THE RESPONSE OF TALL FESCUE AND ITS FUNGAL ENDOPHYTE TO CLIMATE CHANGE

Glade Blythe Brosi
University of Kentucky, glade.brosi@gmail.com

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ABSTRACT OF THESIS

THE RESPONSE OF TALL FESCUE AND ITS FUNGAL ENDOPHYTE TO CLIMATE CHANGE

Tall fescue is the most common cool-season grass in the eastern USA, with broad economic and ecological importance to the region. Tall fescue is known to associate with a fungal endophyte, *Neotyphodium coenophialum*, whose presence can decrease biotic and abiotic stress experienced by the plant. This thesis evaluates the response of tall fescue and the fungal endophyte symbiosis to predicted climate change. I participated in two multi-factor climate change projects where I investigated the response of tall fescue tissue chemistry and growth to various climate change factors. Endophyte-infected (E+) tall fescue had decreased alkaloid production under elevated CO$_2$ but increased alkaloid production under elevated temperatures. Significant differences between E+ and E- (endophyte-free) tall fescue tissue chemistry were also found, suggesting the endophyte interacts with the plant response to abiotic stress. Although several studies have reported benefits of endophyte infection for tall fescue growing under drought stress, my research found no differences between E+ and E- total growth and surprisingly showed increased mortality of E+ individuals under elevated temperature. Taken together, my research indicates that this grass-fungal relationship will respond to climate change, and may produce dramatic and unforeseen results that question the widely believed mutualistic nature of the symbiosis.

KEYWORDS: Tall fescue, endophyte, climate change, abiotic and biotic stress

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Glade Blythe Brosi

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May 16, 2011
THE RESPONSE OF TALL FESCUE AND ITS FUNGAL ENDOPHYTE TO CLIMATE CHANGE

By

Glade Blythe Brosi

Dr. Rebecca McCulley

Dr. Charles Dougherty

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THESIS

Glade Blythe Brosi

The Graduate School
University of Kentucky

2011
THE RESPONSE OF TALL FESCUE AND ITS FUNGAL ENDOPHYTE TO CLIMATE CHANGE

THESIS

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Plant and Soil Science in the College of Agriculture, at the University of Kentucky

By

Glade Blythe Brosi
Lexington, KY

Director: Dr. Rebecca McCulley
Assistant Professor
Lexington, KY

2011

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Chapter 1 - Introduction

Climate change and plant-microbe symbioses

Rising atmospheric CO₂ concentrations, coupled with increases in other greenhouse gases, are predicted to lead to increasing global mean air temperatures (+1.4-5.8°C by 2100) and will likely impact regional precipitation patterns (IPCC, 2007). These changes in climate will impact species interactions and the structure and function of ecosystems around the world (IPCC, 2007). One class of species interaction that is likely to respond to climate change and has widespread ecological effects is that of plant-microbial symbioses.

Plant-microbial symbioses are ubiquitous, and the nature of these relationships can range across a continuum from parasitic to mutualistic (Johnson et al., 1997; Saikkonen et al., 1998; Jones and Smith, 2004; Hirsch 2004; Neuhauser and Fargione, 2004). The degree and nature of these symbioses may be constant through time or they may be sensitive to changes in biotic or abiotic stressors (Thompson 1988; Bronstein 1994; Herre et al., 1999). Symbioses that are mutualistic between plants and microbes are usually based on an exchange of benefits between the two organisms. For example, plant hosts commonly supply nutrients, space, and reproduction for the symbiotic microbe, in return for microbial-mediated advantages against abiotic and biotic stress (Rodriguez et al., 2008; Timmusk and Wanger 1999).
Microbial symbioses that decrease abiotic stress for the plant can have significant positive impacts on their host plants. For example, mycorrhizae and rhizobacteria increase the nutrient acquisition of the host plants they occupy, often conferring significant advantages to the host plant such as increased growth (Hetrick 1989; Bolan 1991; van Loon 2007; Lugtenberg and Kamilova, 2009). Other abiotic stress benefits from plant-microbial mutualisms are associated with tolerance to heat (Redman et al., 2002; Rodriguez et al., 2008; Rodriguez et al., 2004), salt (Rodriguez et al., 2008; Yano-Melo et al., 2003), or drought (Timmusk and Wanger, 1999; Rodriguez et al., 2004; Arshad et al., 2008; Kannadan and Rudgers, 2008). Microbially-mediated abiotic stress tolerance can even allow host plants to survive in environments that they could not without the mutualistic relationship (Redman et al., 2002; Rodriguez et al 2008; Rodriguez and Redman, 2008).

Plant-microbial mutualisms can also reduce biotic stress to the plant by reducing herbivory (Miller et al., 2002; Miller 1986; Hartley and Gange, 2009) or increasing disease resistance of the host plant (Graham and Menge 1982; Volpin et al., 1994) which may alter competitive interactions in the community (Rudgers et al., 2005; 2007). For example, mycorrhizae can alter herbivory of host plants by changing nitrogen availability, which may impact the carbon and nitrogen ratio of the plant in a way that is detrimental to potential herbivores (Gange and West, 1994; Bennett et al., 2006; Hartley and Gange, 2009). Mycorrhizal disease resistance can be the result of mycorrhizae consuming root exudates otherwise used by pathogens (Graham and Menge 1982) or by a mycorrhizae induced defense response (Volpin et al., 1994).
While most plant-microbial mutualisms are species-specific, they can have significant effects across multiple ecological scales, ultimately influencing plant community and ecosystem structure, and function (van der Heijden, 2010; Clay and Hollah, 1999; Rudgers et al., 2004; 2005; 2007; Rudgers and Clay, 2008). By reducing abiotic or biotic stress to the plant, plant-microbial interactions can alter inter- and intra-specific competition (Zobel and Moora, 1995; Allen and Allen, 1990; Hart et al., 2003), plant community structure (Hartnett and Wilson, 2002; van der Heijden, 2010) as well as nutrient cycling (Fogel 1980; van der Heijden, 2010).

Because of the ecological importance of plant – microbe symbioses, knowing their response to climate change is critical in predicting the response of ecosystems to increasing atmospheric CO₂, temperature, and altered precipitation regimes. Previous research has shown that plant-microbial symbioses are sensitive to differences in temperature (Smith and Bowen 1979; Staddon et al., 2002; Smith and Roncadori, 2006), and there is increasing evidence that plant-microbial symbioses may be responsive to climate change (Staddon et al., 2002; Garcia et al., 2008; Rogers et al., 2009). For example, recent work has illustrated elevated CO₂ may increase ectomycorrhizal colonization of some plant species (Garcia et al., 2008) and increase microbial nitrogen fixation in some legumes (Rogers et al., 2009) - both of which may result in alterations in nutrient availability, capture and use in the ecosystems where they occur.

One common type of plant-microbial symbiosis that has received little attention regarding its response to climate change is that of plants and aboveground endophytic fungi.
Endophytic fungi are the most common group of fungal symbionts worldwide; every plant species examined to date has harbored at least one fungal endophyte (Arnold et al. 2000; Faeth and Fagan, 2002). Endophytic fungi are very common in grasses. More than 80 grass genera in all subfamilies of the Poaceae family are capable of forming relationships with fungal endophytes (Clay, 1990; White, 1987). Of the common grass-endophyte relationships, the perennial ryegrass-Neotyphodium lolii and tall fescue-Neotyphodium coenophialum relationships are perhaps the best studied and most well-known.

History of Tall fescue and discovery of its fungal endophyte

Tall fescue, Lolium arundinaceum (Schreber), is a widespread, ecologically and economically important cool-season grass that is known to associate with the endophytic fungus Neotyphodium coenophialum (Morgan Jones-Gams)(Ball et al., 1993). Native to Europe, tall fescue became abundant in the United States after the variety KY-31 was released by the University of Kentucky in 1943 (Fergus, 1952; Fergus and Buckner, 1972). After its release, tall fescue was widely seeded for use as forage, turf, and to control soil erosion, and quickly became the dominant cool-season grass in the southeastern United States, where today it covers more than 14 million hectares (Hill et al., 1990). However, soon after becoming widely established, severe livestock health problems were linked to animal consumption of tall fescue (Cunningham, 1948; Pratt and Haynes, 1950; Goodman 1952; Merriman, 1955), prompting research into the cause of the toxicosis, and ultimately leading to the discovery of the fungal endophyte, N.
**Tall fescue – Neotyphodium coenophialum symbiosis**

*Neotyphodium coenophialum* is an obligate, symbiotic fungal endophyte of tall fescue, depending on its plant host for shelter, nutrients, and reproduction (Siegel 1984; Bacon and Siegel, 1988; Malinowski and Belesky 2006). *Neotyphodium* hyphae grow intercellularly in the aboveground portion of tall fescue. Because *Neotyphodium* is not known to sexually reproduce, transmission of the endophyte only occurs when hyphae colonize fescue seeds during seed set (Welty et al., 1986, Bacon and Siegel, 1988). Infection of seed is not 100% (Welty et al., 1987), and the endophyte may be lost from the seed due to a variety of factors in storage (Siegel et al., 1984; Welty et al., 1987). In general, approximately 80% of individuals within any tall fescue community in the USA will be positive for endophyte infection (Afkhami and Rudgers, 2008; Ball et al., 1993).
In return for space, nutrients, and reproduction, it is generally thought that *Neotyphodium* offers tall fescue many benefits, and the relationship is considered primarily mutualistic in nature (Clay and Schardl, 2002). Endophyte-infected (E+) tall fescue produces three main classes of alkaloids: ergots (ergopeptides), lolines (pyrrolizidine), and peramines (pyrrolopyrazine) (Bush et al., 1997). These alkaloids have been shown to be toxic, or repulsive to mammalian (Bond et al., 1984; Hoveland et al., 1983; Studemann and Hoveland, 1988; Rudgers et al. 2007), avian (Madej and Clay, 1991; Conover and Messmer, 1996), and insect (Bultman et al. 2004; Clay et al. 1985; Johnson et al. 1985) herbivores. Of the three classes of alkaloids, ergot alkaloids seem to more broadly affect mammals, while loline alkaloids specifically affect insects; the peramine alkaloids are non-specific and toxic to all herbivores (Bush et al 1997; Dahlman et al., 1997). Tall fescue infected with *Neotyphodium* has also demonstrated greater resistance to some diseases (Mahmood et al., 1993; Blank and Gwinn, 1992; Gwinn et al., 1992; Gwinn and Galvin 1992) as well as nematodes (West et al., 1988; Elmi et al., 2000).

While *Neotyphodium* infection confers benefits to tall fescue relative to biotic stresses, by reducing herbivory and disease, there is also evidence that endophyte infection increases tolerance of abiotic stress for E+ tall fescue. Arechavaleta et al. (1989) found E+ tall fescue demonstrated drought avoidance by increasing leaf rolling (thus decreasing exposed stomata) compared to E- tall fescue. This same study also found that E+ tall fescue had increased recovery from severe drought compared to E- (Arechevaleta et al., 1989). Richardson et al (1990) reported greater rooting depth of E+ vs. E- fescue while Malinowski and Belesky (1999a) noted differences in E+ and E- root hairs, with
*Neotyphodium* infected tall fescue having longer root hairs and decreased root diameter, which may improve water uptake for E+ tall fescue.

The presence of *Neotyphodium* has also been shown to affect tall fescue physiology in ways that may alter the way the plant recovers from or responds to drought stress. Elmi and West (1995) demonstrated that E+ tillers had increased osmotic adjustment under drought stress compared to E- tall fescue. Richardson et al. (1992) reported increased sugars in the leaf sheaths of drought stressed E+ tall fescue (compared to E-), and Elbersen and West (1996) noted increased water content in the sheaths of E+ (compared to E-) tall fescue, which may act to reduce drying and improve turgor of E+ vs. E- plants (Malinowski and Belesky, 2000).

There are two primary mechanisms that may be responsible for *Neotyphodium* induced drought tolerance for tall fescue (Rodriguez et al., 2004): the first being the activation of tall fescue stress responses by the endophyte, and the second being the production of anti-stress chemicals by the endophyte (Redman et al., 2002). Arechevaleta et al. (1989) suggested that E+ leaves rolling more quickly than E- in response to drought may be evidence for the first type of mechanism. The second mechanism could be the production of anti-stress chemicals by the fungus (Bacon and Hill, 1996); however the only known chemicals produced by *Neotyphodium* are the alkaloids which are not thought to be anti-stress (Siegel and Bush, 1997). Drought-stressed E+ tall fescue has been demonstrated to accumulate loline alkaloids (Bush et al., 1993, 1997) which are water soluble (Bacon 1993) and may influence osmotic adjustment (Bush et al., 1993; Bacon 1993; Malinowski and Belesky, 2000). While there is no consensus of the
mechanisms by which the *Neotyphodium* endophyte confers drought tolerance to tall fescue, the accumulation of alkaloids, sugars, other biochemical compounds and the fungus itself in E+ tall fescue may all play roles in governing the physiological mechanisms that confer abiotic stress tolerance to tall fescue (Malinowski and Belesky, 2000).

Along with improved drought tolerance, *Neotyphodium* infection has been shown to benefit tall fescue under elevated temperature. Marks and Clay (1996) demonstrated that E+ tall fescue photosynthesized at a greater rate than E- when exposed to temperatures above 35°C. These abiotic stress tolerance advantages associated with endophyte symbiosis are thought to be especially important along the edges of tall fescue distribution where abiotic stress is thought to limit tall fescue survival. For example, in the far southern portions of the North American fescue suitability region, where long, hot summers are the norm, E+ tall fescue regularly outperforms E- tall fescue pastures and experiences longer stand persistence (Read and Camp 1986; West et al 1993; Bouton et al., 1993; Shelby and Dalrymple, 1987).

Another abiotic stress for plants is low nutrient availability, and there is evidence that *Neotyphodium* benefits tall fescue in avoiding this type of stress. Endophyte infected tall fescue has demonstrated enhanced nutrient acquisition in poor soils (Malinowski et al., 2000; Malinowski and Belesky, 2000) as well as greater nitrogen use efficiency (Lyons 1990). E+ plants perform better on phosphorous deficient soils (Malinowski and Belesky, 1999), and demonstrate improved acquisition of N and Mg (Vavez-de-Aldana et al.,
Improved uptake of P in E+ tall fescue is thought to be related to greater root exudations and biomass of E+ vs. E- plants (Malinowski and Belsky, 2000).

The influence of Neotyphodium infection on community and ecosystem dynamics

The individual benefits of endophyte infection have been demonstrated to have impacts across multiple ecological scales, from the community (Arechevaleta et al., 1989; Rudgers and Clay, 2008) to the ecosystem (Siegrist et al., 2010; Franzluebbers and Studeman, 2005). Endophyte infection has been shown to alter plant community composition and succession (Clay and Holah, 1999; Clay et al., 2005; Rudgers and Clay, 2008), affect food webs (Rudgers and Clay, 2008; Omancini et al., 2001; 2005) and alter soil microbial communities (Franzluebbers et al. 1999; Jenkins et al. 2006). Although domestic livestock do not appear to be able to differentiate between E+ and E- fescue (Fisher and Burns, 2008), it is clear that several species of insects (Johnson et al., 1985) and voles (Rudgers et al., 2007) can. By impacting herbivore’s choices, consumers generate top-down effects by which the presence of the endophyte in tall fescue may indirectly alter the surrounding plant community composition and succession (Rudgers et al., 2005; 2007). Endophyte-infected tall fescue’s toxicity also influences herbivore communities either through direct death (Johnson et al., 1985), deterrence (Coley et al., 1995; Rudgers et al., 2005; 2007) or delayed development of herbivorous insects (Potter et al, 1992) which may lead to altered insect community diversity (Omacini et al., 2001; Rudgers and Clay, 2008; Finkes et al., 2006) and ecological effects stemming from these changes. Belowground, endophyte-infected tall fescue has demonstrated resistance to
nematodes (Elmi et al., 2000; Kimmons et al., 1990; West et al., 1988) as well as reducing root feeding by white grubs (Patterson et al., 1991; Popay and Lane, 2001).

These various above and belowground impacts of *Neotyphodium* infection and the subsequent influences on plant community composition can significantly alter ecosystem-scale ecological processes (Rudgers and Clay, 2007). The tall fescue – *Neotyphodium* relationship has been shown to influence litter decomposition and nutrient cycling (Siegrist et al., 2010; Franzluebbers and Stuedemann, 2005). Reduced biomass consumption via herbivory, coupled with increased tillering (Arechavaleta et al., 1989), increased rooting depth (Richardson et al., 1990) and greater plant production and competitive ability (Marks et al., 1991) may explain the observed increases in belowground carbon storage in soils of E+ compared to E-fescue stands (Siegrist 2008; 2010; Franzluebbers et al., 1999; Franzluebbers and Stuedemann, 2005).

*The importance of knowing tall fescue’s response to global climate change*

Despite the fact that 20-30% of grasses harbor fungal endophytes (Leuchtmann, 1992), and these symbioses can have substantial effects on ecosystem structure and function (Rudgers et al. 2004), only a few studies have investigated the response of grass-fungal endophyte symbiosis to atmospheric and climate change factors. This previous research has shown that tall fescue is responsive to climate change factors (Greer et al., 1995, 2000; Sasaki et al., 2002, Teughels et al., 1995; Sæbø & Mortensen, 1996; Chen et al., 2007; Marks and Lincoln, 1996; Newman et al., 2003); however few of these studies
considered whether the endophyte was present. Greer et al. (1995;2000), Teughels et al. (1995), and Sasaki et al. (2002) all demonstrated that elevated CO\textsubscript{2} increased tall fescue photosynthesis rates and growth, while Sæbø and Mortensen (1996) reported no increase in tall fescue growth due to increased CO\textsubscript{2} concentrations. Chen et al. (2007) found that elevated CO\textsubscript{2} increased tall fescue growth but that \textit{Plantago} was more responsive than tall fescue when the two species were grown together. Elevated CO\textsubscript{2} increased the root biomass of E+ fescue (relative to E-) but did not influence shoot biomass of either E+ or E- tall fescue (Chen et al. 2007). Marks and Lincoln (1996) investigated the response of fall armyworm to consuming E+ and E- tall fescue grown in 400 or 700 ppm CO\textsubscript{2}; however the only plant parameter measured was \%N (with no significant differences due to endophyte infection or CO\textsubscript{2} treatment). Armyworms reared on E+ tall fescue at the high CO\textsubscript{2} treatment developed more slowly than the other treatments; however the material they were feeding on was not analyzed for alkaloids, so it is difficult to determine the mechanism resulting in delayed growth.

Of all the currently published research (n=8) on tall fescue’s response to climate change only Newman et al. (2003) specifically addressed the endophyte-plant response. Newman et al. (2003) investigated tall fescue response to nitrogen fertilization and elevated CO\textsubscript{2} in regard to chemical composition and digestibility of E+ and E- plants. They found that \textit{Neotyphodium} infected tall fescue had less lignin, increased photosynthesis, and flowered earlier than E- plants when grown with increased N under elevated CO\textsubscript{2}. From an applied, herbivore perspective, Newman et al. reported that elevated CO\textsubscript{2} significantly reduced the forage quality of tall fescue (regardless of
endophyte status) suggesting that the forage will be less nutritious for livestock and wild herbivores under elevated CO$_2$.

The influence of environmental conditions on plants and their associated endophytes is an important focus of both applied and ecological research, especially in widespread and economically important grasses such as perennial ryegrass (*Lolium perenne* L.) and tall fescue. When considering the wide ecological and economic impacts of tall fescue worldwide (Ball et al., 1993; Franzluebers and Stuedemann, 2005; Rudgers et al., 2004), it is obvious that there is a greater need to know more about the response of this plant to climate change. Due to the direct link between grazing animal health and performance and alkaloids produced by *Neotyphodium*, it is striking that no current study has investigated alkaloid response to climate change factors. Because E+ tall fescue has improved resistance to both abiotic and biotic stress, and because this plant is so widely dispersed and ecologically important, the response of this symbiosis to climate change is likely to have significant ecological implications. The overall purpose of my thesis is to determine how the tall fescue grass-fungal symbiosis responds to climate change.

*Experimental Framework*

In order to better understand the response of tall fescue and the tall fescue-*Neotyphodium* symbiosis to climate change, I conducted experiments in two multi-factor field climate change projects: (1) at the Oak Ridge National Laboratory and (2) at the University of Kentucky Spindletop Research Farm. The Oak Ridge experiment was designed to
understand how an “old-field” community might respond to the climate change factors of altered precipitation, and elevated temperature and atmospheric CO$_2$. The Spindletop experiment was designed to understand how elevated temperature and altered precipitation might influence a managed hayfield community. Tall fescue was a component of the vegetative communities in both experiments. The specific objectives and hypotheses associated with these two projects were:

Old Field Climate and Atmospheric Manipulation Experiment

*Objective 1.* Quantify changes in endophyte infection frequency of tall fescue grown in an old-field mixed species community after a five year climate manipulative field experiment.

*Hypothesis.* Endophyte infection frequency will increase under treatments that increase drought and heat stress for the plants due to the reported benefits of endophyte infection for host plants experiencing these conditions.

*Objective 2.* Determine the impacts of the climate change treatments, endophyte status, and their interactions on tall fescue tissue chemistry.

*Hypothesis.* The climate change treatments will interact with endophyte status to affect tall fescue tissue chemistry. Alkaloid production of E+ tall fescue will increase in elevated temperature treatments as has been previously reported by Kennedy and Bush (1983) and as is suggested in Marks and Lincoln’s (1996) experiment. Elevated CO$_2$ will
increase the C:N ratio of tall fescue which may be modified by endophyte status as reported by Newman et al. (2003).

University of Kentucky Forage Climate Change Study

Objective 1. Determine the impact of elevated temperature and altered precipitation on tall fescue growth, tillering, and mortality and evaluate whether these responses are influenced by the endophyte.

Hypotheses.

1a) Tall fescue growth will be seasonally affected by elevated temperature, shifting the optimal growing season to earlier in the spring and later in the fall. The negative effects of increased temperature during the summer months will be reduced by added precipitation.

1b) Due to the benefits of endophyte infection in reducing abiotic stress, E+ tall fescue will grow more, tiller more, and suffer less mortality than E- tall fescue under the treatments that increase drought stress.

Objective 2. To determine the impacts of the climate change treatments on E+ and E- tall fescue tissue chemistry.

Hypothesis. Elevated temperature will increase the alkaloid production of E+ fescue. As reported by Newman et al. (2003) endophyte infection will modify the plant tissue chemistry response to the treatments.
Chapter 2 – Effects of multiple climate change factors on the tall fescue-fungal endophyte symbiosis: infection frequency and tissue chemistry

2.1 Introduction
Atmospheric CO$_2$ concentrations will likely double by 2100, potentially resulting in a 1.4-5.8°C increase in mean air temperatures and modified regional precipitation patterns (IPCC, 2007). These changes in climate will have significant ramifications for species interactions and the structure and function of ecosystems around the world.

One important microbial–plant symbiosis that has received little attention regarding potential climate change response and resulting ecological effects is that of aboveground fungal endophytes in grasses. This type of symbiosis is widespread, occurring in 20-30% of grasses worldwide (Leuchtmann 1992), and the interactions between grass host and endophyte can range from mutualistic to parasitic (Saikkonen et al., 2004; 2006). In species where endophyte symbiosis is thought to be mutualistic, fungal endophytes frequently confer environmental stress tolerance (e.g., to drought (Kannadan and Rudgers, 2008), heat (Rodriguez et al., 1989), low nutrient availability (Lyons et al., 1990), and grazing/herbivory (Miller 1986; Hartley and Gange, 2009)). Thus, the endophyte symbiosis may be particularly important in determining how plant hosts responds to climate change.

A well known example of such a mutualistic symbiosis occurs between tall fescue (*Lolium arundinaceum*) and the clavicipitaceous fungus, *Neotyphodium coenophialum*
Neotyphodium is vertically transmitted through seeds of tall fescue and is estimated to occur in 80% of tall fescue in North America (Ball et al., 1993). Infection with Neotyphodium has been shown to improve drought tolerance (Arachevalta et al., 1989), resistance to herbivory (Bush et al., 1997), and enhance nutrient acquisition and utilization (Malinowski and Belesky, 1999; Rahman and Saiga, 2007) of tall fescue host plants, an internationally important agronomic cool-season grass (Hoveland et al., 1997). Neotyphodium produces alkaloids in E+ tall fescue, which are thought to be the primary mechanism by which environmental stress tolerance and herbivore deterrence is conferred to the plant and may also be responsible for the many ecological consequences associated with this fungal-plant symbiosis. Endophyte infection may be necessary for the long-term survival of tall fescue under certain environmental conditions, such as those which occur in the southeastern region of the USA (Arechevaleta et al., 1989; West et al., 1993).

Despite the fact that we know 20 to 30% of grasses harbor fungal endophytes (Leuchtmann 1992), that these symbioses can have substantial effects on ecosystem structure and function (Rudgers et al., 2004), and that many of the field, climate change projects to date have occurred in grassland ecosystems (Polley et al., 2010), only a few studies have investigated the response of grass-fungal endophyte symbioses to climate change factors [Marks and Lincoln, 1996; Newman et al., 2003; Hunt et al., 2005; Chen et al., 2007], and none of these were performed under true field conditions. Marks and Lincoln (1996) found that insects ate more tall fescue (regardless of endophyte status)
when plants were grown with elevated CO$_2$, and they observed no differences in N content between E+ and E- plants within a CO$_2$ treatment. Similarly, Chen et al. (2007) found no significant differences in biomass accumulation or N acquisition between E+ and E- tall fescue exposed to elevated CO$_2$ in a growth chamber study. However, Newman et al. (2003) and Hunt et al. (2005), both reported that the presence of the endophyte increased plant protein content under elevated CO$_2$ (compared to E-individuals), and speculated that the endophyte plays a significant role in N dynamics. Altered biomass production and/or changes in the chemical composition of that material (e.g., %C and N, hemicellulose, cellulose, and lignin) in response to endophyte presence and interaction with climate change factors may have significant consequences for ecological processes such as rates of herbivory and litter decomposition. Given the vast acreage of tall fescue worldwide, the possible ecological consequences of fungal endophyte infection in this species, and current climate change predictions, a better understanding of how this species and symbiosis will respond is desirable. Here, we address this issue by utilizing an existing field climate manipulation experiment to examine the effects of elevated CO$_2$, atmospheric warming and altered precipitation on a mixed species old field, including tall fescue, in eastern Tennessee, USA.

Because of the well documented benefits of *Neotyphodium* infection for tall fescue under environmental stress, we predicted that the treatments would have an impact on the frequency of endophyte in the tall fescue community and the tissue chemistry of tall fescue. We hypothesized that E+ individuals would survive better under treatments that increase drought stress (e.g., higher temp, less precipitation) and thus increase the
endophyte infection frequency within these tall fescue communities over time. Specifically, we addressed three main questions: 1) Will climate change factors affect the frequency of endophyte infection within tall fescue communities? 2) How will climate change factors interact with the tall fescue-Neotyphodium symbiosis to alter tall fescue tissue chemistry? and 3) Will fungal endophyte alkaloid production respond to climate change?

2.2 Methods

Experimental Design

To examine the effects of climate change on the tall fescue-endophyte symbiosis we utilized an existing field climate manipulation experiment at the Oak Ridge National Laboratory, Tennessee. The Old Field Community Climate and Atmospheric Manipulation (OCCAM) experiment ran from 2002 – 2008 and focused on the response of an old field plant community to three main climate factors: elevated CO₂, elevated temperature, and altered precipitation (Dermondy et al., 2007). This experiment was a random split plot design with three replicate chambers each of the following treatment combinations: ambient CO₂ – ambient temperature (ACAT), ambient CO₂ – elevated temperature (ACET), elevated CO₂ – ambient temperature (ECAT), and elevated CO₂ – elevated temperature (ECET). Each whole plot was split along the north/south axis into two halves: each half received one of two precipitation regimes, either “dry” (2mm H₂O weekly additions) or “wet” (25 mm H₂O weekly additions).
Each whole plot was covered by a 4 m diameter x 2.2 m height polyvinylchloride chamber that excluded rainfall but allowed for air movement. Elevated CO$_2$ was maintained by injecting pure CO$_2$ into the chamber at ambient + 300 ppm. Average CO$_2$ concentrations for the experiment were 396 ± 3 ppm in ambient and 696 ± 10 ppm CO$_2$ in elevated plots (Dermondy et al., 2007). Temperature treatments were maintained using electric heaters and evaporative coolers as outlined by Norby et al., (1997). Because chambers could impact temperature within the plots, ambient temperature plots were based on the current temperature data recorded outside the plot and elevated temperature was ambient +3°C (see Norby et al., 1997 for more detail). Rainfall for the precipitation treatments was collected in the area and applied by hand via metered wands. All treatments started April 2003 and ran continuously until August 2008.

An old-field plant community typical of the southeastern United States (Villalpando et al., 2009) was chosen for the study. Seven species, consisting of grasses, forbs, and legumes were chosen: Canada goldenrod (*Solidago Canadensis* L.), red clover (*Trifolium pretense* L.), sericea lespedeza (*Lespedeza cuneata* Dum. Cours. G.Don), ribwort plantain (*Plantago lanceolata* L.), orchardgrass (*Dactylis glmerata*), broomsedge (*Andropogon virginicus*), and tall fescue. Plants were reared from seed in a greenhouse in 2002 and then transplanted into the plots in July 2002 in a grid pattern so that individuals did not neighbor a conspecific and were approximately 18 cm apart. Approximately 170 individuals were planted per 12.6 m$^2$ plot. For more detailed information see Garten et al., 2008.
**Sampling and Chemical Analysis**

In July 2008, immediately prior to the termination of this multi-year project, one tiller was collected from each individual tall fescue plant in every plot (20 tillers per plot x 12 plots = 240 tillers in total). Tillers were kept cold in transit to the lab where endophyte testing could be performed. Fungal endophyte infection was determined by double blotting the base of each tiller onto nitrocellulose paper and then exposing the paper to an enzyme-linked, endophyte-specific immunosorbent assay (Hill et al., 2002). Endophyte infection frequency was calculated as the percentage of infected tillers per plot. Pre-treatment (July 2002) endophyte infection data were not available (the seed was not saved), but there is no reason to think that infection frequencies differed significantly among plots at time zero (plants were all grown from one seed lot). Sampled endophyte infected and endophyte-free tillers were separated based on assay results into E+ and E- groups for each plot. Tillers were lyophilized using a Botanique 18DX48SA model freeze drier (Botanique Preservation Equipment, Peoria, AZ) and subsequently ground through a 1 mm screen on a Model 4 Wiley Mill (Thomas Scientific Inc., Swedesboro, NJ). Percent cellulose, hemicellulose, and lignin were determined on 5 g sub-samples of the E+ and E- tiller groups using the Van Soest (1963) method with an ANKOM fiber analyzer (Ankom Technology Inc., Fairport, NY). An additional sub-sample of the E+ and E- ground material was further homogenized by ball-grinding and subsequently evaluated for carbon and nitrogen content using a Flash EA1112 elemental analyzer (CE Elantech Inc, Lakewood, NJ).
Because the *Neotyphodium* endophyte produces alkaloids that are not otherwise present in E- tall fescue (Bush et al., 1997), we determined alkaloid content of E+ tillers only. Material was ground through a 1 mm screen on a Wiley Mill in order to determine loline (pyrrolizidine) and ergot (ergopeptides) alkaloids which have been shown to affect insect and mammalian herbivores, respectively (Bush et al. 1997). A modified high performance liquid chromatography (HPLC) fluorescence procedure developed by Yates and Powell (1988) was used to quantify ergot alkaloids (ergovaline, ergovalinine, and total). Separation was performed using an Alltima C18 150 mm x 4.6 mm column with 3µ particle size (Alltech Associates Inc., Deerfield, IL). Solutions used for elution were 75 mM ammonium acetate (A) in water:acetonitrile (3:1, v/v) and acetonitrile (B). Elution gradient was 95:5 (A:B) 1 min; linear change to 60:40 (A:B) during next 15 min and maintained for 5 min; changed to 0:100 (A:B) in 1.5 min and maintained for 5 min; changed to 100:0 (A:B) in 1 min and maintained for 6 min before returning to 95:5. Loline alkaloids (*N*-formyl loline, *N*-acetyl loline, and *N*-acetyl norloline) were quantified using gas chromatography with a flame ionization detector as described in Blankenship et al., (2001).

**Statistical Analysis**

Data were analyzed for treatment differences using a split-plot analysis of variance (ANOVA) (PROC MIXED, SAS Institute, Cary NC, USA) to test for the effects of CO₂, temperature, precipitation, *Neotyphodium* infection and all possible interactions on tall fescue tissue chemistry. Wet and dry split-plots within the main treatment combinations (ACAT, ACET, ECET, ECAT) were treated as repeated measures. All data were
normally distributed and the containment method was used to determine numerator and denominator degrees of freedom. Means comparisons were considered significant at \( p < 0.05 \).

2.3 Results

*Endophyte Infection Frequency and Alkaloid Concentrations*

Elevated CO\(_2\) was the predominant climate change factor affecting endophyte infection frequency (EIF) and alkaloid concentrations of tall fescue. Endophyte infection frequency was significantly higher under elevated CO\(_2\) compared to ambient, (91% vs. 81% infected respectively; Figure 2.1a, \( p=0.032 \)), suggesting that elevated CO\(_2\) promoted the plant-fungal endophyte mutualism over the 6 year study. Surprisingly, EIF was not significantly different under altered precipitation (\( p=0.82 \)), temperature (\( p=0.76 \)), or any treatment interaction (Table 2.1).

For E+ tall fescue tillers, the impacts of CO\(_2\), temperature, and altered precipitation on alkaloid concentrations were mixed. Elevated CO\(_2\) significantly (\( p<0.05 \)) decreased the total concentration of loline (1104 vs. 820 mg/kg in AC vs. EC, respectively, Figure 2.1b) and ergot alkaloids (0.70 vs. 0.48 mg/kg in AC vs. EC, respectively, Figure 2.1c). Conversely, elevated temperature increased loline concentrations from 842 to 1082 mg/kg (AT vs. ET, respectively, \( p=0.045 \), Figure 2.2), but had no effect on ergot alkaloid concentrations (Table 2.1). The wet (vs. dry) precipitation treatment led to a reduction in the loline alkaloids N-actedylnorloline (by 29%, Fig 2c) and N-acetylloline (by 31%, Fig 2.2d) but did not affect (\( p>0.05 \)) N-fomylloline (data not shown) or total loline alkaloid
concentration. For the ergot alkaloids, ergovalinine concentration was marginally reduced in the dry treatment (0.17 mg/kg dry vs. 0.22 mg/kg wet, \( p=0.0522 \), data not shown); however precipitation treatment had no effect on ergovaline or the combined total ergot alkaloid concentration (Table 2.1).

**Tall Fescue Tissue Chemistry**

Elevated CO\(_2\) decreased %N of aboveground tall fescue plant material (1.57% AC vs. 1.24% EC, Figure 1d, \( p=0.024 \)) but had no effect on %C (Table 2.2). Consequently, C:N ratios increased under EC conditions (27 vs. 35 for AC and EC, respectively; Figure 1e). Percent cellulose and hemicellulose, as well as lignin, decreased under elevated CO\(_2\) (%cellulose & hemicellulose = 33.40 AC vs. 30.27 EC, \( p=0.0085 \); %lignin = 5.46% AC vs. 4.04% EC, \( p=0.014 \), Figures 1f & g). Altered precipitation impacted %C and %cellulose & hemicellulose in contrasting ways: %C was higher in dry (42.48%) vs. wet (41.86%) treatments (\( p=0.0018 \), while % cellulose & hemicellulose was higher under wet (32.76) than dry (31.26) (\( p=0.0.041 \), Figure 2.2b).

Despite likely large differences in alkaloid concentrations between E+ and E- tall fescue plants (e.g. 0.48 µg g\(^{-1}\) ergot alkaloids in E+ vs. 0.03 µg g\(^{-1}\) in E-, as shown in Siegrist et al., 2008), *Neotyphodium* infection had no significant impact on %N, C:N, % cellulose & hemicellulose, or lignin (Table 2.2). However, endophyte infection reduced %C in tall fescue tillers (42.60% E- vs. 41.80% E+), but only under elevated CO\(_2\) (\( p= 0.0077 \) for CO\(_2\) * endophyte; Table 2.2).
2.4 Discussion

Because of the well-documented benefits of *Neotyphodium* infection for tall fescue under abiotic stress (Arachevaleta et al., 1989; Malinowski 1999; Rahman and Saiga, 2007), we predicted that after five years of climate treatments the endophyte infection frequency (EIF) of tall fescue would be higher under elevated temperature and under “dry” precipitation treatments. However, we found no support for this hypothesis: in this study there were no significant differences in the EIF of tall fescue between “wet” vs. “dry” treatments or under elevated vs. ambient heat (or in the interaction of these two treatments; Table 2.1). Elevated CO$_2$ was the only climate treatment that produced a significant change in endophyte infection frequency of the tall fescue community.

Multiple studies have shown a ‘fertilizer effect’ of elevated CO$_2$ on plants, where elevated CO$_2$ simultaneously increases growth and decreases plant water stress (Li et al., 2007; Leakey et al., 2009). Given that elevated CO$_2$ frequently improves plant water status and that the benefits of endophyte infection to tall fescue host plants are thought to be most notable under abiotic stress, specifically drought stress (Arechevaleta et al., 1989; West et al. 1993), it is surprising that elevated CO$_2$ increased EIF (we would have predicted the opposite: higher EIF in ambient CO$_2$ conditions). It is possible that in this case the more tall fescue plant growth is stimulated, the more mutualistic and beneficial the endophyte-plant symbiosis is. Interestingly, there was no significant effect of elevated CO$_2$ on tall fescue aboveground biomass over the course of the experiment (Engel et al., 2009).
Tall fescue’s response to the climate treatments may have been influenced by changes in levels of herbivory – which were not monitored during this study. The production of toxic alkaloids by *Neotyphodium* was clearly affected by changes in climate, and these alkaloids are known to have negative effects on the herbivores that consume them (Kindler et al., 1991; Thompson et al., 2001). Elevated CO$_2$ decreased both total ergot and loline alkaloid concentrations by roughly 30%; thus, while the tall fescue community become more endophyte-infected under elevated CO$_2$, lower concentrations of these toxic compounds were contained in that material. Conversely, although elevated temperature did not affect EIF, total loline concentrations increased 28.5% in the endophyte-infected material in this treatment. Likewise, concentrations of two types of N-acetyl loline alkaloids, NANL and NAL, were significantly higher in the dry vs. wet treatment, though EIF was not impacted. The increased concentrations of loline alkaloids in tall fescue material grown under elevated temperature and the “dry” precipitation treatment may be linked to the ability of E+ tall fescue to withstand drought stress and is consistent with prior findings on this topic (e.g., Arechavaleta et al., 1992; Schardl et al., 2007). Drought stress (low water and/or high heat) interacts with the plant-fungal endophyte symbiosis to increase alkaloid production in tall fescue. Since alkaloids are thought to be the primary mechanism by which endophyte infection alters herbivory, plant competition, and ecosystem processes such as litter decomposition (Rudgers et al., 2004) in tall fescue dominated systems, we might predict that under drier and hotter conditions these alkaloid-related effects on ecological processes are likely to be larger, not smaller. However, it is interesting that despite higher concentrations of alkaloids in E+ material and the often-cited advantages of fungal endophyte infection/alkaloid production to tall
fescue, we observed no difference in EIF in the tall fescue communities of the wet and dry treatments. This lack of change in EIF over the 5 year experiment in this particular treatment (hot and dry) cannot be explained and is contrary to current scientific thought on this particular symbiosis (Table 2.1).

Independent of infection with *Neotyphodium*, elevated CO$_2$ altered tall fescue tissue chemistry in some expected ways. For example our results show that elevated CO$_2$ increased the C:N ratio and decreased %N of tall fescue (Figure 2.1) which has been reported in many other plant species and climate change studies (Leakey et al., 2009). While elevated CO$_2$ increases carbon availability to the plant, potentially leading to a subsequent increase in carbon based structural compounds, we found a significant decrease in percent cellulose & hemicelluloses as well as lignin when tall fescue was grown in elevated CO$_2$. Previous work on tall fescue has shown no significant effect of elevated CO$_2$ on either hemicelluloses and cellulose or lignin, but did find that lignin was decreased 14% under elevated CO$_2$ with the addition of nitrogen (Newman et al., 2003). From an herbivore perspective, decreased % cellulose, hemicelluloses and lignin would increase nutritional value. However, the subsequent decrease in %N and increase in C:N ratio under elevated CO$_2$ could offset the impact of CO$_2$ on tall fescue tissue chemistry, resulting in no change to nutritive value. Fewer, but significant effects of the precipitation treatment, endophyte infection, and interactions between CO$_2$ and both temperature and endophyte infection on %C further illustrate the complexity of possible alterations in tall fescue tissue chemistry to changing climate.
These various significant effects on tissue chemistry (both alkaloids and %C, N, cellulose, etc.), coupled with the unexpected and not easily explainable result that EIF was not altered in the elevated temperature and/or dry treatment but did increase under elevated CO₂, clearly indicate that more research is needed to understand how this symbiosis is likely to respond to future climate alterations. Given the extensive acreage of tall fescue worldwide and the fact that the ecological effects of this grass-fungal endophyte symbiosis have been observed at population, community, and ecosystem-scales (Omacini et al., 2005), understanding the response of tall fescue and its endophytic fungi *Neotyphodium* to climate change may be important in predicting not only the responses of grazing livestock and other herbivores but also that of ecological processes such as litter decomposition and nutrient cycling.
Table 2.1: Statistical results (bolding indicates p-values < 0.05) for the variables where fungal endophyte presence was not included in the statistical model (Endophyte infection frequency of the tall fescue community; Total lolines & ergovalines from endophyte infected tall fescue material only).

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Endo Infection Freq</th>
<th>Total Lolines</th>
<th>Total Ergovalines</th>
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<td>p-value</td>
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Table 2.2: Main treatment effects and interactions for tall fescue tissue chemistry data (bolding indicates p-values <0.05).

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<th>p-value</th>
<th>%N</th>
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<th>p-value</th>
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<th>p-value</th>
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<th>p-value</th>
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FIGURE LEGENDS

Fig. 2.1. CO₂ effects on measured tall fescue parameters (means (±S.E.); AC = ambient CO₂ and EC = elevated CO₂ concentrations). a) Endophyte Infection Frequency (EIF); b) total loline alkaloid concentrations; c) total ergovaline alkaloid concentrations; d) %N; e) C:N ratio; f) % cellulose and hemicellulose; g) % lignin. For the alkaloid graphs (b and c), only endophyte infected tillers are shown (as endophyte-free tillers do not have measurable quantities of these compounds). p values are shown in the upper right hand corner of each graph, values of p< 0.05 were considered significant. Error bars are present on Fig 1a but too small to be visible.

Fig. 2.2. The effects of the precipitation treatment (dry = 2mm water additions per week and wet = 25 mm water additions per week) and elevated temperature treatment (ET = elevated temperature; AT = ambient temperature) on measured tall fescue parameters [means (± S.E.)]. a) %C; b) % cellulose and hemicellulose; c) % N-acetyl-norlloline (%NANL); d) % N-acetyl-loline(%NAL); e) total loline concentrations. For the alkaloid graphs (c, d, and e), only endophyte infected tillers are shown (as endophyte-free tillers do not have measurable quantities of these compounds). P values are shown in the upper right hand corner of the graph, values of p< 0.05 were considered significant. Temperature did not have a significant main effect on any of the additional variables measured in the study (Tables 1& 2), though a significant interaction with CO₂ occurred for %C.
Figure 2.1 The effects of the precipitation treatment (dry = 2mm water additions per week and wet = 25 mm water additions per week) and elevated temperature treatment (ET = elevated temperature; AT = ambient temperature) on measured tall fescue parameters.
Fig. 2. The effects of the precipitation treatment (dry = 2mm water additions per week and wet = 25 mm water additions per week) and elevated temperature treatment (ET = elevated temperature; AT = ambient temperature) on measured tall fescue parameters.
Chapter 3 – Does the fungal endophyte *Neotyphodium coenophialum* influence the response of tall fescue to predicted climate change?

3.1 Introduction

Plant-endophyte relationships are widespread and ecologically diverse (Arnold et al., 2000; Faeth and Fagan, 2002), spanning the symbiotic continuum from antagonistic to mutualistic (Saikkonen et al., 1998). Within the plant family *Poaceae*, endophyte relationships are common; more than 80 genera have known relationships with fungal endophytes (Clay 1990; White 1987). Fungal endophyte symbioses can have significant physiological effects on the growth of infected grasses (Belesky and Fedders, 1996), as well as the herbivores that choose to feed on them (Bond et al., 1984), and at a larger scale, these relationships can have significant effects on community (Rudgers et al., 2004; Clay et al., 2005) and ecosystem dynamics (Franzluebbers and Stuedemann, 2005). Amongst the agronomically important cool-season grasses, endophyte mutualisms are thought to be especially important for reducing biotic (Rudgers et al., 2007) and abiotic stress (Arechavaleta et al., 1989).

One such agronomic grass species is tall fescue - a common cool-season grass that covers more than 13 million hectares in the eastern USA (Ball et al., 1993). In this region, tall fescue is used as a forage, for turf, and for erosion control (Ball et al., 1993), but it is also considered an invasive species in natural areas (Rudgers et al., 2005). Tall fescue is a perennial cool-season grass that grows best at temperatures between 20 and 25°C (Volenec et al., 1984). Seasonal growth is influenced by both temperature and available
moisture (Frank et al., 1996; Norton et al., 2006), with peak tall fescue growth rates usually occurring in the spring and late fall months in the southeastern USA (Ball et al., 1993). Approximately 80% of individual tall fescue plants within a population are infected with the endophytic fungus, *Neotyphodium coenophialum* (Ball et al., 1993). This symbiosis has widespread impacts on the ecology i.e., species diversity (Rudgers et al., 2004) and plant succession (Clay et al., 2005), and the economy i.e. livestock production (Hoveland, 1993) of areas where tall fescue grows.

The tall fescue – *Neotyphodium* symbiosis is widely regarded as a mutualism (Clay and Schardl, 2002) and has been described as the “perfect partnership” (Christensen and Voisey, 2006). Endophyte infection has been shown to reduce abiotic and biotic stress for tall fescue, and tall fescue, in turn, provides living space, nutrients, and reproduction for the fungus (Bacon and Hill, 1996). Endophyte mediated biotic stress resistance is primarily achieved through endophyte production of alkaloids in infected fescue. These alkaloids are toxic to livestock, wildlife and various insects (Bush et al., 1997). Endophyte infected tall fescue (E+) also has different physiological responses to abiotic stress than tall fescue that does not have the endophyte (E-) (Marks and Clay 1996; Malinowski). While the mechanisms of *Neotyphodium* conferred abiotic stress resistance are not fully understood, there is significant evidence that E+ tall fescue outperforms E-tall fescue under drought conditions (West et al., 1993; Bouton et al., 1993).

For example, E+ tall fescue has demonstrated increased resistance to drought compared to tall fescue without the endophyte. Arechavaleta et al. (1989) reported that E+ tall
fescue avoided drought conditions by increasing leaf rolling (to reduce stomatal exposure) and recovered more quickly from drought than E- tall fescue. Richardson et al. (1990) reported that E+ tall fescue had deeper root development than E-, which might increase the amount of available soil water to fescue during drought periods. Malinowski and Belesky (1999) reported that E+ tall fescue had longer root hairs than E-, which may also facilitate plant uptake of water, and Marks and Clay (1996) reported that E+ tall fescue is able to photosynthesize at a higher rate than E- at temperatures above 35°C. While the mechanisms producing these various endophyte-associated drought tolerance effects in tall fescue are not well understood, it is clear that E+ tall fescue outperforms E- under hotter and drier conditions (Read and Camp, 1986; Joosi and Combs, 1988; West et al., 1993; Bouton et al., 1993; Shelby and Dalrymple, 1987).

In addition to conferring abiotic stress tolerance, E+ tall fescue is repulsive to some herbivores (Patterson et al., 1991; Popay et al., 2005) and can cause significant health problems for mammalian (Bond et al., 1984; Rudgers et al., 2007) and insect herbivores (Johnson et al. 1985; Bultman et al. 2004) that feed on it. The presence of endophyte-produced toxic alkaloids can significantly alter the feeding, distribution and abundance of individual insects (Omacini et al., 2000), which on a community scale can reduce arthropod diversity (Rudgers and Clay, 2008). Herbivores choosing to feed on material other than E+ tall fescue represent a top down impact on plant communities that has been shown to alter plant succession (Clay et al., 2005; Rudgers et al., 2007) and plant community diversity (Spyreas et al., 2001; Rudgers et al., 2004). The combined effects of reduced herbivory and increased abiotic stress resistance of E+ tall fescue have been
shown to have impacts at the ecosystem scale. For example, studies from Georgia have shown that soil from E+ tall fescue stands exhibited reduced soil microbial activity and greater carbon and nitrogen pools compared to E- (Franzluebbers and Hill 2005, Franzluebbers and Stuedemann 2005).

These endophyte effects may be sensitive to alterations in tall fescue growth and the surrounding environmental conditions. Indeed, in other grass species, environmental conditions are known to be important in determining the nature of endophyte - grass symbioses (Saikkonen et al., 1998; Faeth and Sullivan, 2003). Given that the IPCC report (2007) predicts that by 2100 rising atmospheric CO$_2$ concentrations will likely have altered the world’s climate, increasing mean annual temperatures and altering regional precipitation patterns (IPCC, 2007), it seems likely that grass – fungal endophyte symbioses will be impacted by and respond to these changes. In the eastern USA, where predictions call for an increase in annual temperatures coupled with more intense rainfall events, a prolonged growing season, and possibly more drought (IPCC, 2007), climate change is likely to have implications for the ecologically and economically important tall fescue–*Neotyphodium* symbiosis.

Only a few studies have specifically addressed the role of endophyte infection in tall fescue’s response to climate change, and all of these studies except one (Brosi et al., 2010) have focused solely on elevated atmospheric CO$_2$ conditions. Newman et al. (2003), Teughels et al. (1995), and Sasaki et al. (2002) found that tall fescue grown under elevated CO$_2$ photosynthesized and produced more dry matter than tall fescue grown at
ambient CO$_2$. Newman et al. (2003) also reported significant changes in tall fescue chemical composition and physiology depending on endophyte infection status; E+ tall fescue had less lignin, increased photosynthetic rates, and flowered earlier than E- plants when grown with increased N under elevated CO$_2$ conditions. In 2007, Chen et al. found that E+ tall fescue had increased root biomass compared to E- when grown under elevated CO$_2$; however aboveground biomass was not significantly different between E+ and E- tall fescue. Brosi et al. (2010) used a multi-factor climate change project, which included elevated CO$_2$, temperature, and altered precipitation, to investigate changes in endophyte infection frequency in the tall fescue community and in alkaloid production of E+ tall fescue in response to the manipulated abiotic conditions. Elevated CO$_2$ significantly increased the endophyte infection frequency of tall fescue grown in the old field community but decreased the concentrations of total ergot and loline alkaloids in E+ fescue material. Surprisingly, given the paradigm that endophyte infection enhances tall fescue survival under heat and drought stress, Brosi et al. (2010) measured no change in endophyte infection frequency in treatments promoting these conditions, although elevated temperature did significantly increase loline alkaloid production in E+ material. These results clearly indicate that tall fescue and the endophyte symbiosis are sensitive to changes in climate; however, there are still many unanswered questions regarding the response of this important plant-fungal symbiosis to climate change.

The purpose of this study was to further explore the response of tall fescue and the fungal endophyte symbiosis to elevated temperature and altered precipitation when grown under field conditions. Because tall fescue is a cool-season grass, I hypothesized that elevated
temperature would seasonally alter the growth of tall fescue, increasing growth in the late fall and early spring and reducing fescue growth in the hotter, drier parts of the year (i.e., summer). I predicted that additional summer precipitation would help to reduce the negative aspects of elevated temperature and increase fescue growth (over non-watered plots) during this time. Due to the multiple reports of Neotyphodium mediated drought and heat tolerance, I hypothesized that the fungal endophyte symbiosis would be beneficial to E+ individuals, allowing them to experience greater growth than E- under hotter and drier conditions. I also predicted that E+ individuals would tiller more and suffer less mortality than their E- competitors. I utilized a multi-factor, mixed species climate change project to test these hypotheses.

3.2 Methods

Site Description

This experiment was conducted at the University of Kentucky Spindletop Research Farm in Lexington, Kentucky, USA (38º 10’N, 84º49’W). Prior to beginning this study, the site was a managed hayfield dominated by ‘Select’ endophyte-free tall fescue with a small percentage (< 10%) of red clover (Trifolium pratense), nimblewill (Muhlenbergia schreberi), and Kentucky bluegrass (Poa pratensis).

Historically, this vegetation was cut several times annually for hay production and fertilized sporadically. In March 2008, a relatively level 1800 m² area within this hayfield was sprayed with glyphosate (Roundup Pro; Monsanto, St. Louis, MO, U.S.A.) plowed, disked repeatedly, and then planted to a mixture of hayfield species typical for
the transition zone of the eastern United States (Ball et al., 2007): common toxic endophyte infected and endophyte free (50/50 mixture) ‘Kentucky 31’ tall fescue, Kentucky bluegrass (‘Ginger’), red (Trifolium pretense, ‘Freedom’) and white clovers (Trifolium repens, ‘Patriot’) and Bermuda grass (Cynodon dactylon). Except for the tall fescue seed, which was harvested from research plots at the University of Kentucky (provided by T. Phillips); seed was purchased at a local farm supply store (Woodford Feed, Versailles KY). Seed was homogenized, placed in a conventional planter and then planted at the following rates: E+ tall fescue 11.2kg/ha, E- tall fescue 11.2kg/ha, bluegrass 7.8kg/ha, white clover 2.2kg/ha, and red clover 6.7kg/ha. Bermuda grass plugs from a nearby monoculture stand were transplanted across the whole area in a 25 cm grid pattern in August 2008.

Precipitation at the site is distributed evenly throughout the year with an annual mean of 1163 mm; the 30 year mean annual summer temperature is 23.8°C, with a mean winter temperature of 1.6°C (Ferreira et al., 2010). The soil is mapped as a Bluegrass-Maury silt loam – silty, non-calcareous loess over clayey residuum weathered from phosphatic limestone (Soil Conservation Service, 1967).

Experimental Design

In the spring of 2009, twenty 5.8 m² hexagonal plots were established within the planted study area. The plots, consisting of a uniform mixture of species, were divided into five blocks of four plots each. Each plot within a block was randomly assigned one of four treatments: +Heat, +Precipitation, +Heat+Precipitation or Control.
Treatment Application

Treatments began on May 1, 2009. The temperature of the plant canopy in +Heat plots was maintained at +3°C above ambient, day and night, year-round. Heating was supplied by twelve, 1000W Salamander infrared heaters (Mor Electric Heating Assoc., Comstock Park, MI) arrayed around the plot edge, each pointed toward the ground at the center at a 45° angle. Height of the heaters was maintained at 120 cm above the canopy throughout the experiment. This arrangement allowed for a relatively uniform distribution of heat across the whole area of the plot (see Kimball et al., 2008 for details). The +Precipitation plots received +30% of the long-term mean (+343 mm), applied twice monthly over the growing season in amounts determined by the long-term monthly trends (April-Sept; Table 3.1), during rain events, from rainwater collected on site and applied using metered wands. Aluminum flashing was inserted to a depth of 0.5m around each plot to prevent water from moving horizontally into or out of the plots. The experiment was managed as a hayfield community and, therefore, harvested approximately three times a year with all biomass above 7.6 cm removed. In 2009, the year of this study, the harvests took place June 1, July 21, and September 22.

Sampling and Chemical Analysis

On May 28, 2009, immediately prior to the first harvest, twenty tall fescue tillers were randomly identified in each plot and marked at ground level with a labeled washer. These tillers were cut at 7.6 cm height and cross sections of the base of the cut tiller were blotted onto nitrocellulose paper. Endophyte presence was determined using an enzyme-linked, endophyte specific immunosorbent assay (Hiatt et al., 1999). Endophyte status of
individual tillers was rechecked at each of the three harvests utilizing this same method. Tall fescue growth was measured weekly during the growing season (May 28 –November 11, 2009), bi-weekly during the winter (November 11, 2009 – March 15, 2010), then again weekly from March 15 until May 10, 2010. Using a mobile platform to prevent trampling the plots, pseudostem and leaf growth were measured separately using a metric ruler. Pseudostem growth was measured from the ground up, while leaf growth was measured from leaf origin at the pseudostem to the apex of the leaf; in cases where there was dieback, the measurement ceased at the last visible green material on the leaf (Figure 3.1). When dieback was significant, negative rates of growth were recorded. Plants were considered dead, and recorded as such, when they contained no visible green material aboveground. Total growth (the sum of all measurements, including any dieback) is presented here. I also recorded major instances of herbivory – where > 50% of the aboveground biomass was removed from the tiller.

At each of the three harvest dates, the marked tillers were harvested along with the rest of the plot at a height of 7.6 cm. For the June and July harvest, tillers were kept cold in transit to the lab where the immunosorbent assay could be used to determine (or confirm) endophyte infection status. Results from this test guided the compositing of samples into either E+ or E- fescue pools for each plot. These samples were then oven dried at 55°C for 48 hours after which tiller mass was measured using an analytical balance. The tillers were then combined into groups based on treatment and endophyte status and then ground through a 1 mm screen on a Model 4 Wiley Mill (Thomas Scientific Inc., Slidesboro, NJ). An ANKOM fiber analyzer (Ankom Technology Inc., Fairport, NY) was
used to determine the percent cellulose, hemicellulose, and lignin on 5g sub-samples of the E+ and E- tiller groups using the Van Soest (1963) method. Additionally a sub-sample of the ground material was evaluated for carbon and nitrogen content using a Flash EA1112 elemental analyzer (CE Elantech Inc, Lakewood, NJ).

Because the *Neotyphodium* endophyte is known to produce alkaloids that are not present in E- tall fescue (Bush et al., 1997), I determined the alkaloid content of E+ material only. Previous research has shown that alkaloid concentrations vary seasonally, increasing as the tall fescue plant ages (Belesky and Hill, 1996); I therefore determined alkaloid concentrations for the September harvest only. Like the previous harvests, endophyte infection was checked with an immunosorbent assay; however, for this harvest, tillers were kept at -80°C until they could be freeze-dried. Once freeze dried, composited E+ and E- samples (one each per plot) were treated exactly like the previous harvests: subsamples were ground and then analyzed for tissue chemistry parameters using the Van Soest (1963) method and evaluated for carbon and nitrogen with the elemental analyzer. In addition, E+ subsamples were analyzed for alkaloids. A modified high performance liquid chromatography (HPLC) procedure developed by Yates & Powell (1988) was used on a 0.5g subsample of E+ material to assess the ergot alkaloids - ergovaline and ergovalinine. An Alltima C18 150 mm x 4.6 mm column with 3 μ particle size was used for the separations (Alltech Inc., Nicholasville, KY). Solutions used for elution were 75 mM ammonium acetate (A) in water:acetonitrile (3:1. v/v) and acetonitrile (B). Elution gradient was 95:5 (A:B) 1 min; linear change to 60:40 (A:B) during the next 15 min and maintained for 5 minutes; changed to 0:100 (A:B) in 1.5 min and maintained for 5 min;
changed to 100:0 (A:B) in 1 min and maintained for 6 min before returning to 95:5. A
0.25g subsample of ground material was used to determine the loline alkaloids; \( N \)-formyl loline, \( N \)-acetyl loline, and \( N \)-acetyl norloline. Ethanol-methylene chloride containing quinoline and sodium bicarbonate was used for the extraction and lolines were quantified using gas chromatography following the Blankenship et al. (2001) protocol.

Statistical Analyses

The purpose of this experiment was to determine whether endophyte infection status altered the growth, survivorship, and tissue chemistry of tall fescue exposed to differing temperature and precipitation regimes. Tests for normality were performed on all data, and if needed, data were transformed to adjust their distributions. Because endophyte status was not known until sampling ensued, the growth data was unbalanced for endophyte status. All possible means comparisons were analyzed and comparisons were considered significant at \( p < 0.05 \).

Growth and mortality data were analyzed separately for each of the harvest periods: the early summer (June 1 – July 21, 2009), midsummer (July 22 – Sept 22, 2009), and fall through spring (Sept 23, 2009 – May 10, 2010). Due to relatively high mortality of tillers during the experiment, equal numbers of additional tillers were added to each plot at the beginning of each harvest period as needed. This addition of tillers allowed me to compare data within these periods and kept numbers of tillers high enough to perform robust statistical analyses; however, the addition of new tillers each period meant I was unable to statistically compare data between the periods (i.e., this violated the
assumptions of repeated measures tests across periods for both growth and mortality measurements). Data were analyzed using an repeated measures analysis of variance (ANOVA) (PROC MIXED, SAS Institute, Cary NC, USA) to test for the effects of elevated temperature, added precipitation, date, Neotyphodium infection, and all possible interactions on tall fescue growth. A 2x2 split-plot factorial design was used for the analysis where endophyte status (E+ vs. E-) was the split plot. Temperature, precipitation and their interactions were considered fixed effects, while block, temperature, precipitation and their interactions were included as random effects.

Tillering data collection occurred during two distinct time periods, the fall of 2009 and spring of 2010. A generalized linear mixed model with discrete Poisson response distribution models with repeated measures on each plant was used to determine differences of treatment, endophyte status, and date; all of which were considered fixed effects.

Tissue chemistry was analyzed using a split-plot 2x2 factorial design ANOVA, with heat, precipitation and date as fixed affects and endophyte status (E+ vs. E-) as the split plot. Because alkaloids were only measured in E+ fescue and only in September, the statistical analysis for this dataset was performed as a whole plot, randomized complete block design, where temperature and precipitation were treated as fixed effects and block was included as a random effect.
3.3 Results

*Tissue Chemistry*

All measured tall fescue tissue chemistry parameters were significantly affected by date of harvest (Table 3.2), and interactions between the climate treatments, date, and endophyte status. The significant date effect on tissue chemistry reflects well-known physiological changes associated with tillers maturing throughout the growing season (Burns et al., 2002). Tall fescue tillers harvested in July had the lowest cell structural components, % lignin, and C:N ratio, while the June harvest had the highest amounts of these measured parameters (data not shown). While there were no significant main effects of endophyte infection or the climate treatments for these parameters, under elevated heat, E+ tall fescue had significantly more structural carbohydrates (%cellulose, hemicelluloses and lignin) than E- (60% vs. 57% for E+ vs. E- respectively; Table 3.2; Figure 3.2a). Under elevated temperature, E+ tall fescue also had significantly higher %Lignin than E- (9.9% vs. 7.7% for E+ vs. E- respectively; Table 3.2; Figure 3.3b); however the opposite was true for the +Heat+Precipitation treatments (8.0% vs. 9.8% for E+ vs. E- respectively; Figure 3.3b). Precipitation interacted with endophyte status and date to affect lignin as well (Figure 5b). In July, E+ fescue had lower % lignin under elevated precipitation than E-, while for September this trend was reversed (Figure 3.3b). Endophyte infection status also interacted with date of harvest to alter the C:N ratio of tall fescue material. For the June 1 harvest, the C:N ratio of E+ tall fescue was higher than that of E-; however, for the July and September harvests, the C:N ratio of E+ was lower than E- (Figure 3.3a).
Alkaloid concentrations, specifically ergovaline, N-acetyl nortoline, N-acetyl loline, and total lolines, were significantly increased by elevated temperature, but were unaffected by altered precipitation (Table 3.4; Figure 3.4). Compared to ambient conditions, heated treatments experienced +23-63% increases in alkaloid concentrations, depending on the compound. Ergovalinine and N-formyl loline did not significantly respond to either elevated heat or altered precipitation (Table 3.4).

**Tiller Growth**

Tall fescue growth in early summer (Period 1) was significantly affected by elevated temperature, altered precipitation and interactions between these treatments and with date (Table 3.4). Total growth in the early summer period was reduced by 49% under elevated temperature but was increased by 20% with increased precipitation over the ambient control (Figure 3.5a). Contrary to our prediction that elevated temperature would significantly reduce tall fescue growth during the mid-summer period, the growth of tall fescue was unaffected by either the heat or precipitation treatments during this period 2 (Table 3.4; Figure 3.5b). However, total fescue growth from the fall through the spring was significantly affected by treatment and treatment interactions with date (Table 3.4). In contrast to the early summer period, during the fall through spring, elevated heat significantly increased the growth of tall fescue compared to ambient heat by ~42% (Figure 3.5c). The elevated temperature treatments had higher growth than the combination of elevated temperature and precipitation (Figure 3.5c). For all measurement periods, treatment by date interactions reflect that climate treatments did not significantly affect tall fescue growth until several weeks post-harvest (Table 3.4;
Contrary to our hypothesis that endophyte infection would positively influence tall fescue growth under drought stress, there was no significant effect or interaction of endophyte infection on tall fescue growth for any of the measurement periods (Table 3.5).

**Tillering**

Tall fescue tillering was significantly affected by date and the precipitation treatment. Tall fescue had significantly higher tillering in the spring than in the fall, averaging 3.9 new tillers produced in the spring and 3.2 in the fall ($p = 0.0021$; data not shown). When comparing across time periods, the increased precipitation treatment showed significantly more tall fescue tillering, although this increase was small (4 additional tillers under +Precipitation vs. 3 additional tillers under ambient Precipitation; $p = 0.0277$; Figure 8a). There was no main effect of endophyte status on tall fescue tillering. While date of harvest and precipitation were the only significant ($p > 0.05$) main effects impacting tillering, there were several marginally significant results. There was a trend for increased tillering under elevated heat (4 new tillers in +heat versus 3.2 in ambient (data not shown); $p = 0.0518$). Under elevated temperature ($p = 0.0572$), as well as under additional precipitation ($p = 0.0522$), there was a trend for increased tillering of E+ vs. E-fescue (approximately 1 additional tiller in +Heat and +Precipitation for E+ compared to E-; data not shown). A non-significant three way interaction ($p = 0.0529$) showed increased tillering of E+ under elevated temperature in the spring but not for the fall (data not shown).
Tiller Mass

Tiller mass was significantly affected by elevated temperature, date of harvest, and interactions between elevated temperature and date; however tiller mass was not significantly different based on endophyte infection status or precipitation treatment (Table 3.4). There was no significant difference in tiller weights for the first harvest (Figure 3.6b). However, tiller mass was significantly lower for the July and September harvests under elevated heat compared to ambient (Figure 3.6b).

Herbivory

Herbivory events were recorded on tillers where >50% of the aboveground biomass was removed by an herbivore. Of the 12,800+ tiller measurements for the experiment only 28 herbivory events were recorded, giving a probability of 0.2% that a tiller would experience herbivory. There were no apparent trends for treatment, endophyte status, or date for herbivory events during the experiment (data not shown).

Mortality

Tall fescue mortality was significantly affected by elevated temperature in the mid-summer period and by the interaction between elevated temperature and endophyte status in the early summer (Table 3.5). However, contrary to my hypothesis that endophyte infection would reduce abiotic stress for tall fescue and thereby reduce mortality of E+ tillers growing under elevated heat treatments especially during the hot summer, E+ tall fescue had significantly higher mortality under elevated temperature than E- for the early
summer growing period (54% mortality for E+ vs. 37% for E-; Figure 9a). For the second growing period, tall fescue mortality was 54% higher under elevated heat than ambient conditions ($p = 0.0217$), but there was no interaction with endophyte status (Figure 3.7b). The precipitation treatment had no significant effect on mortality at any point during the experiment, and tall fescue mortality was not significantly affected by climate treatments or endophyte status during the fall – spring period (Table 3.7). While we cannot statistically compare between periods, fescue mortality was highest in the early summer (averaging ~38%) and lowest during the fall through spring period (~10%; data not shown).

3.4 Discussion
Numerous studies have reported benefits of *Neotyphodium* infection for tall fescue growing under and recovering from drought stress (Arachevaleta et al., 1989; Bacon, 1993; West et al., 1993; Marks and Clay, 1996; Malinowski 1999). Because of this previous research, I hypothesized that under elevated temperature, endophyte infection would provide significant growth advantages compared to fescue without the endophyte. However contrary to my hypothesis, my results show no differences between E+ and E-tall fescue growth for any of the climate treatments over the year of study (Figure 3.5), and surprisingly, unpredicted, relatively large differences in mortality. Under elevated temperature, E+ fescue had significantly higher mortality than E- (Figure 3.7a), suggesting there is a disadvantage of endophyte infection under elevated temperature. Endophyte infection did not significantly increase tillering of fescue under ambient conditions (which has been reported by Arachevaleta et al., 1989) but there was a trend
for slightly increased tillering for E+ fescue under elevated temperature (data not shown). Small but significant differences between E+ and E- tall fescue tissue chemistry relative to climate treatments (along with treatment differences for E+ alkaloid production) show that the endophyte and the plant are interacting and responding to the climate treatments (Figures 3.4 & 3.5).

Our climate treatments significantly increased canopy (+2.75°C) and soil surface (+2.66°C) temperatures, and either reduced (by 9.54% under +heat) or increased (by 1.97% under +precipitation) the top 10 cm volumetric soil moisture content of the plots over the course of the study (data not shown). However, the ambient conditions for the year of this study were abnormally cool and moist for the region. During the early summer growing period, the ambient temperature plots only had 3 days with maximum daily temperatures ≥ 30°C; however our manipulated warming of the plots resulted in 14 days ≥ 30°C in the elevated temperature plots. This increase in temperatures resulted in a significant reduction in tall fescue growth during this early summer period, which is consistent with the temperature optimas known for this species (20-25°C; Volenec et al., 1984), and this effect was not eliminated by added precipitation (i.e., the +heat+precipitation treatment had lower total growth than ambient). Surprisingly, despite it being unusually wet, additional precipitation increased tall fescue growth over ambient during this period. For the second growing period, ambient conditions were again relatively cool, with only two days ≥ 30°C; however, elevated temperature plots experienced daytime maximum temperatures ≥ 30°C for approximately 50% of this period. Given this dramatic temperature difference, it is especially noteworthy that our
treatments (including additional precipitation) did not influence tall fescue growth (Figure 3.5b). Marks and Clay (1996) reported benefits of endophyte infection relative to tall fescue photosynthesis $\geq 35^\circ C$. During the entire study period, the maximum temperature in our ambient plots never exceeded $35^\circ C$; however temperature $\geq 35^\circ C$ occurred once for the early summer period and 10 times for the midsummer growing period in the elevated heat treatment (data not shown). Because of these significant differences in temperature, it is surprising that endophyte infection did not influence tall fescue growth relative to climate treatment.

The increased mortality of endophyte infected tall fescue under elevated heat is puzzling and suggests there is a cost to this symbiosis. However, the fact that endophyte infection did not reduce growth rates or indices of nutrient acquisition (i.e. %N) suggests that such ‘symbiotic costs’ may be specific to certain parameters. Costs of endophyte infection have been noted for other grass species. For example, Ahlholm et al. (2002) reported decreased biomass, tillering and seed production of endophyte infected Festuca rubra and Schedonorus pratensis plants growing under low soil nutrients. Saari et al. (2010) noted decreased regrowth after clipping of E+ vs. E- Schedonorus pratensis. For Festuca arizonica, Faeth et al. (2004) documented decreased root and shoot biomass of E+ vs. E-plants, and Faeth and Sullivan (2003) reported decreased biomass and reproduction of endophyte infected tillers. Our results are contradictory to tall fescue literature showing endophyte infected plants experience benefits related to heat and drought tolerance (we saw no evidence for this in the growth data), but instead, the mortality data suggests
elevated temperature is inducing a possible shift of the tall fescue-endophyte symbiosis from mutualistic to parasitic.

Another often-cited benefit of *Neotyphodium* infection for tall fescue is the reduction or re-direction of herbivory through the production of toxic alkaloids. The low degree of herbivory on tall fescue observed in this experiment may possibly explain the overall lack of endophyte effects on fescue growth parameters. Several studies have shown herbivory to be the major significant factor contributing to the frequency of E+ vs. E- fescue within a community (Koh and Hik, 2007; Grundel et al., 2008). However it also clear from my study that endophyte production of alkaloids was responsive to the climate treatments. Concentrations of ergovaline and total loline alkaloids significantly increased under elevated temperature (Figures 3.4a & 3.4b). The increase in total acetyl loline alkaloids under elevated temperature is consistent with prior findings (Brosi et al., 2010) and is likely to be significant to herbivores (especially insects) consuming tall fescue growing under these conditions. Increased ergovaline is also likely to have a significant negative effect on mammalian herbivores consuming tall fescue and may exacerbate warm-season fescue toxicosis. With regard to agricultural impacts, it is also interesting that although elevated temperature did not significantly affect tall fescue midsummer growth rates, tiller mass was significantly reduced under elevated temperature for the July harvest (Figure 8b). This suggests a possible reduction in biomass for haying operations and forage for livestock under hotter conditions. This reduced tiller weight could also mean less carbon storage available for plant use to buffer against future stress. To date, neither tall fescue biomass nor endophyte infection frequency have shown significant responses to the climate treatments.
Because of reported endophyte mediated biotic and abiotic stress resistance, the relationship between tall fescue and *Neotyphodium* is usually considered a mutualism (Clay and Schardl, 2002) and has been reported to be a “perfect partnership” (Christensen and Voisey, 2006). However, our results show that the *Neotyphodium* relationship might not be as beneficial to tall fescue’s response to drought stress as previously thought. Increased mortality of E+ tall fescue under elevated heat could lead to decreased endophyte infection frequencies under climate change scenarios in the southeast; however more research, especially multiple year research, will be needed to determine the long term affects of climate change on the tall fescue community. Increased alkaloid production under elevated temperature is likely to have significant affects on the herbivores that consume E+ tall fescue; while seasonal changes in fescue growth may also impact herbivore communities as well as ecosystem properties such as nutrient cycling. Clearly more research is warranted to elucidate the tall fescue – *Neotyphodium* response to climate change and the implications for this common grass in the future.
Table 3.1: Dates and amounts of precipitation additions for experimental period (using rainwater collected on site).

<table>
<thead>
<tr>
<th>Date</th>
<th>Precip. Added (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jun 3, 2009</td>
<td>25.4</td>
</tr>
<tr>
<td>Jun 10, 2009</td>
<td>28.2</td>
</tr>
<tr>
<td>Jul 12, 2009</td>
<td>28.2</td>
</tr>
<tr>
<td>Jul 22, 2009</td>
<td>28.2</td>
</tr>
<tr>
<td>Aug 11, 2009</td>
<td>28.2</td>
</tr>
<tr>
<td>Aug 28, 2009</td>
<td>28.2</td>
</tr>
<tr>
<td>Sep 10, 2009</td>
<td>25.4</td>
</tr>
<tr>
<td>Oct 1, 2009</td>
<td>25.4</td>
</tr>
<tr>
<td>Apr 16, 2010</td>
<td>25.4</td>
</tr>
<tr>
<td>Apr 29, 2010</td>
<td>25.4</td>
</tr>
<tr>
<td>May 12, 2010</td>
<td>25.4</td>
</tr>
<tr>
<td>May 28, 2010</td>
<td>25.4</td>
</tr>
</tbody>
</table>
Table 3.2: Main treatment effects and interactions for tall fescue tissue chemistry data (bolding indicates p-values < 0.05).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% Cellulose &amp; Hemicellulose</th>
<th>% Lignin</th>
<th>% Carbon</th>
<th>% Nitrogen</th>
<th>C:N ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>df&lt;sub&gt;n,d&lt;/sub&gt; f-value p-value</td>
<td>df&lt;sub&gt;n,d&lt;/sub&gt; f-value p-value</td>
<td>df&lt;sub&gt;n,d&lt;/sub&gt; f-value p-value</td>
<td>df&lt;sub&gt;n,d&lt;/sub&gt; f-value p-value</td>
<td>df&lt;sub&gt;n,d&lt;/sub&gt; f-value p-value</td>
</tr>
<tr>
<td>Endophyte (Endo)</td>
<td>1.16 0.39 0.5398</td>
<td>1.16 0.05 0.8258</td>
<td>1.16 0.50 0.4909</td>
<td>1.16 0.44 0.2473</td>
<td>1.16 0.73 0.4054</td>
</tr>
<tr>
<td>Temperature (Temp)</td>
<td>1.12 2.10 0.1726</td>
<td>1.12 1.25 0.2849</td>
<td>1.12 0.61 0.4481</td>
<td>1.12 1.17 0.3005</td>
<td>1.12 1.26 0.2835</td>
</tr>
<tr>
<td>Precipitation (Precip)</td>
<td>1.12 1.88 0.1958</td>
<td>1.12 0.19 0.6692</td>
<td>1.12 0.12 0.7372</td>
<td>1.12 0.02 0.9003</td>
<td>1.12 0.00 0.9916</td>
</tr>
<tr>
<td>Date</td>
<td>2.64 129.92 &lt;0.0001</td>
<td>2.64 200.41 &lt;0.0001</td>
<td>2.64 178.73 &lt;0.0001</td>
<td>2.64 42.13 &lt;0.0001</td>
<td>2.64 39.85 &lt;0.0001</td>
</tr>
<tr>
<td>Temp * Endo</td>
<td>1.16 5.91 0.0271</td>
<td>1.16 0.17 0.6821</td>
<td>1.16 1.87 0.1909</td>
<td>1.16 0.24 0.6275</td>
<td>1.16 0.15 0.7023</td>
</tr>
<tr>
<td>Precip * Endo</td>
<td>1.16 3.83 0.0682</td>
<td>1.16 3.52 0.0789</td>
<td>1.16 0.13 0.7185</td>
<td>1.16 1.58 0.2274</td>
<td>1.16 1.14 0.3023</td>
</tr>
<tr>
<td>Date * Endo</td>
<td>2.64 0.68 0.5111</td>
<td>2.64 0.16 0.8532</td>
<td>2.64 1.45 0.2422</td>
<td>2.64 2.90 0.0624</td>
<td>2.64 3.98 0.0235</td>
</tr>
<tr>
<td>Temp * Precip</td>
<td>1.12 5.73 0.0339</td>
<td>1.12 0.35 0.5648</td>
<td>1.12 0.32 0.5849</td>
<td>1.12 0.63 0.4418</td>
<td>1.12 0.62 0.4454</td>
</tr>
<tr>
<td>Temp * Date</td>
<td>2.64 3.36 0.0408</td>
<td>2.64 2.37 0.1017</td>
<td>2.64 0.67 0.5171</td>
<td>2.64 1.69 0.1920</td>
<td>2.64 1.46 0.2407</td>
</tr>
<tr>
<td>Precip * Date</td>
<td>2.64 0.71 0.4977</td>
<td>2.64 2.82 0.0671</td>
<td>2.64 1.81 0.1718</td>
<td>2.64 1.84 0.1667</td>
<td>2.64 1.25 0.2935</td>
</tr>
<tr>
<td>Temp<em>Precip</em>Endo</td>
<td>1.16 10.59 0.0050</td>
<td>1.16 6.95 0.0179</td>
<td>1.16 0.01 0.9324</td>
<td>1.16 0.41 0.5313</td>
<td>1.16 0.21 0.6551</td>
</tr>
<tr>
<td>Temp<em>Precip</em>Date</td>
<td>2.64 0.74 0.4807</td>
<td>2.64 3.89 0.0254</td>
<td>2.64 4.77 0.0117</td>
<td>2.64 0.59 0.5581</td>
<td>2.64 0.60 0.5515</td>
</tr>
<tr>
<td>Temp<em>Endo</em>Date</td>
<td>2.64 2.87 0.0639</td>
<td>2.64 1.12 0.3336</td>
<td>2.64 0.07 0.9336</td>
<td>2.64 0.05 0.9492</td>
<td>2.64 0.10 0.9053</td>
</tr>
<tr>
<td>Precip<em>Endo</em>Date</td>
<td>2.64 0.26 0.7695</td>
<td>2.64 3.31 0.0429</td>
<td>2.64 0.26 0.7746</td>
<td>2.64 0.31 0.7353</td>
<td>2.64 0.10 0.9021</td>
</tr>
<tr>
<td>Temp<em>Precip</em>Endo*Date</td>
<td>2.64 1.25 0.2932</td>
<td>2.64 0.51 0.6012</td>
<td>2.64 1.22 0.3008</td>
<td>2.64 0.20 0.8170</td>
<td>2.64 0.28 0.7603</td>
</tr>
</tbody>
</table>
Table 3.3: Statistical results (bolding indicates p-values < 0.05) for parameters where fungal endophyte presence was not included in the statistical model (alkaloid production was measured from endophyte infected material only). Alkaloids were determined for the September harvest only. NANL = N-acetyl norloline, NFL = N-fomyl loline, and NAL = N-acetyl loline.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Ergovaline</th>
<th>Ergovalinine</th>
<th>Total Ergots.</th>
<th>NANL</th>
<th>NFL</th>
<th>NAL</th>
<th>Total Lolines</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>df&lt;sub&gt;n,d&lt;/sub&gt;</td>
<td>f-value</td>
<td>p-value</td>
<td>f-value</td>
<td>p-value</td>
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<tr>
<td>Temperature</td>
<td>1,16</td>
<td>4.79</td>
<td>0.0492</td>
<td>0.71</td>
<td>0.4149</td>
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<td>0.1115</td>
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<tr>
<td>Precipitation</td>
<td>1,16</td>
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<td>0.7554</td>
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<td>0.7492</td>
<td>0.00</td>
<td>0.9477</td>
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<tr>
<td>Temp*Precip</td>
<td>1,16</td>
<td>2.14</td>
<td>0.1696</td>
<td>2.57</td>
<td>0.1349</td>
<td>2.33</td>
<td>0.1531</td>
</tr>
</tbody>
</table>
Table 3.4: Statistical results of climate treatments and endophyte status effects and interactions on tall fescue growth (for the three measurement periods), tillering, and tiller mass (bolding indicates p-values < 0.05).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Total Growth Period 1</th>
<th>Total Growth Period 2</th>
<th>Total Growth Period 3</th>
<th>Tillering</th>
<th>Tiller Mass</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>df, n, f-value, p-value</td>
<td>df, n, f-value, p-value</td>
<td>df, n, f-value, p-value</td>
<td>df, n, f-value, p-value</td>
<td>df, n, f-value, p-value</td>
</tr>
<tr>
<td>Endophyte (Endo)</td>
<td>1.16, 0.03, 0.8550</td>
<td>1.16, 0.00, 0.9643</td>
<td>1.16, 1.60, 0.2245</td>
<td>1.16, 0.02, 0.9039</td>
<td>1.16, 0.28, 0.6025</td>
</tr>
<tr>
<td>Temperature (Temp)</td>
<td>1.12, 74.67, &lt;0.0001</td>
<td>1.16, 1.23, 0.2893</td>
<td>1.16, 90.62, &lt;0.0001</td>
<td>1.16, 0.66, 0.0518</td>
<td>1.16, 15.39, 0.0020</td>
</tr>
<tr>
<td>Precipitation (Precip)</td>
<td>1.12, 30.64, 0.0001</td>
<td>1.16, 0.17, 0.6889</td>
<td>1.16, 6.57, 0.0249</td>
<td>1.16, 6.27, 0.0277</td>
<td>1.16, 3.52, 0.0851</td>
</tr>
<tr>
<td>Date</td>
<td>5.160, 553.51, &lt;0.0001</td>
<td>5.160, 262.22, &lt;0.0001</td>
<td>5.160, 159.36, &lt;0.0001</td>
<td>1.32, 16.51, 0.0003</td>
<td>2.64, 11.35, &lt;0.0001</td>
</tr>
<tr>
<td>Temp * Endo</td>
<td>1.12, 2.39, 0.1420</td>
<td>1.12, 0.82, 0.3791</td>
<td>1.12, 1.65, 0.2168</td>
<td>1.12, 4.20, 0.0572</td>
<td>1.12, 0.20, 0.6603</td>
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<tr>
<td>Precip * Endo</td>
<td>1.16, 0.17, 0.6827</td>
<td>1.16, 0.11, 0.7409</td>
<td>1.16, 0.65, 0.4322</td>
<td>1.16, 4.40, 0.0522</td>
<td>1.16, 0.05, 0.8236</td>
</tr>
<tr>
<td>Date * Endo</td>
<td>5.160, 2.20, 0.0572</td>
<td>5.160, 0.85, 0.2784</td>
<td>5.160, 0.88, 0.6215</td>
<td>1.32, 0.39, 0.5371</td>
<td>2.64, 1.10, 0.3380</td>
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<tr>
<td>Temp * Precip</td>
<td>1.12, 1.11, 0.3138</td>
<td>1.12, 0.82, 0.5791</td>
<td>1.12, 6.70, 0.0237</td>
<td>1.12, 1.7, 0.2997</td>
<td>1.12, 0.12, 0.7313</td>
</tr>
<tr>
<td>Temp * Date</td>
<td>5.160, 22.10, &lt;0.0001</td>
<td>5.160, 1.18, 0.3158</td>
<td>5.160, 3.57, &lt;0.0001</td>
<td>1.32, 1.22, 0.2775</td>
<td>2.64, 3.91, 0.0250</td>
</tr>
<tr>
<td>Precip * Date</td>
<td>5.160, 11.51, &lt;0.0001</td>
<td>5.160, 2.02, 0.0537</td>
<td>5.160, 1.21, 0.2327</td>
<td>1.32, 0.57, 0.4561</td>
<td>2.64, 1.71, 0.1882</td>
</tr>
<tr>
<td>Temp<em>Precip</em>Endo</td>
<td>1.16, 0.00, 0.9905</td>
<td>1.16, 1.76, 0.2030</td>
<td>1.16, 0.20, 0.6624</td>
<td>1.32, 0.50, 0.4885</td>
<td>2.64, 0.25, 0.6235</td>
</tr>
<tr>
<td>Temp<em>Precip</em>Date</td>
<td>5.160, 5.26, 0.0002</td>
<td>5.160, 1.27, 0.2646</td>
<td>5.160, 1.19, 0.2504</td>
<td>1.32, 0.78, 0.3838</td>
<td>2.64, 1.68, 0.1936</td>
</tr>
<tr>
<td>Temp<em>Endo</em>Date</td>
<td>5.160, 0.45, 0.8099</td>
<td>5.160, 0.52, 0.8177</td>
<td>5.160, 0.80, 0.7180</td>
<td>1.32, 4.04, 0.0529</td>
<td>2.64, 1.53, 0.2246</td>
</tr>
<tr>
<td>Precip<em>Endo</em>Date</td>
<td>5.160, 0.49, 0.7807</td>
<td>5.160, 0.39, 0.9100</td>
<td>5.160, 1.19, 0.2479</td>
<td>1.32, 0.47, 0.4901</td>
<td>2.64, 0.65, 0.5253</td>
</tr>
<tr>
<td>Temp<em>Precip</em>Endo*Date</td>
<td>5.160, 1.46, 0.2061</td>
<td>5.160, 1.33, 0.2364</td>
<td>5.160, 0.86, 0.6504</td>
<td>1.32, 2.73, 0.1086</td>
<td>2.64, 1.42, 0.2493</td>
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</table>
Table 3.5: Statistical results of climate treatments and endophyte status and their interactions on tall fescue mortality during each of the three measurement periods, early summer, midsummer, and fall through spring (bolding indicates p-values < 0.05).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mortality</th>
<th>Mortality</th>
<th>Mortality</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Early Summer</td>
<td>Mid Summer</td>
<td>Fall - Spring</td>
</tr>
<tr>
<td></td>
<td>df_n,_d</td>
<td>f-value</td>
<td>p-value</td>
</tr>
<tr>
<td>Endophyte (Endo)</td>
<td>1,16</td>
<td>1.60</td>
<td>0.2337</td>
</tr>
<tr>
<td>Temperature (Temp)</td>
<td>1,12</td>
<td>2.79</td>
<td>0.1205</td>
</tr>
<tr>
<td>Precipitation (Precip)</td>
<td>1,12</td>
<td>0.12</td>
<td>0.7346</td>
</tr>
<tr>
<td>Temp * Endo</td>
<td>1,16</td>
<td>8.11</td>
<td><strong>0.0116</strong></td>
</tr>
<tr>
<td>Precip * Endo</td>
<td>1,16</td>
<td>0.29</td>
<td>0.5991</td>
</tr>
<tr>
<td>Temp * Precip</td>
<td>1,16</td>
<td>0.07</td>
<td>0.7930</td>
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<tr>
<td>Temp<em>Precip</em>Endo</td>
<td>1,16</td>
<td>0.31</td>
<td>0.5874</td>
</tr>
</tbody>
</table>
Figure 3.1: Growth Measurements of tillers. On each measurement date, tillers were measured from the ground up, keeping separate measurements for pseudostem (1+3 on Figure 1) and leaves (2+4+5+6). In cases of dieback, leaves were measured to the last green material as shown below (5).
Figure 3.2: Temperature by endophyte interaction on a) tall fescue structural carbohydrates (cellulose, hemicelluloses & lignin) and b) tall fescue % lignin. Cross-hatch bars indicate E+ fescue (endophyte present) while open bars are E- (endophyte absent). Values are means ± 1 S.E.
Figure 3.3: Endophyte by date interaction affecting tall fescue C:N ratio (a) and precipitation by endophyte by date interaction for tall fescue % lignin (b). Values are means ± 1 S.E.
Figure 3.4: Effect of elevated temperature on a) total lolines and b) ergovaline for endophyte infected tall fescue material harvested in September. Values are means ± 1 S.E.
Figure 3.5: Effects of climate treatments on total tall fescue growth by time period. Values are averaged across endophyte status and represent means ± 1 S.E. Total growth is the sum of all psuedostem and leaf measurements.
Figure 3.6: Effects of climate treatments on tall fescue tillering (a) and the interactive effect of elevated temperature and date on tall fescue tiller mass (b). Values are means ± 1 S.E.
Figure 3.7: Effects of elevated temperature and endophyte status on tall fescue mortality during the early summer growing period (a) and elevated temperature effect on tall fescue mortality for the mid-summer growing period (b). Values are means ± 1 S.E.
Chapter 4: Conclusions

Anthropogenic increases in atmospheric CO$_2$ concentrations contribute to climate change, increasing annual temperatures and altering regional precipitation patterns (IPCC, 2007). My thesis research had two main focuses: 1) the response of the most common cool-season grass in the eastern USA, tall fescue, to climate change; and 2) the role of a fungal endophyte symbiosis in mediating the response of the plant.

Previous research has shown that the fungal endophyte of tall fescue influences the way infected (E+) plants respond to and recover from abiotic stress. Several studies have reported that E+ tall fescue has significant advantages over endophyte free fescue (E-) under drought conditions (Arachevalta et al., 1989; Marks and Clay, 1996; Malinowski and Belesky, 1999), which may be especially important in a future world with higher temperatures and altered precipitation patterns. While previous studies have reported that tall fescue is sensitive to changes in atmospheric CO$_2$ (Teughels et al., 1995; Sasaki et al., 2003), few have considered the effect of endophyte infection in the plant response (Newman et al., 2003, Chen et al., 2007), and no previous study had considered any climate change variable except elevated atmospheric CO$_2$. Because toxic alkaloids produced by E+ tall fescue have significant economic (Hoveland 1993) and ecological (Rudgers et al., 2004) effects, I was particularly interested in quantifying changes in alkaloid production relative to alterations in climate, which to my knowledge had not been previously explored in the scientific literature.
Using a multi-year, multi-factor climate change study at the Oak Ridge National Laboratory, I was able to show that climate change affected the frequency of endophyte infection within tall fescue communities, as well as toxic alkaloid production within E+ individuals. Under elevated CO$_2$ conditions, the proportion of E+, compared to E-, tall fescue tillers increased, while the alkaloid concentrations of E+ individuals decreased. Because multiple studies have shown the benefits of endophyte infection in tall fescue’s response to drought, I was surprised to find that the endophyte infection frequency of tall fescue did not increase under the high heat and low water treatments employed in this experiment. We did, however, find that under elevated temperature and under dry conditions loline alkaloid production increased in E+ individuals, which is consistent with the hypothesis that alkaloids are involved in the plant response to abiotic stress.

For the second part of my thesis, I utilized the University of Kentucky Forage Climate Change Project to investigate the response of tall fescue growth and tissue chemistry to altered temperature and precipitation. I found that these climate treatments seasonally affected the growth of tall fescue; however these growth responses were not dependent on the endophyte status of the plant. In the early summer of 2009 (i.e., June), elevated temperature decreased tall fescue growth while increased precipitation stimulate it. However, from the fall of 2009 through the spring of 2010, elevated temperature significantly increased tall fescue growth and precipitation had no effect. While endophyte infection had no effect on tall fescue growth, it did interact with the climate treatments to affect the tissue chemistry of tall fescue in small but significant ways. These small changes in tissue chemistry may not be significant to herbivores, but they
indicate that the endophyte and climate treatments are interacting in ways that alter the physiology of tall fescue. Similar to the Oak Ridge project, elevated temperature significantly increased alkaloid production in E+ tall fescue. However, elevated temperature also significantly increased the mortality of tall fescue during the summer of 2009, and surprisingly, E+ tillers experienced significantly higher mortality than E- under elevated temperature in the early summer portion of the experiment.

In summary, my results show that tall fescue and its relationship with the fungal endophyte is responsive to predicted climate change. Elevated temperatures are likely to alter the seasonal growth of tall fescue, increasing fall through spring growth but decreasing growth in the early summer. Increasing precipitation in the early summer alleviated some of the negative aspects of elevated temperature on tall fescue growth, but it is noteworthy that the combined treatment (elevated temperature and heat) still exhibited growth below that of ambient conditions during this time period. While endophyte infection did not affect tall fescue growth response to the climate treatments, it is clear that the endophyte influenced tall fescue’s tissue chemistry response to elevated CO₂ and temperature, as well as altered precipitation. The measured changes in alkaloid production, in particular, are likely to have significant effects on herbivores that consume E+ tall fescue in a hotter, potentially drier, and more CO₂-enriched world. The observed increase in mortality of E+ tall fescue under elevated temperature in the early summer is opposed to the bulk of research on the tall fescue-endophyte relationship and merits further research into the response of this symbiosis to climate change. Given the broad geographic distribution of this symbiosis and its economic and ecological importance,
more research, especially multi-year research, is needed to further explore tall fescue’s response to predicted climate change and the response of the fungal endophyte symbiosis.
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VITA

Glade Blythe Brosi
108 Venice Park
Lexington KY, 40503

Born: January 15, 1982. Richmond, KY.

EDUCATION

- B.S. 2008 Agronomy, Department of Agriculture, Eastern Kentucky University

PUBLICATIONS


PRESENTATIONS

Scientific Presentations


Teaching Presentations


Brosi, G.B. What is Climate Change? University of Kentucky GEN 100. Guest Lecture. Fall 2009

AWARDS / RECOGNITION

2010. Tracy Farmer Institute for Sustainability and the Environment annual meeting. Best Poser Award.