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Neotyphodium interactions with a wild grass are driven mainly by endophyte haplotype

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Summary

1. Strong mutualistic associations are expected to arise between host and microbial symbionts when symbionts lose sexuality, and rely strictly on their host for reproduction via vertical transmission. The *Neotyphodium* endophyte is a vertically transmitted, asexual symbiont of pooid grasses. In agronomic grasses, *Neotyphodium* typically interacts mutualistically with its host by increasing drought resistance and other properties. However, the interaction likely depends on host and endophyte genotypic variation, yet little is known how this variation influences host physiological and morphological responses, especially in wild grasses.

2. We used four different *Neotyphodium*-infected maternal lines of a wild grass, Arizona fescue (*Festuca arizonica*). Two lines harboured one haplotype and two lines harboured a different haplotype. We experimentally removed the endophyte from some ramets of the four lines. We grew infected (E+) and uninfected (E–) plants in a greenhouse under varying water availability.

3. We examined the effect of endophyte infection, endophyte and host plant maternal genotype, and water availability on traditional growth parameters. We also measured leaf net photosynthesis and dark respiration, leaf conductance to water vapour, leaf water potential, leaf rolling and stomatal density to provide explanations for differences in biomass production and relative growth rates (RGR).

4. Our general findings show that *Neotyphodium* infection, *Neotyphodium* haplotype and its interaction with host maternal genotype, and varying water availability influence Arizona fescue physiology, growth and biomass production. Based only on infection status, the direction of interaction between endophyte and host is not mutualistic in terms of host growth. Overall, endophyte haplotype and its interaction with host maternal genotype is the most critical, and consistent factor in influencing host growth and physiological outcomes. Variation due to endophyte haplotype in terms of growth and physiological parameters is often greater than that between infected and uninfected hosts.

5. Endophyte–host interactions are likely to be enormously complex because of the genetic and environmental variation that exists in natural populations. The outcome of these interactions in natural grass–endophyte systems is exceedingly difficult to predict based simply on the presence or absence of the endophyte.

Key-words: endophyte, growth rate, haplotype, *Neotyphodium*, photosynthesis

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Introduction

Strong mutualistic associations are expected to arise between host and microbial symbionts when symbionts lose sexuality, and rely strictly on their host for reproduction via vertical transmission (e.g. Ewald 1994; Wilkinson & Schardl 1997). This prediction has been supported for some non-native, but agronomically important, grass cultivars introduced from Eurasia to North America, New Zealand, Australia and South America, and their symbiotic, asexual endophyte, *Neotyphodium*. In these agronomic grasses, particularly tall fescue (*Lolium arundinaceum*), the *Neotyphodium*–grass interaction is generally considered mutualistic (Clay 1988, 1990). Infection generally enhances host plant competitive abilities via increased herbivore and pathogen resistance due to alkaloids produced by the

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fungus (e.g. Clay 1988, 1990), increased above- and below-ground vegetative, and reproductive growth (e.g. Marks, Clay & Cheplick 1991) and increased drought tolerance (e.g. Arachevaleta *et al.* 1989; Elmi & West 1995). Enhanced competitive abilities have been documented for agronomic tall fescue (e.g. Marks *et al.* 1991) and perennial ryegrass (*L. perenne*) (Clay, Marks & Cheplick 1993).

The suite of benefits conferred by *Neotyphodium* infections often result in high frequencies in pastures (Clay 1988) and old fields (Clay, Holah & Rudgers 2005, but see Spyreas, Gibson & Basinger 2001) within a few years, at least for tall fescue (KY 31 cultivar) in North America. Because the endophyte is strictly seed borne, but infections can be lost from seeds (Siegel *et al.* 1984), high frequencies can only be maintained if the interaction is mutualistic (Clay 1988; but see Faeth 2002). However, exceptions to this generalization have been found for tall fescue (e.g. West *et al.* 1993). For another well studied agronomic grass, *L. perenne* L. (perennial ryegrass), the effects of infection on the host plant competitive abilities appear more variable (e.g. Marks *et al.* 1991; Clay *et al.* 1993; Barker, Hume & Quigley 1997). The endophyte does not appear to generally confer drought resistance in perennial ryegrass (e.g. Cheplick, Perera & Koulouris 2000; Cheplick 2007). Increasing evidence suggests that the outcome of the interaction depends on the genotype of the host grass and the haplotype (systemic endophytes are generally haploid, except for those of hybrid origin, where multiple copies of genes often persist; Schardl & Craven 2003) of the endophyte and environmental factors (e.g. Faeth & Sullivan 2003; Saikkonen *et al.* 2004; Müller & Kraus 2005). This dependency is even evident in agronomic grasses, where genetic variation of both host and endophyte are limited due to bottleneck effects and selective breeding. In both agronomic tall fescue and perennial ryegrass, endophyte infection and host plant genotype may affect how the host plant responds to biotic and abiotic factors in the environment. For example, Marks & Clay (1996) found significant infection × plant genotype interactions on carbon exchange rates and leaf conductance to water vapour for 13 different genotypes of tall fescue. They concluded that physiological responses of host plants to fungal endophyte infection depend both on the physiological environment and the genotype of the plant. In 13 perennial ryegrass genotypes, Cheplick *et al.* (2000) found marked host plant genotypic variation in the ability to recover from drought stress while endophyte infection had little or no role. These studies suggest that either plant genotype or environmental factors can affect host physiology and growth of infected agronomic grasses.

Far less is known about the influence of *Neotyphodium* infections in natural populations of wild grasses, especially in terms of drought resistance (e.g. Faeth 2002). *Festuca arizonica* Vasey (Arizona fescue) is a native perennial bunch grass in southwestern USA. *Neotyphodium* infection frequencies in wild populations of Arizona fescue are usually high (Schulthess & Faeth 1998; Saikkonen *et al.* 1999). Despite high frequencies, however, the infection by asexual *Neotyphodium* does not appear to benefit the host, as predicted for asexual symbionts (Ewald 1994; Wilkinson & Schardl 1997). In general, previous experiments indicate that *Neotyphodium* infections in Arizona fescue hosts decrease, rather than increase plant performance in terms of herbivore resistance (e.g. Tibbets & Faeth 1999), growth and reproduction (Faeth & Sullivan 2003), and competitive abilities (Faeth, Saikkonen & Helander 2004), contrary to the mutualistic model of asexual endophytes.

However, genetic variation in *Neotyphodium* endophytes also potentially influences interaction outcomes. Different *Neotyphodium* strains that have been intentionally manipulated or transferred to agronomic tall fescue may alter growth and physiological properties of the host (e.g. Assuero *et al.* 2002). While genetic variation in *Neotyphodium* within agronomic cultivars is relatively low due to selective breeding (Saikkonen *et al.* 1998, 2004, 2006), genetic variation in asexual *Neotyphodium* in wild grass populations varies substantially (e.g. Bony *et al.* 2001; Sullivan & Faeth 2004), although this variation is typically less than that of its outcrossing grass host plant (Sullivan & Faeth 2004). To our knowledge, there have been no tests of how endophyte haplotype in conjunction with host genotype and environmental factors alters host growth and biomass production, gas exchange and water relations in wild grasses infected with *Neotyphodium*.

We assessed the relative performance in terms of growth and biomass production of uninfected (E–) and *Neotyphodium* infected (E+) plants with two endophyte haplotypes within four maternal genotypes of Arizona fescue under two contrasting water availability regimes. We measured traditional growth analysis parameters, including relative growth rates (RGR), above- and below-ground biomass, and below-ground : above-ground biomass ratios. We also measured leaf net photosynthesis and dark respiration, leaf conductance to water vapour, leaf water potential, leaf rolling, and stomatal density to provide explanations for differences in biomass production and RGRs. Our purpose here is to incorporate not just infection status but also variation in endophyte haplotypes within hosts and the environment to determine how these factors interact to influence host growth and physiological parameters.

Materials and methods

THE HOST PLANT – *FESTUCA ARIZONICA*

Festuca arizonica Vasey (Arizona fescue) is a native perennial bunch grass that is widespread in semi-arid Ponderosa pine (*Pinus ponderosa*)/grassland communities above 2000 m elevation in the southwest USA (Kearney & Peebles 1960). *Neotyphodium* infection frequencies in

wild populations of Arizona Fescue are usually high (60%–100%, Schulthess & Faeth 1998).

THE ENDOPHYTE – *NEOTYPHODIUM*

Endophytes of the genus *Neotyphodium* (Morgan Jones & Gams) are obligate seed-borne fungi that commonly form intercellular infections in leaves, culms and inflorescences in many cool-season grasses in the subfamily Pooideae (Saikkonen *et al.* 1998). Arizona Fescue harbours at least three distinct forms of *Neotyphodium*, each likely a unique species (An *et al.* 1992; Sullivan & Faeth 2004). A survey of genetic variation at three microsatellite loci identified multiple haplotypes, including at least five distinct haplotypes of hybrid origin and three of non-hybrid origin (Sullivan & Faeth 2004). Haploid, asexual *Neotyphodium* is represented by two distinct, non-hybrid, haplotypes in the source population of our experimental plants (Sullivan & Faeth 2004). Two of the four naturally infected maternal plants used in our experiments (MD 1 and MD 49) harboured one non-hybrid endophyte haplotype (termed H1) and the other two naturally infected maternal plants (MD 44 and MD 46) harboured the other non-hybrid endophyte haplotype (termed H2). Haplotypes of the experimental plants were confirmed by microsatellite DNA analyses of multiple loci (Sullivan & Faeth 2004).

SEED SOURCES

To test the effect of infection, host maternal genotype and endophyte haplotype, and environmental factors on host growth and physiological parameters, we used *Neotyphodium* infected (E+) and uninfected (E–) Arizona Fescue seeds from four naturally-infected maternal plants (MD 1, MD 44, MD 46 and MD 49) from the same population at Merritt Draw. Merritt Draw is a drainage meadow on the Mogollon Rim (elevation 2500 m) in Arizona. Maternal plants were randomly selected in 1997 from a pool of about 50 infected plants in the population. Initially, infection was determined by a modified tissue print immunoassay (Gwinn, Collins-Shephard & Reddick 1991; Schulthess & Faeth 1998) and later confirmed by staining seeds from each plant after each growing season and examining them for the presence of *Neotyphodium* hyphae (Schulthess & Faeth 1998). The term maternal plant genotype is used because one maternal bunch grass plant was used to generate all samples of that particular Arizona fescue genotype. Seeds from the same maternal plant could be full or half-sibs depending on paternal contribution, or even be related more than 0·5 if selfing occurs, although Arizona fescue is thought to generally outcross (USDA 1988). For simplicity, we hereafter refer to the four maternal seed sources as maternal plant genotypes, and consider the paternal contribution as a random factor, because at least the maternal genome is constant within a given seed group.

Details of the removal of the endophyte, subsequent field plantings and collections of seeds from E+ and E– plants is found in Faeth & Sullivan (2003). Seeds were collected from E– and E+ plants in September 2001, cold-treated for 30 days at 5 °C and then stored at room temperature.

We assumed that the maternal plants, two of which harboured haplotype H1 (MD 1 and MD 49) and two of which harboured haplotype H2 (MD 44 and MD 46), were genetically different from one another. This is a reasonable assumption as Arizona fescue plants form distinct bunches, outcross to produce seed, and do not reproduce via rhizomes (USDA 1988). All four of the selected maternal plants were at least 10 m from one another. Maternal plants with the same haplotype are, at worse, half-sibs and probably less related than half-sibship because of outcrossing and very low dispersal distances of seeds from maternal plants (< 2 m, Sullivan & Faeth 2004).

THE EXPERIMENT

E+ and E– seeds from the four infected maternal plants were planted in individual pots under contrasting water availability regimes to test the effect of endophyte infection, and the influence of host plant maternal genotype and endophyte haplotype on relative performance in terms of growth and biomass production. Three hundred E+ and 300 E– seeds of four genotypes: Arizona fescue, MD 1, MD 44, MD 46 and MD 49 were sown in a 50 : 50 mix of native soil to potting soil (Supersoil and Rod McLellan Company, San Mateo, CA, USA) spread to a depth of about 2 cm over 5 cm of soil on 21 January 2004, 7 October 2003, 6 August 2003 and 22 April 2003, respectively, and watered to field capacity (*c.* 250 mL) in plastic trays. Trays were placed in a growth chamber under a 22 °C/15 °C (day/night) temperature regime with an 18 h photoperiod, during which time they received 400 μmol m^{-2} s^{-1} photosynthetically active radiation (PAR) from a combination of cool white florescence tubes and incandescent bulbs. Soils were watered to field capacity three times per week and seeds were allowed to germinate. After 21 days, seedlings were transplanted from the plastic trays into individual square pots ($11 \times 11 \times 11$ cm, $L \times W \times H$) containing the above native soil/potting soil mixture and allowed to establish for 21 days after which 12 E+ and 12 E– plants were randomly sacrificed for initial above- and below-ground biomass.

Twelve E+ plants and 12 E– plants were randomly placed into each of the two water treatments: high water availability (HW; watered three times per week to field capacity; *c.* 250 mL), or low water availability (LW; watered once a week to field capacity) resulting in a total of 48 plants per plant genotype. Plant infection status was confirmed using a modified tissue print immunoblot (Schulthess & Faeth 1998) at the start of the treatments.

Each of the four half-sib families of Arizona fescue was staggered with regard to their start times in the experiment. Staggering was done to keep the experimental size manageable and to ensure that measurements were made midday (10.00–14.00 h), thus eliminating any possible diurnal variations in plant physiological parameters.

BIOMASS PRODUCTION AND GROWTH

The effect of endophyte infection and water stress on biomass production, and growth parameters was determined over the 49 day period. At the end of the period, plants were divided into roots and above-ground parts, and soil was washed from roots by hand. Tiller number of each plant was not determined in this experiment. Specific leaf mass (SLM) was determined on a subsample of each plant (containing about 25% of the total leaves) using a leaf area meter (Decagon Devices, Pullman, WA, USA). Above-ground biomass was placed in an oven at 60 °C for 24 h and below-ground biomass was placed in an oven at 105 °C for 48 h. Following this drying period, above- and below-ground biomass was determined. Biomass of E+ or E– plants at the initial harvest were randomly paired with respective E+ or E– plants at the final harvest, and the relative growth rate (RGR; rate of biomass gain per biomass) and net assimilation rate (NAR; rate of biomass gain per leaf area) of each plant was estimated using the equations in Xiong, Mueller & Day (2000). Total leaf area. Leaf area ratio (LAR; leaf area per total plant biomass), leaf mass ratio (LMR; leaf biomass per total plant biomass) and below-ground : above-ground biomass ratio (R:S) were also calculated.

LEAF GAS EXCHANGE, DARK RESPIRATION AND WATER POTENTIAL

Rates of net photosynthesis (P_n) and transpiration (E) of one group of leaves on each plant were measured at midday (10.00–14.00 h) on five dates during the experiment. These measurements were made at midday because we anticipated that water limitation treatment effects should be greatest at this time. Measurements were made on the HW treatment 1–2 days after watering on a non-watering day. Measurements were made on the LW treatments the day before a watering day, which ensured that the plants would be at their most stressed. All measurements were made using an opens infra-red gas analyser (IRGA) system (LI-6400, Li-COR, Lincoln, NE, USA). The longest, completely green leaves on a plant were held parallel in the *Arabidopsis* chamber (6400-15 *Arabidopsis* Chamber, Li-COR, Lincoln, NE, USA) filling an area of 0·79 cm^2 for measurements. The CO_2 concentration of air entering the chamber (c_a) was maintained at 380 ppm using the CO_2 injector system (6400-01, CO_2 Injector System, Li-COR, Lincoln, NE, USA). Net photosynthesis, leaf conductance to water vapour (g_L), the intercellular CO_2 concentration (c_i) and dark respiration were calculated using the equation of von Caemmerer & Farqhuar (1981). Instantaneous water-use efficiency (WUE) was calculated as P_n/E in the chamber. Dark respiration was measured after each gas-exchange measurement using the same leaves from the gas-exchange measurement. A black cloth was placed over the leaf chamber and the PAR monitored to ensure that the leaves in the chamber were receiving 0 μmol m^{-2} s^{-1} PAR. Leaf water potential (Ψ_L) was measured after each gas-exchange measurement only on day 42 of the experiment using a pressure chamber (Model 1003, PMS, Corvallis, Oregon, USA) on one of the leaves used in the gas-exchange measurements. The leaf was cut 6 cm from its tip. Leaf water potential was only measured once per maternal plant genotype because Ψ_L does not vary much throughout consistent water treatments and remains relatively constant (Morse, Day & Faeth 2002).

LEAF ROLLING AND STOMATAL DENSITY

Leaf rolling and stomatal density were measured on the first day of the treatments and on the day before plants were harvested. The width of one of the longest green leaves on each plant was measured 5 cm from the tip using a calliper holding the mid-vein in the centre. This measurement was termed actual leaf width. The leaf was then excised 10 cm from the tip, forcibly unrolled, and the opened leaf width was measured 5 cm from the tip using a calliper and holding the mid-vein in the centre. The ratio of actual leaf width to opened leaf width was termed leaf rolling. The same leaf was then used to measure stomatal density using the method of Kubínová (1994). Stomata were present only on the adaxial side of Arizona fescue leaves.

STATISTICAL ANALYSES

Separate analyses of variance (ANOVA) were used to examine the effect of infection (E+ or E–), maternal plant genotype, and water availability treatment effects on biomass production and growth parameters, Ψ_L, leaf rolling and stomatal density. Plant maternal genotype was a nested factor within endophyte haplotype because each plant half-sib genotype was associated with only one of the two endophyte haplotypes. To assess the effect of endophyte haplotype on biomass production and growth parameters, Ψ_L, leaf rolling and stomatal density, we conducted separate ANOVA with only E+ plants, because endophyte-removed plants obviously had no endophyte haplotype associated with them.

Repeated-measures ANOVA were used to examine the effects of infection, plant maternal genotype (nested within endophyte haplotype) and water treatments on the dependent variables P_n, g_L, c_i, WUE and dark respiration over the time course of the experiment

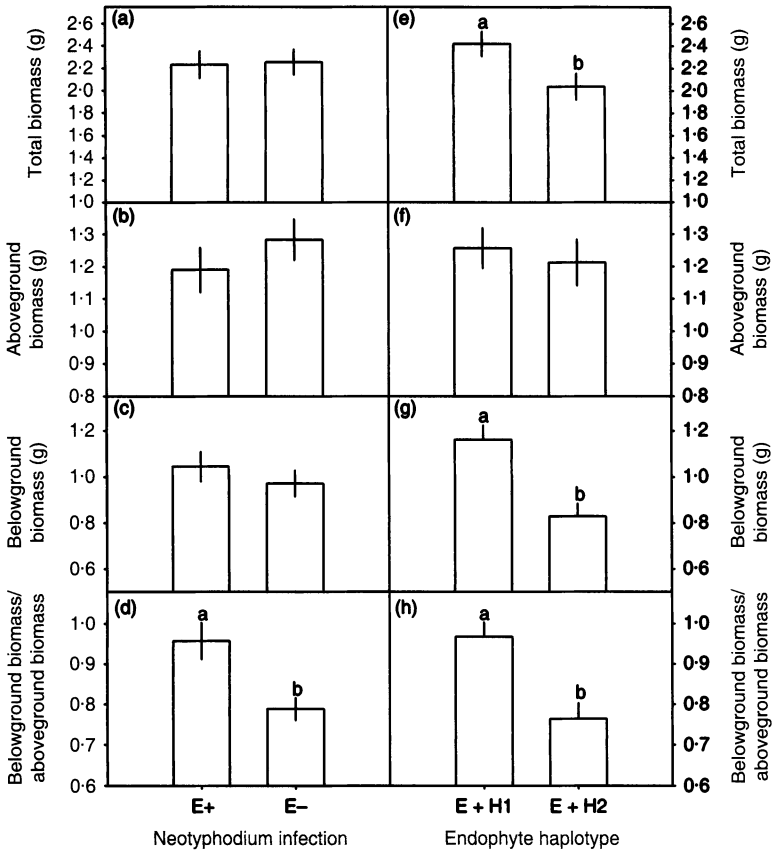

Fig. 1. Total biomass (a), above-ground biomass (b), below-ground biomass (c), and below-ground biomass : above-ground biomass ratio (d) of *Neotyphodium* infected (E+) and uninfected (E–) Arizona fescue and total biomass (e), above-ground biomass (f), below-ground biomass (g), and below-ground biomass : above-ground biomass (h) *Neotyphodium* infected Arizona fescue of the H1 haplotype (E+ H1) and H2 haplotype (E+ H2). Different letters indicate means are significantly different ($P < 0{\cdot}050$).

(SYSTAT 2000). As for the growth and morphological parameters above, we conducted separate repeated measures ANOVA for only E+ plants to determine the effect of endophyte haplotype on P_n, g_L, c_i, WUE and dark respiration over time. We also tested for differences in biomass measurements and physiological parameters among maternal plant genotypes when the endophyte was removed to provide an estimation of variation due to only maternal plant genotype without the complicating factors of infection and endophyte haplotype. All data sets satisfied the assumptions of ANOVA based on homogeneity of variances, normality of errors and independence of errors.

Results

BIOMASS PRODUCTION AND GROWTH

Below- and above-ground, and total biomass did not differ between infected (E+) and uninfected (E–) plants (Table S1, Fig. 1a–c), although E+ plants did produce significantly more below- to above-ground biomass than E– plants (Table S1, Fig. 1d). E– plants had greater LAR than E+ plants but RGR, total leaf area, NAR and SLM did not vary by infection status (Tables S1 & S2). Plant maternal genotype (nested within endophyte haplotype) affected below-ground biomass, root–shoot ratio and RGRs (Table S1, data not shown). As expected, plants under increased water (HW) produced more total biomass, above-ground biomass, as well as below-ground biomass than plants under reduced water (LW) availability (Table S1, data not shown). However, plants under LW availability had a greater below-ground : above-ground biomass ratio than plants in HW availability (Table S1, data not shown). In addition, there were complex two- and three-way interactions between *Neotyphodium* infection status, water availability, plant maternal genotype for total biomass, below- and above-ground biomass, below-ground : above-ground biomass ratio and SLM (Table S1, data not shown).

Whereas infection *per se* had no effect on most growth parameters, endophyte haplotype affected total, below-ground biomass and below-ground biomass : above-ground biomass (Table S3, Fig. 1e,g,h). Furthermore, RGRs and NAR differed significantly between the two endophyte haplotypes (Tables S2 and S3). In general, plants harbouring the H1 endophyte haplotype had greater biomass (Fig. 1e,g), total leaf area and RGRs (Tables S2 and S3) than plants harbouring the H2 endophyte haplotype. Plants infected with the H1 haplotype had greater total leaf area than plants with the H2 haplotype, but this difference was only marginally different ($P = 0{\cdot}09$, Table S3). For infected plants, total and above-ground biomass, RGRs, and total leaf area increased in the HW treatments, as expected (Table S3, data not shown). Endophyte haplotype also interacted with water treatments to affect all growth parameters except LAR (Table S3, data not shown). There was a significant endophyte haplotype effect within plant maternal genotype on below-ground biomass, below-ground biomass : above-ground biomass, NAR and SLM.

When only E– plants were compared, the four maternal plant genotypes varied in below-ground biomass ($F = 3{\cdot}82$, df = 3, 99, $P = 0{\cdot}012$), ratio of below-ground : above-ground biomass ($F = 8{\cdot}44$, df = 3, 99, $P < 0{\cdot}001$) , SLM ($F = 28{\cdot}64$, df = 3, 99, $P < 0{\cdot}001$), leaf water potential ($F = 8{\cdot}27$, df = 3, 99, $P < 0{\cdot}001$) and photosynthesis ($F = 8{\cdot}43$, df = 3, 99, $P < 0{\cdot}001$) (data not shown). Above-ground biomass and other physiological measurements did not vary among the four maternal plant genotypes when the endophyte was removed.

LEAF GAS EXCHANGE, WATER POTENTIAL, LEAF ROLLING AND STOMATAL DENSITY

Neotyphodium infection affected midday P_n, g_s, c_i and dark respiration (Table S4, Fig. 2a,b,c,e). E– plants had higher P_n than E+ plants on four out of five dates (Fig. 2a). E– plants also had higher g_s than E+ plants on four out of five dates (Fig. 2b) and less negative

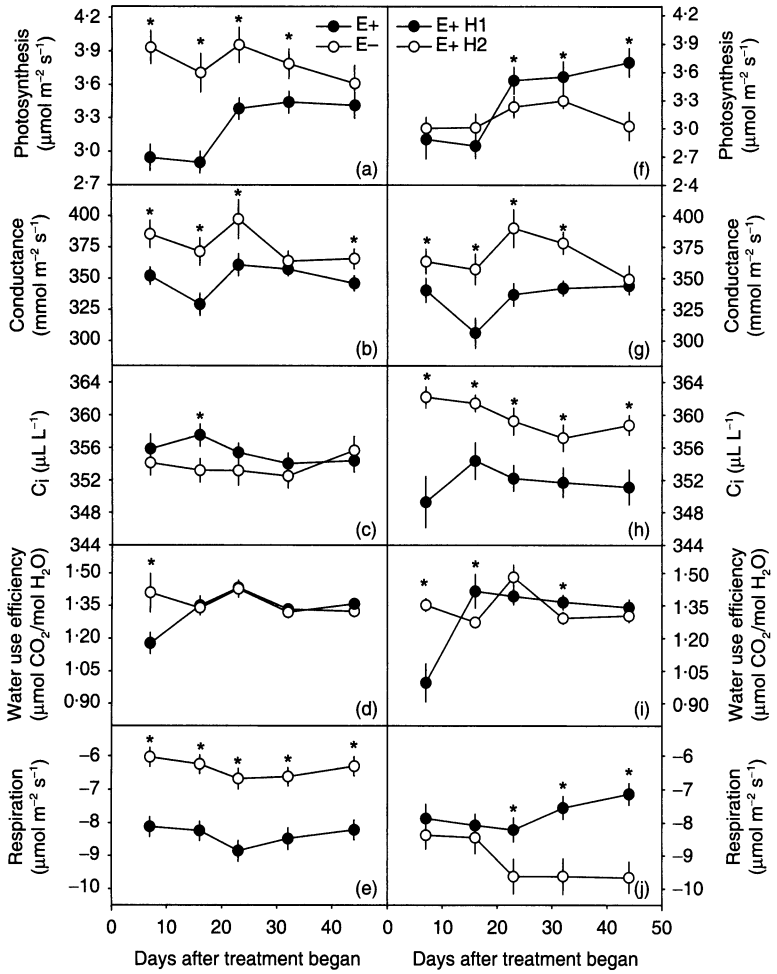

Fig. 2. Net photosynthesis (a), leaf conductance to water vapour (b), intercellular CO_2 (c), water use efficiency (d) and net dark respiration (e) of *Neotyphodium* infected (E+) and uninfected (E–) Arizona Fescue and net photosynthesis (f), leaf conductance to water vapour (g), intercellular CO_2 (h), water use efficiency (i) and net dark respiration (j) of *Neotyphodium* infected Arizona Fescue of the H1 haplotype (E+ H1) and the H2 haplotype (E+ H2). Asterisks indicate means are significantly different at a given sample date ($P < 0{\cdot}050$).

respiration rates than E+ plants on all five dates (Fig. 2e). Internal CO_2 of E+ plants was significantly higher than E– plants on one out of five dates and tended to be higher than E– plants on three out of five dates (Fig. 2c). Water availability affected P_n, g_s, c_i and WUE (Table S4, data not shown). Plants under HW availability had higher P_n and g_s on all five dates (LSD; $P < 0{\cdot}050$; data not shown) and higher WUE on three out of five dates (LSD; $P < 0{\cdot}050$; data not shown) than plants under LW availability, while plants under LW availability had higher c_i than plants under HW availability on four out of five dates (LSD; $P < 0{\cdot}050$; data not shown). In addition, there were complex two- and three-way interactions between *Neotyphodium* infection, water availability, and plant maternal genotype on P_n, g_s, c_i and WUE (Table S4). Midday P_n (Fig. 2a, P_n time effect, df = 4, 640, MS = 2·715, $P = 0{\cdot}003$) and WUE (Fig. 2d, WUE time effect, df = 4, 640, $P = 0{\cdot}05$) varied significantly over the time course of the experiment but the other physiological parameters did not change significantly with time. Infection, water treatment and plant maternal genotype interacted over time in complex two-, three- and four-way interactions to affect some physiological parameters (Table S4).

The presence of *Neotyphodium* also affected leaf water potential (Ψ_L) (Table S5). *Neotyphodium* infected plants had less negative Ψ_L than plants with their endophytes removed. Leaf rolling, but not stomatal density, also varied with infection (Table S5). E+ plants showed greater leaf rolling (a lower actual leaf width : opened leaf width ratio) than E– plants with their endophytes removed. However, infection status did not influence stomatal density.

Endophyte haplotype affected midday P_n, g_s, c_i and dark respiration but not WUE (Table S6, Fig. 2f–h, j). Plants with the H1 haplotype had higher P_n than H2 plants on three out of five dates (Fig. 2f). Infected plants with the H2 haplotype had higher g_s than H1 plants on four out of five dates (Fig. 2g), higher c_i than H1 plants on all five dates (Fig. 2h), and more negative respiration than H1 plants on three out of five dates (Fig. 2j). Endophyte haplotype significantly interacted with levels of water to affect P_n and WUE (Table S6). Midday P_n, g_s (time effect, df = 4, 640, MS =10 397·966, $P = 0{\cdot}022$) and dark respiration (time effect, df = 4, 640, MS = 3·267, $P = 0{\cdot}006$) varied significantly over the course of the experiment (Fig. 2f,g,j).

The two *Neotyphodium* haplotypes also differed in leaf water potential. Infected plants with the H2 haplotypes had more negative Ψ_L than H1 plants (Table S7). Leaf rolling and stomatal density also varied with endophyte haplotypes. Plants harbouring the H1 endophyte rolled leaves more tightly than H2 plants, and H1 plants had higher stomatal density than H2 plants (Table S7). There were no significant endophyte effects within maternal genotype (data not shown).

Discussion

Asexual fungal endophytes like *Neotyphodium* have traditionally been viewed as strong plant mutualists because host grass and fungal reproduction are closely linked through vertical transmission (e.g. Clay 1990; Clay & Schardl 2002). Some of the benefits provided by the endophyte involve physiological changes that increase growth and resistance to drought, especially in arid or semi-arid habitats. Until recently, most studies of endophyte effects on growth, reproduction and physiology considered only infection status – whether grasses are infected or not (e.g. Eerens, White & Lucas 1993; Richardson, Hoveland & Bacon 1993) and used two agronomic grasses, Tall Fescue and Perennial Ryegrass, in empirical tests of the effect of endophytes. Although in agronomic grasses, infection alone may enhance growth (e.g. Cheplick, Clay & Marks 1989; Malinowski *et al.* 1997; Newman *et al.*

2003) and alter physiological parameters (e.g. Marks & Clay 1996; Malinowski *et al.* 1997; Newman *et al.* 2003), more recent studies indicate that plant genotype, endophyte strain and environmental factors may influence the outcome of *Neotyphodium* interactions with its host grass (e.g. Cheplick *et al.* 2000; Bony *et al.* 2001; Faeth, Bush & Sullivan 2002; Cheplick & Cho 2003; Saikkonen *et al.* 2004; Müller & Krauss 2005). For example, Perennial Ryegrass hosts two different taxonomic groups of *Neotyphodium* spp. (Christensen *et al.* 1993; Schardl *et al.* 1994) which may cause variability in the effects of infection and plant genotype × endophyte infection interactions (Hesse *et al.* 2003).

To date the vast majority of studies have involved agronomic grasses, where genetic variation in both the host grass and endophyte is restricted due to selective breeding (e.g. Saikkonen *et al.* 1998). Genetic variation in hosts and endophytes in natural populations is likely much greater, and likely to influence growth and physiological parameters. A recent meta-analysis of endophyte studies (Saikkonen *et al.* 2006) indicates that the agronomic grass systems are poor model systems because they fail to capture the variation in endophyte–host grass interactions that is inherent in wild grass populations and communities. Our results confirm this wide variability in a native grass from wild populations. The outcome of a fungal endophyte interaction with its native host grass, Arizona fescue, is highly dependent on endophyte haplotype, plant maternal genotype and the environmental factor, water availability.

In Arizona fescue, haplotype of the endophyte appears to be the most critical and consistent factor in influencing the growth and biomass production of Arizona fescue plants, as well as physiological outcomes such as leaf water potential, leaf rolling and stomatal density. Endophyte haplotype appears to override infection status, at least in determining growth and several physiological measures. Indeed, E– host plants tended to accumulate more above-ground biomass (Fig. 1b) than E+ plants, supporting previous findings that *Neotyphodium* largely acts antagonistically in Arizona fescue (Faeth & Sullivan 2003; Faeth *et al.* 2004). However, E+ plants apparently allocate more resources to roots than shoots (higher root : shoot biomass ratio, Fig. 1d), which could be advantageous in drought-prone habitats. However, other short- (e.g. Faeth & Sullivan 2003; Faeth *et al.* 2004) and long-term (e.g. Saikkonen *et al.* 1999; Faeth & Hamilton 2006) studies of Arizona fescue show that E+ plants do not survive better as a group than E– plants, even during periods of prolonged drought. Within infected plants, however, the strain of endophyte, even within the same population, greatly influences relative performance and physiological aspects of the host plant. In addition, the many complex interactions involving plant maternal genotype and endophyte haplotype, water availability, and endophyte infection indicate that the direction and magnitude of *Neotyphodium* interactions with this native host grass, even under laboratory conditions, are highly variable and complex.

The variation between endophyte haplotypes is often greater than that between E+ and E– plants for both growth and physiological parameters (Fig 1 and 2). Although the H1 haplotype generally performed better than H2 haplotype, this experiment was conducted under one set of environmental conditions. It is likely that the advantages of harbouring different endophyte haplotypes varies with environmental conditions such as water and nutrient availability, and the presence of other interacting plant and consumer species in natural communities. We might expect that varying environments maintain a suite of endophyte haplotypes commonly found in natural populations of Arizona fescue (e.g. Sullivan & Faeth 2004), much like varying selective pressures may maintain E+ and E– grasses within these populations (Faeth 2002). Indeed, at least the H1 haplotype appears to perform better than E– plants for some growth and physiological parameters in this experiment (Figs 1 and 2).

Genetic background of the maternal host plants results in additional variation in below-ground biomass accumulation, allocation to roots and shoots, and photosynthesis, as indicated by differences among maternal host plant genotypes that are without their endophytes. Host plant genetic variation, even in cultivated grasses, where host genetic variation is reduced relative to wild grasses because of cultivation and selective breeding, can also result in different growth and reproductive performance (e.g. Cheplick *et al.* 2000; Cheplick & Cho 2003).

In these experiments, we used only two non-hybrid endophyte haplotypes (Sullivan & Faeth 2004) from within one population (Merritt Draw) and two maternal plant genotypes for each endophyte haplotype, so caution is required in extrapolating our results to the other host genotype–endophyte haplotype combinations. The number of these combinations within and across native populations is likely profuse. Given that Arizona fescue is thought to generally outcross (USDA 1988), we would expect much greater variation due to maternal plant genotype in natural populations where many different plant maternal genotypes harbour the same endophyte haplotypes. For variation in the endophyte haplotypes, Sullivan & Faeth (2004) showed natural populations of Arizona fescue harbour at least three distinct non-hybrid endophyte and five hybrid haplotypes of *Neotyphodium*, some which occur in the same population. This relatively high degree of genetic diversity in *Neotyphodium*, despite its asexuality, coupled with genetic variation in an outcrossing host grass and environmental variability in natural habitats, suggests that interactions between *Neotyphodium* and native host grasses are enormously variable, and not predictable simply based upon the presence or absence of the endophyte.

PHYSIOLOGICAL AND MORPHOLOGICAL VARIATION

Consistent with a previous physiological study of *Neotyphodium* infection in Arizona fescue (Morse *et al.* 2002), Ψ_L was less negative in E+ plants than E– plants regardless of treatment, and infected plants with H1 endophytes had less negative Ψ_L than plant with H2 endophytes. E+ plants also had more tightly rolled leaves than E– plants regardless of treatment. Rolling leaves more tightly may be the reason for E+ plants maintaining less negative Ψ_L than E– plants. Leaf movements, such as rolling, are common adaptive mechanisms to water stress and drought conditions in plants. These movements help in reducing incident irradiation, leaf temperature and transpiration (Begg 1980; Ehleringer & Forseth 1980) by decreasing the exposed leaf area and g_s (Begg 1980), although the effect of leaf rolling on transpiration is dependent on stomatal distribution, and on the degree and pattern of stomatal opening in rolled leaves. Rolling prevents water loss but also restricts potential carbon gain and may place the plant at a competitive disadvantage especially if adequate soil water is available. Infected plants had significantly lower P_n rates than E– plants on four out of five measuring dates and they tended to produce less above-ground biomass than E– plants regardless of treatment or maternal genotype. Likewise, within E+ plants, H1 plants rolled leaves more tightly than H2 plants, and also had less negative Ψ_L than H2 plants, further supporting the link between increased leaf rolling and less negative Ψ_L. Endophyte haplotype also affected stomatal density and gas exchange rates. Stomata formation in general is primarily controlled by the hormone gibberellic acid (Saibo *et al.* 2003), and endophyte infection in particular may alter other plant hormones, such as auxin (indoleacetic acid; IAA) (de Battista *et al.* 1990; Yue *et al.* 2001) that alter physiological and growth responses. Thus, our results suggest that endophyte haplotype and its interaction with plant genotype also influences levels of plant hormones, as indicated by differences in stomatal density between H1 and H2 endophyte-infected plants.

To our knowledge this is the first study where respiration was measured with respect to endophyte infection and endophyte haplotype. Respiration is an important process in plants where carbohydrates and other molecules are oxidized for the purpose of retrieving the energy stored during photosynthesis and to obtain carbon skeletons used in the growth and maintenance of the plant. *Neotyphodium* infected plants had higher rates of respiration than E– plants, which may be due to endophyte respiration. The endophyte utilizes photosynthates from its host to carry out metabolic processes necessary for its survival and growth, and thus may be increasing the total respiration rates that were measured using an IRGA. Within infected plants, there is also variation in rates of respiration related to endophyte haplotype. Endophyte H2 had greater respiration rates than H1 on three out of the five dates. Thus, respiration is not simply a function of whether plants are infected or not, but also on the specific strain of endophyte inhabiting the host grass. During some periods, the variation in host respiration and other physiological parameters arising from different endophyte haplotypes is greater than that simply based upon infection status (e.g. Fig. 2e,j).

Our general findings show that *Neotyphodium* haplotype and to a lesser extent *Neotyphodium* infection, host plant maternal genotype and environmental conditions influence Arizona fescue physiology, growth and biomass production. The prevailing notion that asexual endophytes interact mutualistically with their hosts based upon studies involving agronomic grasses (e.g. Clay & Schardl 2002) clearly does not consistently hold in this wild grass; instead interactions vary significantly with endophyte haplotype, plant genotype and water availability. Therefore, it is likely that these factors also influence other interactions with the host grass, such as susceptibility to herbivory and pathogens, inter- and intraspecific competition, community structure and ecosystem functions. Nearly all tests of the effects of *Neotyphodium* infections at the community and ecosystem level have involved agronomic grasses, such as Tall Fescue, infected with a single endophyte strain (e.g. Clay *et al.* 2005) Extrapolating from agronomic grass studies to natural populations and communities may be far too simplistic, given that agronomic grasses and their endophytes typically have much less genetic diversity due to selective breeding and genetic bottlenecks (Saikkonen *et al.* 2004, 2006), and are grown in environments that are less variable than natural populations. With the greater genetic diversity in both the endophyte (e.g. Faeth & Sullivan 2003) and its grass host (e.g. Saikkonen *et al.* 2004, 2006), natural populations should exhibit much greater complexities. Based upon our results that endophyte haplotype, plant maternal genotype and environmental factors, in addition to infection, result in highly variable outcomes in terms of host performance and physiology, we conclude it will be extraordinarily difficult to predict effects at the population, community and ecosystem levels.

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Supplementary material

The following supplementary material is available as part of the online article (full text) from http://www.blackwell-synergy.com

Table S1 Summary of ANOVA for the effects of plant genotype (nested within haplotype), infection status and water treatments on biomass and growth parameters (R:S = belowground to aboveground biomass ratio; RGR = relative growth rate; NAR = net assimilation rate; LAR = leaf area ratio; SLM = specific leaf mass).

Table S2 Summary of means and standard errors of *Neotyphodium* infected (E+) and uninfected (E–) Arizona fescue, and *Neotyphodium* infected Arizona fescue haplotype H1 and haplotype H2 growth parameters, leaf water potential, leaf rolling, and stomatal density (RGR = relative growth rate, $g\ g^{-i}\ d^{-1}$; total leaf area, cm^2; NAR = net assimilation rate, $g\ m^{-2}\ d^{-1}$; LAR = leaf area ratio, $cm^2\ g^{-1}$; SLM = specific leaf mass, $mg\ cm^{-2}$; Ψ_L = leaf water potential, MPa; leaf rolling, actual leaf width/opened leaf width; stomatal density, mm^1).

Table S3 Summary of ANOVA of the effects of endophyte haplotype and water treatments on biomass and growth parameters (R:S = belowground to aboveground biomass ratio; RGR = relative growth rate; NAR = net assimilation rate; LAR = leaf area ratio; SLM = specific leaf mass). Only infected plants were included in these analyses.

Table S4 Summary of repeated measures ANOVA for the within-subjects effects of plant genotype (nested within haplotypes), infection status, and water treatment on gas exchange parameters (P_n = leaf net photosynthesis; g_s = leaf conductance to water vapor; C_i; internal CO_2 concentration; WUE = water use efficiency; Resp = leaf dark respiration).

Table S5 Summary of ANOVA for the between-subject effects of plant genotype (nested within haplotypes), infection status, and water treatment on leaf water potential (Ψ_L), leaf rolling, and stomatal density. Within-subject effects involving time are not shown for brevity but are discussed in text.

Table S6 Summary of repeated measures ANOVA for the between-subject effects of endophyte haplotype and water treatment on gas exchange parameters (P_n = leaf net photosynthesis; g_s = leaf conductance to water vapor; C_i; internal CO_2 concentration; WUE = water use efficiency; Resp = leaf dark respiration). Within-subject effects involving time are not shown for brevity but are discussed in text.

Table S7 Summary of ANOVA for the effects of endophyte haplotype and water treatment on leaf water potential (Ψ_L), leaf rolling, and stomatal density.