

Herbivores cause a rapid increase in hereditary symbiosis and alter plant community composition

Keith Clay*, Jenny Holah†, and Jennifer A. Rudgers

Department of Biology, Indiana University, Bloomington, IN 47405

Edited by Nancy A. Moran, University of Arizona, Tucson, AZ, and approved July 19, 2005 (received for review April 13, 2005)

Microbial symbioses are ubiquitous in nature. Hereditary symbionts warrant particular attention because of their direct effects on the evolutionary potential of their hosts. In plants, hereditary fungal endophytes can increase the competitive ability, drought tolerance, and herbivore resistance of their host, although it is unclear whether or how these ecological benefits may alter the dynamics of the endophyte symbiosis over time. Here, we demonstrate that herbivores alter the dynamics of a hereditary symbiont under field conditions. Also, we show that changes in symbiont frequency were accompanied by shifts in the overall structure of the plant community. Replicated 25-m² plots were enriched with seed of the introduced grass, *Lolium arundinaceum* at an initial frequency of 50% infection by the systemic, seed-transmitted endophyte *Neotyphodium coenophialum*. Over 54 months, there was a significantly greater increase in endophyte-infection frequency in the presence of herbivores (30% increase) than where mammalian and insect herbivory were experimentally reduced by fencing and insecticide application (12% increase). Under ambient mammalian herbivory, the above-ground biomass of nonhost plant species was reduced compared with the mammal-exclusion treatment, and plant composition shifted toward greater relative biomass of infected, tall fescue grass. These results demonstrate that herbivores can drive plant-microbe dynamics and, in doing so, modify plant community structure directly and indirectly.

dynamics | grasslands | herbivory | endophyte

Most prior research on symbiotic interactions has focused on the pairwise dynamics between host and symbiont without considering the broader biological community (1–3). However, symbionts can alter the phenotype of their host in ways that may affect interactions with other species as well as the relative fitness of each partner. Some symbionts can increase their hosts' susceptibility to predation (4). Other symbionts protect hosts from consumers. For example, gut bacteria in locusts produce phenolics that defend these insects from fungal infection (3). Similarly, actinomycete bacteria associated with leaf-cutting ants may guard fungal symbionts from pathogen attack (5). Both endophytic and mycorrhizal fungi can reduce herbivore and pathogen damage to their plant hosts (6–10). Investigations of species interactions extrinsic to the symbiosis may provide insights into factors that influence the long-term dynamics of symbioses and lead to fixation, loss, or intermediate frequency of the resident symbiont over time.

In hereditary symbioses, genomes of both partners are coinherited. Therefore, these symbionts are linked directly to evolutionary changes in their host populations (11, 12). Hereditary symbionts are transmitted across generations through eggs, seeds, or clonal propagules, and rarely through sperm. They include a diversity of interactions (13) and are especially well known in arthropods and their obligate associations with vertically transmitted bacteria (3, 14). Also, hereditary fungal symbionts may be widespread in seed plants, including the families Casuarinaceae, Cistaceae, Convolvulaceae, Fabaceae, Pinaceae, and Poaceae (15–18). These hereditary symbionts are distinguished from seed-infecting fungi, which are not vertically transmitted (19). Grasses that are infected by fungal endophytes that are vertically transmitted through seeds are a familiar example (20). Seed-transmitted fungal endophytes may

alter host phenotypes by producing bioactive secondary compounds. In contrast to many symbiotic systems, these plant-endophyte interactions are facultative, allowing for experimental investigations of factors affecting the dynamics of the symbiosis over time.

Here, we focus on the widespread symbiosis between tall fescue grass (*Lolium arundinaceum*) and the endophytic fungus *Neotyphodium coenophialum*, which grows systemically in above-ground tissues and is vertically transmitted through seeds. The association between Pooidae grasses and hereditary endophytes is estimated to be ≈ 40 million years old, and vertically transmitted *N. coenophialum* originated through hybridization among sexual *Epichloë* species, which are capable of horizontal transmission by ascospores (21). To our knowledge, there is no known mechanism or evidence of horizontal transmission of the endophyte in tall fescue (22). Endophyte infection has been reported to enhance competitive ability (23), increase drought tolerance (24, 25), and improve nutrient acquisition (26), but it can act as a pathogen or commensal in some circumstances (27–29). Also, the alkaloids produced by the tall fescue endophyte can deter herbivores and granivores (30). Various compounds are produced, including ergovaline and other ergot alkaloids, loline alkaloids, and peramines; endophyte-free plants lack these compounds (31).

From its Mediterranean center of origin, tall fescue has attained a worldwide distribution in a diversity of managed and unmanaged habitats (32, 33). It has been the subject of much research, given its ecological and economic dominance ($>15,000,000$ hectares in the eastern United States, with an average of 80% of the grass infected by the endophyte; ref. 34), but it may not be typical of all grass-endophyte interactions (27), especially those that are characterized by little or no alkaloid production (31).

Endophyte-infection frequency in tall fescue varies considerably among natural and managed populations. Understanding the factors that generate this variation may provide insights into the maintenance of this and other endophyte associations. At most sites, tall fescue is infected at a high rate. However, endophyte-free sites and sites with low levels of infection ($<25\%$) exist also. For example, in the United States, where tall fescue is introduced, 58% of 1,483 samples from 26 states were infected by the endophyte (35). In Kentucky, 97% of 200 fields spanning 42 counties were infected, with mean infection frequencies varying 67–100% (36). In six Illinois sites, infection frequency varied 68–100% (37). A variable but high endophyte frequency is typical of native European and African tall fescue as well. In Britain, 15 sites had a mean infection rate of 64%; 9 of the sites had mean infection levels of $\geq 85\%$, but 3 sites were endophyte-free (37). In Finland, endophyte-infection frequency averaged 98% in 13 of 15 sampled sites, whereas the other 2 sites were endophyte-free (38). In France, infected plants were present at 27% of 41 sampled sites, although a higher

This paper was submitted directly (Track II) to the PNAS office.

Abbreviation: MANOVA, multiple ANOVA.

*To whom correspondence should be addressed. E-mail: clay@indiana.edu.

†Present address: Graduate School of Education, George Mason University, Fairfax, VA 22030.

© 2005 by The National Academy of Sciences of the USA

incidence of infection (43% of 14 sites) was detected in seed samples from European botanical gardens (39). In Spain, 15 of 17 seed accessions contained the endophyte, with a mean infection frequency ranging 7–100% (40). Last, 99 of 104 collections of tall fescue that were sampled from Morocco, Tunisia, and Sardinia (Italy) were infected at a mean rate of 77% (41). In most of these studies, widely spaced tillers were sampled to estimate infection frequency. Although this method does not necessarily provide a direct estimate of the frequency of infected vs. uninfected plants, it does estimate the relative biomass or cover of infected plants. Surveys of endophyte-infection frequency have been conducted also in other regions and with other grass species, and they demonstrate similar variability in infection frequencies (42–49).

Why do populations vary in infection rate? For endophyte-free populations, founder effects are the most likely cause. The endophyte in tall fescue is completely vertically transmitted (22). Therefore, if founding seeds are endophyte-free, and there is no long-distance seed dispersal, the population should remain endophyte-free. Also, several studies have demonstrated that fungal viability in seeds declines over time depending on environmental conditions (50, 51). Long-term dormancy in the seed bank or seed storage can also result in loss of infection and, thus, produce a patchwork uninfected populations within a matrix of highly infected populations. More interesting are situations in which infection rates are high but <100%. One possible mechanism is imperfect vertical transmission, such that each new generation of seedlings has a lower level of infection than the parental populations (29). Models suggest that metapopulation dynamics could also maintain high levels of infection as long as vertical transmission is 100% effective, even if the symbiont is not mutualistic (52). Also, populations at <100% endophyte frequency may be in a transitional state that will eventually reach 100% infection. Last, and perhaps most important, intermediate infection rates may reflect variation in the selective advantage (or disadvantage; see ref. 53) of endophyte symbiosis in spatially or temporally heterogeneous environments (27). Three previous studies with tall fescue (reviewed in ref. 30) showed that endophyte frequency increased over time, with more rapid increases in populations beginning at a lower endophyte frequency. However, ecological conditions were not manipulated experimentally to determine the factors that cause endophyte frequency to change.

The objective of this study was to identify the ecological mechanisms affecting the dynamics of endophyte symbiosis over time. Specifically, we tested the hypothesis that herbivory increases the frequency of the endophyte in populations of tall fescue. By independently reducing insect and vertebrate herbivory, we were able to assess the relative importance of these two groups of herbivores. Also, we tested the hypothesis that changes in infection level in tall fescue would be associated with concomitant changes in plant community composition. This work provides a mechanistic link between a previous study showing divergence in the plant community between highly (>90%) and low (<5%) infected tall fescue plots (34) and several studies documenting resistance of endophyte-infected tall fescue to individual mammalian or invertebrate species. The list of herbivores that are affected by endophytes in grasses includes >40 invertebrate species, as well as vertebrates such as cattle, horses, goats, sheep, deer, rabbits, voles, rats, and birds (see refs. 30 and 54–56). Tests of these hypotheses contribute to our understanding of the community-level consequences of plant–microbe symbiosis, and they determine the ecological importance of herbivore pressure in driving the long-term dynamics of symbiosis.

Materials and Methods

Site. We established 60 field plots (5 × 5 m) during the summer of 1996 in Bloomington, IN (North 39°13'9", West 086°32'29"). The site supported herbaceous perennial vegetation, including tall fescue, which was maintained by mowing two or three times per

year. Our treatments manipulated mammalian and insect herbivory in a 2 × 2 factorial design ($n = 15$ plots per treatment, randomly assigned), with a fence treatment plus supplemental vole (*Microtus* spp.) removal to reduce mammals or an unfenced treatment to allow ambient mammalian herbivory, and we used Malathion insecticidal spray to deter insects or a water-spray treatment to allow ambient insect herbivory. The area was plowed and disked. In fenced plots, steel hardware cloth (120 cm in width, 1.25-cm mesh) was inserted into trenches (40–50 cm in depth) and attached to steel corner posts to inhibit burrowing mammals. Aluminum flashing (30 cm in width) was attached above the cloth to inhibit climbing by small mammals. Chicken wire (90 cm in width, 5-cm mesh) was attached above the flashing to deter larger mammals.

Seedling Establishment. *L. arundinaceum* seeds were sown in September of 1996. The endophyte was eliminated by long-term storage of infected seeds at room temperature, which reduces endophyte viability but not seed viability. Seeds were several generations removed from the storage treatment and came from parents that freely cross-pollinated (*L. arundinaceum* is self-incompatible), allowing for homogenization of the plant genetic background with respect to the endophyte treatment. Other plant species colonized plots from the seed bank, vegetative fragments, or dispersal from surrounding areas. Endophyte-infected and uninfected seeds were combined to create a 50:50 seed mixture (seed source, germination rate, and confirmation of infection according to the method described in ref. 34) and sown at 112.5 g per plot. To estimate initial endophyte frequency, a random sample of 20 seedlings per plot was collected in November of 1996, grown in a greenhouse, and examined microscopically.

Endophyte Frequency. Changes in endophyte frequency were quantified by repeatedly sampling experimental grassland plots enriched with tall fescue seed at an initial endophyte frequency of 50%. There is no contagious spread or loss of the endophyte from adult plants (20); therefore, any changes in infection result from differential survival, clonal growth, and/or reproduction of individual plants. Each June and October (June 1997–June 2001), ≈50 *Lolium* tillers were collected from each plot. Each plot was divided into a 5 × 5 grid, and two tillers were collected at random per grid square. In October of 2000, 100 tillers were collected (four per grid square). To score infection, a thin cross section of each tiller was placed on a nitrocellulose membrane with known positive and negative controls and treated with dye-linked monoclonal antibodies specific for the fungal endophyte (57) (Fig. 1). Two researchers scored membranes independently and without knowledge of treatment. Ambiguous blots (<5% of total) were reexamined under a dissecting scope. Initial endophyte frequency (angular-transformed) was analyzed with factorial ANOVA (PROC GLM 2000, version 8.1; SAS Institute, Cary, NC). For each census date, the change in endophyte frequency was calculated as the present endophyte frequency minus initial endophyte frequency. The change in endophyte frequency, including the repeated effect of census date and all interactions, was analyzed with repeated-measures multiple ANOVA (MANOVA). If we detected significant interactions between treatments and time, we used a (two-tailed) Dunnett's test of difference from the control to test for differences among treatments for each census date while controlling for multiple comparisons; a Tukey's honestly significantly different test yielded similar results. Data met assumptions of normality (Shapiro–Wilks test) and equality of variances (Levene's test) after angular transformation.

Vole Trapping and Insecticide Application. Voles (*Microtus* spp.) have been identified as the most important vertebrate herbivores in local tall fescue grasslands (58). Voles were trapped in all plots 10 times during the experiment (Fig. 2B) and nine additional times in fenced plots to ensure reductions in vole abundance. On these nine additional dates, a mean of 0.53 (±0.08 SE) voles were removed per

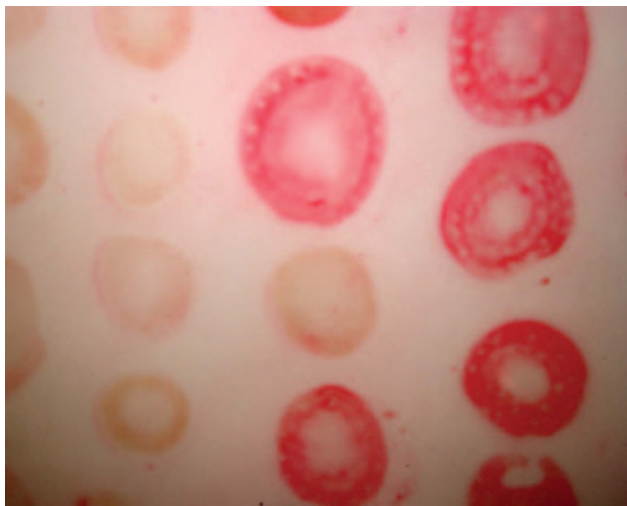


Fig. 1. Tissue-print immunoblot analyses. Red circles indicate *N. coenophialum*. Hyphal cross sections are clustered around vascular bundles of *L. arundinaceum*. Pink circles indicate uninfected tillers.

plot per date. Live traps were baited with rolled oats, set during the evening, and checked the next morning. Each plot typically received two live traps per trapping date. Voles were removed from fenced plots and released into adjacent unfenced plots. Most voles were *Microtus ochrogaster* (prairie vole), with 10–20% *Microtus pennsylvanicus* (meadow vole). Vole number per trap was determined for each plot and square-root-transformed to meet assumptions of normality and equality of variances. Data were analyzed with repeated-measures MANOVA, as described above.

Beginning in the spring of 1997, Malathion was sprayed every 2 weeks (April 1 to first frost) on one-half of the plots. At the same time, an equal volume of water was applied to non-insecticide-treated plots. Malathion is a nonsystemic, contact organophosphate insecticide that has no effects on plant growth (23, 59). Insecticide (or water) application was performed with an engine-powered sprayer (113.6-liter tank, 400 ml of Malathion per tank). Each plot was sprayed for 40 s by using a continuous sweeping motion. Sweep-net sampling assessed arthropod abundance three times during the experiment. Each plot was swept 25 times with a 15-cm (diameter) net. Arthropods were identified to order in the laboratory and classified as herbivores (a majority of Coleoptera, Homoptera, Hemiptera, Orthoptera, and Lepidoptera) or predators (mainly Aranae, Odonata, and Hymenoptera). Sweep-net samples of arthropods that were collected in nearby plots at the same site were identified to family, genus, and/or species. These data showed that herbivores constituted 100% of the collected Homoptera, Lepidoptera, and Orthoptera individuals, \approx 99% of the Coleoptera, and 71% of Hemiptera. The total number of herbivorous (or predaceous) individuals per plot per census was analyzed with repeated-measures MANOVA, as described above. Data met assumptions of normality and equality of variances.

Plant Composition. To assess the effects of treatments on tall fescue as well as other plant species in the community, we harvested subplots of above-ground biomass in June of 2001 at the conclusion of the experiment. Plant cover was visually estimated over the course of this study, but only results from above-ground biomass harvests at the end of the study are reported here. Above-ground biomass was harvested from four 0.5×0.5 -m quadrats per plot according to the method described in ref. 34. Biomass was sorted into tall fescue, nonfescue grasses, or forbs and then dried and weighed. Other common grasses besides tall fescue included *Agrostis alba*, *Bromus commutatus*, *Dactylis glomerata*, *Elymus repens*,

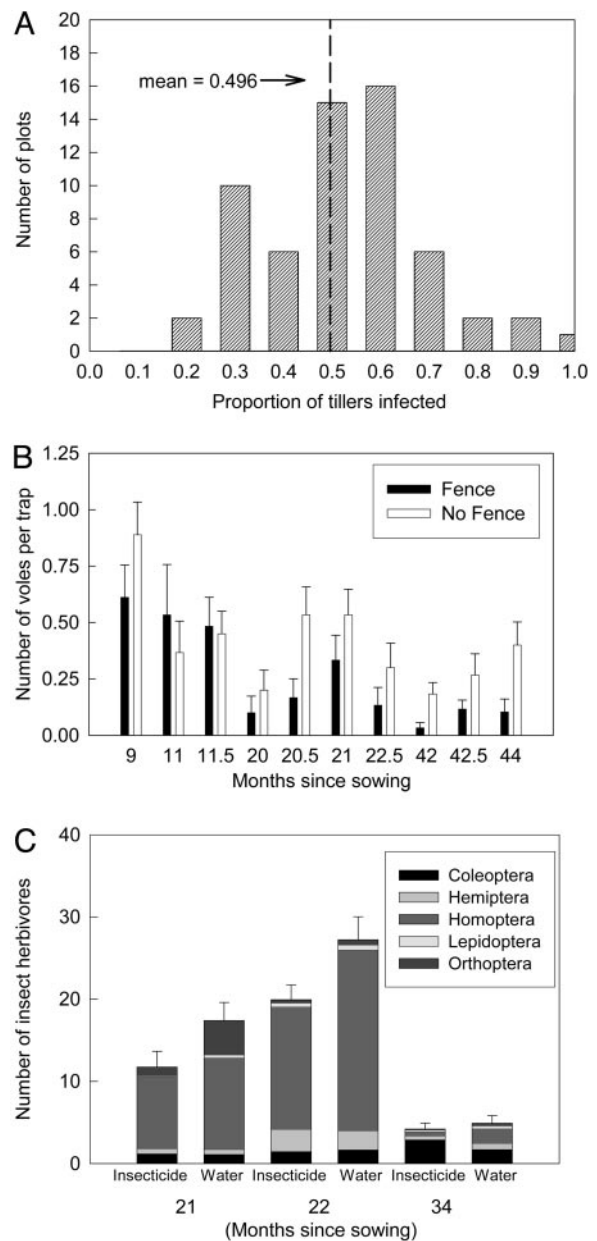


Fig. 2. Efficacy of experimental treatments. (A) Distribution of the initial proportion of infected tillers per plot, which did not deviate significantly from 0.5 (mean, 0.496; 95% confidence interval, 0.452–0.540), by using the bias-corrected accelerated bootstrap with 10,000 resamples. (B) The number of voles per trap for fenced vs. unfenced plots across 10 census dates. (Fence, $F_{1,55} = 13.1$, $P = 0.0007$; fence \times time, $F_{9,47} = 0.6$, $P = 0.8$.) (C) The number of herbivorous insects collected per plot for plots sprayed with the insecticide (Malathion) vs. plots sprayed with water for three census dates. Within a bar, data are divided by insect orders. (Insecticide, $F_{1,56} = 6.7$, $P = 0.012$; insecticide \times time, $F_{2,55} = 1.3$, $P = 0.3$.) Error bars indicate means \pm SE. The insecticide did not affect voles ($F_{1,55} = 0.9$, $P = 0.3$; fence \times insecticide, $F_{1,55} = 0.04$, $P = 0.8$), nor did fencing affect herbivorous arthropods ($F_{1,56} = 1.6$, $P = 0.2$; fence \times insecticide, $F_{1,56} = 0.7$, $P = 0.4$) or predaceous arthropods ($F_{1,56} = 1.7$, $P = 0.2$; fence \times insecticide, $F_{1,56} = 0.61$, $P = 0.4$).

Elymus villosus, and *Poa pratensis*. Of these grasses, all but one (*B. commutatus*) have been reported to be infected with systemic, endophytic fungi (60), but in southern Indiana, we have found only *E. villosus* to be infected (44). Dominant forbs included *Ambrosia trifida*, *Cirsium arvense*, *Conyza canadensis*, *Plantago lanceolata*, *Rumex crispus*, *Solidago* spp., and *Vernonia altissima*. Biomass data

were analyzed in combination with mixed-model MANOVA. When effects were significant in MANOVA, we proceeded with mixed-model ANOVA for each type of biomass measured (tall fescue, nonfescue grasses, or forbs), including the random effect of plot nested within fence \times insecticide (PROC MIXED, SAS Institute). Assumptions of normality and equality of variances were met after log transformation of all response variables.

Tall Fescue Fitness. To examine the effects of herbivores on the fitness of tall fescue plants, we used above-ground biomass of tall fescue as our fitness estimate. Like many perennial grasses, tall fescue spreads largely through clonal growth, with infrequent seedling establishment as soon as dense swards are formed (61, 62). Thus, biomass constitutes a particularly important component of fitness. Seed and pollen production can also contribute to tall fescue fitness, especially by means of dispersal into new habitats, and seed (but not pollen) production increases the fitness and dispersal of the endophyte. We did not directly measure seed or pollen production of tall fescue in this experiment, but two related and concurrent experiments demonstrated that tall fescue biomass was highly correlated with the number of inflorescences (seed heads) that were produced. In the same site as used in this study, by using the same seed stock, 100% E⁺ or 100% E⁻ seeds were planted in alternating 30 \times 30-m plots during 2000 (total of 16 plots). The Pearson's correlation coefficient for biomass vs. inflorescence number during June of 2003 was $r = 0.83$ ($P < 0.0001$, $n = 128$ subplots measuring 0.5 m²). The endophyte status of the plot did not affect the slope of this relationship ($F = 0.39$, $P = 0.53$). Similarly, in an experiment using related seed stock at another site 3-km distant (details are given in ref. 34), the Pearson's correlation coefficient for biomass vs. inflorescence number was $r = 0.92$ ($P < 0.0001$, $n = 160$ subplots measuring 0.5 m²). Again, the presence of the endophyte did not affect this relationship ($F = 2.11$, $P = 0.15$). Other studies (63–65) also have found that the endophyte increases seed production and survival in tall fescue.

Results

Initial Endophyte Frequency. We estimated endophyte-infection frequency of the plots as the proportion of tall fescue tillers that were infected by the endophyte. Across all plots, our estimate of the initial proportion of infected tillers did not deviate significantly from 0.5 (Fig. 2A). Initial infection rate was consistent among plots regardless of their subsequent treatment with insecticide (insecticide: $F_{1,56} = 1.2$, $P = 0.3$; fence \times insecticide: $F_{1,56} = 0.1$, $P = 0.8$). In contrast, fenced plots had significantly higher initial infection than unfenced plots (mean \pm SE; unfenced, 0.44 ± 0.02 ; fenced, 0.56 ± 0.03 ; $F_{1,56} = 8.3$, $P = 0.006$). This pattern is the reverse of that expected if herbivores preferentially consumed uninfected tall fescue seeds or seedlings. Nevertheless, initial differences had no effect on infection dynamics over time as evidenced by the fact that initial endophyte frequency was independent of final endophyte frequency (Pearson's correlation coefficient, $r = -0.03$, $P = 0.8$).

Treatment Effectiveness. The fencing treatment reduced the vole capture rate significantly (Fig. 2B). Similarly, the insecticide treatment reduced herbivorous insects significantly (Fig. 2C). Predaceous arthropods were reduced also by the insecticide (mean \pm SE; water treatment, 4.8 ± 0.6 ; insecticide, 2.5 ± 0.4 ; $F_{1,56} = 25.8$, $P < 0.0001$), but herbivorous insects still remained fewer than in the water-treated plots (Fig. 2C). Because neither treatment was 100% effective, our experiment underestimates the impact of voles and herbivorous insects on infection frequency.

Dynamics of the Symbiosis. Across all plots, endophyte-infection frequency (as estimated by the proportion of tillers with the endophyte) increased from an initial level of 50% to 67% over 54 months (Fig. 3). Infection frequencies among treatments diverged over time as indicated by the time \times fence \times insecticide interaction

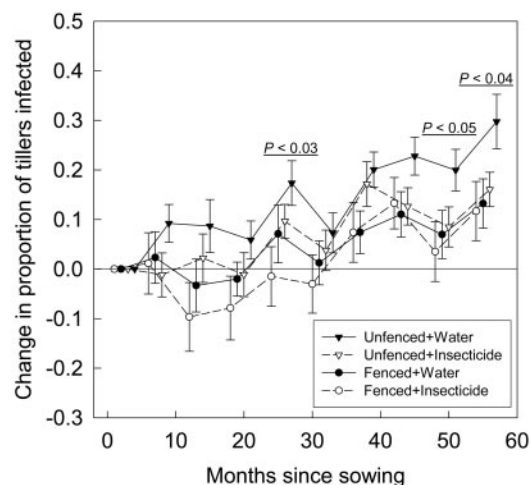


Fig. 3. The change in endophyte frequency among treatments. The change in frequency was determined by subtracting the initial proportion of tillers infected in that plot from the proportion of tillers infected on each date. The change in proportion is bounded by -0.5 and 0.5 (0% and 100% infected, respectively). Over time, infection increased in all plots (time, $F_{8,47} = 29.6$, $P < 0.0001$). Treatments diverged over time (fence \times insecticide \times time interaction, $F_{8,47} = 2.8$, $P = 0.01$). No main effects or two-way interactions were significant (fence, $F_{1,54} = 3.1$, $P = 0.08$; fence \times time, $F_{8,47} = 1.4$, $P = 0.2$; insecticide, $F_{1,54} = 1.9$, $P = 0.2$; insecticide \times time, $F_{8,47} = 0.8$, $P = 0.6$; fence \times insecticide, $F_{1,54} = 0.43$, $P = 0.5$). Symbols show means \pm SE and are slightly offset to show error bars clearly. P values indicate a significant difference between the dual herbivore-exclusion treatment (fenced plus insecticide) and the control (unfenced plus water) for each date.

(Fig. 3). Specifically, endophyte frequency increased more under ambient herbivory (unfenced plus water, 30%) than in the fenced plus insecticide treatment (12%), and this difference was significant when averaged across all census dates (orthogonal contrast, $F_{1,54} = 5.1$, $P = 0.03$). When treatment effects were decomposed by census date, the difference between the control plots and the dual herbivore-exclusion plots was significant on multiple, but not all, censuses, as shown in Fig. 3. Neither the main effect of fencing nor that of insecticide was statistically significant (Fig. 3), demonstrating that the interactive effects of mammalian and invertebrate herbivores were driving the dynamics of the symbiosis. Also, there was evidence of seasonal fluctuations, with a large increase in infection frequency between spring and fall censuses across all treatments in 1998 and 1999 (months 25 and 37, respectively). This increase could indicate an advantage of endophyte infection under summer drought stress, as has been reported frequently (24, 47).

Plant Community Composition and Tall Fescue Fitness. Changes in plant community structure, measured as above-ground biomass at the end of the study, accompanied changes in infection within experimental plots. All plots were initially established under the same conditions, and treatments were assigned randomly with respect to the preexisting seed bank. Therefore, any differences in plant composition were attributable to the herbivory treatments. Tall fescue biomass was 58% greater in unfenced plots than in fenced plots (Fig. 4A). The significant increase in tall fescue biomass arising from greater numbers and/or larger sizes of individual plants in the unfenced plots suggests that tall fescue experiences fitness benefits in the presence of mammalian herbivores. In contrast to tall fescue biomass, the biomass of forbs (Fig. 4B) and nonfescue grasses (fenced, 41.4 ± 6.6 g; unfenced, 39.6 ± 3.8 g; $F_{1,56} = 3.9$, $P = 0.05$) declined in unfenced plots. With the possible exception of *E. villosus* (which was not assessed), the nonfescue grass biomass was endophyte-free. Although fencing shifted the relative biomass of species, it had no effect on total plant biomass,

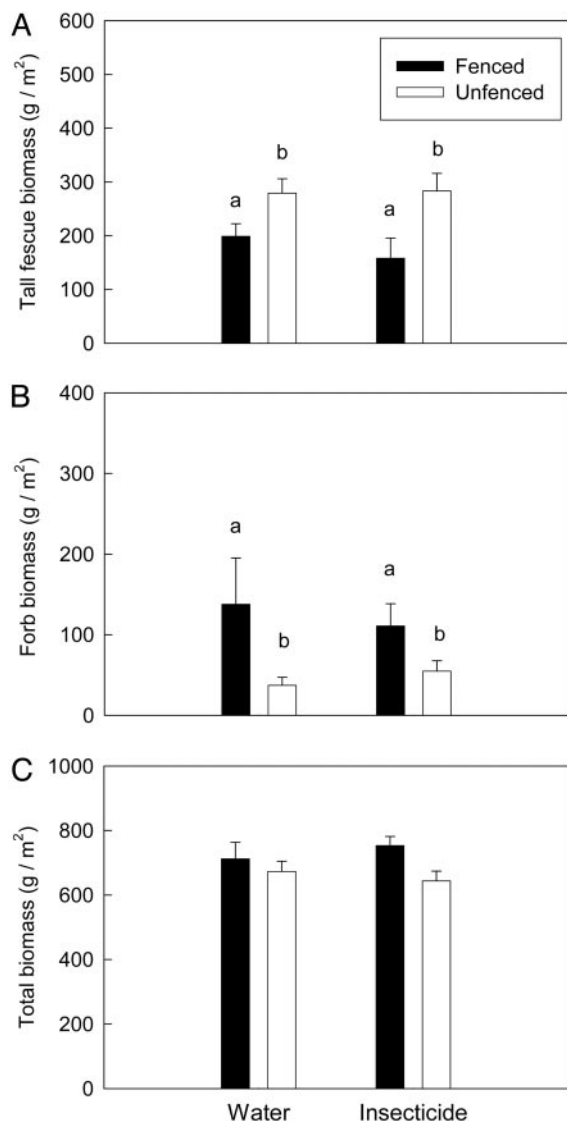


Fig. 4. Plant composition among treatments. Fencing significantly affected tall fescue biomass (fence, $F_{1,56} = 4.4$, $P = 0.04$) (A) and forb biomass (fence, $F_{1,56} = 4.4$, $P = 0.04$) (B) but not total biomass (fence, $F_{1,56} = 3.0$, $P = 0.1$) (C). Total biomass was calculated as the sum of tall fescue, forb, nonfescue grasses, and thatch biomass. Error bars show means \pm SE. Different letters indicate significant differences among treatments. The insecticide treatment did not significantly affect the biomass of tall fescue ($F_{1,56} = 2.1$, $P = 0.2$; insecticide \times fence, $F_{1,56} = 1.8$, $P = 0.2$), forbs ($F_{1,56} = 0.6$, $P = 0.4$; insecticide \times fence, $F_{1,56} = 0.1$, $P = 0.8$), nonfescue grasses ($F_{1,56} = 0.7$, $P = 0.4$; insecticide \times fence, $F_{1,56} = 0.0$, $P = 0.9$), or total biomass ($F_{1,56} = 0.0$, $P = 0.9$; insecticide \times fence, $F_{1,56} = 1.6$, $P = 0.2$).

which is a measure of primary productivity (Fig. 4C). In contrast to fencing, the insecticide did not affect plant composition (Fig. 4), suggesting that any changes in endophyte frequency due to insect herbivory were not accompanied by significant shifts in the plant community.

Discussion

Our results demonstrate that biological pests significantly increase the frequency of a hereditary symbiont over time. This experimental study identifies herbivory as an important mechanism in the dynamics of hereditary symbiosis. The increase in frequency of the *Neotyphodium* endophyte in tall fescue grass was 2.5 times greater in control plots with herbivores than in the dual mammalian and invertebrate herbivore-exclusion treatment. This effect was pre-

sumably in part due to the herbivore-deterrent alkaloids present only in endophyte-infected plants (31). Although we did not quantify alkaloids in this study, their production in endophyte-infected tall fescue is well documented (31, 66).

The importance of herbivores in the dynamics of plant-microbe interactions may be widespread. Although our results provide the first evidence for long-term impacts of herbivores on a hereditary symbiont, other studies have found strong interactions between herbivores and horizontally transmitted symbionts such as mycorrhizal fungi, pathogens, and other endophytic fungi (7, 67–69). For example, in one of the few long-term studies of herbivore effects, high densities of scale insects reduced colonization by mycorrhizal fungi on pinyon pines that were susceptible to scale attack (70). However, with herbivores and mycorrhizal fungi, the effects are typically in the opposite direction of the results presented here, in which the herbivores suppress, rather than promote, the symbiont. The direction of the herbivore effect may hinge on how the symbiont alters the herbivore resistance of its host.

The increase in symbiont frequency that occurred even when both mammalian and insect herbivores were reduced (a 12% increase) suggests that even low levels of herbivory may benefit infected plants. Our exclusion treatments were not 100% effective, and other groups of plant consumers (such as plant pathogens, nematodes, and mollusks) were not experimentally manipulated. Also, other benefits of the endophyte, such as enhanced drought tolerance or nutrient acquisition, may also have contributed to increasing endophyte frequency in the dual exclusion plots (24, 25). However, in the absence of interactive effects with our treatments (e.g., plants treated with insecticide have increased drought tolerance or reduced pathogen attack), these alternative benefits and uncontrolled consumption of tall fescue would have been spread evenly over all replicates. Thus, the 2.5-fold difference in infection frequency (30% vs. 12%) represents the effect of our experimental reduction in vertebrate and invertebrate herbivory. Had this experiment continued longer, endophyte frequency may have reached fixation (100% of plants with the endophyte), but metabolic costs of infection, variation in environmental conditions (including herbivore pressure), and/or loss of infection from dormant seeds could maintain variation in infection frequency (28, 29).

Not only were the dynamics of the symbiont altered by herbivores, but the relative biomass of the host plant shifted also. Mammalian herbivory increased tall fescue biomass by 58% in unfenced plots compared with fenced plots. Biomass is an important component of fitness for this clonal species, and it is also highly correlated with inflorescence production and, therefore, reproductive fitness. Thus, when the endophyte is present, tall fescue clearly benefits from mammalian herbivory. We estimated the percentage of total live biomass that consisted of infected tall fescue by multiplying the percentage of total biomass that was tall fescue in fenced plots (38%) vs. unfenced plots (58%) by their mean endophyte-infection frequencies. In fenced plots, 26% of the total biomass was infected tall fescue, vs. 39% in unfenced plots (a 48% increase). Given the lower initial infection in unfenced plots, this is a conservative estimate of the increase in endophyte-infected biomass. This result is relevant to previous research on the possible benefits to plants from herbivory (71–73) and shows that herbivores can increase the competitive dominance of toxic plants. However, in the case of tall fescue, the herbivore resistance traits are not intrinsic to the plant but are rather provided by the endophyte symbiont. Although herbivory may be harmful to individual plants, it is clearly advantageous to infected tall fescue populations (74).

Mammalian herbivory not only increased the biomass of tall fescue but also reduced the biomass of forb and nonfescue grass species in the community. In contrast to mammalian herbivory, insect herbivory did not affect tall fescue biomass or plant composition, although it did affect endophyte frequency in combination with mammalian herbivory. Therefore, the change in endophyte frequency was not the only factor altering composition of the plant

community. These results suggest that mammalian herbivores affect plant composition both directly by preferentially consuming nonfescue plants as well as indirectly by altering endophyte frequency. It has been found that voles consume significantly more nonfescue plants in plots of 100% endophyte-infected tall fescue than in endophyte-free plots (J.A.R., S. P. Orr, and K.C., unpublished data). The observed shift in plant composition was consistent with ref. 34, which reported reduced species richness and increased tall fescue biomass in experimental grasslands, with 100% infected tall fescue compared with endophyte-free tall fescue. Results presented here demonstrate that herbivores modify community structure by increasing the biomass of herbivore-resistant, endophyte-infected tall fescue.

Although our focus was on tall fescue and changes in the plant community, our results point to an important role of herbivores for the fitness of the fungal endophyte. Because it has no free-living or contagious stage, *N. coenophialum* can increase its fitness in only two ways: by clonal spread of its host plants or vertical transmission to seeds. Our data suggest that both mechanisms are enhanced by herbivory. The percentage of biomass infected with the endophyte increased most in the presence of herbivores, and biomass is highly correlated with seed production in this system. Our calculations in the above paragraph suggest that fungal fitness is 48% higher in the presence (unfenced plots) than absence (fenced plots) of mammalian herbivores. There was no evidence for a net cost to the fungus of symbiosis with tall fescue because the frequency of endophyte infection increased over time in all of our experimental treatments.

Our results have broad implications for understanding the success of invasive species. Plants invading novel habitats may frequently suffer less damage from pests and parasites than native species (75–77). Also, invasive plants may possess novel chemistry

to which resident species are not adapted (78). In the case of tall fescue, the mixture and quantities of endophyte alkaloids may represent formidable barriers to resident herbivores. However, there have been no comparable studies examining the interaction among the endophyte symbiont, herbivores, and the resident plant community in tall fescue or in any other grass species. It would be useful to replicate this experiment at multiple locations around the world where tall fescue occurs. In particular, it would be useful to determine whether European and African herbivores are more adapted to endophyte-infected tall fescue. In our experiment, the relative biomass of infected tall fescue was enhanced by herbivores, suggesting that this grass may be better able to invade novel habitats with high levels of herbivore pressure. More generally, our results confirm the important role of mammalian herbivores (particularly voles) in shaping the composition and dynamics of plant communities (79–81).

Considering the role of species interactions extrinsic to symbioses, such as predation or competition, may prove to be crucial to understanding the long-term dynamics of host–symbiont interactions, the fixation or loss of hereditary symbioses, the evolution of mutualism, and the mechanisms driving associated changes in community structure. Here, we show that herbivory reduced the biomass of symbiont-free tall fescue, as well as other plant species, and increased the biomass of symbiotic tall fescue.

We thank J. Bever, C. Lively, M. Wade, S. Louda, J. Maron, C. Schardl, S. Strauss, W. Van der Putten, and K. Whitney for critical review of the manuscript; W. Hollin for processing the tissue-print immunoblots; S. Zachariah for field and laboratory assistance; and a large group of undergraduates for help in the field and the laboratory. This work was supported by National Science Foundation Grants DEB-9727116 (to K.C.) and DBI-0200385 (to J.A.R.).

- Law, R. & Dieckmann, U. (1998) *Proc. R. Soc. London Ser. B* **265**, 1245–1253.
- Mouton, L., Dedeine, F., Henri, H., Bouletreau, M., Profizi, N. & Vavre, F. (2004) *Genetics* **168**, 181–189.
- Dillon, R. J. & Dillon, V. M. (2004) *Annu. Rev. Entomol.* **49**, 71–92.
- Moore, J. (2002) *Parasites and the Behavior of Animals* (Oxford Univ. Press, Oxford).
- Currie, C. R., Scott, J. A., Summerbell, R. C. & Malloch, D. (2003) *Nature* **398**, 701–704, and correction (2003) **423**, 461.
- Gange, A. C. & West, H. M. (1994) *New Phytol.* **128**, 79–87.
- Gehring, C. A. & Whitham, T. G. (2002) in *Mycorrhizal Ecology*, eds. van der Heijden, M. & Sanders, I. (Springer, Heidelberg), pp. 295–320.
- Arnold, A. E., Mejia, L. C., Kyllo, D., Rojas, E. I., Maynard, Z., Robbins, N. & Herre, E. A. (2003) *Proc. Natl. Acad. Sci. USA* **100**, 15649–15654.
- Gwinn, K. D. & Gavin, A. M. (1992) *Plant Dis.* **76**, 911–914.
- Clay, K., Cheplick, G. P. & Marks, S. (1989) *Oecologia* **80**, 374–380.
- Maynard Smith, J. & Szathmari, E. (1995) *The Major Transitions in Evolution* (Oxford Univ. Press, Oxford).
- Ewald, P. (1987) *Ann. N.Y. Acad. Sci.* **503**, 295–306.
- Douglas, A. E. (1994) *Symbiotic Interactions* (Oxford Univ. Press, Oxford).
- Moran, N. A. & Telang, A. (1998) *Bioscience* **48**, 295–304.
- Braun, K., Romero, J., Liddell, C. M. & Creamer, R. (2003) *Mycol. Res.* **107**, 980–988.
- Kucht, S., Grob, J., Hussein, Y., Grothe, T., Keller, U., Basar, S., Konig, W. A., Steiner, U. & Leister, E. (2004) *Planta* **219**, 619–625.
- Petrini, O. (1991) in *Microbial Ecology of Leaves*, eds. Andrews, J. H. & Hirano, S. S. (Springer, New York), pp. 179–197.
- Ernst, M., Mendgen, K. W. & Wirsig, S. G. R. (2003) *Mol. Plant–Microbe Interact.* **16**, 580–587.
- Neergaard, P. (1979) *Seed Pathology* (MacMillan, London).
- Clay, K. (1990) *Annu. Rev. Ecol. Syst.* **21**, 275–297.
- Schardl, C. L., Leuchtman, A. & Spiering, M. J. (2004) *Annu. Rev. Plant Biol.* **55**, 315–340.
- Schardl, C. L. & Clay, K. (1997) in *The Mycota V Part B*, eds. Carroll, G. C. & Tudzynski, P. (Springer, Berlin).
- Clay, K., Marks, S. & Cheplick, G. P. (1993) *Ecology* **74**, 1767–1777.
- Elmi, A. A. & West, C. P. (1995) *New Phytol.* **131**, 61–67.
- Bouton, J. H., Gates, R. N., Belesky, D. P. & Owsley, M. (1993) *Agron. J.* **85**, 52–55.
- Malinowski, D. P., Alloush, G. A. & Belesky, D. P. (2000) *Plant Soil* **227**, 115–126.
- Saikkonen, K., Wali, P., Helander, M. & Faeth, S. H. (2004) *Trends Plant Sci.* **9**, 275–280.
- Cheplick, G. P., Clay, K. & Marks, S. (1989) *New Phytol.* **111**, 89–97.
- Ravel, C., Michalakakis, Y. & Charnet, G. (1997) *Oikos* **80**, 18–24.
- Clay, K. (1996) *Res. Popul. Ecol.* **38**, 191–201.
- Clay, K. & Schardl, C. (2002) *Am. Nat.* **160**, S99–S127.
- Ball, D. M., Pedersen, J. F. & Lacey, G. D. (1993) *Am. Sci.* **81**, 370–379.
- Clay, K. (1994) in *Biotechnology of Endophytic Fungi of Grasses*, eds. Bacon, C. W. & White, J. F. (CRC, Boca Raton, FL), pp. 73–86.
- Clay, K. & Holah, J. (1999) *Science* **285**, 1742–1744.
- Shelby, R. A. & Dalrymple, L. W. (1987) *Plant Dis.* **71**, 783–786.
- Siegel, M. R., Johnson, M. C., Varney, D. R., Nesmith, W. C., Buckner, R. C., Bush, L. P., Burrus, P. B., II, Jones, T. A. & Boling, J. A. (1984) *Phytopathology* **74**, 932–937.
- Spyreas, G., Gibson, D. J. & Basinger, M. (2001) *J. Torrey Bot. Soc.* **128**, 25–34.
- Saikkonen, K., Ahlholm, J., Helander, M., Lehtimäki, S. & Niemeläinen, O. (2000) *Ecography* **23**, 360–366.
- Leyronas, C. & Raynal, G. (2001) *Ann. Appl. Biol.* **139**, 119–127.
- Oliveira, J. A. & Castro, V. (1997) *Genet. Resour. Crop Evol.* **44**, 519–522.
- Clement, S. L., Elberson, L. R., Youssef, N. N., Davitt, C. M. & Doss, R. P. (2001) *Crop Sci.* **41**, 570–576.
- Large, E. C. (1954) *Plant Pathol.* **3**, 6–11.
- White, J. F., Jr. (1987) *Plant Dis.* **71**, 340–342.
- Clay, K. & Leuchtman, A. (1989) *Mycologia* **81**, 805–811.
- Holder, T. L., West, C. P., Turner, K. E., McConnell, M. E. & Piper, E. L. (1994) *Crop Sci.* **34**, 252–254.
- Koga, H., Tsukiboshi, T. & Uematsu, T. (1995) *Nippon Sochi Gakkai-Shi* **40**, 373–380.
- Lewis, G. C., Ravel, C., Naffaa, W., Astier, C. & Charnet, G. (1997) *Ann. Appl. Biol.* **130**, 227–238.
- Wennstrom, A. (1996) *Ecography* **19**, 377–381.
- Li, B., Zheng, X. & Sun, S. (1997) in *Neotrophodum/Grass Interactions*, eds. Bacon, C. W. & Hill, N. S. (Plenum, New York), pp. 69–73.
- Rolston, M. P., Hare, M. D., Moore, K. K. & Christensen, M. J. (1986) *N. Z. J. Exp. Agric.* **14**, 297–300.
- Welty, R. E., Azevedo, M. D. & Cooper, T. M. (1987) *Phytopathology* **77**, 893–900.
- Saikkonen, K., Ion, D. & Gyllenberg, M. (2002) *Proc. R. Soc. London Ser. B* **269**, 1397–1403.
- Faeth, S. (2002) *Oikos* **98**, 25–36.
- Breen, J. P. (1994) *Annu. Rev. Entomol.* **39**, 402–423.
- Clement, S. L., Elberson, L. R., Bosque-Perez, N. A. & Schotzko, D. J. (2005) *Entomol. Exp. Appl.* **114**, 119–125.
- Latch, G. C. M. (1993) *Agric. Ecosyst. Environ.* **44**, 143–156.
- Hiatt, E. E. I., Hill, N. S., Bouton, J. H. & Mims, C. W. (1997) *Crop Sci.* **37**, 1265–1269.
- Fortier, G. M., Bard, N., Jansen, M. & Clay, K. (2000) *J. Wildl. Manag.* **64**, 122–128.
- Brown, V. K., Leijn, M. & Stinson, C. S. A. (1987) *Oecologia* **72**, 377–381.
- Rudgers, J. A., Koslow, J. M. & Clay, K. (2004) *Ecol. Lett.* **7**, 42–51.
- Eriksson, O. & (1992) *Oikos* **63**, 439–448.
- Snyder, R. W. (1978) in *Plant Relations in Pastures*, ed. Wilson, J. R. (Commonwealth Sci. Indust. Res. Org., East Melbourne, Australia), pp. 253–269.
- Rice, J. S., Pinkerton, B. W., Stringer, W. C. & Undersander, D. J. (1990) *Crop Sci.* **30**, 1303–1305.
- Clay, K. (1998) in *Population Biology of Grasses*, ed. Cheplick, G. P. (Cambridge Univ. Press, Cambridge, U.K.), pp. 255–285.
- Rudgers, J. A., Mattingly, W. B. & Koslow, J. M. (2005) *Oecologia*, in press.
- Lyons, P. C., Plattner, R. D. & Bacon, C. W. (1986) *Ecology* **232**, 487–489.
- Faeth, S. H. & Hammon, K. E. (1997) *Ecology* **78**, 820–827.
- Hatcher, P. E. (1995) *Biol. Rev.* **70**, 639–694.
- Thaler, J. S., Owen, B. & Higgins, V. J. (2004) *Plant Physiol.* **135**, 530–538.
- Gehring, C. A., Cobb, N. S. & Whitham, T. G. (1997) *Am. Nat.* **149**, 824–841.
- Owen, D. F. & Wiegert, R. G. (1981) *Oikos* **36**, 376–378.
- McNaughton, S. J. (1983) *Oikos* **40**, 329–336.
- Paige, K. N. & Whitham, T. G. (1987) *Am. Nat.* **129**, 407–416.
- Belsky, A. J. (1987) *Am. Nat.* **127**, 870–892.
- Keane, R. M. & Crawley, M. J. (2002) *Trends Ecol. Evol.* **17**, 164–170.
- Mitchell, C. E. & Power, A. G. (2003) *Nature* **421**, 625–627.
- Carpenter, D. & Cappuccino, N. (2005) *J. Ecol.* **93**, 315–321.
- Callaway, R. M. & Aschehoug, E. T. (2000) *Science* **290**, 521–523.
- Howe, H. F. & Brown, J. S. (1999) *Ecology* **80**, 1776–1781.
- Howe, H. F., Brown, J. S. & Zorn-Armold, B. (2002) *Ecol. Lett.* **5**, 30–36.
- Ostfeld, R. S., Manson, R. H. & Canham, C. D. (1997) *Ecology* **78**, 1531–1542.