Oecologia
DOI 10.1007/s00442-010-1685-2

PLANT-ANIMAL INTERACTIONS - ORIGINAL PAPER

Fungal endophytes of native grasses decrease insect herbivore preference and performance

Kerri M. Crawford · John M. Land · Jennifer A. Rudgers

Received: 14 March 2010 / Accepted: 2 June 2010

Abstract Endophytic fungal symbionts of grasses are well known for their protective benefit of herbivory reduction. However, the majority of studies on endophyte–grass symbioses have been conducted on economically important, agricultural species—particularly tall fescue (*Lolium arundinaceum*) and perennial ryegrass (*Lolium perenne*)—raising the hypothesis that strong benefits are the product of artificial selection. We examined whether fungal endophytes found in natural populations of native grass species deterred insect herbivores. By testing several native grass–endophyte symbiota, we examined phylogenetic signals in the effects of endophytes on insects and compared the relative importance of herbivore and symbiotum identity in the outcome of the interactions. Preference was assessed using three herbivore species [*Spodoptera frugiperda* (Lepidoptera), *Schistocerca americana* (Orthoptera), *Rhopalosiphum padi* (Hemiptera)] and ten native symbiota, which spanned seven grass genera. We also assessed herbivore performance in a no choice experiment for five native symbiota against *S. frugiperda*. We compared greenhouse and laboratory trials with natural levels of herbivory measured in experimental field populations. In all cases, we included the agronomic grass species, *L. arundinaceum*, to compare with results from the native grasses. Both in the field and in experimental trials, herbivores showed a significant preference for endophyte-free plant material for the majority of native grasses, with up to three times lower herbivory for endophyte-symbiotic plants; however, the degree of response depended on the identity of the herbivore species. Endophyte presence also significantly reduced performance of *S. frugiperda* for the majority of grass species. In contrast, the endophyte in *L. arundinaceum* had few significant anti-herbivore effects, except for a reduction in herbivory at one of two field sites. Our results demonstrate that the mechanisms by which native symbionts deter herbivores are at least as potent as those in model agricultural systems, despite the absence of artificial selection.

Keywords Plant–herbivore interactions · Herbivore defenses · Native grasses · *Lolium arundinaceum* · Alkaloids

Communicated by Roland Brandl.

K. M. Crawford (✉) · J. M. Land · J. A. Rudgers
Department of Ecology and Evolutionary Biology, Rice University, 6100 Main Street, Houston, TX 77005, USA
e-mail: kcrawford@rice.edu

Introduction

Plants associate with a variety of microbes, including pathogens, mycorrhizal fungi, rhizosphere bacteria, and endophytes (Dighton et al. 2005; Hardoim et al. 2008; Franche et al. 2009). Not only do these microbial symbionts have direct effects on plant growth but they can also have notable indirect effects on herbivores (Clay 1990; Barbosa et al. 1991; Gehring and Whitham 2002). Although the impacts of plant–microbe symbioses on herbivores can range from detrimental to beneficial (Hartley and Gange 2009), in the case of fungal endophytes in grasses, negative consequences for herbivores have been considered typical.

Class 1 endophytes (family *Clavicipitaceae*) grow asymptomatically in above-ground plant tissues for at least part of their life cycle (Rodriguez et al. 2009), and show a variety of mechanisms for protecting hosts against herbivores (Clay 1990; Dugassa-Gobena et al. 1998; Bultman et al.

Published online: 29 June 2010

2004). Most notably, endophytes produce toxic alkaloids that can deter herbivores or reduce herbivore performance (Siegel et al. 1990; Clay and Schardl 2002). Both constitutive and wound-inducible resistance have been reported (Boning and Bultman 1996; Gonthier et al. 2008). Additionally, endophytes have also been shown to affect plant resource allocation in ways that increase the host plant's ability to compensate for herbivory (Sullivan et al. 2007).

To date, few controlled studies with native grass–endophyte symbiota have tested for anti-herbivore benefits, and even fewer have tested native symbiota against more than one species of herbivore. The majority of studies have focused on a small subset of the grass species known to host endophytic fungi, most notably the agronomically important species tall fescue (*Lolium arundinaceum*) and perennial ryegrass (*Lolium perenne*) (Saikkonen et al. 2006). Agricultural grasses are propagated in more controlled environments than occur in natural ecosystems, which could affect their genetic diversity and limit selection pressures from external forces. In addition, it has been proposed that agricultural practices may artificially select for endophytes with enhanced herbivore deterrence (Saikkonen et al. 2006). It is largely unknown if the degree of herbivore deterrence in agricultural grasses is significantly greater than in native grasses because few native grasses have been examined in controlled experiments. Twelve studies have specifically addressed whether or not endophytes in native grasses deter herbivores, including nine experimental studies (Cheplick and Clay 1988; Siegel et al. 1990; Bazely et al. 1997; Clement et al. 1997, 2005; Miles et al. 1998; Tibbets and Faeth 1999; Brem and Leuchtmann 2001; Afkhami and Rudgers 2009) and three observational studies (Clay et al. 1985; Lopez et al. 1995; Saikkonen et al. 1999). Together, these studies examined 17 genera and 32 species of grasses (13 of which were in the genus *Festuca*), which is a small subset of the more than 300 grass species documented to host class 1 endophytes and the ~2,000 species that are estimated to be hosts (Leuchtmann 1992).

In these studies, preference for endophyte-symbiotic versus endophyte-free grasses appears to depend on the identity of both the endophyte–host grass symbiotum and the herbivore species. For example, *Rhopalosiphum padi* aphids damaged more endophyte-free *Festuca subverticillata* than endophyte-symbiotic *F. subverticillata*, while a common grasshopper species, *Romalea guttata*, preferentially consumed endophyte-symbiotic *F. subverticillata* over endophyte-free (Afkhami and Rudgers 2009). Similarly, *Spodoptera frugiperda* caterpillars showed no preference for either endophyte-symbiotic or endophyte-free *Brachypodium sylvaticum* (Brem and Leuchtmann 2001), but a strong preference for endophyte-free *F. subverticillata* (Afkhami and Rudgers 2009).

Unlike herbivore preference, patterns for herbivore performance on native grass species hosting endophytic fungi appear to be more consistent, with the majority of studies showing negative effects of endophyte symbiosis, including increased mortality (Clay et al. 1985; Cheplick and Clay 1988; Tibbets and Faeth 1999; Brem and Leuchtmann 2001), reduced mass (Clay et al. 1985; Cheplick and Clay 1988; Lopez et al. 1995; Bazely et al. 1997; Brem and Leuchtmann 2001), decelerated development time (Clay et al. 1985; Cheplick and Clay 1988; Brem and Leuchtmann 2001), and decreased fitness (Tibbets and Faeth 1999).

The context-dependent nature of the interaction between the grass–endophyte symbiotum and herbivores suggests that further experimentation should be conducted using a suite of grasses and a suite of herbivores to test for the generality of effects in a single experiment. Few studies have simultaneously examined the response of one herbivore to multiple grass–endophyte symbiota (Clay et al. 1985; Cheplick and Clay 1988; Siegel et al. 1990; Clement et al. 1997, 2005) or the responses of multiple herbivores to one grass–endophyte symbiotum (Clement et al. 2005; Afkhami and Rudgers 2009). Furthermore, only six studies (Siegel et al. 1990; Clement et al. 1997, 2005; Saikkonen et al. 1999; Brem and Leuchtmann 2001; Afkhami and Rudgers 2009) experimentally manipulated the presence of the endophyte. Non-experimental studies, which pair naturally endophyte-symbiotic with naturally endophyte-free plants, confound the genetic background of the host plant with endophyte presence, preventing the assignation of causality to endophyte presence.

Here, we assessed insect herbivore responses for ten native grass species that host endophytic fungi in natural populations. We used experimental disinfection to decouple endophyte status and host plant genotype, and we conducted both laboratory and field assays of herbivore preference. We also measured herbivore performance for a subset of the grasses using a single model herbivore. To our knowledge, this is the first study to assess the context-dependency of endophyte symbiosis on herbivore responses across both multiple host–endophyte symbiota and multiple herbivore species, which is essential for characterizing the relative importance of these primary drivers of conditionality in the interaction. We also compared results from native grasses with the agronomically important, but non-native, species, tall fescue (*L. arundinaceum*). Specifically, we addressed the following questions. (1) Does endophyte presence reduce herbivore preference or performance? (2) To what degree does endophyte-mediated resistance vary with herbivore identity? (3) To what degree does endophyte-mediated resistance depend on symbiotum identity, and, specifically, do native grasses differ from a model agronomic grass?

Materials and methods

Study system

To test for generalities in herbivore deterrence across native grass species, we evaluated ten grass species native to the United States, each with their natural endophyte symbiont. Grasses spanned five subtribes in the Poaceae (Table 1). The presence of lolines, known anti-insect compounds that are produced by endophytes (Schardl et al. 2007), was evaluated for a subset of the grass species in each subtribe (Table 1). For comparison with natives, we also tested the non-native tall fescue symbiotum (*L. arundinaceum–Neotyphodium coenophialum*), the most commonly studied grass–endophyte system. Tall fescue is native to Europe and North Africa, but has become abundant in North America; it is estimated that 75–80% of North American tall fescue plants host the fungal endophyte (Ball et al. 1993; Clay and Holah 1999).

Endophyte treatment

Endophyte elimination

To separate effects of the endophyte from plant genotype, we created symbiont-free plants by heating endophyte-symbiotic seeds in a drying oven at 60°C (Table 1). In the greenhouse, we then propagated plants clonally and reduced possible side effects of the heat treatment by starting cloned plants at the same initial size. We deviated from this protocol for two species. First, due to low levels of natural endophyte colonization, seeds of *Ammophila breviligulata* were not heat treated, but instead seedlings were artificially inoculated with the endophyte and controls were sham-inoculated following methods in Tintjer and Rudgers (2006). Second, because it is an annual/short-lived perennial that is difficult to propagate clonally, *Agrostis hyemalis* was subjected to seed heat treatment for 6 min in a 62°C water bath. Treated seeds were grown in the greenhouse until seed production; this second generation of seeds, produced in common environment, was then used to plant the experimental treatments. Altogether, these methods homogenized plant genetic background with respect to the endophyte treatment. Only experimentally-treated, endophyte-free (or successfully inoculated endophyte-symbiotic) plants were used in the experiments.

Plant propagation

Following heat treatment, seeds were surface sterilized in 50% bleach and rinsed with sterile water. Then, we placed seeds on 2% water agar in 9 cm Petri plates for cold stratification at 4°C for 2–4 weeks. Germinated seedlings were transplanted to pots filled with ProMix BX (Premier Horticulture, Quakertown, PA) potting soil and grown in the greenhouse with daily watering (max. temp, 25°C; min. temp, 13°C, no supplemental light). Plants were split to produce equal sized clones for use in the laboratory and field experiments. Material for the laboratory experiment was maintained in the greenhouse in 10 cm square plastic pots. Field plants were grown in 115 ml pots (Conetainers, Stuewe and Sons, Canby, OR) until experimental populations were planted.

Endophyte detection

Following cloning, plants were checked twice for endophyte-status prior to experimentation. We removed a single leaf from each plant and applied aniline blue-lactic acid stain to thin sections of the inner leaf sheath (Bacon and White 1994). Stained tissue was examined under a compound brightfield microscope (Leica Microsystems, Wetzlar, Germany) at 200–400×.

Preference trials

Study organisms

We tested three generalist herbivores, the fall armyworm, *S. frugiperda*, the American grasshopper, *Schistocerca americana* , and the bird cherry oat aphid, *R. padi*. *S. frugiperda* is a common agricultural pest native to eastern North America that is frequently used in laboratory experiments due to its broad diet, lack of mobility, and ease of maintenance in artificial colonies (Hardy et al. 1985; Clay and Cheplick 1989; Bultman and Conard 1998). Similarly, the aphid *R. padi* is commonly used as a model phloem-feeder in grass studies, and has a cosmopolitan distribution. Few studies have tested orthopteran herbivores despite the fact that they are common, widespread, generalists that feed on grasses (but see Lopez et al. 1995; Saikkonen et al. 1999; Afkhami and Rudgers 2009). Therefore, the inclusion of preference trials utilizing native *S. americana* provided a novel comparison for herbivore choice.

Source material

Larvae of *S. frugiperda* were obtained from a commercial supplier (Bio-Serv, Frenchtown, NJ). Larvae arrived at second and third instars on Bio-Serv sterilized Lepidoptera diet trays, and remained at approximately 22°C until most had developed to the fourth instar. Larvae were starved for 2 h prior to the choice experiment. The *S. americana* (supplied by S. Behmer, Texas A&M University) were fed a uniform diet of wheatgrass (*Triticum aestivum*) (Quality

Table 1 Grass species with corresponding taxonomy, collection information, duration of heat treatment for endophyte disinfection [either in convection oven at 60°C (days) or water bath at 62°C (min)], and use in experiments indicated by *X*

Tribe	Subtribe	Code	Grass species	Endophyte species	Lolines?	GPS coordinates	Heat treatment	Performance	Preference	Field trial location (date)
Poeae	Agrostidinae	AGHY	*A. hyemalis*	*E. amarillans*	Yes[a]	31°29′52″N 94°46′46″W	6 min	X		TX (1 May 08)
Poeae	Agrostidinae	AGPE	*A. perennans*	*E. amarillans*	No	39°14′31″N 86°13′08″W	7–10 days	X	X	IN (12 Sep 09)
Poeae	Agrostidinae	AMBR	*A. breviligulata*	*E.* sp.	No	44°51′31″N 86°03′56″W	inoc.	X		*Not tested*
Poeae	Aveninae	CIAR	*C. arundinacea*	*N.* sp.	*nt*	39°14′54″N 86°13′05″W	12 days	X	X	IN (12 Sep 09)
Triticeae	Hordeinae	ELRI	*E. villosus*	*E. elymi*	No	39°14′58″N 86°13′01″W	6–7 days	X		IN (1–15 Aug 09)
Triticeae	Hordeinae	ELVI	*E. virginicus*	*E. elymi*	No	31°29′46″N 94°46′08″W	6–9 days	X	X	TX (1–15 Aug 09)
Poeae	Loliinae	FESU	*F. subverticillata*	*N.* sp.	No	39°14′19″N 86°13′04″W	10–12 min	X	X	IN (26–28 Jun 09)
Poeae	Poinae	POAL	*P. alsodes*	*N.* sp.	Yes	39°14′29″N 86°13′07″W	7–10 days	X		IN (21 May 09)
Poeae	Poinae	POAU	*P. autumnalis*	*N. PauTG-1*	Yes	31°30′07″N 94°47′18″W	11–18 days	X	X	TX (31 Jul 08)
Poeae	Poinae	POSY	*P. sylvestris*	*N. PsyTG-1*	No	39°14′22″N 86°12′55″W	6–7 days	X		IN (21 May 09)
Poeae	Loliinae	LOAR	*L. arundinaceum**	*N. coenophialum*	*nt*[b]	39°14′48″N 86°13′06″W	7 days	X	X	IN (26–28 Jun 09)
						31°29′49″N 94°44′36″W				TX (19 Jun 08)

Endophyte species are either *Epichloë* or *Neotyphodium*. All grasses were obtained from native populations (GPS coordinates for seed collections) with the exception of *L. arundinaceum* (*), which were collected from two naturalized populations. *A. breviligulata* was artificially inoculated to impose the endophyte treatment following Tintjer and Rudgers (2006). P. Nagabhyru and C. Shardl generously provided loline data. Symbiota that were not tested for lolines are indicated as *nt*

[a] Some endophyte-symbiotic individuals produce lolines

[b] Our accessions were not tested, but LOAR typically produces lolines

Feed and Garden Inc., Houston, TX, USA) after hatching and prior to the initiation of the experiment. The *R. padi* were collected from populations that naturally colonized grasses (primarily *Poa autumnalis*) in the Rice University greenhouse complex. Care was taken to ensure aphids were collected from endophyte-free grasses. Additionally, a separate experiment was conducted using *R. padi* collected from both endophyte-symbiotic and endophyte-free individuals (details below) to compare preferences by aphid diet history.

Choice-tests with S. americana and S. frugiperda

Experimental feeding arenas were constructed from 25 cm × 12 cm × 9 cm (length × width × height) plastic containers and stocked with two leaves: one endophyte-symbiotic and one endophyte-free from the same host grass species. Leaves were paired by length and morphology to control for possible effects of leaf age. Within a trial, each leaf was collected from a unique greenhouse-grown plant genotype. Wet sponges (2 cm × 2 cm × 2 cm) were placed on the end of the leaves to retain moisture and prevent wilting. To begin the experiment, third or fourth instar *S. frugiperda* or second or third instar *S. americana* were placed in the center of the arenas, equidistant between the leaves. Individual herbivores were only used in one trial to ensure that independence among replicates was maintained. Because the two herbivores differed in their feeding rates, *S. frugiperda* was allowed to feed for 24 h or until 50% of the total plant material was consumed, and *S. americana* was allowed to feed for 48 h or until 50% of total plant material was consumed. Termination at 50% consumption was deemed necessary to ensure that herbivores did not consume indiscriminately. Sample sizes for each symbiota × herbivore combination varied with the availability of plant and insect material (Table 2). Trials

where the herbivore did not choose either leaf were excluded from analyses.

To measure damage, we scanned leaf clippings before the inception of the trial and afterwards and used ImageJ computer imaging software (Rasband 2009) to obtain measurements of leaf surface area. Initial and final scans were compared to calculate the percentage surface area consumed. Preference of *S. americana* and *S. frugiperda* for endophyte-symbiotic or endophyte-free leaves was analyzed using mixed-model ANOVA with the fixed effects of plant species, endophyte treatment, and the plant × endophyte interaction, and the random effect of trial nested within plant species (Proc MIXED, SAS Institute 2009). When the plant species × endophyte treatment interaction was significant, we then tested endophyte effects within each plant species. Models met assumptions of normality of residuals and homogeneity of variance following arcsine square root transformation of percentage surface area consumed.

Choice-tests with R. padi

To test for aphid preference an experimental set-up similar to methods in Clement et al. (1992) was employed. Equal lengths of endophyte-symbiotic and endophyte-free leaves were placed in 10 cm Petri dishes, which were lined with damp filter paper to retain moisture. Three aphids (final instar and/or wingless adults) were placed equidistant between the leaves. Individual aphids were only used once during the course of the feeding trials. Seven grass species were tested—non-native *L. arundinaceum* and six natives, including five that were tested with *S. frugiperda* and *S. americana* as well as one additional *Agrostis* species (*A. hyemalis*). The number of trials for each species depended on the availability of plant material (Table 3).

To determine preference, after 24 h, we counted the number of the original three aphids feeding on each leaf. Some aphids did reproduce, but for a more conservative measure of preference, only original aphids were counted. Preference for endophyte-free plant material was assessed by scoring the number of trials in which endophyte-free plant material was preferred, defined as more of the original aphids feeding on the endophyte-free leaf than endophyte-symbiotic leaf. Aphids that were not feeding on either leaf when the data was recorded were excluded from the aphid counts. Trials in which no original aphids were observed on leaves and trials resulting in equal numbers of original aphids on endophyte-symbiotic and endophyte-free leaves were not included. Preference for each species was analyzed using chi-square tests to test the null hypothesis that the endophyte symbiosis did not affect *R. padi* preference (Proc FREQ, SAS Institute 2009).

Table 2 Results for the effect of endophyte symbiosis on preference of *S. frugiperda* and *S. americana*

Grass	Herbivore	E+	E−	*n*	*F*
AGPE	*S. frugiperda*	21.11 ± 3.81	48.61 ± 3.81	30	**18.33*****
	S. americana	7.64 ± 2.12	10.71 ± 2.67	30	3.05
AMBR	*S. frugiperda*	7.04 ± 5.58	11.47 ± 5.58	14	1.04
	S. americana	5.72 ± 2.68	4.29 ± 1.52	15	0.05
CIAR	*S. frugiperda*	36.17 ± 4.67	42.79 ± 4.67	20	1.08
	S. americana	22.58 ± 9.04	26.69 ± 8.43	9	0.18
ELRI	*S. frugiperda*	22.86 ± 4.26	22.21 ± 4.26	24	0.01
	S. americana	21.98 ± 4.25	23.77 ± 3.49	22	1.25
ELVI	*S. frugiperda*	17.45 ± 6.30	13.25 ± 6.30	11	0.34
	S. americana	27.38 ± 4.02	12.27 ± 3.63	12	**9.24***
FESU	*S. frugiperda*	17.32 ± 4.45	30.41 ± 4.45	22	2.82
	S. americana	20.24 ± 4.01	20.82 ± 4.62	23	0.02
POAL	*S. frugiperda*	19.79 ± 4.79	34.07 ± 4.79	19	**5.11***
	S. americana	33.60 ± 3.79	41.63 ± 3.81	28	**5.16***
POAU	*S. frugiperda*	18.20 ± 3.88	50.94 ± 3.88	29	**51.04*****
	S. americana	21.77 ± 3.58	34.14 ± 4.59	30	**6.94***
POSY	*S. frugiperda*	29.15 ± 5.79	50.32 ± 5.79	13	**5.80***
	S. americana	16.42 ± 2.31	24.27 ± 12.17	3	0.01
LOAR	*S. frugiperda*	15.12 ± 3.81	11.71 ± 3.81	30	0.82
	S. americana	5.94 ± 2.13	9.72 ± 2.59	31	1.63

Means (±s.e.) are the untransformed percentages of leaf area consumed for endophyte-symbiotic (E+) and endophyte-free (E−) treatments

F ratios shown from general linear mixed-models with significance indicated in *bold* as * $P < 0.05$, ** $P < 0.005$, or *** $P < 0.0005$

Aphid diet history

The endophyte-status of the aphid source population may influence herbivore preference for endophyte-symbiotic grasses. For example, aphids collected from endophyte-free plants may disproportionally prefer endophyte-free grasses relative to aphids collected from endophyte-symbiotic grasses. To test for this effect, greenhouse-reared aphids collected from both endophyte-symbiotic and endophyte-free *P. autumnalis* were reciprocally challenged with endophyte-symbiotic and endophyte-free *P. autumnalis* leaves following methods above. We chose *P. autumnalis* because endophyte symbiosis affected aphid preference in prior trials. There were twenty replicates for each aphid source population × diet history. To test for an effect of the endophyte-status of the aphid source populations, trials were scored based on the preference of the aphids for either endophyte-symbiotic or endophyte-free plant material, as described above. A Fisher's exact test evaluated the null hypothesis that aphid source population (either endophyte-symbiotic or endophyte-free) had no effect on aphid

Table 3 The percentage of trials where endophyte-free leaves were preferred over endophyte-symbiotic leaves by *R. padi* aphids

Grass	Percentage of trials	*n*	χ^2
AGHY	32.61	46	**5.57***
AGPE	61.11	54	2.67
ELVI	43.59	39	0.64
FESU	42.86	21	0.43
POAL	100	16	–[a]
POAU	72.22	18	**3.56**†
LOAR	57.14	14	0.29

Chi-square tests shown for the null hypothesis that aphids showed no preference, with significance indicated in *bold* as † $P < 0.06$, * $P < 0.05$, ** $P < 0.005$, or *** $P < 0.0005$

[a] No aphids preferred endophyte-symbiotic POAL; a chi-square test could not be conducted

preference for endophyte-symbiotic versus endophyte-free plants (Proc FREQ, SAS Institute 2009).

Performance trials

Study organisms

To test herbivore performance on endophyte-symbiotic and endophyte-free grasses, we chose a subset of five native species, each from a separate subtribe, as well as non-native *L. arundinaceum*. On 5 Febraury 2009, after the clones had grown for 2–4 months in the greenhouse, we placed a single fourth instar *S. frugiperda* on each individual, and enclosed the entire plant in a 0.85 mm polypropylene perforated plastic bag (Hubert Company, Harrison, OH, USA). Sample sizes varied with the availability of material (Table 4). Before the experiment was initiated, larvae were weighed for initial wet mass to the nearest 0.1 mg.

Response variables

At two time points (3 and 6 days), we recorded survival, and removed the caterpillars to measure larval mass to the nearest 0.1 mg. After 6 days (approx. 40% of the duration of the larval life stage), the larvae had heavily damaged several of the plants. To prevent starvation before pupation, we transferred larvae to a wheat-germ based diet medium (Bio-Serv, Frenchtown, NJ). We recorded the date of pupation for each larva and measured pupal wet mass. Larvae that were not recovered dead or alive were excluded from survival analyses ($n = 13$ of 248 total). The experiment was terminated on 29 February 2009, after all larvae had either pupated, died, or were unrecovered.

Statistical analysis

We tested for treatment effects on *S. frugiperda* performance using ANOVA including the fixed effects of plant species, endophyte treatment, and the plant × endophyte interaction (Proc GLM, SAS Institute 2009). When the plant × endophyte treatment interaction was significant, we then tested endophyte effects within each plant species. The following response variables were examined: larval mass at 3 and 6 days, days to pupation, and pupal weight. Initial larval mass did not differ among species or treatments (endophyte treatment—$F_{1,236} = 0.22$, $P = 0.64$; grass species—$F_{5,236} = 0.93$, $P = 0.46$; endophyte treatment × grass species—$F_{5,236} = 0.30$, $P = 0.92$). Therefore, any effect of larval mass on the responses was distributed evenly across treatments. Analysis of larval mass at 3 days did not differ from analysis of larval mass at 6 days, so we present only the 6 days results. Following standards used by other herbivore performance studies (Clay et al. 1985; Cheplick and Clay 1988; Brem and Leuchtmann 2001), we are considering a longer (or decelerated) development time disadvantageous to the herbivore. Analyses met the assumptions of normality of residuals and homogeneity of variances, except for the analysis of days to pupation, which was log transformed to correct normality. Survival on each species was analyzed using Fisher's exact tests for the null hypothesis that the endophyte symbiosis did not influence *S. frugiperda* survival (Proc FREQ, SAS Institute 2009).

Field trials

Field experimental design

We created field populations for each species at the same locations where seeds were collected at the Indiana University Research and Teaching Preserve at Lily Dickey Woods (Nashville, IN) or at the Stephen F. Austin Experimental Forest (Nacogdoches, TX) (Table 1). *L. arundinaceum* plots were established in both Texas and Indiana from seeds collected from locally naturalized populations at each site. Therefore, Texas plots contained *L. arundinaceum* collected from Texas and Indiana plots contained *L. arundinaceum* collected from Indiana, thus representing independent tests of endophyte presence in this species. Each population consisted of 20 plants (0.5 m apart) arranged in a 4 × 5 grid, with 5 replicate 100% endophyte-free populations and 5 replicate 100% symbiotic populations. The only experiment that deviated was *L. arundinaceum* in Texas, for which replication was ten rather than five because we were concerned about high rates of plant predation by voles. Adult plants were added to the natural matrix of vegetation to maintain ambient

Table 4 Results for the effect of endophyte symbiosis on the performance of *S. frugiperda*

Grass	Response	E+	E−	Stats
AGPE	Percentage survival	10 (20)	82.35 (17)	**<0.0001**
	Larval mass (mg)	60.64 ± 19.31 (16)	78.23 ± 10.28 (16)	0.65
	Days to pupation	16.00 ± 4.00 (2)	18.57 ± 0.49 (14)	3.00
	Pupal weight (mg)	317.55 ± 39.25 (2)	336.54 ± 7.11 (14)	0.72
CIAR	Percentage survival	78.57 (14)	93.33 (15)	0.33
	Larval mass (mg)	115.26 ± 9.14 (14)	231.07 ± 22.41 (16)	**20.66*****
	Days to pupation	17.73 ± 0.92 (11)	13.93 ± 0.30 (14)	**20.24*****
	Pupal weight (mg)	237.46 ± 20.37 (11)	320.54 ± 5.63 (14)	**19.02*****
ELRI	Percentage survival	100 (18)	89.47 (19)	0.49
	Larval mass (mg)	138.81 ± 6.37 (18)	172.93 ± 13.28 (20)	**5.00***
	Days to pupation	16.06 ± 0.42 (18)	15.12 ± 0.35 (17)	3.08
	Pupal weight (mg)	307.79 ± 9.96 (18)	312.09 ± 10.31 (17)	0.09
FESU	Percentage survival	94.12 (17)	100 (6)	1.00
	Larval mass (mg)	92.78 ± 7.16 (17)	111.76 ± 12.71 (7)	1.89
	Days to pupation	18.94 ± 0.31 (16)	17.83 ± 0.31 (6)	**4.30**†
	Pupal weight (mg)	363.78 ± 9.36 (16)	347.32 ± 13.44 (6)	0.89
POAU	Percentage survival	28 (25)	85.71 (21)	**<0.0001**
	Larval mass (mg)	24.10 ± 2.53 (22)	45.26 ± 3.65 (21)	**23.08*****
	Days to pupation	23.29 ± 0.29 (7)	22.11 ± 0.32 (18)	**4.40***
	Pupal weight (mg)	370.10 ± 12.67 (7)	363.35 ± 10.75 (18)	0.13
LOAR	Percentage survival	100 (31)	100 (32)	–[a]
	Larval mass (mg)	139.85 ± 6.28 (30)[b]	140.29 ± 6.69 (32)	0.00
	Days to pupation	16.74 ± 0.23 (31)	16.56 ± 0.22 (32)	0.31
	Pupal weight (mg)	325.36 ± 7.27 (31)	331.87 ± 7.77 (32)	0.37

Untransformed means and standard errors for the herbivore responses on endophyte-symbiotic (E+) and endophyte-free (E−) plants are presented, followed by the total sample size in parentheses. Fisher's exact test was used to test the null hypothesis of no interaction between endophyte-status and survival (*P* values reported in the stats column). ANOVA was used for all other statistical tests (*F* ratios reported with statistical significance at † $P < 0.06$, * $P < 0.05$, ** $P < 0.005$, and *** $P < 0.0005$, respectively)

[a] No aphids died on LOAR during the course of the experiment, so a Fisher's exact test could not be conducted

[b] There was a missing larva that was found later and included in the pupal measures

levels of plant competition. Plots were positioned a minimum of 5 m apart to minimize seed dispersal between plots, and the endophyte treatment was assigned to plot location at random. Within a plot, each plant had the same endophyte-status but represented a unique host genotype; thus, all plots had the same initial level of plant genotypic diversity. Due to high local densities of deer, plots were fenced (1.8 m T-posts plus deer netting). In Texas, we planted during 9–10 November 2007. In Indiana, we planted during 7–10 April 2008, excepting for *L. arundinaceum*, which was planted during 27–29 September 2007 (corresponding to the target timing for tall fescue planting in the Midwestern US).

Response variables

After the plants had been established for 6–21 months, we assessed natural levels of herbivore damage using two methods for each of the 200 plants per grass species. First, we visually estimated the total percentage of damage for the whole plant, subdividing the damage into two types: haustellate damage (mainly hemipterans) and mandibulate damage (mainly lepidopterans and orthopterans). Haustellate damage included spotting or stippling of leaves caused by the localized destruction of chlorophyll by enzymes at feeding site (leaf chlorosis) which is typically associated with aphids and leafhoppers, as well as silvery or bronze leaf scarring caused by thrips (Thysanoptera). It is possible that haustallate damage was underestimated; however, underestimation should not alter the relative differences between endophyte-symbiotic and endophyte-free plots. Mandibulate damage included holes chewed in leaves, consumption of entire leaves, consumption of distinct portions of leaves, and notches in the margins of leaves. For leaves that were consumed entirely or chewed from the tips, the nearest, similarly sized, undamaged leaf was compared to assist in estimating

undamaged leaf area. For each species, visual estimates were calibrated against quantitative estimates using transparent grids placed over the leaves; grid squares were counted to estimate both total leaf area and damaged area for a random subset of 47–98 individual leaves per species. For the second method, we chose the first 20–30 leaves encountered per plant from a haphazardly chosen location and scored each leaf for the presence of herbivory to calculate the percentage of leaves with damage. Based on repeated censuses, herbivory estimates were obtained during peak damage levels; these dates varied among species and locations (Table 1).

Statistical analysis

Plot was the unit of replication; therefore, the effect of the endophyte treatment was tested over variation between plots using mixed-model ANOVA with the fixed factor of endophyte treatment and the random effect of plot (Proc MIXED, SAS Institute 2009). We analyzed the experiment for each species separately for clarity of presentation and because laboratory trials led to the a priori expectation of strong effects of symbiotum identity on herbivore responses to endophyte presence.

Results

Herbivore preference

S. frugiperda preferred endophyte-free plant material over endophyte-symbiotic plant material for four of nine native grass species and displayed no preference for either endophyte-symbiotic or endophyte-free material for the remaining five grass species (Table 2). Preference for non-symbiotic plants was most pronounced in the Poinae subtribe, with *S. frugiperda* preferring endophyte-free leaves from all three *Poa* species. The largest difference was observed for *P. autumnalis*, where *S. frugiperda* consumed nearly three times more non-symbiotic than symbiotic material ($F_{1,28} = 51.04$, $P < 0.0001$). In addition, twice as much endophyte-free *Agrostis perennans* material was consumed ($F_{1,29} = 18.33$, $P = 0.0002$), and 70% more plant material from endophyte-free *Poa alsodes* ($F_{1,18} = 5.11$, $P = 0.0364$) and *Poa sylvestris* ($F_{1,12} = 5.08$, $P = 0.0330$). In trials with *L. arundinaceum*, *S. frugiperda* showed no preference for either endophyte-symbiotic or endophyte-free material ($F_{1,29} = 0.82$, $P = 0.3713$).

S. americana preferred endophyte-free plants of *P. autumnalis* and *P. alsodes* (Table 2), consuming 55% more endophyte-free *P. autumnalis* ($F_{1,29} = 6.94$, $P = 0.0134$) and 24% more endophyte-free *P. alsodes* ($F_{1,27} = 5.16$, $P = 0.0314$). In trials using *Elymus virginicus*, a significant preference for endophyte-symbiotic material was observed, with grasshoppers consuming more than twice as much endophyte-symbiotic material as endophyte-free material ($F_{1,11} = 9.24$, $P = 0.0113$). Despite careful pairings, post-hoc analysis of *E. virginicus* leaf size showed that paired endophyte-free leaves had 38% more surface area than endophyte-symbiotic leaves ($F_{1,22} = 5.35$, $P = 0.0305$), which were generally narrower than endophyte-free leaves of the same length. Therefore, *S. americana* preference for endophyte-symbiotic *E. virginicus* leaves was confounded with a smaller initial leaf area; however, it seems unlikely that grasshopper preference for leaves with the endophyte would occur only because of their smaller size. *S. americana* showed no preference for either endophyte-symbiotic or endophyte-free *L. arundinaceum* ($F_{1,30} = 1.63$, $P = 0.2121$).

Of the six native grass species tested, only one species (*P. alsodes*) showed an endophyte-mediated reduction in preference by the aphid *R. padi* (Table 3). The aphids never preferred endophyte-symbiotic leaves of *P. alsodes*; thus, no statistical tests were required (or possible). There was a trend for aphids to prefer endophyte-free *P. autumnalis*, with aphids preferring symbiont-free material in 72% of trials $\left(\chi^2_{1,17} = 3.56, P = 0.0593\right)$. In contrast, *R. padi* showed a significant preference for endophyte-symbiotic *A. hyemalis*, with aphids preferring symbiotic leaves in ~70% of the trials $\left(\chi^2_{1,45} = 5.57, P = 0.0183\right)$. Endophyte symbiosis had no effect on *R. padi* preference for non-native *L. arundinaceum* $\left(\chi^2_{1,13} = 0.29, P = 0.5930\right)$.

Aphid preference for endophyte-free plant material was not dependent on whether they consumed endophyte-symbiotic or endophyte-free *P. autumnalis* prior to the feeding trials ($P = 1.00$). Aphids from both endophyte-symbiotic and endophyte-free plants consumed endophyte-free leaves of *P. autumnalis* in 75% of the trials. Therefore, we do not believe using *R. padi* from only endophyte-free plants biased our results.

Herbivore performance

Two of five native plant-endophyte symbiota showed strong effects of endophyte presence on *S. frugiperda* survival. Larval survival was significantly higher on endophyte-free than on symbiotic plants for *A. perennans* ($P < 0.0001$) and *P. autumnalis* ($P < 0.0001$) (Table 4). More than 80% of the *S. frugiperda* larvae survived on endophyte-free plants of both species, while only 10% survived on symbiotic *A. perennans* and 28% survived on symbiotic *P. autumnalis*. No *S. frugiperda* reared on either endophyte-symbiotic or endophyte-free *L. arundinaceum* died during the course of the experiment.

S. frugiperda had significantly greater mass when reared on endophyte-free plants for three out of five native species

(Table 4). On endophyte-free *Cinna arundinacea*, *S. frugiperda* weighed twice as much as larvae reared on symbiotic plants ($F_{1,28} = 20.66$, $P < 0.0001$). Significant differences were also detected for *P. autumnalis,* where larvae on endophyte-free plants weighted 89% more than larvae on symbiotic plants ($F_{1,41} = 23.08$, $P < 0.0001$). On average, the larvae on symbiotic *P. autumnalis* lost mass over the course of the trial. *S. frugiperda* were 24% larger when reared on endophyte-free *Elymus villosus* ($F_{1,36} = 5.00$, $P = 0.0317$). In contrast to responses for native grasses, larvae raised on *L. arundinaceum* did not differ in mass when reared on endophyte-symbiotic versus endophyte-free plants ($F_{1,60} = 0.00$, $P = 0.9617$).

Larvae reared on endophyte-free plants of two of the five native grasses developed to pupae more quickly than those reared on symbiotic plants (Table 4). In particular, *S. frugiperda* on endophyte-free *C. arundinacea* pupated 27% (~4 days) more quickly than larvae on symbiotic plants ($F_{1,23} = 20.24$, $P = 0.0002$). On endophyte-free *P. autumnalis*, larvae developed 5% (~1 day) more quickly ($F_{1,23} = 4.40$, $P = 0.0471$). There was a trend for larvae on endophyte-free *F. subverticillata* to develop 6% (~1 day) more quickly ($F_{1,20} = 4.30$, $P = 0.0513$). Finally, one native symbiotum showed endophyte effects on pupal mass. Pupae reared on endophyte-free *C. arundinacea* weighed, on average, 35% more than pupae reared on symbiotic plants ($F_{1,23} = 19.02$, $P = 0.0002$) (Table 4). Endophyte symbiosis did not affect the developmental rate ($F_{1,61} = 0.31$, $P = 0.5799$) or pupal mass ($F_{1,61} = 0.37$, $P = 0.5435$) of caterpillars reared on *L. arundinaceum.*

Field herbivory

Endophyte presence significantly reduced insect herbivory for six of nine native symbiota grown in field plots (Table 5). Within the subtribe Agrostidinae, the presence of the endophyte decreased total damage by 69% for *A. hyemalis* ($F_{1,9} = 2.19$, $P = 0.0082$) and 42% for *A. perennans* ($F_{1,9} = 6.71$, $P = 0.0323$). Herbivory was reduced by >50% for all the three members of the Poinae (*P. alsodes*—$F_{1,9} = 24.56$, $P = 0.0012$; *P. autumnalis*—$F_{1,9} = 19.10$, $P = 0.0024$; *P. sylvestris*—$F_{1,9} = 37.37$, $P = 0.0002$). *C. arundinacea* (Aveninae) also benefited from symbiosis, with total damage decreasing by 48% ($F_{1,9} = 7.96$, $P = 0.0226$). Insect herbivory was reduced on non-native, symbiotic *L. arundinaceum*, but only at the Texas location ($F_{1,19} = 16.10$, $P = 0.0012$), where there were twice as many plots compared to the other experiments. This result could reflect a weaker effect of the endophyte in *L. arundinaceum* relative to native species-one that is only detected with the greater statistical power conferred by higher sample sizes.

As in the laboratory, symbiota varied in the type of herbivory they affected in the field. Endophyte symbiosis in the Poinae decreased mandibulate herbivory (*P. alsodes*—$F_{1,9} = 21.99$, $P = 0.0227$; *P. autumnalis*: $F_{1,9} = 39.65$, $P = 0.0002$; *P. sylvestris*—$F_{1,9} = 10.83$, $P = 0.0013$), whereas symbioses in the Agrostidinae deterred haustellate herbivory (*A. hyemalis*—$F_{1,9} = 9.69$, $P = 0.0144$; *A. perennans*—$F_{1,9} = 5.79$, $P = 0.0405$), suggesting a phylogenetic signal of symbiotum for herbivore deterrence. *C. arundinacea*, our sole representative of the Aveninae, showed endophyte-mediated reductions in both haustellate ($F_{1,9} = 4.98$, $P = 0.0669$) and mandibulate herbivory($F_{1,9} = 3.25$, $P = 0.1103$), although both herbivory types were only marginally significant. In general, the type of herbivory most strongly affected by the endophyte was also the more abundant type occurring on that symbiotum; thus, differences could also reflect statistical power to detect treatment effects.

Discussion

Our results demonstrate the general pattern that endophytic fungi confer anti-herbivore benefits to native grasses. For seven of the ten native grass species examined, which spanned four genera, we found at least one case whereby endophyte-symbiotic individuals reduced herbivore preference or performance relative to endophyte-free individuals (Table 6). Results from laboratory trials were largely consistent with data collected on levels of natural herbivory in experimental field plots, which suggests the protective benefit of reduced herbivory is strong in native symbiota (Table 6). This work is important because it is the first experimental test to include a taxonomically broad array of native symbiota and herbivores, thereby facilitating the detection of general patterns.

Interestingly, we found little evidence that the endophyte in the non-native and agronomic host, *L. arundinaceum*, conferred anti-herbivore benefits to its host, with the exception of field data from one of two locations. Contrary to the hypothesis that agriculture has artificially selected for endophytes with improved herbivore deterrence, our data suggest that endophytic fungi that occur in native grasses benefit their hosts through reductions in herbivore preference and performance at least as much and often more than endophytic fungi in the most studied, agronomically important grass species. Our results conflict with many other studies on tall fescue, which have shown strong, endophyte-mediated insect resistance (reviewed by Saikkonen et al. 2006; Rudgers and Clay 2007). Unlike the *L. arundinaceum* from agronomic plots, which are typically used in other studies, our plants came from two naturalized, roadside populations. Although this difference in source

Table 5 Results for the effect of endophyte symbiosis on insect herbivory in experimental field populations

Grass (location)	Response	E+	E−	*F*
AGHY	% Leaves damaged	64.9 ± 9.4	83.2 ± 10.4	1.64
(TX)	Visual total % damage	6.3 ± 2.7	20.5 ± 2.7	**2.19***
	Haustellate % damage	5.7 ± 3.0	18.4 ± 3.0	**9.69***
	Mandibulate % damage	0.6 ± 0.5	2.1 ± 0.5	0.90
AGPE	% Leaves damaged	71.3 ± 4.8	89.8 ± 4.7	**7.44***
(IN)	Visual total % damage	15.0 ± 4.1	25.8 ± 4.1	**6.71***
	Haustellate % damage	11.8 ± 3.6	20.4 ± 3.6	**5.97***
	Mandibulate % damage	2.0 ± 0.4	2.9 ± 0.4	1.63
CIAR	% Leaves damaged	75.4 ± 4.1	88.3 ± 4.0	**5.04**†
(IN)	Visual total % damage	20.2 ± 4.3	39.1 ± 4.3	**7.96***
	Haustellate % damage	2.4 ± 0.8	4.4 ± 0.8	4.98
	Mandibulate % damage	16.8 ± 5.9	33.6 ± 5.9	3.25
ELRI	% Leaves damaged	4.2 ± 1.3	0.7 ± 1.4	3.46
(IN)	Visual total % damage	30.7 ± 4.5	34.2 ± 4.6	0.11
	Haustellate % damage	22.8 ± 4.3	25.9 ± 4.3	0.10
	Mandibulate % damage	7.8 ± 0.8	8.2 ± 0.8	0.35
ELVI	% Leaves damaged	n/a	n/a	n/a
(TX)	Visual total % damage	9.3 ± 2.3	11.0 ± 2.3	0.42
	Haustellate % damage	4.0 ± 1.1	7.1 ± 1.1	3.22
	Mandibulate % damage	4.2 ± 1.2	1.9 ± 1.2	1.15
FESU	% Leaves damaged	45.0 ± 5.5	44.7 ± 5.5	0.01
(IN)	Visual total % damage	10.6 ± 1.6	9.2 ± 1.6	0.29
	Haustellate % damage	7.6 ± 1.2	6.6 ± 1.2	0.30
	Mandibulate % damage	3.0 ± 0.4	2.7 ± 0.4	0.19
POAL	% Leaves damaged	22.9 ± 4.4	43.3 ± 4.6	**10.16***
(IN)	Visual total % damage	2.6 ± 1.2	11.3 ± 1.3	**24.56****
	Haustellate % damage	1.3 ± 1.0	4.1 ± 1.0	1.78
	Mandibulate % damage	1.0 ± 1.4	7.2 ± 1.4	**21.99*****
POAU	% Leaves damaged	34.0 ± 3.8	69.4 ± 3.8	**44.18*****
(TX)	Visual total % damage	7.7 ± 1.4	17.5 ± 1.4	**19.10****
	Haustellate % damage	5.8 ± 1.1	8.1 ± 1.1	2.46
	Mandibulate % damage	2.0 ± 1.0	9.6 ± 1.0	**39.65*****
POSY	% Leaves damaged	17.4 ± 2.6	33.2 ± 2.6	**23.55*****
(IN)	Visual total % damage	2.7 ± 0.9	8.4 ± 0.9	**37.37*****
	Haustellate % damage	1.1 ± 0.7	3.7 ± 0.7	4.15
	Mandibulate % damage	1.5 ± 0.7	4.2 ± 0.7	**10.83*****
LOAR	% Leaves damaged	36.6 ± 4.1	34.0 ± 4.2	0.19
(IN)	Visual total % damage	9.0 ± 1.2	8.5 ± 1.2	0.74
	Haustellate % damage	6.1 ± 0.9	5.0 ± 1.0	3.52
	Mandibulate % damage	2.9 ± 0.4	3.5 ± 0.4	1.39
LOAR	% Leaves damaged	n/a	n/a	n/a
(TX)	Visual total % damage	7.2 ± 2.7	13.1 ± 2.8	**16.10****
	Haustellate % damage	2.1 ± 0.6	5.7 ± 0.7	**15.38****
	Mandibulate % damage	4.7 ± 2.7	4.9 ± 2.8	1.82

Data show the untransformed means and standard errors for endophyte-symbiotic (E+) and endophyte-free (E−) plants. Sample size was 5 plots per endophyte treatment (20 individuals per plot), excepting *L. arundinaceum* (LOAR) in Texas, which had 10 plots. General linear mixed-models were used for statistical tests, and significance is indicated by † $P < 0.06$, * $P < 0.05$, ** $P < 0.005$, or *** $P < 0.0005$

population could explain the lack of significant effects, it is likely that the populations in our study escaped from nearby cultivated areas, as commonly occurs with *L. arundinaceum* (Raloff 2003). Another possible explanation for our conflicting results is that endophyte-mediated resistance to *S. frugiperda*, in particular, appears to be relatively weaker and more variable for *L. arundinaceum* than for other grass-endophyte symbiotum (Ball et al.

2006). Regardless of the mechanism underlying these differences among tall fescue studies, our results show clearly that native symbiota can show endophyte-mediated resistance to herbivory that is equal if not stronger than resistance conferred by the tall fescue endophyte, both in prior work and in our current study.

We detected phylogenetic patterns in both the type and magnitude of herbivore resistance conferred by endophyte symbiosis. Four of the ten native grass-endophyte symbiota benefited from reduced herbivore preference in both laboratory trials and the field. These symbiota represented two subtribes, the Agrostidinae (*A. perennans*) and the Poinae (*P. alsodes*, *P. sylvestris*, and *P. autumnalis*), neither of which have previously been tested for endophyte-mediated herbivore deterrence. We also observed endophyte-mediated resistance to herbivory in the field for the related *A. hyemalis* (Agrostidinae), and this species was only tested against aphids in the laboratory. The endophyte did not deter herbivores in either the lab or the field for *A. breviligulata* (also Agrostidinae), which typically receives low amounts of herbivore damage in natural populations (1–15% per plant K. Crawford personal observation). Therefore, the endophyte in *A. breviligulata* may primarily confer other benefits to its host, such as increased drought tolerance or nutrient uptake (see also Emery et al. 2010).

Unexpectedly, we found no effect of the endophyte on herbivore preference in either laboratory or field experiments on *F. subverticillata*. In contrast, a previous study of *F. subverticillata* found that both *S. frugiperda* and *R. padi* preferred endophyte-free plants (Afkhami and Rudgers 2009). Afkhami and Rudgers tested a single population of *F. subverticillata* that was collected from the Indiana University Experimental Field at Bayles Road, Bloomington, Indiana (39°13′12″N, 86°32′33″W), which was not tested either in the laboratory or the field for this study. Therefore, it is possible that there exists a strong influence of grass and/or endophyte genotype on herbivore deterrence for this species; strong genotypic effects are not uncommon among grass-endophyte symbiota (Cheplick and Faeth 2009). In addition, sample sizes for *F. subverticillata* in our laboratory trials were small, which may have limited our ability to detect an effect. For example, there was a trend for *S. frugiperda* larvae to develop more quickly on endophyte-free *F. subverticillata* than on endophyte-symbiotic.

In addition to the conditionality arising from grass phylogenic history, the effects of endophyte symbiosis on herbivore preference also depended on the identity of the herbivore. Of the three herbivore groups tested, the aphids were the least responsive, suggesting that *R. padi* may not be a good model herbivore for detecting generalized insect deterrence in native grasses. Additionally, future tests for endophyte-mediated aphid deterrence would benefit from using caged plants rather than cut leaves for increased realism. In contrast to aphids, both of the mandibulate herbivore species tested (*S. frugiperda* caterpillars and *S. americana* grasshoppers) were typically deterred by the same symbiota, suggesting similar mechanisms may underlie endophyte-mediated resistance to these insect species. All three herbivore species were deterred by only two congeneric species, *P. alsodes* and *P. autumnalis*. While *P. sylvestris* did not have significantly reduced damage by *S. americana* in the laboratory, this was likely due to a lack of power (low sample size)

Table 6 Summary of results for herbivore preference and performance across the symbiota tested in this study

Grass	Laboratory herbivore preference			Herbivore performance				Field herbivore preference		
	S. frugiperda	*S. americana*	*R. padi*	Percentage survival	Larval mass	Days to pupation	Pupal weight	Haustellate damage	Mandibulate damage	Total damage
AGHY	−	0	+	n/a	n/a	n/a	n/a	−	0	−
AGPE	n/a	n/a	0	−	0	0	0	−	0	−
AMBR	0	0	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
CIAR	0	0	n/a	0	−	−	−	0	0	−
ELRI	0	0	n/a	0	−	0	0	0	0	0
ELVI	0	+	0	n/a	n/a	n/a	n/a	0	0	0
FESU	0	0	0	0	0	−†	0	0	0	0
POAL	−	−	−	n/a	n/a	n/a	n/a	0	−	−
POAU	−	−	−†	−	−	−	0	0	−	−
POSY	−	0	n/a	n/a	n/a	n/a	n/a	0	−	−
LOAR	0	0	0	0	0	0	0	−	0	−

Significantly negative effect of endophyte presence denoted by (−), significantly positive effect by (+), and no significant effect by (0)

n/a indicates the symbiota was not tested for a given response variable

† Marginal significance

as damage was 33% lower in symbiotic plants; furthermore, symbiotic *P. sylvestris* did show lower mandibulate damage in the field. This pattern suggests that insect herbivores may be broadly deterred by endophytes in the Poinae, whereas deterrence in other clades may tend toward greater specificity against particular insect species or feeding guilds.

While most herbivores were deterred by endophyte symbiosis, endophyte presence increased herbivore preference in experimental trials for two species: *A. hyemalis* and *E. virginicus*. Endophyte-symbiotic *A. hyemalis* was preferred by aphids in the laboratory, but received significantly less haustellate damage in the field. These results contrast with the previous conclusion that *R. padi* is not affected by the endophyte in this species (Siegel et al. 1990). The effect of endophytes on the reproduction of *R. padi* can be dependent on the genotype of the aphid (Bieri et al. 2009), suggesting that some aphid genotypes are less sensitive to the presence of endophytes than others. Aphids less sensitive to the negative effects of endophytes may not show a preference for endophyte-free versus symbiotic plants. Therefore, it is possible that aphid genotype may explain our conflicting results. The difference between field and laboratory results may also reflect a complex relationship between aphid preference and performance. For example, aphids may prefer symbiotic plants when presented with a choice, but are lower in abundance on symbiotic plants in the field due to reduced performance. Alternatively, the majority of haustellate damage in the field was likely caused by species other than *R. padi*, which we used as a model haustellate herbivore but was not commonly observed in our field plots. For *E. virginicus*, preference for endophyte-symbiotic plants was observed for the grasshopper, *S. americana*. There was also a strong trend for both *S. frugiperda* and *R. padi* to prefer endophyte-symbiotic leaves of *E. virginicus* and a trend for higher haustellate damage to endophyte-symbiotic plants in the field. Increased herbivore preference for symbiotic grasses appears generally uncommon, although two native grasshoppers (*Encoptolophus costalis* and *R. guttata*) preferred symbiotic *F. subverticillata* in similar laboratory choice trials (Afkhami and Rudgers 2009). One possible mechanism for the reversed preference in these two host grass species is that symbiotic individuals of *A. hyemalis* and *E. virginicus* have increased nutrient quality relative to endophyte-free plants. For example, endophyte-symbiotic tall fescue (*L. arundinaceum*) can have higher nitrogen (Lyons et al. 1990) and phosphorus (Malinowski et al. 2000) content than endophyte-free individuals. Alternatively, herbivores could be displaying compensatory feeding in the presence of the endophyte.

In contrast to herbivore preference, herbivore performance was reduced by endophyte symbiosis for four of five native grass species. Overall, negative effects of the endophytes tended to occur before pupation, which is likely to be of most benefit to the grass. In fact, for endophyte-symbiotic *A. perennans* and *P. autumnalis*, fewer than 30% of larvae survived to pupation. This percentage survival was similar to the survival of *S. frugiperda* on other native symbiota, although only one of these studies experimentally manipulated endophyte presence (Clay et al. 1985; Cheplick and Clay 1988; Brem and Leuchtmann 2001). *S. frugiperda* preference and performance were not strongly linked within grass species. The one exception to this pattern was *P. autumnalis* which had strong negative effects on both preference and performance. Interestingly, the endophyte in *C. arundinacea*, which did not influence the preference of herbivores in the laboratory, did reduce the performance of *S. frugiperda* and also reduced damage levels in the field. This field result could reflect a primary reduction in performance rather than preference. However, it remains unknown whether reduction in larval mass represents an effect of larvae consuming less (which would benefit the plant) or simply assimilating less plant material. Similarly, endophyte-infected *E. villosus* negatively influenced some aspect of herbivore performance relative to endophyte-free individuals, but did not influence herbivore preference; however, this species did not show endophyte-mediated reductions in herbivore damage in the field. Finally, *S. frugiperda* reared on symbiotic *F. subverticillata* did not perform differently than larvae reared on endophyte-free plants, excepting a marginally significant negative effect on developmental time. This is consistent with previous work showing that this symbiotum does not affect *S. frugiperda* larval or pupal weight (Afkhami and Rudgers 2009). However, other endophyte-symbiotic species of *Festuca* are reported to have negative effects on survival, mass gain, and development of *S. frugiperda* (Cheplick and Clay 1988), as well as on leaf-cutter ants, *Acromyrmex versicolor* (Tibbets and Faeth 1999). We do note that we did not control for the sex of herbivores used in either the preference or performance tests. Further studies testing how sex mediates the effect of endophyte symbiosis on herbivore performance could yield important information on how endophytes influence the structure of herbivore populations.

A likely mechanism underlying endophyte-mediated anti-herbivore benefits is the production of fungal alkaloids, which have been shown to both deter insect herbivores and reduce their performance (reviewed in Schardl et al. 2007). The effect of endophyte symbiosis on herbivore preference was particularly strong for *P. alsodes* and *P. autumnalis,* and the endophyte in *P. autumnalis*

significantly decreased most measures of herbivore performance. The endophytes present in our accessions of these two species are documented to produce loline alkaloids, including the derivatives *N*-acetyl norloline (NANL) in *P. alsodes* and *N*-formyl loline (NFL), *N*-methyl loline (NML), Loline and NANL in *P. autumnalis* (P. Nagabhyru and C.L. Schardl, unpublished data). These derivatives have different biological activities (reviewed in Schardl et al. 2007), and further studies are needed to document their effects on specific herbivores. Lolines have not been detected in *P. sylvestris*. Symbiota that had no effect on herbivore preference or performance (*E. virginicus* and *F. subverticillata*) produced no detectable lolines (P. Nagabhyru and C. Schardl, unpublished data). Finally, *E. amarillans* present in *Agrostis* species reduced haustellate herbivory to both *A. hyemalis* and *A. perennans* in the field, and reduced damage by *S. frugiperda* to *A. perennans* in the laboratory. Lolines were detected in some but not all *A. hyemalis* plants (P. Nagabhyru and C. Schardl, unpublished data), but has not been detected in *A. perennans*. This suggests the possibility that some herbivores respond to different defensive compounds, such as peramine—a known insect herbivore deterrent that is also synthesized by grass endophytes (Schardl et al. 2007). Finally, although long-term studies with *L. arundinaceum* have revealed that the presence of the endophyte alters the composition of neighboring plant species (Rudgers et al. 2007; Rudgers and Clay 2008), thus far we have not detected similar effects in our field populations (unpublished data). Therefore, differences in herbivory between symbiotic and symbiont-free plots are not currently an indirect response to changes in the surrounding vegetation.

Altogether, our results expand current understanding of the effects of native grass-endophyte symbioses on herbivores by demonstrating phylogenetic signal in endophyte-mediated resistance and context-dependency on herbivore identity. Specifically, herbivore preference was conditional on both the herbivore species and on the identity of the grass-endophyte complex. Herbivore performance was more consistently negatively influenced by endophytes than was preference, and negative impacts were most apparent before pupation. By examining a range of responses across a suite of grass species and a variety of generalist herbivores, this study highlights the general pattern that endophytes in native grasses confer defenses against herbivores to their hosts. Importantly, these benefits were stronger in magnitude than the effects of endophyte-mediated herbivore resistance in the non-native and agronomic grass, *L. arundinaceum*, for which large community and ecosystem consequences of endophyte symbiosis have been reported. Thus, results here suggest that endophytes in native grasses have the potential to cause similarly broad-scale community and ecosystem impacts.

Acknowledgments We would like to thank S. Behmer and his lab for supplying the grasshoppers used in this experiment, and P. Nagabhyru and C. Schardl for providing loline data for our plant accessions. The Rice Undergraduate Honors Research in Ecology and Evolutionary Biology class of 2009 provided useful discussion, and S. Clement, A. Davitt, C. Schardl, and P. Nagabhyru provided insightful comments on the text. K. Clay, C. Rudolf, D. Saenz, and R. Thill provided logistical support at the field sites, and L. Albert, S. Hammer, E. Seifert, and S. Ziegler assisted with field data collection. KMC was supported by a National Science Foundation Graduate Research Fellowship and a Rice University Presidential Fellowship. JAR was supported by the endowed Godwin Professorship at Rice University and National Science Foundation DEB 054278.

References

Afkhami ME, Rudgers JA (2009) Endophyte-mediated resistance to herbivores depends on herbivore identity in the wild grass *Festuca subverticillata*. Environ Entomol 38:1086–1095

Bacon CW, White JF (1994) Stains, media, and procedures for analyzing endophytes. In: Bacon CW, White JF (eds) Biotechnology of endophytic fungi of grasses. CRC Press, Boca Raton, pp 47–56

Ball DM, Pedersen JF, Lacefield GD (1993) The tall fescue endophyte. Am Sci 81:370–379

Ball OJP, Coudron TA, Tapper BA, Davies E, Trently D, Bush LP, Gwinn KD, Popay AJ (2006) Importance of host plant species, *Neotyphodium* endophyte isolate, and alkaloids on feeding by *Spodoptera frugiperda* (Lepidoptera: Noctuidae) larvae. J Econ Entomol 99:1462–1473

Barbosa P, Krischik VA, Jones CG (1991) Microbial mediation of plant–herbivore interactions. Wiley, New York

Bazely DR, Vicari M, Emmerich S, Filip L, Lin D, Inman A (1997) Interactions between herbivores and endophyte-infected *Festuca rubra* from the Scottish islands of St Kilda, Benbecula and Rum. J Appl Ecol 34:847–860

Bieri AP, Härri SA, Vorburger C, Müller CB (2009) Aphid genotypes vary in their response to the presence of fungal endosymbionts in host plants. J Evol Biol 22:1775–1780

Boning RA, Bultman TL (1996) A test for constitutive and induced resistance by tall fescue (*Festuca arundinacea*) to an insect herbivore: impact of the fungal endophyte, *Acremonium coenophialum*. Am Midl Nat 136:328–335

Brem D, Leuchtmann A (2001) *Epichloe* grass endophytes increase herbivore resistance in the woodland grass *Brachypodium sylvaticum*. Oecologia 126:522–530

Bultman TL, Conard NJ (1998) Effects of endophytic fungus, nutrient level, and plant damage on performance of fall armyworm (Lepidoptera:Noctuidae). Environ Entomol 27:631–635

Bultman TL, Bell G, Martin WD (2004) A fungal endophyte mediates reversal of wound-induced resistance and constrains tolerance in a grass. Ecology 85:679–685

Cheplick GP, Clay K (1988) Acquired chemical defenses in grasses—the role of fungal endophytes. Oikos 52:309–318

Cheplick GP, Faeth SH (2009) Ecology and evolution of the grass–endophyte symbiosis. Oxford University Press, Oxford

Clay K (1990) Fungal endophytes of grasses. Annu Rev Ecol Syst 21:275–297

Clay K, Cheplick GP (1989) Effect of ergot alkaloids from fungal endophyte-infected grasses on fall armywork (*Spodoptera frugiperda*). J Chem Ecol 15:169–182

Clay K, Holah J (1999) Fungal endophyte symbiosis and plant diversity in successional fields. Sci 285:1742–1744

Clay K, Schardl C (2002) Evolutionary origins and ecological consequences of endophyte symbiosis with grasses. Am Nat 160:S99–S127

Clay K, Hardy TN, Hammond AM (1985) Fungal endophytes of grasses and their effects on an insect herbivore. Oecologia 66:1–5

Clement SL, Lester DG, Wilson AD, Pike KS (1992) Behavior and performance of *Diuraphis noxia* (Homoptera: Aphididae) on fungal endophyte-infected and uninfected perennial ryegrass. J Econ Entomol 85:583–588

Clement SL, Wilson AD, Lester DG, Davitt CM (1997) Fungal endophytes of wild barley and their effects on *Diuraphis noxia* population development. Entomol Exp Appl 82:275–281

Clement SL, Elberson LR, Bosque-Perez NA, Schotzko DJ (2005) Detrimental and neutral effects of wild barley—*Neotyphodium* fungal endophyte associations on insect survival. Entomol Exp Appl 114:119–125

Dighton J, White JF Jr, Oudemans P (2005) The fungal community: its organization and role in the ecosystem. CRC Press, Boca Raton

Dugassa-Gobena D, Raps A, Vidal S (1998) Influence of fungal endophytes on allelochemicals of their host plants and the behaviour of insects. Med Fac Landbouw Univ Gent 63:333–337

Emery SM, Thompson D, Rudgers JA (2010) Variation in herbivory and drought tolerance between native populations and cultivars of *Ammophila breviligulata*, a dominant sand dune grass. Am Midl Nat 163:186–196

Franche C, Lindstrom K, Elmerich C (2009) Nitrogen-fixing bacteria associated with leguminous and non-leguminous plants. Plant Soil 321:35–39

Gehring CA, Whitham TG (2002) Mycorrhizae–herbivore interactions: population and community consequences. In: van der Heijden MGA, Sanders IR (eds) Mycorrhizal ecology. Springer, Heidelberg, pp 295–320

Gonthier DJ et al (2008) Stroma-forming endophyte *Epichloe glyceriae* provides wound-inducible herbivore resistance to its grass host. Oikos 117:629–633

Hardoim PR, van Overbeek LS, van Elsas JD (2008) Properties of bacterial endophytes and their proposed role in plant growth. Trends Microbiol 16:463–471

Hardy TN, Clay K, Hammond AM (1985) Fall armyworm (Lepidoptera, Noctuidae)—a laboratory bioassay and larval preference study for the fungal endophyte of perennial ryegrass. J Econ Entomol 78:571–575

Hartley SE, Gange AC (2009) Impacts of plant symbiotic fungi on insect herbivores: Mutualism in a multitrophic context. Annu Rev Entomol 54:323–342

Institute SAS (2009) The SAS system for Windows, release 9.1.3. SAS Institute. Cary, North Carolina

Leuchtmann A (1992) Systematics, distribution, and host specificity of grass endophytes. Nat Toxins 1:150–162

Lopez JE, Faeth SH, Miller M (1995) Effect of endophytic fungi on herbivory by redlegged grasshoppers (Orthoptera: Acrididae) on *Arizona fescue*. Environ Entomol 24:1576–1580

Lyons PC, Evans JJ, Bacon CW (1990) Effects of the fungal endophyte *Acremonium coenophialum* on nitrogen accumulation and metabolism in tall fescue. Plant Physiol 92:726–732

Malinowski DP, Alloush GA, Belesky DP (2000) Leaf endophyte *Neotyphodium coenophialum* modifies mineral uptake in tall fescue. Plant Soil 227:115–126

Miles CO et al (1998) Endophytic fungi in indigenous Australasian grasses associated with toxicity to livestock. Appl Environ Microbiol 64:601–606

Raloff J (2003) Cultivating weeds: Is your yard a menace to parks and wild lands? Sci News 163:232

Rasband WS (2009) ImageJ, U.S. National Institutes of Health, Bethesda, Maryland, USA, http://rsb.info.nih.gov/ij/

Rodriguez RJ, White JF, Arnold AE, Redman RS (2009) Fungal endophytes: diversity and functional roles. New Phytol 182:314–330

Rudgers JA, Clay K (2007) Endophyte symbiosis with tall fescue: how strong are the impacts on communities and ecosystems? Fungal Biol Rev 21:107–124

Rudgers JA, Clay K (2008) An invasive plant-fungal mutualism reduces arthropod diversity. Ecol Lett 11:831–840

Rudgers JA, Holah J, Orr SP, Clay K (2007) Forest succession suppressed by an introduced plant-fungal symbiosis. Ecology 88:18–25

Saikkonen K, Helander M, Faeth SH, Schulthess F, Wilson D (1999) Endophyte-grass-herbivore interactions: the case of *Neotyphodium* endophytes in *Arizona fescue* populations. Oecologia 121:411–420

Saikkonen K, Lehtonen P, Helander M, Koricheva J, Faeth SH (2006) Model systems in ecology: dissecting the endophyte-grass literature. Trends Plant Sci 11:428–443

Schardl CL, Grossman RB, Nagabhyru P, Faulkner JR, Mallik UP (2007) Loline alkaloids: currencies of mutualism. Phytochem 68:980–996

Siegel MR et al (1990) Fungal endophyte-infected grasses—alkaloid accumulation and aphid response. J Chem Ecol 16:3301–3315

Sullivan TJ et al (2007) Symbiont-mediated changes in *Lolium arundinaceum* inducible defenses: evidence from changes in gene expression and leaf composition. New Phytol 176:673–679

Tibbets TM, Faeth SH (1999) *Neotyphodium* endophytes in grasses: deterrents or promoters of herbivory by leaf-cutting ants? Oecologia 118:297–305

Tintjer T, Rudgers JA (2006) Grass-herbivore interactions altered by strains of a native endophyte. New Phytol 170:513–521