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The Distribution of the Epiphytic Fungus *Atkinsonella texensis* and its Effects on the Performance of its Plant Host, *Nassella leucotricha*

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The Distribution of the Epiphytic Fungus *Atkinsonella texensis* and its Effects on the Performance of its Plant Host, *Nassella leucotricha*

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Dedication

To my parents Jody and Bill Maas

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The Distribution of the Epiphytic Fungus *Atkinsonella texensis* and its Effects on the Performance of its Plant Host, *Nassella leucotricha*

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I investigated the distribution of the epiphytic fungus *Atkinsonella texensis* and its effects on the performance of its plant host, *Nassella leucotricha*. I conducted surveys to determine the effects of small-scale heterogeneity and host plant density on the rate of disease incidence in the host plant population. In all three sites surveyed, the incidence of disease was highest beneath woody canopies and lowest in the open. Where *Nassella* was growing beneath woody plants, there was a positive relationship between the incidence of infection and *Nassella* density. In contrast, no relationship was detected between *Nassella* density and the incidence of infection in the open grassy areas.

The effects of *A. texensis* on plant size and resistance to insect herbivores were studied in a natural population of *Nassella*. Infection had no effect upon the proportion of leaves damaged by grasshoppers, suggesting that the alkaloids produced by *A. texensis* do not spread throughout the plant. Infection significantly increased host plant vegetative size (leaf number), perhaps by diverting resources normally used for plant reproduction

to vegetative growth. Relative amounts of herbivory, however, were not higher on these larger, infected plants.

The effects of *A. texensis* on its host's reproduction, size, resource allocation patterns, competitive abilities, and tolerance of herbivory were studied in a greenhouse experiment. *Atkinsonella texensis* sterilized *Nassella*; infected plants produced fungal stromata in place of inflorescences. Infection was found to have no effect on the total above-ground biomass produced by *Nassella*. Instead, infection altered resource allocation: infected pairs allocated less to fungal reproduction than uninfected plants did to plant reproduction. As a result, infected plants produced more vegetative biomass than uninfected plants. The effect of simulated herbivory was independent of the effects of infection and competition on *Nassella.* Because infection also did not reduce the amount of herbivore damage in the field, infection appears to have no beneficial effects on *Nassella*. Therefore, *A. texensis* is a parasite, unlike many of its close relatives. The relationship between *Nassella* and *A. texensis* may represent the earliest stage in the evolution of the mutualisms that now exist between similar fungi and their plant hosts.

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Chapter 1: Introduction

Unlike most plant-fungus relationships, infections of plants by fungi of the tribe Balansieae (Clavicipitaceae, Ascomycota) are often thought to be mutualistic. At least 80 genera of plants host fungi of this tribe; they include C_3 and C_4 grasses, sedges, and rushes (Clay 1988). Most of these fungi are endophytic, with intercellular hyphae, and many of them are transmitted vertically, from mother to seed (Clay 1988). Previous studies have found that, or found evidence suggesting that, infection increases plant size (Clay *et al*. 1989, Clay 1990, Rice *et al*. 1990) and plant competitive ability (Marks *et al*. 1991, Clay *et al*. 1993, Brem and Leuchtmann 2002), deters insect herbivores (Clay *et al*. 1985, Cheplick and Clay 1988, Bultman *et al*. 2004), and increases plant tolerance of drought (Arachevaleta *et al*. 1989), heat (Marks and Clay 1996), low soil fertility (Malinowski and Belesky 2000), and other environmental stresses (reviewed by Clay and Schardl 2002). Most of these studies have involved endophyte-infected pasture grass cultivars such as tall fescue (*Festuca arundinacea*) and perennial ryegrass (*Lolium perenne*).

Some of the species in the tribe Balansieae are epiphytic rather than endophytic, that is, their hyphae grow on the leaf and stem surfaces (Clay and Schardl 2002). In central Texas savannas, the perennial bunchgrass *Nassella (Stipa) leucotricha* is frequently infected by an epiphytic member of this tribe, *Atkinsonella texensis*. Like some of the other epiphytic and endophytic species in its tribe, *A. texensis* sterilizes its host; infected plants produce fungal stromata (fungal fruiting bodies) instead of seed-bearing culms and seeds. It is not known whether the relationship between an epiphyte and its host can be mutualistic; only a few studies have examined the effects of epiphytic fungi on host plant performance (Clay 1984, Fowler and Clay 1995, McCormick *et al*.

2001). Therefore, the purpose of this study was to describe the distribution of *A. texensis* in central Texas savannas and to determine whether the symbiosis between *Nassella* and *A. texensis* is a parasitism or a mutualism.

Central Texas savannas are characterized by the presence of clusters of woody plants separated by open grassy areas. Soil temperatures, soil organic content, water availability, light levels, and the density and composition of the herbaceous vegetation all vary among these open grassy areas, the edges of the woody plant clusters, and under the clusters (Anderson *et al*. 2001, Wayne and Van Auken 2004), often on a scale of meters or less. Small-scale environmental heterogeneity such as this can cause an uneven distribution of infectious diseases (Burdon 1987).

However, diseases like *A. texensis* can also be unevenly distributed within the area occupied by the host population due to host plant characteristics (*e.g.*, host density), or biotic factors (*e.g.*, dispersal agents), or from factors intrinsic to the fungal populations itself (*e.g.*, density-dependence of fungal reproduction) (Agrios 1997). Many of these factors have been well-studied, but there have been few studies of the complex interactions between small-scale environmental heterogeneity, host plant characteristics, and disease incidence in natural plant populations (but see Augspurger and Kelly 1984, Jarosz and Burdon 1988, Garcia-Guzman *et al.* 1996, McCormick *et al.* 2001). Therefore, in chapter 2, I quantify the relationship between spatial position vis-à-vis woody plant clusters, *Nassella* density, and infection incidence (presence/absence of *A. texensis*) at three representative savanna sites in central Texas.

Because of the importance of herbivory in natural plant populations, plants have developed a variety of defenses against insect herbivores, including the formation of symbiotic relationships with the alkaloid-producing fungi of the tribe Balansieae (reviewed by Clay and Schardl 2002). The fungus subsists on sugars and amino acids

produced by the plant, while the plant is assumed to be protected from herbivory by alkaloidal mycotoxins (Clay *et al*. 1985, Cheplick and Clay 1988, Bultman *et al*. 2004). However, most studies of these plant-fungal symbioses have been of endophyte-infected forage grasses (*e.g.*, *Lolium perenne*), in which fungal infection is apparently always protective.

A few studies of endophyte-infected wild grass species have failed to find protective effects of infection by related fungi (Lopez *et al*. 1995, Saikkonen *et al*. 1999), but some have also found protective effects as well (*e.g*., Clay *et al*. 1985, Brem and Leuchtmann 2001). It has been suggested that variation in alkaloid levels may be responsible for these conflicting results (Saikkonen *et al*. 1999). It was not known whether infection by an epiphyte like *A. texensis* would deter native insect herbivores, thereby reducing the amount of herbivore damage to infected plants in a natural plant population. In chapter 3, I describe the relationship between infection by *A. texensis* and levels of herbivory by grasshoppers, the most common source of visible damage to *Nassella* plants in natural populations.

The effects of *A. texensis* on other measures of host plant performance (plant size, resource allocation, competitive response, and tolerance of herbivory) were also not known. Previous studies of related fungi suggested that infection increased plant size and host plant competitive ability, and perhaps altered the allocation of plant resources to vegetative growth (Clay 1984, Clay 1990, Fowler and Clay 1995, Pan and Clay 2002). Studies of related endophytes also found that infected plants did not replace damaged tissue as quickly as uninfected plants, suggesting that infection reduced host tolerance of herbivory (Belesky and Fedders 1996, Cheplick 1998, Bultman *et al*. 2004). Therefore, in chapter 4, I examine the joint effects of infection by *A. texensis*, simulated grasshopper

herbivory, and competition from a co-occurring grass on *Nassella* size and resource allocation.

Chapter 2: The distribution of *Nassella leucotricha* and its epiphytic fungus, *Atkinsonella texensis*, in central Texas savannas

ABSTRACT

The abundance of *Nassella leucotricha* and its rate of infection by its fungal pathogen, *Atkinsonella texensis* (Balansieae, Clavicipitaceae, Ascomycota), varied spatially in three savanna sites in central Texas. In two of the three sites, *Nassella* was much more abundant at the edge of clusters of woody plants than in the open grassy areas between them. In all three sites, the incidence of disease was highest beneath woody canopies and lowest in the open. Where *Nassella* was growing under or near woody plants, there was a positive relationship between the incidence of infection and *Nassella* density. In contrast, there was no relationship between *Nassella* density and the incidence of infection in the open grassy areas between clusters, possibly because infected plants there did not produce infectious fungal spores. Environmental conditions in the open might have affected the stroma adversely; the results are also compatible with an Allee effect on fungal mating.

INTRODUCTION

The incidence of fungal pathogens within natural plant populations often varies spatially (Burdon *et al.* 1989). Not uncommonly, disease is only found in some patches within the area occupied by the host population (Burdon 1987). This variation in infection incidence can arise from environmental (abiotic) heterogeneity, host plant characteristics (*e.g.*, host density), other biotic factors (*e.g.*, dispersal agents), and from factors intrinsic to the fungal populations itself (*e.g.*, density-dependence of fungal reproduction) (Agrios 1997). Many of these factors have been well-studied, but complex interactions between small-scale environmental heterogeneity, host plant characteristics, and disease incidence in natural plant populations have seldom been studied (but see Augspurger and Kelly 1984, Jarosz and Burdon 1988, Garcia-Guzman *et al.* 1996, McCormick *et al.* 2001).

Nassella leucotricha (Poaceae), a perennial bunchgrass very common in central Texas savannas (Fowler and Dunlap 1986, Fowler 1988), is regularly infected by the epiphytic fungus *Atkinsonella texensis* (Balansieae, Clavicipitaceae, Ascomycota). These savannas are characterized by the presence of clusters of woody plants separated by open grassy areas. Soil temperatures, soil organic content, water availability, light levels, and density and composition of herbaceous vegetation all vary among these open grassy areas, the edges of the woody plant clusters, and under the clusters (Anderson *et al*. 2001, Wayne and Van Auken 2004), often on a scale of meters or less. An earlier descriptive study (Fowler and Clay 1995) suggested that *Nassella* infection rates were higher under woody plant clusters than in the open (cluster edges were not separated out in this descriptive study). The relationship between *Nassella* density and infection incidence was not examined in that study. In this study, I quantify the relationship between spatial

position vis-à-vis woody plant clusters, *Nassella* density, and infection incidence at three representative sites in central Texas.

METHODS

Study system and organisms

The vegetation of the Edwards Plateau of central Texas is primarily savanna; woody species are often aggregated in large clusters and surrounded by open grassy patches ranging in size from under five meters to over 100 meters (M. Maas, pers. obs.). The region has hot summers, mild winters, and little seasonal pattern in precipitation (Riskind and Diamond 1988). The savannas of the eastern Edwards Plateau are usually dominated by clusters of *Quercus fusiformis* (Plateau live oak) and *Juniperus ashei* (Ashe juniper) in a matrix of midgrasses, shortgrasses, and other herbaceous plants (Fowler and Dunlap 1986, Gabbard 2003). The dominant grass species include *Nassella leucotricha* (Texas wintergrass) as well as *Schizachyrium scoparium* (little bluestem), *Bouteloua spp.* (grama grasses), *Bothriochloa ischaemum* (King ranch bluestem), and *Aristida spp.* (three-awn grasses).

Nassella leucotricha (Trin. & Rupr.) Pohl, hereafter *Nassella*, is a C_3 perennial bunchgrass whose range extends from southwestern Oklahoma to northeastern Mexico (Hicks *et al.* 1990, White and Van Auken 1996). *Nassella* is amphicarpic, producing both cleistogamous and chasmogamous flowers (Call and Spoonts 1989). Cleistogamous flowers are self-fertilized and are located at the base of culms. Chasmogamous flowers are potentially outcrossed and are located on aerial panicles. *Nassella* is frequently infected by an ascomycetous fungus, *Atkinsonella texensis* (Balansieae, Clavicipitaceae, Ascomycota). The infection is epiphytic (Leuchtmann and Clay 1988, Morgan-Jones and

White 1989); it first occurs on the leaves just above the basal meristems and later surrounds reproductive culms and immature chasmogamous inflorescences.

Once *A. texensis* has successfully infected a host plant, it produces stromata (fungal fruiting bodies) that bear conidia (asexual spores). Because *A. texensis* is heterothallic, conidia from one mating type must be exchanged with conidia from another mating type before ascospores (sexual spores) can be produced on the stromata (Morgan-Jones and White 1989). The mechanism of horizontal transmission (contagious spread) of ascospores between host plants has not been tested. Insects may play a role in transferring infection, but the sexual spores are more likely dispersed between plants by water (Fowler and Clay 1995).

Atkinsonella texensis sterilizes host plants because stromata form in place of chasmogamous inflorescences, but some studies have reported that infected plants can still produce cleistogamous seeds (Clay and Jones 1984, Fowler and Clay 1995). However, I have been unable to find any infected *Nassella* plants that produce cleistogamous seeds (M. Maas, pers. obs.). Previous work also indicates that this type of fungal infection may increase host plant growth and competitive ability (Clay 1990, Marks *et al*. 1991, Clay *et al.* 1993), and reduce damage by herbivores (Cheplick and Clay 1988, Saikkonen *et al.* 1998, Bultman *et al.* 2004).

Experimental design

In May 2003, I selected three savanna sites on the eastern Edwards Plateau in central Texas: Lady Bird Johnson Wildflower Center (LBJ), Pedernales Falls State Park (PED), and the Balcones Canyonlands National Wildlife Refuge (SIM) (Table 2.1). At LBJ the woody dominant was *Juniperus ashei*; at PED and SIM the woody dominant was *Quercus fusiformis*.

At each site I selected a 200m x 200m area containing both uninfected and infected *Nassella*, in which I randomly placed the centers of either 100 or 200 circular plots 2m in diameter. Plots that partly fell outside a site's boundary (usually indicated by barbed wire or a fence) were discarded from the sample. I sampled 194 plots at LBJ (6 plots fell outside its boundary), 100 plots at PED, and 97 plots at SIM (3 plots fell outside its boundary), for a total of 391 plots. I conducted this survey during the month of May because infection status was most easily determined then; in May uninfected plants produced seed-bearing culms while infected plant produced culms bearing fungal fruiting bodies.

At each randomly located plot, I recorded patch type as either 1) under tree canopy, 2) at the edge of tree canopy, or 3) in an open grassy patch. 'Tree' in this context includes large *J. ashei* individuals, despite their multi-trunked physiognomy. A plot was classified as under tree canopy if the entire plot was under a tree's canopy and within its drip line. A plot was classified as being at the edge of tree canopy if any part of the plot was under a tree's drip line. A plot was classified as in an open grassy patch if the entire plot was positioned outside of the drip line. If a plot was at the edge or under tree canopy, I also recorded the dominant woody species providing the majority (> 50%) of the canopy cover directly above the plot. Dominant canopy type was then later classified as either "*J. ashei*-dominated' or as 'hardwood-dominated'.

I also carefully inspected each plot for the presence of *Nassella*. *Nassella* was recorded as present if at least one *Nassella* plant was present in the plot. If *Nassella* was present, I also measured the density of *Nassella* in the plot. Because it was difficult to measure density by counting the number of individual plants in each plot, I instead measured density by recording the density of *Nassella* tillers. Plots covered by less than 50% of *Nassella* tillers were classified as 'sparse', while plots covered by 50% or more of

Nassella tillers were classified as 'dense'. Infection incidence (presence/absence of infection) was then determined for each plot; plots were recorded as 'infected' if at least one culm bearing a fungal fruiting body was present. Only plots with at least one *Nassella* plant present were included in the analyses of *Nassella* density and infection incidence.

Statistical analyses of dominant canopy type

To determine whether the composition of the dominant canopy type differed among sites (*i.e.* whether there were significantly more plots under or at the edge of *J. ashei* canopy at LBJ than at PED or at SIM), I used Pearson's chi-square test (χ^2) or Fisher's Exact Test (p) (Fisher's Exact Test was used when cells had low expected values) to analyze three separate 2X2 tables comparing pairs of sites. Multiple testing was taken into account by using Bonferroni's correction (Sokal and Rohlf 1995). Therefore each pairwise comparison was done with a α-level of 0.017, to yield an overall α-level of 0.05. Plots in open patches were not included in analyses of dominant canopy type.

Statistical analyses of *Nassella* density

To examine the relationship between site, patch type, and *Nassella* density, I used a saturated log-linear model with three main effects: site (LBJ, PED, SIM), patch type (under, edge, open), and *Nassella* density (sparse, dense). The saturated model included all main effects and all interaction terms. The highest order interaction (the three-way interaction between site, patch type and *Nassella* density) was then removed from the model to determine if this more parsimonious model was as good a fit to the observed

distribution of data as the saturated model. Goodness-of-fit was measured by the likelihood ratio test (*G*). Because the likelihood ratio test was significant, the relationship between patch type and *Nassella* density was analyzed for each site separately.

To examine how the relationship between *Nassella* density and patch type varied among sites, I used Pearson's chi-square test (χ^2) or Fisher's Exact Test (p) to analyze three separate 2X3 contingency tables (one table per site). If there was a significant departure from random, the 2X3 table was broken down into three 2X2 tables, comparing pairs of patch types. Multiple testing was taken into account by using Bonferroni's correction (Sokal and Rohlf 1995). Each pairwise comparison was done with a α-level of 0.017, to yield an overall α-level of 0.05.

Statistical analyses of infection incidence

Due to the small number of plots with dense *Nassella* under a tree canopy at all three sites, I could not construct a saturated log-linear model to explore the relationship between site, patch type, *Nassella* density, and infection incidence. Instead, I explored the relationship between these main effects by constructing three separate log-linear models. Only plots with *Nassella* present were included in these models.

The first of the three log-linear models explored the relationship between site (LBJ, PED, SIM), patch type (under, edge, open), and infection incidence (present, absent). The saturated model included all main effects and all interaction terms. The highest order interaction (the interaction between site, patch type, and infection incidence) was then removed from the model to determine if the more parsimonious model was as good a fit to the observed distribution of data as the saturated model. Goodness-of-fit was again measured by the likelihood ratio test (*G*). Because the likelihood ratio test was non-significant I removed the highest order interaction term from

the model. A Wald statistic was then calculated for all interaction terms remaining in the more parsimonious model (*W*).

The second log-linear model explored the relationship between site, *Nassella* density (sparse, dense), and infection incidence. Again, the likelihood ratio test indicated that the highest order interaction (the interaction between site, *Nassella* density, and infection incidence) could be removed from the saturated model. As before, a Wald statistic was calculated for all interaction terms remaining in the more parsimonious model (*W*).

Because these first two log-linear models found that infection incidence varied among sites (*i.e.*, there was a significant interaction between site and infection incidence), I used Pearson's chi-square test (χ^2) to analyze three separate 2X2 tables, comparing pairs of sites. Multiple testing was taken into account by using Bonferroni's correction (Sokal and Rohlf 1995). Therefore each pairwise comparison was done with a α-level of 0.017, to yield an overall α-level of 0.05.

Because the first two log-linear models found that the relationship between patch type and infection incidence and the relationship between *Nassella* density and infection incidence did not vary among sites, I combined the data collected at all three sites to explore the relationship between patch type, *Nassella* density, and infection incidence. A third log-linear model was used to explore this relationship. In this instance, though, I was unable to remove any effects from the saturated log-linear model. I therefore examined the relationship between *Nassella* density and infection incidence for each patch type separately. I used Pearson's chi-square test (χ^2) or Fisher's Exact Test (p) to analyze the three 2X2 contingency tables (one table per patch type).

RESULTS

Dominant canopy type

Composition of the dominant canopy type varied significantly among sites (Figure 2.1). Approximately 70% of plots at LBJ that were under or at the edge of a tree were beneath *Juniperus ashei*-dominated canopy; at both PED and SIM, less than 10% of plots under or at the edge of a tree were beneath *J. ashei*-dominated canopy (LBJ to PED comparison, df = 1, $\chi^2 = 72.74$, $p < 0.0001$; LBJ to SIM comparison, df = 1, $\chi^2 = 48.46$, $p < 0.0001$; PED to SIM comparison, $p = 0.9542$).

Nassella density

The best log-linear model of *Nassella leucotricha* density included the three-way interaction between site, patch type, and *Nassella* density (Figure 2.2). (The model generated by removing the three-way interaction was significantly different from the saturated model: df = 4, $G = 18.83$, $p = 0.0008$.) In other words, the relationship between patch type and *Nassella* density varied among sites. Therefore the relationship between *Nassella* density and patch type was tested in each site separately. At both LBJ and SIM, there was a significant relationship between patch type and *Nassella* density (LBJ, df = 2, $\chi^2 = 14.65$, $p = 0.0007$; PED, $p = 0.1443$; SIM, df = 2, $\chi^2 = 5.87$, $p = 0.0532$). Because this relationship was significant at LBJ and SIM, three separate pairwise comparisons were made, for each of the two sites.

At LBJ, *Nassella* was approximately five to seven times more likely to be dense in a plot at the edge of a tree canopy or in the open patches than it was in a plot under a tree canopy (under to edge comparison, df = 1, $\chi^2 = 8.06$, $p = 0.0045$; under to open comparison, df = 1, $\chi^2 = 14.59$, $p = 0.0001$; edge to open comparison, df = 1, $\chi^2 = 2.54$, p

= 0.1113). At SIM, *Nassella* density increased from 'open' plots to plots under canopies to plots at the edge of canopies. However, only the 'edge' to 'open' comparison was significant at SIM (under to edge comparison, df = 1, χ^2 = 0.49, p = 0.4850; under to open comparison, p = 0.2367; edge to open comparison, df = 1, χ^2 = 5.83, p = 0.0157). At PED, *Nassella* density was also highest in plots at the edge of canopies, but this trend was not statistically significant.

Infection incidence

The model generated by removing the three-way interaction between site, patch type, and infection incidence was not significantly different from the saturated model (Table 2.2). The relationship between patch type and infection incidence did not vary from site to site. However, this more parsimonious model demonstrated that there was a significant relationship between site and infection incidence as well as between patch type and infection incidence. The highest incidence of infection was found at SIM, with over 50% of plots with *Nassella* containing at least one infected plant (LBJ to PED comparison, df = 1, χ2 = 6.2871, p =0.0122; LBJ to SIM comparison, df = 1, χ2 = 28.445, p < 0.0001; PED to SIM comparison, df = 1, χ2 = 4.3231, p =0.0376) (Figure 2.3), and plots with at least one infected *Nassella* plant were more likely to occur under or at the edge of a tree canopy (Figure 2.4).

The model generated by removing the three-way interaction between site, *Nassella* density, and infection incidence was also not significantly different from the saturated model (Table 2.3). Tthe relationship between *Nassella* density and infection incidence also did not vary from site to site. As before, this more parsimonious model demonstrated that there was a significant relationship between site and infection incidence. This model found a significant relationship between *Nassella* density and

infection incidence; infection was more likely to be found in plots with dense *Nassella* (Figure 2.4).

The relationship between *Nassella* density and infection incidence was then found to vary among patch types because the model generated by removing the three-way interaction between patch type, *Nassella* density, and infection incidence was significantly different from the saturated model (df = 2, G = 10.10, p = 0.0064). Therefore the relationship between *Nassella* density and infection incidence was tested in each patch type separately. For plots located at the edge of a tree canopy or under a tree canopy, infection was approximately twice as likely to occur in plots with dense *Nassella* as in plots with sparse *Nassella* (under, p = 0.0570; edge, df = 1, χ^2 = 15.87, p < 0.0001) (Figure 2.4). However, for plots located in the open grassy patches, infection was just as likely to occur in plots with dense *Nassella* as in plots with sparse *Nassella* (df = 1, χ^2 = 0.3887, p = 0.5330).

DISCUSSION

Effect of patch type on the distribution of *Nassella leucotricha*

In all three study sites, *Nassella leucotricha* was present in all three patch types: under woody plant clusters, at their edges, and in the open grassy patches between them. This habitat breadth is unusual among grasses in these savannas: the other common perennial grass species in these savannas, such as *Bouteloua rigidiseta* and *Bothriochloa ischaemum*, are rarely found beneath woody canopies (Fowler and Dunlap 1986, Gabbard 2003). Since all of the common perennial grass species except *Nassella* are also C_4 species (Hicks *et al*. 1990), the ability to live under woody plant canopies may be related to the differences between C_3 and C_4 physiology.

Although *Nassella* was present throughout the spatial mosaic, plots at the edge of a tree canopy were more likely to have a high density of *Nassella* tillers than plots in the open or under the canopy. These results are compatible with those of Fowler and Clay (1995), although they did not separate edge patches from patches under woody plants. Abiotic conditions at the edge of woody canopies may be the most favorable for *Nassella*. Plants at the edge of a woody canopy receive fewer hours of direct sunlight than plants in open grassy patches, but more than plants under a woody canopy. This intermediate amount of light may optimize the balance between sunlight for photosynthesis and water-stress reduction by shade.

At the LBJ study site, plots in open grassy patches were also likely to have a high density of *Nassella* tillers. This site had a plant community that differed from the plant communities of the PED and SIM study sites in several other ways, as well. *Juniperus ashei* was much more common in the woody plant clusters, and *Quercus fusiformis* correspondingly less common than at the other two sites. The LBJ site also was the only site in which *Opuntia* spp., a sign of overgrazing (Ueckert and McGinty 2004), were common (M. Maas, pers. obs.). It also had other evidence of disturbance (a road and a trash pile). This suggests that abundant *Nassella* in open grassy areas may be a sign of disturbance (overgrazing and/or vehicle traffic) in the recent past. This hypothesis is supported by casual observation of other *Nassella*-dominated sites in the region (N. Fowler, pers. comm., M. Maas, pers. obs.).

Effect of patch type on the incidence of *Atkinsonella texensis*

At all three sites, *Nassella* was more likely to be infected by *Atkinsonella texensis* when growing under or at the edge of a tree canopy than when growing in an open grassy patch. *Nassella* plants growing in open grassy patches, which receive more hours of

direct sunlight each day, may be less susceptible to infection, if, for example, they are less likely to have a film of water on their leaves. A film of water is necessary from most fungal spores to germinate and to penetrate the leaf surface (Agrios 1997). A study of a related grass endophyte (*Epichloë sylvatica*, Balansieae, Clavicipitaceae) found that the incidence of fungal infection was up to six times higher in the shade than in the open (Meijer and Leuchtmann 2000). Studies of unrelated fungi have also found that plants are more likely to be infected by fungal pathogens under shaded conditions (Jarosz and Burdon 1988, Jarosz and Levy 1988).

While the direct effects of shading on the probability that a spore will infect a plant are the most likely explanation for the higher incidence of infection of *Nassella* beneath woody canopies, at least six other explanations are possible. Lower soil nitrogen or organic content in the open (Anderson *et al.* 2001, Wayne and Van Auken 2004) might have reduced the nitrogen content of *Nassella*, which might in turn have made it less susceptible to infection (Wennström and Ericson 1992). Secondly some genetic differentiation in infection resistance between *Nassella* plants growing in different patch types is possible, but unlikely. Thirdly, infection incidence might reflect differential survival of infected plants in different patch types. In general, infection increases vegetative size (M. Maas, chapters 3 and 4), which would increase survival rates of infected plants. However, it is possible that under the conditions present in the open grassy patches, especially during summer droughts, infected plants might be more likely to die than uninfected plants if infection increases plant water loss. A similar explanation could be drawn involving nitrogen, if nitrogen is consistently lower in the open areas (Wayne and Van Auken 2004). A field study in Michigan of *Danthonia spicata* found that the incidence of infection by *A. hypoxylon* was lower in low-ammonia patches, perhaps because infected plants had a higher nitrogen demand (to support fungal tissue)

than uninfected plants (McCormick *et al.* 2001). The authors suggested that infected plants were excluded from low-ammonia locations because of the high-nitrogen demands of the fungus, as supported by higher nitrogen content of infected plant tissue compared to uninfected plant tissue. A fifth possible explanation involves lifespan. If average lifespan is shorter in the open (independent of infection), plants there simply have fewer years in which they are exposed to infection; while it is likely that plants in the open are shorter-lived, because they are smaller, it is not known whether adults can become infected throughout their life. A sixth explanation involves differences in the production of infectious spores, as discussed below.

Effect of host density on the incidence of *Atkinsonella texensis*

At all three sites, but only in plots under or at the edge of a woody canopy, the incidence of infection was higher in plots with dense *Nassella* tillers than in plots with sparse *Nassella* tillers. A positive relationship between host density and fungal disease incidence has been reported by many other researchers (reviewed by Burdon and Chilvers 1982, Augspurger and Kelly 1984, Thrall and Jarosz 1994, Garcia-Guzman *et al.* 1996). The likely cause is density dependence in fungal spore dispersal. In many instances fungal spores move only a meter or two from the inoculum source (Alexander 1990, Roche *et al.* 1995). As the distance between plants decreases, infectious spores are more likely to reach a host plant (Burdon 1987).

However, at all three sites, in plots located in open grassy areas, there was *Nassella* density and infection incidence were not related, implying that a plant without a nearby host is as likely to become infected as one with an infected plant neighbor. This could occur if plants in the open are not being infected by spores produced from infected plants in the open, but by spores dispersing from the woody plant clusters. (This would

also account for the low incidence of infection in the open.) Infectious spores might not be produced in the open for at least two possible reasons. First, stromata produced in the open may become too hot or too desiccated to produce infectious spores. White *et al.* (1993) suggested that stromata are not adapted for absorption and conduction of water, unlike plant tissues, and are therefore susceptible to overheating and desiccation. Second, because infected plants are at such low densities in the open, an Allee effect may be operating, that is, the fungus on one plant has such a low probability of exchanging conidia with the fungus of a different mating type on another plant that infectious spores are not formed in the open. *Atkinsonella texensis* has a heterothallic mating system that requires conidia exchange (Morgan-Jones and White 1989). To my knowledge, this is only the second suggestion of the presence of an Allee effect in a fungus (see Garrett and Bowden 2002). Whatever the cause of a failure of spore production in the open, it seems to be the most likely cause of the absence of density dependence in infection rates in the open. Experiments to test these hypotheses are needed.

Table 2.1. Descriptions of each site surveyed in this study.

Site	County	Latitude	Longitude	Soil type	Grazing History	Fire History	Vegetation
LBJ	Travis	30º 10' 58" N	97º 52' 0" W	Speck-Tarrant Association (shallow clay loam with underlying limestone rock)	Ungrazed for previous 20 years	No fire for at least 70 years	*Juniperus ashei* dominated grassland matrix
PED	Blanco	30º 19' 10" N	98º 14' 37" W	Hensley Association (shallow loam with underlying limestone rock)	Ungrazed for at least 30 years	Prescribed burns within the last 6 years	*Quercus fusiformis* dominated grassland matrix
SIM	Burnet	30º 39' 26" N	98º 4' 55" W	Eckrant Association (shallow clay with underlying limestone rock)	Ungrazed for previous 9 years	3 cool season burns in most areas within last 3 years	*Quercus fusiformis* dominated grassland matrix

Table 2.2. Maximum likelihood analysis of the relationship between site, patch type, and infection incidence.

Source	df	*W*	p
Site	2	14.11	0.0009
Patch type	2	6.58	0.0372
Infection incidence	1	8.97	0.0027
Site*Patch type	4	31.74	<0.0001
Site*Infection incidence	2	36.36	**<0.0001**
Patch type*Infection incidence	2	38.55	**<0.0001**
Likelihood Ratio	4	$G = 7.89$	0.0955

Table 2.3. Maximum likelihood analysis of the relationship between site, *Nassella* density, and infection incidence.

Source	df	*W*	p
Site	2	28.62	< 0.0001
Nassella density	1	42.89	< 0.0001
Infection incidence	1	2.10	0.1472
Site**Nassella* density	2	26.34	<0.0001
Site*Infection incidence	2	33.72	**<0.0001**
Nassella abundance*Infection incidence	1	17.38	**<0.0001**
Likelihood Ratio	2	$G = 1.27$	0.5305

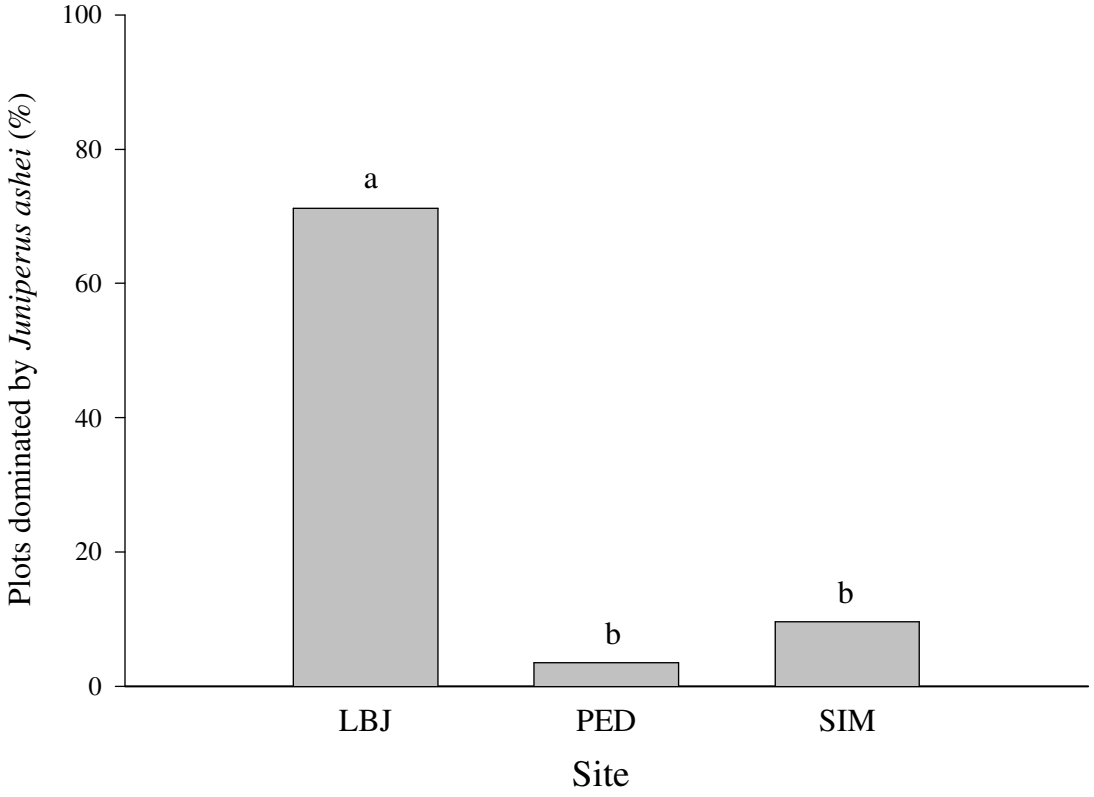

Figure 2.1. Percentage of plots under or at the edge of *Juniperus ashei*-dominated canopy at each site. Because there was a significant two-way interaction between site and dominant canopy type, pairwise comparisons between sites were conducted. Sites sharing letters were not significantly different at the adjusted α-level of 0.017.

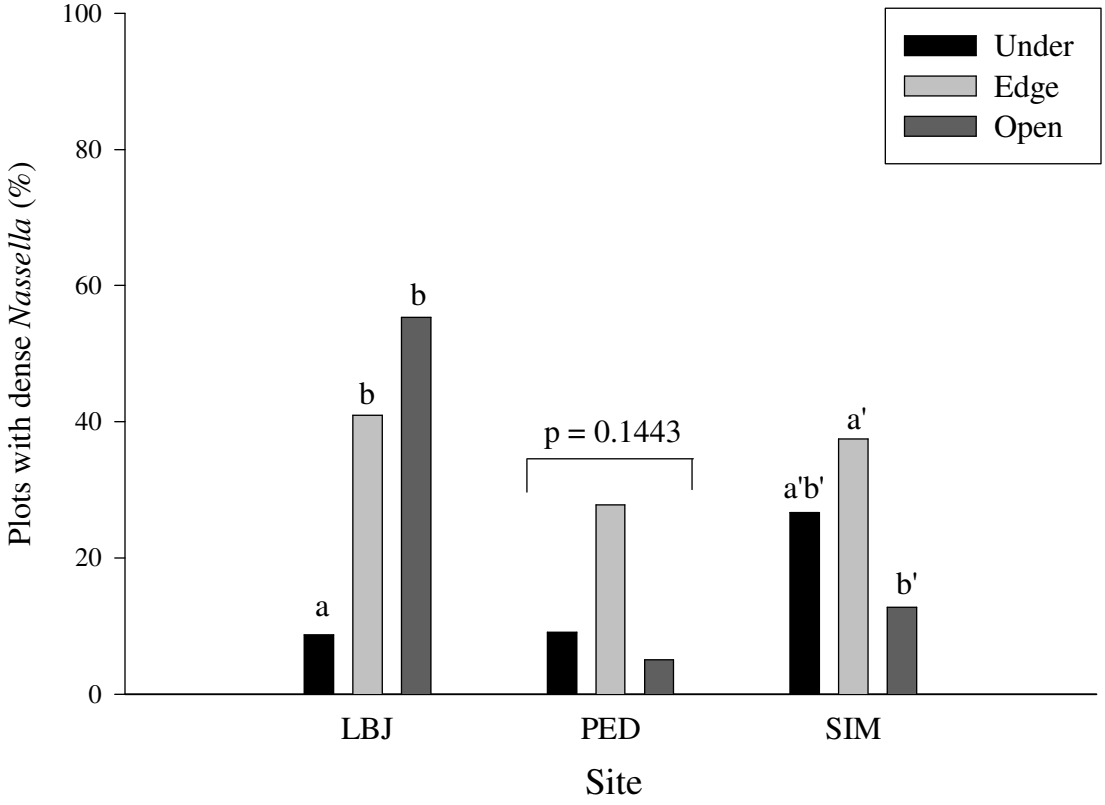

Figure 2.2. Percentage of plots of each patch type with dense *Nassella*, at each site separately. Because there was a significant three-way interaction between site, patch type, and *Nassella* density, the relationship between patch type and *Nassella* density was examined for each site separately. If there was significant relationship between patch type and *Nassella* density at a site, pairwise comparisons between patch types were conducted. Patch types sharing letters were not significantly different at the adjusted α-level of 0.017. Shared letters indicate only that the pair of patch types does not differ within that site; no paired comparisons were made of patch types between sites.

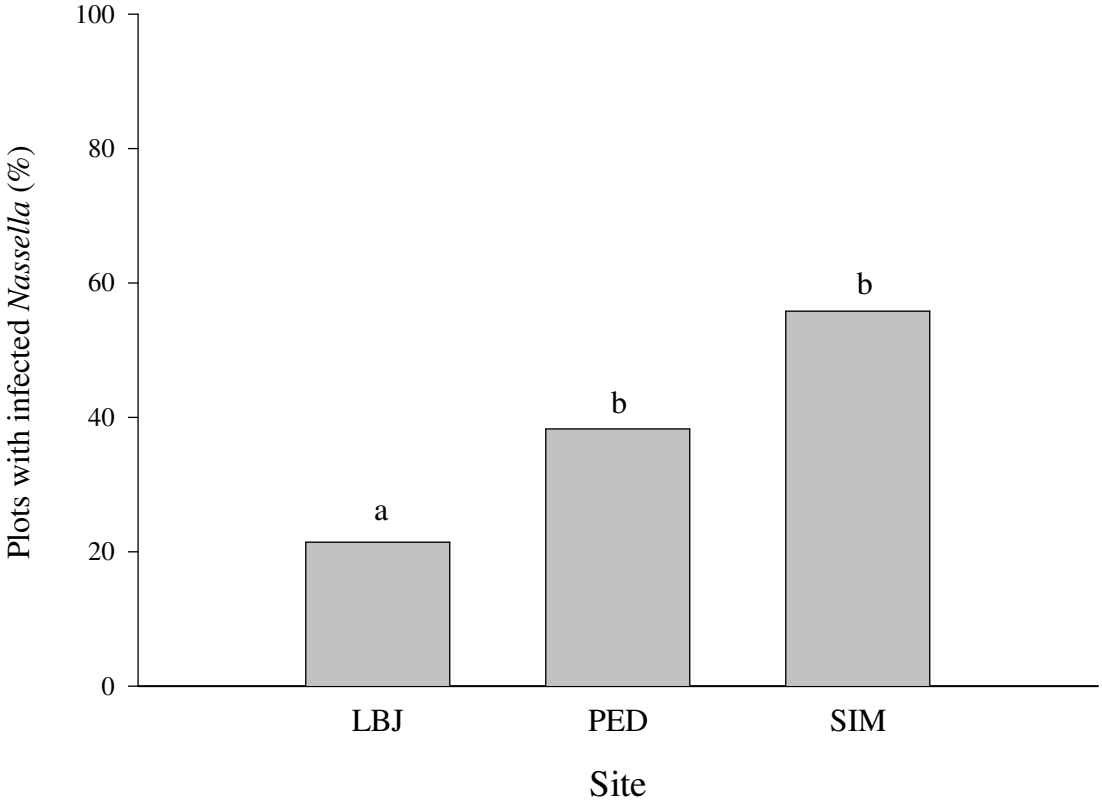

Figure 2.3. Percentage of plots with infected *Nassella* at each site. Because there was a significant two-way interaction between site and infection incidence, pairwise comparisons between sites were conducted. Sites sharing letters were not significantly different at the adjusted α-level of 0.017.

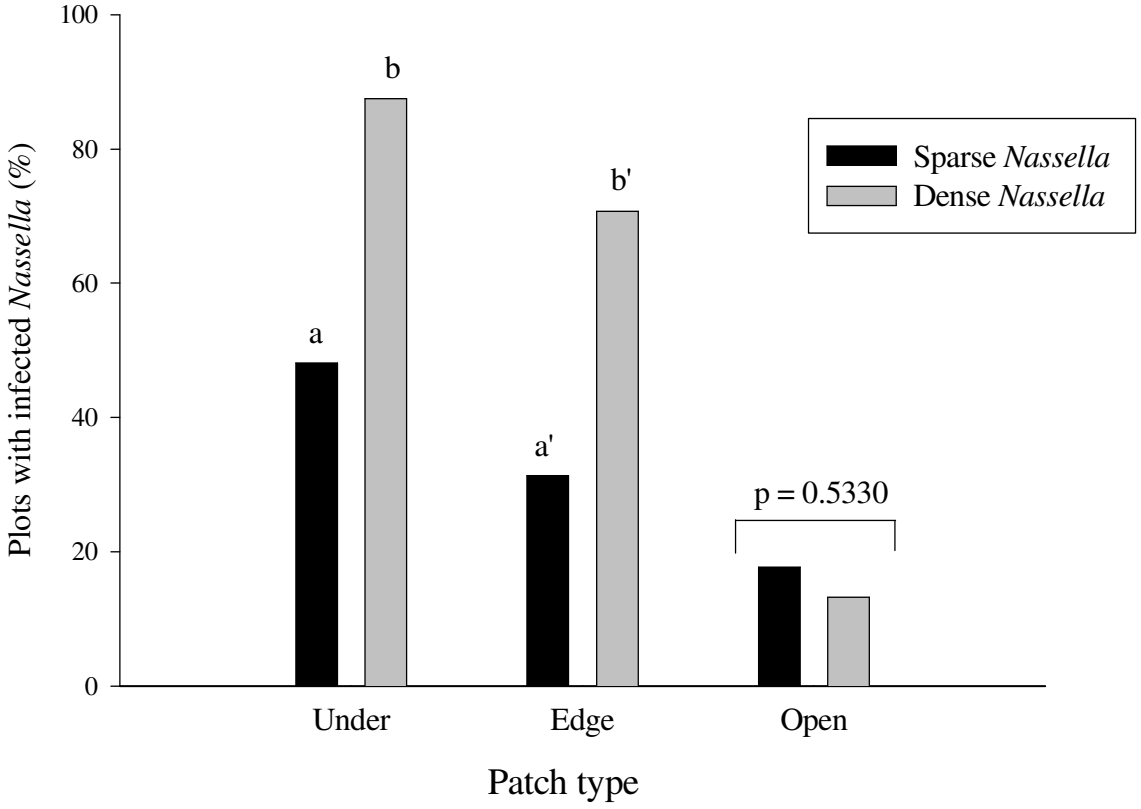

Figure 2.4. Percentage of plots with infected *Nassella* for plots with sparse and dense *Nassella* in each patch type. Because there was a significant three-way interaction between patch type, *Nassella* density, and infection incidence, the relationship between *Nassella* density and infection incidence was examined for each patch type separately. Bars with different letters were significantly different at the α-level of 0.05. No comparisons were made of bars types between patch types.

Chapter 3: Levels of herbivore damage in a natural population of *Nassella leucotricha*: effects of infection by *Atkinsonella texensis* and small-scale environmental heterogeneity

ABSTRACT

Nassella leucotricha, a perennial bunchgrass common in central Texas savannas, is often infected by the epiphytic fungus *Atkinsonella texensis* (Balansieae, Clavicipitaceae, Ascomycota). Closely related endophytic fungi are known to deter insect herbivores from eating their plant hosts. This study was undertaken to determine whether infection by *A. texensis* reduces herbivory on *Nassella* in the field. However, infection was found to have no effect upon the proportion of leaves damaged by grasshoppers, suggesting that the alkaloids produced by *A. texensis* do not spread throughout the plant, but instead remain associated with the fungal mycelia at the bases of the leaves, where grasshoppers rarely feed. Infection significantly increased host plant vegetative size (leaf number), perhaps by diverting resources normally used for plant reproduction to vegetative growth. Relative amounts of herbivory, however, were not higher on these larger, infected plants. The symbiotic relationship between *A. texensis* and *Nassella* may therefore be parasitic; *A. texensis* completely sterilizes its host, but does not provide protection from grasshoppers, a major herbivore of this grass.

INTRODUCTION

Insect herbivory can have a significant effect on a plant; it can influence its biochemistry (Bowers and Stamp 1993, Baldwin and Ohnmeiss 1994), growth and survival (Crawley 1983, Doak 1992, Ehrlén 1995, Hickman and Hartnett 2002), and competitive interactions (Clay *et al*. 1993, Taylor *et al*. 1997, Van der Wal *et al*. 2000). Because of the importance of herbivory in natural plant populations, plants have developed a variety of defenses against insect herbivores. Examples include surface defenses (*e.g*. lignin, silica, trichomes), secondary plant chemicals (*e.g*. phenolics and tannins), and the formation of symbiotic relationships with insects (*e.g*. ant defenders) (reviewed by Crawley 1983). Symbiotic relationships with alkaloid-producing fungi of the tribe Balansieae (Clavicipitaceae, Ascomycota) may also provide protection against herbivores (reviewed by Clay and Schardl 2002).

The symbiotic relationship between the host plant and the alkaloid-producing fungus is usually assumed to be mutualistic; the fungus subsists on sugars and amino acids produced by the plant (Clay and Schardl 2002), while the plant is assumed to be protected from herbivory by alkaloidal mycotoxins (Clay *et al*. 1985, Cheplick and Clay 1988, Bultman *et al*. 2004). However, most studies of these plant-fungal symbioses have been of agronomic grasses (*e.g*., *Lolium perenne*), in which fungal infection is apparently always protective. A few studies of wild grass species have failed to find protective effects of infection by related fungi (Lopez *et al*. 1995, Saikkonen *et al*. 1999), but some have also found protective effects (*e.g*., Clay *et al*. 1985, Brem and Leuchtmann 2001), including one study of *Atkinsonella texensis* and *Nassella leucotricha* (Clay *et al*. 1985). Variation in alkaloid levels could be responsible for these conflicting results (Saikkonen *et al*. 1999). Alkaloid production can depend on host plant genotype, host plant age, season, and abiotic conditions (reviewed by Clay and Schardl 2002).

A host plant might also benefit from the fungal infection through an increase in tolerance to a wide variety of environmental stresses, such as drought (Arachevaleta *et al*. 1989, Lewis *et al*. 1997, Malinowski *et al*. 1997), heat (Marks and Clay 1996), low soil fertility (Malinowski and Belesky 2000), and competition (Marks *et al.* 1991, Clay *et al*. 1993, Brem and Leuchtmann 2002). Studies have also shown enhanced growth in infected plants in the absence of such environmental stresses (Clay *et al*. 1989, Clay 1990, Rice *et al*. 1990). If infection promotes plant size, and larger plants are more apparent to insect herbivores (Feeny 1976), then infection could actually increase damage by insect herbivores.

Nassella leucotricha, a perennial bunchgrass common in central Texas savannas (Fowler and Dunlap 1986, Fowler 1988), is often infected by the epiphytic fungus *A. texensis* (Balansieae, Clavicipitaceae, Ascomycota) (Leuchtmann and Clay 1988, Morgan-Jones and White 1989). Clay *et al*. (1985) reported that, in a study using greenhouse-grown plants and fall armyworms (*Spodoptera frugiperda*), larvae fed infected plants grew more slowly than larvae fed uninfected plants. These authors therefore suggested that infection by *A. texensis* could protect *Nassella* from herbivory. However, it was not known whether infected *Nassella* actually experienced lower levels of herbivory from native herbivores in natural populations. This study was therefore designed to measure the relationship between fungal infection and herbivory by grasshoppers, the most common source of visible damage to these plants. Because infection may alter plant size, which could in turn affect herbivore behavior, plant size was also measured. Finally, the relationship between small-scale environmental heterogeneity and insect herbivory was also examined.

METHODS

Study site and organisms

Two surveys were conducted in 2001 and 2003 at a site in Pedernales Falls State Park on the Edwards Plateau in central Texas (30º 19' 10" N, 98º 14' 37" W). The vegetation at the study site was typical for the region with clusters of woody plants, primarily *Quercus fusiformis* (Plateau live oak) and *Juniperus ashei* (Ashe juniper), scattered in a matrix of grasses and other herbaceous plants (Fowler and Dunlap 1986, Gabbard 2003). Soil temperatures, soil organic content, water availability, light levels, and the density and composition of the herbaceous vegetation all vary between open grassy patches, the edges of the woody plant clusters, and under the clusters (Anderson *et al*. 2001, Wayne and Van Auken 2004), often on a scale of meters or less. One of the dominant grass species at the site is *Nassella leucotricha* (Texas wintergrass); it grows in all three patch types (patches under woody clusters, patches at the edge of woody clusters, and in the open grassy patches) that characterize central Texas savannas (M. Maas chapter 1).

Nassella leucotricha (henceforth referred to as *Nassella*) is a perennial bunchgrass. *Nassella* is amphicarpic, producing both chasmogamous and cleistogamous flowers. Cleistogamous flowers are self-fertilized and are located at the base of culms. Chasmogamous flowers are potentially outcrossed and are located on aerial panicles. Chasmogamous flowers produced by *Nassella* are susceptible to infection by *Atkinsonella texensis*, a fungal species that belongs to the tribe Balansieae (Clavicipitaceae, Ascomycota) (Leuchtmann and Clay 1988, Morgan-Jones and White 1989). The Balansieae are systemic and perennial fungi that often produce intercellular hyphae in leaf and stem tissues. They infect at least 80 genera, including C_3 and C_4 grasses, sedges, and rushes (Clay 1988). Species that occur intercellularly are

endophytic. In contrast, a few species in the tribe are epiphytic, producing hyphae that grow on the surface of young leaves and meristems. The genus *Atkinsonella* is epiphytic, first occurring in the upper leaf bases and then enveloping the culm and immature chasmogamous inflorescence (Morgan-Jones and White 1989). Eventually a stroma (a fungal fruiting body) that resembles a bird dropping forms in place of the chasmogamous inflorescence. *Atkinsonella* therefore sterilizes host plants by causing the abortion of chasmogamous inflorescences.

2001 experimental design

To determine the effect of fungal infection on plant size and whether infection reduces or increases the amount of plant tissue eaten by insect herbivores, I conducted a field survey at the state park in a 300m X 300m area in a site that contained both uninfected and infected plants. Because infected plants were commonly found growing in the patches under tree canopies, I established plots under the canopies of 50 randomly selected trees. Herbivore damage was measured in 10 of these plots in each of 5 months: May, June, July, August, and October 2001; each plot was used only once. Each month, I randomly collected 4 plants (2 uninfected and 2 infected) from each of the 10 plots randomly assigned to that month. Plants were clipped at the base and their green leaves were examined one by one. Each leaf was scored as having grasshopper 'bites' or not. I examined *Nassella* leaves for evidence of grasshopper herbivory because grasshoppers are one of the most widespread and damaging agricultural pests in central Texas (Patrick and Davis 2004), and because it was easy to determine whether a *Nassella* leaf had been 'bitten' by a grasshopper. Common grasshoppers found in central Texas and observed at Pedernales Falls State Park include the differential grasshopper (*Melanoplus*

differentialis) and the red-legged grasshopper (*Melanoplus femurrubrum*). However, I did not identify which species of grasshopper were 'biting' *Nassella* leaves.

2001 analyses of plant size

The response variable 'plant size', measured as the total number of green leaves produced by each plant, was analyzed using a mixed-model ANOVA. The explanatory terms included in the ANOVA were month, plot (nested within month), infection and the interaction between month and infection (PROC GLM, SAS 1990). Type I sums of squares were used to better understand the effect of infection on plant size after accounting for the effects of month and plot on plant size. Month, infection and their interaction were treated as fixed effects while plot nested within month was treated as a random effect. The effect of 'month' was tested over the mean square error of 'plot nested within month'. The response variable 'plant size' was log-transformed for each plant to correct for its skewed distribution. Because there was a significant effect of month on plant size, all possible pairwise comparisons between months were made using Fisher's least significant difference test (LSD). Multiple testing was taken into account by using Bonferroni's correction (Sokal and Rohlf 1995). Therefore, the analyses of month to month differences were evaluated with an α-level of 0.005, to yield an overall α-level of 0.05.

2001 analyses of herbivore damage

For each *Nassella* plant, I also calculated the response variable 'herbivore damage' by dividing the number of leaves with grasshopper 'bites' by the total number of leaves. The explanatory terms included in the ANCOVA were log-transformed plant size, month, plot nested within month, infection and all possible two-way and three-way

interactions (PROC GLM, SAS 1990). The term 'log-transformed plant size' was treated as a covariate and placed as the first term in the model to better understand the effects of the other terms in the model after accounting for size. Month and infection were treated as fixed effects while plot nested within month was treated as a random effect. The effect of 'month' was tested over the mean square error of 'plot nested within month'. All of the interaction terms in the model that involved plant size were non-significant and were therefore dropped from the model. Because there was a significant effect of month on herbivore damage all possible pairwise comparisons between months were made using Fisher's least significant difference test (LSD). Therefore, the analyses of month to month differences were evaluated with an α-level of 0.005, to yield an overall α-level of 0.05.

The analysis of herbivore damage revealed that infection did not significantly reduce the proportion of leaves with grasshopper 'bites'. A higher proportion of leaves produced by infected plants were 'bitten' by a grasshopper than were leaves produced by an uninfected plant (0.3049 vs. 0.2862, respectively). However, the difference between the proportion of infected leaves eaten and uninfected leaves eaten (0.01866) was non-significant. A *post-hoc* power analysis was conducted to determine the minimum size of an effect of infection that would have been detectable, given the sample size used in this study. To determine the minimum detectable difference in herbivore damage between infected and uninfected plants I increased the proportion of leaves 'bitten' for each infected plant and decreased by an equal amount the proportion of leaves 'bitten' for each uninfected plant until the difference was large enough for infection to become a significant term in the model. However, the proportion of leaves 'bitten' was not allowed to fall below zero.

2003 experimental design

In 2001, plots were placed exclusively under one patch type (patches under tree canopy). Therefore, the effect of patch type on plant size and levels of herbivore damage was not explored. To better understand the effect of patch type on these two variables, I conducted an additional survey in June 2003 at the same site at Pedernales Falls State Park that was used in 2001. I selected a smaller area (200m X 200m) in this site and randomly placed 27 larger plots within this area. Within each plot, I randomly selected one uninfected *Nassella* plant from each of the following patch types: 1) under tree canopy, 2) at the edge of tree canopy (directly under the dripline), and 3) out in the open grassy patches. 'Tree' in this context includes large *Juniperus ashei* individuals, despite their multi-trunked physiognomy. I collected all of the above-ground vegetative biomass for each plant. I later measured plant size by counting the total number of green leaves produced by each plant. The number of these leaves with 'grasshopper bites' was also recorded.

2003 analyses of plant size

To determine the effect of plot and patch type (under, edge and open) on plant size (total number of green leaves), I used ANOVA (PROC GLM, SAS version 8.0). The response variable 'plant size', measured as total number of green leaves, was log-transformed for each plant to correct for its skewed distribution.

2003 analyses of herbivore damage

As in 2001, for each uninfected *Nassella* plant I calculated the proportion of leaves damaged by herbivores by dividing the number of green leaves with grasshopper 'bites' by the total number of green leaves. However, the response variable 'herbivore

damage' was not normally distributed in 2003, and no transformation could be found to correct for its skewed distribution. Therefore, the effect of plot and the effect of patch type (under, edge and open) on herbivore damage were tested separately using two Kruskal-Wallis tests. To test the effect of plant size on herbivore damage, plants were classified as either small (1-5 leaves) or large (more than 5 leaves). A Kruskal-Wallis test was also used to compare the amount herbivore damage experienced by small and large plants.

RESULTS

2001 analyses of plant size

Census month and infection both had significant effects on plant size (Table 3.1). *Nassella* plants were, on average, significantly larger in May (Figure 3.1). During the following months (June to October), average plant size steadily decreased. Infected plants were, on average, significantly larger than uninfected plants during all months of the study. Plot also had a significant effect on average plant size; in May, for example, average plant size ranged from approximately 20 green leaves in one plot to approximately 75 green leaves in another plot (Figure 3.2).

2001 analyses of herbivore damage

Plant size did not significantly affect the proportion of leaves with herbivore damage (Table 3.2). Herbivory damage did vary significantly, though, from month to month; there was a significant increase in the mean proportion of green leaves with grasshopper 'bites' from May to June (Figure 3.3). During the remaining summer months (July and August), some additional damage occurred. Differential senescence in late summer of damaged leaves reduced the mean proportion of damaged green leaves

back to the May value. Herbivore damage also varied significantly from plot to plot, indicating that, within a given month, there was a great deal of spatial variation in the mean proportion of leaves 'bitten' by a grasshopper (Figure 3.4).

Infection, however, did not significantly reduce the mean proportion of leaves with grasshopper 'bites'; infected plants were just as likely to be 'bitten' by a grasshopper as uninfected plants. A higher mean proportion of leaves produced by infected plants showed signs of insect herbivory than leaves produced by uninfected plants (0.30 vs. 0.29, respectively), but the difference between the mean proportion of infected leaves eaten and the mean proportion of uninfected leaves eaten (0.02) was not significant at the $\alpha = 0.05$ level. A *post-hoc* power analysis determined that infection would have become a significant term in the model if the mean difference between uninfected and infected *Nassella* plants had been equal to or greater than 0.04.

2003 analyses of plant size

Uninfected plants were, on average, much smaller in size in 2003 than in 2001 (Figure 3.5). As in 2001, plot had a significant effect on plant size (Table 3.3, Figure 3.6). Average plant size ranged from approximately one leaf in one plot to approximately 16 leaves in another plot. Patch type, though, did not have a significant effect on plant size. In all three patch types, average plant size was approximately three to four leaves.

2003 analyses of herbivore damage

The amount of herbivore damage was, on average, much higher in 2003 than in 2001 (Figure 3.5). Grasshopper 'bites' were found on a significantly higher mean proportion of leaves produced by smaller plants (1-5 leaves) than on leaves produced by larger plants (greater than 5 leaves) ($\chi^2 = 18.99$, df = 1, $p < 0.0001$, Table 3.4). As in

2001, herbivore damage also varied significantly from plot to plot, indicating that there was a great deal of spatial variation in the mean proportion of leaves damaged by grasshoppers (χ^2 = 28.97, df = 18, p = 0.0488, Table E). However, patch type did not have a significant effect on herbivore damage (χ^2 = 0.30, df = 2, p = 0.8587, Table 3.6); approximately 90 percent0 of green leaves in all three patches were 'bitten' by grasshoppers.

DISCUSSION

Infection status and plant size

Nassella leucotricha plants infected by the epiphytic fungus *Atkinsonella texensis* were significantly larger than uninfected plants, a result consistent with the (non-significant) effect of infection of plant size reported by Fowler and Clay (1995) and the significant effect on plant size I measured in a greenhouse experiment (M. Maas chapter 4). A similar increase in plant size due to fungal infection has been found in other grass species infected with other members of the same fungal tribe (Clay *et al*. 1989, Clay 1990, Clay *et al*. 1993), although most of these fungi are endophytic, not epiphytic as *A. texensis* is. It is likely that, as I found in a greenhouse study (M. Maas chapter 4), the increase in *Nassella* size probably represented a shift in resource allocation by infected plants towards greater investment in vegetative tissues, because fungal reproductive tissues were less 'expensive' (i.e., less dry mass) to produce than plant reproductive tissues. However, other explanations are also possible, such as increased tolerance to environmental stresses such as drought or disease (Arachevaleta *et al*. 1989, Gwinn and Gavin 1992, Malinowski and Belesky 2000).

Infection status and herbivore damage

A previous study suggested that infection by *A. texensis* would be adaptive if infected *Nassella* plants were less damaged by insect herbivores than healthy plants (Clay *et al*. 1985). Since this hypothesis had never been tested in the field, I measured amounts of grasshopper damage on uninfected and infected *Nassella* plants in a natural population. Uninfected *Nassella* plants did not have more visible grasshopper damage (bite marks) than infected plants; the (very weak and non-significant) trend was even in the other direction. Evidently infection did not deter grasshoppers from eating infected plants.

In the previous study by Clay *et al.* (1985), lepidopteran larvae (*Spodoptera frugiperda*) fed infected leaves of *Nassella* grew more slowly than larvae fed uninfected *Nassella* leaves. There are at least three possible reasons for the discrepancy between their results and mine. **First**, it could be that grasshoppers are affected by the fungus just as lepidopteran larvae are, but were not deterred from eating infected plants because they could not sense ('taste') the presence of the fungus, even though *A. texensis* is known to produce alkaloids (Leuchtmann and Clay 1988, Clay and Cheplick 1989). However, fungal alkaloids are readily detected by many herbivores (Clay and Cheplick 1989, Siegal *et al*. 1990, Bush *et al*. 1997). It is possible, but unlikely, that lepidopteran larvae are simply more sensitive to these alkaloids than grasshoppers are. A **second** possibility is that, under natural field conditions, concentrations of compounds present only in infected plants are substantially lower than they were in greenhouse-grown plants used by Clay *et al*. (1985), too low for the grasshoppers to respond to. A study by Lyons *et al*. (1986) found that the concentration of alkaloids in greenhouse-grown tall fescue (*Festuca arundinacea*) was significantly higher as a result of nitrogen fertilizer applications, and

several studies have found that alkaloid concentrations can vary widely depending on host plant characteristics and abiotic conditions (reviewed by Clay and Schardl 2002).

The present study is one of the first studies of the effects of fungal infection on herbivory conducted in the field. The only comparable field study (Brem and Leuchtmann 2001), that did find a relationship between fungal infection status and rates of herbivory, was of a grass species infected by an endophytic fungus. Endophytic fungi grow throughout a plant's tissues, whereas *A. texensis*, an epiphytic species, grows most densely at the bases of young leaves and is sparse and scattered on mature leaf blades (Leuchtmann and Clay 1988). Therefore, a **third** possible explanation for the absence of an effect of infection on herbivory is that the compounds associated with infection are present only where the fungus itself is present, below the sections of the leaf readily accessible to grasshoppers. When Clay *et al*. (1985) fed harvested leaves to the lepidopteran larvae, they may have included the infected leaf bases. If this is correct, one would expect to find that insects that eat leaf bases would be deterred by fungal infection. Note, however, that for this third explanation to be correct, *A. texensis* must not be releasing alkaloids into the vascular tissue of *Nassella*, another possible difference between *A. texensis* and related endophytic species.

Plant size and herbivore damage

Because infected plants were larger, they could, in theory, have been more apparent (Feeny 1976) to herbivores. More apparent plants could have disproportionately more herbivore damage. No such relationship between plant size and herbivore damage occurred in 2001. It is likely that larger plants were in fact no more apparent. Plants of *Nassella* were often surrounded by conspecific individuals, the whole group forming a single patch of *Nassella*. Patch size, not individual plant size, may determine the

apparency of *Nassella*, as it does for other grasses and herbaceous species (Kareiva 1985, Bach 1988); this hypothesis remains to be tested for *Nassella*. Herbivores may also be responding to other spatial patterns in this spatially complex system.

Only in the 2003 study of uninfected *Nassella* plants were levels of herbivore damage found to be significantly higher on smaller plants. It is not clear whether plant size is the cause or the effect. This was a very dry spring: cumulative rainfall in nearby Johnson City, Texas was only 25 centimeters from January 2003 to May 2003, versus a 29 year average (1971 to 2000) of 34 centimeters for these months (National Climatic Data Center 2005). Apparently *Nassella*, like virtually every plant species, was drought-stressed that spring. Plants that were physiologically stressed may have been more attractive to insect herbivores (Louda and Collinge 1992). If smaller plants were more 'stressed' than larger plants that spring, they might have been more attractive to grasshoppers. Alternatively, rather than plant size determining herbivory, herbivory may have determined plant size: perhaps the smaller plants were smaller because they had suffered more damage and, in a dry year, could not compensate for the damage.

Patch type, plant size, and herbivore damage

Savannas are characterized by small-scale environmental heterogeneity. In these central Texas savannas, soil temperatures, soil organic content, water availability, and surface light levels can vary between the patch types defined by the presence of woody canopy cover (Anderson *et al*. 2001, Wayne and Van Auken 2004). Although many of these environmental variables are related to the distribution and density of insect herbivores (Joern 1982, Bach 1984, Dudt and Shure 1994), no relationship was found between patch type and herbivore damage. This portion of the study was conducted in the very dry spring of 2003. In the same year, *Nassella* plants were on average very

small and no relationship between *Nassella* size and patch type was found, even though a previous study (Fowler and Clay 1995) had found that *Nassella* plants growing at the edge of canopies were, on average, larger than plants in open, a difference consistent with casual observation (M. Maas, pers. obs., N. Fowler, pers. obs.). Perhaps the severe drought reduced the differences among patch types. Perhaps food was in such short supply that grasshoppers foraged wherever food was available, regardless of patch types. It would therefore be premature to say that the spatial mosaic of these savannas, which affects the composition and physiognomy of the plant species, soil, and so many other ecological variables, does not also affect herbivore damage.

Spatial and temporal variation from other sources

In addition to the factors targeted for study (infection status, plant size, and patch type), the results of these studies also provide some estimates of the magnitude of temporal and spatial variation from other sources. As mentioned above, *Nassella* plants were, on average, much smaller and the levels of herbivore damage were, on average, much higher in 2003, a very dry year, than in 2001. The 2001 study also provides an estimate of month-to-month variation in cumulative herbivore damage and in plant size. Both of these varied substantially among months. Most *Nassella* growth occurred before June; it is a C_3 species and grows in the winter and spring when most other native grasses are dormant (Hicks *et al*. 1990). Most herbivore damage in 2001 occurred in June. Many central Texas grasshopper species emerge in early summer, and in normal years, their populations peak in size in June and July (Patrick and Davis 2004). During July and August 2001, plant size decreased as normal summer leaf die-back progressed and little additional herbivore damage occurred. In October, as growth resumed, cumulative herbivore damage on the new leaves was low.

There was also a great deal of within-month, plot-to-plot variation in plant size and herbivore damage in the 2001 study. This variation in plant size may reflect environmental factors that differ among trees (plots) such as light levels and understory species, while the variation in herbivore damage probably reflects the patchy distribution of grasshoppers. In 2003, though, there was less plot-to-plot variation. Plants were uniformly small and herbivore damage was uniformly high throughout the site that year. The drought may have reduced the amount of spatial variability in plant size and herbivore damage so that the plot-to-plot differences were not as evident.

Table 3.1. Results of the analysis of plant size in 2001. The denominator of the F value that tested the effect of 'Month' was 'Plot(Month)'; all other terms in the model were tested using the mean square error of the model.

Source	DF	Type I SS	Mean Square	F value	p
Month	4	42.8370	10.7092	13.07	**< 0.0001**
Plot (Month)	45	36.8612	0.8191	2.09	**0.0005**
Infection	1	10.8402	10.8402	27.72	**< 0.0001**
Month*Infection	4	0.1199	0.0300	0.08	0.9893
Residual	145	56.7024	0.3911	------	--------

Table 3.2. Results of the analysis of herbivore damage in 2001. The denominator of the F value that tested the effect of 'Month' was 'Plot(Month)'; all other terms in the model were tested using the mean square error of the model.

Source	DF	Type I SS	Mean Square	F value	p
Plant size	1	0.0246	0.0246	1.51	0.2200
Month	4	2.6022	0.6505	23.82	**< 0.0001**
Plot (Month)	45	1.2290	0.0273	1.67	**0.0119**
Infection	1	0.0070	0.0070	0.43	0.5148
Month*Infection	4	0.0581	0.0145	0.89	0.4712
Residual	144	2.3484	0.0163	------	--------

Table 3.3. Results of the analysis of plant size in 2003.

Source	DF	Type I SS	Mean Square	F value	p
Plot	18	28.4240	1.5791	2.42	**0.0117**
Patch type	2	1.0114	0.5057	0.78	0.4680
Residual	36	23.4705	0.6520	------	------

Table 3.4. The effect of plant size on herbivore damage in 2003.

Size	**Mean proportion 'bitten'**
Small (1-5 leaves)	0.95
Large (> 5 leaves)	0.83

Table 3.5. The range of mean values for the effect of 'plot' on herbivore damage in 2003.

Plots	**Mean proportion 'bitten'**
Plot with highest average value	1.00
Plot with lowest average value	0.56

Table 3.6. The effect of 'patch type' on herbivore damage in 2003.

Patch type	**Mean proportion 'bitten'**
Under	0.95
Edge	0.88
Open	0.90

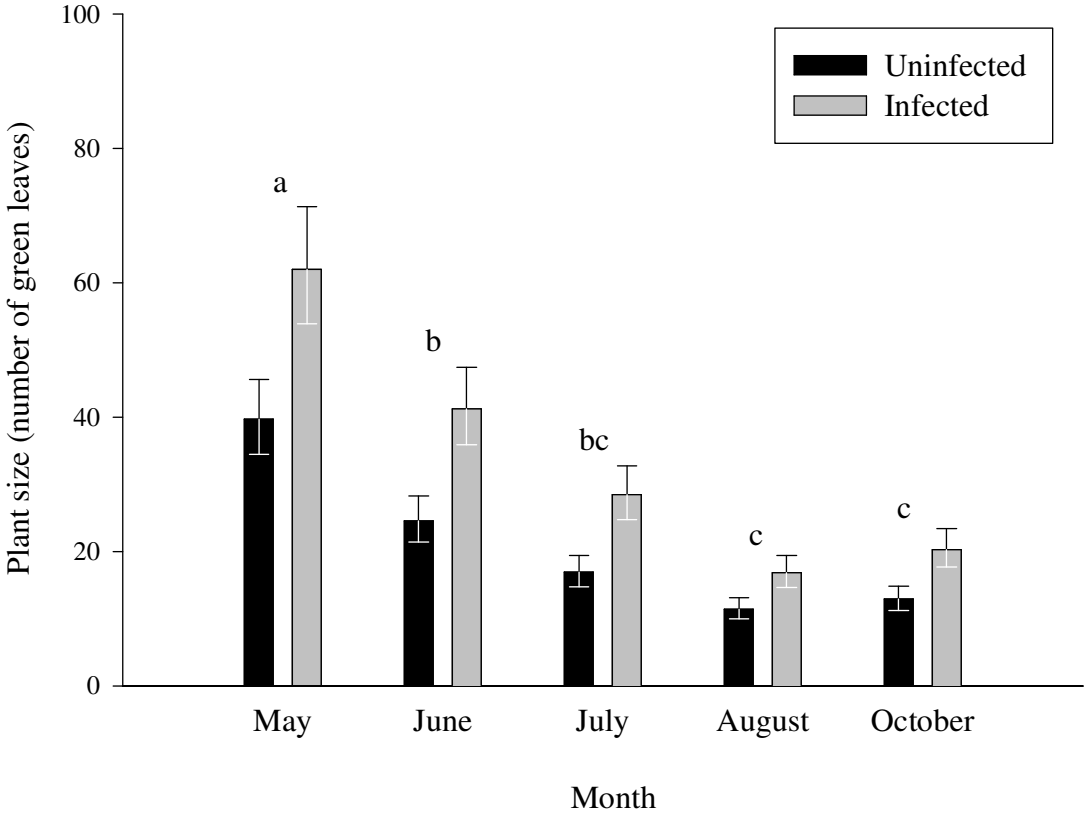

Figure 3.1. Plant size for uninfected and infected *Nassella* plants during each month of the 2001 study. Months sharing letters were not significantly different at the adjusted α-level of 0.005. Column heights are the back-transformed means of log-transformed values. Upper and lower error bars are also back-transformed (*i.e.*, upper bar: back transformation of [mean + 1 S.E. of log-transformed values]; lower bar: back transformation of [mean - 1 S.E. of log-transformed values]). Therefore the upper and lower error bars are not symmetrical.

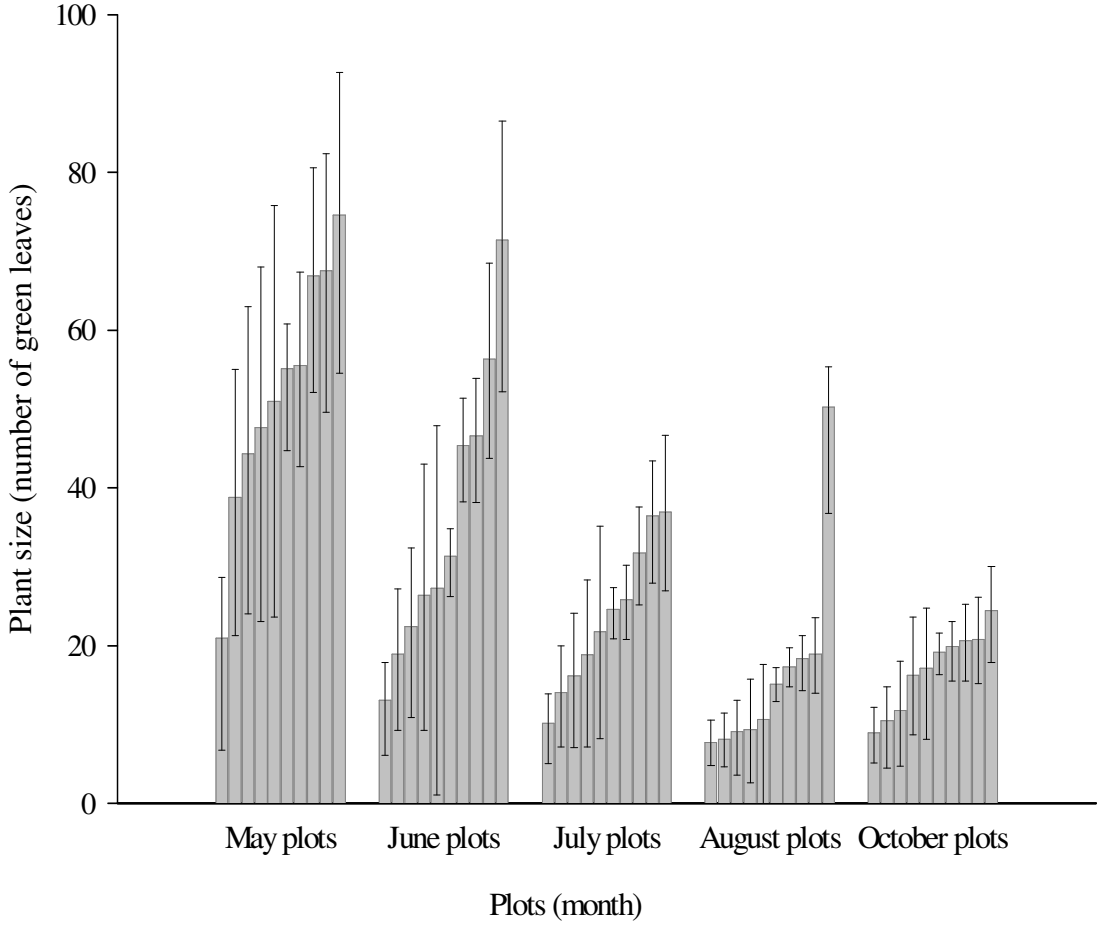

Figure 3.2. Plant size for *Nassella* plants collected from the 10 plots assigned to each month of the 2001 study (different plots were used each month). Column heights are the back-transformed means of log-transformed values. Upper and lower error bars are also back-transformed (*i.e.*, upper bar: back transformation of [mean + 1 S.E. of log-transformed values]; lower bar: back transformation of [mean - 1 S.E. of log-transformed values]). Therefore the upper and lower error bars are not symmetrical.

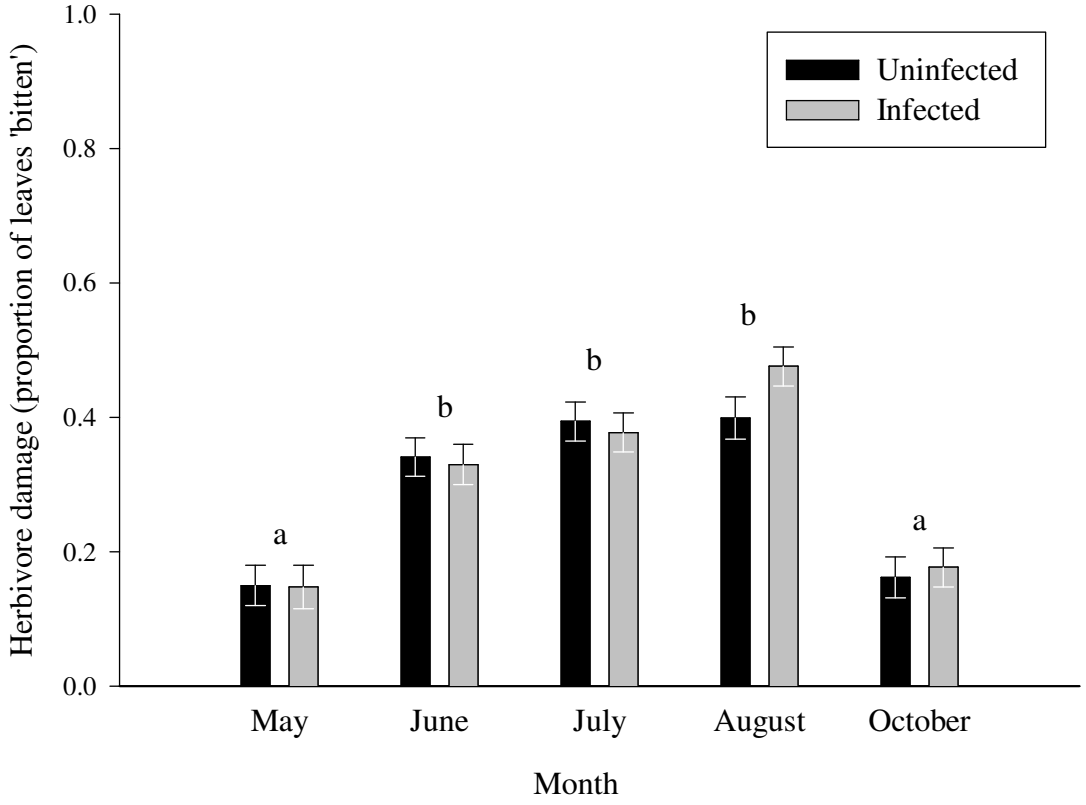

Figure 3.3. Herbivore damage for uninfected and infected *Nassella* plants during each month of the 2001 study. Months sharing letters were not significantly different at the adjusted α-level of 0.005. Column heights are the back-transformed means of log-transformed values. Upper and lower error bars are also back-transformed (*i.e.*, upper bar: back transformation of [mean + 1 S.E. of log-transformed values]; lower bar: back transformation of [mean - 1 S.E. of log-transformed values]). Therefore the upper and lower error bars are not symmetrical.

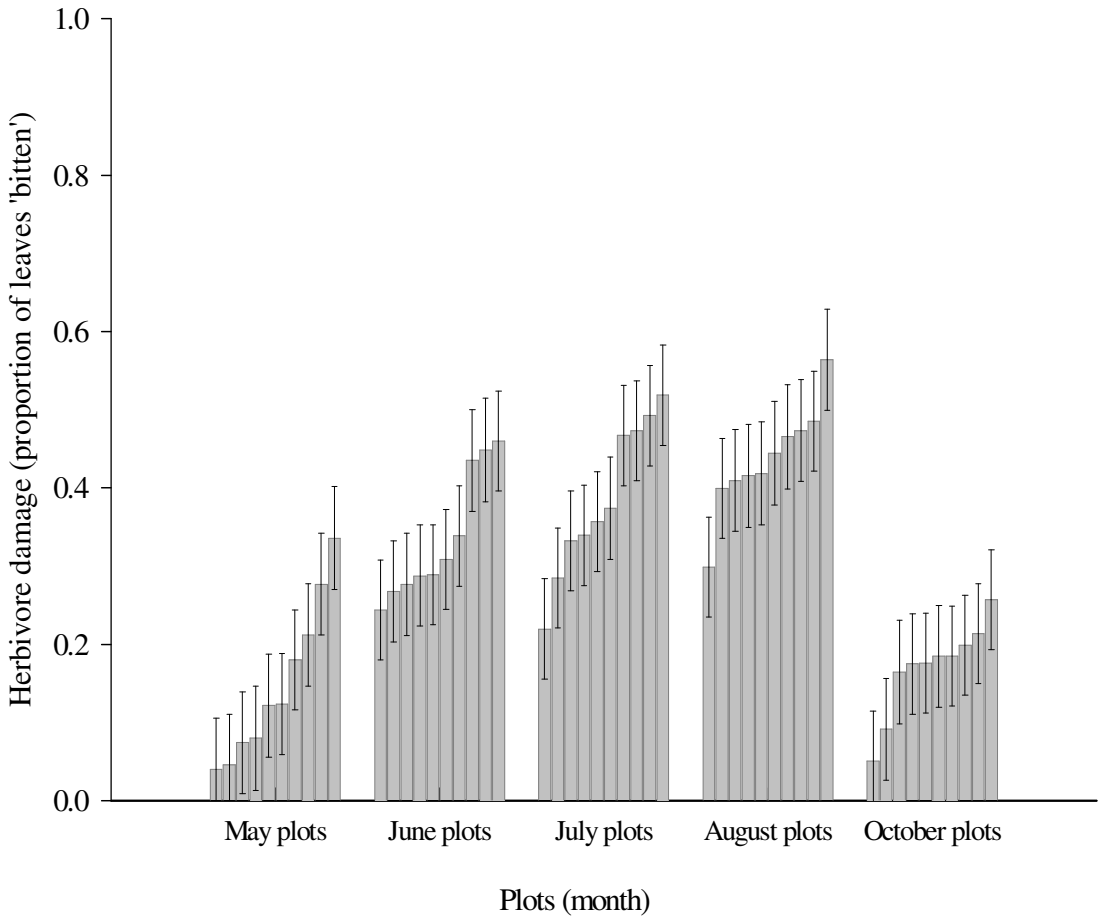

Figure 3.4. Herbivore damage for *Nassella* plants collected from the 10 plots assigned to each month of the 2001 study (different plots were used each month). Column heights are the back-transformed means of log-transformed values. Upper and lower error bars are also back-transformed (*i.e.*, upper bar: back transformation of [mean + 1 S.E. of log-transformed values]; lower bar: back transformation of [mean - 1 S.E. of log-transformed values]). Therefore the upper and lower error bars are not symmetrical.

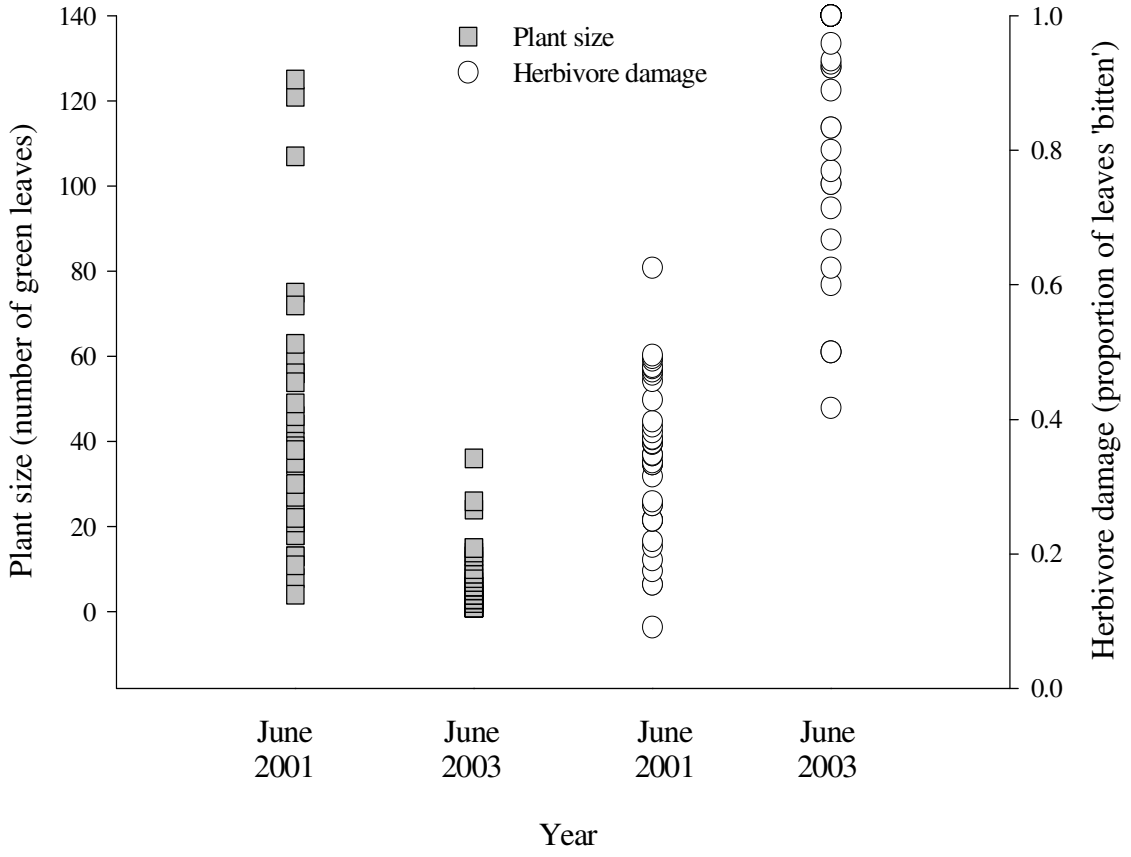

Figure 3.5. Year to year differences in plant size and herbivore damage for *Nassella*. Each box (for plant size) and each circle (for herbivore damage) represent the data collected from one plant.

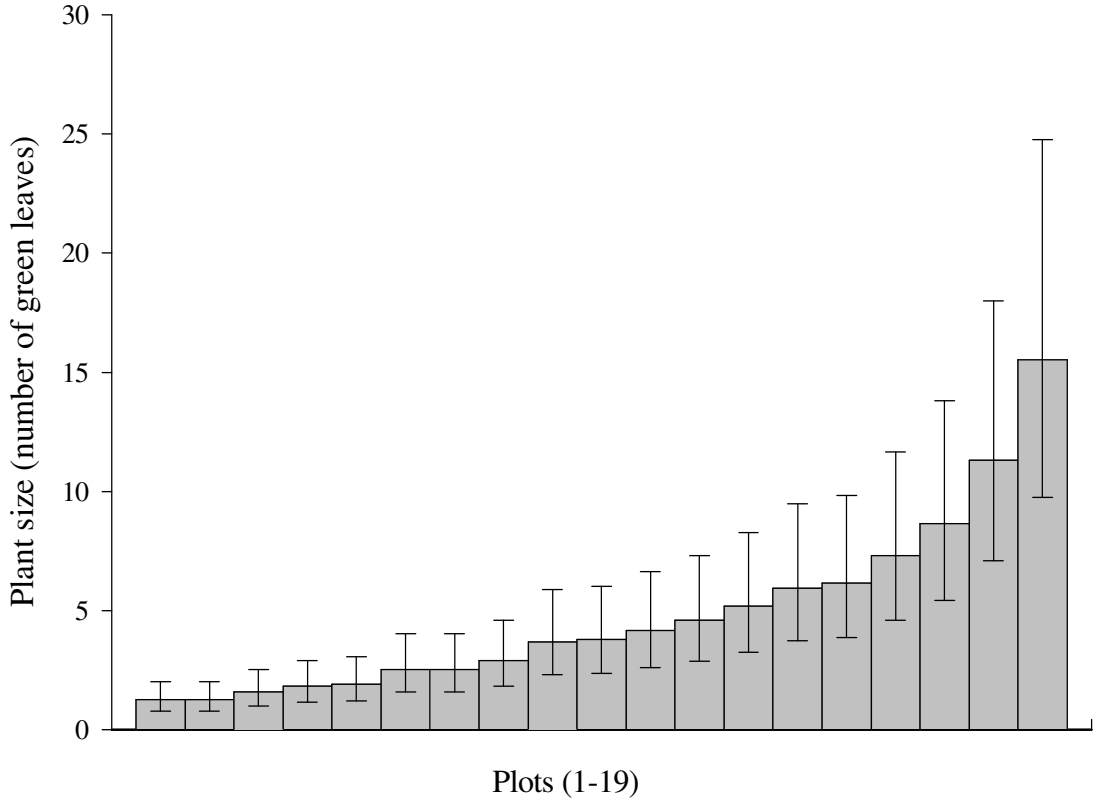

Figure 3.6. Plant size for *Nassella* plants collected from the 19 plots used in the 2003 study. Column heights are the back-transformed means of log-transformed values. Upper and lower error bars are also back-transformed (*i.e.*, upper bar: back transformation of [mean + 1 S.E. of log-transformed values]; lower bar: back transformation of [mean - 1 S.E. of log-transformed values]). Therefore the upper and lower error bars are not symmetrical.

Chapter 4: Joint effects of competition, simulated herbivory, and infection by *Atkinsonella texensis* on the growth and resource allocation of *Nassella leucotricha*

ABSTRACT

The native perennial bunchgrass *Nassella leucotricha* is frequently infected by the host-specific epiphytic fungus *Atkinsonella texensis* (Balansieae, Clavicipitaceae, Ascomycota) in central Texas. *Atkinsonella texensis* sterilizes its host; infected plants produce fungal stromata in place of inflorescences. Previous studies of related fungi and their grass hosts have suggested that infection may increase plant size. However, infection was found to have no effect on the total above-ground biomass produced by *Nassella*. Instead, infection altered resource allocation: infected pairs allocated less to fungal reproduction (plant culms + fungal stromata) than uninfected plants did to plant reproduction (plant culms + plant inflorescences and seeds): ~25% to fungal reproduction versus ~50% to plant reproduction. As a result, infected plants had more vegetative biomass than uninfected plants.

Previous studies of related fungi and their grass hosts have also suggested that infected plants may be less tolerant of defoliation, but less affected by competition. However, the effect of simulated herbivory (clipping to mimic grasshopper grazing) was independent of the effects of infection and competition on *Nassella*, and the effects of competition were independent of the effects of infection and herbivory. Because a previous study found that infection also does not reduce the amount of herbivore damage in the field, infection therefore appears to have no beneficial effects on *Nassella*. Therefore, *A. texensis* is a complete parasite, unlike many of its close relatives. The relationship between *Nassella* and *A. texensis* may therefore represent the earliest stage in

the evolution of the mutualisms that now exist between similar fungi and their plant hosts.

INTRODUCTION

Unlike most plant-fungus relationships, infections of plants by fungi of the tribe Balansieae (Clavicipitaceae, Ascomycota) are often thought to be mutualistic. At least 80 genera of plants host fungi of this tribe; they include C_3 and C_4 grasses, sedges, and rushes (Clay 1988). Most of these fungi are endophytic, with intercellular hyphae, and many of them are transmitted vertically, from mother to seed (Clay 1988). Previous studies have found that, or found evidence suggesting that, infection increases plant size (Clay *et al*. 1989, Clay 1990, Rice *et al*. 1990) and plant competitive ability (Marks *et al*. 1991, Clay *et al*. 1993, Brem and Leuchtmann 2002), deters insect herbivores (Clay *et al*. 1985, Cheplick and Clay 1988, Bultman *et al*. 2004), and increases plant tolerance of drought (Arachevaleta *et al*. 1989), heat (Marks and Clay 1996), low soil fertility (Malinowski and Belesky 2000), and other environmental stresses (reviewed by Clay and Schardl 2002). Most of these studies have involved pasture grass cultivars such as tall fescue (*Festuca arundinacea*) and perennial ryegrass (*Lolium perenne*).

Some species in the tribe Balansieae are epiphytic rather than endophytic, that is, their hyphae grow on the leaf and stem surfaces (Clay and Schardl 2002). In central Texas, the perennial bunchgrass *Nassella (Stipa) leucotricha* is infected by an epiphytic member of this tribe, *Atkinsonella texensis*. Like some, but not all, of the other epiphytic and endophytic species in this tribe, *A. texensis* always sterilizes its host in the field (M. Maas chapter 2), although this may not be true in greenhouse-grown plants (Fowler and Clay 1995). Infected plants produce fungal stromata on what would have been their chasmogamous flowering culms, and no cleistogamous seeds. Previous studies of

Nassella have found that plants infected by *A. texensis* suffer as much damage from grasshoppers as uninfected plants do (M. Maas chapter 3); that infected plants tend to occupy a narrower range of micro-habitats (M. Maas chapter 2); that greenhouse-grown infected plants reduce lepidopteran larvae growth rates more than uninfected plants do (Clay *et al*. 1985); and that infection may increase vegetative size and tiller number (Fowler and Clay 1995). Studies of other epiphytic species have found that infection increases host plant growth rates and survival, reduces the growth of insect herbivores, and promotes host plant competitive ability (Clay 1984, Clay *et al*. 1985, Cheplick and Clay 1988).

In this study I measured the joint effects of infection by *A. texensis*, simulated grasshopper herbivory, and competition from a co-occurring grass on *Nassella* size and resource allocation. The latter was included to distinguish between an increase in total size and a shift in resource allocation from reproductive to vegetative biomass. Simulated herbivory was included to test the hypothesis that infection makes plants less able to replace tissue removed by herbivores, and therefore less tolerant of herbivory, than uninfected plants. This is one of a few studies that examines the effect of epiphytic infection on host competitive response and tolerance of herbivory.

METHODS

Study organisms

Nassella leucotricha (henceforth referred to as *Nassella*) is a perennial bunchgrass common in savannas of the Edwards Plateau of central Texas (Fowler and Dunlap 1986), although its range extends from Oklahoma to northeastern Mexico (Hicks *et al*. 1990, White and Van Auken 1996). *Nassella* is amphicarpic, producing both cleistogamous

and chasmogamous flowers and seeds (Call and Spoonts 1989). Cleistogamous flowers are self-fertilized and are located at the base of culms. Chasmogamous flowers are potentially outcrossed and are located on aerial panicles.

Chasmogamous flowers produced by *Nassella* are susceptible to infection by *Atkinsonella texensis*, a fungal species in the tribe Balansieae (Clavicipitaceae, Ascomycota) (Leuchtmann and Clay 1988). *Atkinsonella texensis* is epiphytic, first occurring in the upper leaf bases and then enveloping the culms and immature chasmogamous inflorescences produced by *Nassella* (Morgan-Jones and White 1989). Eventually a stroma (a fungal fruiting body) that resembles a bird dropping forms in place of the chasmogamous inflorescence. *Atkinsonella* therefore sterilizes *Nassella* by causing the abortion of chasmogamous inflorescences. Cleistogamous seeds are not produced by infected plants either (M. Maas pers. obs.). The mechanism of horizontal transmission (contagious spread) between host plants is not known. Insects may play a role in transferring infection, but spores could be wind-transmitted (Morgan-Jones and White 1989).

I selected *Bothriochloa ischaemum*, a perennial Eurasian bunchgrass, as the competitor species because it is common in the same central Texas savannas where *Nassella* is found and it is easy to grow. A pilot study conducted in 2000 found that *Nassella* plants grown with two or more *B. ischaemum* individuals had a high mortality rate. Therefore in this study *Nassella* plants assigned to the 'neighbor' competitive treatment were grown with just one *B. ischaemum* individual in their pot.

Experimental design

To test the effects of infection, competition and herbivore damage on *Nassella* growth and reproduction, I conducted a factorial experiment with two levels of infection

(uninfected or infected), two levels of competition (alone or with one neighbor), and two levels of simulated herbivory (unclipped or clipped), for a total of eight treatment combinations. Each treatment combination was replicated 20 times for a total of 160 target plants.

To generate the 160 target plants needed for this experiment, I transplanted approximately 60 uninfected and 60 infected *Nassella* plants from Pedernales Falls State Park into a greenhouse in the summer of 2001. In February 2002, these plants were divided by hand into smaller individuals, planted in 20 oz. containers, and allowed to recover from transplant shock. Eighty uninfected and 80 infected target plants of similar size (5-10 tillers) were then randomly selected and watered and fertilized as needed. Some of these 160 plants were genetic clones; plants were randomly assigned to treatment combinations and blocks without regard for genetic identity. It was not possible to obtain enough *Nassella* plants to avoid the use of clones entirely.

To generate the 80 'neighbor' plants needed for this experiment, I planted *B. ischaemum* seeds (collected from Pedernales Falls State Park) in early December 2001. These seeds germinated in late December 2001, and 80 of these seedlings were randomly selected and planted in 20 oz. containers in the greenhouse. These plants were also watered and fertilized as needed.

In April 2002, all plants were transplanted into one gallon pots containing either one *Nassella* and one *B. ischaemum* plant (neighbor present level of the competition treatment) or only one *Nassella* plant (neighbor absent level of the competition treatment). A full-factorial design was used, with four factors: 2 levels of infection, 2 levels of competition, 2 levels of simulated herbivory, and 20 blocks. Each block contained one pot of each treatment combination, so there was no replication of treatment

combination within a block. Plants and pots were randomly assigned to the competition and the herbivory treatments and to blocks.

A field study conducted in 2001 found that approximately 50% of *Nassella* leaves had grasshopper 'bites' (M. Maas chapter 3). In June 2002 I clipped the experimental plants to simulate this level of herbivory. Each plant assigned to the 'clipped' level of the simulated herbivory treatment had the upper half of half of its leaves (*i.e.*, 1/4 of its leaf area) removed with scissors.

I harvested target *Nassella* plants in August 2002 by clipping them at the base. Plants were then dried, and then their above-ground vegetative structures and reproductive structures were weighed separately. For both uninfected and infected plants, above-ground vegetative biomass was equal to the combined weights of their brown and green tillers. However, because fungal infection prevented seed production, reproductive biomass was determined differently for uninfected and infected plants.

I was unable to collect all seeds produced by each uninfected plant. I therefore used a two-step linear regression to estimate the weight of seeds produced by each uninfected plant. Total culm weight of each plant was used to estimate total seed number of each plant, which was then used to estimate total seed weight of each plant. Reproductive biomass of each plant was then calculated by summing the weights of its inflorescence-bearing culms and its estimated total seed weight. For each infected plant, reproductive biomass was calculated by summing the weights of its stroma-bearing plant culms and its stromata. Therefore, for infected plants, the response 'reproductive biomass' was a measure of resources (plant and fungal) devoted to fungal reproduction.

Above-ground total biomass of each plant was then calculated for each plant by summing its above-ground vegetative and reproductive biomass. Therefore, if a plant was infected, its total biomass was the sum of all above-ground fungal and plant biomass.

Below-ground biomass was not collected during this experiment. To explore how uninfected and infected plants allocated resources, the response variable 'resource allocation' was calculated for each plant by dividing its reproductive biomass by its total biomass.

Analyses of vegetative biomass

The response variable 'vegetative biomass' was analyzed using a mixed-model ANOVA (PROC GLM, SAS 1990). Infection, competition and simulated herbivory were treated as fixed effects in the model. 'Block' and the two-way and three-way interactions between 'block' and other terms in the model were treated as random effects. Because treatment combinations within each block were not replicated, the four-way interaction between 'block' and the other three main effects became the error term. The three-way interactions between 'block' and the other main effects were not significant and were dropped from the model.

In the final model (the model without the three-way interactions involving 'block'), the F-values of 'block', of all two-way interactions, and of the remaining three-way interaction had the mean square error of the model as their denominators. Because block was considered to be a random effect, the F-values of the three treatment main effects had the corresponding block X treatment mean square as their denominator.

The significant two-way interactions between infection and competition and between infection and simulated herbivory were explored using Fisher's least significant differences (LSD) to compare the means of different treatment combinations. The significant three-way interaction between infection, competition and simulated herbivory was explored in the same way. Multiple testing was taken into account by using Bonferroni's correction (Sokal and Rohlf 1995). Therefore, the analyses of the two-way

interactions were done with an α-level of 0.025, to yield an overall α-level of 0.05. The analysis of the three-way interaction was done with an α-level of 0.004, to yield an overall α-level of 0.05.

Analyses of reproductive biomass, total biomass, and resource allocation

The three response variables 'total biomass', 'reproductive biomass', and 'resource allocation' were analyzed separately using the same mixed-model ANOVA as before (PROC GLM, SAS 1990). Infection, competition, and simulated herbivory were again treated as fixed effects. 'Block' and the two-way and three-way interactions between 'block' and other terms in the model were again treated as random effects. As before, the four-way interaction between 'block' and the other main effects in the model became the error term.

All three-way interactions were not significant and were dropped from the models. For each analysis, the F-values of 'block' and of all two-way interactions had the mean square error of the model as their denominators. Because block was considered to be a random effect, the F-values of the treatment main effects had the corresponding block X treatment mean square as their denominator.

For the response variable 'reproductive biomass', the significant two-way interaction between infection and competition was explored using Fisher's least significant differences (LSD) to compare the means of different treatment combinations. Multiple testing was taken into account by using Bonferroni's correction (Sokal and Rohlf 1995). Therefore, the analysis of the two-way interaction was done with an α-level of 0.025, to yield an overall α-level of 0.05.

RESULTS

Analyses of vegetative biomass

There was a marginally significant three-way interaction between infection, simulated herbivory, and competition (Table 4.1), because clipping had a significant effect only upon infected plants grown alone; infected plants grown alone that were clipped produced less above-ground vegetative biomass than expected. There was also a consistent effect of the interaction between infection and competition (Table 4.1, Figure 4.1). Uninfected plants grown with a neighbor were, on average, approximately 4g smaller than uninfected plants grown alone, while infected plants were, on average, 6g smaller when grown with a neighbor. That is, competition had a significantly stronger effect on infected plants than on uninfected plants. This analysis, though, examined the effect of competition by measuring the absolute decrease in average plant size due to competition. If the effect of competition had been measured by the relative decrease in average plant size, there would have been no difference between uninfected and infected plants; plants grown with neighbors, regardless of infection status, were, on average, 50% smaller than plants grown alone.

Infection and competition both had significant main effects on mean above-ground vegetative biomass (Table 4.1, Figure 4.1). Infected *Nassella* plants were, on average, 55% larger than uninfected plants. Plants grown with neighbors were, on average, 50% smaller than plants grown alone. Simulated herbivory, though, had no significant main effect on mean vegetative biomass (Table 4.1).

Analyses of reproductive biomass

There was a significant interaction between the effects of infection and competition (Table 4.2, Figure 4.2). Competition reduced the average amount of reproductive biomass produced by uninfected *Nassella* plants by approximately 7 grams. Competition reduced the average amount of reproductive biomass produced by infected *Nassella* plants by approximately 5 grams. Therefore, competition had a stronger absolute effect on uninfected plants than on infected plants. If the effect of competition on reproductive biomass had been measured by the relative decrease in biomass, there would have been no difference between uninfected and infected plants; plants grown with neighbors, regardless of infection status, reduced their average production of reproductive biomass by approximately 70 percent.

Infection and competition both had a significant main effect on reproductive biomass (Table 4.2, Figure 4.2). Infection reduced the average amount of reproductive biomass produced by *Nassella* by approximately 4 grams (a 55% relative decrease). Competition reduced the average amount of reproductive biomass produced by *Nassella* by approximately 6 grams (a 70% relative decrease). Simulated herbivory did not affect the average amount of reproductive biomass produced by uninfected and infected plants (Table 4.2).

Block also had a significant main effect on reproductive biomass (Table 4.2). In other words, unknown and uncontrolled variation within the greenhouse affected the reproduction of *Nassella*. However, the effect of this variation on reproductive biomass did not differ between uninfected and infected plants or between plants grown alone and with a neighbor or between plants that were clipped and unclipped.

Analyses of total biomass

The response variable 'total biomass' was measured for each plant by summing its above-ground vegetative and reproductive biomass. Infection status did not have a significant main effect on total above-ground biomass (Table 4.3, Figure 4.3). Both uninfected and infected plants produced, on average, approximately 13g of vegetative and reproductive tissue. However, both competition and simulated herbivory did have a significant main effect on total above-ground biomass. Competition reduced the average amount of total above-ground biomass produced by *Nassella* by approximately 11 grams (a 60% relative decrease) (Figure 4.3). Simulated herbivory reduced the average amount of total above-ground biomass produced by *Nassella* by approximately 1 gram (an 8% relative decrease) (Figure 4.4). The effect of competition and simulated herbivory on total above-ground biomass was not affected by fungal infection. Block had a marginally significant main effect on total above-ground biomass, and the effect of competition and simulated herbivory on total above-ground biomass was significantly different from block to block (Table 4.3).

Analyses of resource allocation

The response variable 'resource allocation' was calculated by dividing each plant's reproductive biomass by its total above-ground biomass. Both infection and competition significantly affected the proportion of resources that plants allocated to reproduction (Table 4.4, Figure 4.5). Infected plants devoted, on average, approximately 20% of their total above-ground biomass to reproduction (stroma-bearing culms and culms) while uninfected plants devoted, on average, approximately 50 percent. Plants

grown with competitors allocated, on average, approximately 30% of their total above-ground biomass to reproduction, while plants grown alone devoted 45 percent.

Competition did not have a significantly stronger effect on infected plants than on uninfected plants; both infected and uninfected plants decreased the mean proportion of resources allocated to reproduction by approximately 15 percent (Table 4.4, Figure 4.5). Simulated herbivory did not significantly affect resource allocation (Table 4.4). Block had a marginally significant effect on resource allocation (Table 4.4). The effect of competition and herbivory on resource allocation also did not differ among blocks.

DISCUSSION

Infection and resource allocation

Nassella leucotricha plants infected by the epiphytic fungus *Atkinsonella texensis* produced significantly more plant *vegetative* above-ground biomass, a result consistent with the (non-significant) effect of infection on plant size reported by Fowler and Clay (1995) and the significant effect of infection on *Nassella* leaf number I measured in a field experiment (M. Maas chapter 3). Studies of related endophytic and epiphytic fungi have also found that infection increases host plant size in the absence of herbivores, pathogens, and other environmental stresses, assuming that fungal non-reproductive biomass is negligible (Clay 1984, Clay 1990, Rice *et al*. 1990). For example, Clay (1984) found that *Danthonia spicata* plants infected by *A. hypoxylon* produced more tillers than uninfected plants. However, most of these studies have not measured fungal or plant reproductive biomass.

All infected *Nassella* plants were sterilized by the infection, producing *fungal* reproductive structures (stroma-bearing culms and stromata) instead of *plant* reproductive

structures (seed-bearing culms and seeds). The average biomass of fungal reproductive structures produced by infected plants was significantly less than the average biomass of plant reproductive structures produced by uninfected plants. Thus, on average, an infected plant allocated fewer resources to fungal reproduction than an uninfected plant did to plant reproduction. However, uninfected and infected plants produced, on average, the same amount of total above-ground biomass (including fungal biomass). Therefore, infected plants were not larger overall than uninfected plants; they merely allocated a greater proportion of their total biomass to vegetative biomass. In other words, fungal infection shifted the allocation of resources; instead of producing 'costly' seeds, infected *Nassella* plants produced 'cheap' fungal stromata and diverted 'extra' resources to vegetative growth.

The fungal partner sterilizes its plant host in many of the symbioses involving related fungi. Shifts in resource allocation similar to those observed in this study could explain why infected plants are larger than uninfected plants in many of these symbioses. This hypothesis has not been tested for symbioses other than *Nassella – A. texensis*. However, infection by the related endophytic fungus *Epichloë glyceriae* caused the host grass *Glyceria striata* to produce more stolons and fewer tillers than uninfected plants without altering total biomass, a different type of shift in resource allocation (Pan and Clay 2002).

These sterilizing fungi are sometimes considered 'beneficial' and not a drain on host plant resources (Clay 1984, Kelley and Clay 1987, Pan and Clay 2002). However, infected *Nassella* plants allocated about 25% of their total above-ground biomass to fungal reproduction. The cost of seed production was larger (~50% of an uninfected plant's above-ground biomass), but this 'cost' increased the host plant's fitness.

Studies of related endophytic fungi have found that infection may increase host plant size by reducing the incidence of other fungal diseases (Gwinn and Gavin 1992), by increasing host tolerance of environmental stresses such as drought (Arachevaleta *et al*. 1989, Bacon 1993, Marks and Clay 1996, Cheplick *et al*. 2000, Malinowski and Belesky 2000), and by deterring insect herbivores (Clay *et al*. 1985, Cheplick and Clay 1988, Brem and Leuchtmann 2001, Bultman *et al*. 2004). In this study, though, the increase in plant size was entirely due to a shift in the allocation of resources from plant reproduction to vegetative growth. This shift occurred in spite of the fact that infected plants produced more (stroma-bearing) culms than uninfected plants produced (seed-bearing) culms. The increase in the number of culms could have been caused by a plant growth hormone released by the fungus, as a closely related fungus (*Balansia epichloë*) was found to produce auxin in vitro (Porter *et al*. 1985).

Infection and herbivory tolerance

Infection by *A. texensis* does not appear to deter grasshopper herbivores (M. Maas chapter 3), and this study suggests that it does not increase the host's tolerance of herbivory either. The effects of clipping on total above-ground biomass were independent of the effects of infection and competition. This result is not consistent with studies of related endophytes, which have found that infected plants do not replace tissue as quickly as uninfected plants (Belesky and Fedders 1996, Cheplick 1998, Bultman *et al*. 2004). Bultman *et al*. (2004) found that infected tall fescue (*Festuca arundinacea*) damaged by herbivores had elevated levels of fungal alkaloid (induced resistance), but lower tolerance of herbivory, than uninfected plants damaged by herbivores. The lack of evidence for a similar effect of infection by *A. texensis* on *Nassella* may indicate that the relationship between *A. texensis* and *Nassella* is less tightly co-evolved.

Infection and competitive response

Competitive response, defined as the change in the performance of a target plant as neighboring plants alter resource abundance (Goldberg 1990), was measured in this study. The *absolute* competitive response of a target plant was measured as (size alone) – (size grown with competitor[s]). The *relative* competitive response of a target plant was measured as (size alone – size with competitor)/(size alone). The relative response is particularly useful when comparing different target species (Goldberg and Scheiner 1993), or, as here, plants of different morphologies.

Competition had the same effect on total biomass of uninfected and infected plants: both uninfected and infected plants were about 11g and about 50% smaller when they were grown with a competitor. Because uninfected and infected plants were about the same size, their absolute and relative responses to competition did not differ. Infection altered resource allocation, as discussed above, but this was independent of competition: competition did not affect resource allocation at all.

Because infected and uninfected plants did differ in vegetative and in reproductive biomass, their absolute responses to competition differed from their relative responses. The absolute effect of competition on vegetative biomass was greater in infected plants, but this was only because infected plants produced more vegetative biomass than uninfected plants. Similarly, the absolute effect of competition on reproductive biomass was greater in uninfected plants because they produced more reproductive biomass. However, both uninfected and infected plants had the same relative responses to competition; both had about 50% less vegetative biomass and 70% less reproductive biomass when grown with a competitor.

Competition and infection therefore operated independently on *Nassella*; infection did not improve host plant competitive response. This result is not consistent with those

of studies of cultivars of the forage grass species *Festuca arundinacea*, *F. rubra*, and *Lolium perenne* (Marks *et al*. 1991, Clay *et al*. 1993). However, a study of two species of wild endophyte-infected grasses (*Brachypodium sylvaticum* and *B. benekenii*) found that infected plants were sometimes better and sometimes worse competitors than uninfected plants, depending on host plant age (Brem and Leuchtmann 2002). In that study, the fungal species did not always sterilize its host, and therefore when it had a positive effect upon the competitive ability of its host the symbiosis was a mutualism. In contrast, *A. texensis* did not affect the competitive ability of its host but always sterilized it (and did not reduce levels of grasshopper herbivory, M. Maas chapter 3), making this symbiosis a parasitism.

Parasitism versus mutualism

Some of the other fungi in the tribe Balansieae form true mutualisms with their plant hosts (*e.g*., *Neotyphodium lolii*/*Lolium perenne*, and *N. coenophialum*/*F. arundinacea*) (Clay and Schardl 2002). The symbiosis between *Nassella* and *A. texensis* may represent the earliest stage in the evolution of the relationships between Balansieae species and their plant hosts. *Atkinsonella texensis* not only reduces plant fitness to zero by sterilizing its host, but provides no other benefits to its host. *Atkinsonella texensis* does not even help protect its host from herbivory, although that would increase the fitness even of a sterilizing fungus. *Atkinsonella texensis* is still a true parasite in all respects. Therefore, the symbiosis between *Nassella* and *A. texensis* may resemble the relationship that once existed between present mutualists like *N. coenophialum* and *F. arundinacea*.

Table 4.1. Results of the analyses of above-ground vegetative biomass. The denominator of the F value that tested the effect of 'Infection' was 'Block*Infection'. The denominator of the F value that tested the effect of 'Competition' was 'Block*Competition'. The denominator of the F value that tested the effect of 'Herbivory' was 'Block*Herbivory'. All other terms in the model were tested using the mean square error of the model.

Source	**df**	**Type III SS**	**Mean Square**	**F value**	**P**
Block	19	96.8391	5.0968	1.13	0.3421
Infection	1	385.7031	385.7031	106.60	**<0.0001**
Competition	1	1080.6642	1080.6642	306.29	**<0.0001**
Herbivory	1	20.8947	20.8947	3.28	0.0859
Block*Infection	19	68.7465	3.6182	0.80	0.6992
Block*Competition	19	67.0360	3.5282	0.78	0.7217
Block*Herbivory	19	120.9841	6.3676	1.41	0.1482
Infection*Comp	1	35.5134	35.5134	7.86	**0.0064**
Infection*Herbivory	1	29.1897	29.1897	6.46	**0.0131**
Competition*Herb	1	8.4181	8.4181	1.86	0.1763
Inf*Comp*Herb	1	17.5695	17.5695	3.89	0.0523
Residual	76	343.3882	4.5183	-----	--------

Table 4.2. Results of the analyses of reproductive biomass. The denominator of the F value that tested the effect of 'Infection' was 'Block*Infection'. The denominator of the F value that tested the effect of 'Competition' was 'Block*Competition'. The denominator of the F value that tested the effect of 'Herbivory' was 'Block*Herbivory'. All other terms in the model were tested using the mean square error of the model.

Source	**df**	**Type III SS**	**Mean Square**	**F value**	**p**
Block	19	424.6486	424.6486	2.57	**0.0019**
Infection	1	321.3801	321.3801	44.13	**<0.0001**
Competition	1	1563.6568	1563.6568	131.15	**<0.0001**
Herbivory	1	4.4165	4.4165	0.38	0.5450
Block*Infection	19	138.3701	7.2826	0.84	0.6571
Block*Competition	19	226.5333	11.9228	1.37	0.1671
Block*Herbivory	19	220.9341	11.6281	1.34	0.1859
Infection*Comp	1	43.1445	43.1445	4.96	**0.0288**
Infection*Herbivory	1	14.3057	14.3057	1.65	0.2035
Competition*Herb	1	15.2113	15.2113	1.75	0.1899
Residual	77	669.6226	8.6964	-----	--------

Table 4.3. Results of the analyses of above-ground total biomass. The denominator of the F value that tested the effect of 'Infection' was 'Block*Infection'. The denominator of the F value that tested the effect of 'Competition' was 'Block*Competition'. The denominator of the F value that tested the effect of 'Herbivory' was 'Block*Herbivory'. All other terms in the model were tested using the mean square error of the model.

Source	**df**	**Type III SS**	**Mean Square**	**F value**	**p**
Block	19	421.6671	22.1930	3.98	**<0.0001**
Infection	1	2.9318	2.9318	0.39	0.5419
Competition	1	5244.1579	5244.1579	323.60	**<0.0001**
Herbivory	1	44.5238	44.5238	4.87	**0.0399**
Block*Infection	19	144.3573	7.5978	1.36	0.1717
Block*Competition	19	307.9108	16.2058	2.91	**0.0005**
Block*Herbivory	19	173.7484	9.1447	1.64	0.0674
Infection*Comp	1	0.3711	0.3711	0.07	0.7971
Infection*Herbivory	1	2.6259	2.6259	0.47	0.4946
Competition*Herb	1	0.997559	0.997559	0.18	0.6735
Residual	77	429.3849	5.5764	-----	--------

Table 4.4. Results of the analyses of resource allocation. The denominator of the F value that tested the effect of 'Infection' was 'Block*Infection'. The denominator of the F value that tested the effect of 'Competition' was 'Block*Competition'. The denominator of the F value that tested the effect of 'Herbivory' was 'Block*Herbivory'. All other terms in the model were tested using the mean square error of the model.

Source	**df**	**Type III SS**	**Mean Square**	**F value**	**p**
Block	19	0.9622	0.0506	1.64	0.0666
Infection	1	1.8037	1.8037	92.41	**<0.0001**
Competition	1	0.8207	0.8207	36.05	**<0.0001**
Herbivory	1	0.0059	0.0059	0.22	0.6478
Block*Infection	19	0.3709	0.0195	0.63	0.8690
Block*Competition	19	0.4325	0.0228	0.74	0.7680
Block*Herbivory	19	0.5179	0.0273	0.88	0.6023
Infection*Comp	1	0.0219	0.0219	0.71	0.4016
Infection*Herbivory	1	0.0675	0.0675	2.19	0.1431
Competition*Herb	1	0.0547	0.0547	1.77	0.1867
Residual	77	2.3731	0.0308	-----	--------

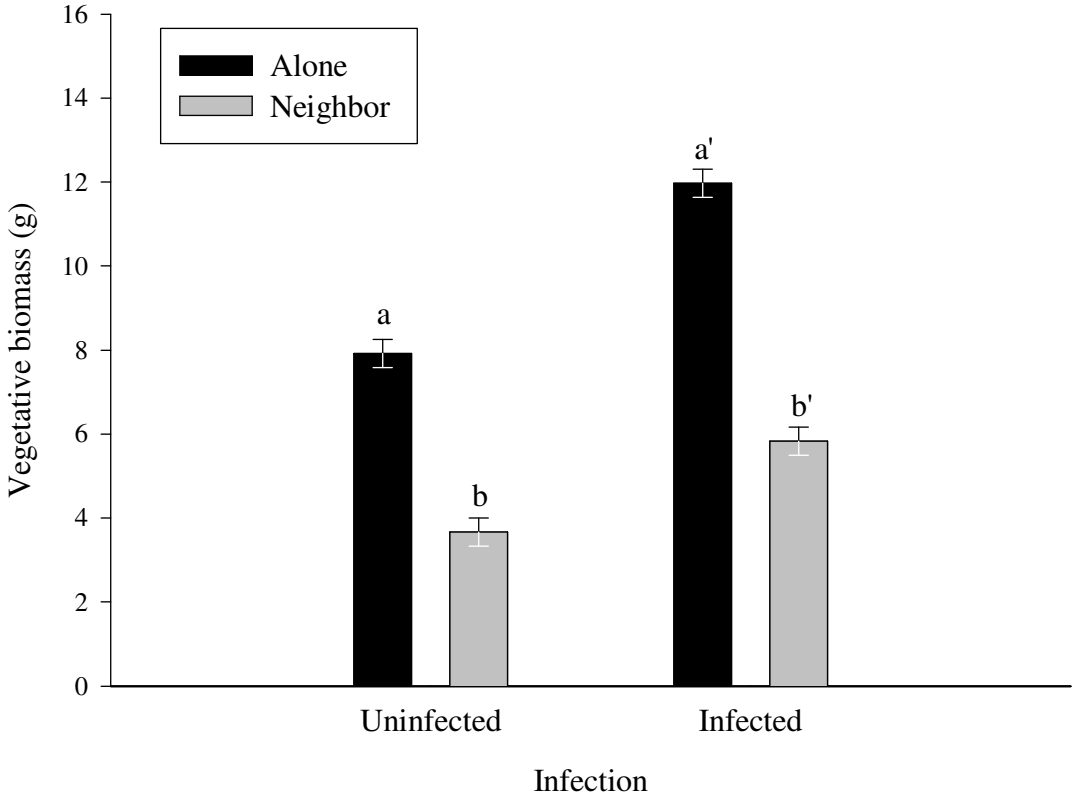

Figure 4.1. Effects of infection and competition on mean above-ground vegetative biomass. Upper and lower error bars are ± 1 standard error. Pairwise comparisons were made to test for significant differences among different treatment combinations. Comparisons were only made between plants with the same infection status (i.e., comparisons were not made between uninfected and infected plants). Plants sharing letters are not significantly different at the α-level of 0.025.

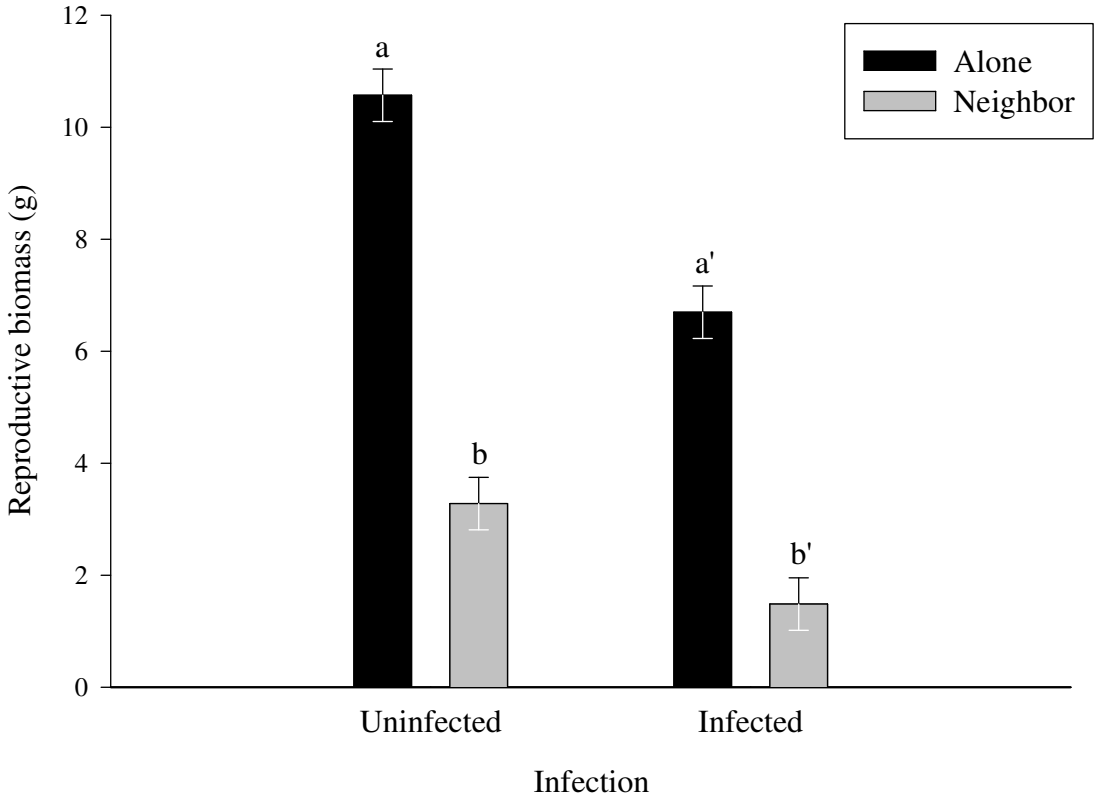

Figure 4.2. Effects of infection and competition on mean reproductive biomass. Upper and lower error bars are ± 1 standard error. Pairwise comparisons were made to test for significant differences among different treatment combinations. Comparisons were only made between plants with the same infection status (i.e., comparisons were not made between uninfected and infected plants). Plants sharing letters are not significantly different at the α-level of 0.025.

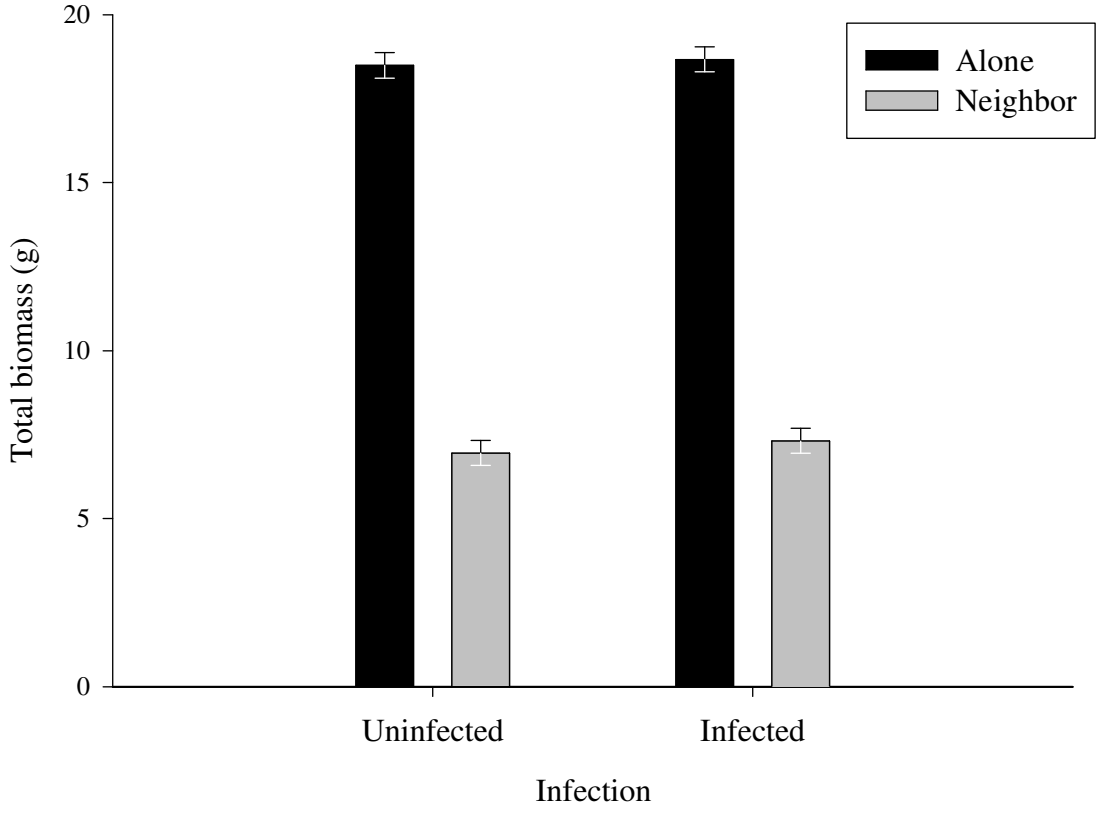

Figure 4.3. Effects of infection and competition on mean above-ground total biomass. Because the effect of competition on total biomass did not differ between uninfected and infected plants, no pairwise comparisons were made. Upper and lower error bars are ± 1 standard error.

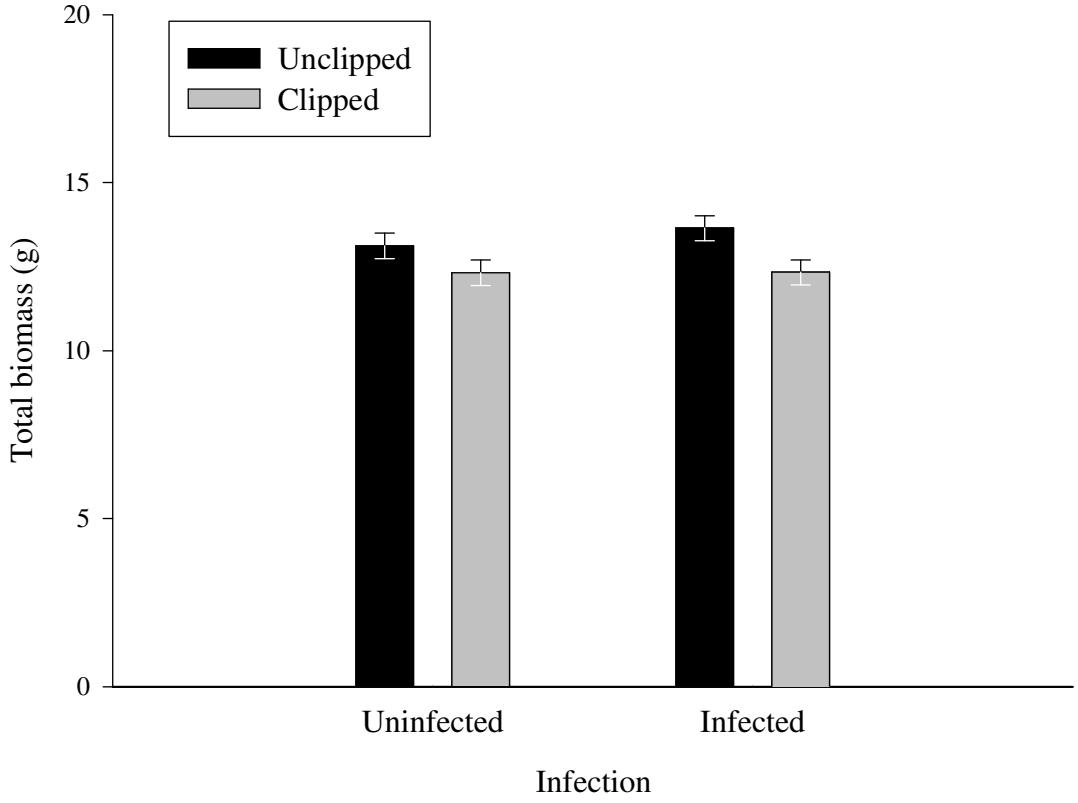

Figure 4.4. Effects of infection and simulated herbivory on mean above-ground total biomass. Because the effect of simulated herbivory on total biomass did not differ between uninfected and infected plants, no pairwise comparisons were made. Upper and lower error bars are ± 1 standard error.

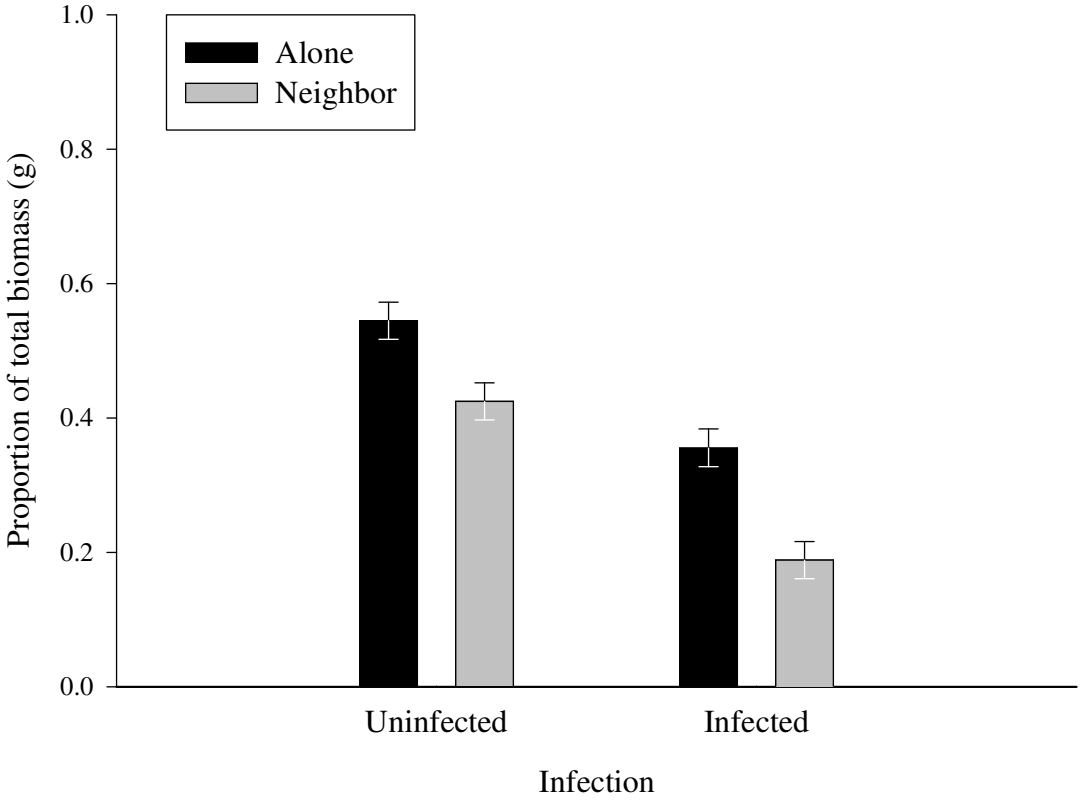

Figure 4.5. Effects of infection and competition on mean resource allocation to reproduction (resource allocation to reproduction = reproductive biomass/total biomass). Because the absolute effect of competition on resource allocation did not differ between uninfected and infected plants no pairwise comparisons were made. Upper and lower error bars are ± 1 standard error.

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Vita

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