Hybridization of *Neotyphodium* endophytes enhances competitive ability of the host grass

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Summary

• Associations with microbial symbionts may lead to niche differentiation of their host. Vertically transmitted *Neotyphodium* endophytes of grasses often hybridize in nature. Infection by these hybrid symbionts may result in different host–plant phenotypes from those caused as a result of infection by nonhybrid symbionts. Observations of wild Arizona fescue (*Festuca arizonica*) populations show that hybrid *Neotyphodium*-infected (H+) grasses dominate in resource-poor environments, whereas nonhybrid endophyte-infected (NH+) grasses dominate in environments with more resources. We studied the hypothesis that hybridization of endophytes increases stress tolerance of the host.

• To test whether hybridization of *Neotyphodium* affects performance and competitive abilities of the host depending on resources, we conducted a glasshouse experiment where competition, nutrients and watering were manipulated.

• H+ plants had greater wet biomass than NH+ and endophyte-free plants, when grown in competition, but only in low-water and low-nutrient treatments. By contrast, NH+ plants did not perform better than H+ or endophyte-free plants regardless of the treatment combination.

• Our results suggest that hybridization of symbiotic *Neotyphodium* endophytes may increase competitive potential of the host in stressful environments and that this hybridization may be underlying niche expansion of Arizona fescue in the environments with low resources.

Introduction

Niche differentiation is the process by which natural selection drives competing species or individuals into different patterns of resource use (Hutchinson, 1957; MacArthur & Levins, 1964). Most research has focused on niche reduction caused by negative species interactions, but the effects of positive species interactions on niche differentiation have received considerably less attention (Bruno *et al.*, 2003; Warren *et al.*, 2011). By contrast to competition and predation, positive interactions, such as mutualism, can expand the realized ecological niche of a species by conferring benefits such as increased tolerance to abiotic and biotic stresses (Bruno *et al.*, 2003; Afkhami & Strauss, 2011). Partner-generated niche shifts could also lead to niche differentiation within a species, if individuals that associate with partners have different niches from those that do not (Afkhami & Strauss, 2011).

One potential source causing niche shifts in plant populations are microbial symbionts living asymptomatically within tissues of the host plant. These symbionts, called endophytes, are known to alter host phenotypes (Saikkonen *et al.*, 2006, 2010). The variation from microbial symbiosis may arise from different sources. First, differential fitness, imperfect transmission (e.g. sensu Ravel *et al.*, 1997) and migration can create populations with mixed infection frequencies, with part of the population carrying the symbiont while other individuals remain uninfected (Cheplick & Cho, 2003; Cheplick, 2004; Faeth, 2009). Secondly, within the infected part of plant populations, plants may be infected by various genetic strains of the symbionts that differentially alter host phenotypes. For example, symbionts such as asymptomatic, strictly vertically transmitted *Neotyphodium* grass endophytes may hybridize (Selosse & Schardl, 2007), and infection by these hybrid symbionts may result in different plant phenotypes from those caused as a result of infections by nonhybrid symbionts (Hamilton *et al.*, 2009).

Most grass populations are mixtures of uninfected grasses and grasses infected with endophytes (see, e.g., Lewis *et al.*, 1997; Saikkonen *et al.*, 2000; Wali *et al.*, 2007; Cheplick & Faeth, 2009). In some grass species, such as Arizona fescue (*Festuca arizonica*), the infecting endophytes are often a mixture of hybrid (H+) and nonhybrid (NH+) endophytes (Sullivan & Faeth, 2008; Hamilton *et al.*, 2009). About two-thirds of infections in cool season grasses are of hybrid origin (Schardl & Craven, 2003). It has been suggested that hybridization provides an infusion of genetic variation that renders the host plant more tolerant of abiotic and biotic stresses (Schardl & Craven, 2003). However, this hypothesis remains largely untested.

Contrary to the general dominance of H+ endophytes in most grass species, NH+ endophytes dominate most of Arizona fescue populations. On average, Arizona fescue populations consist of 55% NH+, 15% H+ and 30% uninfected (E−) grass individuals (Sullivan & Faeth, 2008; Hamilton *et al.*, 2009). A possible explanation for the observed frequencies of endophtye infections in Arizona fescue is that H+, NH+ and E− grasses respond differently to varying environmental factors. There is some observational support of this hypothesis. H+ plants are more common in habitats with low nutrients and moisture, whereas NH+ plants are more prevalent in the areas with higher soil nutrients and moisture (Sullivan & Faeth, 2008; Hamilton *et al.*, 2009).

Sullivan & Faeth (2008) found that H+ hosts produce higher volume : mass ratios than NH+ hosts in moisture- and nutrient-poor habitats, but not in habitats with plentiful resources. They suggested that this change in plant architecture by H+ plants may be a response to plant density, as H+ plants are typically located under dense tree canopy and likely experience greater intra- and interspecific competition for resources than NH+ plants in less stressful environments. In addition, Hamilton *et al.* (2010) found that hybrid endophytes increase survival of grass hosts in stressful habitats and concluded that infection by H+ endophytes may increase the fitness of the plants in habitats with scarce resources.

We tested the effects of hybridization of *Neotyphodium* endophytes on the growth and performance of Arizona fescue with and without competition under varying amounts of water and nutrients. To study performance of plant and plant–endophyte combinations found in the natural populations, NH+, H+ and E− plants were compared. To separate the effects of endophyte infections from plant responses, we also compared plants infected with endophytes (H+ and NH+) with those whose endophyte had been experimentally removed (H− and NH−). Based on the hypothesis by Schardl & Craven (2003) and the past research (Sullivan & Faeth, 2008; Hamilton *et al.*, 2010), we expected that H+ plants perform better than H−, NH+ and E− plants when water and nutrients are scarce and the plants are competing, and that NH+ plants perform better than NH−, H+ and E− plants when there is no competition and water and nutrients are abundantly available.

Materials and Methods

Arizona fescue and *Neotyphodium*

Arizona fescue (*Festuca arizonica* Vasey) is a dominant, perennial native grass in the southwestern USA and is frequently infected by H+ and NH+ *Neotyphodium* endophtyes. The hybrid forms of the endophyte in Arizona fescue are most likely a result of hybridization between *Epichloë* spp. (a sexually reproducing, close relative of *Neotyphodium*) and *Neotyphodium* spp., presumably via a parasexual process where hyphae and genomes fuse (Schardl *et al.*, 1994; Tsai *et al.*, 1994; Moon *et al.*, 2004). In DNA sequencing, multiple gene copies indicate *Neotyphodium* hybridization (H+), whereas single gene copies

indicate a NH+ *Neotyphodium* species (Schardl & Craven, 2003; Sullivan & Faeth, 2004). Five different haplotypes of H+ endophyte and three different haplotypes of NH+ endophyte have been found in natural Arizona fescue populations (Sullivan & Faeth, 2004).

Glasshouse experiment

Seeds from known H+ plants and NH+ plants, and naturally endophyte-free (E) plants were collected from natural populations in Arizona. From H+ and NH+ plants, endophytes were removed in two ways to produce endophyte-free, hybrid (H−) and nonhybrid (NH−) seeds: infected plants were treated with fungicides and then seeds collected from these plants (see Faeth & Sullivan, 2003 for details); or by long-term storage of seeds at room temperature (Welty & Azevedo, 1985; Rolston *et al.*, 1986; Wheatley *et al.*, 2007). Infected (H+ and NH+), and naturally (E−) and manipulated (H− and NH−) endophyte-free Arizona fescue seeds were planted in a random order in the field in Flagstaff, AZ, in 2001. Seeds produced by these plants were collected in autumn 2009 and stored at −21°C until the beginning of the experiment in November 2010. Seeds of the five endophyte infection categories were sown in 0.75 dl pots in regular potting soil and grown in a glasshouse in natural light at 24°C. Within each infection category, we used seed mixtures from at least five maternal plants to randomize the effect of plant genotype. In these mixtures, the seeds originating from infected maternal plants (H+ and NH+) contained a random set of endophytic haplotypes (five different haplotypes of H+ endophytes and three different haplotypes of NH+ endophyte). After c. 1 month's growth, the grasses were replanted so that there were either two plants (competition treatment) or one (control treatment) plant growing in 3 dl pots. The glasshouse was set to 17°C night : 23°C day temperature conditions with natural lighting. In the competition treatment, NH+ and H+ plants were tested against each other, and in competition with H−, NH− and E− plants (E− vs H+, E− vs NH+, H+ vs E−, H+ vs H−, H+ vs NH+, H− vs H+, NH+ vs E−, NH+ vs H+, NH+ vs NH−, NH− vs NH+). All plants were watered twice a week and fertilized twice a month (20 : 20 : 20 (N : P : K), with micronutrients) until February 2011 when water and nutrient treatments began. Each competition pairing and individually growing plants were grown in four treatment combinations (high nutrients and high water; high nutrients and low water; low nutrients and low water; and low nutrients and high water) in each of the 12 completely randomized replicates. Altogether, there were 480 pots in the experiment. Pots assigned to high- and low-nutrient treatments were fertilized with a liquid fertilizer [20 : 20 : 20 (N : P : K), with micronutrients] once a week or once in 2 months, respectively. Pots were watered two to three times a week so that plants in the high-water treatment conditions received twice as much water as those in the low-water treatment. These amounts of watering for Arizona fescue are known from previous studies to achieve distinct differences in growth in the glasshouse (e.g. Faeth *et al.*, 2004) and in the field (Faeth & Sullivan, 2003).

After 3 months, the number of live tillers was counted and three tillers per plant were removed and weighed. Based upon the mass and number of the tillers, we estimated the living wet biomass of the grasses. This plant tissue was also used to verify the infection status of the plants using an immunoblot assay to detect monoclonal antibodies specific to *Neotyphodium* (Phytoscreen Immunoblot Kit #ENDO7973; Agrostics, Watkinsville, GA, USA). The NH+ and H+ infection status of the plants was further analyzed from a random sample of 10 NH+ and 10 NH− plants using PCR (Sullivan & Faeth, 2004) and from an additional random sample of five NH+ and five H+ plants by sequencing the PCR products. The expected endophyte status was confirmed for each plant. At the end of 4.5 months of growth, all plants were harvested and their roots were washed with water. All plant parts were then dried at 65°C and the above- and below-ground dry biomass of each plant was determined.

Statistical methods

The experimental design was a randomized complete block design, where location of each pot with different treatments (endophyte status, competition, watering and fertilization) was completely randomized within each whole block. In order to address the hypotheses presented in the introduction, the data were analyzed in two ways: we lumped each competing infection category together (e.g. all E− plants combined together that were competing); and we analyzed competition individually against each individual infection category (e.g. E− plants competing against H+ and NH+ plants were analyzed separately). A mixed model, assuming a normal distribution for the response variables shoot dry biomass, shoot wet biomass, root dry biomass, root : shoot ratio and number of tillers, was used to model the effects of endophyte infection, competition, fertilization and watering. The response variables shoot dry biomass, shoot wet biomass, root dry biomass and root : shoot ratio were log-transformed, and the response variable number of tillers was square-root-transformed to meet the expectations of the statistical analysis. *A priori* hypotheses-related pairwise comparisons were performed using a least-squares *post hoc* test.

Results

Main effects

Dry biomass, wet biomass and number of tillers were significantly lower in the low-nutrient and low-watering treatments than in the high-nutrient and high-watering treatments, and lower in plants that were competing than in those that were not competing (Supporting Information, Tables S1, S2). Root biomass and root : shoot ratio were significantly higher in the low-nutrient treatment than in the high-nutrient treatment (Tables S1, S2). Most of the response variables had significant two-way interactions between competition, fertilization and watering (Tables S1, S2). Endophyte infection had an overall effect on root dry biomass and number of tillers; H+ infected

grasses had significantly higher root biomass and number of tillers than E− grasses. Furthermore, H+ grasses had significantly higher root dry biomass than NH− grasses (Tables S1, S2).

### Performance of plant and plant–endophyte combinations in the competition treatment

Plants infected with H+ endophytes performed better than grasses infected with NH+ endophytes in terms of shoot dry biomass (t_{587} = 2.14, P = 0.036, Fig. 1), shoot wet biomass (t_{583} = 2.23, P = 0.026, Fig. 2) and the number of tillers (t_{590} = 2.03, P = 0.042, Fig. 3). H+ plants grew better than E− grasses in terms of shoot wet biomass (t_{584} = − 3.4, P = 0.002, Fig. 2), and the number of tillers (t_{590} = −1.95, P = 0.052, Fig. 3) but only when plants were competing in low-water and low-nutrient treatments. No other differences were found among plant and plant–endophyte combinations when the plants were competing.

The effects of endophyte infections in the competition treatment

Hybrid endophyte infection (H+ vs H−) increased shoot wet biomass (t_{583} = 2.32, P = 0.021, Fig 2) and root dry biomass (t_{598} = 1.98, P = 0.056, Fig. 4) of the host grass when the plants were competing in low-water and low-nutrient treatments. The H+ endophyte did not affect any of the response variables in any other treatment combinations when the grasses were competing. The nonhybrid endophyte (NH+ vs NH−) did not affect performance of the host in any treatment combinations when the grasses were competing.

### The effect of endophyte status on specific competitors

There was a significant interaction between the endophyte status of the competitors (E− vs H+, E− vs NH+, H+ vs E−, H+ vs H−, H+ vs NH+, H− vs H+, NH+ vs E−, NH+ vs H+, NH+ vs
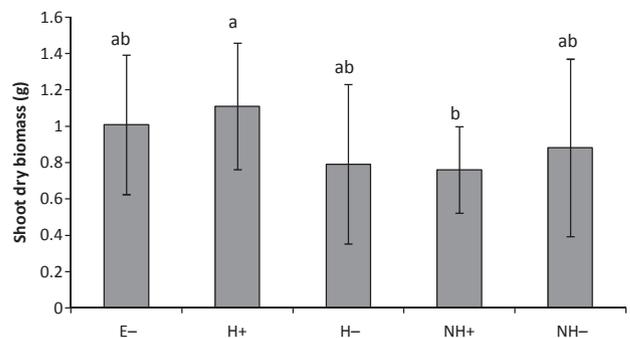
Fig. 1 Back-transformed estimated means of Arizona fescue (*Festuca arizonica*) shoot dry biomass and 95% confidence limits of grasses competing in low-water and low-nutrient treatments. Different letters indicate significant differences ($P < 0.05$) between endophyte infection categories. E−, uninfected grasses; H+, hybrid endophyte infected grasses; H−, manipulatively hybrid endophyte-free grasses; NH+, nonhybrid endophyte-infected grasses; NH−, manipulatively nonhybrid endophyte-free grasses.
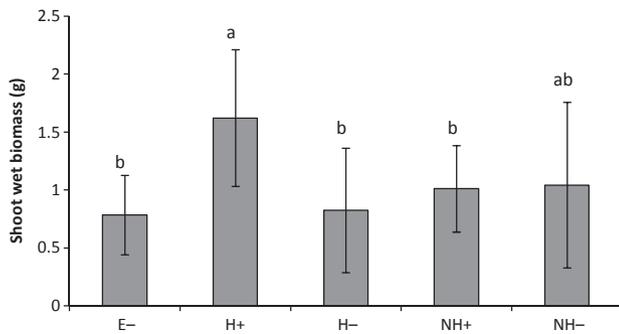
Fig. 2 Back-transformed estimated means of Arizona fescue (*Festuca arizonica*) wet above-ground biomass and 95% confidence limits of grasses competing in low-water and low-nutrient treatments. Different letters indicate significant differences ($P < 0.05$) between endophyte infection categories. E−, uninfected grasses; H+, hybrid endophyte infected grasses; H−, manipulatively hybrid endophyte-free grasses; NH+, nonhybrid endophyte-infected grasses; NH−, manipulatively nonhybrid endophyte-free grasses.
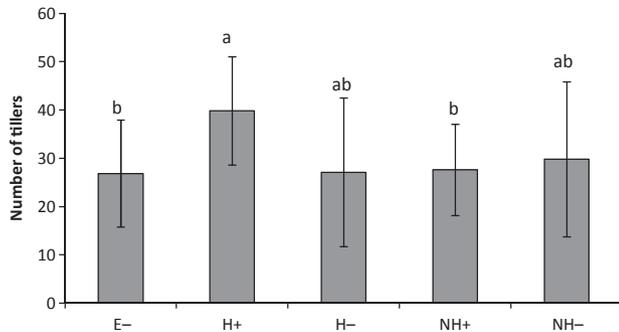
Fig. 3 Back-transformed estimated mean number of Arizona fescue (*Festuca arizonica*) tillers and 95% confidence limits of plants competing in low-water and low-nutrient treatments. Different letters indicate significant differences ($P < 0.05$) between endophyte infection categories. E−, uninfected grasses; H+, hybrid endophyte infected grasses; H−, manipulatively hybrid endophyte-free grasses; NH+, nonhybrid endophyte-infected grasses; NH−, manipulatively nonhybrid endophyte-free grasses.
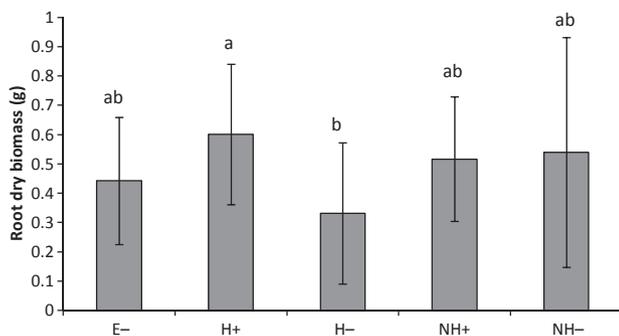

Fig. 4 Back-transformed estimated means of Arizona fescue (*Festuca arizonica*) root dry biomass and 95% confidence limits of grasses competing in low-water and low-nutrient treatments. Different letters indicate significant differences ($P < 0.05$) between endophyte infection categories. E−, uninfected grasses; H+, hybrid endophyte infected grasses; H−, manipulatively hybrid endophyte-free grasses; NH+, nonhybrid endophyte-infected grasses; NH−, manipulatively nonhybrid endophyte-free grasses.

NH−, NH− vs NH+), watering (high and low) and nutrients (high and low) in the root biomass ($F_{9,415} = 244$, $P = 0.01$). However, no *a priori* hypotheses-related differences were found in the pairwise comparisons (All *a priori* hypotheses-related P-values > 0.05). No other significant differences were found when the plants were competing.

Performance of plants and plant–endophyte combinations – no competition

All plant groups (NH+, NH−, H+, H− and E−) performed equally well in terms of shoot dry biomass, shoot wet biomass, root dry biomass, root : shoot ratio and number of tillers when the plants were not competing. No differences were found in any treatment combinations (high nutrients and high water, high nutrients and low water, low nutrients and low water and low nutrients and high water).

Discussion

Our results support the hypothesis of increased host performance of H+ plants when resources are scarce. There was increased performance of H+ grasses compared with other plant and plant–endophyte combinations found in the natural populations (NH+ and E−) in almost every response variable measured, but only when competing in low-water and low-nutrient treatments. Hybrid endophyte infection was verified to increase above-ground wet biomass and root dry biomass of the host when competing in low-water and low-nutrient treatments by comparing infected plants (H+) with those whose endophyte had been experimentally removed (H−). Our analysis confirmed that the status of the specific competitors did not matter. In other words, H+ plants were equally superior competitors against all other plants (NH+, H− and E−) in low-water and low-nutrient treatments. When not competing, or competing in other treatment combinations (high water and low nutrients, low water and high nutrients, high water and high nutrients), H+ endophyte did not appear to benefit the host grass. In contrast to expectations, the NH+ endophyte did not affect performance of the host compared with other grasses, regardless of treatment. Our results suggest that symbiont-conferred protection against biotic and abiotic stresses may be underlying the observed niche expansion of Arizona fescue infected by H+ endophyte in the environments with low resources (e.g. Hamilton *et al.*, 2009).

The competitive dominance of H+ Arizona fescues in low-resource environments may result from novel or extra genes in hybrid strains (e.g. Schardl & Craven, 2003; Moon *et al.*, 2004). Hybridization has been suggested to be advantageous for the hosts, especially in marginal habitats at the edge of the host range (Rieseberg, 1997; Schardl & Craven, 2003). The advantages may result from higher genetic variation in the H+ endophytes, which, in turn, increase tolerance to biotic and abiotic stresses (Schardl & Craven, 2003). The mechanism by which the novel genes of H+ improve competitive potential of the infected host is unclear. Several hypotheses explaining improved competitive potential of *Neotyphodium*-infected grasses have been

proposed. These include changes in nutrient metabolism (Lyons *et al.*, 1990), plant hormone–endophyte interactions (De Battista *et al.*, 1990) and osmotic adjustment by the endophyte (Elmi & West, 1995).

Neither NH+ nor H+ endophyte infections improved growth of the host when plants were not competing, contrary to reports in the general literature, which suggest sweeping benefits of *Neotyphodium* infections (Saikkonen *et al.*, 2010; Faeth & Saari, 2011). However, our findings are in line with previous studies of Arizona fescue where the endophyte does not appear to benefit the host plant, at least in experiments with no competition (Sullivan & Faeth, 2008; Hamilton *et al.*, 2010). In general, endophyte infections have been demonstrated to have variable effects in the growth of the host plant, depending on the plant species, and especially on the plant and endophyte genotypes in question (Cheplick *et al.*, 1989; Cheplick, 1998; Faeth & Sullivan, 2003; Hunt *et al.*, 2005). The combinations of conditions that result in greater growth of endophyte-infected plants are not fully understood.

Because we mainly found NH+ endophytes to have neutral effects on the host, our findings fail to explain the overall high frequencies of NH+ infections in natural Arizona fescue populations (Schulthess & Faeth, 1998; Sullivan & Faeth, 2008; Hamilton *et al.*, 2009). It is possible that NH+ and H+ endophytes affect other characteristics of the host than those measured in this experiment. For example, increased incidence of fungal pathogens has been suggested to limit the distribution of H+ hosts (Hamilton *et al.*, 2010). Also different effects of H+ and NH+ endophytes on reproductive strategies have been reported (Sullivan & Faeth, 2008). In general, *Neotyphodium* infections have been suggested to increase resistance of the host against herbivores, seed predators, and plant pathogens. However, these benefits, as well as some others (e.g. resistance to fire), are not found in Arizona fescue (e.g. Saikkonen *et al.*, 1999; Tibbets & Faeth, 1999; Faeth & Sullivan, 2003; Neil *et al.*, 2003; Faeth *et al.*, 2004; Hamilton *et al.*, 2010).

Thus, the question of how high frequencies of NH+ infections are maintained in natural Arizona fescue populations remains unanswered. One explanation for the persistence of high NH+ infection rates, and the repeatedly failed attempts to find positive effects of this endophyte on the host, is that NH+ endophyte infections are infrequently mutualistic, and the positive effects only occur at certain times, such as periods of severe and prolonged droughts or rapid population decline (Faeth, 2002; Morse *et al.*, 2002). We also acknowledge that our experiments may have failed to capture long-term selective pressures associated with a long-lived host plant and its symbiont. Furthermore, in more natural settings in the field, the outcome of the interactions between Arizona fescue and NH+ and H+ endophytes may differ.

Until recently, hybridization has been viewed as destructive force, at least in terms of maintaining species diversity in communities (e.g. Rhymer & Simberloff, 1996; Mallet, 2005). However, hybridization can also be a creative force, increasing diversity and allowing species to persist in marginal habitats (Rieseberg, 1997). At least one of the parental species of H+ endophytes in Arizona

fescue is *Epichloë*, which, when horizontally transmitted, is highly pathogenic. Thus, we propose here that the occasional presence and genetic input from the pathogen *Epichloë*, and subsequent hybridization, may be necessary to maintain the mutualistic interaction of *Neotyphodium* with it host grass in natural populations, at least in some environments.

In conclusion, our results support the hypothesis (Schardl & Craven, 2003) that hybridization by endophytes may lead to increased survival of the host plant in stressful environments. To fully assess the impact of hybridization of this symbiont and the consequences to expanding its host's niche, long-term experiments in the field conditions are necessary. Nonetheless, our results suggest that interactions between plants and microbes may have an important role in colonization, metapopulation dynamics and plant community structure.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Table S1 Summary of the main effects

Table S2 Back-transformed estimated means and 95% CLs for variables with some significant differences

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