

## Asexual endophytes and associated alkaloids alter arthropod community structure and increase herbivore abundances on a native grass

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### **Abstract:**

Despite their minute biomass, microbial symbionts of plants potentially alter herbivory, diversity and community structure. Infection of grasses by asexual endophytic fungi often decreases herbivore loads and alters arthropod diversity. However, most studies to date have involved agronomic grasses and often consider only infection status (infected vs. uninfected), without explicitly measuring endophyte-produced alkaloids, which vary among endophyte isolates and may impact consumers. We combined field experiments and population surveys to investigate how endophyte infection and associated alkaloids influence abundances, species richness, evenness and guild structure of arthropod communities on a native grass, *Achnatherum robustum* (sleepygrass). Surprisingly, we found that endophyte-produced alkaloids were associated with increased herbivore abundances and species richness. Our results suggest that, unlike what has been found in agronomic grass systems, high alkaloid levels in native grasses may not protect host grasses from arthropod herbivores, and may instead more negatively affect natural enemies of herbivores.

**Keywords:** *Achnatherum robustum* | alkaloids | arthropod diversity | community genetics | community structure | defensive mutualism | endophytes | evenness | herbivory | *Neotyphodium*

### **Article:**

#### **Introduction**

Understanding what determines the diversity and structure of natural communities has long been a goal of community ecologists. In recent decades, researchers have begun to consider symbiotic microbes as potential players in structuring communities. Nearly all primary producers in plant communities harbour microbial symbionts in some form, and symbiotic microbes such as

mycorrhizal fungi can have surprisingly strong effects on plant and consumer species diversity and ecosystem properties (van der Heijden *et al.* 1998, 2008) even though their biomass constitutes a miniscule fraction of the community.

One group of microbial symbionts, the fungal endophytes, has received relatively little attention concerning their effects on consumer communities (Hartley & Gange 2009). Fungal endophytes are common, abundant and diverse inhabitants of the above-ground tissues of most plant species (e.g. Cheplick & Faeth 2009). Most of these endophyte infections are localized and horizontally transmitted. However, many cool-season pooid grasses are infected with *Neotyphodium*, an asexual fungal endophyte that systemically infects the host grass and is transmitted vertically by hyphae growing into seeds. As variable and maternally transmitted components, *Neotyphodium* can be viewed within the context of community genetics, where heritable variation within plant species has cascading effects at the community level (e.g. Whitham *et al.* 2003; Hughes *et al.* 2008). The community level effects of *Neotyphodium* on plant (Clay & Holah 1999) and arthropod diversity (Omacini *et al.* 2001; Rudgers & Clay 2008) have been tested with non-native agronomic grasses in containers or old fields, with interesting results. However, these studies examined only the effect of infection status, without considering variation in alkaloid concentrations. Natural grass communities are typically mosaics of uninfected and infected grasses, and *Neotyphodium* isolates vary genetically within and among populations of the same grass species (e.g. Sullivan & Faeth 2004), with alkaloid production varying with endophyte haplotype (Cheplick & Faeth 2009). Studies of how variation in *Neotyphodium* haplotypes and their changes in host properties affect the diversity and structure of native consumer communities are scarce.

Asexual endophytes have the potential to alter structure and diversity of consumer communities by inducing dramatic alterations to the phenotypes of their host plants. Because they are vertically transmitted, asexual endophytes are conventionally viewed as strong mutualists as endophyte and host fitness are tightly linked (Clay 1990; Schardl & Clay 1997; Clay & Schardl 2002). *Neotyphodium* infections may cause a suite of phenotypic changes that benefit their plant hosts, including increased competitive abilities, resistance to abiotic stresses and enhanced nutrient uptake (e.g. Faeth & Bultman 2002; Muller & Krauss 2005). These benefits from infection stem from *Neotyphodium* altering biochemical (e.g. Rasmussen *et al.* 2008), physiological (e.g. Morse *et al.* 2002) and morphological (e.g. Malinowski & Belesky 1999) properties of the host. However, the most renowned and often-cited benefit of infection is increased resistance to herbivores via the production of toxic alkaloids (Clay 1988; Clay & Schardl 2002). *Neotyphodium* endophytes can produce four different types of alkaloids, each with varying biological activity against invertebrate and vertebrate herbivores (Leuchtman *et al.* 2000; Schardl *et al.* 2004). Thus, endophytes are viewed as ‘acquired defenses’ (Cheplick & Clay 1988) or ‘defensive mutualists’ (Clay 1988) of grasses, which often lack their own chemical defenses against herbivores. Reduction of herbivory is expected to

benefit the host grass and concomitantly increase fitness of the vertically transmitted endophyte (Saikkonen *et al.* 1998; Schardl *et al.* 2004) but see Faeth & Sullivan (2003).

Increased resistance of grasses to herbivory via endophyte alkaloids has been demonstrated primarily in laboratory bioassays. Field tests of endophyte-associated resistance to herbivory rarely measure alkaloids and generally involve introduced agronomic grass cultivars (e.g. Faeth 2002; Saikkonen *et al.* 2006). Studies involving native grasses are relatively scarce and short in duration, and results range from increased (Koh & Hik 2007) to decreased herbivore resistance (Saikkonen *et al.* 1999; Tibbets & Faeth 1999). Notably, in natural grass communities, the types and levels of alkaloids vary greatly (Cheplick & Faeth 2009).

Asexual endophytes and their alkaloids not only directly affect herbivores but can also indirectly affect higher consumer abundances and diversity through trophic cascades (Cheplick & Faeth 2009). Studies involving the agronomic grasses perennial ryegrass (*Lolium perenne*) (de Sassi *et al.* 2006), tall fescue (*Lolium arundinaceum*) (Finkes *et al.* 2006; Rudgers & Clay 2008) and Italian ryegrass (*Lolium multiflorum*) (Omacini *et al.* 2001) show that infection and associated alkaloids can dramatically alter insect herbivore and natural enemy (parasites and predators) abundances and species richness. To date, however, it is unknown how endophyte infection and varying alkaloids interact to influence arthropod abundances, diversity and feeding guild structure in native grasses or natural communities.

In this study, we used both a survey of a natural population and a controlled field experiment to test how *Neotyphodium* infection and alkaloid production affect arthropod community structure on *Achnatherum robustum* (sleepygrass), a native grass known for its toxic effects due to ergot alkaloids associated with *Neotyphodium* infection. We asked how endophyte infection in general, and the associated variation in alkaloid production specifically, affect arthropod abundances, richness, evenness and trophic structure. First, we sampled arthropods from naturally occurring sleepygrass and correlated arthropod abundance, diversity and trophic structure with infection status and alkaloid levels. Second, we conducted a 3-year factorial field experiment with plants that varied in endophyte infection and alkaloid content while also manipulating soil moisture, a key limiting factor that can influence both plant and higher trophic level responses to *Neotyphodium* infection (Morse *et al.* 2002; Bultman & Bell 2003; Faeth & Sullivan 2003). In this experiment, we compared three classes of plants: (1) those without endophytes (E<sup>-</sup>), and therefore also without alkaloids, (2) plants infected with an endophyte that produced no alkaloids (E+A<sup>-</sup>) and (3) plants infected with an endophyte that produced high levels of alkaloids (E+A<sup>+</sup>). By using a whole-community sampling approach in this native endophyte–host grass system, we address the question of how these microbial symbionts and their alkaloids influence the diversity, structure and composition of natural communities.

## Materials and methods

### Study system

*Achnatherum robustum* (Vasey) Barkworth [= *Stipa robusta* (Vasey) Scribn. = *Stipa vaseyi* Scribn.] (Pooideae: Tribe Stipeae) commonly known as sleepygrass, is a perennial bunchgrass native to the western United States in semi-arid pine/fir grasslands above 2500 m. The name sleepygrass is derived from the plant's long-known narcotizing effects on livestock (Bailey 1903), which are caused by ergot alkaloids produced by *Neotyphodium* endophytes (Petroski *et al.* 1992). The primary ergot alkaloids produced by sleepygrass are lysergic and isolysergic acid amides, ergonovine and ergonovinine. These may be produced in very high concentrations ( $> 150 \mu\text{g g}^{-1}$ ) but the levels are highly variable within and among infected sleepygrass populations, with some infected plants producing no alkaloids at all (Faeth *et al.* 2006). Ergot alkaloids in general are deterrent and toxic to both vertebrate and invertebrate herbivores, at least based on observations and bioassay studies (Siegel *et al.* 1990).

### Observational field study

In October 2002, we haphazardly selected and marked 100 naturally occurring sleepygrass plants in the Lincoln National Forest near Cloudcroft, New Mexico USA. Of these, 79 plants are included in this study because 7 could not be found in later visits and 14 were spatially distinct and therefore possibly from a separate population. We collected plant samples for analysis of infection status and alkaloid concentrations. Leaf tissue was cut 1 cm above the ground and kept on ice in the field. *Neotyphodium* infection status of all plants was determined by tissue print immunoblot (modified from Gwinn *et al.* 1991), using at least 3 tillers per plant. Remaining tissue was freeze-dried and ground to a powder in a Wiley Mill for ergot alkaloid analysis. Analyses of ergot alkaloids (ergonovine, ergonovinine, lysergic acid amide, isolysergic acid amide) was performed by HPLC as described in Faeth *et al.* (2006).

In May 2003, we measured plant size (height and basal diameter were measured in the field and used to estimate plant volume as a cylinder) and sampled arthropods from all 79 plants. Arthropods were sampled by vacuuming from each plant (the entire plant was vacuum-sampled) using a Vortis Insect Suction Sampler (Burkard Manufacturing, Hertfordshire, UK), and immediately preserved in 70% ethanol. Arthropods were counted, sorted by morphospecies, keyed to family, and assigned to feeding guilds [herbivore, natural enemy (predators and parasitoids), detritivore and omnivore] with the exception of thrips which were classified by morphospecies and feeding guild (all thrips were considered herbivores), but not keyed to family. Because mites (Acari) may be omnivores, herbivores, or predators depending upon individual species, we excluded mites from analyses. The dataset thus comprised mostly insects, with a few families of spider. We estimated biomass of each morphospecies as  $W = aL^b$ , where  $W$  is estimated biomass,  $L$  is body length, and  $a$  and  $b$  are constants specific to given taxa (Hodar 1996). We verified this method by regression of calculated biomass against empirically determined dry weights for 41 representative specimens ( $P < 0.0001$ ,  $R^2 = 0.85$ ).

### Experimental study

To test the effect of infection status and alkaloid levels on arthropod abundances and species richness, we designed a 3-year field experiment using plants grown from seeds from three maternal plant genotypes: uninfected (E<sup>-</sup>), infected and producing alkaloids (E+A<sup>+</sup>) and infected but producing no alkaloids (E+A<sup>-</sup>). Maternal plants were collected in the field (from the site where the observational study of the natural population was conducted), and alkaloid concentrations were measured as described above. Experimental plants were germinated from seed from the maternal plants and grown in a green house for 6–8 months in native soil. A plot at the Arboretum of Flagstaff, Flagstaff, AZ, USA was prepared by disking in May 2003 to remove existing vegetation. The original plot was in a natural and previously undisturbed semi-arid Ponderosa pine-grassland habitat, harbouring native plant species and dominated by native grasses. The plot was covered with a weed barrier (Dalen<sup>®</sup>, Dalen Products, Inc. Knoxville, TN) that prevents growth of unwanted plants but is permeable to water and nutrients, and then covered in a layer of pine bark chips to ameliorate any temperature changes caused by the weed barrier.

In the summer of 2003, E<sup>-</sup>, E+A<sup>-</sup> and E+A<sup>+</sup> plants were randomly assigned positions in the plot, and planted 2 m apart into holes cut in the weed barrier. The experiment was a full factorial experiment with two levels of water. The two water treatments were ambient precipitation and supplemented water (drip irrigation, 8 L per plant per day). All infection-alkaloid status and treatment combinations were replicated 13 times for a total of 78 plants. Treatments began in the summer of 2003 and continued through 2007.

To confirm infection status, seeds were collected in 2007, stained, and examined for the presence of characteristic hyphae in the seed embryo. All plants from E<sup>+</sup> maternal plants remained infected save one, and all plants from E<sup>-</sup> maternal plants remained uninfected. To confirm alkaloid levels, small tissue samples were collected from each plant, freeze-dried and analysed for ergot and total alkaloid concentration (per methods described above). None of the E<sup>-</sup> or E+A<sup>-</sup> plants had any detectable alkaloids. All but one E+A<sup>+</sup> plants showed high levels of ergot alkaloids [mean =  $33.7 \pm 8.09$  SE p.p.m., range (22.4–89.4 p.p.m.)]. The one E<sup>+</sup> plant with no alkaloids was the same plant that appeared to have lost *Neotyphodium* infection, and was excluded from all analyses.

Arthropods were sampled with an insect vacuum sampler (see above) in May 2006 and May 2007, the peak period of arthropod abundances (Faeth 2009, Faeth & Shochat 2010). Unlike in the observational study, a uniform volume (1750 cm<sup>3</sup>, the volume of the vacuum aperture) of each plant was suctioned for 10 s from the centre of the plant. Thus, the collection from each plant represents a uniform volumetric sample and estimates density of arthropods per plant. Arthropods were identified to at least family and assigned to guilds based upon family or genus descriptions as detailed above. We also estimated arthropod biomass using the methods described above. Plant size was measured each growing season using height and basal diameter during the growing season, and then harvesting, drying and weighing aboveground biomass at the end of each growing season.

## Data analyses

### Linear models

We used several statistical methods to analyse arthropod abundance and diversity data. First, we used linear models to test for relationships between plant infection/alkaloid status and arthropod response variables, including total species richness and total, herbivore, detritivore and natural enemy (predator and parasitoid) abundance and biomass. We also tested for relationships between infection/alkaloids and abundances of insects from particular groups of interest: dominant herbivore families (Cicadellidae, Miridae, Delphacidae and Aphididae), which are expected to respond strongly to endophytes (Hartley & Gange 2009), non-Hemipteran sucking herbivores (Thysanoptera) and the dominant detritivore group (Collembola). All assumptions of anova were tested and, where needed, data were transformed to approximate the normal distribution. One data point in the observational set was excluded as an outlier because a large aggregation of coccinellid beetles was collected with the arthropod sample. All linear analyses were performed using jmp 7 (SAS Institute 2007, SAS Institute Inc., Cary, NC, USA) and Systat 10 (SPSS Institute 2000, SPSS, Inc., Chicago, IL).

In the experimental study, our main question is whether endophyte infection *per se* (i.e. E+ or E) or their associated alkaloids affect arthropod abundances and richness. Therefore, we performed anova comparing the three plant types (E−, E+A− and E+A+), and constructed two planned linear contrasts to (1) compare arthropod abundances on E− and E+ (grouping together all infected plants, regardless of alkaloid status) and (2) compare alkaloid-producing plants (E+A+) and alkaloid-free plants (A− plants, regardless of infection status). We focus our analyses on these ecologically pertinent contrasts. Because arthropods were sampled on a per unit volume basis and thus we estimated density of arthropods per plant, we did not use plant size as a covariate in the analyses presented here. We note, however, that inclusion of plant size as a covariate does not qualitatively alter the results of the planned contrasts. Also, because analyses for number of individuals and biomass were concordant with those for number of individuals, we report only results from number of individuals here for brevity.

In the observational study of the natural population, nearly all (76 of 79) of the plants were infected with *Neotyphodium*, with alkaloid levels of infected plants ranging from 0 to 168 p.p.m. Therefore, rather than concentrate on comparisons of infected and uninfected plants, we focused on patterns associated with alkaloid concentrations in the natural population. We used least-squares regression to model the relationship between alkaloid concentration and each of the following variables: arthropod abundance, biomass, richness, evenness, Shannon Diversity Index, and total and relative abundances of herbivores and natural enemies. We used anova to test if these response variables differed between plants with and without alkaloids. Evenness was calculated as Hurlbert's Probability of Interspecific Encounter (PIE) (Hurlbert 1971). Because we sampled entire plants in the observational study, and plant size influences the abundance and diversity of associated arthropods, plant size was included as a covariate in all models for the

observational study (therefore, multiple regression and ancova), except those with relativized response variables (e.g. relative abundance), to account for possible effects of habitat size on arthropod communities. However, the results of our analyses are qualitatively the same if anova is used rather than ancova (no changes to what variables are significant).

## **Rarefaction**

Comparing differences in taxonomic richness between groups with unequal sampling sizes or number of sampled individuals can be problematic due to the relationship between sampling effort and observed richness (Gotelli & Colwell 2001). Therefore, we used rarefaction to compare cumulative morphospecies richness and diversity between groups. In the experimental study, because plants varied widely in sampled arthropod abundance, rarefaction was used to compare richness and evenness (as Hurlbert's PIE) among E<sup>-</sup>, E+A<sup>-</sup> and E+A<sup>+</sup> grasses. In the field survey, because we had highly uneven sample sizes (five without alkaloids, 74 with alkaloids), we used rarefaction to estimate richness assuming we had sampled only five plants with alkaloids. For rarefaction analysis, we used Ecosim 7 (Gotelli & Entsminger 2000) to run 1000 Monte Carlo simulations to estimate the cumulative richness of plant groups in each study. Groups were considered significantly different if the mean richness of the group with smaller sample size did not overlap the 95% confidence intervals of the rarefied richness of the group with larger sample size (Gotelli & Entsminger 2000).

## **Multivariate analyses**

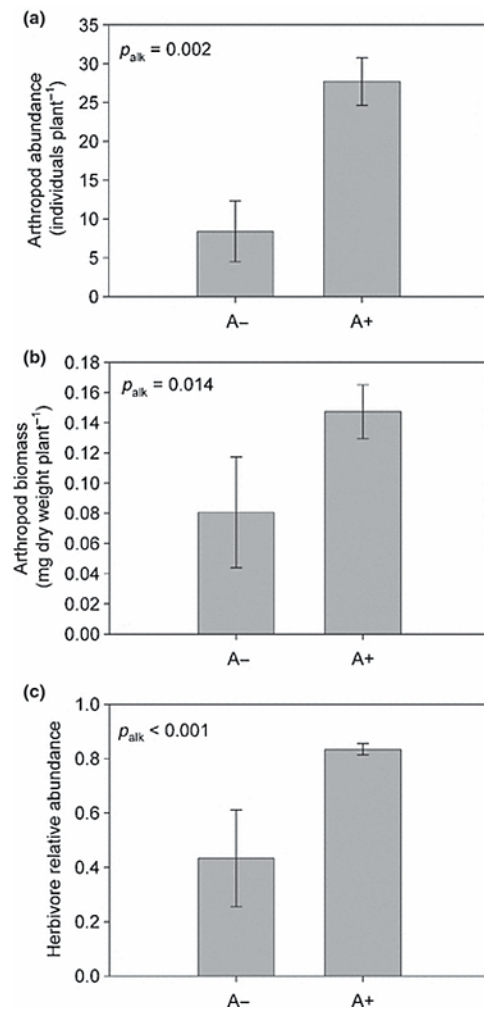
To examine multivariate relationships between alkaloid concentration, infection status and arthropod community composition, we analysed the similarity of morphospecies abundances among all plants sampled. Distances between samples in morphospecies community space were generated using the Sørensen dissimilarity index [aka Bray-Curtis or percent dissimilarity, calculated as  $1 - 2W/(A + B)$  where W is the sum of shared morphospecies abundances and A and B are the sums of morphospecies abundances in individual sample units; Sørensen 1948] using the pc-ord software package (McCune & Mefford 2006). We conducted multi-response permutation procedures (MRPP) to determine if samples exhibited greater community similarity than expected by chance when grouped by alkaloid presence/absence (observational study), or alkaloid presence/absence and infection status (experimental study). We also ordinated samples via non-metric multidimensional scaling (NMDS) and used least-squares regression to test for relationships between resulting axes of community structure and alkaloid concentrations.

## **Results**

### **Observational study**

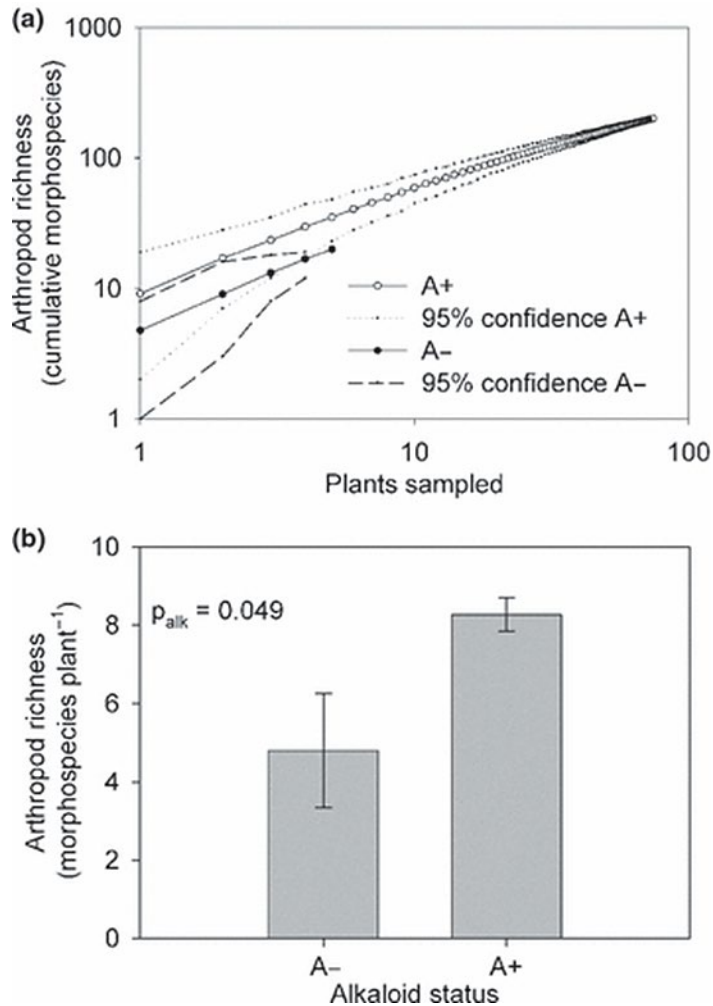
We collected 2155 arthropods from the 79 plants in the natural population. In our multivariate regression model, alkaloid concentration significantly but weakly predicted arthropod abundance ( $p_{\text{model}} < 0.0001$ ,  $p_{\text{alk}} = 0.035$ ,  $R^2_{\text{model}} = 0.26$ ,  $R^2_{\text{alk}} = 0.03$ ) and richness ( $p_{\text{model}} <$

0.0001,  $p_{\text{alk}} = 0.015$ ,  $R^2_{\text{model}} = 0.36$ ,  $R^2_{\text{alk}} = 0.05$ ), with higher alkaloids associated with greater richness and total abundances. Results using morphospecies richness agreed with those for the Shannon Diversity Index, so only species richness data are reported. Regression analysis found no relationship between alkaloid concentration and arthropod community biomass, morphospecies evenness, or relative abundances of herbivores or natural enemies, but abundance of herbivores showed a marginally significant positive relationship with alkaloids ( $p_{\text{alk}} = 0.055$ ). In anova tests, plants with alkaloids had greater arthropod abundance ( $p_{\text{alk}} = 0.002$ ,  $p_{\text{volume}} < 0.0001$ ), biomass ( $p_{\text{alk}} = 0.014$ ,  $p_{\text{volume}} < 0.0001$ ), richness ( $p_{\text{alk}} = 0.049$ ,  $p_{\text{volume}} < 0.0001$ ) and abundance of herbivores ( $p_{\text{alk}} = 0.001$ ,  $p_{\text{volume}} < 0.0001$ ) than alkaloid-free plants, but did not differ in evenness, abundance of natural enemies, or abundance of any subgroup of herbivores analysed separately (Figs 1 and 2). anova revealed that plants with alkaloids had greater relative abundance of herbivores ( $P < 0.0001$ ) and ratio of herbivores to natural enemies ( $P = 0.015$ ) than plants without alkaloids (Fig. 1c).





**Figure 1.** Relationships between alkaloid concentration in plant tissues and (a) arthropod abundances, (b) biomass and (c) herbivore relative abundances, based on the observational study. (Graphs show mean  $\pm$  SE).



**Figure 2.** Relationship between alkaloid concentration and arthropod species richness in the observational data, as determined by (a) rarefaction and (b) anova.

Rarefaction analyses agreed with ancova tests: the 95% confidence intervals of rarefied estimates for plants with alkaloids did not overlap with observed values for plants without alkaloids, indicating that species richness was lower on A- plants (Fig. 2).

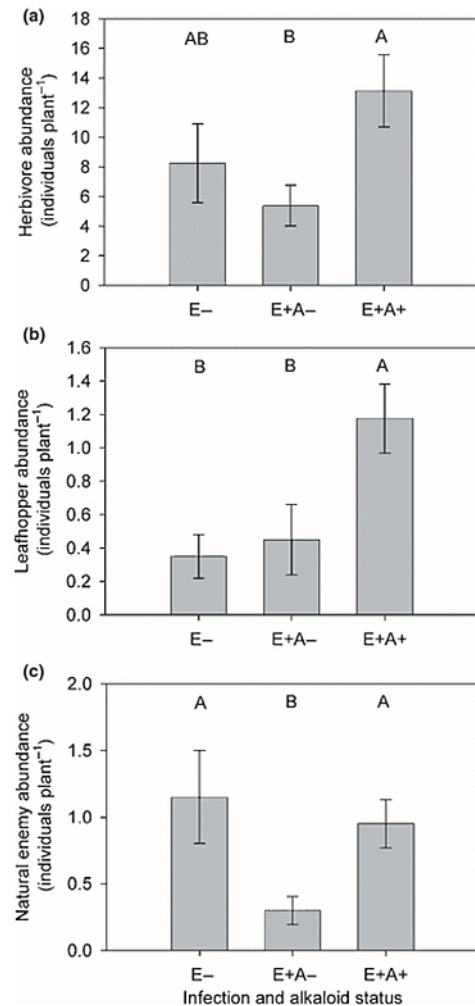
Alkaloid content was a poor predictor of arthropod species or guild community composition according to our multivariate analyses. MRPP analysis showed no consistent differentiation between communities associated with alkaloid-containing and alkaloid-free plants ( $P = 0.052$ , effect size  $A = 0.006$  where  $A$  ranges from 1 when samples are identical within groups to 0 when heterogeneity within groups equals expectation by chance). NMDS converged on three dimensions (stress = 18.40). Relationships between alkaloid concentration and NMDS axis

scores were tested using least-squares regression and no significant relationships were found ( $R^2 < 0.1$ ,  $P > 0.1$ ).

## Experimental study

### Species abundances and biomass

In 2006 and 2007, 11236 and 7515 arthropods, respectively, were collected and identified from sleepygrass plants in the experimental plot. anova comparing the three plant types (E+A+, E+A– and E–) for 2006 found differences in abundances of natural enemies ( $P = 0.044$ ), the dominant herbivore family Cicadellidae ( $P = 0.009$ ) and marginal differences in abundances of herbivores as a whole ( $P = 0.074$ ; Fig. 3). Other than the Cicadellidae, no family/order that we analysed individually differed among groups. There were no significant differences in 2007 [although detritivores were marginally more abundant on E+A+ plants than on the other plant types ( $P = 0.075$ )]. No significant differences or interactions due to water treatment were found.

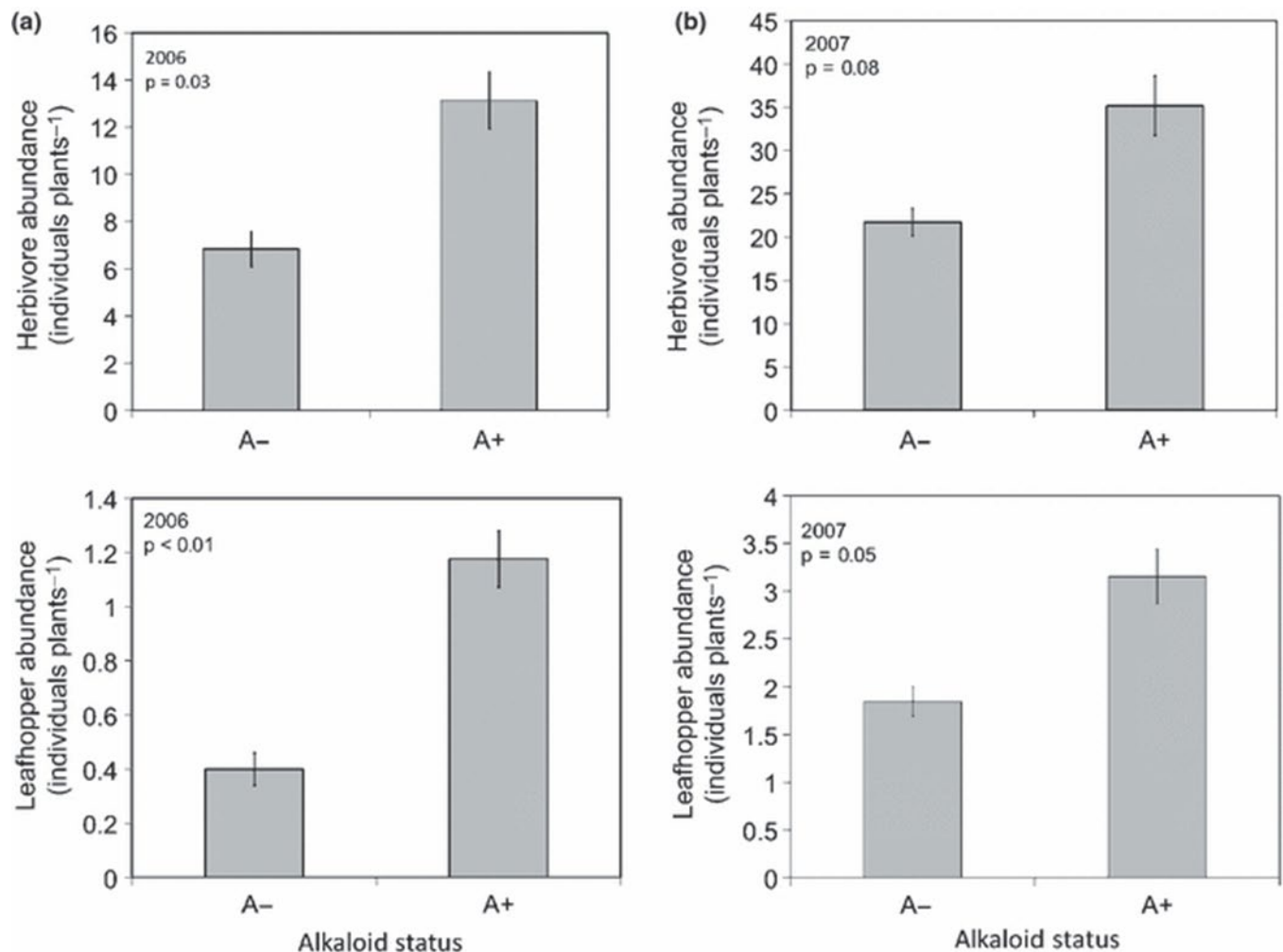


**Figure 3.** Mean (± SE) of (a) number of herbivores per plant, (b) number of leafhoppers (Cicadellidae) per plant and (c) number of natural enemies (predators and parasites) per plant on

E<sup>-</sup>, E+A<sup>-</sup> and E+A<sup>+</sup> plants in the experimental study in 2006. Different letters above bars indicate significant differences ( $P < 0.05$  for panels (b, c);  $P < 0.10$  for panel (a); Tukey HSD *post hoc* test of multiple means).

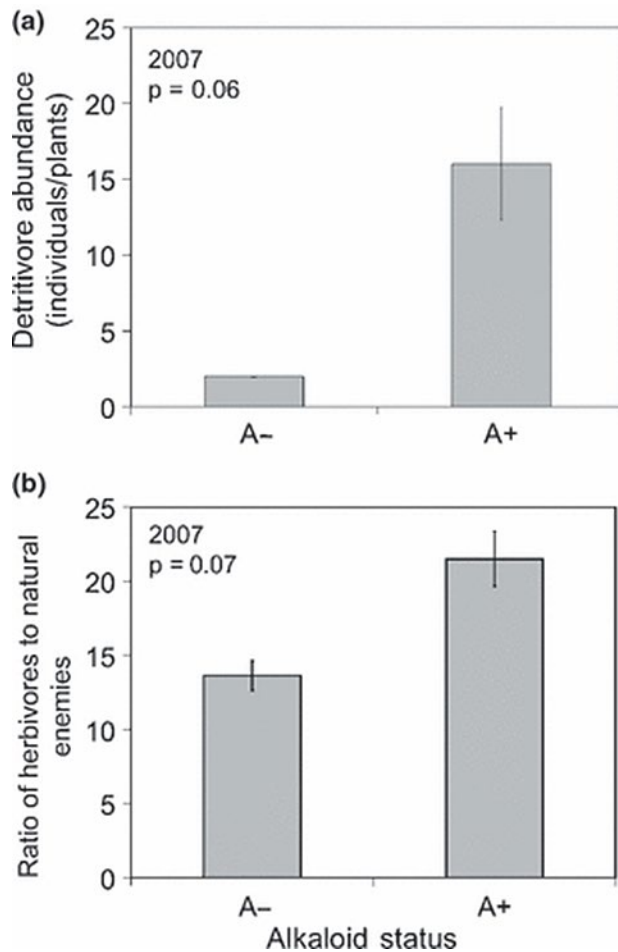
Linear contrasts examining infection status (E<sup>+</sup> vs. E<sup>-</sup> irrespective of alkaloid status) found no significant differences in any of the variables tested in either year [although natural enemies were marginally greater on E<sup>-</sup> plants ( $P = 0.078$ ) in 2006 but not 2007].

The most consistent patterns emerged when we performed linear contrasts examining alkaloid status (A<sup>+</sup> vs. A<sup>-</sup> irrespective of infection status). We found that, in both years, A<sup>+</sup> plants had greater abundances of the dominant herbivore family (Cicadellidae,  $p_{2006} = 0.002$ ,  $p_{2007} = 0.050$ ) and herbivores overall ( $p_{2006} = 0.031$ ,  $p_{2007} = 0.085$ ) compared with A<sup>-</sup> plants (Fig. 4). In addition, in 2007 only, the abundance of detritivores and the ratio of herbivores to natural enemies was marginally greater on A<sup>+</sup> plants ( $P = 0.066$ ,  $P = 0.060$  respectively; Fig. 5).



**Figure 4.** Mean ( $\pm$  SE) number of herbivores per plant (upper panels), and number of leafhoppers (Cicadellidae) per plant (lower panels) in 2006 (a, left panels) and 2007 (b, right

panels), in the experimental study. Graphs show results of planned contrasts between A+ and A− plants.



**Figure 5.** Mean ( $\pm$  SE) of (a) number of detritivores and (b) ratio of herbivores to natural enemies in the experimental study in 2007. Graphs show results of planned contrasts between A+ and A− plants.

Rarefaction analyses showed that, in 2006, alkaloid-containing plants (E+A+) had higher overall species richness and higher herbivore richness than alkaloid-free plants (E− and E+A−) (Table 1). This pattern did not hold true in 2007, when richness was equivalent in all groups (Table 1). Patterns in species evenness were more complex and varied more among trophic groups and years (Table 1).

**Table 1.** Effect of infection and alkaloid status on species richness and evenness of arthropods (grouped by feeding guild) that were associated with sleepygrass in 2006 and 2007, based on rarefaction

	2006	2007

Species richness										
Total	E−	=	E+A−	<	E+A+	E−	=	E+A−	=	E+A+
	31		29		45	39		49		61
Herbivores	E−	=	E+A−	<	E+A+	E−	=	E+A−	=	E+A+
	20		18		28	21		22		30
Predators	E−	=	E+A−	=	E+A+	*		E+A−	>	E+A+
	4		3		4	1		8		7
Parasites	E−	=	E+A−	=	E+A+	E−	<	E+A−	=	E+A+
	5		3		7	6		10		12
Species evenness										
Total	E−	<	E+A−	>	E+A+	E−	>	E+A−	<	E+A+
	0.156		0.426		0.328	0.694		0.45		0.78
Herbivore	E−	<	E+A−	>	E+A+	E−	<	E+A−	<	E+A+
	0.26		0.426		0.327	0.529		0.55		0.64
Predators	*		*		*	*		E+A−	>	E+A+
	0.9		0.833		0.643	*		0.97		0.909
Parasites	E−	=	E+A−	=	E+A+	E−	<	E+A−	=	E+A+
	0.729		0.615		0.701	0.625		0.76		0.719

Significant differences ( $P < 0.05$ ) indicated by inequality signs (< or >). There were too few omnivore and detritivore species for meaningful comparisons and in some cases (designated by \*) for predators. Values for richness or Hulbert's PIE (probability of interspecific encounter) are beneath each comparison. Uninfected, E−; infected with no alkaloids, E+A−; infected with alkaloids, E+A+.

For both the experimental and observational studies, Sørensen indices of guild relative abundance showed that pooled arthropod communities were more similar among alkaloid-free plants than either arthropod community was to alkaloid-containing plants (Appendix S2).

## Discussion

Although systemic, asexual endophytic fungi in grass constitute a minute fraction of the total biomass in a community, they may impart profound changes on plant (Clay & Holah 1999) and animal abundances and diversity (Omacini *et al.* 2001; Finkes *et al.* 2006; Rudgers & Clay 2008) and ecosystem functions (Rudgers *et al.* 2004). In our study of *Neotyphodium* inhabiting the native grass, *A. robustum*, infection also influences arthropod and feeding guild abundances and diversity. Furthermore, by considering alkaloid levels as well as endophyte infection, we were able to show that variation in alkaloid production by endophytes is a possible mechanism for endophyte-associated changes to the arthropod community. Of particular importance is that plants infected with high alkaloid-producing endophytes generally harboured more herbivorous insects, in contrast to studies of introduced, agronomic grass systems (Omacini *et al.* 2001; Rudgers & Clay 2008) and contrary to the prevailing concept that endophytes act primarily as defenses of host grasses against herbivores (Cheplick & Clay 1988; Clay & Schardl 2002). The *Neotyphodium* literature has an interesting history of contrasting stories emerging from agronomic and native grass systems (Hartley & Gange 2009), and our results add a community level insight to the ongoing dialogue.

In both the field survey and experimental study, grasses infected with alkaloid-producing *Neotyphodium* endophytes had greater arthropod richness and abundances than plants without alkaloids. In addition, three-way anova in the experimental study showed that most of the differences in abundances and richness were due to differences between infected plants that varied in alkaloid production rather than between E+ and E- plants, indicating that it is not endophyte infection *per se* that influences arthropod abundances and richness, but rather whether infection results in alkaloid production.

In our experimental study, the alkaloid concentrations (among those plants that had alkaloids) ranged from 22.4 to 89.4 p.p.m. It is clear from our observational study and previous work (Faeth *et al.* 2006), that alkaloid variation among infected plants in natural populations spans a much wider range than that encompassed by our experiment, so caution is required when interpreting our experimental results. Nonetheless, results from our observational field study [with alkaloid levels spanning a wide range (0 to >150 p.p.m.)] suggest that genetic variation in the maternally inherited endophyte and its influence on alkaloid levels may be an important trait in shaping differences in arthropod diversity and abundances. In terms of overall species richness, herbivore richness and arthropod community similarity, this variation in host phenotype mediated by endophyte alkaloid production appears to overwhelm variation due simply to whether plants are infected or not, the usual standard of comparison in grass endophyte studies (e.g. Cheplick & Faeth 2009). While factors other than endophyte haplotype, including plant genotype, nutrient availability and prior herbivore-induced damage, may influence plant alkaloid levels, in the sleepygrass system endophyte haplotype appears to be the primary determinant of alkaloid concentrations (Faeth *et al.* 2006). Thus, maternally inherited endophytes in grasses and their variable alkaloids appear to cause community-wide changes, much like genetic variation in

host plants that alter host properties and have cascading effects through the community and ecosystem (e.g. Hughes *et al.* 2008).

Studies involving agronomic grasses have shown that *Neotyphodium* infection can alter abundances and diversity of the arthropod community. Omacini *et al.* (2001) found that arthropod communities associated with E+ agronomic Italian ryegrass exhibit reduced herbivore abundances, a shortened food chain, and slightly lower diversity than communities found on E- plants. Working with agronomic perennial ryegrass, Harri (2007) and de Sassi *et al.* (2006) demonstrated lower herbivore abundances on infected grasses. Finkes *et al.* (2006) showed that E+ agronomic tall fescue plots had lower diversity of spiders and altered evenness. Most recently, Rudgers & Clay (2008) found decreased total diversity and herbivore abundance associated with endophyte-infected agronomic tall fescue in old field environments. While these studies did not measure alkaloids, endophytes in these agronomic grasses generally produce high levels of alkaloids, and variation in alkaloid production is greatly reduced.

In contrast, our results from both field survey and experimental study using native sleepygrass demonstrate increased herbivore abundances and diversity associated with infected plants with high levels of ergot alkaloids. In another recent study with the native grass *Festuca arizonica*, herbivore abundances were also higher on endophyte-infected plants (Faeth & Shochat 2010). This seems counter to the defensive mutualism hypothesis, where host grasses enlist endophytes and their alkaloids for protection against herbivores, and is especially puzzling because ergot alkaloids are known to be deterrent and toxic to insect herbivores, at least in bioassays using generalist insects (e.g. Siegel *et al.* 1990; Siegel & Bush 1997). We did not directly measure herbivory, so it is possible that alkaloids are indeed protective by reducing rates of invertebrate or vertebrate herbivory. However, E+A+ plants in the experimental study tended to have equal or less biomass than E- and E+A- plants at the end of each growing season (data in grams dry weight, 2006: E+A+ =  $26.70 \pm 2.11$ ; E- =  $37.00 \pm 3.0$ ; E+A- =  $31.70 \pm 2.03$ ; 2007: E+A+ =  $27.45 \pm 2.50$ ; E- =  $27.37 \pm 4.22$ ; E+A- =  $30.84 \pm 4.58$  Faeth *et al.*, in review), suggesting that infection by high alkaloid-producing endophytes does not reduce overall herbivory. Interestingly, another study measuring herbivore damage on a native grass found no reduction in herbivory of E+ compared with E- plants (Tintjer & Rudgers 2006), further underscoring that endophyte-plant-consumer interactions in native grasses may not be completely represented by studies of introduced, agronomic grasses.

We propose two possible explanations for the positive association of herbivorous insects with E+A+ grasses. First, most previous studies directly testing insect deterrence and toxicity of alkaloids have been conducted with generalist agricultural pest insects and infected agronomic grasses (e.g. Faeth & Saikkonen 2007), leading to the expectation that endophytes deter herbivores. However, in natural communities, many insect herbivores are specialists that may be able to detoxify plant defensive chemicals, or even require them for locating, ovipositing and developing on host plants (Faeth 2002). It is possible that insects feeding on E+A+ plants are

specialized to tolerate ergot alkaloids. The vast quantity of arthropods (>20 000 specimens; see Table S1) in our study precluded identification of specimens to species level, so we cannot definitively group specimens into specialist or generalist classes as would be required to test the above hypothesis. Understanding the interplay between herbivore host-specificity and endophyte effects on arthropod communities is an important goal for future research.

An alternative explanation for the increase in herbivore abundances on alkaloid-containing grasses is that natural enemies of herbivorous insects may be more sensitive to allelochemicals such as alkaloids than the herbivores themselves, or herbivores may sequester alkaloids while feeding as defense against their natural enemies. Indeed, some parasitoids of herbivores on grasses show delayed development and increased mortality due to endophytic alkaloids consumed by their insect hosts (e.g. Bultman *et al.* 1997), and consumption of alkaloids by herbivores can have more severe effects on parasitoids of those herbivores than on the herbivores themselves (Barbosa *et al.* 1991). Thus, E+A+ plants may provide enemy-reduced space for some herbivorous insects. This hypothesis is consistent with our result that E+A+ plants had higher ratio of herbivores to natural enemies than A- plants in the field survey and in the second year of the experimental study. Further tests will be required to elucidate the mechanism underlying the higher herbivore abundances and richness on E+A+ plants. Nevertheless, it is clear that the defensive mutualism hypothesis may not apply universally to endophytes in wild grass communities.

Our results also indicate endophyte-related changes in species richness and evenness. In both the field survey and first year of the experimental study, arthropod richness was higher on E+A+ plants (Fig. 2, Table 1) than on plants without alkaloids. Infection not only affected species richness but, in our experimental study, also shifted evenness of arthropod communities and individual feeding guilds, an important but often overlooked component of diversity (Smith & Wilson 1996). Evenness for the total arthropod community was greatest on E+A- plants in 2006 and least on the same plants in 2007, indicating dramatic year to year in changes in the evenness component of arthropod diversity. Apparently, as has been found with agronomic grasses (Finkes *et al.* 2006; Harri 2007), endophytes can dramatically alter diversity of the associated arthropod community of this native grass, although these changes vary from season to season, underscoring the importance of long-term studies of the effects of endophytes inhabiting perennial grasses.

Asexual endophytes and their associated alkaloids change abundances and diversity of arthropods associated with sleepygrass in ways that are counter to prevailing notions of endophyte-host relationships. Instead of reduced herbivore abundances predicted by the defensive mutualism hypothesis, we found consistently higher herbivore abundances, and in some cases higher species diversity, on E+A+ plants. In addition, by considering not just infection status, but also alkaloid concentrations, we were able to show that changes in arthropod communities are associated with alkaloids, rather than infection *per se*. Strikingly, the effect of alkaloids in this native system are the opposite of what is expected based on agronomic systems



and conventional ideas of endophytes as defensive mutualists. Overall, our results demonstrate that effects of *Neotyphodium* endophytes on herbivore abundances and arthropod communities in native grasses differ from, and may be more complex than, patterns that have been observed in agronomic grasses.

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## **Supporting Information**

**Appendix S1** Arthropod taxa collected from sleepygrass during the course of the experimental study.

**Appendix S2** Sørensen distances among arthropod communities found on plant types varying in infection (E+ or E–) and alkaloid status (A+ or A–) in the (a) observational and (b) experimental study.

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