

Effects of hybrid and non-hybrid *Epichloë* endophytes and their associated host genotypes on the response of a native grass to varying environments

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Abstract:

Asexual *Epichloë* endophytes are prevalent in cool season grasses, and many are of hybrid origin. Hybridization of asexual endophytes is thought to provide a rapid influx of genetic variation that may be adaptive to endophyte–host grass symbiota in stressful environments. For Arizona fescue (*Festuca arizonica*), hybrid symbiota are commonly found in resource-poor environments, whereas non-hybrid symbiota are more common in resource-rich environments. There have been very few experimental tests where infection, hybrid and non-hybrid status, and plant genotype have been controlled to tease apart their effects on host phenotype and fitness in different environments. We conducted a greenhouse experiment where hybrid (H) and non-hybrid (NH) endophytes were inoculated into plant genotypes that were originally uninfected (E–) or once infected with either the H or NH endophytes. Nine endophyte and plant genotypic group combinations were grown under low and high water and nutrient treatments. Inoculation with the resident H endophyte enhanced growth and altered allocation to roots and shoots, but these effects were greatest in resource-rich environments, contrary to expectations. We found no evidence of co-adaptation between endophyte species and their associated host genotypes. However, naturally E– plants performed better when inoculated with the hybrid endophyte, suggesting these plants were derived from H infected lineages. Our results show complex interactions between endophyte species of hybrid and non-hybrid origin with their host plant genotypes and environmental factors.

Keywords: Abiotic stress | Endophyte | *Festuca arizonica* | Hybridization | Inoculation | Plant genotype

Article:

Introduction

Most, if not all, plants have symbiotic partnerships with microorganisms that may expand their realized niche and enable them to persist in otherwise marginal or inhospitable habitats or expand into novel ones. For example, mycorrhizal partnerships with ancient plants are thought to have facilitated the transition of aquatic plants to terrestrial habitats more than 400 million years ago (e.g., [1]). All modern plants appear to be associated with below and aboveground symbiotic non-pathogenic viruses, bacteria, and fungi that can alter host phenotypes, expand ecological realized niches, and alter fitness (e.g., [2]).

One group of symbiotic plant microorganisms that have garnered increasing attention is the endophytic fungi. Endophytic fungi are ubiquitous and diverse across plant species and are usually found in aboveground tissues but also in roots (e.g., [3]). Most of these endophytes are horizontally transmitted via spores and produce localized infections, with a wide range of effects on the host plant [3]. In contrast, cool season grasses in the subfamily Pooideae are often infected with clavicipitaceous endophytes in the genus *Epichloë* that are systemic, asexual, and vertically transmitted by hyphae growing into seeds (these anamorphic or asexual forms were formerly placed in the genus *Neotyphodium* [4]). Because of the tight linkage between host and endophyte reproduction, vertically transmitted *Epichloë* endophytes are thought to act more mutualistically than horizontally transmitted endophytes. 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., [3, 5]).

Whereas it has been well established that asexual *Epichloë* endophytes can radically alter host phenotype and increase fitness in some grasses, especially agronomic cultivars, accumulating evidence suggests that the strength and direction of asexual *Epichloë* endophyte interactions with their hosts in wild grasses are highly variable (e.g., [6, 7, 8]). There are three sources of variation that may change interaction outcomes: (1) endophyte strain or species, (2) host plant genotype, and (3) the local abiotic (e.g., soil nutrients and moisture) and biotic (e.g., the presence of herbivores and natural enemies of herbivores) environments. Recent molecular studies show remarkable genetic variation in *Epichloë* endophytes (e.g., [4]) across host grass species but also within a given grass species (e.g., [9]). Host phenotypic differences stemming from different endophyte strains may even be greater than that from infection itself (e.g., [10]). 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. About two thirds of asexual *Epichloë* endophytes across species are of hybrid origin [4, 11]. Hybridization probably occurs when sexual, haploid *Epichloë* endophytes co-occurring in the same plant fuse to produce asexual, heteroploid (incomplete polyploidy) *Epichloë* endophyte species [11]. Ecologically, these hybrids are thought to be fitter in a wider range of biotic and abiotic environments, like some plant hybrids (e.g., [12]), because they express traits from both parental species [13]. 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 [9, 14].

Because asexual *Epichloë* endophytes are thought to be largely transmitted maternally (e.g., [13, 15]), endophyte species and strains may have long ecological associations with specific plant maternal genotypes [9, 16]. At the phylogenetic level, certain *Epichloë* species appear to have co-evolved with their host grass species by common descent (e.g., [17]). At the ecological

level, high specificity between endophyte and plant genotype might be promoted when more compatible endophyte–plant genotype combinations are selected by varying environments after sexual recombination of the host plant [16, 18, 19]. Thus, a high degree of co-adaptation between endophyte and host plant genotype is expected for *Epichloë* endophytes, especially asexual ones that are vertically transmitted. Support for genetic compatibility between endophyte and host genotypes comes from inoculation experiments of fungal strains into a native grass [16] as well as the relatively poor success of moving novel endophytes from native grasses into cultivated grasses for better agronomic production and tolerance to stressful biotic and abiotic environments (e.g., [18]).

If endophyte strains and species show fidelity to specific plant genotypes, then disentangling the effects of endophyte and plant genotype under varying environments becomes particularly challenging. To study endophyte strain or species effects, most studies rely on removing the endophyte and then comparing performance of infected (E+) plants with their uninfected counterparts in different environments (e.g., [10]). But if different endophytic taxa, such as H and NH endophytes, are associated with certain plant genotypes, then this design cannot adequately test the effects of these associated plant genotypes. Alternatively, to study plant genotype effects, most studies have examined performance of various plant genotypes infected with the same endophyte strain (e.g., [20]). But this approach does not include different endophyte species or strains. Another way to separate the effects of endophyte genotype and associated plant genotypes is to inoculate the host genotypic groups from which endophytes had been removed with their resident and non-resident endophytes and then compare growth or reproductive performance under controlled environmental conditions [3, 9].

However, this approach has been limited because endophyte removal and then inoculation with various endophyte species or strains is technically challenging in native grasses. We know of only two studies where different endophyte types have been re-inoculated into different plant accessions of a native grass. Saikkonen et al. [16] manipulated grass–endophyte strain combination in a long-term garden experiment. They found that inoculation success, endophyte transmission to the next generation, and beneficial effects of the endophyte on host reproduction depended on endophyte and host genetic compatibility. Oberhofer et al. [9] inoculated seedlings from four populations of the woodland grass, *Hordeylmus europaeus*, that were rendered endophyte-free with hybrid and non-hybrid endophyte strains or left endophyte-free. They found that infection with either hybrid or non-hybrid endophytes increased growth, but each infection type had different effects on reproduction.

Unlike *Hordeylmus europaeus* and most other native grass species (e.g., [21]), Arizona fescue (*Festuca arizonica*) populations are dominated by plants with non-hybrid *Epichloë* infections or, to lesser extent, plants that are endophyte-free [22, 23]. Hosts infected with hybrid species prevail primarily in areas where soils have lower nutrients and water availability. Recent experiments show that hybrid infected plants (hereafter H+ plants) were better competitors than non-hybrid infected plants (hereafter NH+ plants) but only when water and nutrients were limiting, supporting the hypothesis that infection by hybrid *Epichloë* endophyte may expand ecological niches especially in marginal habitats [14]. However, these experiments did not use plant genotypic groups with endophytes removed and then inoculated with resident or alien H

and NH infections so that the effects of infection, endophyte type, and plant genotype associated with specific endophytes could be controlled.

To test the relative roles of endophyte infection, hybridization, and plant genotypes associated with specific endophyte on host grass performance in varying environments, we performed a greenhouse experiment with plant genotypic groups inoculated with their resident or alien endophyte or remaining endophyte-free (a total of nine different endophyte-associated plant genotype combinations). We then measured various growth parameters as well as relative allocation to roots and shoots for plants grown in resource-poor (low water and low soil nutrients) or resource-rich (high water and high soil nutrient) conditions. We specifically asked (1) if re-infection with the resident endophyte improves plant performance, (2) whether endophyte species or associated plant genotypes or their interactions drive plant responses to variable environments, and (3) if co-adaptation occurs between combinations of endophyte species and their associated host genotypic groups.

Materials and methods

Arizona fescue and *Epichloë* species

Arizona fescue (*Festuca arizonica* Vasey), in the subfamily Pooideae, is a dense, perennial bunchgrass that reproduces by seed allogamously and is native and widespread in the southwestern USA and in northern Mexico [24]. Arizona fescue grows in semiarid ponderosa pine–bunchgrass communities above 2000-m elevation [24], where soils are low in nutrients and seasonal and yearly droughts are common [5]. Arizona fescue is frequently infected by either a non-hybrid (NH) or hybrid (H) endophyte. The non-hybrid endophyte is *Epichloë typhina* subsp. *poae* var. *huerfana* (formerly *Neotyphodium huerfanum*, [4]). Hereafter, we refer to this endophyte taxon as NH (for non-hybrid). The hybrid endophyte (hereafter, H) is *Epichloë tembladera* (formerly *Neotyphodium tembladera*—asexual *Epichloë* were formerly placed in the genus *Neotyphodium* but were recently absorbed into the genus *Epichloë* [4]). The hybrid endophyte in Arizona fescue has resulted from hybridization between co-occurring *Epichloë typhina* and *E. festucae* endophytes [21]. *E. tembladera* is found across host grass species and across continents suggesting multiple and independent hybridization events between *E. typhina* and *E. festucae* [25]. However, in 30 years 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 [22]. Both endophytes are asexual, vertically transmitted, and obligate symbionts (no free-living stages), but their hosts remain facultative as endophyte-free (hereafter E– plants) plants are found in nature.

Inoculation experiment

To test the roles of H and NH endophyte and their associated plant genotypes on host grass performance in resource-poor and resource-rich environments, we inoculated seedlings of different genotypic origins (half-sib families) with the H or NH endophyte. Seeds were collected from plants that were originally infected with the H or NH endophyte or were naturally endophyte-free (E–). The H and E– plants were collected from a study site in Clints Well, Arizona, USA, whereas the NH plants were collected from a nearby study site in Merritt Draw,

Arizona, USA. In 2009, some of the seeds from the H⁺ and NH⁺ plants were heat-treated to remove the resident endophyte, thus becoming H⁻ and NH⁻ seeds. Seedlings were grown from these H⁻, NH⁻, and E⁻ seeds in 2009 and then germinated in pots and planted in a field plot at The Arboretum of Flagstaff in 2010. Subsequently, seeds used in this experiment were collected from multiple individuals of these three plant types in 2013. Thus, all plants from whence seeds were derived in this experiment were several years removed from any extraneous effects of experimental endophyte removal or transplanting.

E⁻ plant maternal genotypes have unknown origin in terms of infection. They may have originated from plant accessions that have never been infected by either endophyte species. Alternatively, they may have once harbored the H or NH endophyte or some other *Epichloë* species (unlikely, since no other *Epichloë* species has been discovered in Arizona fescue) and subsequently lost the H or NH endophyte. Systemic endophytes can be “lost” either by imperfect transmission where hyphae fail to grow into seeds [26] or randomly lost from the seed, seedling, or adult stage by environmental factors such as excessive heat [27].

At least five maternal plants of each plant category (H⁻, NH⁻, and E⁻) were used as seed sources to randomized variation among individual plants within a given plant category. Twenty seeds from each of the five maternal plants (half-sib families) were used in each group (H⁻, NH⁻, E⁻). From each group of 100 seeds from each maternal plant, a sample of 10 seeds were stained (Rose Bengal solution containing 5 % NaOH for 48 h) and examined microscopically for the presence of fungal hyphae to confirm their endophyte-free status before inoculation.

It is important to note that we are testing the effect of half-sib families of the host grass that are associated with each original infection category (H, NH, and E⁻) and not the effect of specific plant genotypes. While there are limitations to this approach, it has been used effectively to test the relative effects of infection and infection type and the plant lineages associated with them (e.g., [28, 29]). Because Arizona fescue, like many pooid grasses harboring endophytes, are allogamous, at each generation there is a paternal contribution to the grass genotype, which may (e.g., [30]) or may not (e.g., [31]) destabilize the host–endophyte mutualism. If the effects of paternal genetic contribution or other random factors such as occasional horizontal transmission (e.g., [15]) overwhelm plant maternal lineages that are associated with either the H or NH endophyte, then we would expect no differences in growth parameters among the categories of plants without their endophytes (H⁻, NH⁻, and E⁻). If, however, these plant categories differ in host growth measures in controlled environments, then this result would suggest that plant lineages associated with the H or NH endophyte or E⁻ plant groups are genetically distinct.

Seedlings of the three plant genotypic groups (H⁻, NH⁻, E⁻) that were originally infected with the resident endophyte (H or NH) or natural endophyte-free (E⁻) were then inoculated with H or NH endophytes, or left endophyte-free. Inoculations resulted in nine plant genotype and endophyte combinations (H⁻ × H⁺, H⁻ × NH⁺, H⁻ × E⁻, NH⁻ × H⁺, NH⁻ × NH⁺, NH⁻ × E⁻, E⁻ × H⁺, E⁻ × NH⁺, E⁻ × E⁻). For example, H⁻ × H⁺ represented seedlings that were originally infected by the hybrid endophyte, had the endophyte removed, and then inoculated with their resident hybrid endophyte. NH⁻ × H⁺ represented seedlings that were originally infected by the non-hybrid endophyte, had the endophyte removed, and then inoculated with the non-resident hybrid endophyte.

To inoculate seedlings, lemma and palea were peeled from seeds and seeds were then surface-sterilized. Seeds were germinated on potato dextrose agar (PDA) plates in a growth chamber at 22 °C with 12/12 h for day and night cycle. Several plates with fresh fungal mycelia of each endophyte type (ground with a pestil in sterile water and spread on PDA surface 4 days prior to inoculations) were used as inoculum. Seedlings (5–7-mm tall) in each plant genotype group were inoculated with hybrid mycelium, and other seedlings were inoculated with non-hybrid mycelium via insertion into a vertical slit at a shoot primordial zone under sterile conditions [9, 32]. Each inoculation was performed with a new, sterile hypodermic needle under a laminar flow hood with a dissecting scope by puncturing the seedling and inserting a portion of mycelium into the wound with great care not to break the fragile stem. Inoculated seedlings were kept for a minimum of 1 week on the agar plate before being planted into soil. Infection status of seedlings was tested by using Phytoscreen Immunoblot Kit (Agrinostics, GA, USA). The plants in each category that remained uninfected after testing were used as H⁻, NH⁻, and E⁻ plants. Note that these plants underwent the same wounding treatments and transplantation as successfully inoculated plants.

Greenhouse experiment

The nine combinations of plant genotype and endophyte species combinations (H⁻ × H⁺, H⁻ × NH⁺, H⁻ × E⁻, NH⁻ × H⁺, NH⁻ × NH⁺, NH⁻ × E⁻, E⁻ × H⁺, E⁻ × NH⁺, E⁻ × E⁻) were planted in pots with potting soil and grown in a greenhouse in natural light at 24 °C beginning in May 2013. We cloned inoculated and endophyte-free plants by separating three tillers per clone and planting individually in 3-dl pots with Metro Mix 360 Sun Gro Horticulture Canada Ltd. soil mixture in October 2013. After cloning, all plants were clipped to the same height (10 cm). Three weeks after clipping, similar size plants were selected for the experiment. The greenhouse was set to 20 °C night/25 °C day temperature conditions with natural lighting.

Water and nutrient treatments started in November 2013. Each combination of plant genotypic groups and endophyte species was randomly grown under two treatments (high nutrients and high water; low nutrients and low water) with the target of 10 plants per endophyte/plant genotype combination and treatment (180). We combined water and nutrients into single treatments to parallel a long-term field experiment (Saari et al. unpublished data) also testing the effects of H and NH endophytes but without inoculations. Some plants did not survive inoculation and cloning, and the final number of plants in the experiment was 151. Pots assigned to high- and low-nutrient treatments were fertilized with a fertilizer [20:20:20 (N/P/K), with micronutrients] (Southern Agricultural Insecticides, Inc.) twice a month or once in 4 months, respectively. Pots were watered twice a week so that plants in the high water treatment conditions received 2× water as those in the low water treatment. These conditions of watering and fertilization for Arizona fescue are known from previous studies to achieve distinct differences in growth in the greenhouse and accurately simulated high and low resource conditions, respectively, in the field (e.g., [14]). Pot location was randomized each week to prevent any microclimate differences in growth. After 4 months, we recorded number of tillers and plant height. All plants were then harvested and their roots were washed with water. After all plants were dried at 65 °C, aboveground and belowground dry biomass for each plant was measured. To verify the infection status of the plants at the end of the experiment and before

harvesting, an immunoblot assay with specific monoclonal antibodies (Phytoscreen Immunoblot Kit no. ENDO7973; Agrostics, Watkinsville, GA, USA) was used to confirm endophyte status for each plant.

Statistical analysis

To test the effect of infection by the resident endophyte on its respective host genotypic group, we used ANOVA (Systat 13.0) with infection and treatment as independent variables to test their effect on the various growth measurements. Because root/shoot allocation is ratio, we arcsine square root-transformed this variable before analysis. We analyzed the effect of the NH and H endophyte on their respective plant genotypic groups separately since we are interested here in only how the resident endophyte affects host growth when reinstated in its associated host plant genotypic group. All assumptions of normality and homogeneity of variances were tested and met.

We also used ANOVA to test the effect of associated plant genotype, endophyte species, and treatment and their interactions on the various growth parameters. Here, we analyzed only infected plants ($H^- \times H^+$, $H^- \times NH^+$, $NH^- \times NH^+$, $NH^- \times H^+$, $E^- \times H^+$, $E^- \times NH^+$) because we are interested in testing the effect of the two endophyte species in their resident host plant genotypic groups and in the two other host plant genotypic groups to determine if endophytes are co-adapted to their resident plant genotypic groups. All assumptions of normality and homogeneity of variances were tested and met. Because root/shoot allocation is a ratio, we arcsine square root-transformed this variable before analysis.

Because we are interested in the relative roles of endophyte species and associated plant genotypes, we show plant growth responses separately for endophyte species and for associated plant genotypes. We used Tukey's HSD for post hoc comparisons among means for endophyte species and associated plant genotypes within each treatment.

Results

Resident infection effects

Inoculation of the hybrid endophyte into plants (H^-) that originally harbored this endophyte increased shoot and total dry biomass but not plant height, number of tillers, or root dry biomass (Table 1, Fig. 1). The increase in shoot biomass is also reflected in a decreased allocation to roots (lower root/shoot ratio) for $H^- \times H^+$ plants. However, in contrast to the prevailing notion that hybrid endophytes should have their greatest effect in stressful, resource limited environments, inoculation of the resident hybrid endophyte had its largest positive effect on shoot and total biomass and root/shoot ratio in the high soil nutrient and water treatment (significant infection \times treatment interactions, Table 1, Fig. 1). As expected, higher soil nutrients and water increased all growth parameters, regardless of infection status or type.

Table 1. Analysis of variance results for the effect of infection and water and nutrient treatments for non-hybrid and hybrid endophytes in *Festuca arizonica*

	df	Plant height		Tiller number		Shoot dry biomass		Root dry biomass		Total dry biomass		Root/shoot	
		F	Pvalue	F	Pvalue	F	Pvalue	F	Pvalue	F	Pvalue	F	Pvalue
Non-hybrid endophyte													
Infection (I)	1	1.259	0.267	9.978	<0.01	7.338	<0.01	0.898	0.348	3.869	0.055	1.347	0.252
Treatment (T)	1	33.157	<0.01	316.432	<0.01	1205.41	<0.01	99.119	<0.01	567.059	<0.01	106.384	<0.01
I × T	1	1.024	0.317	2.833	0.099	2.276	0.138	0.278	0.600	1.200	0.279	0.834	0.366
Error	49												
Hybrid endophyte													
Infection (I)	1	0.526	0.473	1.275	0.266	20.291	<0.01	0.103	0.750	7.981	<0.01	29.264	<0.01
Treatment (T)	1	5.821	0.021	143.790	<0.01	203.070	<0.01	51.723	<0.01	158.594	<0.01	48.901	<0.01
I × T	1	0.346	0.560	0.754	0.391	6.660	0.014	3.084	0.087	6.499	0.015	16.748	<0.01
Error	37												

Significant ($p < 0.05$) p values are in bold

Table 2. Analysis of variance results for the effect of endophyte species, host plant genotypic group, and water and nutrient treatments for infected plants of Arizona fescue

	df	Plant height		Tiller number		Shoot dry biomass		Root dry biomass		Total dry biomass		Root: Shoot	
		F	Pvalue	F	Pvalue	F	Pvalue	F	Pvalue	F	Pvalue	F	P
Endophyte species (E)	1	2.277	0.135	4.937	0.029	10.474	<0.01	1.736	0.191	6.821	0.011	2.936	0.090
Plant genotypic group (P)	2	24.549	<0.01	22.989	<0.01	0.230	0.795	7.103	<0.01	2.587	0.081	0.517	0.598
Treatment (T)	1	27.337	<0.01	499.491	<0.01	522.880	<0.01	203.158	<0.01	456.238	<0.01	89.980	<0.01
E × P	2	10.525	<0.01	15.371	<0.01	2.010	0.141	15.186	<0.01	6.635	<0.01	13.790	<0.01
E × T	1	0.144	0.706	0.973	0.327	3.084	0.083	1.142	0.228	2.552	0.114	2.150	0.146
P × T	2	0.335	0.716	11.004	<0.01	0.609	0.546	4.379	0.016	0.694	0.502	6.893	<0.01
E × P × T	2	0.344	0.710	6.949	<0.01	1.885	0.158	3.915	0.024	2.552	0.084	1.335	0.269
Error	82												

Significant ($p < 0.05$) p values are in bold

There were no clear patterns of the increased benefits to host genotypic groups via inoculation with their resident endophyte. For example, infection of the H plant genotypic group with its resident H endophyte did not increase number of tillers, shoot, root, or total dry biomass compared to the H plant genotypic group inoculated with the non-resident NH endophyte ($H^- \times NH^+$). Indeed, the $H^- \times NH^+$ combination had greater plant height (Fig. 2a) and higher root biomass than the $H^- \times H^+$ combination in the high water, high nutrient treatment (Fig. 2d). Likewise, inoculation of the NH genotypic group with the resident NH endophyte did not generally improve host performance relative to the NH genotypic group infected with the non-resident H endophyte ($NH^- \times H^+$). All growth parameters in both treatments were equivalent for $NH^- \times NH^+$ and $NH^- \times H^+$ combinations except for fewer number of tillers for the non-resident endophyte ($NH^- \times H^+$) compared to the resident endophyte $NH^- \times NH^+$ (Fig. 2b).

When E^- plants that were naturally endophyte-free (E^-) were inoculated with the H endophyte, plants generally performed better than E^- plants inoculated with the NH endophyte (Fig. 2). In the low water, low nutrient treatment, $E^- \times H^+$ plants had more tillers and greater root and total biomass than $E^- \times NH^+$ plants (Fig. 2b–e). Similarly, in the high water, high nutrient treatment,

$E^- \times H^+$ plants had more tillers and greater root and total biomass than $E^- \times NH^+$ plants (Fig. 2b–e).

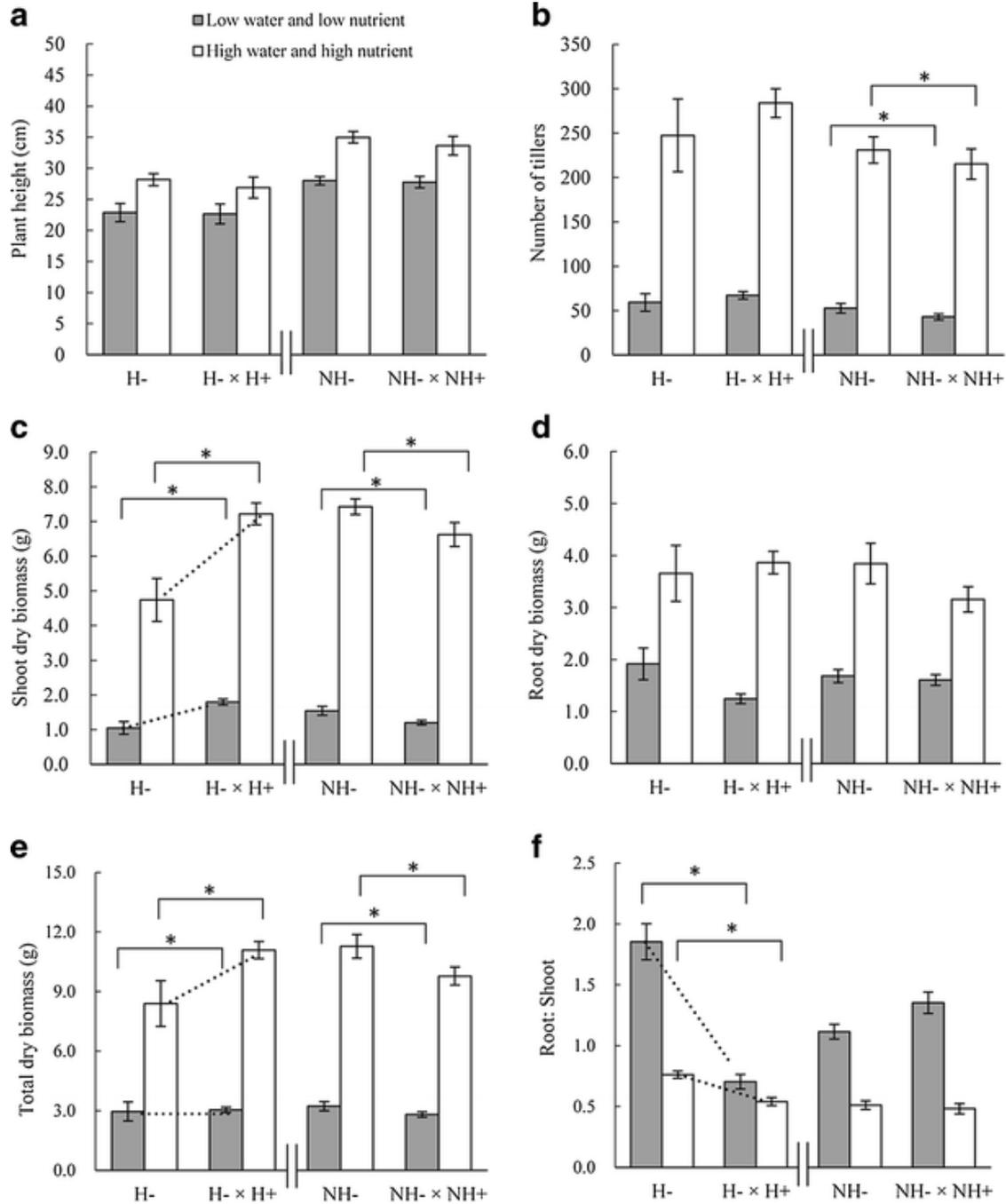


Figure 1. Means (\pm SE) of growth parameters **a** plant height; **b** number of tillers; **c** shoot dry biomass; **d** root dry biomass; **e** total dry biomass; **f** root/shoot ratio for plants with their resident endophyte removed (H^- ; NH^-) and inoculated ($H^- \times H^+$; $NH^- \times NH^+$) in the two treatments. Asterisks above columns indicate significant differences ($p < 0.05$) in growth parameters within a treatment between the endophyte-free plant genotypic group and the same genotypic group with the resident endophyte inoculated. Dotted lines between bars indicate a significant interaction between endophyte infection and treatment within a given associated plant genotype

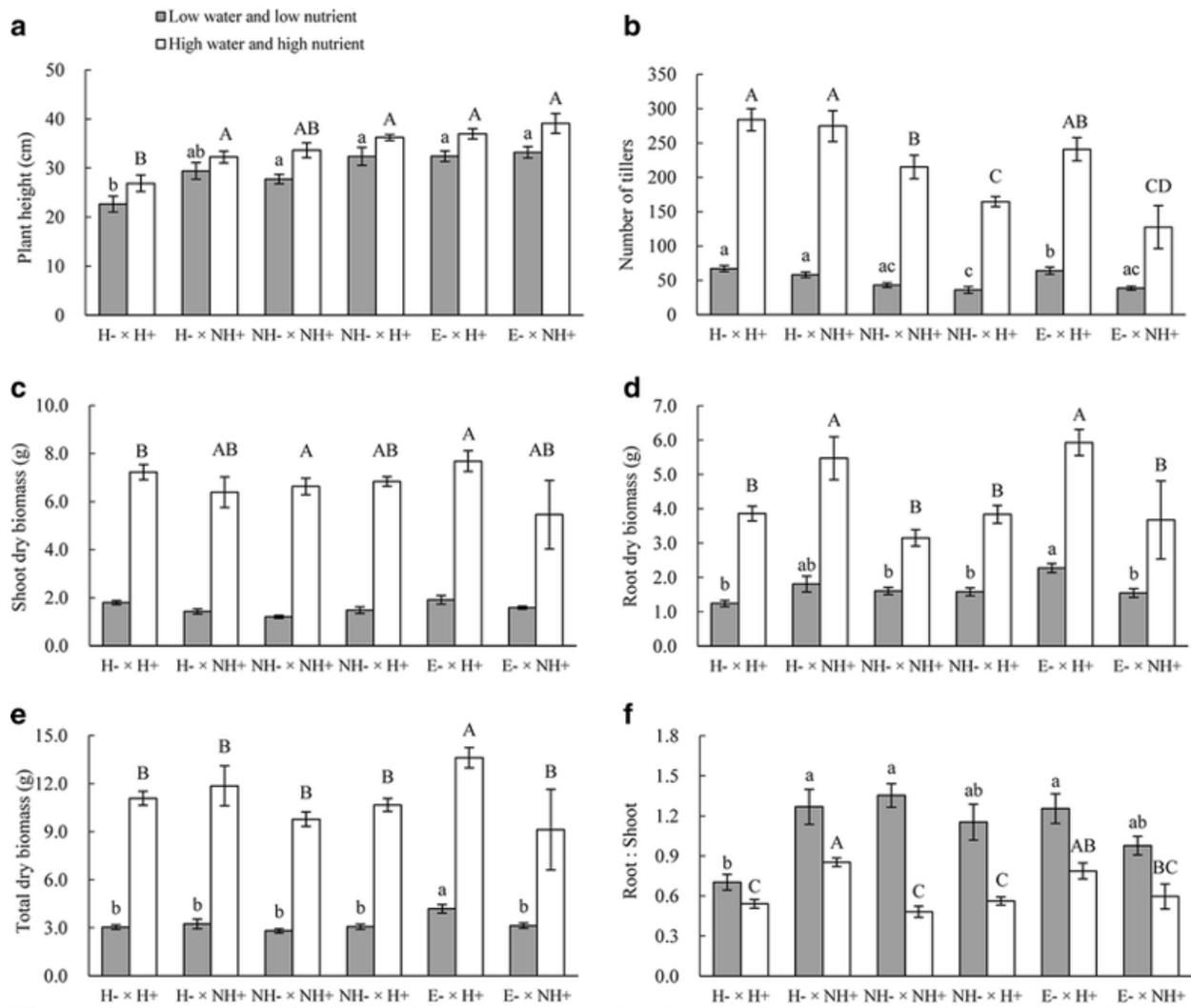


Figure 2. Means (\pm SE) of growth parameters **a** plant height; **b** number of tillers; **c** shoot dry biomass; **d** root dry biomass; **e** total dry biomass; and **f** root/shoot ratio for plants inoculated with their resident and with the non-resident endophyte in the two treatments. *Different letters* above columns indicate significance differences (Tukey HSD test for multiple comparisons) among infected plants with different endophyte species for each treatment (*small letters* for pairwise comparisons in the low water, low nutrient treatment; *capital letters* for the high water, high nutrient treatment)

Associated plant genotypic effects

Plant genotypic group affected plant height, number of tillers, and root dry biomass and marginally affected total dry biomass (Table 2). When plant genotypic group effects are examined separately from the effect of endophyte, there are differences among the three genotypic groups in their response to the two treatments (Fig. 3). The H⁻ plant genotypic group showed less height and shoot dry biomass than either the E⁻ or NH⁻ plant genotypic groups in the low water, low nutrient treatment (Fig. 3a, c). H⁻-associated plant genotypes also had less

plant height and shoot biomass than E⁻- and NH⁻-associated genotypes in the high water, high nutrient treatment (Fig. 3a, c) and less total biomass (Fig. 3e) than the E⁻ genotype. Alternatively, H⁻-associated plant genotypes had higher root/shoot ratio than E⁻ and NH⁻ plants in the low water, low nutrient treatment and H⁻ had higher root-shoot ratio than NH⁻ plants in the high water, high nutrient treatment (Fig. 3f).

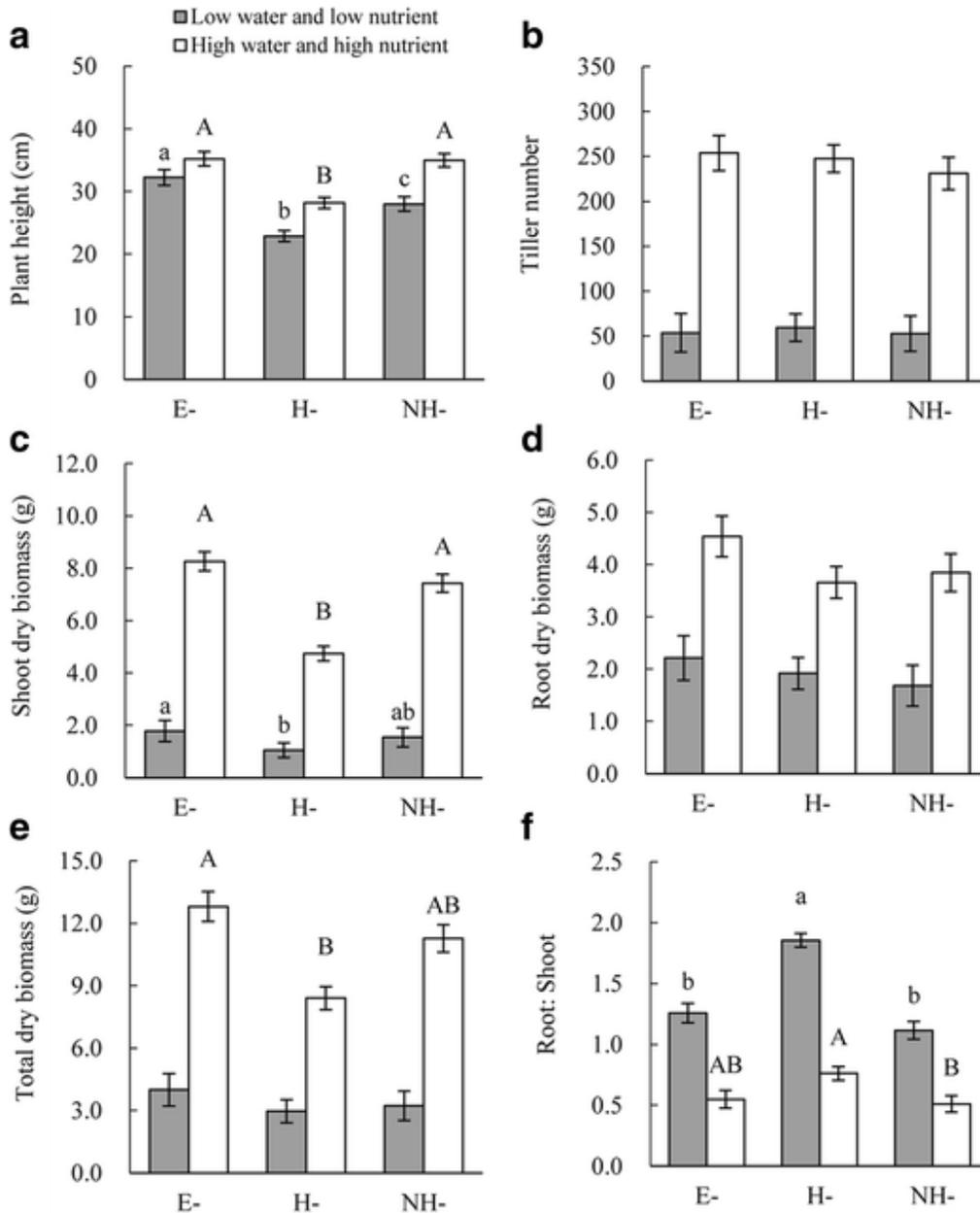


Figure 3. Means (\pm SE) of growth parameters **a** plant height; **b** number of tillers; **c** shoot dry biomass; **d** root dry biomass; **e** total dry biomass; and **f** root/shoot ratio for the three associated plant genotypes (E⁻, H⁻, NH⁻) without their endophytes in the two treatments. *Different letters* above columns indicate significance differences (Tukey HSD test for multiple comparisons) among associated plant genotypes for each treatment (*small letters* for pairwise comparisons in the low water, low nutrient treatment; *capital letters* for the high water, high nutrient treatment)

Discussion

Infections by *Epichloë* endophytes are well known for profoundly changing host phenotypes of agronomic and wild grasses such that growth, reproduction, and survival are often enhanced. Often, these alterations in host phenotype lead to positive effects on host fitness [33, 34, 35]. This is especially thought to be the case for asexual, vertically transmitted *Epichloë* endophytes (formerly *Neotyphodium* endophytes [4]) because vertical transmission implies strong mutualistic interactions (e.g., [13, 27]). But there is growing realization that the effects of infection by *Epichloë* endophytes are contingent upon variation in endophyte species or strain, host plant genotype, and biotic and abiotic environmental factors (e.g., [3, 10, 36]), similar to other well-studied plant–microbe symbioses (e.g., [37, 38]). In some cases, especially for wild grass populations, infection by asexual endophytes may even lead to detrimental effects on host performance and hence fitness (e.g., [27]).

Effects of infection by hybrid and non-hybrid endophytes

Our results indicate that returning the resident endophyte to the plant genotypic groups that once harbored the endophyte does not necessarily enhance performance and depends on endophyte species and environmental conditions. Inoculating the hybrid endophyte into plant genotypes originally associated with the H endophyte resulted in enhanced aboveground growth and total biomass, suggesting a positive effect of this endophyte species on growth and performance. This endophyte also shifted allocation of host growth to shoot biomass relative to root biomass. In contrast, inoculating the non-hybrid endophyte into plant genotypes originally associated with the NH endophyte either did not affect growth parameters or did so in a negative fashion for number of tillers and shoot and total biomass. This negative effect of the NH endophyte is consistent with previous studies that show infection by this endophyte often leads to reduced growth and reproduction [27] and decreased competitive abilities [39]. Therefore, it remains puzzling why infection by the NH endophyte is much more common in natural populations of Arizona fescue than either hybrid infected or uninfected grasses [22]. We discuss possible explanations below (see “Frequency of Hybrid and Non-hybrid Endophytes in Nature” section below).

We also did not find support for the hypothesis that hybridization in *Epichloë* endophytes enhances their host grass abilities to grow and survive in stressful or harsh environments [11, 40] and thus expand their realized niche [9, 41]. Inoculation of the H endophyte into the plant genotypes associated with the H endophyte did enhance shoot and dry biomass, but this effect was significantly more pronounced in the high water, high nutrient than the low water, low nutrient treatment (Table 1, Fig. 1 c, e). The H endophyte did, however, have a stronger effect on reducing root/shoot ratio in the low resource compared to the high resource environment (Fig. 2f), but it is not clear if reduced allocation to root growth would be advantageous in harsh environments. To the contrary, usually plants in stressful environments allocate more to roots in order to increase competitive abilities to uptake scarce resources [5]. In competition experiments, Saari and Faeth [14] found that H infected plants outcompeted their H– counterparts but NH infected plants did not outcompete NH– plants, consistent with our results that infection by the H endophyte, but not the NH endophyte, improves plant growth. However, unlike our results, they

found that H⁺ plants outcompeted NH⁺ plants and E⁻ grasses based on some growth measures (but not others) but only when water and nutrients were limited. However, their study involved competition whereas our study was competition-free. Saari and Faeth [14] also did not control plant genotypic groups by inoculation, which may explain differences in outcomes. In a study involving another wild host grass, *Hordeylmus europaeus*, Oberhofer et al. [9] inoculated seedlings from four populations that were made endophyte-free with different hybrid and non-hybrid endophyte taxa. They found that infection with either hybrid or non-hybrid endophytes generally increased growth, but endophyte type had varying effects on reproduction. They also did not find support that the hybrid endophyte increased host performance over wider range of environments, purportedly by virtue of additional genes acquired during hybridization [11]. Therefore, at least for these two grass species where inoculation experiments have been performed and plant genotypic group has been examined, the niche expansion hypothesis via hybridization of *Epichloë* symbiosis does not seem to be supported.

Co-adaptation of endophyte and host plant

Because asexual *Epichloë* endophytes are thought to be strictly vertically and transmitted via seeds (but see [15]), we expect a high degree of fidelity and co-adaptation between endophyte strain or species and plant maternal genotype (e.g., [3, 9, 16, 19]). For infected plants, it is clear that host performance depends on endophyte species, plant genotypic group, and environmental factors and the complex two- and three-way interactions among them. However, our results do not show that the resident H or NH endophyte provides any growth advantage over the non-resident endophyte as would be expected if the maternal plant genotype and endophyte species are co-adapted or lineages co-evolved (e.g., [17]) (Fig. 2). Infection by the resident H endophyte did not enhance, and may have instead reduced, growth compared to infection with the non-resident NH endophyte in the H plant genotypic group. Similarly, the resident NH endophyte did not generally improve host performance relative to the non-resident H endophyte in the NH plant genotypic group. Both endophyte species are also compatible and viable in their non-resident plant genotypic group, as well as in naturally E⁻ plants, further indicating a lack of co-adaptation of endophyte species and plant genotype.

Oberhofer et al. [9] also found evidence for compatibility of endophyte types across plant accessions but only weak co-adaptation of hybrid and non-hybrid endophytes to their respective host plant genotypes in wild populations of *Hordeylmus europaeus*. The two endophytes that showed most compatibility by somewhat improving growth of their resident host genotypes were non-hybrid endophytes, one of which is capable of sexual reproduction. They suggested that the lack of co-adaptation indicates relatively recent colonization events of the host grass and its endophytes in the geographical range of the grass [42]. In contrast, for Arizona fescue, the NH endophyte provided the least benefit to its respective resident host genotypic group. Neither the NH nor the H endophyte in Arizona has been observed to have sexually reproduced [23]. Thus, the lack of co-adaptation is even more puzzling. One explanation, suggested by Faeth and Sullivan [27] and Oberhofer et al. [43], is that many of the non-hybrid, and even the hybrid, *Epichloë* endophytes that were traditionally viewed as strictly vertically transmitted may be capable of horizontal transmission via hyphae or other propagules such as conidia and spores (e.g., [15, 44]). Although such transmission has not been observed in nature for Arizona fescue, the ability to experimentally inoculate non-resident endophytes and the compatibility of

endophyte species across plant genotypic groups, plus the lack of specificity in benefits between resident endophytes and plant genotypes, points to contagious spread of *Epichloë* endophytes, even those considered as strictly vertically transmitted. *E. tembladerae*, the hybrid endophyte found in *Festuca arizonica*, is for example known to occur in at least another 19 different plant species on the South American continent [21] which may suggest the presence of a horizontal transmission pathway at least at some point in evolutionary history. Alternatively, the wide distribution of *E. tembladerae* may have also resulted from multiple and independent hybridization events between *E. typhina* and *E. festucae*, parental species that are widespread geographically among host grass species [25].

Frequency of Hybrid and Non-hybrid Endophytes in Nature

The frequency of asexual *Epichloë* endophyte infections in natural and agronomic grass populations has often been used to infer relative fitness advantages of harboring the endophytes (e.g., [45, 46]). Higher frequencies of infection were thought to be reflective of greater fitness advantages over uninfected conspecific hosts. Likewise, within and across grass species, observed higher frequencies of vertically transmitted hybrid relative to non-hybrid infections stimulated the hypothesis that hybrid endophytes increased fitness more so than non-hybrid endophytes [11, 40]. Yet, accumulating evidence suggests that for Arizona fescue as well as for some other wild grasses, non-hybrid and hybrid infection frequency does not match fitness measures. For Arizona fescue, infection frequencies are 55 % NH infected, 15 % H infected, and 30 % uninfected (E⁻) individuals on average across populations [22, 23]. These frequencies do not correspond at all to our experimental results and to previous studies (e.g., [27]), where the NH endophyte is less beneficial, and apparently, even harmful, compared to the H endophyte. Furthermore, the H infected grasses are more commonly found in the most stressful environments (low soil moisture and nutrients), but our experimental results suggest H infected grasses should grow best in more resource-rich environments. Oberhofer et al. [9] also found a mismatch between the observed hybrid and non-hybrid endophyte infection frequencies and distribution and their experimental results measuring relative fitness advantages. Our results, as well as those of Oberhofer et al. [9] suggest that relative frequency and distribution among habitats cannot be readily used to gauge the relative advantage of H⁺, NH⁺, and E⁻ plants. Lack of correspondence between frequency, distribution, and fitness for asexual *Epichloë* endophyte infections can result from variation in transmission rates [27, 47, 48], metapopulation dynamics [49], weak or transient selection [50], or as discussed above, occasional horizontal transmission.

Another explanation for the relative low frequency of H infected grasses in natural populations, despite their better growth, at least in some environments, than NH⁺ plants is that H⁺ grasses more readily lose their endophyte than NH⁺ grasses. Systemic endophytes can be lost in several ways from infected hosts: (1) via imperfect transmission where hyphae fail to grow into seeds (e.g., [51]), (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 [3]. That E⁻ plants benefitted more from inoculation by the hybrid than non-hybrid endophyte in terms of root and total dry biomass (Fig. 2d, e) suggests that the E⁻ plant genotypic group may have originally been infected by the hybrid endophyte. The E⁻ plants were originally from the same grass population with the H endophyte, and thus they might have a long co-evolutionary history. However, countering this argument is that H⁻ and E⁻ plants in our experiment appear to be less similar to

each other in growth parameters than E⁻ and NH⁻ plants (Fig. 2). A comparison of transmission rates of hybrid and non-hybrid endophytes might shed additional light on whether H⁻ plants are more likely than NH⁻ plants to lose their endophytes.

In conclusion, whereas we found that growth parameters in Arizona fescue depend on endophyte species, host plant genotypic groups, environmental factors, and the complex interactions among them, we do not find support that hybridization of endophytes leads to fitness advantages of the host in stressful environments. To the contrary, infection by hybrid endophytes appears to increase performance only in resource-rich environments. We also did not find support for co-adaptation between endophyte species and host genotype as expected for asexual, vertically transmitted symbionts. These results suggest that the linkage between supposedly asexual endophyte species and their host grass genotypes may be much more fluid than previously thought.

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