Endophyte-Mediated Resistance to Herbivores Depends on Herbivore Identity in the Wild Grass *Festuca subverticillata*

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ABSTRACT   Understanding factors that affect the context dependency of species interactions has been identified as a critical research area in ecology. The presence of symbionts in host plants can be an important factor influencing the outcome of plant–insect interactions. Similarly, herbivore identity can alter the outcome of plant–symbiont interactions. Symbiotic foliar fungal endophytes confer resistance to herbivores in economically important agronomic grasses, in part through the production of alkaloids. Although endophytes are common in nature, relatively little is known about their effects on herbivores of native, wild grass species, and a recent meta-analysis suggested that endophytes are only beneficial in agronomic settings. In this study, we performed choice trials for five insect species and a greenhouse experiment with one species to assess effects of the fungal endophyte *Neotyphodium* sp. on herbivores of the wild grass *Festuca subverticillata*. In feeding trials, endophyte presence altered the preference of all five insect species tested. However, the magnitude and direction of preference varied among species, with *Pterophylla camellifolia* (F.), *Spodoptera frugiperda* (J. E. Smith), and *Rhopalosiphum padi* L. preferring endophyte-disinfected plants and *Encoptolophus costalis* (Scudder) and *Romalea guttata* (Houttuyn) preferring endophyte-symbiotic plants. Despite reducing insect preference, the endophyte had no significant effect on *S. frugiperda* performance in a no-choice greenhouse experiment and did not increase plant growth in response to this herbivore. Our results show that endophyte-mediated resistance to herbivory depends strongly on herbivore identity and suggest that the fitness consequences of endophyte symbiosis for host plants will be context dependent on the local composition of insect herbivores.

KEY WORDS   context-dependent interactions, food choice, *Neotyphodium*, *Epichloë*, *Spodoptera frugiperda*

Many plants harbor symbiotic foliar fungal endophytes that may alter the suitability of host tissue for insect consumption (Barbosa et al. 1991, Clay 1996, Bazely et al. 1997, Gehring and Whitham 2002). These fungi reside in above-ground tissues and have been documented in the majority of plant species examined to date, including mosses, ferns, gymnosperms, and herbaceous and woody angiosperms (Bacon and White 2000). Accumulating evidence suggests that foliar endophytes can alter insect herbivore consumption, performance, and preference (Clay 1990, Saikkonen et al. 1998). For example, endophytes reduced the performance of insect herbivores on sedges (Clay et al. 1985) and deterred gall-forming insects on trees (Faeth and Hammon 1997, Wilson and Carroll 1997). Endophytic fungi associated with morning glories produce ergoline alkaloids, which are known to function in anti-herbivore defense (Kucht et al. 2004). Similarly, in grasses (Poaceae), symbiotic fungi of the genera *Neotyphodium* and *Epichloë* (Clavicipitaceae) can confer resistance to herbivory by producing alkaloids (Breen 1994, Clay 1996, Miles et al. 1998, Brem and Leuchtmann 2001, Clay et al. 2005, Ball et al. 2006, Gonthier et al. 2008) while gaining nutrients from the plant (Thrower and Lewis 1973, Clay and Schardl 2002).

Systemic fungal endophytes are found in at least 80 genera and 300 species of cool-season and warm-season grasses (Saikkonen et al. 2006). However, current understanding of how fungal endophytes affect herbivory primarily comes from studies on a few economically important, cultivated C$_3$ grasses, particularly tall fescue (*Lolium arundinaceum*) and perennial ryegrass (*Lolium perenne*). In fact, these two species comprised >80% of studies in a recent meta-analysis (Saikkonen et al. 2006). For both perennial ryegrass and tall fescue, endophyte-mediated herbivore resistance has been found for a diversity of insect taxa including at least 23 species from 10 families and 5 orders (Saikkonen et al. 1998, Meister et al. 2006, Rudgers and Clay 2007).

It has been suggested that artificial selection on agronomic grasses may have selected for highly ben-

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eficial endophytes that confer resistance to herbivory, whereas wild grasses may not experience similarly strong selection from native herbivores (Saikkonen et al. 2006). In support of this idea, there seems to be little effect of endophyte symbiosis on herbivores of wild *Festuca arizonica* (Arizona fescue), the grass for which the majority of empirical studies on wild endophyte symbioses have been conducted (Lopez et al. 1995, Tibbets and Faeth 1997, Saikkonen et al. 1999). However, the vertical transmission (passed from maternal plant to seeds) of many endophytes is predicted to select against pathogenicity—and potentially favor mutualism—because the reproductive success of the symbiont is directly tied to the fitness of the host (Fine 1975, Ewald 1987, Gundel et al. 2008). Indeed, some evidence supports the defensive mutualist hypothesis (Clay 1988) because insect herbivore deterrence has been found for at least five species of wild grasses, including reductions in the survival and growth of locusts on *Festuca rubra* (Bazely et al. 1997), declines in the densities of aphids on wild barley (Clement et al. 1997), decreases in the percentage of aphids choosing symbiotic *Poa autumnalis* (Siegel et al. 1990), and improvements in resistance to the Hessian fly (*Mayetiola destructor*) in wild barley (Clement et al. 2005). Furthermore, fall armyworms (*Spodoptera frugiperda*) raised on endophyte-infected *Brachypodium sylvaticum* had significantly lower larval weights, longer times to pupation, and lower survival (Brem and Leuchtmann 2001) than on endophyte-free plants, and the endophyte in *Glyceria striata* provided inducible resistance to *R. padi* aphids (Gonthier et al. 2008). For the majority of native, wild grasses, however, nothing is known about how endophytes affect insect herbivores.

In both wild and agronomic systems, the benefits of endophytes for insect resistance are likely to be context dependent. In fact, a recent meta-analysis showed that the outcome of grass-endophyte symbiosis varied substantially among specific combinations of plant and endophyte genotypes and with nutrient availability to the plant (Saikkonen et al. 2006). Less is known about the degree to which endophyte-mediated resistance varies with the identity of the herbivore, particularly in nonagronomic systems. Understanding factors that affect the context dependency of species interactions has been identified as a critical research area in ecology (Agrawal et al. 2007, Bronstein 1994).

It is likely that insect herbivores will vary in their response to the presence of endophytes, making the outcome of the plant–endophyte interaction dependent on the local herbivore context. For example, endophyte symbiosis in tall fescue reduced reproduction of the bird cherry-oat aphid (*Rhopalosiphum padi*) but enhanced performance of fall armyworm (*Spodoptera frugiperda*) in one study (Bultman and Bell 2003). Prior studies have hypothesized that herbivore identity and natural history (e.g., generalist versus specialist, aposematic versus cryptic, mandibulate versus haustellate) may influence herbivore sensitivity to endophyte presence (Rudgers and Clay 2007). However, for most of the wild grass–endophyte systems that have been investigated, only one or two

insect herbivores have been challenged with symbiotic and symbiont-free plants, making it difficult to draw broad generalizations. Even in *Festuca arizonica*, arguably the best studied wild grass–endophyte system, only three species of insect herbivores (the leaf-cutting ant *Acromyrmex versicolor* and the grasshoppers, *Melanoplus femurrubrum* and *Xanthippus corallipes*) have been tested in the published literature (Lopez et al. 1995, Saikkonen et al. 1999, Tibbets and Faeth 1999). In addition, many prior studies have compared naturally symbiotic and symbiont-free plants, which confounds endophyte presence and plant genotype in their effects on insect deterrence. Even for agricultural grasses, few studies have evaluated herbivore preference, herbivore performance, and plant performance for the same herbivore species.

Here we evaluated the degree to which insect resistance varied with herbivore identity in a wild grass–endophyte symbiosis. We conducted a series of choice trials and a greenhouse experiment to examine the role of a fungal endophyte (*Neotyphodium* sp.) in herbivore deterrence and performance on the wild grass *Festuca subverticillata.* We studied insect preference for naturally symbiotic versus experimentally disinfected plants for five insect herbivores: a lepidopteran (*Spodoptera frugiperda* Smith), three orthopterans (*Pterophylla camellifolia* F., *Encoptolophus costalis* Scudder, and *Romalea guttata* Houttuyn), and an aphid (*Rhopalosiphum padi* L.). These feeding trials addressed the following question: (1) does endophyte-mediated insect deterrence vary among herbivore species? In a no-choice greenhouse experiment, we exposed endophyte-symbiotic and experimentally disinfected plants to the generalist herbivore *S. frugiperda* to link herbivore preference, herbivore performance, and plant performance for this insect because *S. frugiperda* has been the most commonly used species for assays involving grass endophytes. We addressed two additional questions. (2) Is herbivore performance affected by endophyte presence? and (3) Does endophyte presence influence the performance of plants challenged with herbivory? Our results show that insect preference varies strongly among the herbivore species tested, ranging from an 80% preference for endophyte-free to a 90% preference for endophyte-symbiotic, and showing substantial context dependency on herbivore identity. Given this variation in herbivore preference, results suggest that single species assays may be insufficient for understanding endophyte effects in wild grasses. Furthermore, results from the no-choice experiment indicate that herbivore preferences may not translate into differences in plant or herbivore performance.

Materials and Methods

Study System

Festuca subverticillata (nodding fescue) is a perennial C_3 grass common to forest understory habitats throughout the eastern and central United States and frequently hosts a systemic fungal endophyte, *Neoty-*

Table 1. Species collection and feeding trial information

Species	Common name	Collection location	Stage	N	Trial length (h)
Encoptolophus costalis	Dusty grasshopper	Stephen F. Austin Experimental Forest (Nacogdoches, TX) 31°29′52″ N, 94°46′46″ W	Adult	21	1
Pterophylla camellifolia	Common true katydid	Stephen F. Austin Experimental Forest (Nacogdoches, TX) 31°29′52″ N, 94°46′46″ W	Nymph	15	4
Rhopalosiphum padi	Bird cherry-oat aphid	Rice University (Houston, TX) 29°41′07″ N, 95°24′10″ W	Apterous	10 (3)[a]	24
Romalea guttata	Eastern lubber grasshopper	Angelina National Forest (Zavalla, TX) 31°05′23″ N, 94°19′21″ W	Nymph	11	4
Spodoptera frugiperda	Fall armyworm	Purchased commercially (Bio-Serv, Frenchtown, NJ)	Third Instar	37	1

[a] Each independent trial was replicated three times.

N, no. of independent feeding trials.

phodium sp. (Clay and Leuchtmann 1989; Leuchtmann and Clay 1990; Afkhami and Rudgers 2008; Rudgers et al. 2009). *Neotyphodium* endophytes are asexual anamorphs of *Epichloë* endophytes that lack mechanisms of horizontal transmission and are exclusively vertically transmitted through the seeds (Clay and Schardl 2002). On 27 June 2006, *F. subverticillata* seeds were collected from the Indiana University Experimental Field at Bayles Road, Bloomington, IN (39°13′12″ N, 86°32′33″ W). This natural population consistently had 95–100% of seed-bearing tillers symbiotic with the endophyte ($n = 31$ randomly selected plants on 27 March 2003, $n = 17$ on 18 June 2004, and $n = 20$ on 18 July 2005). After collection, seeds were dried at room temperature for 1 wk and placed in an envelope for storage at 4°C, because the endophyte may be lost from seeds stored at high temperature and high humidity (Siegel et al. 1984).

Experimental Disinfection

We removed the endophyte from a subset of seeds by heat treating at 60°C for 6 d (February 2007). This method is commonly used for endophyte removal (Bultman et al. 2006, Rudgers and Swafford 2008), and we optimized the length of heat treatment for *F. subverticillata* by experimentally determining the minimum number of days required for endophyte elimination in this species (unpublished data). Experimental removal is used because comparisons of naturally symbiotic and endophyte-free plants confound plant genotype and endophyte status. Some studies have raised the concern that endophyte removal may be unrealistic if endophytes and plant lineages are highly coevolved (Lehtonen et al. 2005, Wäli et al. 2007). However, pollen from endophyte-free plants can fertilize seeds from endophyte-symbiotic plants, which decouples endophyte symbiosis from the plant genetic lineage. Furthermore, imperfect vertical transmission, which has been documented for *F. subverticillata* (Afkhami and Rudgers 2008), is a natural process that generates plant offspring lacking the endophyte from within symbiotic plant lineages. Although heat treatment could have nontarget effects on plant growth or performance, we had similar rates of germination for heat-treated and untreated seeds, sug-

gesting heat treatment effects were minimal. In addition, for experiments where plant performance was measured, we used a round of vegetative reproduction (cloning to generate plants of similar size) to separate any differences in plant performance from the initial heat treatment. This method has been commonly used in endophyte studies (Faeth and Sullivan 2003, Morse et al. 2007).

After heat treatment, we cold stratified all seeds at 4°C on wet perlite for 2 wk before planting. On 20 February 2007, we planted seeds directly into 1.5-liter pots (15 cm depth and diameter; Hummert International, St. Louis, MO) containing Metromix 200 soil (Sun Gro Horticulture Distribution, Bellevue, WA). Seedlings were grown in a greenhouse (≈23°C, watered to saturation daily, no supplemental light) and after 1 mo were scored for endophyte presence microscopically using rose bengal stain following Belanger (1996). The heat treatment successfully disinfected all treated plants.

Insect Herbivores

All five insect species included in this study (Table 1) overlap in distribution with *F. subverticillata* but have varied life histories. *Pterophylla camellifolia* (true katydid) is a cosmopolitan species present throughout the United States that uses cryptic coloration and camouflage (Marshall 2006). Although its mixed diet includes deciduous tree leaves, *P. camellifolia* has been observed consuming C_3 grasses in the field (J.A.R., personal observervation). In addition, we included two grasshoppers: *Encoptolophus costalis* (dusky grasshopper) occurs throughout central and western North America and predominately consumes grasses, including C_3 grass likely to host endophyte, with as little as 1% of its diet consisting of forbs in nature (Bailey and Riegert 1971, 1973), and *Romalea guttata* (Eastern lubber grasshopper) occurs in the southeastern United States and commonly consumes forbs and woody plants (Jones et al. 1989). Unlike *E. costalis*, *R. guttata* is aposematically colored and produces a frothy secretion from modified metathoracic spiracles to deter predators (Eisner et al. 1971). We also tested two species that have been commonly used in herbivory assays and experiments with grass endo-

phytes. *Spodoptera frugiperda* (fall armyworm) is a common, generalist herbivore that occurs throughout the United States, as well as Central and South America (Ashley et al. 1989). *Rhopalosiphum padi* (bird cherry-oat aphid), unlike the other herbivores in this study, is a phloem feeder with haustellate mouthparts. This species is cosmopolitan and often uses grasses as summer hosts (Dixon 1971, Wiktelius et al. 1990). We observed both *S. frugiperda* and *R. padi* on *F. subverticillata* in the field.

For the experiments, we obtained *S. frugiperda* commercially (Bio-Serv, Frenchtown, NJ) and collected and identified orthopterans from several locations in northern Texas (Table 1). Apterous *R. padi* were collected from a population subsisting on grasses and herbaceous plants in the Rice University greenhouses (Houston, TX). To avoid bias, the aphids were not collected from *F. subverticillata* plants.

Choice Experiments

***Spodoptera frugiperda* and Orthopteran Choice Trials.** Before trials, the orthopterans (*P. camellifolia, E. costalis*, and *R. guttata*) were maintained in 46-cm^3 lumite screen cages (Bioquip, Rancho Domiguez, CA) with ample food (mixed forbs and grasses other than *F. subverticillata*) and water (saturated sponges) for 2–4 d. *S. frugiperda* were maintained on commercial nutrient media (diet F9179B; wheat germ and corn cob base) until the third larval instar. All insects were kept at 23–25°C under 16:8 L:D conditions.

We placed each insect into an individual transparent plastic box (16 by 11 by 4 cm) between two food items: a 10-cm leaf segment of a randomly selected endophyte-symbiotic *F. subverticillata* and the same sized leaf segment from a randomly selected, disinfected *F. subverticillata* plant. Cut leaves have been commonly used for assaying food preference and performance in prior experiments, including in grass–endophyte studies (Hardy et al. 1986, Clay 1988, Bultman and Bell 2003, Ball et al. 2006, Bultman et al. 2006). Clearly, plant quality may be altered in detached leaves compared with whole plants; however, these assays can still inform our understanding of insect preference, especially when preferences are strong. Because it is possible that clipping the leaf segments could induce plant or endophyte defenses, our methods did not distinguish between induced and constitutive mechanisms of insect resistance.

Before each feeding trial we measured the total area of both leaf segments by scanning them on a flatbed scanner and calculating total area with Scion Image (Alpha 4.0.3.2, Frederick, MD). We attached each leaf segment to a water-saturated sponge with an insect pin to maintain the moisture of the food items. At the end of each trial, leaf segments were rescanned and measured, and percentage damage was calculated.

The availability of each insect species in the field determined the number of choice trials conducted (Table 1). Because it was important to maintain a choice in food items throughout the trials, the length of time of the trial was determined by the typical feeding voracity of the species. The length of feeding trials remained constant within each species (Table 1), and timing started when the herbivore began consumption of either food item. If an insect did not consume any food within 24 h, we terminated the trial to avoid possible leaf desiccation. Termination of trials was rare (no more than three terminations per species). Those insects were returned to the holding cage and tested 2–3 d later. Otherwise, each individual insect and each individual plant were tested only once.

Aphid Choice Trials (Laboratory). After collection from the greenhouse, we immediately tested *R. padi* preference by placing five apterous aphids into a large petri plate (diameter = 10 cm) between two food items: a 3-cm leaf segment from an endophyte-symbiotic *F. subverticillata* plant and the same sized leaf segment from a disinfected plant. This method has been used in prior aphid studies on grasses (Bultman et al. 2006). We covered the bottom of the plate with moist filter paper and used insect pins to attach the leaf segments to water-saturated sponges. Trials were conducted under 23–25°C, 16:8 L:D conditions. We tested leaves from 10 randomly selected symbiotic plants and 10 randomly selected disinfected plants. Each pair of endophyte-symbiotic and disinfected plants was replicated three times with a new set of five aphids. The plates were undisturbed for 24 h, after which time we scored aphid preference by counting the number of adults with their stylet inserted into each leaf segment. For each trial, we calculated the proportion of the aphids that were feeding on each endophyte treatment (aphids that did not select a leaf segment were not included). For each plant pair, we averaged the three replicate trials before analysis.

Aphid Choice (Greenhouse). We conducted a complementary greenhouse-based assessment for *R. padi.* Whereas the laboratory trials provided detailed information under controlled conditions, the greenhouse experiment allowed us to gain longer term data for live plants rather than cut leaves. We assigned endophyte-symbiotic and disinfected plants to random positions in a greenhouse (≈23°C, watered to saturation daily) with no supplementary light. Plants were spaced 35 cm apart to reflect a natural density for *F. subverticillata* (mean distances between plants ± SE, measured in three populations in southern Indiana = 28 ± 4 ($n = 30$), 38 ± 11 ($n = 13$), and 52 ± 7 cm ($n = 35$); range = 9–238 cm). During early development, seedlings were sprayed once with a 2% formulation of insecticidal soap (Garden Safe Brand; Schultz, Bridgeton, MO) to keep aphid damage low. After seedlings produced 5–10 tillers (20 March 2007; 3 wk after the insecticide application), leaves were rinsed thoroughly with water, and aphids from a population previously established in the greenhouse were allowed to colonize without restriction. After 2 mo, we counted the number of live and senesced tillers and leaves, individual aphid damage sites, aphids at each life stage (nymphs, adults, or alates), and parasitized aphid mummies. Because of the rarity of some life stages and lack of differences in endophyte effect among stages, aphid counts were summed across all life stages.

Statistical Analyses. For the laboratory feeding trials, we used paired *t*-tests (two-tailed; $\alpha = 0.05$) with the independent factor of endophyte treatment (SAS Institute 2004). For the orthopteran and *S. frugiperda* trials, the response variable was percentage damage to each leaf segment. For the aphid laboratory trials, we used the percentage of aphids with their stylet inserted into each leaf segment. In the aphid greenhouse experiment, we analyzed the number of aphid damage sites and total aphid counts using a general linear model with the fixed factor of endophyte treatment and leaf count as a covariate to account for size differences among plants. We also included the interaction between the covariate and endophyte treatment to assess whether the relationship between plant size and aphid response depended on the endophyte treatment. Both aphid damage sites and aphid number required square-root transformation to normalize residuals.

Spodoptera frugiperda Greenhouse Experiment

After 3 mo of growth in a greenhouse, a subset of 15 endophyte-symbiotic and 15 disinfected plants were split into two equal-sized clones (5–10 tillers per clone). This procedure allows the assessment of plant performance (e.g., growth, survival) to be separated from any potential side effects of the initial heat treatment to remove the endophyte by a round of vegetative reproduction and has been commonly used in endophyte studies (Faeth and Sullivan 2003, Morse et al. 2007). Each clone was planted into a 1.5-liter pot containing Metromix 200 soil and enclosed in a mesh bag of 0.5-mm fiberglass. After 1 mo, we counted the number of live tillers on each plant to capture initial differences in plant size before the herbivory treatment.

We randomly assigned one of each clone pair to receive herbivory by *S. frugiperda* larvae. We placed a third instar on each plant and monitored survival. We measured larval wet weights after 10 d and measured pupal wet weights (PL303; Mettler Toledo, Westminster, CO). Because larval mortality was low (<10%), there was insufficient data to statistically test for effects of the endophyte on *S. frugiperda* survival. Six weeks after initiation of the experiment, we counted live and senesced tillers, senesced and damaged leaves, and harvested above and below-ground biomass. Roots were washed in a U.S. Standard Sieve (No. 18, 1-mm opening), and plant biomass was dried to constant mass at 60°C in a convection oven.

Statistical Analyses. To assess plant performance, we used factorial multivariate analysis of variance (MANOVA; SAS Institute 2004) with the fixed factors of endophyte treatment, herbivory treatment, the endophyte × herbivory interaction, and the random effect of clone. Response variables included the proportion of dead leaves per tiller, final tiller number, and plant biomass (above-ground, below-ground, total, and root:shoot). Although transformation of tiller count and proportion senesced leaves could not normalize the residuals, analysis of rank-transformed data

as well as nonparametric ANOSIM with a Bray-Curtis distance measure yielded qualitatively similar results. After significant overall effects in the MANOVA ($\alpha = 0.05$), we conducted ANOVA on the individual response variables (SAS Institute 2004). For response variables that could not be normalized, we confirmed the ANOVA qualitative results with a distribution-free randomization tests (with 9,999 iterations) (Manly 1991; ANOVA embedded in SAS randomization macro, Cassell 2002). For the subset of clones in the herbivory treatment, herbivore performance response variables (larval and pupal mass) and the number of leaves damaged per tiller were analyzed with ANOVA including the fixed factor of endophyte treatment.

Results

Choice Experiments

Does Endophyte-Mediated Insect Deterrence Vary Among Herbivore Species? The endophyte altered the food preference of all five herbivores tested. However, the magnitude and direction of preference varied highly among the insect species.

Orthopteran and *S. frugiperda* Trials. *Pterophylla camellifolia* and *S. frugiperda* preferred endophyte-disinfected plants: *P. camellifolia* consumed 80% less area of endophyte-symbiotic tissue compared with endophyte-disinfected tissue (Fig. 1A; $t_{1,14} = -4.34$, $P < 0.001$), and *S. frugiperda* consumed 40% less area of endophyte-symbiotic tissue (Fig. 1B; $t_{1,36} = -2.123$, $P = 0.040$). Conversely, *E. costalis* and *R. guttata* preferred endophyte-symbiotic plants: *E. costalis* consumed >50% more area of endophyte-symbiotic tissue compared with endophyte-disinfected tissue (Fig. 1C; $t_{1,20} = 2.1$, $P = 0.024$), and *R. guttata* consumed 90% more area of endophyte-symbiotic tissue (Fig. 1D; $t_{1,10} = 2.55$, $P = 0.015$).

Aphid (*R. padi*) Trials. In laboratory choice trials, on average 2.7 original aphids attached to disinfected leaf segments whereas only 1.6 aphids attached to symbiotic tissue, suggesting a preference against endophyte-symbiotic tissue. This difference was not statistically significant ($t_{1,9} = 1.5$, $P = 0.195$), but the statistical power was low (power = 0.27). Similarly, during our one time count of aphids in the greenhouse preference experiment, the total number of aphids (adults, nymphs, winged, and mummies) on plants in the greenhouse experiment did not significantly differ between endophyte-symbiotic and disinfected plants but trended toward a preference for disinfected plants (E+ mean aphids = 4.2 ± 2.1 SE, E− aphids = 23.2 ± 6.1; $F_{1,36} = 0.6$, $P = 0.446$). In addition, larger plants had more aphids (covariate, leaf number: $F_{1,36} = 15.9$, $P < 0.001$; leaf number × endophyte $F_{1,36} = 0.61$, $P = 0.439$). However, in the greenhouse experiment, the amount of aphid damage to leaves was significantly lower (by 65%) for plants with the endophyte than for experimentally disinfected plants (Fig. 2A; $F_{1,36} = 10.3$, $P = 0.003$). The damage increased more rapidly with plant size (estimated by the number of leaves) in disinfected plants than in endophyte-symbiotic plants
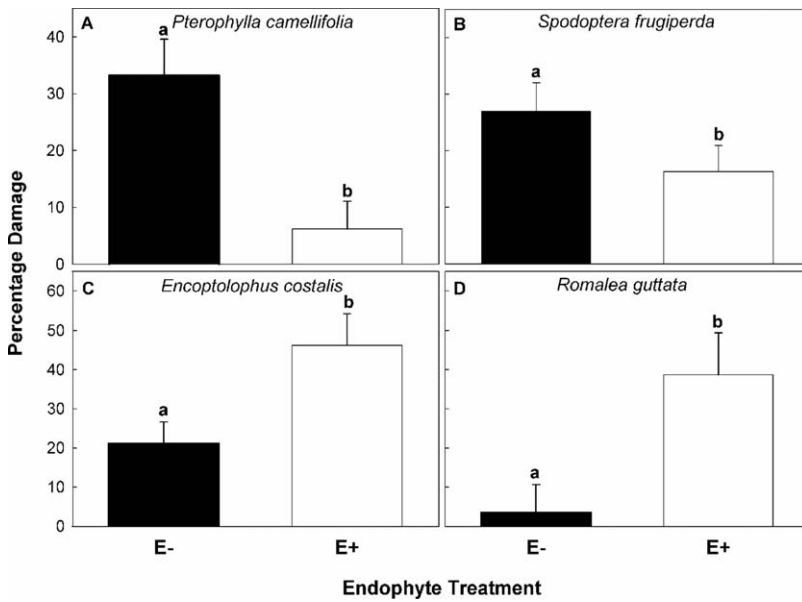
Fig. 1. Laboratory feeding trials. Effect of the endophyte *Neotyphodium* sp. on percentage leaf damage to *F. subverticillata* by four insect species. Filled bars indicate disinfected plants; open bars indicate endophyte-symbiotic plants. *P. camellifolia* (A) and *S. frugiperda* (B) preferred disinfected tissue. *E. costalis* (C) and *R. guttata* (D) preferred symbiotic tissue. Significant differences are denoted by different letters within an herbivore species ($P < 0.05$).

(significant interaction between the number of leaves and the endophyte treatment, $F_{1,36} = 6.39$, $P = 0.016$). In combination, these results suggest that *R. padi* preferred endophyte-free plants and that the effects of endophyte deterrence were stronger for feeding damage than for numbers of aphids.

Spodoptera frugiperda Greenhouse Experiment

Is Herbivore Performance Affected by Endophyte Presence? *Spodoptera frugiperda* raised on endophyte-symbiotic versus experimentally disinfected *F. subverticillata* did not significantly differ in larval mass (Table 2; $F_{1,19} = 0.5$, $P = 0.497$) or pupal mass (Table 2; $F_{1,15} = 0.5$, $P = 0.489$).
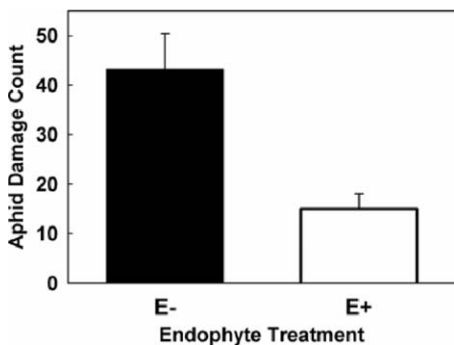

Fig. 2. Aphid greenhouse experiment. Endophyte-disinfected plants had significantly more aphid damage sites (per plant) than symbiotic plants ($F_{1,36} = 10.3$, $P = 0.003$). Filled bars indicate disinfected plants; open bars indicate endophyte-infected plants.

Does Endophyte Presence Influence the Performance of Plants Challenged With Herbivory? Herbivory by *S. frugiperda* significantly reduced combined estimates of plant performance (Pillai's trace = 0.60, $F_{5,23} = 6.9$, $P < 0.001$). Herbivore-exposed plants had significantly lower above-ground biomass (by 25%, $F_{1,28} = 27.7$, $P < 0.001$) and total biomass (by 20%, $F_{1,28} = 13.7$, $P < 0.001$; Fig. 3A).

Neither endophyte presence (Pillai's trace = 0.15, $F_{5,23} = 0.79$, $P = 0.571$) nor the interaction between the endophyte and herbivory (Pillai's trace = 0.08, $F_{5,23} = 0.4$, $P = 0.857$) affected combined estimates of plant performance. Endophyte disinfection did not significantly affect any plant responses in the individual ANOVA (Table 2); in fact, the maximum difference between endophyte-symbiotic and endophyte-free plants was 31% (for proportion of dead leaves per tiller). Plant genotype (clone identity) significantly influenced all biomass measurements (Table 2; MANOVA Pillai's trace = 2.90, $F_{140,135} = 1.3$, $P = 0.049$), suggesting that plant genotype was more important to growth than the presence of the endophyte.

Discussion

Although surveys have documented the presence of endophytes in many, diverse wild grasses (White 1987, Clay and Leuchtmann 1989, Afkhami and Rudgers 2008, Rudgers et al. 2009), and many studies have shown that endophytes deter herbivores in agronomic systems (Madej and Clay 1991, Conover 1998, Bultman et al. 2006), experimental tests of endophyte effects on herbivory in natural systems have been rare (Saikkonen et al. 2006). Here, we found that the fungal

Table 2. Results from a mixed-model ANOVA of the *S. frugiperda* greenhouse experimental data, including factors of endophyte infection, herbivory, and genotype

Response variable	Endophyte			Herbivory			Endophyte × herbivory			Genotype (clone)		
	df	F	P	df	F	P	df	F	P	df	F	P
Senesced leaves per tiller	1,27	0.4	0.548	1,27	1.5	0.240	1,27	0.5	0.501	28,27	0.9	0.612
Live tiller count	1,28	0.4	0.536	1,28	2.5	0.129	1,28	0.1	0.879	28,28	0.6	0.924
Above-ground biomass	1,28	2.4	0.131	1,28	27.7	**<0.001**	1,28	0.3	0.517	28,28	4.2	**<0.001**
Below-ground biomass	1,35	0.3	0.595	1,35	2.1	0.157	1,35	1.0	0.318	30,35	3.9	**<0.001**
Total biomass	1,28	1.2	0.284	1,28	13.7	**<0.001**	1,28	0.7	0.402	28,28	3.7	**<0.001**
Root:shoot ratio	1,28	0.5	0.495	1,28	8.3	**0.008**	1,28	0.2	0.701	28,28	2.0	**0.035**
Leaf damage (per tiller)	1,28	0.4	0.511	—	—	—	—	—	—	—	—	—
Herbivore larval mass (g)	1,19	0.5	0.497	—	—	—	—	—	—	—	—	—
Herbivore pupal mass (g)	1,15	0.5	0.489	—	—	—	—	—	—	—	—	—

Significant *P* values (<0.05) are in bold.

endophyte associated with the native, wild grass *F. subverticillata* altered herbivore preference in a series of food choice trials. Importantly, whereas all five insects tested showed strong preferences, responses to the endophyte were highly specific to the herbivore species examined. This study is among the first to show how the ecological consequences of endophyte symbiosis vary with insect identity in a nonagronomic system. Strong variation among herbivore species, ranging from an 80% preference for endophyte-free to a 90% preference for endophyte-symbiotic, suggests that the benefits of endophytes for herbivore deterrence will depend highly on the local herbivore context.

Context-Dependent Endophyte-Mediated Resistance to Insect Herbivores. The effect of endophytes on herbivores resistance in *F. subverticillata* is clearly conditional on herbivore identity. Our results suggest that the interaction between endophytes and their host plants may be too complex to rely on single species assays for information on endophyte-mediated herbivore resistance. The majority of research examining the effects of endophytes on herbivory has focused on two insect species: *S. frugiperda* and *R. padi* (Saikkonen et al. 1998), which in our study were both deterred by endophyte symbiosis (as was *P. camellifolia*). However, an additional two species, *E. costalis*

Fig. 3. *S. frugiperda* greenhouse experiment: Effect of the endophyte *Neotyphodium* sp. and herbivory on total biomass of *F. subverticillata.* Bars show means ± SE. Filled bars indicate disinfected plants; open bars indicate symbiotic plants.

and *R. guttata*, significantly preferred endophyte-symbiotic tissue. Assaying only *S. frugiperda* and *R. padi* could lead to the simplifying generalization that endophyte symbiosis deters herbivores when, in fact, endophyte symbiosis provides context-dependent benefits to plant hosts.

Although our results show that effects of the endophyte depended strongly on herbivore identity, the mechanisms underlying these preferences remain unclear and merit future study. We suggest several hypotheses for these results. First, herbivores may have differential tolerance to a chemical deterrent produced by the endophyte. Endophyte-mediated deterrence of insects is often attributed to the production of peramine alkaloids (Clay and Schardl 2002, Schardl et al. 2007). However, Siegel et al. (1990) found no evidence for peramine, loline, lolitrem B, or ergovaline alkaloid production by the endophyte in the *F. subverticillata* plants they examined. Although sample sizes for plant chemistry were low (three to six individuals tested), the endophyte in *F. subverticillata* may in fact lack these common mycotoxins. Similarly, Brem and Leuchtmann (2001) found that endophyte symbiosis reduced *S. frugiperda* performance and weakly deterred damage to the wild grass *Brachypodium sylvaticum*, although this endophyte (*Epichloë sylvatica*) also did not produce any of the typical anti-herbivore alkaloids. They suggested that an unknown endophyte-produced compound could cause resistance and deterrence; this may also be the case for *F. subverticillata.* Consistent with this hypothesis, recent work with *L. perenne* has documented a wide range of changes in plant chemistry when symbiotic with the endophyte (Rasmussen et al. 2008). The variable effect of endophyte symbiosis on herbivores in this study could result, in part, from differential tolerance to endophyte-produced secondary compounds.

Second, although much attention has been given to the role of alkaloids in endophyte-mediated effects on herbivores (Schardl et al. 2007), endophytes could also alter insect preferences by changing insect phagostimulants or host nutrient content. For example, in other grasses, endophytes are known to increase nutrient uptake (Malinowski et al. 2000), host nitrate content (Lyons et al. 1990, Rasmussen et al. 2008), and
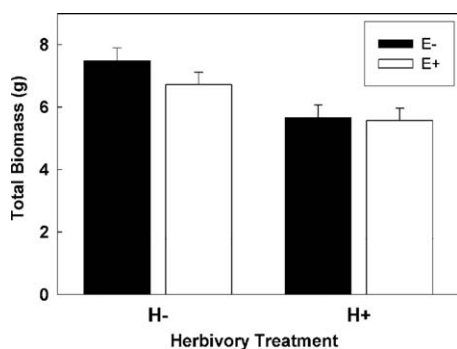
drought tolerance (Malinowski et al. 2005, Kannadan and Rudgers 2008). These effects could explain why grasshoppers preferred endophyte-symbiotic tissue, e.g., if they were insensitive to chemical deterrents and attracted by higher nitrogen levels. Another species of grasshopper, *Xanthippus corallipes,* showed increased mean relative growth rates on endophyte-symbiotic Arizona fescue compared with naturally endophyte-free plants (Saikkonen et al. 1999), which is consistent with the idea that endophyte symbiosis may increase tissue quality or nutritional value for grasshoppers, specifically. In a separate study, the grasshopper *Melanoplus femurrubrum* consumed equal amounts Arizona fescue tissue with high and low densities of endophyte but had significantly greater assimilation of tissue with high endophyte density in no choice trials (Lopez et al. 1995). Additional work is needed to identify possible changes in nutrient content or phagostimulants caused by endophyte presence.

Third, preferences did not seem to depend on differences in insect natural history, such as whether herbivores were aposomatic or cryptic, consumed grass as a large or small part of their diet, were generalists or specialists, or had haustellate or mandibulate mouthparts. Future studies focusing on particular aspects of herbivore natural history with increased replication at that level could provide additional insight.

Fourth, although the ranges of all herbivores included in this study overlap with the range of *F. subverticillata* and some of these insects have been observed consuming *F. subverticillata* in nature, the degree to which these herbivores depend on and interact with this host species is unclear in most cases. Importantly, even if *F. subverticillata* was a novel host for some of these herbivores, all insects expressed strong preference, signifying the potential importance of endophyte symbioses in noncoevolved interactions among hosts, symbionts, and herbivores. A future study explicitly examining endophyte-mediated deterrence in herbivores that range in their level of dependence and experience with a host (from specialist to generalist to novel herbivore) would be particularly interesting.

Endophyte Effects on Insect Herbivore and Plant Performance. Although endophyte symbiosis deterred feeding by *S. frugiperda* in our choice experiments, presence of the endophyte had no effect on *S. frugiperda* performance in our no-choice greenhouse experiment. In contrast to our results, Cheplick and Clay (1989) found that, although endophyte presence in *F. subverticillata* had no effect on survival of *S. frugiperda*, both larval and pupal mass were reduced relative to naturally uninfected plants. The most obvious difference between these studies is our use of experimentally disinfected plants compared with the naturally endophyte-free plants used by Cheplick and Clay (1989), which confounded plant genotype with endophyte status. This highlights the importance of experimental manipulation for detecting endophyte effects on herbivores. In addition, insect preference may not be tightly linked to performance for *S. frugiperda* and other herbivores. For example, Ball et al. (2006) found endophyte-mediated deterrence of *S. frugiperda* without decreased performance for both tall fescue and perennial ryegrass, and Bultman et al. (2006) showed that some strains of endophytes in tall fescue deterred *R. padi* individuals but did not influence aphid population size. Thus, decoupled preference and performance may not be an uncommon phenomenon for insect herbivores and adds to the complexity of understanding the consequences of endophyte symbiosis.

Not surprisingly, given the lack of an endophyte effect on *S. frugiperda* damage, plant performance was also unaffected by the endophyte symbiosis in these no choice trials. However, our result that symbiotic benefits depended on herbivore identity in the choice trials suggest that more work is needed with other herbivore species to assess how the benefits of endophyte symbiosis depend on herbivore identity in the no-choice experiments. However, benefits other than herbivore resistance may be important for explaining the high frequency of endophyte symbiosis observed in *F. subverticillata* populations in nature.

Conclusions. While the outcome of species interactions often depends on biotic and abiotic conditions, in many cases, little is known about the factors influencing this context dependency (Agrawal et al. 2007, Bronstein 1994). In this study, we showed endophyte-mediated herbivore resistance strongly depends on herbivore identity in the wild grass *F. subverticillata.* Our results suggest that in native, wild grasses, the effects of endophyte symbiosis may be highly insect species specific.

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