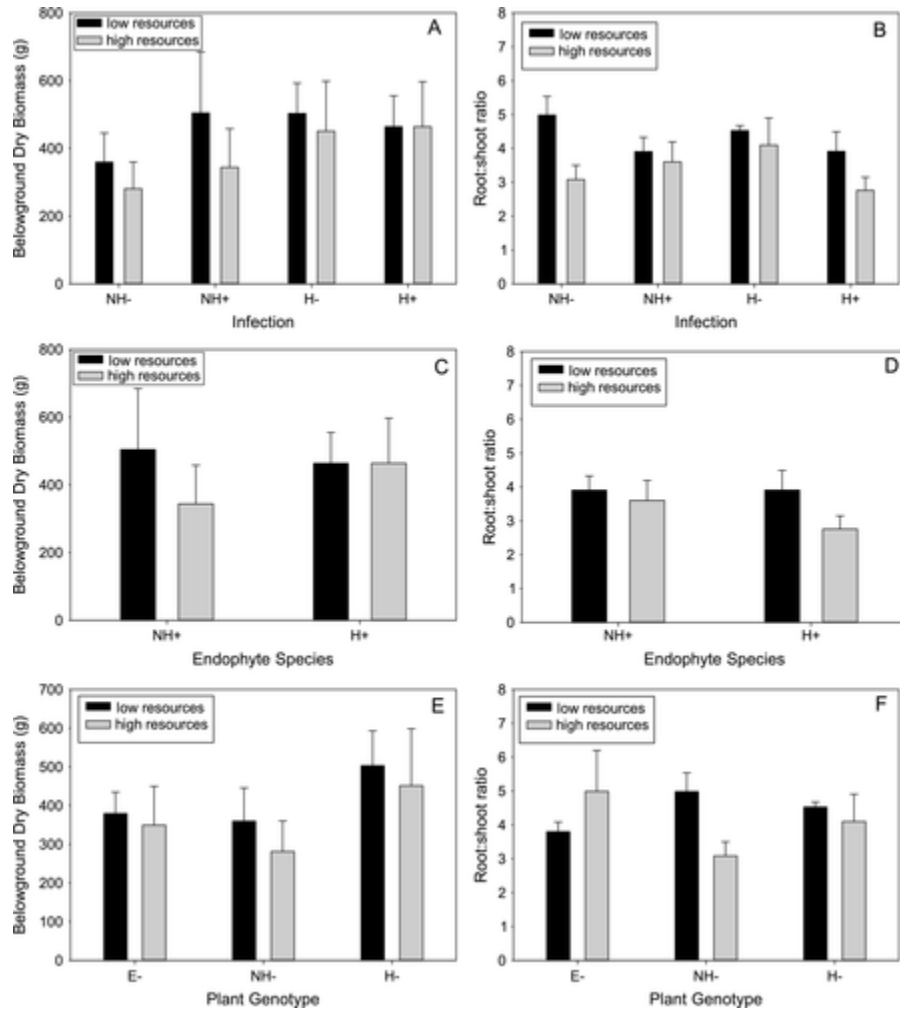


Figure 4. Estimate mean (\pm SE) of dry belowground biomass (A) and root:shoot ratio (B) between NH⁻ and NH⁺ plants and H⁻ and H⁺ plants, between NH⁺ and H⁺ plants (C, D), and among E⁻, NH⁻ and H⁻ plants (E, F) in low and high resource treatments.

At the end of the experiment, plant genotype also marginally affected seed biomass in the low resource treatment ($F = 2.26$, $df = 2, 41$, $P = 0.08$) and significantly so for the high resource treatment ($F = 3.46$, $P = 0.04$). In the low resource treatment, the difference among plant genotypes was largely due to H⁻ plants having marginally greater seed biomass than NH⁻ plants ($P = 0.06$; Fig. 2A). In the high resource treatment, the E⁻ plants had greater seed biomass than NH⁻ ($P = 0.03$) but not H⁻ plants (Fig. 2B).

Table 3. Analysis of variance results for the effect of block, endophyte species (H⁺ and NH⁺) and nutrient and watering treatments (high and low) on growth and reproductive parameters for Arizona fescue plants

	df	Above-ground dry biomass		Seed dry biomass		Reproductive effort		df	Root dry biomass		Root : Shoot	
		F	P	F	P	F	P		F	P	F	P
Between subjects												
Block (B)	1	8.67	<0.01	4.49	0.04	7.22	<0.01	1	4.86	0.04	1.70	0.21
Species (S)	1	2.70	0.10	1.92	0.17	1.89	0.17	1	0.18	0.67	0.62	0.44
Treatment (T)	1	11.21	<0.01	9.94	<0.01	5.23	0.02	1	0.44	0.51	1.92	0.18
S × T	1	0.01	0.93	0.01	0.93	0.05	0.83	23	0.30	0.59	0.79	0.38
Error	58											
Within subjects												
Year (Y)	3 (2)	23.24	<0.001	0.36	0.70	1.94	0.15					
Y × B	3 (2)	4.68	<0.01	6.21	<0.01	8.04	<0.01					
Y × S	3 (2)	7.16	<0.001	2.78	0.07	2.17	0.12					
Y × T	3 (2)	6.74	<0.001	2.34	0.10	0.80	0.79					
Y × T × S	3 (2)	0.59	0.62	0.44	0.65	0.24	0.24					
Error	174 (116)											

Notes. Within subjects degrees of freedom vary because above-ground biomass was measured for 4 yr, whereas reproduction did not begin until the second year of monitoring. Root biomass was measured once at the end of the experiment for a subset of the surviving plants. Significant ($P < 0.05$) or marginally significant ($0.10 > P > 0.05$) P -values are in bold.

Table 4. Analysis of variance results for the effect of block, plant genotype (H⁻, NH⁻ and E⁻) and nutrient and watering treatments (high and low) on growth and reproductive parameters for Arizona fescue plants

	df	Above-ground dry biomass		Seed dry biomass		Reproductive effort		df	Root dry biomass		Root : Shoot	
		F	P	F	P	F	P		F	P	F	P
Between subjects												
Block (B)	1	6.40	0.01	2.36	0.13	5.93	0.02	1	0.25	0.62	0.31	0.58
Genotype (G)	2	3.18	<0.05	6.32	<0.01	13.39	<0.001	2	1.05	0.36	0.07	0.93
Treatment (T)	1	0.33	0.57	0.14	0.71	1.58	0.21	1	0.51	0.48	0.42	0.52
G × T	2	2.65	0.08	0.42	0.66	0.04	0.96	2	0.02	0.98	1.38	0.26
Error	89							38				
Within subjects												
Year (Y)	3 (2)	20.85	<0.001	0.72	0.49	2.50	0.08					
Y × B	3 (2)	5.41	0.01	0.43	0.65	0.70	0.50					
Y × G	6 (4)	1.42	0.20	1.54	0.19	2.25	0.07					
Y × T	3 (2)	0.26	0.86	2.44	0.09	3.07	0.05					
Y × T × G	6 (4)	1.33	0.24	1.38	0.24	0.25	0.91					
Error	267 (178)											

Notes. Within subjects degrees of freedom vary because above-ground biomass was measured for 4 yr, whereas reproduction did not begin until the second year of monitoring. Root biomass was measured once at the end of the experiment for a subset of the surviving plants. Significant ($P < 0.05$) or marginally significant ($0.10 > P > 0.05$) P -values are in bold.

At the end of the experiment, plant genotype affected reproductive effort in both low ($F = 5.35$, $df = 2, 41$, $P < 0.01$; Fig. 3A) and high resource treatments ($F = 5.48$, $df = 2, 47$, $P < 0.01$; Fig. 3B). In the low resource treatment, E⁻ ($P = 0.07$) and H⁻ ($P < 0.01$) plants had higher reproductive effort than NH⁻ plants. Similar differences occurred in the high resource treatment, with E⁻ ($P = 0.02$) and H⁻ ($P = 0.02$) plants showing a greater reproductive effort than NH⁻ plants (Fig. 3B).

Discussion

Hybridization in nature is common among many plant and animal taxa, and also occurs in microbes although the consequences are far less known for microbial hybridization (Schardl and Craven 2003). Hybridization can have wide-ranging effects on fitness of plants and animals. In some cases, hybrid offspring are less fit due to reduced pre- and post-zygotic viability or sterility. In others, hybrids may be more fit than either parental type in marginal habitats where neither parent can persist (e.g., Riesberg et al. 2003). For *Epichloë* endophytes, hybridization has been proposed as mechanism for a rapid infusion of genetic variation that may thwart Müller's ratchet in asexual endophytes (Selosse and Schardl 2007) or increase success in a wider range of stressful environments (Schardl and Craven 2003). Whereas recent advances in molecular genetics show that hybrids have larger genomes and a greater variety of genes, especially those for producing alkaloids (e.g., Schardl et al. 2012), there have been few tests that hybridization in an endophyte renders that host grass-endophyte symbiotum more fit in a wider range of habitats. Those that have tested the HFH have been relatively short term and conducted in the greenhouse or growth chambers.

The effect of endophyte infection

Whether they are of non-hybrid or hybrid origin, asexual and seed-borne *Epichloë* endophytes are postulated to have positive effects on growth and reproduction, and hence fitness, of their host grasses through a variety of mechanisms (e.g., Clay and Schardl 2002, Cheplick and Faeth 2009). One of the primary benefits of endophyte infection is greater resistance to drought (e.g., Ren et al. 2014) and nutrient stress (e.g., Malinowski et al. 1999). Generally, our long term field experiment confirms that infection by the non-hybrid and hybrid endophyte increases growth and reproductive effort compared to their respective uninfected counterparts (Tables 1 and 2). However, these effects of infection vary over time and are dependent on nutrient availability. Overall, the non-hybrid endophyte appeared to have the greatest positive effects on growth and reproduction relative to its uninfected counterpart (Table 1). However, by the end of the experiment, H⁺ plants had greater above-ground and seed biomass than H⁻ plants, but only in the rich resource treatment. Likewise, NH⁺ plants had greater seed (but not above-ground) biomass and greater reproductive effort than NH⁻ plants but only in resource rich environments. Thus, it appears that both endophyte species may be beneficial to the host but not in water- or nutrient-deficient environments as proposed for seed borne endophytes in general, and for hybrid *Epichloë* endophytes in particular (e.g., Cheplick and Faeth 2009).

These results generally parallel those for previous greenhouse experiments for Arizona fescue. Jia et al. (2016) found that inoculating the H endophyte into plants from which the endophyte was removed enhanced most growth parameters but only in resource-rich environments.

However, inoculating the NH endophyte into NH⁻ plants did not enhance growth as we found here in our field experiment. However, Jia et al.'s (2016) experiment was short term (<6 months) and the effects of endophytes we observed in our field experiment were not evident until the second year or after.

Endophyte species effects

In our long term field experiment, we found some support for the HFH. Generally, H⁺ plants had greater above-ground and seed biomass and greater reproductive effort than NH⁺ plants but these differences were not evident until the fourth year of the experiment (Figs. 1, 2, 3A, B). But the growth advantages of H⁺ plants compared to NH⁺ plants were significant in the final year only in the high resource treatment (Fig. 1B), contrary to the HFH that predicts that grasses infected with hybrid endophytes should be relatively more adaptive in stressful, resource-poor environments (Schardl and Craven 2003). H⁺ plants did tend to have greater seed biomass (Fig. 2A, B) and higher reproductive effort (Fig. 3A, B) than NH⁺ plants, but again these differences were not more evident in low resource environments as predicted by the HFH.

In the only other experiments with hybrid and non-hybrid endophyte inhabiting a wild grass of which we are aware, Oberhofer et al. (2013) experimentally removed endophytes from *Hordeylmus europaeus* and then inoculated plants with different hybrid and non-hybrid strains. Hybrid and non-hybrid infection generally increased host growth but had variable effects on reproduction in greenhouse experiments. They did not find strong support for the HFH as the hybrid endophyte did not enhance host performance more than the non-hybrid endophyte in drought or non-drought treatments. Furthermore, they found that the non-hybrid endophyte was more common in drier field sites relative to the hybrid endophyte, corroborating their greenhouse experiments, but not supporting the prediction of HFH that hybrid endophytes confer higher fitness to host grasses than non-hybrid endophytes in stressful environments.

For Arizona fescue, the distribution of hybrid and non-hybrid endophytes seems to support the HFH. Hybrid infected grasses are more common in drier, nutrient-poor sites (Sullivan and Faeth 2008, Hamilton et al. 2009). However, unlike Oberhofer et al. (2013), results from our current field experiment as well as a previous greenhouse experiment (e.g., Jia et al. 2016) do not match expectations from the observed distribution. One explanation is that Jia et al.'s (2016) experiment and the one here were competition-free experiments. In a greenhouse experiment, Saari and Faeth (2012) found that hybrid-infected Arizona fescue outcompeted NH infected plants but only in resource-poor environments. However, another explanation is that hybrid endophytes do not increase fitness in stressful environments as envisioned by HFH. Rather there are other factors, such as interactions with herbivores and natural enemies (e.g., Saari et al. 2014) or relative fitness measures related to plant genotype that dictate the distribution of hybrid infected plants in nature.

Plant genotype effects

Because asexual endophytes are maternally transmitted, certain plant genotypes, at least maternal genotypes (H and NH plants are sometimes found within the same population and are therefore likely to cross-pollinate) should be associated with different endophyte species (e.g., Jia

et al. 2016). We found support for this prediction, as plant genotypes associated with NH⁺, H⁺, and E⁻ plants varied significantly in terms of above-ground and seed biomass and reproductive effort (Table 4). Thus, the fitness of plant genotypes associated with hybrid and non-hybrid endophytes and naturally uninfected plants likely interact with, and may even subsume (e.g., Jia et al. 2016), the effects of infection generally or hybrid or non-hybrid infection status specifically.

Furthermore, analyses of pairwise differences show that all of the significant differences stem from differences in growth and reproduction between E⁻ and NH⁻ plants or NH⁻ and H⁻ plants in both resource-poor and -rich treatments. There were no significant differences between E⁻ and H⁻ plant genotypes for either growth or reproduction across treatments. This result supports the hypothesis that E⁻ plant genotypic group may have been derived from once H infected plants that randomly lost their infection. Infection can be lost from plants due to (1) imperfect transmission where hyphae fail to grow into seeds (e.g., Ravel et al. 1997), (2) unviable hyphae in seeds due to excessive heat or long-term storage, or (3) from random loss of hyphae from ramets of adult, perennial grasses (Cheplick and Faeth 2009). The explanation for the observed lower frequency of H relative to NH plants in nature may reside in a scenario based less upon relative fitness and more on random events or physiological mechanisms.

Conclusions and Caveats

Our long term experiment provides only partial support for the HFH in that hybrid infected plants grow and reproduce better than NH plants. However, these benefits occur generally in resource-rich environments, which is counter to the same hypothesis. Although our field experiment is far longer in duration and more realistic (in a natural field setting) than previous greenhouse experiments, we caution that even a 4-yr experiment cannot fully assess the relative fitness benefits of *Epichloë* endophytes inhabiting perennial grasses that may live for decades. Although our experiment measured both long term growth and seed production, we also acknowledge that these parameters are only surrogates for relative fitness. Furthermore, our experiment was a no-competition experiment, and the benefits of hybrid endophytes may be manifested via enhancement of intra- or interspecific competitive abilities. Nonetheless, our experiment reinforces the now rather consistent findings (e.g., Saikkonen et al. 2006, Saari and Faeth 2012), that endophyte species or strain, plant genotype and environmental factors interact in complex ways to influence the consequences for host grass fitness.

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Literature Cited

- Abdala-Roberts, L., and Mooney, K. A.. 2014. Ecological and evolutionary consequences of plant genotypic diversity in a tri-trophic system. *Ecology* **95**: 2879– 2893.
- Cheplick, G. P. 2015. *Approaches to plant evolutionary ecology*. Oxford University Press, New York, New York, USA.
- Cheplick, G. P., and Faeth, S. H.. 2009. *The ecology and evolution of the grass-endophyte symbiosis*. Oxford University Press, New York, New York, USA.
- Clay, K., and Schardl, C. L.. 2002. Evolutionary origins and ecological consequences of endophyte symbiosis with grasses. *American Naturalist* **160** (Suppl.): S99– S127.
- Crutsinger, G. M., Collins, M. D., Fordyce, J. A., Gompert, Z., Nice, C. C., and Sanders, N. J.. 2006. Plant genotypic diversity predicts community structure and governs an ecosystem process. *Science* **313**: 966– 968.
- Faeth, S. H. 2009. Asexual microbial symbionts alter reproductive allocation and herbivory over time in their native perennial grass hosts. *American Naturalist* **173**: 554– 566.
- Faeth, S. H., and Sullivan, T. J.. 2003. Mutualistic asexual endophytes in a native grasses are usually parasitic. *American Naturalist* **161**: 310– 325.
- Faeth, S. H., and Saari, S.. 2012. Fungal endophytes of grasses and arthropod communities: lessons from plant-herbivore interactions. *Fungal Ecology* **5**: 364371.
- Gentile, A., Rossi, M. S., Cabral, D., Craven, K. D., and Schardl, C. L.. 2005. Origin, divergence, and phylogeny of *Epichloë* endophytes of native Argentine grasses. *Molecular Phylogenetics and Evolution* **35**: 196– 208.
- Hamilton, C. E., Faeth, S. H., and Dowling, T. E.. 2009. Distribution of hybrid fungal symbionts and environmental stress. *Microbial Ecology* **58**: 408– 413.
- Hartley, S. E., and Gange, A. C.. 2009. Impacts of plant symbiotic fungi on insect herbivores: mutualism in a multitrophic context. *Annual Review of Entomology* **54**: 323– 342.
- Hughes, A. R., Inouye, B. D., Johnson, M. T. J., Underwood, N., and Vellend, M.. 2008. Ecological consequences of genetic diversity. *Ecology Letters* **1**: 609– 623.
- Iannone, L. J., Novas, M. V., Young, C. A., De Battista, J. P., and Schardl, C. L.. 2012. Endophytes of native grasses from South America: biodiversity and ecology. *Fungal Ecology* **5**: 357– 363.
- Jia, T., Oberhofer, M., Shymanovich, T., and Faeth, S. H.. 2016. Effects of hybrid and non-hybrid *Epichloë* endophytes and their associated host genotypes on the response of a native grass to varying environments. *Microbial Ecology* **8**: 633– 641.

- Kearney, T. H., and Peebles, R. H.. 1960. *Arizona flora*. University of California Press, Berkeley, California, USA.
- Leuchtman, A., Bacon, C. W., Schardl, C. L., White, J. F., and Tadych, M.. 2014. Nomenclatural realignment of *Neotyphodium* species with genus *Epichloë*. *Mycologia* **106**: 202– 215.
- Malinowski, D., Brauer, D. K., and Belesky, D. P.. 1999. The endophyte *Neotyphodium coenophialum* affects root morphology of tall fescue grown under phosphorous deficiency. *Journal of Agronomy & Crop Science* **183**: 53– 60.
- Morse, L. J., Faeth, S. H., and Day, T. A.. 2007. *Neotyphodium* interactions with a wild grass are driven mainly by endophyte haplotype. *Functional Ecology* **21**: 813– 822.
- Moy, M., Belanger, F., Duncan, R., Freehoff, A., Leary, C., Meyer, W., Sullivan, R., and White, J. F..2000. Identification of epiphyllous mycelial nets on leaves of grasses infected by clavicipitaceous endophytes. *Symbiosis* **28**: 291– 302.
- Neuhauser, C., Andow, D. A., Heimpel, G. E., May, G., Shaw, R. G., and Wagenius, S.. 2003. Community genetics: expanding the synthesis of ecology and genetics. *Ecology* **84**: 545– 558.
- Oberhofer, M., Gusewell, S., and Leuchtman, A.. 2013. Effects of natural hybrid and non-hybrid *Epichloë* endophytes on the response of *Hordelymus europaeus* to drought stress. *New Phytologist* **201**: 242– 253.
- Oberhofer, M., and Leuchtman, A.. 2012. Genetic diversity in epichloid endophytes of *Hordelymus europaeus* suggests repeated host jumps and interspecific hybridizations. *Molecular Ecology* **21**: 2713– 2726.
- Oberhofer, M., and Leuchtman, A.. 2014. Horizontal transmission, persistence and competition capabilities of *Epichloë* endophytes in *Hordelymus europaeus* grass hosts using dual endophyte inocula. *Fungal Ecology* **11**: 37– 49.
- Ravel, C., Michalakakis, Y., and Charmet, G.. 1997. The effect of imperfect transmission on the frequency of mutualistic seed-borne endophytes in natural populations of grasses. *Oikos* **80**: 18– 24.
- Reekie, E. G. 1999. Resource allocation, tradeoffs, and reproductive effort in plants. Pages 173– 193 in T. M. Vuorisalo and P. K. Mutkainen, editors. *Life history evolution in plants*. Kluwer Academic Publishers, Dordrecht, The Netherlands.
- Ren, A., Wei, M., Yin, L., Wu, L., Zhou, Y., Li, X., and Gao, Y.. 2014. Benefits of a fungal endophyte in *Leymus chinensis* depend more on water than on nutrient availability. *Environmental and Experimental Botany* **108**: 71– 78.

- Rieseberg, L. H. 1997. Hybrid origin of plant species. *Annual Review of Ecology and Systematics* **28**: 359– 389.
- Rieseberg, L., Raymond, O., Rosenthal, D. M., Lai, Z., Livingstone, K., Nakazato, T., Durphy, J. L., Scharzbach, A. E., Donovan, L. A., and Lexer, C.. 2003. Major ecological transitions in wild sunflowers facilitated by hybridization. *Science* **301**: 1211– 1216.
- Saari, S., and Faeth, S. H.. 2012. Hybridization of *Neotyphodium* endophytes enhances competitive ability of the host grass. *New Phytologist* **195**: 231– 236.
- Saari, S., Higgins, M., Richter, S., Robbins, M., and Faeth, S. H.. 2014. Bottom-up regulates top-down: the effects of hybridization of grass endophytes on an aphid herbivore and its generalist predator. *Oikos* **123**: 545– 552.
- Saikkonen, K., Faeth, S. H., Helander, M., and Sullivan, T. J.. 1998. Fungal endophytes: a continuum of interactions with host plants. *Annual Review of Ecology and Systematics* **29**: 319– 343.
- Saikkonen, K., Lehtonen, P., Helander, M., Koricheva, J., and Faeth, S. H.. 2006. Model systems in ecology: dissecting the endophyte-grass literature. *Trends in Plant Science* **11**: 428– 433.
- Schardl, C. L., and Craven, K. D.. 2003. Interspecific hybridization in plant-associated fungi and oomycetes: a review. *Molecular Ecology* **12**: 2861– 2873.
- Schardl, C. L., Young, C. A., Faulkner, J. R., Florea, S., and Pan, J.. 2012. Chemotypic diversity of epichloae, fungal symbionts of grasses. *Fungal Ecology* **5**: 331– 344.
- Schulthess, F. M., and Faeth, S. H.. 1998. Distribution, abundances, and associations of the endophytic fungal community of Arizona fescue (*Festuca arizonica*). *Mycologia* **90**: 569– 578.
- Selosse, M., and Schardl, C. L.. 2007. Fungal endophytes of grasses: Hybrids rescued by vertical transmission? An evolutionary perspective. *New Phytologist* **173**: 452– 458.
- Sullivan, T., and Faeth, S. H.. 2008. Local Adaptation in *Festuca arizonica* infected by hybrid and nonhybrid *Neotyphodium* endophytes. *Microbial Ecology* **55**: 697– 704.
- Tsai, H.-F., Liu, J. S., Christensen, M. J., Latch, G. C. M., Siegel, M. R., and Schardl, C. L.. 1994. Evolutionary diversification of fungal endophytes of tall fescue grass by hybridization with *Epichloë* species. *Proceedings of the National Academy of Sciences USA* **91**: 2542– 2546.
- Heijden, M. G. A., Klironomos, J. N., Ursic, M., Moutoglis, P., Streitwolf-Engel, R., Boller, T., Wiemken, A., and Sanders, I. R.. 1998. Mycorrhizal fungal diversity determines plant biodiversity, ecosystem variability and productivity. *Nature* **396**: 69– 72.
- Whitham, T. G., et al. 2003. Community and ecosystem genetics: a consequence of the extended phenotype. *Ecology* **84**: 559– 573.