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INVESTIGATIONS INTO HOST-SPECIFIC INTERACTIONS AND LOCAL ADAPTATION  
IN THE MYCORRHIZAL SYMBIOSIS

A Thesis Presented

by

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to

The Faculty of the Graduate College

of

The University of Vermont

In Partial Fulfillment of the Requirements  
for the Degree of Master of Science  
Specializing in Biology

October, 2014

Accepted by the Faculty of the Graduate College, The University of Vermont, in partial fulfillment of the requirements for the degree of Master of Science specializing in Biology.

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## ABSTRACT

Mycorrhizal fungi are soil-borne organisms that form symbiotic associations with the majority of land plants. These fungi gather and exchange mineral nutrients with plants for photosynthetically derived carbohydrates. Mycorrhizal fungi can also confer other benefits onto plants, e.g. defense against pathogens, improved water relations, tolerance to heavy metal toxicity and herbivory. The influence of mycorrhizal fungi on plant mineral nutrition and response to stress suggests that these organisms may have a role to play sustainable agriculture as well as in bioremediation and ecosystem restoration.

In contributing to this important research, I investigated host-specific interactions between mycorrhizal fungi and the sex morphs of the gynodioecious perennial herb *Polemonium foliosissimum* (Polemoniaceae) and their mycorrhizal associates in the field. I hypothesized that the genders of this species differed in their associations with mycorrhizal fungi in benefits received. I performed a full factorial simulated herbivory experiment and evaluated the extent of mycorrhizal colonization in the roots as well as the concentrations of nutrients in leaf tissue. Mycorrhizal colonization and leaf nutrient concentrations did not differ between the genders nor were influenced by the experimental treatments. This suggests that the genders of *Polemonium foliosissimum* do not interact differently with mycorrhizal fungi, and thus do not represent different “hosts”.

Also, I investigated local adaptation of mycorrhizal associations by exploring the effect of large herbivore grazing on plant-mycorrhizal associations. I hypothesized that grazing by large herbivores results in locally adapted symbioses that enhance plant response to herbivory. I grew the perennial bunchgrass *Themeda triandra* (Poaceae) in inoculum prepared from soils collected from three field exclosures with differing histories of large herbivore exclusion in the Kenya Long Term Exclosure Experiment. I conducted a full factorial simulated herbivory experiment in which plants were subject to two clipping events over the course of 5-months, and evaluated plant regrowth as well as mycorrhizal colonization for plants in the experiment. Plants grown in inoculum from exclosures in which large herbivores have had access produced more root mass when mycorrhizal fungi were present. Further, I found equivalent biomass production of clipped and non-clipped plants in inoculum prepared from the exclosure with only native large herbivore access while equivalent biomass production was not found in the substrate prepared from areas with a history of large herbivore exclusion. This suggests that mycorrhizal fungi mediate plant growth and response to herbivory in this system.

## ACKNOWLEDGEMENTS

I would like to thank my adviser Alison Brody for her guidance and patience over the last several years. Too many times I've juggled more than I could handle and Alison was always understanding when times were tough. If she hadn't had patience I would never have made it this far. Alison was my adviser and became my friend, I hope that we stay in contact and work together again in the future.

I would also like to thank my committee members, Jeanne Harris and Jim Vigoreaux. Jeanne- thank you so much for the example you set and your many tips on how to survive in science. Our research interests are similar and I hope that in the future we solve some of the mysteries behind symbiotic plant-microbe interactions. Jim, thank you as well for your support, humor, and suave demeanor. You were always able to reduce the temperature in any room by at least 5C.

I would also like to thank Renee Petipas, and Gretel Clarke for their help at every part of this process. Renee, has been my other academic sister, who I've always respected for her determination and drive. She got me into this in the first place, and I'll have to remember that always, I guess. I interviewed with Renee for a work-study position that started this whole thing. The interview went something like this....

Renee: do you like science?

Jon: yes.

Renee: how do you feel about working 36 hours straight?

Jon: I can do that.

Renee: ok you're hired.

Gretel has always been my academic older sister, and I've looked up to her since day one, and I've probably been her academic annoying younger brother for the most part.

Next, I would like to thank my family, for their support and for putting up with my absence as I worked ceaselessly to forward my academic and career goals. I sacrificed a lot of time with them to accomplish these things- and I hope it pays off. They are more important to me than anything else in this world.

I would like to thank a couple of special friends, whose company has energized me and improved my life beyond measure. Jordan Armstrong and Amanda Northrop, I hope we stay close friends for all time.

I would also like to thank a few people who have made great contributions to my research, Josef Gorres, Joel Tilley, Alan Howard, Ruth Mickey, the greenhouse staff: Colleen Armstrong, Thomas Doubleday and Dave Heleba, Christina Feng out in RMBL for your help collecting data, and Rogelio Zimbron and Shannon Prior for your help in the lab.

To my close friends in the Department, Samantha Alger, Cristian Dambros, Allyson Degrassi, Allison Neal, Katie Miller, Nabil Nasser, Andrew Nguyen- thanks so much for being there and making the Department such a great and fun place to spend the majority of my time.

I would also like to thank the faculty and staff of the Biology Department, for your intelligence, creativity, and mentorship. Most especially Sara Helms-Cahan and Nicholas Gotelli and Joseph Schall. Thanks as well to administrative staff, who have always been so helpful and who have taken care of us all. Kimberly Hess at the Graduate College- thank you for your help for the past year as I've inched my way toward submitting this thesis! I also would like to thank the people who are not on this list who I will kick myself for not mentioning later after I've had more sleep.

Thanks finally to my best friend Vinny, He's always been my biggest fan and supporter, and most reliable companion.

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## CHAPTER 1: INTRODUCTION

“Many of the benefits sought by living things are disproportionately available to cooperating groups” –Robert Axelrod and William D. Hamilton, 1981

### 1.1. Background

Nearly 70% of vascular plants form symbiotic associations with arbuscular mycorrhizal fungi (AMF), (Brundrett 2009). These soil-borne fungi colonize their host plants' roots, and exchange nutrients (and potentially other benefits) for photosynthetically derived carbohydrates (Smith and Read 2008).

AMF are obligate biotrophs in that they receive all of their organic carbon from plant hosts and are unable to complete their life cycle in the absence of a host. AMF have no known sexual cycle, though recently the genes for meiotic machinery as well as sex pathway-related genes have been identified (Halary et al., 2011, 2013) indicating the presence of a possible “cryptic” sexual cycle. Nevertheless, no sexual activity has been observed in AMF in over 100 years of observation (Smith and Read 2008). AMF are thus considered possibly the most ancient asexual organisms; fossil records date their existence to approximately 450mya (Taylor 1995).

AMF can initiate colonization of roots by germination of multi-nucleate spores and via root fragments bearing AMF structures as well as by extraradical mycelium (Klironomos and Hart 2002 and references therein). Subsequent to colonization, the fungi extend intraradical hyphae within their plant host, constructing nutrient exchange sites (arbuscules) and storage structures (vesicles). The fungi also extend extraradical

mycelium into the surrounding soil, with which they extract nutrients for exchange with their plant host. The plant in turn, transfers hexose carbohydrates to the fungal partner, which the fungus uses for growth, maintenance, reproduction, and nutrient acquisition (Smith and Read, 2008). Genetically similar AMF isolates can anastomose (Giovannetti et al., 2001), which can result in the establishment of large functional networks in the soil surrounding plant hosts. The mycelium from this network can establish colonization units within multiple host plants, and individual host plants can be colonized by many genetically different fungi, creating what is referred to as a common mycorrhizal network.

The benefit to a plant of being mycorrhizal is generally considered to be enhanced nutrient acquisition. Acquisition and transfer of P from the soil is the primary effect of mycorrhizal colonization in these terms, though studies also indicate that Zn, Cu, and N acquisition and transfer via fungal partners is also prevalent (Smith and Read, 2008). Mycorrhizal association can also benefit plants in the form protection from pathogens (Newsham et al., 1995), drought stress (Augé 2001), heavy metal tolerance (Turnau et al., 2010 and references therein, or enhanced response to herbivory (Hetrick et al., 1990, Kula et al., 2005, Bennett and Bever 2007). The cost of the association to the plant is generally considered to be C allocation to the fungal partner(s). Mycorrhizal fungi can consume up to 20% of a plants' photosynthetic product (Jakobsen and Rosendahl 1990). Plant allocation of C to fungal partners may or may not be compensated for in nutritional benefits gained by the fungus, and in some cases, mycorrhizal colonization can lead to growth deficits in plant hosts (Johnson et al., 1997, Jones and Smith 2004). In fact, although generally considered a mutualism, enormous variability in plant mycorrhizal

responsiveness has been observed (Smith and Read 2008). The net effects of mycorrhizal colonization are influenced by many factors, including environmental conditions, e.g. soil pH and nutrient status, as well as plant and fungal genotypes (Johnson et al., 1997). These factors influence the cost-benefit relationship between the plant and fungal partners, giving rise to what has been referred to as a “mycorrhizal phenotype” which lies on a continuum between mutualism and parasitism of plants (Johnson et al., 1997).

The symbiosis may in part be maintained by control mechanisms on the side of both the plant and fungal partners (Kiers and van der Heijden 2006). In fact, regulatory mechanisms are now coming to light; specifically, plants and fungi have been found to confer increased benefits upon partners that prove more beneficial (Bever et al., 2009, Kiers et al., 2011); supporting the notion of “partner choice” and “sanctions” as mechanisms of ensuring partner cooperation in the symbiosis (Kiers and van der Heijden, 2006). Effective control mechanisms such as these could allow for proliferation of plant and fungal partners that prove most beneficial to one another in stable environmental conditions, giving rise to optimal associations. **The objective of my thesis was to investigate two areas in which optimal associations between plant and fungal partners could be found: 1) host-specific interactions, and 2) local adaptation within the association.**

## **1.2. Host-specific interactions between AMF and plants**

In natural systems, many plant and mycorrhizal species can coexist (Vandenkoornhuyse et al., 2002); however, plant hosts do not respond uniformly to

AMF. Plant hosts range from constitutively non-mycorrhizal to so-called “obligate” symbionts (Smith and Read 2008) in that only some show a high degree of dependence on AMF. Possibly due in part to differential AMF dependence of hosts, different AMF and plant pairings can influence plant growth positively or negatively (Klironomos 2003).

In gynodioecious plant species, populations consist of both hermaphrodite and female (male-sterile) individuals (Richards 1997). A recent meta-analysis revealed that females in gynodioecious species generally have an advantage over hermaphrodites in terms of flower production, fruit set, seed production, seed size, and seed germination (Shykoff et al., 2003). Females generally produce smaller flowers (Shykoff et al., 2003) and do not invest in pollen production; these resource savings may represent excess available carbon for exchange with AMF, in which case, the sexes of gynodioecious plants may represent different “hosts” to AMF. The interaction between the sexes of gynodioecious plants and mycorrhizal fungi is largely unexplored, and the potential for the sexes of gynodioecious plants to act as different hosts to AMF remains speculative (Varga 2010).

In the only gynodioecious species in which specificity of the mycorrhizal symbiosis has been explored in depth, *Geranium sylvaticum* (Geraniaceae), sex-specific responses to mycorrhizal colonization were found in flowering, aboveground biomass production and P content, mycorrhizal colonization (Varga and Kytoviita 2010a), floral diameter and visitation by dipterans (Varga and Kytoviita 2010b), and seed germination (Varga et al., 2013).

During the summer of 2011, I performed a field experiment in Gothic, CO, at the Rocky Mountain Biological Laboratory. I investigated the interactions between AMF and

the sex morphs of the gynodioecious perennial herb *Polemonium foliosissimum* (Polemoniaceae). Specifically, I asked: do the sexes of *P. foliosissimum* differ in reliance on mycorrhizal fungi for nutrient acquisition or in response to herbivory? I hypothesized that female persistence in gynodioecous populations of *P. foliosissimum* is aided by greater benefits received by females from mycorrhizal symbionts compared to hermaphrodites. I predicted that under ambient field conditions, females would have higher nutrient concentrations in leaf tissue than hermaphrodites, and that this would be correlated with the extent of mycorrhizal colonization of roots. Further, I predicted that mycorrhizal associations are less beneficial to hermaphrodites; and that herbivory would result in reduced extent of mycorrhizal colonization and leaf nutrient concentrations in hermaphrodites, as hermaphrodites would preferentially allocate carbon to fitness vice mycorrhizal associates. To test this, I evaluated mycorrhizal colonization of roots collected from plants in the field, representing the “ambient” condition, and conducted a full factorial simulated herbivory experiment, in which I evaluated mycorrhizal colonization, leaf tissue nutrient concentrations, and plant fitness subsequent to clipping. Sex-specific differences in colonization or benefit conferred on plants by AMF would suggest that the sex-morphs represent different hosts for AMF.

### **1.3. Local adaptation of the symbiotic association**

Because of the numerous factors influencing mycorrhizal associations, the realized benefit to the plant and to its fungal partner of the association will inevitably be very context dependent, and may vary by year, season, or environmental variability

(Jones and Smith 2004). For example, the lifetime fitness of long-lived perennials may be determined by their ability to withstand erratic harsh environmental conditions, e.g. nutrient or water limitation (Jones and Smith 2004). Further, benefit may only be apparent under certain conditions (e.g. drought (Augé 2001), pathogenicity (Newsham et al., 1995), herbivory (Hetrick et al., 1990, Kula et al., 2005, Bennett and Bever 2007), or pollution (Turnau et al., 2010 and references therein) or only during certain part(s) of the plant lifespan (Jones and Smith 2004).

Dispersal limitation of plants and AMF, and preferential allocation of resources to more beneficial partners could result in repeated interactions over generations in which the fitness interests of the partners become aligned (Kiers and van der Heijden 2006). It is possible then, that selection has favored mycorrhizal traits or genotypes that are most beneficial to plant hosts under the environmental conditions within which they are found (Klironomos 2003, Read 2002) resulting in associations that are adapted to their local environment.

During the academic years of 2012-2013, I conducted an experiment in the University of Vermont greenhouse. I investigated local adaptation of mycorrhizal associations by evaluating plant growth and response to herbivory when grown in association with AMF from areas of differing grazing pressures. My work took advantage of the Kenya Long-term Exclosure Experiment (KLEE) located at the Mpala Research Centre (MRC) in central Kenya. KLEE consists of exclosure plots that have been managed with different combinations of large mammalian herbivores since 1995 (Young et al., 1998).

Specifically I asked two over-arching questions: (1) Is plant growth and compensatory response to herbivory mediated by AMF from areas differing in grazing history? (2) Do these AMF differ functionally in extent to which they colonize their hosts and enhance host plant nutrient acquisition?

I hypothesized that excluding large herbivores for nearly two decades has resulted in functional changes in the AMF communities. I predicted that AMF communities from areas subject to herbivory would enhance plant compensatory response to herbivory while, in contrast, AMF from areas protected from herbivores would not enhance plant compensatory response. Further I predicted that functional differences in AMF communities would be evident in the extent of colonization of roots and in plant nutrient acquisition; such that areas of large herbivore access would be more similar to one another other in these regards, than either would be to areas in which large herbivores have been excluded. To test this, I grew seedlings of the bunchgrass *Themeda triandra* in inocula prepared from these soils, and conducted a full factorial simulated herbivory experiment. I assessed the influence of AMF from areas of differing grazing histories on plant growth and compensatory response to herbivory by comparing plants grown in sterilized substrate, to plants grown in substrate amended with a grazing history-specific mycorrhizal inoculum, when clipped or non-clipped conditions. I assessed functionality of the AMF communities from areas of differing grazing histories by evaluating mycorrhizal colonization and leaf tissue nutrient concentration of clipped and non-clipped plants.

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**CHAPTER 2: LACK OF SEX-SPECIFIC DIFFERENCES IN MYCORRHIZAL  
ASSOCIATIONS AND RESPONSE TO HERBIVORY IN THE  
GYNODIOECIOUS HERB *POLEMONIUM FOLIOSISSIMUM***

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**2.1. Abstract**

Sex-differential mycorrhizal associations between female and hermaphrodite sex morphs in gynodioecious plants may contribute to the maintenance of females. However, the role of these plant-symbiotic fungi in the maintenance of females is extremely limited. Here, we asked whether the sexes of the gynodioecious perennial herb *Polemonium foliosissimum* (Polemoniaceae) differed in their associations with mycorrhizal fungi in the field, and whether differential benefit from mycorrhizal associations could be detected in plant response to herbivory. We collected plant roots to compare mycorrhizal colonization between the sexes under ambient field conditions, and conducted a simulated herbivory experiment, in which we collected plant roots and leaf tissue to compare mycorrhizal colonization and leaf nutrient concentrations between the sexes and also evaluated several measures of fitness for plants in the experiment.

Mycorrhizal colonization did not differ between sexes in either ambient field conditions, or subsequent to clipping. In addition, leaf nutrient concentrations did not differ between the sexes nor was influenced by clipping. Clipping influenced percent root length colonization and vesicle colonization in a plant-size dependent manner, in that larger clipped plants had higher values for both measures, while the inverse was true for smaller clipped plants when compared to non-clipped plants. The proportion of fruits set, and the proportion of fruits parasitized by a pre-dispersal seed predator (*Hylemya* sp.) were higher in hermaphrodites, but this and other measures of plant fitness, were not correlated with mycorrhizal colonization. Our findings suggest that the sexes of *P. foliosissimum* do not differentially associate with mycorrhizal fungi. Further, we found no evidence that mycorrhizae differentially benefit the sexes under conditions of herbivory. Taken together, our results do not support the idea that sex-differential mycorrhizal associations contribute to the maintenance of females in *P. foliosissimum*.

## 2.2. Introduction

Most flowering plants are hermaphrodites. However, gynodioecy is a relatively common breeding system wherein populations consist of both hermaphrodite and female (male-sterile) individuals (Richards 1997). In gynodioecious plants, female fitness is garnered only through the production of seeds, while the fitness of hermaphrodites is comprised of both female (seed set) and male (seed siring) contributions. According to theory, for females to persist, they must compensate for the lack of male function with increased fitness (Lewis 1941, Charlesworth and Charlesworth 1978). A recent meta-

analysis revealed that females in gynodioecious species generally have an advantage over hermaphrodites in terms of flower production, fruit set, seed production, seed size, and seed germination (Shykoff et al., 2003). Females generally produce smaller flowers (Shykoff et al., 2003) and do not invest in pollen production; these resource savings may be reallocated to increased seed production or quality.

Sex-specific interactions with other species may also lead to fitness advantages for females (Ashman 2006, Spigler and Ashman 2012). Sex-specific interactions with other species have been observed in hermaphrodite-biased seed predation (Marshall and Ganders 2001), nectar-robbing (Zhang et al., 2009) and predispersal seed predation (Collin et al., 2002).

Largely, unexplored, however, are interactions between the sexes of gynodioecious plants and mycorrhizal fungi (Varga 2010). Nearly 70% of vascular plants engage in symbiosis with arbuscular mycorrhizal fungi (AMF), (Brundrett 2009). In these associations, the fungi colonize plant roots and provide soil nutrients to plants in exchange for photosynthetic carbon compounds. AMF can provide up to 100% of a plant's required phosphorous (Smith et al., 2004) and consume up to 20% of the plant's photosynthetic product (Jakobsen and Rosendahl 1990). AMF may influence several plant characteristics and interactions that could differentially contribute to the fitness of the sexes in a dimorphic plant species. These include flower size and pollinator visitation (Gange and Smith 2005), flower phenology (Lu and Koide 1994), resistance to pathogens (Newsham et al., 1995) and improved fitness under conditions of resource limitation (Johnson et al., 2010). Further, AMF can influence plant response to herbivory, though

responses vary widely by fungal and host combinations (Klironomos et al., 2004, Kula et al., 2005, Bennett and Bever 2007).

The potential for AMF to influence the persistence of females in gynodioecious plants remains speculative (Varga 2010). In the only gynodioecious species in which sex-specificity of the mycorrhizal symbiosis has been explored in depth, *Geranium sylvaticum* (Geraniaceae), sex-specific responses to mycorrhizal colonization were found in flowering, aboveground biomass production and P content, mycorrhizal colonization (Varga and Kytoviita 2010a), floral diameter and visitation by dipterans (Varga and Kytoviita 2010b), and seed germination (Varga et al., 2013).

Here, we investigated the relationship between sexes of the gynodioecious perennial herb *P. foliosissimum* and their mycorrhizal symbionts. Specifically, we asked: do the sexes of *P. foliosissimum* differ in reliance on mycorrhizal fungi for nutrient acquisition or in response to herbivory? We hypothesized that female persistence in gynodioecous populations of *P. foliosissimum* is aided by greater benefits received by females from mycorrhizal symbionts compared to hermaphrodites. We predicted that under ambient field conditions, females would have higher nutrient concentrations in leaf tissue than hermaphrodites, and that this would be correlated with the extent of mycorrhizal colonization of roots. Further, we predicted that mycorrhizal associations are less beneficial to hermaphrodites; and that herbivory would result in reduced extent of mycorrhizal colonization and leaf nutrient concentrations in hermaphrodites, as hermaphrodites would preferentially allocate carbon to fitness vice mycorrhizal associates. To test this, we evaluated mycorrhizal colonization of roots collected from plants in the field, representing the “ambient” condition, and conducted a full factorial

simulated herbivory experiment, in which we evaluated mycorrhizal colonization, leaf tissue nutrient concentrations, and plant fitness subsequent to clipping.

### **2.3. Materials and methods**

*Polemonium foliosissimum* A. Gray var. *mole* (Greene) is a long-lived, herbaceous perennial (Polemoniaceae) that occurs in New Mexico, Colorado, and southern Utah (Anway 1968). Our work was conducted in June through August of 2011 at the Rocky Mountain Biological Laboratory (RMBL) in Gothic, Gunnison County, Colorado, USA (lat. 38°57'N, long. 106°59'W, alt. 2883m), where the sex-ratio was ca. 50% female. In nearby populations, the sex ratios vary from 0 to 64% female (Clarke 2014).

#### **2.3.1. Mycorrhizal colonization of the sexes of *P. foliosissimum* in ambient field conditions**

To examine the relationship between *P. foliosissimum* sex-morphs and mycorrhizal fungi, we first compared mycorrhizal colonization of females and hermaphrodites by sampling roots just after flowering began and scoring percent colonization (hereafter referred to as “ambient colonization”). We sampled roots by taking three soil cores from each of 20 plants (10 hermaphrodites and 10 females, in pairs matched by size) using a 2.5cm x 20cm soil corer. The cores were taken 5 cm from the base of each plant and spaced evenly around the plant unless rocks prohibited coring to the full depth; in which

case, cores were distributed around the plant as evenly and to as full a depth as possible. The three samples per plant were pooled into one composite sample for analysis. We extracted *P. foliosissimum* roots from the pooled samples by matching roots of the composite sample with roots of *P. foliosissimum* from excised plants. Extracted roots ranged in size from .20mm-.50mm in diameter.

We stained roots in a 5% ink and vinegar solution, (Pelikan Royal Blue Ink 4001; Pelikan, Hannover, Germany) using the methods described by Vierheilig et al., (1998). Roots were cleared in 10% KOH for 24h at room temperature, rinsed with tap water, bleached in 3% hydrogen peroxide for 20min, rinsed again, acidified with 1% HCl for one minute and then placed directly in 5% ink and vinegar at room temperature for 24h. Stained roots were then rinsed and stored in water acidified with a few drops of vinegar until scoring. To score AMF colonization, roots were placed on slides and mycorrhizal structures counted using a Leica DM E compound light microscope at 200x and 400x magnification. The percent of root length colonized by mycorrhizal fungi was quantified using the magnified intersections method (McGonigle et al., 1990). The number of intersections inspected was limited by the amount of extracted root material that matched *P. foliosissimum* morphology. A minimum of seventy intersections per plant were inspected; however, in a few cases, fewer intersections were inspected due to insufficient root material; overall intersections: Mean=80.94, SE=4.49. Putative mycorrhizal nutrient exchange sites; namely, arbuscules, arbusculate coils, and coiled hyphae (Smith and Read 2008), were observed in stained root segments and are consolidated into one variable “Nutrient exchange site colonization” (NEXC), calculated as the number of intersections containing these structures divided by the total number of intersections viewed\*100.

Vesicle colonization (VC) was likewise determined, and overall percent root length colonized (PRLC) was calculated as the percentage of non-negative intersections.

Hermaphrodites, with higher nutrient investment in reproductive structures, may be more stressed when growing in nutrient poor conditions (reviewed by Ashman 2006), and thus may be more reliant on AMF for enhanced nutrient acquisition in conditions of low nutrient availability. To determine if females and hermaphrodites inhabit soil microsites with differing nutrient availability that might affect their relationship with AMF, we collected and pooled three soil samples 5cm from around the base of three plants of each gender using a 2.5 x 20cm soil corer as described previously. The plants were matched in size representing three size classes, 4-9 stems, 10-18 stems, and 19+ stems (n=3 per sex). Samples were air-dried at room temperature, ground to pass through a 2.00mm sieve. Soil samples were analyzed by University of Maine Analytical Laboratory (University of Maine, Orono, ME, USA). Soil samples were analyzed for soil pH, % organic matter, P, K Ca, Mg, S, B, Cu, Fe, Mn, and Zn. Organic matter was determined by loss on ignition and nutrients were extracted using the modified Morgan method. Phosphorous content was determined colorimetrically; all other nutrients were determined by ICP-OES.

### **2.3.2. Mycorrhizal colonization, leaf nutrient concentration, and fitness of the sexes subsequent to simulated herbivory**

To determine if the relationship between the *P. foliosissimum* sex-morphs and mycorrhizal fungi differed in response to herbivory, we simulated herbivore damage by clipping plants early in the flowering season and then measured mycorrhizal colonization

and leaf P concentration. At the outset, we chose quadruplets of similarly sized plants (two females and two hermaphrodites) and randomly assigned one of each gender to be clipped and the other to serve as an unclipped control. There were 40 total plants in the experiment (20 females and 20 hermaphrodites); ranging in size from four to 31 stems. Plants at the study site had been identified and permanently marked in previous years and so information regarding gender for many plants was known prior to blooming. Gender was confirmed and the sex of plants not previously identified was determined as plants came into bloom. In all cases, care was taken to select plants that showed little or no signs of previous herbivory or damage, and that were separated by all other *P. foliosissimum* plants by at least 20cm.

Although *P. foliosissimum* usually flowers by the first week of July (Zimmerman 1980, Brody 1997), 2011 was a year of high, late-season snowpack plants did not begin blooming until mid-July. We took root samples and clipped plants at the outset of flowering, after gender could be confirmed for all plants. Due to differences in flowering phenology, root samples were taken and plants were clipped on July 16<sup>th</sup> and July 27<sup>th</sup>, 2011. For plants assigned to the clipping, we clipped exactly half of the stems. We selected stems haphazardly, subjectively attempting to reduce the plant's aboveground biomass by one half. In cases where the total number of stems was uneven, we took care to remove a number of stems that would reduce the plant's above-ground mass by approximately one half. Stems were cut to 4 inches above the ground; a height that is typical of deer herbivory on this plant.

To examine the effects of clipping on mycorrhizal colonization and leaf nutrient content, we collected root samples and quantified mycorrhizal colonization (hereafter

referred to as “post-clipping colonization”), and collected leaf tissue for nutrient analysis from all plants at the end of the experiment (ca. three weeks after the plants were clipped). Root samples were collected and processed as described previously. To conduct leaf tissue analysis, we collected the youngest, fully expanded leaves and removed the petiole and rachis (Cornelissen et al., 2003). Leaves were dried at 55°C to a constant weight and shipped to the University of Vermont, Burlington, VT, USA. Leaf tissue was then re-dried at 65°C overnight prior to being ground and analyzed. Tissue sampling was not performed for one plant, as this would have required removing most of the remaining leaf tissue from that plant. All remaining plants from the herbivory experiment had leaves sampled for nutrient analysis (N=39). The University of Vermont Agriculture and Environmental Testing Laboratory performed leaf tissue analysis by nitric acid digestion and ICP-OES. Nutrients analyzed included Ca, P, K, Mg, Al, Fe, Mn, Zn, and S.

We evaluated fitness of all plants involved in the study (those used to evaluate ambient colonization, as well as clipped and non-clipped plants from the simulated herbivory experiment). We haphazardly chose a subset of two to three stems per plant, and counting the total number of fruits on them. From within the total number of fruits, we differentiated between fruits that contained seeds (referred to as “set”) and fruits that bore no seeds (referred to as “aborts”). From within fruits set, we considered fruits to be “destroyed” if they bore seeds showing evidence of parasitism by the pre-dispersal seed predator (*Hylemya* sp.). We calculated whole plant estimates of total fruits, proportion fruit set, and proportion fruits destroyed by multiplying values from the subset through the total number of reproductive stems per plant. We calculated seeds per fruit (SPF) as the average number of seeds per non-destroyed, set fruit (referred to as “good” fruits).

We also calculated the total number of seeds per plant (SPP) by multiplying the SPF by the estimated number of good fruits per plant.

### **2.3.3. Statistical analysis**

Plant size may differently influence female and hermaphrodite fitness, possibly interacting with resource availability (reviewed in Ashman 2006); we therefore suspected that reliance on AMF could also differ over plant size in a sex-specific manner.

Therefore, plants size (number of stems) was included as a covariate in all analyses.

To determine if mycorrhizal colonization differed between the genders or was influenced by clipping, we evaluated ambient and post-clipping mycorrhizal colonization of plants by analyzing PRLC, VC, and NEXC as response variables in two- and three-way MANCOVA's respectively, followed by separate ANCOVA's for each response. For ambient colonization, factors included plant gender, size, and their interaction; and for post-clipping colonization, factors included plant gender, experimental treatment (clipped vs. non-clipped), plant size, and all interactions. For ambient colonization, we had insufficient root material with which to calculate measures of colonization for three plants; these plants were not included in the analysis (N=17). For post-clipping colonization, the data from two plants were lost due to mislabeling (N=38). We compared microsite soil characteristics between the sexes by analyzing soil pH, % organic matter, P, K Ca, Mg, S, B, Cu, Fe, Mn, and Zn content of soils using two-way univariate ANCOVA's. To determine if leaf nutrient accumulation differed between the genders or was influenced by clipping, we analyzed nutrient concentrations by three-way

MANCOVA; factors included plant gender, experimental treatment (clipped vs. non-clipped), plant size, and all interactions. To determine if AMF colonization was correlated with leaf nutrient content, we examined correlations between mycorrhizal colonization and nutrient concentrations using only plants for which both nutrient content and post-clipping mycorrhizal colonization data were available (N=37).

We evaluated the influence of plant sex, and the experimental treatments (core vs. clip vs. control) on the five measures of plant fitness by three-way MANCOVA followed by univariate ANCOVA's; factors included plant sex, experimental treatment, plant size, and all interactions. Eight plants were not located or had died during the experiment, or were incorrectly identified during the fitness data collecting process, therefore N=52 for fitness data analysis. To analyze the correlation between mycorrhizal colonization and plant fitness, we combined the post-clipping colonization data set with the plant fitness data set, and analyzed data for plants which both colonization and fitness data were collected (N=32).

Post-clipping vesicle colonization (VC) and proportion fruits destroyed data were logit transformed to meet assumptions (Warton and Hui 2011). Total fruits, seeds per fruit, and seeds per plant were log transformed prior to analysis. Multivariate analyses of covariance (MANCOVA), analyses of covariance (ANCOVA), and Tukey's HSD tests were performed in R version 3.2.0 (R Core Team, 2013).

## 2.4. Results

### 2.4.1. Colonization of the sexes of *P. foliosissimum* in ambient field conditions

Ambient mycorrhizal colonization was not influenced by sex (Wilks'  $\Lambda=0.9548$ , approx.  $F_{(3,11)}=0.1738$ ,  $P=0.9119$ ), or plant size (Wilks'  $\Lambda=0.7366$ , approx.  $F_{(3,11)}=1.3114$ ,  $P=0.3199$ ). Percent root length colonization (PRLC) for females and hermaphrodites were  $53.35\pm 11.83$  and  $52.33\pm 14.02$  respectively (Mean $\pm$ SD), (Figure 1). The soils below plants of the different sexes and size classes were similar, in that two-way ANCOVA's revealed no differences in pH, % organic matter, P, K Ca, Mg, S, B, Cu, Fe, Mn, and Zn content by sex (all  $P>0.35$ ) or size (all  $P>0.18$ ).

### 2.4.2. Mycorrhizal colonization, leaf nutrient concentration, and fitness of the sexes subsequent to simulated herbivory

Subsequent to clipping, mycorrhizal colonization was again not influenced by sex (Wilks'  $\Lambda=0.9374$ , approx.  $F_{(3,28)}=0.6229$ ,  $P=0.6062$ ), or plant size (Wilks'  $\Lambda=0.9734$ , approx.  $F_{(3,28)}=0.2554$ ,  $P=0.8568$ ), nor was it influenced by clipping treatment (Wilks'  $\Lambda=0.8643$ , approx.  $F_{(3,28)}=1.4653$ ,  $P=0.2453$ ) (Figure 2). However, mycorrhizal colonization was found to be marginally influenced by the treatment\*plant size interaction (Wilks'  $\Lambda=0.7707$ , approx.  $F_{(3,28)}=2.7772$ ,  $P=0.0597$ ). Subsequent three-way ANCOVA's revealed a significant effect of the treatment\*plant size interaction on percent root length colonization ( $F_{(1,30)}=5.6756$ ,  $P=0.0273$ ) and vesicle colonization

( $F_{(1,30)}=6.8559$ ,  $P=0.0137$ ). Larger clipped plants had higher values of both PRLC and VC than non-clipped plants, while the inverse was true for smaller plants (Figure 3a and 3b respectively).

Leaf tissue nutrient concentrations did not differ by plant sex (Wilks'  $\Lambda=0.6618$ , approx.  $F_{(9,23)}=1.3059$ ,  $P=0.2869$ ), or plant size (Wilks'  $\Lambda=0.5541$ , approx.  $F_{(9,23)}=2.0562$ ,  $P=0.0788$ ), or clipping treatment (Wilks'  $\Lambda=0.7048$ , approx.  $F_{(9,23)}=1.0702$ ,  $P=0.4198$ ). Plant leaf nutrient concentrations were also not highly correlated with mycorrhizal colonization (all  $r^2 \leq 0.14$ ).

Measures of plant fitness were marginally influenced by plant sex (Wilks'  $\Lambda=0.7739$ , approx.  $F_{(5,36)}=2.1031$ ,  $P=0.0875$ , Figure 4), and significantly influenced by experimental treatment (Wilks'  $\Lambda=0.5956$ , approx.  $F_{(10,72)}=2.1295$ ,  $P=0.0327$ ), and plant size (Wilks'  $\Lambda=0.5359$ , approx.  $F_{(5,36)}=6.2356$ ,  $P=0.0003$ ). Univariate three-way ANCOVA's for the five fitness measures revealed sex as a significant factor for proportion fruit set ( $F_{(1,40)}=7.1395$ ,  $P=0.0109$ ), proportion fruit destroyed ( $F_{(1,40)}=6.7073$ ,  $P=0.0133$ ), and as a marginally significant factor for seeds per fruit ( $F_{(1,40)}=4.0078$ ,  $P=0.0521$ ). Hermaphrodites had higher proportion fruits set, and fruits destroyed, compared to females (Student's T-test, Table 1). Further, univariate three-way ANCOVA's revealed that experimental treatment, and plant size, were significant factors for total fruits (treatment:  $F_{(2,40)}=10.0947$ ,  $P=0.0003$ ; plant size:  $F_{(1,40)}=30.8773$ ,  $P<0.0001$ ), and seeds per plant (treatment:  $F_{(2,40)}=3.2915$ ,  $P=0.0475$ ; plant size:  $F_{(1,40)}=15.8270$ ,  $P=0.0003$ ). Logically, clipped plants, with approximately half of their stalks removed, had fewer total fruits than cored plants, or non-clipped plants (Tukey's HSD, Table 2). Clipped plants also had fewer seeds per plant than non-clipped plants;

however, cored plant seeds per plant did not differ from either clipped or non-clipped plants (Tukey's HSD, Table 2); likely due to slightly lower (not significantly), proportion fruit set of cored plants below both clipped and non-clipped plants (Table 2). Also logically, plants with more stalks produced more total fruits and seeds per plant than plants with fewer stalks. Finally, the five fitness measures were not highly correlated with mycorrhizal colonization (all  $r^2 \leq 0.072$ ).

## 2.5. Discussion

Female plants must compensate for the loss of male fitness to be maintained alongside hermaphrodites (Lewis 1941, Charlesworth and Charlesworth 1978). The ways in which they do so are varied and include producing greater numbers and/or higher quality seeds (Shykoff et al., 2003). We hypothesized that in gynodioecous populations of *P. foliosissimum*, female persistence is aided by greater benefits received by females from mycorrhizal symbionts compared to hermaphrodites. Contrary to our expectations, we found that the extent of mycorrhizal colonization did not differ between the sexes in ambient field conditions. Also, we found that subsequent to simulated herbivory, the extent of mycorrhizal colonization and leaf nutrient concentrations were similar between the sexes and not differently influenced by simulated herbivory.

It may be that a sex-specific relationship between the sexes of *P. foliosissimum* and AMF could not be detected utilizing the methods that we employed. The duration of the current study was limited by flowering phenology and the time available to perform the study. The life span of arbuscules is considered to be approximately 7 days

(Alexander et al., 1988); however, arbuscules may persist for much longer periods in herbaceous woodland species (Brundrett and Kendrick, 1990). Our study took place over ca. three weeks, and so effects of clipping on the extent of colonization may not have been manifested in such a short time. Also, nutrient acquisition by plants via mycorrhizal partners can be responsible for the majority of plant P uptake independent of a growth response, (Smith et al., 2004); suggesting that direct nutrient acquisition by plants is suppressed or at least decreased in the presence of mycorrhizal fungi (Smith and Read 2008). If females receive relatively more P from AMF associates than hermaphrodites, this would have gone undetected in our study as we did not have non-mycorrhizal plants in the field with which to compare the colonized plants.

With the use of clonal propagation and non-mycorrhizal plants, Varga and associates have been able to detect sex-specific differences in the mycorrhizal associations of *G. sylvaticum* (Varga and Kytoviita, 2010a, Varga and Kytoviita, 2010b, Varga et al., 2013). Similar to findings in this study, the sexes of *G. sylvaticum* were not differentially colonized by mycorrhizal fungi in the field, and simulated herbivory did not influence mycorrhizal colonization of *G. sylvaticum* (Varga et al., 2009). However, contrary to this study, females had significantly less plant P content at the end of the experiment (regardless of clipping) and the average number of seeds per flower in females of *G. sylvaticum* was highly correlated with arbuscule colonization in non-clipped plants. The authors attribute the reduction of P in female tissue to allocation of P to seeds (females produced more seeds than hermaphrodites). In contrast, fitness measures, including total fruit production, fruit set, seeds per fruit, and seeds per plant, are not correlated with mycorrhizal colonization in either of the sexes of *P*.

*foliosissimum*, (Appendix A) nor were plant functional traits of flower size, whole leaf size, specific leaf area, stalk length, and stalk diameter (Appendix A).

We found that the percent of fruits parasitized by the pre-dispersal seed predator (*Hylemya* sp.) was higher in hermaphrodites than in females (Table 1); supporting findings of Clarke (2014) in this same species, and supporting the hypothesis of the influence of differential multi-species interactions between the sexes as a contributor to female maintenance in gynodioecious species (Ashman 2006). Unexpectedly, we found that hermaphrodites had higher fruit set than females (Table 1); a finding that is in contrast with findings of Clarke (2014), and also in contrast with the findings of Shykoff et al. (2003) regarding gynodioecious plant species. In the current study, we took care to evaluate fitness of the sexes across a range of plant sizes. Plant size may influence hermaphrodite fruit set more so than female fruit set (Ashman 2006); and indeed in the current study, there was a trend of size-dependent fruit set for hermaphrodites among non-clipped plants, with larger plants having lower fruit set, although this was not statistically significant (Appendix A, Figure A6). It may be that the sexes of *P. foliosissimum* differ in fruit set throughout their life cycles, with mature females having higher average fruit set than mature hermaphrodites.

Also unexpectedly, we found that clipping influenced mycorrhizal colonization of *P. foliosissimum* in a plant size-dependent manner (Figure 3). Tropical trees have been found to associate with different communities of AMF as they mature (Husband et al., 2002a, 2002b); and the associations of mature trees may differ in benefit from those of seedlings (Kiers et al., 2000). As a long-lived perennial, different size-classes (ages) of *P. foliosissimum* may also associate with different AMF communities, leading to different

responses on the part of AMF in conditions of reduced plant C availability. Also, root samples were collected from a standard distance from the base of all plants, regardless of size (see methods). It is likely that larger plants have more root material, this sampling protocol may have lead to bias in measures of root colonization in that samples taken from larger plants, may have relatively more root material of the correct plant present. We took precautions against this by carefully comparing roots in samples with roots taken directly from *P. foliosissimum* plants excised prior to the study.

In summary, our findings suggest that the mechanism resulting in a different relationship between sexes of *G. sylvaticum* and their mycorrhizal associates is not present in *P. foliosissimum*. Because of the numerous factors influencing mycorrhizal associations (Johnson et al., 1997), the realized benefits of AMF associations will inevitably be context dependent, and may vary by year, season, or with environmental variability (Jones and Smith 2004). Thus, a realistic approach is to consider the relative short-term benefits of the association in terms of plant biomass production, nutrient acquisition, or response to stress (Jones and Smith 2004, Smith and Read 2008). However, the lifetime fitness of long-lived perennials, like *P. foliosissimum*, may be determined by their ability to withstand erratic harsh environmental conditions, e.g. nutrient or water limitation (Jones and Smith 2004), and thus, the benefit from AMF association may only be apparent under certain conditions or only during certain part(s) of the plant lifespan (Jones and Smith 2004). Therefore, the presence of other, yet to be identified benefits of the association, could still prove to be sex-specific in *P. foliosissimum* and other plant species with alternative reproductive systems. To our knowledge, this study represents only the second gynodioecious plant species within

which sex-specificity of the mycorrhizal symbiosis has been investigated in depth. These findings are therefore of great value to others researching the symbiosis in alternative plant sexual systems.

## **2.6. Acknowledgments**

The support of many people made this project possible. Primarily the staff and scientists at RMBL, and members of the Brody Lab. Special thanks to JBG's MSc. committee and to the UVM Biology Department for constructive criticism of the analysis and interpretation of the results, and to Alan Howard and Ruth Mickey for help with the statistical analysis. JBG was supported by an NSF REU, a UVM CALS FUSE and a UVM Honors College Grant.

## 2.7. Figures and tables

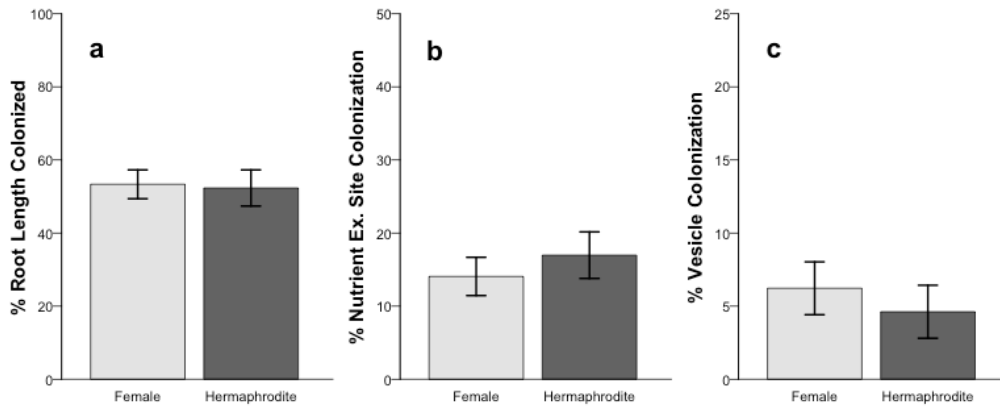


Figure 1: Ambient mycorrhizal colonization. Mycorrhizal colonization did not differ between the sexes for (a) percent root length colonized, (b) percent nutrient exchange site colonization, or (c) percent vesicle colonization. Values are means  $\pm$  SE. Y-axes scaled to accommodate values.

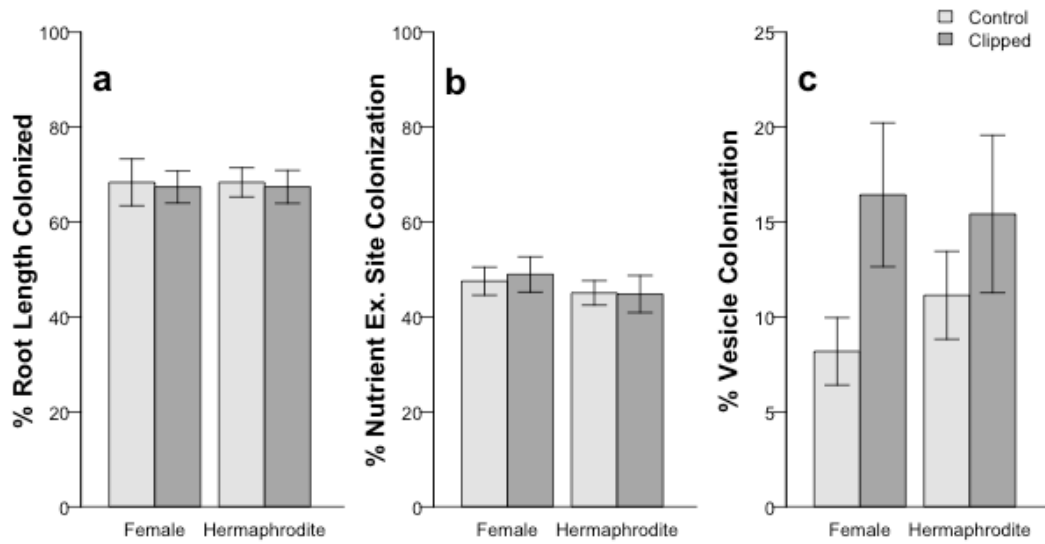


Figure 2: Post-clipping mycorrhizal colonization. Mycorrhizal colonization did not differ between the sexes for (a) percent root length colonized, (b) percent nutrient exchange site colonization, or (c) percent vesicle colonization. Values are means  $\pm$  SE. Y-axes scaled to accommodate values.

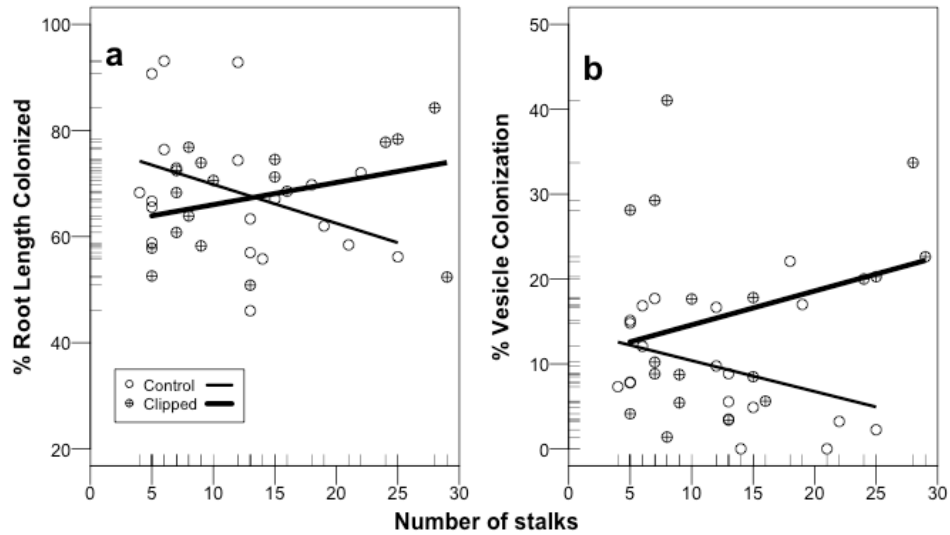


Figure 3: Post-clipping mycorrhizal colonization by size (number of stalks) and treatment. The interaction between clipping treatment and plant size was significant for (a) percent root length colonized,  $F_{(1,30)}=5.6756$ ,  $P=0.0273$ , and (b) percent vesicle colonization  $F_{(1,30)}=6.8559$ ,  $P=0.0137$ . For both (a) and (b), larger clipped plants were generally more colonized than non-clipped plants while the opposite was true for smaller plants. Y-axes scaled to accommodate values.

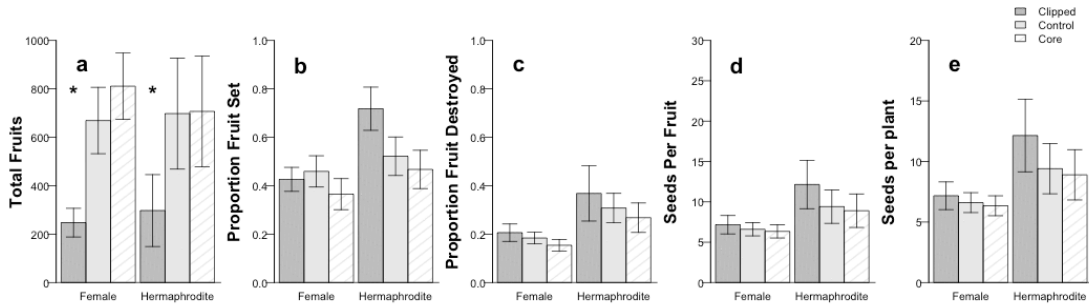


Figure 4: Fitness measures. Measures of plant fitness were marginally influenced by plant sex (Wilks'  $\Lambda=0.7739$ , approx.  $F_{(5,36)}=2.1031$ ,  $P=0.0875$ ), and significantly influenced by experimental treatment (Wilks'  $\Lambda=0.5956$ , approx.  $F_{(10,72)}=2.1295$ ,  $P=0.0327$ ). Univariate three-way ANCOVA's for the five fitness measures revealed sex as a significant factor for proportion fruit set ( $F_{(1,40)}=7.1395$ ,  $P=0.0109$ ), proportion fruit destroyed ( $F_{(1,40)}=6.7073$ ,  $P=0.0133$ ), and as a marginally significant factor for seeds per fruit ( $F_{(1,40)}=4.0078$ ,  $P=0.0521$ ). Hermaphrodites had higher proportion fruits set, and fruits destroyed, compared to females. Further, univariate three-way ANCOVA's revealed that experimental treatment, was a significant factor for total fruits ( $F_{(2,40)}=10.0947$ ,  $P=0.0003$ , asterisks in above figure).

Table 1: Fitness responses by sex. Measures of plant fitness were marginally influenced by plant sex (Wilks'  $\Lambda=0.7739$ , approx.  $F_{(5,36)}=2.1031$ ,  $P=0.0875$ ). Univariate three-way ANCOVA's for the five fitness measures revealed sex as a significant factor for proportion fruit set ( $F_{(1,40)}=7.1395$ ,  $P=0.0109$ ), proportion fruit destroyed ( $F_{(1,40)}=6.7073$ ,  $P=0.0133$ ), and as a marginally significant factor for seeds per fruit ( $F_{(1,40)}=4.0078$ ,  $P=0.0521$ ). Means across sexes bearing different letters are significantly different (Student's T-test).

	Female (n=29)		Hermaphrodites (n=23)	
	Mean	SE	Mean	SE
Total fruits	567.76a	96.94	596.86a	117.25
Proportion fruit set	41.87a	3.25	55.16b	4.45
Proportion fruit destroyed	18.23a	1.70	30.80b	4.14
Seeds per fruit	6.72a	0.54	9.92a	1.27
Seeds per plant	825.37a	221.84	915.00a	185.88

Table 2: Fitness responses by experimental treatment. Measures of plant fitness were significantly influenced by experimental treatment (Wilks'  $\Lambda=0.5956$ , approx.  $F_{(10,72)}=2.1295$ ,  $P=0.0327$ ). Univariate three-way ANCOVA's revealed that experimental treatment was a significant factor for total fruits ( $F_{(2,40)}=10.0947$ ,  $P=0.0003$ ), and seeds per plant ( $F_{(2,40)}=3.2915$ ,  $P=0.0475$ ). Means across experimental treatments bearing different letters are significantly different (Tukey's HSD).

	Cored Plants (n=18)		Clipped Plants (n=16)		Non-Clipped Plants (n=18)	
	Mean	SE	Mean	SE	Mean	SE
Total fruits	758.94a	147.32	266.09b	64.20	681.90a	122.97
Prop. fruit set	41.62a	3.80	53.57a	5.70	48.70a	4.93
Prop. fruit destroyed	21.11a	3.36	26.68a	5.03	23.90a	3.27
Seeds per fruit	7.62a	1.07	9.03a	1.42	7.85a	1.05
Seeds per plant	904.63ab	195.27	533.85a	178.15	1119.75b	339.69

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**CHAPTER 3: PLANT GROWTH AND RESPONSE TO HERBIVORY IS  
DIFFERENTLY INFLUENCED BY ARBUSCULAR MYCORRHIZAL FUNGI  
FROM AREAS OF DIFFERING GRAZING HISTORY**

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**3.1. Abstract**

Stable environments can give rise to arbuscular mycorrhizal fungal (AMF) associations that are adapted to local conditions. Large herbivores influence the composition and function of AMF communities within the soil, which in turn influence plant growth and compensatory response to herbivory. Here we tested the hypothesis that grazing by large herbivores results in locally adapted symbioses that enhance plant response to herbivory. We grew the perennial bunchgrass, *Themeda triandra* Forssk, in inoculum prepared from soils collected from three field exclosures with different histories of large herbivore grazing, and conducted a full factorial simulated herbivory experiment in which plants were subject to two clipping events over the course of 5-months. To investigate the influence of AMF, we grew plants in sterilized or unsterilized substrates, supplemented with a non-mycorrhizal microbial filtrate. Plant growth, aboveground P accumulation, and compensatory response to clipping, were affected differently by AMF from areas of

differing grazing histories. Plants grown with AMF from an area protected from large herbivores for 17 years, were depressed in early aboveground growth, inhibited in compensatory response, and showed no benefit from the association in terms of biomass production or P acquisition. Plants grown with AMF from an area in which large native herbivores grazed also showed depressed early aboveground growth, but allocated more biomass belowground, had higher P concentration in aboveground tissue, and compensated for clipping in terms of aboveground- and total biomass production. Plants grown with AMF from an area in which cattle were grazed in addition to native herbivores had the greatest extent of mycorrhizal colonization in roots, and showed enhanced biomass production and higher P concentrations in aboveground tissue, but were inhibited in compensatory response. Our findings indicate that differing large herbivore grazing for nearly two decades has resulted in functional changes in AMF communities, and lead to differential mediation of plant growth, P acquisition, and response to herbivory in this system. Our findings suggest that 17 years of differing grazing pressure is adequate for local adaptation between plants and AMF to arise.

### **3.2. Introduction**

In East African savannas, vertebrate herbivores are important drivers of plant productivity and community composition (McNaughton 1985, 1988; Hobbs 1996, Frank et al., 1998, Veblen and Young 2010, Pringle et al., 2011, Porensky et al., 2013, Young et al., 2013). Recent studies also demonstrate that vertebrate herbivores affect the diversity and abundance of soil-dwelling microbes—particularly, arbuscular mycorrhizal fungi

(AMF) that form symbioses with plants (Bethlenfalvay and Dakessian 1984, Eom et al., 2001, Frank et al., 2003, Murray et al., 2010). It is, indeed, likely that the responses of plants to grazing is mediated by AMF (Hetrick et al., 1990, Kula et al., 2005, Bennett and Bever 2007) and yet little is known about the links between grazing intensity and compensatory responses of plants to grazing vis-à-vis locally adapted relationships with AMF. Functional differences between AMF assemblages from areas with different grazing pressure have been evidenced by differences in the extent of mycorrhizal colonization (Bethlenfalvay and Dakessian 1984, Frank et al., 2003), and effects on plant biomass production (Frank et al., 2003).

Nearly 70% of vascular plants form symbiotic associations with arbuscular mycorrhizal fungi (AMF), (Brundrett 2009). In these associations, the fungi colonize plant roots and act as extended root systems, gathering inorganic nutrients from the soil and transporting them to roots in exchange for photosynthetic carbon (Smith and Read 2008). AMF can be critical to plant nutritional status, providing up to 100% of a plant's required phosphorous (Smith et al., 2004) in exchange for up to 20% of the plant's photosynthetic product (Jakobsen and Rosendahl 1990).

AMF also provide other benefits to plant hosts, e.g. protection from pathogens (Newsham et al., 1995), drought (Augé 2001), and heavy metal toxicity (Turnau et al., 2010 and references therein). AMF can also enhance plant regrowth following herbivory (Hetrick et al., 1990, Kula et al., 2005, Bennett and Bever 2007). The influence of AMF on plant response to herbivory may vary widely by host and fungal species combinations (Klironomos et al., 2004, Kula et al., 2005, Bennett and Bever 2007), and thus herbivore-mediated changes in AMF communities could provide important feedbacks on plant

response to herbivory. For example, AMF species and community assemblages may become adapted to local environmental conditions that, in turn, result in increased biomass production (Frank et al., 2003) or tolerance to nutrient limitation (Johnson et al., 2010).

Here we examined how mycorrhizal assemblages from areas with different grazing histories influence plant growth and response to simulated herbivory.

Specifically we asked two over-arching questions: (1) Is plant growth and compensatory response to herbivory mediated by AMF from areas differing in grazing history over the past 17 years? (2) Do these AMF differ functionally in extent to which they colonize their hosts and enhance host plant nutrient acquisition? Our work took advantage of the Kenya Long-term Exclosure Experiment (KLEE) located at the Mpala Research Centre (MRC) in central Kenya. KLEE consists of exclosure plots that have been managed with different combinations of large mammalian herbivores since 1995 (Young et al., 1998). We hypothesized that excluding large herbivores for nearly two decades has resulted in functional changes in the AMF communities. We predicted that AMF communities from areas subject to herbivory would enhance plant compensatory response to herbivory while, in contrast, AMF from areas protected from herbivores would not enhance plant compensatory response. Further we predicted that functional differences in AMF communities would be evident in the extent of colonization of roots and in plant nutrient acquisition; such that areas of large herbivore access would be more similar to one another other in these regards, than either would be to areas in which large herbivores have been excluded. To test this, we grew seedlings of the bunchgrass *Themeda triandra* (hereafter *Themeda*) in inocula prepared from these soils, and

conducted a full factorial simulated herbivory experiment. We assessed the influence of AMF from areas of differing grazing histories on plant growth and compensatory response to herbivory by comparing plants grown in sterilized substrate, to plants grown in substrate amended with a grazing history-specific mycorrhizal inoculum, when clipped or non-clipped conditions. We assessed functionality of the AMF communities from areas of differing grazing histories by evaluating mycorrhizal colonization and leaf tissue nutrient concentration of clipped and non-clipped plants.

### **3.3. Materials and methods**

#### **3.3.1. Study system**

To investigate the influence of mycorrhizal fungi from areas with different grazing histories on host plant compensatory growth, we took advantage of the Kenya Long Term Exclosure Experiment (KLEE). The Kenya Long-term Exclosure Experiment is located at the Mpala Research Centre (MRC) on the Laikipia plateau (37E, 0N: 1800m elevation) in central Kenya. KLEE was established in 1995 and consists of 200x200m exclosure plots designed to permit access by different combinations of large mammalian herbivores (Young et al. 1998). The area in and around KLEE is semi-arid grassland which receives 500-600mm of rainfall annually. The dominant soil type within the area is a clay vertisol (Young et al., 1998).

Resident native large mammalian herbivores include several species of large ungulate herbivores, e.g. cape buffalo (*Syncerus caffer* Sparrman), eland (*Taurotragus*

oryx Pallas), Grevy's zebra (*Equus grevyi* Oustalet), Burchell's zebra (*Equus burchelli* Gray), oryx (*Oryx beisa* Ruppell), hartebeest (*Alcelaphus buselaphus jacksoni* Pallas), Grant's gazelle (*Gazelle granti* Brooke), and steinbuck (*Raphicerus campestris* Thunberg); and the mega-herbivores elephant (*Loxodonta africana* Blumenbach) and giraffe (*Giraffa camelopardalis* Linnaeus), (Young et al., 1998). In addition to native large herbivores, the area is also managed for cattle grazing (*Bos taurus indicus* Linnaeus). Within plots managed for cattle access, cattle are grazed in 2hr 'runs', 6-8 times per year, to simulate the stocking rate of the ranch, which is 0.1 to 0.2 cattle/ha (Young et al., 2005).

To investigate if grazing history influences AMF communities and their effects on host plant response to simulated herbivory, we collected rhizospheres of a common grass, *Themeda triandra*, from three grazing treatments in KLEE: 1) "0" plots from which all large herbivores have been excluded 2) "MW" plots that allow access by mega-herbivores (elephants and giraffe) as well as native ungulates, and 3) "MWC" which are grazed by cattle in addition to mega-herbivores and native ungulates. We collected the rhizospheres of *Themeda* plants in May of 2011 from within each of these KLEE treatments, using a 10x10cm square frame placed around a single plant and then extracting the block of soil within that frame to a depth of 15cm. The rhizospheres of two neighboring *Themeda* plants were sampled from three different locations collected haphazardly across each of the three grazing treatments (6 total samples per grazing treatment). Samples were air-dried and shipped in sealed plastic bags to the University of Vermont.

### 3.3.2. Experimental design

To stimulate production of fresh spores of the native fungal communities for use as experimental inocula, we prepared trap cultures using the collected soils (Morton et al., 1995). Each trap culture was prepared by homogenizing the roots and soil of two neighboring *Themeda* plant samples, and mixing 650mL of this composite sample 1:1 with autoclaved sand (60min at 121°C). All roots were cut into 1.0cm segments and soil aggregates were ground to 2.00mm or less. The trap cultures were seeded with 85-90 Sorghum-sudan seeds and grown in the greenhouse for three months under a 12hr photoperiod with average day and nighttime temperature ranges of 22°-24°C and 17°-20°C respectively. Upon harvest, shoots were removed and roots were cut into <2.0cm pieces and homogenized with their soil. The three trap cultures per grazing treatment were then homogenized into one composite sample per grazing treatment; hereafter referred to as the “experimental inocula”.

To assess if AMF from the three KLEE treatments differed in their effects on plant response to herbivory, we conducted a simulated herbivory experiment which was set up in a factorial design: 3 grazing treatments (representing the three enclosure treatments in KLEE from which *Themeda* rhizospheres were collected) × 2 substrate treatments consisting of 1) substrate amended with experimental inoculum, hereafter referred to as “mycorrhizal” or 2) substrate amended with sterilized inoculum (autoclaved at 121°C for 60 minutes), hereafter referred to as “non-mycorrhizal” × 2 herbivory treatments (clipped vs. non-clipped); resulting in 12 treatment combinations. All experimental pots received 20ml of a grazing treatment-specific microbial filtrate to

reintroduce a portion of the non-AMF soil microbial community (Koide and Li 1989). The microbial filtrate was prepared for each grazing treatment by blending unsterilized inoculum with distilled water at a ratio of 180g of soil per liter of water, passed through a 10 $\mu$ m sieve.

Seeds of *Themeda* were collected at the study site at the time of soil collection. Seeds were collected from outside of the KLEE plots but within ca. 200m from KLEE, in an area with a grazing history similar to MWC (native herbivores and cattle grazing). Seeds were germinated in a growth chamber using a 12:12hr day:night cycle with temperatures of 30°C day and 20°C night. All seedlings had 2-3 green leaves measuring 2-2.5 inches in length when they were transplanted into experimental pots. Experimental pots were 6.4cm diameter x 12.7cm depth, 315mL deeppots, (D19L; Stuewe and Sons Inc., Tangent, OR, USA). The growth substrate for each experimental pot consisted 300 mL of a 1:1 mixture of autoclaved sand and calcined clay (Turface; Industrial Materials Corp., Deerfield, IL, USA) into which 10mL of grazing treatment-specific experimental inoculum (either mycorrhizal or non-mycorrhizal) was thoroughly mixed (3% v/v). Nineteen plants were used for each treatment combination, for a total of 342 plants.

Plants were grown in the greenhouse under a 12hr photoperiod with average day and nighttime temperature ranges of 22°-24°C and 17°-20°C respectively. One week after transplanting, eleven plants were replaced with fresh seedlings due to lack of establishment. All plants were supplemented with 35mL of low P fertilizer (17-4-17, Jack's Pure Water LX, J.R. Peters, Inc. Allentown, PA USA) at the end of the first week and fertilized every four weeks after that until the last month of growth prior to harvest, in which they were not fertilized.

We employed a severe but infrequent clipping design to best approximate what might occur in nature (*sensu* Danckwerts 1993, and Hodgkinson et al., 1989 respectively). We clipped plants to 2.5cm in height twice after seven weeks of growth and again, 10 weeks later. . Leaves that were alive and taller than 2.5cm were clipped and weighed after drying at 65°C for 72-84hrs. Leaves that appeared senesced were not clipped but left for collection at harvest for evaluation of total biomass production.

At the end of the 22 weeks, plants were harvested and roots were separated from shoots. Aboveground material was dried at 65°C for 72-84hrs, and weighed. Roots were rinsed using distilled water, gently dried with a paper towel, and allowed to air-dry for 2-4hrs prior to fresh weights being taken. To assess AMF colonization of plants, approximately 0.15g of roots were cut haphazardly from the fresh root material; effort was made to select from all areas of the root material evenly. Roots were then placed in 70% ethanol and stored at 4°C until processing. The remaining root material was dried at 65°C for 72-84hrs and weighed. To calculate the total dry weight of roots, wet:dry ratios of the oven-dried roots were calculated and applied to the root masses separated for evaluation of AMF colonization. Because it was impossible to process 210 plants all at once, we harvested plants over a two-week period after randomly assigning harvest date among plants in all treatments. The total length of the experiment was thus 22-24 weeks for all treatments.

To assess AMF colonization, roots were stained using 5% ink and vinegar solution (v/v), (Sheaffer Skrip Bottled Ink, black, product SHF94231, Sheaffer Pen, Shelton, CT, USA; methods in Vierheilig et al., 1998). Five samples per non-mycorrhizal treatment combination were stained and counted; all samples from the mycorrhizal

treatments were stained and counted. The percent of root length colonized by AMF was quantified using the gridline intersect method (Giovannetti and Mosse 1980). The number of intersections ranged from 61 to 262;  $\bar{X}=164.87 \pm 40.04\text{sd}$ . Only roots with intact cortical tissue were scored for colonization (Brundrett 2004); these roots were typically  $\leq 0.28\text{mm}$  in diameter. Larger roots had a coarse appearance and, although mycorrhizal hyphae were visible in few instances, no other AMF structures were observed. The primary putative nutrient exchange sites are arbuscules, arbusculate coils, and densely coiled hyphae (Smith and Read 2008). Arbuscules were rarely observed and therefore, arbuscules, arbusculate coils, and coiled hyphae were tabulated together and are referred to as “Nutrient exchange site colonization” (NEXC), calculated as the number of intersections containing these structures divided by the total number of intersections viewed\*100. Spherical and oblong vesicle-like structures observed within roots were tabulated together as vesicle colonization (VC). Percent root length colonization (PRLC) was calculated as the percentage of non-negative intersections.

Leaf tissue was later re-dried at 65°C overnight prior to being ground and analyzed for P concentration. For several clipped plants, we had insufficient aboveground material at harvest to conduct nutrient analysis. To have enough tissue, all plants were randomly paired within treatments and their aboveground tissues combined for analysis. This resulted in 7-9 samples per treatment combination for analysis. Tissue nutrient analysis was conducted by the University of Vermont Agriculture and Environmental Testing Laboratory, by nitric acid digestion and ICP-OES.

### 3.3.3. Statistical analysis

We evaluated the influence of AMF from areas of differing grazing history on plant growth and P acquisition by analyzing above- and belowground biomass production, total biomass production, root:shoot ratio (R:S) and aboveground tissue P concentration at harvest of non-clipped plants as response variables in separate two-way ANCOVA's. Factors included grazing history (0, MW, MWC), substrate (AMF+ vs. AMF-), and their interaction, with harvest date included as a covariate. We also evaluated aboveground growth prior to, and between clipping events, by analyzing the dry weight of removed material at both clipping events and the aboveground dry weight of clipped plants at harvest by two-way ANOVA and ANCOVA's. The dry weight of the clipped material at first clipping was added to the model as a covariate in the analysis of the 2<sup>nd</sup> clipping dry weight. The 2<sup>nd</sup> clipping dry weight and the harvest date were added as covariates in analysis of the dry weight of the aboveground material collected at harvest. We evaluated compensatory response to clipping by combining the clipped and non-clipped plant data sets and introducing the clipping factor into the model, resulting in three-way ANCOVA's. For clipped plants, total biomass included the dry weights of both clipping events combined with the biomass of the plant at harvest. For non-clipped plants, the total biomass produced was simply the dry weight of the plant at harvest. Lastly, we evaluated the extent of mycorrhizal colonization of roots by analyzing PRLC, NEXC, VC as response variables in two-way ANCOVA's for each response variable. Differences in means between mycorrhizal and non-mycorrhizal plants within grazing history treatments, and clipped and non-clipped plants were evaluated by orthogonal LS

Means Contrasts tested against the model errors. PRLC and NEXC were logit and arcsine square root transformed respectively to meet assumptions of normality. Only three of the thirty plants evaluated from the non-mycorrhizal treatments were colonized (PRLC Mean=0.001, SD=0.002). Therefore all plants in the non-mycorrhizal treatments were considered un-colonized. All analyses were performed in JMP® Pro version 10.0.2 (SAS Institute Inc, 2012).

### 3.4. Results

#### 3.4.1. The influence of AMF on growth and aboveground P of non-clipped plants

Plant biomass and P accumulation of plants grown with AMF from 0 were not different from 0- plants (Table 3, Figure 5). However, plant growth and P accumulation were differently influenced by AMF from MW and MWC (two-way MANCOVA, grazing history\*substrate interaction: Wilks'  $\Lambda=0.8290$ , approx.  $F_{(8,188)}=2.3101$ ,  $P=0.0219$ ). Plants in MW+ and MWC+ produced 46 and 45% more belowground biomass, and had 28 and 16% higher P concentrations than plants in MW- and MWC- respectively (Table 3, Figure 5). Plants in MWC+ also produced 29% more aboveground biomass than plants in MWC-, resulting in a 36% increase in total biomass production (Table 3, Figure 5). For plants in MW+, the increase in total biomass (5%) was not statistically significant; this was likely due to a marginal decrease (-14%;  $F_{(1,197)}=3.0829$ ,  $P=0.0807$ ) in aboveground biomass of plants in MW+ below that of plants in MW-. Plants in MW+ thus had 70% higher R:S ratio than MW- plants, whereas the R:S ratio of

plants in the other treatments (0 and MWC) did not differ with or without mycorrhizae (Table 3, Figure 5).

### **3.4.2. The influence of AMF on plant growth prior to- and between clipping events**

In the seven weeks prior to the first clipping event, plants in 0 and MW substrates produced 59 and 42% less aboveground biomass when mycorrhizal, respectively, while aboveground production by plants in MWC did not differ with or without mycorrhizae (Table 4, Figure 6). During the ten weeks between the first and second clipping events, regrowth of clipped MW+ plants exceeded that of MW- plants by 44%, while regrowth of plants in 0 and MWC substrates did not differ with or without mycorrhizae (Table 4, Figure 6). In the 5-7 weeks between the second clipping event and harvest, clipped MW plants produced similar aboveground biomass with or without mycorrhizae, while 0+ plants produced marginally more aboveground biomass (14%;  $F_{(1,98)}=3.5923$ ,  $P=0.0610$ ), and MWC+ plants produced marginally less aboveground biomass (-12%;  $F_{(1,98)}=3.4707$ ,  $P=0.0655$ ) than 0- and MWC- plants respectively (Figure 6).

### **3.4.3. The influence of AMF on plant response to simulated herbivory**

Compensation was evident in three treatments: 1) clipped plants in 0- produced similar aboveground production as non-clipped 0- plants ( $F_{(1,197)}=3.3797$ ,  $P=0.0675$ , Figure 7); 2) clipped plants in MW+ produced similar above and total biomass as non-clipped MW+ plants ( $F_{(1,197)}=0.0172$ ,  $P=0.8959$ ;  $F_{(1,197)}=2.6581$ ,  $P=0.1046$ , respectively,

Figure 7); and 3) clipped plants in MWC- produced similar above- and total biomass as non-clipped MWC- plants ( $F_{(1,197)}=0.0100$ ,  $P=0.9206$ ,  $F_{(1,197)}=1.5507$ ,  $P=0.2145$ , Figure 7). Clipping did not influence P concentrations of aboveground tissue for plants in 0 substrate or MW substrates (Table 5, Figure 7). However, clipping resulted in a 15% reduction in aboveground P of plants in MWC- ( $F_{(1,84)}=4.0983$ ,  $P=0.0461$ ), and an 18% increase in aboveground P of plants in MWC+ ( $F_{(1,84)}=8.6871$ ,  $P=0.0041$ , Figure 7).

#### **3.4.4. Colonization of roots by AMF from areas of differing grazing history**

Grazing history significantly affected all measures of mycorrhizal colonization: percent root length colonization (PRLC), vesicle colonization (VC), nutrient exchange site colonization (NEXC), (MANCOVA: Wilks'  $\Lambda=0.4974$ , approx.  $F_{(6,198)}=13.7900$ ,  $P<0.0001$ ). PRLC was highest in plants of MWC (M=57.5%, SE=2.33, Figure 8a), followed by 0 (M=46.18%, SE=2.36), and MW (M=36.0%, SE=2.27). VC also differed significantly among grazing history treatments with identical ranking as PRLC (MWC: 37.26%, SE=1.81, 0: M=23.23%, SE=1.73, MW: 15.43, SE=1.96, Figure 8b). NEXC was similar in plants of MW (M=14.9%, SE=1.17) and MWC (M=16.7%, SE=1.43), and both were higher than plants of 0 (M=10.8%, SE=1.12, Figure 8c). Overall, clipping resulted in an increase in nutrient exchange site colonization ( $F_{(1,103)}=8.3395$ ,  $P=0.0047$ ); however, LS Means Contrasts revealed significant differences between clipped and non-clipped plants for MWC only ( $F_{(1,102)}=4.9621$ ,  $P=0.0281$ , Figure 8c). Clipping did not affect PRLC or VC.

### 3.5. Discussion

Our findings suggest that differing large herbivore grazing for nearly two decades has resulted in functional changes in AMF communities. Our results supported our predictions in some respects and produced some unexpected patterns. We predicted that clipped plants grown in inoculum from areas historically subject to large mammalian herbivory would compensate for herbivory. Likewise, we predicted that plants grown in inoculum from areas protected from large herbivores for 17 years would show a reduced compensation response. These predictions were partially supported. Clipped plants grown in unsterilized inoculum from areas that have included only large native herbivores compensated for damage; producing equivalent aboveground- and total biomass compared to non-clipped controls. Also, plants grown in inoculum from areas protected from large herbivores for the past 17 years did not compensate. However, compensation was not detected in plants grown in inoculum from areas in which cattle were grazed in addition to the large native herbivores.

Investigations employing entire AMF communities are rare (Borowicz 2013), but reveal that native fungal assemblages can enhance regrowth from herbivory compared to non-mycorrhizal controls (Hetrick et al., 1990). The interaction between plants and mycorrhizal fungi is influenced by several factors, including environmental conditions, and plant and fungal genotypes (Johnson et al., 1997, Jones and Smith 2004). Recent findings indicate that plants can preferentially allocate carbon to more beneficial partners (Bever et al., 2009, Kiers et al., 2011). Changes in the AMF community composition in areas subject to grazing (Bethlenfalvai and Dakessian, 1984, Frank et al., 2003, Eom et

al., 2001, Murray et al., 2010) may therefore be due to selection by plants for partners that are more beneficial in an environment of chronic herbivory. The ability to select for mycorrhizal partners that enhance growth under conditions of herbivory may be the mechanism by which locally-adapted plant-fungal associations could arise (Read 2002, Klironomos 2003, Friesen et al., 2011).

The effects of AMF on compensatory responses were not uniform across grazing treatments. Plants grown in substrates from 0 and MWC were able to compensate for clipping regardless of the presence of AMF and, in fact, growth was reduced by AMF. Although there were no observable advantages or disadvantages of AMF association in non-clipped plants, the carbon required to support AMF likely still represented a carbon drain which inhibited plant recovery from clipping. In contrast to plants in 0 substrate, plants in MWC substrate appear to be reliant on AMF for growth and P acquisition; as plants in MWC+ responded positively to AMF in terms of biomass production and P accumulation (Figure 5), also clipped plants in the non-mycorrhizal MWC substrate showed evidence of P reserve run-down (Figure 7). However, clipping resulted in a significant increase in NEXC of roots (Figure 8) and in P concentration of aboveground tissue (Figure 7) of mycorrhizal plants in MWC substrate, which was not associated with enhanced regrowth from clipping (Figure 7). The high level of mycorrhizal colonization in MWC (Figure 8) may have played a role in increased biomass production and P accumulation of non-clipped plants, but appears to have become parasitic when plants were clipped, as evidenced by an inhibited compensatory response, and excess accumulation of P in the aboveground tissue. In sum, AMF in 0 and MWC had neutral and positive effects on growth and P accumulation of plants when non-clipped

(respectively), but these associations appear to have become parasitic when the plants were clipped.

The only grazing history substrate in which AMF appeared to enhance compensatory response to simulated herbivory was that of MW. Plants in MW+ substrate showed compensation for clipping in terms of aboveground and total biomass production (Figure 7). Even though the presence of AMF resulted in reduced growth early in the experiment (Figure 6), non-clipped plants in MW+ produced similar total biomass, had higher root-shoot ratios, and had higher aboveground P concentrations compared to non-mycorrhizal plants (Figure 5). Enhanced root growth may help fortify plants to withstand herbivory (McNaughton and Chapin 1985), and excess accumulation of P may enhance plant compensatory response (Oyarzabal and Oesterheld 2009). AMF associations in MW substrate may then enhance plant compensation by facilitating a change in allocation of plant resources to belowground structures, and enhancing reserve P prior to clipping. Clipped MW+ plants also produced more aboveground biomass in the 10 weeks between clipping events than non-mycorrhizal plants; however, this cannot be attributed to a compensatory response to clipping in this experiment, as this may instead have been compensation for earlier reduced growth.

We anticipated that *Themeda* grown in substrates from MW and MWC would respond similarly to grazing, as grazing of *Themeda* does not differ between these plots in the field (Odadi 2004). However, the addition of cattle to areas in which large native herbivores graze appears to have a different effect on the functioning of AMF than that of the native herbivores alone. Despite facing similar grazing pressure in the two treatments, *Themeda* may be involved in different plant-plant competitive interactions in MWC than

MW. In KLEE, cattle grazing results in decreased grass cover (Young et al., 2005) and increased plant species diversity and aggregation (Porensky et al., 2013); in these conditions, enhanced growth may be more beneficial than enhanced response to herbivory.

Although our findings suggest a role for mycorrhizal fungi in plant compensation in the MW substrate, we cannot exclude the possibility that other factors or interactions were also involved. Firstly, we were unable to acquire *Themeda* seeds from within the different grazing history treatments, and so seeds were collected from east of the KLEE plots, in an area with a grazing history similar to MWC (native large herbivores and cattle grazing). *Themeda* plants from areas of differing grazing histories in KLEE may respond differently to clipping (Oosterheld and McNaughton 1988), it is therefore possible that seedlings grown in the MWC substrate in this experiment provide the most specific match between plant genotype and fungal associations, and that growth of these seedlings in substrates from 0 and MW represent “foreign” pairings between plants and AMF.

Secondly, we compared growth of plants in unsterilized substrate with that of plants grown in sterile substrate supplemented with a non-mycorrhizal microbial filtrate (Read 2002, Koide and Li 1989). The purpose of this design is to re-introduce a portion of the background non-mycorrhizal microbial community into a sterile substrate to establish non-AMF control plants (Read 2002). Caveats of this experimental design include 1) the potential effects of autoclave sterilization on nutrient availability or soil structure (Trevors 1996), 2) possible unbalancing of non-mycorrhizal microbial abundance or composition between the treatments, and 3) the inability to simulate the interactive effects of mycorrhizal fungi with other soil microbes (Read 2002). The use of a small

amount of soil as experimental inoculum (as in the current study) aids in diluting the potential effects of nutrient release due to sterilization (Brinkman et al., 2010); further, fertilization during the course of the experiment can also reduce differences in nutrient availability caused by sterilization. However, the density of soil organisms in experimental microcosms is likely smaller than in field soil, and thus realistic effects may not be possible to achieve if they are density dependent, or dependent on interactions within or between soil organisms (Brinkman et al., 2010).

Thirdly, *Themeda* growth (Anderson et al., 2006, 2007) and responses to clipping (Anderson et al., 2006) are influenced by substrate texture. The soils in which these plants and fungi occur in KLEE are vertisols with high clay content (Young et al., 1998). Our use of a nearly 1:1 sand and clay substrate may have resulted in different physiological responses of plants to clipping in the greenhouse and thus altered their relationship with AMF symbionts.

Finally, host-specific stimulation of spore production has been observed (Hetrick and Bloom, 1986, Sanders and Fitter 1992, Johnson et al., 1992, Bever et al., 1996, Eom et al., 2000, Bever 2002), and so the use of trap pot culturing with a mycotrophic host to stimulate AMF spore production could have lead to changes in the relative abundances of spores of AMF species. Further, the bulk soil collections that were used to prepare the trap pots could have captured roots, spores, or extraradical mycelium from other non-target plant species; all of which can act as mycorrhizal propagules (Hart and Klironomos 2002 and references therein). Another approach would have been to use bulk soil alone as experimental inoculum. However, this would not have alleviated the problem of including roots, spores, and mycelium of non-target plant. Further, due to temporal

variation in spore production (Bentivenga and Hetrick 1992) the use of bulk soil as inoculum could bias the relative abundance of fresh, viable AMF spores in favor of those species producing more propagules at the time of the soil collection. Also, seedlings may associate with different communities of AMF as they mature (Husband et al., 2002a, 2002b), and the AMF species associating with mature plants may not be the most beneficial for seedlings of the same species (Kiers et al., 2000). In the current study, plants in the field from which bulk soils were collected were mature, whereas we utilized seedlings for the experiment, and thus using only viable AMF propagules in bulk-soil collections from the field may have biased colonization of seedlings due to age-specific dynamics. Despite these caveats, Eom et al., (2000) found that significant host-specificity in mycorrhizal spore community composition detected in field collected soils was also detected in spores isolated from trap cultures prepared with field-collected soils. Further, Bever et al. (1996) found that AMF community compositions from trap pots were similar to associated field soils.

A microcosm approach to mycorrhizal research, should be designed to allow the reassembly of communities of organisms for experimentation, while maximizing relevance and minimizing confounding factors (Read 2002). Our intent in utilizing trap pot culturing to stimulate production of viable spores, was to circumvent temporal and plant age-specific dynamics in viable propagule density by stimulating the production of fresh spores representative of the local diversity present in the soil collected directly beneath *Themeda* plants in the field. With no apriori knowledge regarding which members of the AMF community would impart optimum benefits to *Themeda* seedlings, we considered the best approach to be to provide seedlings with access to viable AMF

propagules representing the diversity of AMF present in the rhizospheres of *Themeda* in the field.

In conclusion, we found evidence that large herbivore grazing has influenced mycorrhizal associations, and that these associations differently influence plant growth and response to clipping. This work supports others (Read 2002, Johnson et al., 2010, Antunes et al., 2011) in that ecologically specific associations should be taken into consideration in mycorrhizal research. Our results suggest that a period of 17 years of protection from large mammalian herbivory, is sufficient to result in functional changes in the association between plants and AMF, possibly resulting in optimized associations under the environmental conditions. Our results have important implications to a highly distributed agriculturally important species. *Themeda* is one of the most abundant plant species in the understory in this study area (Young et al., 1998). A graminaceous perennial with a considerable distribution across Africa, Asia, Australia, and the Middle East (reviewed by Snyman et al., 2013), *Themeda* is an important forage species for wild and domesticated large herbivores (reviewed in Dell'Acqua et al., 2012 and Snyman et al., 2013). In the field, *Themeda* is regarded as sensitive to grazing, in that it decreases in abundance under heavy grazing (Heady 1966). The sensitivity of *Themeda* to grazing may be due to run-down of carbon reserves (Danckwerts, 1993), and is influenced by soil texture (Anderson et al., 2006, 2007), clipping severity (Hodgkinson et al., 1989, Nie et al., 2009), and recovery time (Oesterheld and McNaughton 1988). Our results indicate that growth and response to herbivory of *Themeda* is also influenced by AMF, which are in turn differently influenced by grazing history. It is important that future investigations

into the influence of AMF on plant response to herbivory consider ecologically-specific associations between plants and AMF communities.

### **3.6. Acknowledgements**

This project would not have been possible without the help of several people, primarily members of the Brody Lab and the UVM Greenhouse staff. Special thanks to JBG's MSc. committee and to the UVM Biology Department for constructive criticism of the analysis and interpretation of the results, and to Alan Howard for help with the statistical analysis. Our work was supported by the National Science Foundation (NSF DEB) and by the Mpala Research Center. The Kenya Long-term Exclosure Experiment (KLEE) has been made possible with grants from the James Smithson Fund of the Smithsonian Institution, The National Geographic Society (4691-91), The National Science Foundation (LTREB BSR-97-07477, 03-16402, and 08-16453), and the African Elephant Program of the U.S. Fish and Wildlife Service (98210-0-G563). JBG was also supported by UVM URECA and a UVM CAS APLE Grant.

### 3.7. Figures and tables

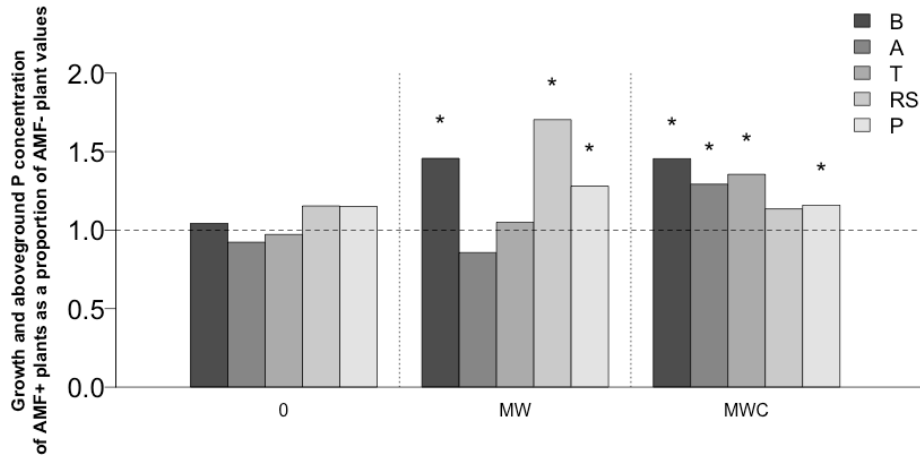


Figure 5: The influence of AMF from areas of differing grazing history on growth and aboveground P concentrations of non-clipped plants. Mean biomass and aboveground P concentrations of AMF+ plants calculated as a proportion of AMF- plant means;  $1 - \frac{[(\text{AMF- treatment mean}) - (\text{AMF+ treatment mean})]}{\text{AMF- treatment mean}}$ . Values above 1.0 indicate that mycorrhizal plant means were higher than non-mycorrhizal plant means. Asterisks indicate significant difference between mycorrhizal and non-mycorrhizal means (LS Means Contrasts,  $P < 0.05$ ). B=belowground, A=aboveground, T=total biomass, RS= R:S ratio, P= P concentration of aboveground tissue.

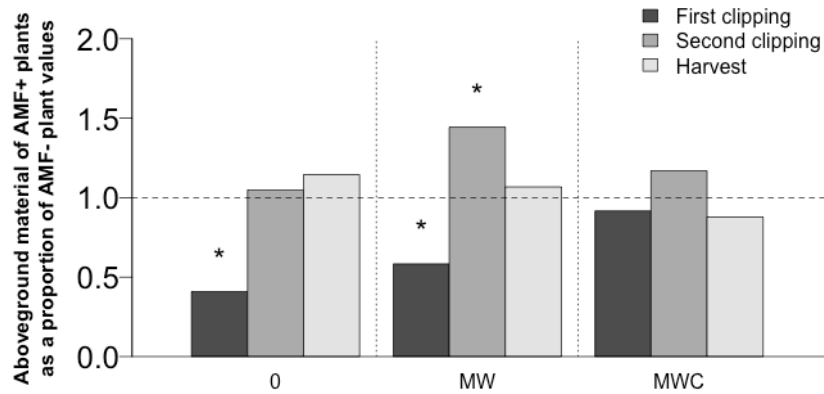


Figure 6: The influence of AMF from areas of differing grazing history on plant aboveground growth prior to- and between clipping events. Mean aboveground biomass of AMF+ plants prior to- and between clipping events, presented as a proportion of AMF- plant means;  $1 - [(AMF- \text{ treatment mean}) - (AMF+ \text{ treatment mean})] / AMF- \text{ treatment mean}$ . Values above 1.0 indicate that AMF+ plant means were higher than AMF- plant means. Asterisks indicate significant difference between mycorrhizal and non-mycorrhizal means (LS Means Contrast,  $P < 0.05$ ).

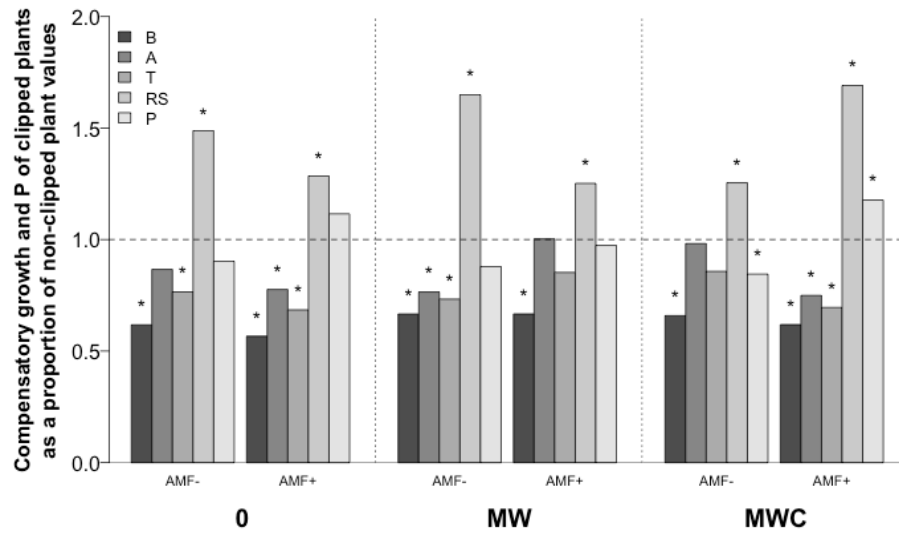


Figure 7: The influence of AMF from areas of differing grazing histories on plant compensatory response to simulated herbivory. Mean biomass and aboveground P concentrations of clipped plants, presented as a proportion of non-clipped plant means;  $1 - [(\text{Non-clipped treatment mean}) - (\text{Clipped treatment mean})] / \text{Non-clipped treatment mean}$ . Values above 1.0 indicate that clipped plant means were higher than non-clipped plant means. Asterisks indicate significant difference between clipped and non-clipped means (LS Means Contrasts,  $P < 0.05$ ), legend as in Figure 5.

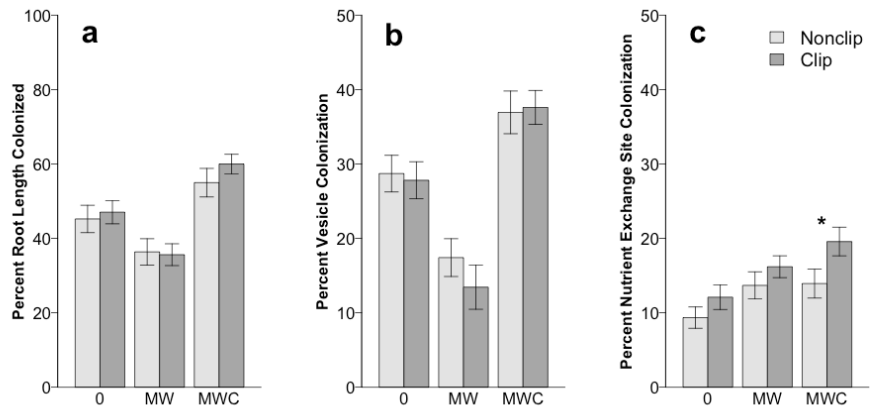


Figure 8: Colonization of roots by AMF from areas of differing grazing history (mean $\pm$ SE). **(a)** percent root length colonization (PRLC), **(b)** percent vesicle colonization (VC), and **(c)** percent nutrient exchange site colonization (NEXC). Asterisk indicates significant difference between clipped and non-clipped means (LS Means Contrast,  $P < 0.05$ ). Y-axes scaled to accommodate values.

Table 3: Orthogonal LS Means Contrast results for contrast of AMF+ vs. AMF- plant growth and aboveground P concentrations of non-clipped plants. Biomass contrasts were tested against three-way ANCOVA model error (denom. df 197) for each response respectively; P concentration was tested against three-way ANOVA model error (denom. df 84) for P concentration.

	Below		Above		Total		R:S		P	
	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>
0	0.04	NS	1.37	NS	0.44	NS	1.61	NS	3.12	NS
MW	12.84	0.0004	3.08	NS	0.28	NS	20.90	<0.0001	17.26	<0.0001
MWC	14.42	0.0002	7.63	0.0063	12.54	0.0005	1.30	NS	4.58	0.0352

Table 4: Orthogonal LS Means Contrast results for contrast of AMF+ vs. AMF- aboveground material collected at the first and second clipping events and at harvest. First clipping contrasts were tested against two-way ANOVA model error (denom. df 100); second clipping and harvest contrasts were tested against two-way ANCOVA model errors (denom. df's 99 and 98 respectively).

	First clipping		Second clipping		Harvest	
	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>
0	30.65	<0.0001	0.28	NS	3.59	NS
MW	8.69	0.0040	15.98	0.0001	0.85	NS
MWC	0.27	NS	2.86	NS	3.47	NS

Table 5: Orthogonal LS Means Contrast results for contrast of clipped vs. non-clipped plant growth and aboveground P concentrations. Biomass contrasts were tested against three-way ANCOVA model error (denom. df 197) for each response respectively; P concentration was tested against three-way ANOVA model error (denom. df 84) for P concentration.

	Belowground		Aboveground		Total		R:S		P	
	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>
0+	30.41	<0.0001	7.01	0.0087	18.19	<0.0001	9.19	0.0028	2.57	NS
0-	25.03	<0.0001	3.38	NS	12.29	0.0006	20.59	<0.0001	1.30	NS
MW+	13.67	0.0003	0.02	NS	2.66	NS	8.48	0.0040	0.25	NS
MW-	7.37	0.0072	7.78	0.0058	9.34	0.0026	15.98	<0.0001	2.84	NS
MWC+	18.02	<0.0001	8.63	0.0037	14.92	0.0002	44.64	<0.0001	8.69	0.0041
MWC-	6.48	0.0117	0.01	NS	1.55	NS	4.22	0.0412	4.10	0.0461

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## CHAPTER 4: CONCLUSIONS AND NEXT STEPS

### 4.1. Conclusions

Mycorrhizal fungi are soil-borne organisms that form symbiotic associations with the majority of land plants. These fungi gather and exchange mineral nutrients with plants for photosynthetically derived carbohydrates. Mycorrhizal fungi can also confer other benefits onto plants, e.g. defense against pathogens, improved water relations, tolerance to heavy metal toxicity and herbivory. The influence of mycorrhizal fungi on plant mineral nutrition and response to stress suggests that these organisms may have a role to play in sustainable agriculture as well as in bioremediation and ecosystem restoration.

In order to harness this potential and manage it sustainably, we must develop our understanding of mycorrhizal fungi at every level, from transmission genetics to functional specificity of the associations in natural and managed ecosystems. Regulatory mechanisms are now coming to light; specifically, plants and fungi have been found to confer increased benefits upon partners that prove more beneficial. Further, local environmental conditions may select for optimal associations between plants and fungi in the environment within which they are found.

In contributing to this important research, I investigated host-specific interactions of AMF by exploring the interaction between the sex morphs of the gynodioecious perennial herb *Polemonium foliosissimum* (Polemoniaceae) and their mycorrhizal associates in the field. I will refer to this investigation as “Experiment 1”. My findings

suggest that the sexes of *P. foliosissimum* do not interact differently with mycorrhizal fungi, and thus do not represent different “hosts”.

Secondly, I investigated local adaptation of mycorrhizal associations by exploring the effect of large herbivore grazing on plant-mycorrhizal associations. I will refer to this as “Experiment 2”. My findings from this experiment suggests that mycorrhizal fungi differently mediate plant growth and response to herbivory in this system.

## **4.2. Next steps**

### **4.2.1. Experiment 1**

I detected no differential benefit of mycorrhizal colonization between the sexes of *P. foliosissimum*. That does not indicate that there is truly no difference in benefit received by the different sexes. The sexes may still differ in benefit from mycorrhizal associations, despite lack of differences in root colonization or leaf tissue nutrient concentrations; in fact, these measures are only a proxy for benefit. The next experiment to be conducted in this system should be to compare responses of plants grown with and without mycorrhizal colonization. Varga and associates have found sex-specific responses to mycorrhizal colonization in the gynodioecious plant *Geranium sylvaticum* (references within Chapter 2) by clonally propagating plants of the different sexes, and growing them in mycorrhizal and non-mycorrhizal conditions. If this approach is pursued with *P. foliosissimum*, it is critical that the clones be grown in association with mycorrhizal assemblages native to the same geographic area, and hopefully the same

actual population, from which the plant tissues are collected. This would result in pairing the plant genotype with the AMF genotypes with which they are most accustomed to associating.

Other questions that could be addressed simultaneously are 1) does the composition of the fungal community colonizing the roots of the sexes differ? 2) does the extent of external mycelial growth by fungi colonizing roots of the two sexes differ? 3) does the extent of colonization, or composition of species colonizing roots differ over the season or over the life of the plants? and 4) does 'benefit', as defined by the researcher, differ between the sexes over the life of the plant? I am not familiar with plant propagation methods; these methods and any possible influences of the propagation process on the plants or their relationship with mycorrhizal fungi should be researched.

If in a future investigation, roots of *P. foliosissimum* are sampled and stained for quantifying mycorrhizal colonization, samples of individual plants should be limited to no more than one or two (well-spaced) samples per season. I took core samples from one plant twice within one week's time, and the plant subsequently died. It may be possible to take an early season sample, and a late season sample from the same plant without killing it- but I did not test this. Also, root clearing and staining trials involving warm KOH should be pursued. I had great difficulty clearing the field roots while on-site in RMBL. I had no fume hood or warm water bath to work with, and so I chose the most innocuous clearing and staining method that I could employ. I believe better clearing of the roots can be achieved with warm KOH, either in a warm water bath (~80C) for several days, or in an autoclave (121C) for 20-45 minutes.

#### 4.2.2. Experiment 2

Despite findings of differential growth and response to herbivory when grown with AMF from areas differing in grazing history, whether these differences are truly due to local adaptation of AMF, and whether these findings can be extrapolated to the field, remains to be seen. An important next step toward determining whether local adaptation of AMF to herbivory is taking place, is to determine to what extent the diversity of AMF in the roots of plants in the greenhouse corresponds to the diversity of AMF in roots collected in the field.

A caveat of my experimental design was the use of trap pots to stimulate propagule production for the inoculum of the experiment. The primary concern with this method is that AMF fungi can show differential sporulation with different host plants (see references within Chapter 3). This leads to the concern that species propagule abundance in trap pots may differ from the field, which may lead to differences in the extent of colonization by fungi from that which is seen in the field. If these concerns are valid, it may be that species diversity within the roots in my greenhouse experiment differs from that of plants in the field. To my knowledge it has not been shown experimentally, that 1) differential intra-specific propagule production occurs in trap pots when compared to similar species assemblages in the field, or 2) the diversity of AMF colonizing host plant roots changes subsequent to a trap culturing. Comparison of AMF diversity within the greenhouse roots of my experiment to that of roots collected in the field could provide very important information regarding the effect of trap culturing on

the diversity of AMF species that colonize roots, and would be an important finding to the field of mycorrhizal research.

Next, a full field experiment should follow findings such as this, in order to confirm their applicability in natural systems. The microcosm approach, while insightful, lacks much ecological realism. In nature, plants interact with AMF via common mycorrhizal networks (CMN) in which both plant and fungi are simultaneously associating with multiple partners. This is an issue that is not generally taken into consideration in microcosm experiments. Plants could respond much differently to simulated herbivory when they are connected to other plants via this symbiotic network. *Themeda* may interact with other plants via the CMN, and perhaps gain increased benefits from the association over conditions in which other partners are not present. This opposite might also be true- when connected to a CMN, *Themeda* may enhance the response of its neighboring plants. Our understanding of nutrient movement through these networks is very limited, but they appear to play a role in plant response to colonization (Walder et al., 2012). Although investigating the influence of the CMN in the field is conceptually very challenging, a more realistic microcosm experiment could be developed which employed such a network. For more information, consider Walder et al., (2012).

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## APPENDIX A; SUPPLEMENT TO CHAPTER 2

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## A.1. PLANT ACCOUNTABILITY INFORMATION

The same plants are not used in every response analyzed and thus the plants contributing to the number of experimental units in each analysis (the “n”) may not be clear in the text of the chapter. The purpose of this section is to account for all experimental plants used in each of the separate data analyses.

**Soil microsite analysis:** a micro-site analysis was performed in which soil collected from the base of three female and hermaphrodite plants within the site were analyzed. The three plants of each sex were matched in size representing three size classes, 4-9 stalks, 10-18 stalks, and 19+ stalks (n=3 per sex).

**Dry weight, whole leaf size and specific leaf area of clipped plants:** The dry weight of entire stalks was acquired for all clipped plants (n=20). The clipped stalks of one plant inadvertently froze in the refrigerator prior to my being able to remove leaves for measurements of SLA and WLS (Plant #22, Female, 15 stalks). This plant is not included in the analysis; SLA and WLS data was available for all remaining plants (n=19).

**Early season “ambient” mycorrhizal colonization:** For three plants, I was unable to acquire more than 35 intersections of data with which to calculate measures of colonization. These plants are not included in the analysis; therefore n=17 for pre-clip colonization data.

**Late season mycorrhizal colonization:** All plants of the clipping experiment were sampled (20 females and 20 hermaphrodites); however, two plants' sampling bags were mislabeled and so were not distinguishable from one-another (#110 Hermaphrodite, Clipped, 6 stalks; and #111 Female, Clipped, 14 stalks) and were removed from the analysis, all remaining plant samples were accounted for (n=38).

**Late season nutrient analysis:** Plant #102 (Hermaphrodite, Clipped, 5 stalks) had only one full stalk remaining at the end of the experiment, the other stalk had been damaged, and so sampling for nutrient analysis was not performed as this would have required removing most of the remaining leaf tissue from this plant. All remaining plants from the herbivory experiment had leaves sampled for nutrient analysis (n=39).

**Analysis of flower size correlation with mycorrhizal colonization:** Only plants for which both colonization data and flower size data were available were evaluated for colonization. This included 33 plants.

**Late season whole leaf size and specific leaf area:** All plants from the clipping experiment were sampled (20 females and 20 hermaphrodites), all samples are accounted for (n=40). I also sampled 7 cored plants (4 females and 3 hermaphrodites), but was not able to sample more and so cored plants were not included in the analysis.

**Late season stalk length and diameter:** Stalk lengths averages were calculated as the average of three randomly selected stalks. Plant #102 (Hermaphrodite, clipped, 5 stalks) had only one full length stalk, the other stalk had been damaged, and so this plant's length and diameter data were excluded from analysis. Plant #3 (Female, clipped, 5 stalks), plant #43 (Female, control, 6 stalks), and plant #97 (Female, cored, 5 stalks) had experienced recent herbivory and had less than 2 full-length stalks available, these plants were excluded from measurement. Plant #33 (Female, clipped, 7 stalks) had only 2 stalks available due to recent natural herbivory. The length and diameter of these two stalks were averaged and included in the analysis. Plant #29 (Hermaphrodite, control, 5 stalks) was not found. Plants #100 (Hermaphrodite, clipped, 8 stalks) and #110 (Hermaphrodite, clipped, 6 stalks) died after the final root coring and were not measured. All remaining plants had 3 stalks available for measurement, therefore n=53.

**Fitness measures:** Two plants #100 (Hermaphrodite, Clipped, 8 stalks) and #110 (Hermaphrodite, Clipped, 6 stalks) died subsequent to the final coring for colonization and so fruit data and samples were not taken. Plant #83 (Hermaphrodite, Clipped, 21 stalks) was not found. The cored plants were also sampled; therefore, fitness data is available for n=57 plants.

## **A.2. *POLEMONIUM FOLIOSISSIMUM* FUNCTIONAL TRAIT ANALYSIS**

### **A.2.1. Materials and methods**

#### **A.2.1a. Clipped plant material dry weight, whole leaf size, and specific leaf area**

Clipped plant material was collected as described in Chapter 2. The base of the clipped stalks were wrapped in a moist paper towel and placed in an unclosed plastic ziplock bag. Plants were thus placed in a fridge at 6°C until processing. I then dried the clipped plant material for 84h at 55°C to a constant weight. Several of the leaves of one female plant were damaged while refrigerated prior to weighing; this plant has therefore been excluded from leaf measurements and tissue nutrient analysis. Three hermaphrodites and three females were selected to have their leaf tissue analyzed for nutrient content. These individuals were selected according to the plant size classes described in the chapter for soil collection for soil nutrient analysis. Leaf tissue used for analysis was selected according to Cornelissen et al. 2003 in that only the youngest, fully expanded leaves were used, with petiole and rachis removed. Leaf nutrient content was analyzed by the University of Maine Analytical Laboratory, and determined by dry ashing, acid digestion, and inductively coupled plasma spectrometry. Using the clipped plant material, I chose two of the youngest, fully expanded leaves for analysis of WLS and SLA and measured them according to Cornelissen et al. 2003 by digitally scanning the leaves and using ImageJ software (ImageJ version 1.42q, National Institute of Health,

2009) to calculate the area of the leaves. SLA was calculated by dividing the area of the leaf by its oven-dry mass, expressed in  $\text{mm}^2\text{mg}^{-1}$ .

#### **A.2.1b. Average stalk length and diameter**

Three stalks per plant were haphazardly selected for measurement and their values averaged to calculate the average stalk diameter and height per plant. In a few cases, the number of stalks available for measurement was less than three; however, only plants with at least two stalks available were measured. Stalk diameter and height were measured following the plant height and specific stalk density protocols described in Cornelissen et al. 2003. Plant height was measured as the distance between the ground and the highest photosynthetic tissue, not including inflorescence. Three diameter measurements were taken from evenly along a 10cm section of the stalk at one-third the full height and averaged.

#### **A.2.1c. Late season whole leaf size and specific leaf area**

Following the protocols described in Cornelissen et al. 2003, I sampled two young, fully expanded leaves for analysis of whole leaf size (WLS) and specific leaf area (SLA). Leaves were held in moist paper towels in a sealed plastic bag at  $6^\circ\text{C}$  until processing (no more than 12 hours). Leaves with petioles and rachis attached were digitally scanned and their areas determined using ImageJ software (ImageJ version 1.42q, National Institute of Health, 2009). Leaves were then dried at  $55^\circ\text{C}$  to a constant

weight and SLA calculated by dividing the area of each leaf by its oven-dry mass, expressed in  $\text{mm}^2\text{mg}^{-1}$ . The two leaves' values were averaged to calculate the average SLA per plant.

#### **A.2.1d. Flower dimensions**

Three flowers per plant were haphazardly chosen for measurement. The length and width of sepals, petals, and corollas were measured using calipers per guidance of Dr. Alison Brody. These measurements were averaged per plant.

#### **A.2.1e. Statistical analysis**

For each group of response variables measured, analyses initially proceeded as multivariate analyses of covariance (MANCOVA), with the exception dry weight of clipped plant material and leaf tissue analysis. Dry weight was only one variable and so MANOVA not applicable, leaf tissue analysis by MANCOVA resulted in error in R program, possibly due to low number of samples, and so was investigated by separate ANCOVA's. Effects that were found to be significant or marginally so were then investigated by analysis of covariance (ANCOVA) with no subsequent correction for multiple comparisons applied unless noted.

Multivariate analyses of covariance (MANCOVA), analyses of covariance (ANCOVA), and Tukey's HSD tests performed in R version 3.2.0 (R Core Team, 2013). Assumption of normality of residuals was verified by graphical analysis of qqPlots in R

and Shapiro-Wilk test in JMP version 10.0.2 (SAS Institute Inc, 2012). Tests for homogeneity of variance were conducted in JMP. Transformations of data are reported for cases in which serious departures from assumptions were identified. All means are reported with  $\pm$ SD. Clipped plant SLA data were inverse-transformed to meet assumption of normality.

Stalk length and diameter were measured over all three treatments (clip, core, and control) whereas whole leaf size and specific leaf area were measured for clipped and control plants only. Due to the difference in sample size, stalk length and diameter was analyzed by MANCOVA separately from WLS and SLA. Factors included plant sex (female, hermaphrodite), experimental treatment (clipped or control), plant size (number of stalks) and all interactions. Stalk length data were inverse transformed to meet assumption of normality. Correlation between mycorrhizal colonization and stalk length, stalk diameter, whole leaf size, and specific leaf area was evaluated by Pearson's correlation coefficient.

A three-way MANCOVA of the six flower dimensions was performed with the main effects plant sex (female, hermaphrodite), experimental treatment (clipped, cored, control), and plant size (number of stalks) as a covariate, and included all interactions. Separate three-way ANCOVA's were then performed to evaluate the influence of these factors on flower size. Subsequently, corolla width was found to be significantly influenced by sex and the treatment by number of stalks interaction. In order to describe these effects, separate two-way ANCOVA's were performed within each treatment data set with sex and plant size as the main effects. Correlation between mycorrhizal

colonization and flower size measurements was evaluated by Pearson's correlation coefficient.

## A.2.2. Results

### A.2.2a. Clipped plant dry weight, WLS, SLA, and nutrient analysis

A two-way ANCOVA revealed no difference in dry weight of plants of the two sexes ( $F_{(1,16)}=1.1972$ ,  $P=0.2901$ , Table A1). Predictably, dry weight of clipped material increased with increasing number of stalks ( $F_{(1,16)}=25.8495$ ,  $P=0.0001$ ). The sex\*plant size interaction was marginally significant ( $F_{(1,16)}=4.4302$ ,  $P=0.0515$ , Figure A1) in that hermaphrodites with more stalks tended to weigh more than females with more stalks. Two-way MANCOVA revealed that neither whole leaf size (WLS) nor specific leaf area (SLA) of the clipped plants were influenced by sex or plant size (Wilks'  $\Lambda=0.9097$ , approx.  $F_{(2,15)}=0.7441$ ,  $P=0.4919$  and Wilks'  $\Lambda=0.9734$ , approx.  $F_{(2,15)}=0.2052$ ,  $P=0.8167$  respectively, Table A1). Two-way ANCOVA evaluated at level of significance  $\alpha=0.025$  (Bonferroni correction  $\alpha/2$ ), revealed that whole leaf size was also marginally influenced by the sex\*number of stalks interaction ( $F_{(1,15)}=4.7075$ ,  $p=0.0465$ ), in that large female plants tended to have smaller leaves than large hermaphrodites (Figure A1).

Two-way ANCOVA's revealed that Mg, and Fe were significantly influenced by plant sex ( $F_{(1,5)}=8.4444$ ,  $P=0.0336$ , and  $F_{(1,5)}=7.6191$ ,  $P=0.0398$  respectively) with females having higher values in both cases. Cu and Al concentrations were marginally influenced by plant sex  $F_{(1,5)}=4.8696$ ,  $P=0.0784$ , and  $F_{(1,5)}=5.4312$ ,  $P=0.0672$

respectively, again females had higher values in both cases. Further, Al concentration was significantly influenced by plant size,  $F_{(1,5)}=6.7622$ ,  $P=0.0482$ , and Fe was marginally influenced by plant size ( $F_{(1,5)}=5.3282$ ,  $P=0.0691$ ), in both Al, and Fe, concentrations decreased with increasing plant size.

#### **A.2.2b. Average stalk length and diameter and late season WLS and SLA**

Three-way MANCOVA revealed that neither stalk length nor diameter were influenced by sex, plant size, or experimental treatments (Wilks'  $\Lambda=0.9190$  approx.  $F_{(2,40)}=1.7639$ ,  $P=0.1844$ , Wilks'  $\Lambda=0.9312$ , approx.  $F_{(2,40)}=1.4782$ ,  $P=0.2402$ , and Wilks'  $\Lambda=0.9577$ , approx.  $F_{(4,80)}=0.4369$ ,  $P=0.7816$  respectively, Table A2). Similarly, the separate three-way MANCOVA revealed that neither whole leaf size nor specific leaf area were influenced by sex, plant size, or experimental treatments (Wilks'  $\Lambda=0.9695$ , approx.  $F_{(2,31)}=0.484$ ,  $P=0.6189$ , Wilks'  $\Lambda=0.9891$ , approx.  $F_{(2,31)}=0.1714$ ,  $P=0.8433$ ), and Wilks'  $\Lambda=0.9322$ , approx.  $F_{(2,31)}=1.1282$ ,  $P=0.3365$ ). No leaf or stalk measurements were highly correlated with mycorrhizal colonization (all  $r^2 \leq 0.10$ ).

#### **A.2.2c. Flower dimensions**

Three-way MANCOVA revealed that flower characteristics were significantly influenced by sex (Wilks'  $\Lambda=0.2938$ , approx.  $F_{(6,37)}=14.0828$ ,  $P<0.0001$ , Figure A2) and significantly influenced by plant size (Wilks'  $\Lambda=0.7189$ , approx.  $F_{(6,37)}=2.4115$ ,  $P=0.0456$ ). The experimental treatments did not directly influence flower characteristics

(Wilks'  $\Lambda=0.8889$ , approx.  $F_{(12,74)}=0.3742$ ,  $P=0.9687$ ); however, flower characteristics were significantly influenced by the treatment\*plant size interaction (Wilks'  $\Lambda=0.5562$ , approx.  $F_{(12,74)}=2.1004$ ,  $P=0.0269$ ). Subsequent three-way ANCOVA's revealed significant effects of sex on petal length ( $F_{(1,42)}=64.9048$ ,  $P<0.0001$ ), petal width ( $F_{(1,42)}=50.4629$ ,  $P<0.0001$ ), and corolla width ( $F_{(1,42)}=13.1722$ ,  $P=0.0008$ ) in that hermaphrodites had larger measurements in all cases (Table A3). Sepal and corolla lengths were marginally influenced by sex ( $F_{(1,42)}=3.8542$ ,  $P=0.0563$  and  $F_{(1,42)}=3.0537$ ,  $P=0.0879$  respectively). Again, hermaphrodites had larger values for both measures (Table A3). Sepal width did not differ by sex ( $F_{(1,42)}=1.7235$ ,  $P=0.1964$ ). Sepal length was found to be significantly influenced by plant size ( $F_{(1,42)}=7.7302$ ,  $P=0.0081$ , (Figure A3) in that larger plants tended to have longer sepals. No other flower dimensions were influenced by plant size (all  $P\geq 0.1693$ ). Corolla width was revealed to be significantly influenced by the treatment\*plant size interaction ( $F_{(2,42)}=6.2726$ ,  $P=0.0041$ ). To explore this effect, separate two-way ANCOVA's were performed within each of the three treatment datasets with the main effects of sex and plant size. The corolla widths of control plants did not differ by sex or plant size ( $F_{(1,15)}=0.1831$ ,  $P=0.6748$ , and  $F_{(1,15)}=0.0686$ ,  $P=0.7969$  respectively) whereas clipped plants and cored plants responded differently to their respective treatments. The corolla widths of clipped plants were influenced by both sex and plant size ( $F_{(1,12)}=9.1115$ ,  $P=0.0107$ , and  $F_{(1,15)}=6.2317$ ,  $P=0.0283$ ). Likewise, the corolla widths of cored plants were influenced by both sex and plant size ( $F_{(1,15)}=11.0144$ ,  $P=0.0047$ , and  $F_{(1,15)}=7.2541$ ,  $P=0.0167$ ) (Figure A4). No flower measurements were highly correlated with mycorrhizal colonization (all  $r^2\leq 0.10$ ).

### A.2.3. Discussion

Marginal differences were found in clipped plant material dry weight and whole leaf size (Figure A1). Differences in leaf nutrient concentrations by plant sex and size were also found in clipped plant nutrient concentrations; however, the end of season sampling of the entire study site revealed that specific leaf area, whole leaf size, stalk length, stalk diameter, and nutrient concentrations did not differ by plant sex or size nor was influenced by the experimental treatments (Table A2, also see chapter contents). I therefore considered the early marginal differences to be due to either a) sampling error, in that the sample sizes were quite small, or b) temporal variation in plant phenology. Flower characteristics differed between females and hermaphrodites for three dimensions: petal length, petal width, and corolla width, with females have the larger dimensions in all three cases (Figure A2).

Interestingly, plant size influenced two flower dimensions: sepal length (Figure A3) and corolla width (Figure A4). Sepals appear to be longer in larger plants (regardless of sex). The effect of plant size on corollas was quite complex in that it was only manifested in stressed plants. The corolla widths of control plants did not differ by sex or plant size; however, both above and belowground stress (clipping and coring the plants) resulted in significant differences in corolla width by sex and by plant size (Figure A4). While the effect of plant size is significant in both the clipped and cored plants, the slopes of the lines are opposite one another in that corolla width of clipped plants decreases with plant size for both sexes, while that of cored plants increases with plant size similarly for both sexes (Figure A4).

#### **A.2.4. Conclusion**

The marginal differences in dry weight and flower size of clipped plants, as well as the differences in leaf tissue nutrient concentrations suggests that the two sexes may differ phenologically, growing at different rates. However, these data must be taken with extreme caution, as the sample sizes employed were quite small (nutrient analysis results are based on n=3 per sex). These tests should be repeated with a larger sample size before investing in further investigations.

It appears that *P. foliosissimum* flower size responds to stress in a plant-size specific manner, and that the direction and magnitude of this response is influenced by the type of stress, and the sex of the plant respectively. In my investigation, I was concerned primarily with the influence of mycorrhizal fungi on these and other characters of the sexes of *P. foliosissimum*. Mycorrhizal colonization was not correlated with plant functional traits and so I did not delve further into these interesting differences. It would be very interesting to grow plants in both mycorrhizal and non-mycorrhizal to investigate whether these differences are influenced by mycorrhizal fungi. I hope that these data may be useful to myself or another researcher in future investigations.

#### **A.2.5. Literature Cited**

Cornelissen, J.H.C., Lavorel, S., Garnier, E., Dias, S., Buchmann, N., Gurvich, D.E., Reich, P.B., ter Steege, H., Morgan, H.D., van der Heijden, M.G.A., Pausas, J.G., Poorter, H. 2003. A handbook of protocols for standardized and easy measurement of plant functional traits worldwide. *Australian Journal of Botany* 51:335-380.

### A.2.6. Figures and tables

Table A1: Plant trait measurements at clipping (Means±SD). Two-way ANCOVA revealed no difference in dry weight of plants of the two sexes ( $F_{(1,16)}=1.1972$ ,  $P=0.2901$ ). Two-way MANCOVA revealed that neither whole leaf size (WLS) nor specific leaf area (SLA) of the clipped plants were influenced by sex (Wilks'  $\Lambda=0.9097$ , approx.  $F_{(2,15)}=0.7441$ ,  $P=0.4919$ ).

Plant trait	Female	Hermaphrodite
Dry weight (g)	10.18±5.45 (n=10)	12.77±10.67 (n=10)
Whole leaf size (mm <sup>2</sup> )	957.65±280.65 (n=9)	1069.24±321.22 (n=10)
Specific leaf area (mm <sup>2</sup> /mg)	29.31±9.22 (n=9)	26.27±5.74 (n=10)

Table A2: Plant trait measurements at end of the experiment (Means±SD). Three-way MANCOVA revealed that neither stalk length nor diameter were influenced by sex (Wilks'  $\Lambda=0.9190$  approx.  $F_{(2,40)}=1.7639$ ,  $P=0.1844$ ). Similarly, the separate three-way MANCOVA revealed that neither whole leaf size nor specific leaf area were influenced by sex (Wilks'  $\Lambda=0.9695$ , approx.  $F_{(2,31)}=0.484$ ,  $P=0.6189$ ).

Plant trait	Female	Hermaphrodite
Stalk length (cm)	59.75±10.19 (n=27)	61.42±9.56 (n=26)
Stalk diameter (cm)	3.94±0.91 (n=27)	3.75±0.77 (n=26)
Whole leaf size (mm <sup>2</sup> )	596.85±127.11 (n=20)	574.91±102.44 (n=20)
Specific leaf area (mm <sup>2</sup> /mg)	19.59±2.40 (n=20)	20.19±1.77 (n=20)

Table A3: Flower dimension table of means±SD with connecting letters (Tukey’s HSD). Three-way MANCOVA revealed that flower characteristics were significantly influenced by sex (Wilks’  $\Lambda=0.2938$ , approx.  $F_{(6,37)}=14.0828$ ,  $P<0.0001$ ). Subsequent three-way ANCOVA’s revealed significant effects of sex on petal length ( $F_{(1,42)}=64.9048$ ,  $P<0.0001$ ), petal width ( $F_{(1,42)}=50.4629$ ,  $P<0.0001$ ), and corolla width ( $F_{(1,42)}=13.1722$ ,  $P=0.0008$ ) in that hermaphrodites had larger measurements in all cases. Sepal and corolla lengths were marginally influenced by sex ( $F_{(1,42)}=3.8542$ ,  $P=0.0563$  and  $F_{(1,42)}=3.0537$ ,  $P=0.0879$  respectively). Again, hermaphrodites had larger values for both measures. Sepal width did not differ by sex ( $F_{(1,42)}=1.7235$ ,  $P=0.1964$ ).

Flower Dimension	Female (n=28)	Hermaphrodite (n=26)
Sepal length	7.13±0.58a	7.44±0.67a
Sepal Width	2.22±0.24a	2.30±0.19a
Petal Length	8.18±0.74a	10.37±0.94b
Petal Width	5.78±0.51a	7.08±0.79b
Corolla Length	3.86±0.39a	4.05±0.40a
Corolla Width	4.63±0.45a	5.09±0.55b

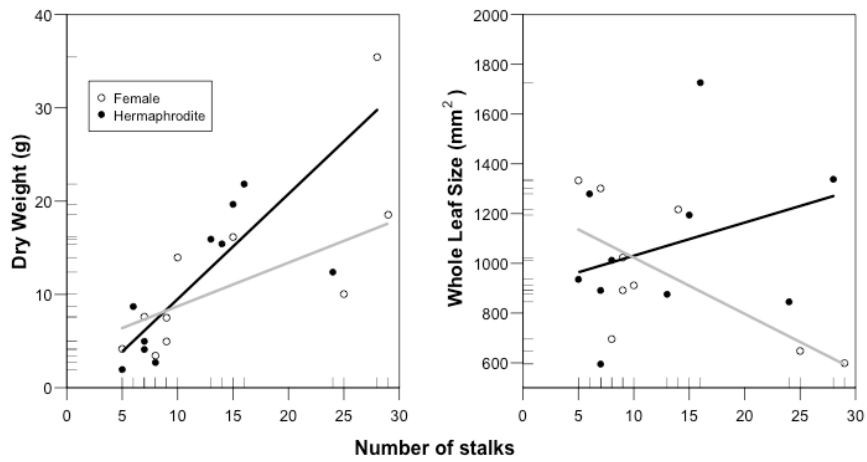


Figure A1: Interaction of sex and plant size (number of stalks) on dry weight (a) and whole leaf size (b) of clipped material. One-way ANCOVA revealed that plant dry weight was marginally influenced by the sex\*number of stalks interaction ( $F_{(1,16)}=4.4302$ ,  $p=0.0515$ ). One-way ANCOVA evaluated at level of significance  $\alpha=0.025$  (Bonferroni correction  $\alpha/2$ ), revealed that whole leaf size was also marginally influenced by the sex\*number of stalks interaction ( $F_{(1,15)}=4.7075$ ,  $p=0.0465$ ).

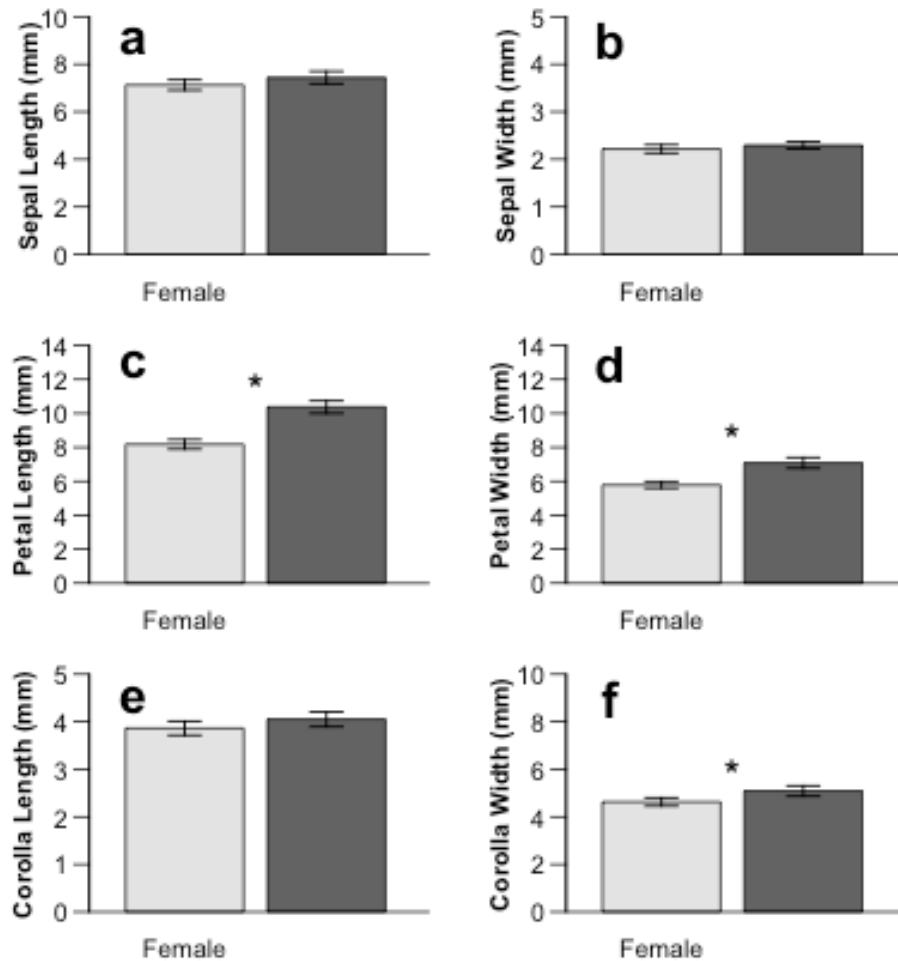


Figure A2: Three-way MANCOVA revealed that flower characteristics were significantly influenced by sex (Wilks'  $\Lambda=0.2938$ , approx.  $F_{(6,37)}=14.0828$ ,  $P<0.0001$ ). Asterisks indicate significant differences between female and hermaphrodites (Tukey's HSD). Female and hermaphrodite flowers differed in petal length (c), petal width (d) and corolla width (f). No differences were found between female and hermaphrodite plants in sepal length (a), sepal width (b) or corolla length (e).

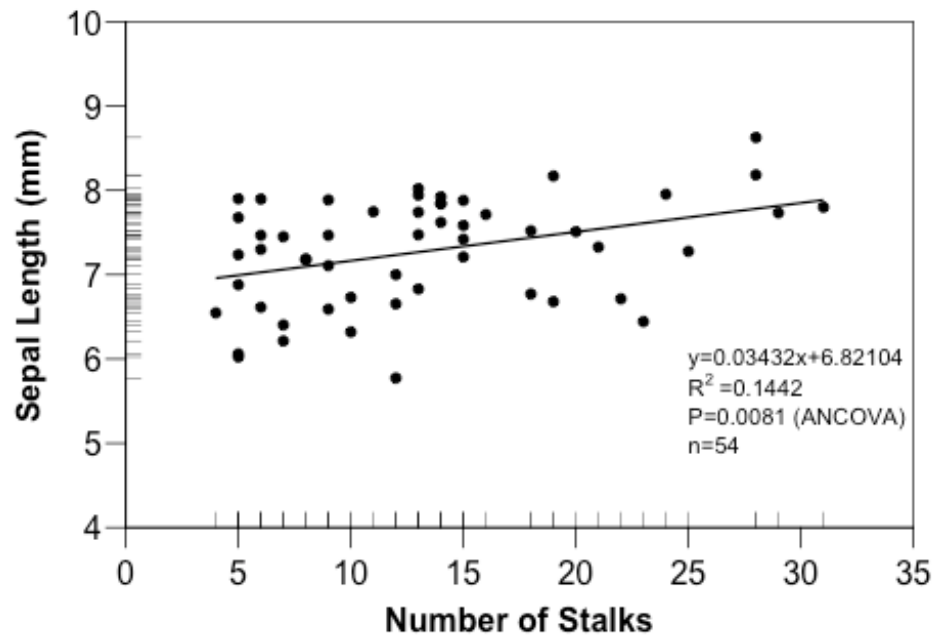


Figure A3. Sepal length by plant size. Sepal length was found to be significantly influenced by plant size (three-way ANCOVA,  $F_{(1,42)}=7.7302$ ,  $P=0.0081$ ) in that larger plants tended to have longer sepals. In the above figure, the equation and  $R^2$  values are from the simple linear regression of sepal length on plant size. The P value is from the three-way ANCOVA.

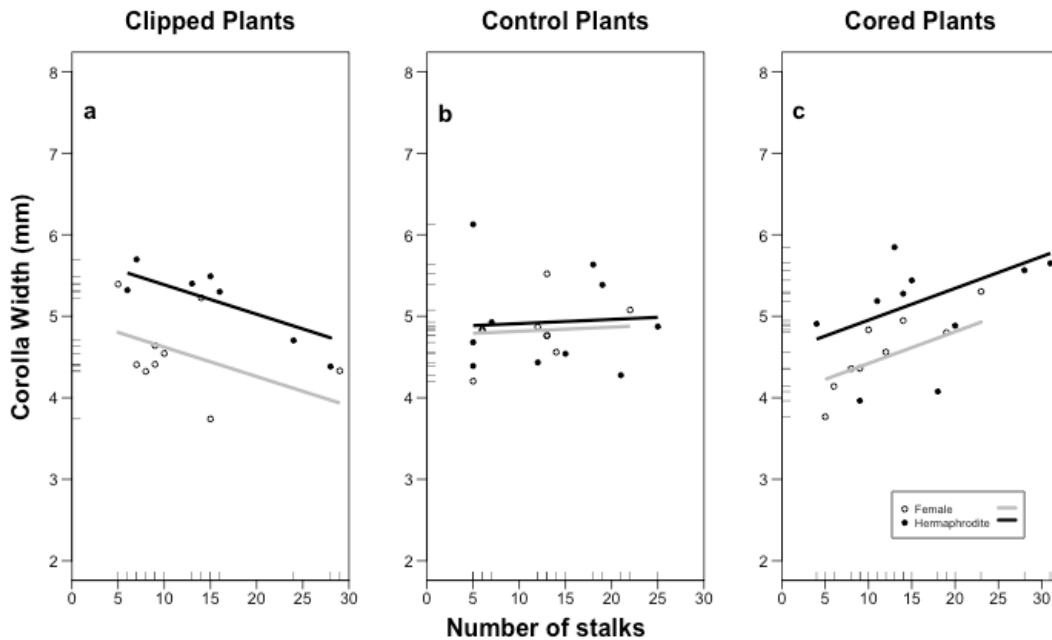


Figure A4. Corolla width by sex and plant size. Corolla width was significantly influenced by the treatment\*plant size interaction (three-way ANCOVA,  $F_{(2,42)}=6.2726$ ,  $P=0.0041$ ). Separate two-way ANCOVA's were then performed within each of the three treatment datasets with main effects of sex and plant size, and their interaction. The corolla widths of control plants (b) did not differ by sex or plant size ( $F_{(1,15)}=0.1831$ ,  $P=0.6748$ , and  $F_{(1,15)}=0.0686$ ,  $P=0.7969$  respectively) whereas clipped plants (a) and cored plants (c) responded differently to their respective treatments. The corolla widths of clipped plants were influenced by both sex and plant size ( $F_{(1,12)}=9.1115$ ,  $P=0.0107$ , and  $F_{(1,15)}=6.2317$ ,  $P=0.0283$ ). Likewise, the corolla widths of cored plants were influenced by both sex and plant size ( $F_{(1,15)}=11.0144$ ,  $P=0.0047$ , and  $F_{(1,15)}=7.2541$ ,  $P=0.0167$ ).

### A.3. FITNESS TERMINOLOGY TREE

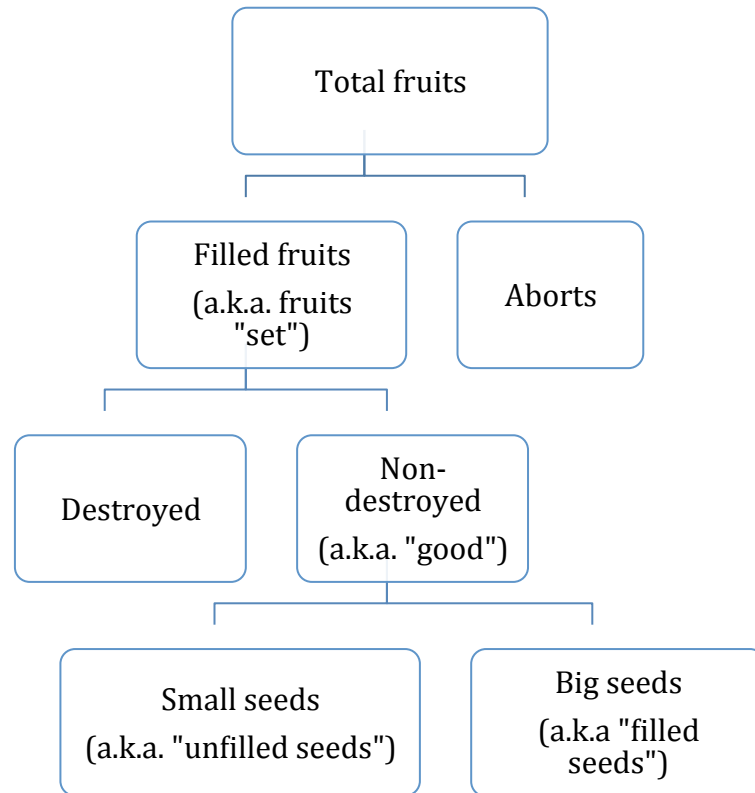


Figure A5: *Polemonium foliosissimum* fruit and seed terminology tree with fitness calculations.

Fitness calculations:

1. Total fruits per plant
2. Proportion fruit set = filled fruits/total fruits
3. Proportion fruit destroyed = destroyed/filled fruits
4. Seeds per fruit = (small seeds+ big seeds in lab)/non-destroyed fruits in lab
5. Estimated total number of seeds per plant = # seeds per fruit \* estimated total number of “good” (non-destroyed) fruits on entire plant

#### A.4. WSP SITE SOIL CHARACTERISTICS

Table A4: Site soil characteristics (Means±SD). The soils below plants of the different sexes and size classes were similar; two-way ANCOVA's revealed no differences in pH, % organic matter, P, K Ca, Mg, S, B, Cu, Fe, Mn, and Zn content by sex (all  $P>0.35$ ) or size (all  $P>0.18$ ). The values in this table are averaged over sex and size classes to describe the characteristics of the site (N=6). Sample collection and processing is described in the chapter.

Soil characteristic	Mean±SD
pH	6.55±0.16
Organic matter (%)	8.12±0.79
P (lb/A)	5.87±2.50
K (% Sat)	8.27±6.22
Ca (% Sat)	78.97±8.12
Mg (% Sat)	12.77±2.07
S (ppm)	7.33±1.21
B (ppm)	0.73±0.12
Cu (ppm)	0.24±0.13
Fe (ppm)	2.50±0.69
Mn (ppm)	3.92±0.42
Zn (ppm)	22.02±22.83

### A.5. SUPPLEMENT FITNESS FIGURE

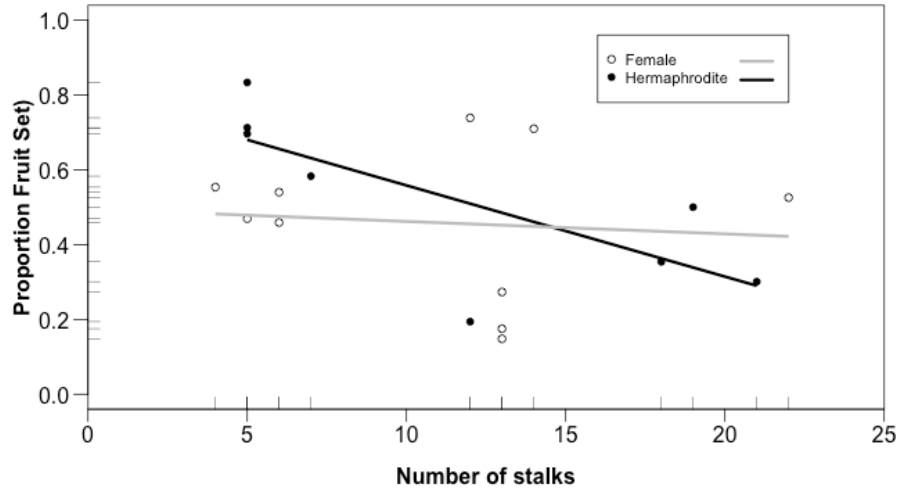


Figure A6: Proportion fruit set of non-clipped plants by sex and number of stalks. The proportion fruit set of non-clipped plants was evaluated separately from cored and clipped plants by two-way ANCOVA in R; factors included sex, the number of stalks, and their interaction. Proportion fruit set of non-clipped plants did not differ by sex ( $F_{(1,14)}=0.4646$ ,  $P=0.5066$ ), but was marginally influenced by plant size ( $F_{(1,14)}=3.6273$ ,  $P=0.0776$ ). The interaction of sex and plant size was not significant ( $F_{(1,14)}=0.4646$ ,  $P=0.5066$ ).  $N=8$ , and  $10$  for hermaphrodites and females respectively. There was a trend of size-dependent fruit set for hermaphrodites among non-clipped plants, with larger plants having lower fruit set. The sexes of *P. foliosissimum* may differ in fruit set throughout their life cycles, with mature females having higher average fruit set than mature hermaphrodites.

## A.6. SUMMARY OF ARCHIVED DATA FILES FOR CHAPTER 2

I have reviewed these files in September 2014 prior to publication of the thesis, and ensured that the codes function properly and reproduce the same statistical values and figures as shown in the chapter when run in R version 3.2.0 (R Core Team, 2013). I could not find the code file for constructing figure 3. I believe that I accidentally saved over it.

For many data files, the spreadsheets include variables that are not used in the analysis as coded. I left the data for these additional variables in the spreadsheet in case a future researcher may be interested in their values. The variables used by the code are the ones that correspond to the analyses as reported in the chapter. I am by no means an expert in R coding. I taught myself the majority of the coding for statistical analysis using on-line resources. I learned much regarding coding for figures in a colloquium, but likewise learned a lot by searching for answers to my questions on the internet. Due to my relative inexperience in coding in R, the coding that I employed may not be easy to interpret to new R users, or to experienced R users. Please refer to on-line resources and the help tool in R.

The files in this list are grouped by content, with headers in bold. All files in this list will be uploaded to the archive with the thesis document, following the guidelines of the Graduate College.

**General site data**

GPS data for WSP.xlsx .....	GPS coordinates and reference to associated RMBL data file
Soildata.csv .....	Microsite soil analysis data
Soil analysis code in R.R .....	Code for microsite soil analysis
Jon Gonzalez 2011 Pf Experiment Plant Inventory .....	Inventory of experiment plants

**Root colonization data**

Preclipcolr.csv .....	Ambient colonization data
Preclipcol analysis code in R.R .....	Code for ambient colonization analysis and figure
Finalcolcorrect.csv .....	Post clipping colonization data
Postclipcol analysis code in R.R... ..	Code for post clipping colonization analysis and figure

**Leaf tissue nutrient analysis data**

Nutscomplete.csv .....	Plant tissue nutrient data
Nutsandcolcomplete.csv .....	Final colonization and tissue nutrient analysis data
Tissue nutrient analysis code in R.R.....	Code for plant tissue nutrient analyses

**Fitness data**

Fitness.csv .....	Fitness data
Fitnesscolcombined.csv .....	Fitness and colonization data combined
Fitnesscontrol.csv.....	Fitness data for control plants only for supplemental figure
Fitness analysis code in R.R .....	Code for fitness analyses and figures

**Plant functional trait data**

Dryweight.csv .....	Clipped plant dry weight data
---------------------	-------------------------------

Slaxform.csv .....	Clipped plant WLS and inverse transformed SLA data
Clipped plant traits code in R.R.....	Code for clipped plant trait analysis and figures
Clippedtissue.csv .....	Clipped plant leaf tissue analysis data
Clipped plant nutrient analysis.R.....	Code for analysis of clipped plant leaf tissue
Eosleaf.csv .....	End of season leaf trait data (WLS and SLA)
Eosld.csv .....	End of season stalk trait data (length and diameter)
Finalcolleaf.csv .....	End of season WLS and SLA data, with colonization
Finalcolstem.csv.....	End of season stalk length and diameter data, with colonization
Eos functional trait analysis in R.R.....	Code for end of season functional trait analysis
Flowersize.csv.....	Flower size data
Flowersizeclip.csv.....	Flower size data for clipped plants for separate ANCOVA
Flowersizecontrol.csv .....	Flower size data for control plants for separate ANCOVA
Flowersizecore.csv.....	Flower size data for cored plants for separate ANCOVA
Flowercol.csv .....	Flower size and colonization data combined
Flowersize analysis code in R.R.....	Code for flower size analyses and figures
Corolla size by treatment.R.....	Code for Corolla size interaction figure

### **Images**

15F True roots Colonization .....	Images of colonization in roots of a 15stalk Female
Confirmed Pf roots.....	Roots from excised plants
Excised plant 6.13.11 .....	Plant excised in Gothic townsite
Excised plant 6.14.11 .....	Plant excised in Gothic townsite
Extraction photos .....	Images of root extraction

**APPENDIX B; SUPPLEMENT TO CHAPTER 3**

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### **B.1. *THEMEDA* GERMINATION TRIAL**

In November, 2011 I performed a small germination experiment in the beginning of November which included simply taking 20 light colored seeds and 20 dark colored seeds and placing them in square petri dishes in paper towels. The paper towels were folded such that they filled the square bottom of the dish and filled the dish when closed. 20 seeds were placed in each dish, 10 of which were placed a few folds within the paper towel and the other 10 were placed on top of the paper towel. The dishes were placed in the lab growth chamber for 2 weeks, checking the water daily and watering with a sprayer every 2-3 days, just to keep the paper towels moist. The chamber setting was 12:12hr day:night, 30°C day and 20°C night. After 11 days 3 of 20 dark seeds germinated, and no light seeds germinated. 2 of the 3 germinated seeds were from within the paper towel and the third was on top.

In December, 2011 I started a second larger germination experiment based on previous smaller experiment. This experiment was intended to germinate seeds for a pilot experiment for KLEE Herbivory experiment. This germination experiment included the following 4 treatments,

- a. “Gshaken”: 300ppm gibberellic acid (GA3) in distilled water in sand on the shaker
- b. “Gnoshake”: seeds soaked in 300 ppm GA3 in distilled water without shaking
- c. “Water”: seeds soaked in distilled water alone

- d. “Dry”: a control which included no water or GA3, but instead allowing the seeds to sit at room temp while dry.

An equal number of light colored seeds and dark colored seeds were tried, total of 400 light colored seeds and 400 dark colored seeds. All treatments will be for 18hrs, after which the seeds will be placed in square petri dishes as described for previous germination experiment, to include half of the seeds being placed within a few layers of paper towel and the other half on top of the paper towel. All petri dishes were placed in Dr. Yolanda Chen’s growth chamber on Dec 31, 2011. 300ppm GA3 was prepared using .3g of GA3 in 500mL water. Shaken seeds: the sand used for this was autoclaved at 121°C for 60min on the dry cycle. 125ml of sand was placed in a 500ml cylindrical plastic container which fit the shaker. The seeds were placed in small nylon bags which were tied at the ends using string. 50 seeds were placed in each bag. The bags were placed on top of the sand and 125ml of prepared GA3 solution was added to the container. The container was sealed and shaken vigorously to ensure that the bags were distributed within the sand. The container was the placed on the shaker. Seeds in GA3 or distilled water alone: 50 seeds each were placed in small 100ml beakers and 30ml of either GA3 solution or distilled water was added to each according to the treatments. Seeds left dry: 100 dark seeds and 100 light seeds were left at room temp in square petrous dishes throughout the 18hr duration.

No light colored seeds germinated and the bulk of germination occurred within the first 9 days, with no further germinant observed at the 12 day mark. There is no statistical difference between Water and Gnoshake, though water germinated slightly

more seeds; however, Gnoshake germinated slightly faster. The Gshaken treatment appeared to inhibit/delay germination, though the difference does not appear to be statistically significant although much fewer Gshaken seeds germinated overall. On day 15 two new germinant were observed, one in DD2 and one in SD1, the trial was terminated at this time. Overall germination % in order of germination success: Water:13.0%, Gnoshake: 11.8%, Dry: 9.0%, Gshaken:7.0%.

## B.2. STATISTICAL ANALYSIS TABLES

Statistics from the tables that follow are employed in Chapter 3 as included within this Thesis. All analyses were conducted in JMP® Pro10.0.02. For all analyses, the model is presented following R coding syntax as follows:

<b>Symbol</b>	<b>Meaning</b>
~	Fit the preceding response(s) onto the following model
*	Include factor preceding and immediately after in a full factorial manner
+	Include the following factor as a covariate

### **Factor coding:**

KLEE	Grazing history treatment, including KLEE 0, MW, and MWC
AMF	Substrate treatment, including AMF+ and AMF-(W)
Clipping	Clipping treatment (Clip vs non-clipped)

### **Response coding:**

Dwroot	total root dry weight
Totalshoot	total shoot mass produced (including material removed at clippings)
Totalbio	dwroot+totalshoot
R:S	Root:shoot ratio at harvest

LogotPRLC+            Logit transformed PRLC values, with the lowest value added to all values in order to correct for zero values. Logit transformation is not possible with zero values (Warton and Hui 2011, Ecology).

ArcsinsqrtNEXC        Arcsine square root transformed NEXC data.

Table B1: MANCOVA for control plants, used to evaluate the influence of AMF on plant growth in non-clipped conditions. Model:  $\text{dwroot, totalshoot, totalbio, R:S} \sim \text{KLEE*AMF+Harvest date}$ , with experimental N for each treatment combination. These data correspond to the biomass measures reported in Figure 5 of Chapter 3. Experimental N also corresponds to Table 3 of Chapter 3.

Effect	Wilks $\Lambda$	Approx F	NumDF	DenDF	P value
Model	0.5133	2.8886	24	329.14	<0.0001
KLEE	0.7847	3.0275	8	188	0.0032
AMF	NA	Exact F 5.1084	4	94	0.0009
KLEE*AMF	0.8290	2.3101	8	188	0.0219
Harvest Date	NA	Exact F 3.7476	4	94	0.0071

Experimental N for this analysis.

MW	Myc +	19
MW	Myc-W	18
MWC	Myc +	18
MWC	Myc-W	15
O	Myc +	16
O	Myc-W	18

Table B2. Experimental N associated with biomass analysis of clipped plants prior to and between clipping events, as reported in Figure 6 and Table 4 of Chapter 3.

MW	Myc +	19
MW	Myc-W	16
MWC	Myc +	18
MWC	Myc-W	17
O	Myc +	18
O	Myc-W	18

Table B3. MANCOVA for measures of mycorrhizal colonization. Model: LogitPRLC+, VC, arcsinsqrtNEXC~ KLEE\*Clipping+Harvest date, with experimental N for each treatment combination. These data correspond to Figure 8 in Chapter 3.

Effect	Wilks $\Lambda$	Approx F	NumDF	DenDF	P value
Model	0.3138	7.8937	18	280.5	<0.0001
KLEE	0.4974	13.7900	6	198	<0.0001
KLEE*clip	0.9860	0.2332	6	198	=0.9653
Clipping	NA	Exact F 3.4784	3	99	=0.0188
Harvest Date	NA	Exact F 14.9333	3	99	<0.0001

Experimental N for this analysis.

MW	Clip	19
MW	No-Clip	19
MWC	Clip	18
MWC	No-Clip	18
O	Clip	18
O	No-Clip	16

Table B4. Univariate ANCOVA table for measures of mycorrhizal colonization. Models fit separately: LogitPRLC+, VC, arcsinsqrtNEXC~ KLEE\*Clipping+Harvest date, experimental N corresponds to that of analysis for Table B3.

Response	KLEE		Clipping		KLEE*Clipping		Harvest date	
	$F_{(2,101)}$	$P$	$F_{(1,101)}$	$P$	$F_{(1,101)}$	$P$	$F_{(1,101)}$	$P$
PRLC	22.14	<0.0001	0.37	0.5425	0.32	0.7293	2.82	0.0964
VC	36.10	<0.0001	0.21	0.6478	0.46	0.6346	1.62	0.2066
NEXC	6.11	0.0031	8.25	0.0050	0.31	0.7317	0.64	0.4247

Table B5. Full experimental N for compensation analysis. These data correspond to the biomass measures reported in Figure 7 and Table 5 of Chapter 3.

MW	Myc +	Clip	19
MW	Myc +	No-Clip	19
MW	Myc-W	Clip	16
MW	Myc-W	No-Clip	18
MWC	Myc +	Clip	18
MWC	Myc +	No-Clip	18
MWC	Myc-W	Clip	17
MWC	Myc-W	No-Clip	15
O	Myc +	Clip	18
O	Myc +	No-Clip	16
O	Myc-W	Clip	18
O	Myc-W	No-Clip	18

Table B6. Experimental N for P concentration analysis. These data correspond to the P concentration analysis reported in Figure 5 and Figure 7 of Chapter 3.

MW	Myc +	Clip	8
MW	Myc +	No-Clip	9
MW	Myc-W	Clip	7
MW	Myc-W	No-Clip	8
MWC	Myc +	Clip	9
MWC	Myc +	No-Clip	8
MWC	Myc-W	Clip	7
MWC	Myc-W	No-Clip	7
O	Myc +	Clip	9
O	Myc +	No-Clip	8
O	Myc-W	Clip	8
O	Myc-W	No-Clip	8

### **B.3. 2010 SORGHUM SUDAN EXPERIMENT REPORT**

The following experiment was conducted in 2010, and was the precursor to the experiment involving *Themeda triandra* (Chapter 3). This report was submitted, as is, for academic credit for undergraduate and advanced undergraduate research. Formatting has been modified to comply with Graduate College Master's thesis requirements.

#### **THE INFLUENCE OF UNGULATE GRAZING HISTORY AND SOIL BIOTA ON PLANT TOLERANCE TO SIMULATED HERBIVORY**

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#### **Abstract**

Arbuscular mycorrhizal fungi (AMF) colonize plant roots and perform many integral functions in support of plant health. In this study, I explored the effects of ungulate herbivores, AMF, and their plant hosts by examining how the history of grazing affects the diversity of AMF present in soils and how the AMF communities, in turn, affect the

ability of plants to tolerate simulated herbivory. Soils were acquired from experimental plots in Kenya where different ungulate herbivory treatments have been in place for over 15 years. AMF spores were isolated from the soils and categorized morphologically to compare species diversity among treatments, and a full factorial experiment was performed in which plants were inoculated with fungi from different herbivory treatments and subject to simulated herbivory. Unexpectedly, plants grown without mycorrhizae outperformed mycorrhizal plants in measures of plant growth. This result maybe due in whole or in part to differences in the microbial communities, the presence of non-AMF septate fungi within the mycorrhizal treatments, host-specific interactions between plants and mycorrhizal partners, and possible detrimental effects of AMF communities upon plant hosts.

## **Introduction**

The Kenya Long-term Exclosure Experiment (KLEE), established in 1995 (Young et al. 1998), offers the opportunity for studies to be performed on the biological impacts of ungulate grazing by comparison of land plots managed for both grazed and ungrazed conditions, and offers a unique opportunity for consideration of the composition and functionality of arbuscular mycorrhizal fungi (AMF) communities that have been subject to differing biotic pressures.

Arbuscular mycorrhizal fungi form symbiotic relationships with land plants (Helgason and Fitter 2005). The fungi penetrate roots of plants and extend thread-like hyphae into the surrounding soil (Allen 1991). Hyphae act, in effect, as extensions of the

root system of plants, gathering inorganic nutrients from the soil and transporting them to roots in exchange for photosynthetic carbon (Allen 1991). In addition to providing access to soil nutrients, mycorrhizae affect their plant hosts in other ways as well, e.g., by increasing plant resistance to pathogens and herbivores (Newsham et al. 1995, Kula et al. 2005), stimulating photosynthesis and the rate of nutrient cycling (Hart and Klironomos 2002), and enhancing soil aggregation and available water (Milleret et al. 2009). Numerous studies have examined the role of mycorrhizae on plant defense against herbivores, and the results have been conflicting. Ungulate herbivory has been seen to both increase and reduce AMF colonization of roots as well as decrease AMF diversity (Gehring and Whitham 1995, Eom et al. 2001, Bethlenfalvay and Dakessian 1984). Field AMF communities have been seen to increase compensatory re-growth of plants following insect herbivory (Kula et al. 2005) while AMF inoculation has been seen to decrease re-growth following insect herbivory in the greenhouse (Bennett and Bever 2007).

Here, I explored the effect of ungulate herbivores on mycorrhizal diversity and compared the influence of mycorrhizal communities from areas subject to different combinations of ungulate herbivore pressure on plant response to herbivory. I hypothesized that differing ungulate herbivore pressures in the KLEE plots has resulted in a change in the mycorrhizal communities, and that these AMF communities offer differing benefits to their plant partners in regards to response to herbivory. To examine these hypotheses, I compared mycorrhizal diversity between soils collected from areas in KLEE that have been subject to different ungulate herbivory treatments for over 15 years to include areas where ungulates have been excluded, where only cattle have been

allowed access, and where ungulates have been allowed to move freely. Additionally, I explored the importance of the mycorrhizae for plant tolerance to herbivory.

## **Methods**

### **Soils Sampling**

Soil samples were collected from the Kenyan Long-term Exclosure Experiment (KLEE), located at the Mpala Research Centre (MRC) on the Laikipia plateau (37E, 08N: 1800m elevation) in central Kenya. The area around MRC is semi-arid grassland which receives 500-600mm of rainfall annually. The dominant soil type within the area sampled is a clay vertisol (Young et al. 1998). Herbivore exclosures have been in place in KLEE since 1995. Barriers to herbivores include large fences with electric wire and posts to mark areas for cattle grazing. Rhizosphere samples were obtained in April of 2010, using a standardized 10x10cm template extracting a block of soil to a depth of 15cm. Samples were collected exclusively from under *Pennisetum stramineum*, a common C4 bunchgrass to control for possible species-specific AMF interactions. The soils of three *P. stramineum* were sampled from three locations per herbivory treatment. The samples from within locations were pooled resulting in three representative samples per treatment. Samples were air dried and shipped in sealed plastic bags to Dr. Brody's laboratory at the University of Vermont.

### **Trap Cultures**

Several factors inhibit the use of collected field soil for taxonomic identification of AMF spores. Seasonal fluctuations in spore abundance, parasitism by soil organisms, and

chemical activity within the soil may lead to a reduction in viable spores necessary for taxonomic identification (Morton et al. 1995). Trap culturing involves the use of field-collected soil as media upon which a mycotrophic host plant is grown in controlled greenhouse conditions; this provides an environment in which the fungi are stimulated to sporulate. The use of trap cultures is essential to produce viable spores for taxonomic identification as well as to ensure representation of fungi that may not have matured prior to the acquisition of field samples (Morton et al. 1995).

In this experiment, trap cultures were established in order to ensure adequate species representation for experimental inocula and for the quantification of AMF diversity. Soil and root samples received from Kenya were homogenized and mixed with equal parts autoclaved sand (60min at 121°C). This mixture was seeded with 80-100 sorghum-sudan seeds (a highly mycotrophic C4 grass). Trap cultures were grown in the greenhouse for 3 months under a 12hr photoperiod with average day and nighttime temperature ranges of 20°-24°C and 17°-19.5°C respectively. Plants were watered as needed and fertilized with a low phosphorus fertilizer (17:4:17) twice throughout growth when signs of nutrient stress were apparent. Upon harvest, shoots were removed and roots were cut into 1.0-2.0cm pieces and homogenized with their soil for use as inoculum and spore extraction. In this manner, each sample was cultured in individual pots for three months and upon harvest, 200g (dry weight) of the trap pot material from each of the three locations were pooled into one composite sample for each KLEE herbivory treatment. The three composite samples were then partitioned for use as live inoculum, sterile inoculum, and for microbial filtrate.

## **AMF spore extraction and identification**

Spores were isolated from homogenized 50ml samples of soil using the wet sieving and decanting method described in Gerdemann and Nicolson (1963). Spores collected on the 38 $\mu$ m sieve were transferred in DI water to a Petri dish with a gridded bottom. Ten grids (of 36) were chosen at random and the presence and description of mycorrhizal spores recorded. Spores were grouped by morphology and mounted on slides with polyvinyl-lacto-glycerol (PVLG) and stained with Melzers reagent to confirm identification. Identifications were made by comparison of key morphological characteristics against the on-line taxonomic web resource provided by the International Culture Collection of (Vesicular) Arbuscular Mycorrhizal Fungi (INVAM) (<http://invam.caf.wvu.edu/index.html>) as well as through consulting Dr. Joseph Morton, the director of INVAM.

## **Experimental design**

The experiment was set up in a factorial design: 3 Inoculum sources  $\times$  2 AMF treatments  $\times$  2 clipping treatments. The inoculum sources consisted of the three herbivory treatments in KLEE (0,C,MWC). AMF consisted of AMF+ and AMF-, representing AMF inocula and sterilized non-AMF inocula respectively. The clipping treatment consisted of clipped and unclipped plants. Each treatment combination consisted of 10 experimental units, for a total of 120 units. The growth medium for the experiment consisted of a 1:1 mixture of sand and calcined clay (Turface; Industrial Materials Corp.,

Deerfield, IL, USA). Growth medium was sterilized by autoclaving for 60 min at 121°C. Three hundred mL was then mixed with 10g (dry mass) root and soil inoculum and placed into a 315mL deep-pot (D19L; Stewed and Sons Inc., Tangent, OR, USA). Eight sorghum-sudan seeds were placed evenly 3.5cm from the top of the pots and covered with 2.0cm of the remaining inoculum mix. Non-AMF inocula were prepared by autoclaving a portion of the composite soil, allowing for control of the non-mycorrhizal effects of soil particle size and nutrient composition. In order to equalize non-AMF microbial differences between AMF+ and AMF- treatments, a portion of the microbial community was reintroduced into the AMF- treatments by supplementing with a microbial filtrate (Koide and Li 1989), using a 1:6 soil to distilled water ratio (gym). Twenty-five mL of a 10µm-filtered washing of the respective AMF+ soils was applied to the non-mycorrhizal pots. The pots were placed at random in the greenhouse and grown under the following conditions: 12hr photoperiod, day and nighttime temperature ranges of 20°-24°C and 17°-19.5°C respectively. Plants were watered as needed and fertilized with a low phosphorus fertilizer (17:4:17) once at four weeks from seeding. In order to standardize the number of plants per pot, the pots were thinned to three plants, with seedlings selected to allow equal spacing between remaining plants. Plant height was measured weekly throughout the experiment and the average height per pot recorded. Weekly height averages were natural log transformed for normalization of data and pre-clipping daily growth rate was calculated using the following formula:

$$\frac{\ln(\text{Height Week 6}) - \ln(\text{Height Week 1})}{\text{Number of days}}$$

Likewise, post-clipping growth rate was calculated as the difference between the natural log of the height at harvest and the height post-clipping over the number of days elapsed.

On the sixth week the pots designated for clipping were clipped to 2.5cm. The clipped material was dried at 85°C for 48hrs and weighed. After ten weeks of growth, the pots were harvested and roots and shoots separated. Roots were gently extracted from the soil and rinsed with distilled water to remove debris. Above and belowground biomass was measured by drying and weighing root and shoot material.

### **AMF colonization**

To quantify mycorrhizal colonization, 0.03g of roots (dry weight) were rehydrated using a method prescribed by Dr. Joseph Morton of INVAM (personal communication). Dried roots were placed in 5mL distilled water with one drop of surfactant (un-scented dish soap) for 4 days. Rehydrated roots were then stained with 0.05% trypan blue using the methods of Phillips and Hayman (1970); modified as described in the on-line mycorrhizal methods resource provided by INVAM (<http://invam.caf.wvu.edu>). Root colonization was scored using the magnified intersections method (McGonigle et al. 1990).

### **Statistical analysis**

I performed a two-way ANOVA (JMP, Version 8.0, SAS Institute 2009) to examine the main effects and interactions of inoculum source and mycorrhizal inoculation on growth rate prior to clipping and clipped biomass. I performed a full factorial ANOVA to examine main effects and all possible interactions of inoculum source, mycorrhizal

inoculation (AMF), and simulated herbivory (clipping) on post-clipping growth rate, final above and below ground biomass, and root to shoot ratio.

## **Results**

### **AMF species richness**

We found that the number of AMF species present between two KLEE herbivory treatments were significantly different ( $F_{(5,17)}=4.42$ ,  $p = 0.02$ ). The number of species identified was significantly higher in the ungrazed KLEE treatment (0) than in the treatment, which allowed full ungulate access (MWC), with a mean of  $10.0 \pm 0.3$  and  $8.2 \pm 0.49$  for 0 and MWC respectively.

### **Plant responses to herbivory**

#### **Pre-clipping growth rate and biomass removed at clipping**

Mycorrhizal inoculation significantly influenced pre-clipping growth rate ( $F_{(5,119)}=3.819$ ,  $p = 0.0002$ ) with AMF- treatments out-growing AMF+ treatments (mean= $0.033 \pm 0.001$  and  $0.027 \pm 0.001$  respectively); however, there was no inoculum source treatment effect ( $F_{(5,119)}=3.819$ ,  $p = 0.8673$ ). The interaction between KLEE inoculum source and mycorrhizal inoculation was also not significant ( $F_{(5,119)}=3.819$ ,  $p = 0.13$ ). Biomass of clipped shoot material was significantly higher in non-mycorrhizal treatments ( $F_{(5,59)}=3.422$ ,  $p = .0008$ ). Clipped biomass was also positively correlated to height at clipping ( $n=60$ ,  $R^2=0.80$ ,  $p < 0.0001$ ).

### **Post-clipping growth rate**

I used a three-way ANOVA to examine main effects and all possible interactions of inoculum source, mycorrhizal inoculation (AMF), and simulated herbivory (clipping). Clipping treatment significantly influenced rate of growth after clipping ( $F_{(11,119)}$ ,  $p < 0.0001$ ), with clipped plants growing faster than unclipped plants (mean= $0.057 \pm 0.001$  and  $0.003 \pm 0.0002$ ). There was a significant AMF  $\times$  clipping interaction, ( $p = 0.0016$ ), with AMF- treatments re-growing from clipping faster than AMF+ treatments (mean= $0.032 \pm 0.004$  and  $0.028 \pm 0.003$ ).

### **Above and belowground biomass at harvest**

Total above ground biomass produced during the course of the experiment was also calculated and is described in the following section. A three-way ANOVA was performed to assess treatment effects on above and belowground dry weight at harvest. Clipping resulted in significantly lower above and belowground biomass ( $p < 0.0001$  for both responses). Unclipped AMF- plants produced significantly more above ground biomass than unclipped AMF+ plants ( $p = 0.01$ , mean= $0.37 \pm 0.021$  and  $0.22 \pm 0.014$ ); and although not significant, this same relationship between the AMF- and AMF+ treatments was also seen in the below ground biomass of unclipped plants (mean= $0.22 \pm 0.012$  and  $0.16 \pm 0.010$ ). In both above and belowground biomass, mycorrhizal inoculation significantly influenced the response ( $p < 0.0001$  for both responses) with AMF- plants producing more biomass than AMF+ plants (Above: AMF- mean= $0.23 \pm 0.018$ ; AMF+

mean=0.15±0.012; Below: AMF- mean=0.18,±0.009; AMF+ mean=0.14±0.007). The three-way interaction of AMF × inoculum source × clipping was not significant for either above or belowground biomass ( $p = 0.37$ , and  $0.48$  respectively).

To get a sense of total plant productivity, total above ground biomass produced was calculated by combining the biomass removed at clipping with the final above ground biomass at harvest. Clipping resulting in significantly less biomass ( $p < 0.0002$ ), and mycorrhizal inoculation again significantly influenced the response ( $p < 0.0001$ ) in that AMF- treatments produced more total above ground biomass than AMF+ treatments (mean=0.30±0.015 and 0.19±0.010).

### **Root to Shoot Ratio**

Clipping significantly influenced root to shoot (R:S) ratio ( $F_{(11,119)}=14.19$ ,  $p < 0.0001$ ) in that clipped plants had higher R:S than unclipped plants (mean=1.43±0.057 and 0.72±0.022). Mycorrhizal inoculation also significantly influenced R:S ( $p = 0.005$ ) with AMF- treatments having lower R:S than AMF+ treatments (mean=0.99±0.057 and 1.16±0.068).

### **AMF Root colonization**

I used a two-way ANOVA to examine main effects and interactions of inoculum sources and clipping on AMF root colonization of a subsample of roots harvested from the experiment. The influence of the inoculum source × clipping interaction on

mycorrhizal colonization was nearly significant ( $p = .09$ ). Roots from clipped C and 0 treatments had higher mycorrhizal colonization than those of unclipped C and 0 treatments, while roots from the clipped MWC treatment had lower colonization than those of the unclipped MWC treatment. I also quantified root colonization by non-mycorrhizal septate fungi. Mean septate colonization percentage was  $76.3\% \pm 1.79\%$  and was not significantly affected by inoculum source, clipping, or the inoculum source  $\times$  clipping interaction ( $F_{(5,29)}=3.028$ ,  $p = 0.90$ ).

## **Discussion**

### **AMF Species richness of KLEE treatments**

We found a significant difference in the number of AMF species present between the 0 and MWC treatments. The difference in AMF communities between these treatments supports my hypothesis that differing ungulate herbivore pressures has resulted in a change in the mycorrhizal communities in KLEE. Ungulate grazing has been found to reduce AMF species richness in other studies as well (Eom et al. 2001, Bethlenfalvay and Dakessian 1984). Changes in plant nutrient availability and allocation due to herbivory and the cost of association with mycorrhizal partners may have caused changes in the mycorrhizal community. AMF species with higher carbon requirements or otherwise lower competitive ability may not be able to persist in these conditions.

## **Growth and response to herbivory**

AMF are widely considered to be beneficial to their plant hosts. The presence of AMF can increase the availability of soil nutrients, enhance resistance to soil pathogens, and improve water relations (Allen 1991, Newsham et al. 1995, Kula et al. 2005, Milleret et al. 2009). Therefore, we expected that plants in the AMF+ treatments would out-perform AMF- treatments. Surprisingly inoculation with mycorrhizae negatively affected plant growth in all responses measured. This result was unexpected, and may be due in part to an imbalance in the microbial community between AMF+ and AMF- treatments, the presence of septate fungi within the AMF+ treatments, possible host-specific interactions between fungal communities and plant hosts, and possible negative effects of AMF.

## **Microbial organisms**

Nematodes and micro arthropods were observed in samples taken of the inoculum used in this experiment. Both of these groups can have an effect on both plant growth and mycorrhizal vigor. Disruption of extraradical hyphae by microscopic arthropods can cause a reduction of the positive effects of AMF colonization (Klironomos et al. 1999). Nematodes may feed on plant roots or root hairs (Paul 2007), and some fungal-feeding nematodes have been shown to reduce AMF colonization and increase occurrence of parasitized, non-viable spores when present in high densities (Smith and Read 2008). In my experiment, I attempted to supplement the sterilized AMF- treatments with a portion

of the microbial community (<10µm) present in the AMF+ treatments in order to equalize potential non-AMF effects of these communities on plant growth. Large nematodes and micro arthropods can be greater than 10µm in size (Paul 2007), and therefore, may have been excluded from the AMF- microbial filtrate, leading to a possible imbalance of beneficial and parasitic organisms between treatments.

Identification and quantification of soil microbes, including septate fungi, was not within the scope of this experiment.

### **Colonization of roots by non-AMF fungi**

I found the roots from the AMF+ treatments of this experiment to be colonized not only with AMF fungi, but also with a high amount of septate fungi (Fig. 1). The function of these organisms is not well understood, although they are prevalent in stressed environments and may co-occur with AMF (Mandyam and Jumpponen 2005). Septate fungi may exist along a mutualism-parasitism continuum (Smith and Read 2008); studies performed to determine the nature of the relationship between septate fungi and plant hosts have yielded results ranging from pathogenic to pseudomycorrhizal (Jumpponen and Trappe 1998). Septate fungi were excluded from the AMF- treatments due to the sterilization procedure employed to eliminate AMF fungi; therefore, AMF- treatments may have had an advantage over AMF+ treatments.

### **Host-specificity**

Although mycorrhizal fungi are thought to be generalists, host-specificity, between plant and fungal partner has been described several times in the literature, though the degree of this specificity appears to depend on the particular plant and AMF combination observed (van der Heijden et al. 2002), meaning that AMF communities present in soil may offer increased benefits to plants with which they have a history of growth. In this experiment, soils were collected from rhizospheres of the plant *Pennisetum stramineum*. Seeds for this plant were not available for this experiment and so *Sorghum bicolor* var. *sudanense*, a commercially available mycotrophic nurse plant related to similar plants of the region, was chosen as a substitute. It may be that a native grass species, paired with its local fungus would have reaped the maximum benefit of the symbiosis in this experiment.

### **Mutualism/parasitism**

It is also possible that the mycorrhizal association may have negatively affected both plant growth and health. I found lower growth rates for mycorrhizal plants (compared to non-mycorrhizal controls), lower biomass, and higher root to shoot ratios. Although AMF are generally considered to be mutualistic, with plant and fungal partners both benefiting from the association, AMF are known to function along a mutualism-parasitism continuum with AMF associations capable of affecting plant growth responses negatively (Johnson, et al. 1997). The association between mycorrhizal fungi and plant host is

beneficial to plants only if the cost of the relationship in carbon allocation to the fungal partner does not outweigh the benefit received by the plant in regards to increased nutrient availability or reduced carbon allocation to root development (Johnson et al. 1997). In this experiment, AMF+ treatments had significantly higher root to shoot ratios than those of the AMF- treatments, meaning that AMF+ plants produced less above ground biomass per mass root material than plants of the AMF- treatments. This is inconsistent with studies that show that AMF association reduces the root to shoot ratio of plants (Smith and Read 2008). Root to shoot ratio is considered an indicator of plant health, with higher root to shoot ratios indicating growth conditions which are less than favorable (Harris 1992).

### **Conclusion**

Due to the complex interactions between plant hosts, mycorrhizae, and soil microorganisms, extreme care must be taken when designing experiments that attempt to assess the functionality of mycorrhizae. In this experiment, I did not employ adequate controls with which to isolate the influence of mycorrhizae on plant growth responses and thus the differences between KLEE inoculum sources, though not statistically significant, cannot be attributed to the mycorrhizal component of the experiment.

A loss of AMF diversity within a community is concerning considering their role in ecosystem diversity and productivity (van der Heijden et al. 1998, Maherali and Klironomos 2007).

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#### B.4. SUMMARY OF ARCHIVED DATA FILES FOR CHAPTER 3

The master growth and biomass data file contains growth and biomass data for all plants of the experiment. This file is a record of all biomass data collected, and was not itself used in statistical analyses. Subsets of this dataset were used to assemble separate files to meet statistical analysis objectives. The files used in statistical analyses are indented below the “Master growth and biomass data file” entry.

The experiment originally included a fully sterilized treatment (AMF-), to which no microbial wash was added. We deemed this treatment non-biologically relevant, and consider it an error to have included it in the original experimental design. The data for these plants were removed from the data set and are archived here, in the file “Biomass of sterile treatment.csv”. The data for this treatment are also found in the “Master growth and biomass data file”, which houses all growth and biomass data for the experiment.

File name with extension

Contents

##### **General information**

AMF STRUCTURE DIAGNOSIS KEY.xlsx .....	Diagnostic key with images embedded
Master growth and biomass data file.xlsx.....	All growth and biomass data
Biomass and colonization data.xlsx.....	Plant nutrient and root colonization data
Biomass analysis file.csv .....	Biomass data used in analysis
Biomass of sterile treatment.csv .....	Data of fully sterilized treatment that was removed
Nutrient and colonization data.xlsx .....	Plant nutrient and root colonization data
Colonization data wash plants .....	Colonization date for the AMF-(W) plants

**2010 *Sorghum sudan* experiment**

2010 Herbivory experiment.xlsx ..... Growth and biomass data

2010 Experiment colonization data.xlsx..... AMF colonization data

**Images**

THEMEDA ROOT COLONIZATION IMAGES..... Images of root colonization

Sorghum sudan root colonization images ..... Images of root colonization

## APPENDIX C; SUPPLEMENTAL INFORMATION

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## C.1. SPORE EXTRACTION PROTOCOL 2010

This protocol was developed in 2010, and was the primary method used to extract and isolate arbuscular mycorrhizal spores from soil samples in the effort to evaluate the community composition of AMF within areas of interest in the Mpala Research Center. This protocol was developed primarily with reference to the INVAM protocol.

1. Lay down several layers of newspaper, crush sample and chop with scissors-thoroughly homogenize-use 50ml plastic cups to scope out sample.
2. Label tape with ID and place on 50ml cup (mark with an X so we can keep track of which samples have been done)
3. Fill blender with 200ml of DI-place 50ml of soil in blender-pulse on low speed for five seconds (twice for a total of ten seconds of blending)
4. Use two sieves 500 and 38 (500 on top and 38 on bottom)
5. Rinse blender cover and 50ml cup then spray out blender (use hose with spray nozzle) into sieves.
6. Spray top sieve-push down clay bits.
7. Bring 38 to tap-gentle flow rinse material (mud and spores towards bottom of sieve) and scrape material into 50ml cup (same labeled cup)
8. Fill 50ml tubes with 15ml 20% (FIRST) then push tube below and fill with 15ml 80%
9. Slowly drop contents (important to make sure spores are not sunk to the bottom without being suspended) into the tubes-four tubes per 50 ml sample-continuously mix sample with little bits of water to keep materials suspended.
10. After tubes are filled (make sure divide 50mls evenly- Place in centrifuge for one minute at 2600-3000)
11. Use 38 sieve pour contents out of tube-38 will capture even smallest spores-use spatula to scrape all bits down and rinse into glass Petri dish (transfer label)

For further information, see the following sources:

INVAM:

<http://invam.caf.wvu.edu/methods/spores/extraction.htm>

Brundrett:

<http://mycorrhizas.info/resource.html>

Sucrose gradient (table sugar):

60g of sugar dissolved in 300ml solution = 20% sucrose w:v

180g of sugar dissolved in 300ml solution = 60% sucrose w:v

## C.2. INK AND VINEGAR STAINING PROTOCOL 2012

WARNING: GLOVES MUST BE WORN DURING CLEARING AND RINSING, GLOVES AND LAB COAT ADVISABLE THROUGHOUT PROCEDURE, REVIEW MSDS FOR KOH BEFORE BEGINNING

### A. SUPPLIES NEEDED

- Gloves
- Lab coat (advisable)
- 20mL tubes
- Histological cassettes
- Nylon mesh (use Five Star nylon paint strainer in lab)
- Forceps
- Long stainless steel tongs
- Test tube rack
- Water bath
- 250mL beaker
- KOH and ink waste containers
- 10 % KOH solution w/v
  - a. ex: mix 100g KOH with 800mL distilled H<sub>2</sub>O and bring final volume to 1L
- 5% Ink in 5% acetic acid (household vinegar) solution v/v
  - a. ex: mix 5mL Black Sheaffer ink with 95mL household vinegar; final volume= 100mL

### B. OVERVIEW OF STEPS

1. Clear roots
2. Rinse x3
3. Stain
4. Rinse for 20min
5. View/store

### C. CLEARING AND STAINING METHOD

1. Set warm water bath to 85°C (2-3 hours in advance)
2. Carefully rinse roots with distilled water to cleanse them of soil and debris. Cover roots with 10%KOH in 20mL tubes and place into warm water bath for 30min (time/temp may vary depending on roots)
3. Drain KOH into waste container and rinse roots with distilled water 3 times, use nylon mesh to cover the end of tube to prevent loss of roots
4. Cut small pieces of nylon mesh and enclose roots with mesh, transfer to histological cassettes, label cassettes with pencil

5. Place 200mL of ink/vin solution into large Erlenmeyer flask and place onto heat plate in fume hood. Bring to boil (takes ~10min). This volume is good for up to 12 cassettes.
6. Drop cassettes into boiling solution for 5min, remove flask from heat and extract cassettes with tongs, place into beaker filled with distilled water. Once ink solution has cooled, pour into waste container.
7. Using forceps, place roots back into 20mL tubes
8. Cap end of tube with nylon mesh and using squeeze bottle filled with distilled H<sub>2</sub>O, repeatedly rinse roots for 20minutes, this step may require addition of a few drops of vinegar to squeeze bottle to avoid loss of stain in fungal structures (one change of water every 3-4 minutes should suffice)
9. Roots can be viewed immediately and/or stored by adding distilled H<sub>2</sub>O to vials to cover roots (12-15mL).

#### **D. References**

This protocol is based on Vierheilig et al., 1998. Ink and vinegar, a simple staining technique for arbuscular-mycorrhizal fungi. *Appl Environ Microb* 64(12):5004-5007

Also refer to Vierheilig et al., 2005. An overview of methods for the detection and observation of arbuscular mycorrhizal fungi in roots. *Physiol Plantarum* 125:393-404

### C.3. TRYPAN BLUE STAINING PROTOCOL 2010

Root clearing and staining protocol for greenhouse grown plants (*Sorghum-sudan*), using Trypan Blue stain. Developed July 2010 from INVAM guidelines.

WARNING: GLOVES MUST BE WORN DURING THIS PROCEDURE, LAB COAT ADVISABLE THROUGHOUT PROCEDURE. REVIEW MSDS FOR KOH AND TRYPAN BLUE BEFORE BEGINNING

#### A. SUPPLIES NEEDED

- Gloves
- Lab coat
- 20mL tubes
- Water bath
- 10 % KOH
- 1% HCl (acidified water)
- Hydrogen peroxide
- **.05% Trypan blue solution**, this is composed of a mixture of lactoglycerol and 0.4% trypan blue. A sample recipe is as follows and results in a final quantity of 500ml.
  - a. 437.5mL of lactoglycerol
    - i. Lactoglycerol is composed of a 1:1:1 mixture of DI water, lactic acid, and glycerol (145.8ml each).
  - b. 62.5mL of .4% Trypan blue
- **De-stain solution**, de-stain is acidified glycerol, which is composed of glycerol, distilled water, and HCL. A sample recipe is as follows and results in a final quantity of 1L.
  - a. 500mL glycerol
  - b. 450mL distilled water
  - c. 50mL 1% HCL

#### B. OVERVIEW OF STEPS

1. Clear
2. Bleach
3. Acidify
4. Stain
5. De-stain

#### C. CLEARING AND STAINING METHOD

6. Set warm water bath to 85°C
7. Carefully rinse roots with distilled water to cleanse them of soil and debris. Cut roots into 1cm segments and place into 20ml vials. Cover roots with 10%KOH and place vials into warm water bath at 85°C for 30min.

8. Drain KOH into a waste container and rinse roots with distilled water 3 times (the orange handled net screen in the shop is excellent for this).
9. If necessary, (ie. field collected roots) bleach roots by adding hydrogen peroxide to the vials to cover the roots and allow to sit at room temperature for 45 minutes, after which rinse the roots three times with distilled water. If not required, proceed directly to next step (acidification).
10. Prior to staining with trypan blue, roots must be acidified. In order to acidify the roots, add 1% HCL to cover roots. The vials are then allowed to sit at room temperature for 30 minutes.
11. Drain the HCL from the vials (no rinsing in this step) and replaced with .05% trypan blue solution to cover the roots.
12. Place vials in a warm water bath at 85°C for four hours, after which drain the trypan blue solution from the vials into a waster container and replace with acidified glycerol in order to leach excess stain (this is 'de-staining').

#### **D. References**

INVAM: <http://invam.wvu.edu/methods/mycorrhizae/staining-roots>

Mark Brundrett: <http://mycorrhizas.info/method.html#am1>

Place all vials in cooler at 4°C until processing (per INVAM).

#### **C.4. THEMEDA TRIANDRA ROOT STAINING PROTOCOL**

This method was developed in February 2013, modified from the “INK AND VINEGAR STAINING PROTOCOL 2012”, for the purpose of clearing and staining *Themeda triandra* roots of greenhouse grown plants for quantifying the extent of mycorrhizal colonization. The recipes and many of the steps from the INK AND VINEGAR protocol are followed, with the following modifications:

**Clearing and Staining protocol**, Total time to clear and stain: 72hrs (3 days)

1. From stored sample, weigh out .15g wet weight roots (wet with EtOH) Rinse free of EtOH x3 with DI
2. Place in 10% KOH (8mL of KOH) at room temperature (RT) for 48h. Gently stir with a metal spatula to better disperse the roots in KOH.
3. Rinse x3 with DI
4. Place in 8mL H<sub>2</sub>O<sub>2</sub> at RT for 20min. Gently stir the roots with a metal spatula to disperse them in the H<sub>2</sub>O<sub>2</sub>
5. Rinse x3 with DI
6. Place in 6mL ink:vin solution in warm water bath at 85C for 24h
7. Rinse x6 over 20min in DI then store in DI at 4C.

**Rinsing Protocol**

The protocol below is specifically for rinsing roots post-staining; however, this applies for rinsing prior to and after KOH step, as well as after H<sub>2</sub>O<sub>2</sub> step.

1. Rinse 5 tubes at a time, using approximately 8ml of distilled water
2. Using a piece of paint strainer material, secure it tightly along one side of the first tube and loosely fold it back over the opening so that there is a fully open area for air flow.
3. Slowly pour the ink out of the tube and into a waste container
4. Holding the tube upright, gently spray distilled water back through the paint strainer mesh to push roots back into the tube that may be caught in the mesh, use forceps as a last resort.
5. Perform 3 full rinses on the first tube then refill the tube with fresh distilled water, using a small stainless steel spatula, gently swirl the roots in the tube in order to increase root exposure to the water and allow more ink to be rinsed from the roots; allow the tube to stand while working on the remaining tubes.
6. Rinse forceps and spatula in a beaker with distilled water between uses, being careful to check and remove any roots clinging to them
7. After the 5<sup>th</sup> tube is done, go back to the first and then rinse all again once each.
8. Repeating step 7, each tube should be rinsed a total of 6 times in 20 minutes (3 initial rinses and then 3 more rinses in rotation).
9. Following the 6<sup>th</sup> rinse, refill the tubes with ~8mL DI and cap them.
10. Dry the exterior of the tubes with a paper towel and place in the 4C fridge to await analysis.

## C.5. BLUEBERRY ROOT STAINING PROTOCOL 2013

This protocol was developed for an investigation performed by Dr. Alison Brody in support of Mr. Ben Waterman's research at his farm in Johnson, Vermont. Roots were collected by myself and Dr. Brody from blueberry plants in the field at Mr. Waterman's farm, following the root-coring method described in Chapter 2 of this Thesis. Roots were brought to Dr. Brody's lab at UVM, whereupon roots matching blueberry morphology (as per Mr. Waterman's guidance) were isolated from soil samples and stored in 90% ethanol at 4C prior to clearing and staining (approximate time in ethanol: 3 months). Please note, this protocol was found to be optimal for the roots of this sampling period. Plant age, seasonality, and environmental variability may influence root clearing and staining quality. Staining trials, in which the steps of this procedure are varied, should be conducted to calibrate the protocol prior to quantifying mycorrhizal colonization of the roots.

In June of 2014, I conducted a staining comparison trial, in which I compared this protocol to Trypan Blue (TB) and Chlorazol Black E (CBE) at standard concentrations. CBE stained structures more fully than either ink or TB, but it also stained a lot of background material, possibly cell wall components, which resulted in some difficulty in differentiating fungal structures from background staining in some cases. Background staining was not a problem for either ink or TB, but in ink and TB, the structures were faintly stained in comparison to CBE. So, it may be that by using Ink or TB, we are overlooking some structures that do not pick up the stain as well.

In conclusion, the results from the staining comparison suggest that at standard concentrations, the CBE performs better for general quantification because it appears to bind to the tissue more fully. I think another staining trial is in order, using an increased concentration of black ink, and a longer de-staining time with the CBE.

For additional information regarding staining methods, refer to:

Brundrett, M.C., Y. Piche, and R.L. Peterson. 1983. A new method for observing the morphology of vesicular-arbuscular mycorrhizae. *Canadian Journal of Botany*. 62:2128-2134

Vierheilig et al., 1998. Ink and vinegar, a simple staining technique for arbuscular-mycorrhizal fungi. *Appl Environ Microb* 64(12):5004-5007.

Vierheilig et al., 2005. An overview of methods for the detection and observation of arbuscular mycorrhizal fungi in roots. *Physiol Plantarum* 125:393-404

Blueberry root staining protocol, using ink and vinegar technique.

Total time to clear and stain: 1+ days

8. From stored sample, separate approximately 1/2-3/4 of root material most closely resembling blueberry root per description (Ben Waterman)
9. Rinse free of EtOH x3 with DI
10. Divide sample into 2 cassettes, secured by nylon and labeled.
11. Add cassettes to 200ml 10% KOH in a 1L Erlenmeyer flask (no more than 10 cassettes).
12. Autoclave flask and contents for 45 min within a secondary autoclavable container, covering opening of flask with aluminum foil
13. Within fume hood, remove cassettes from flask and rinse x3 with DI
14. Remove roots from cassettes and place in 15ml centrifuge tubes
15. Treat roots with 8mL H<sub>2</sub>O<sub>2</sub> at room temperature for 20min
16. Rinse x3 with DI
17. Add 8mL ink:vin solution, place in warm water bath at 85C for 24h
18. Rinse x6 over 20min in DI then store in DI at 4C until viewing

## C.6. SUMMARY OF SUPPLEMENTAL ARCHIVED DATA FILES

File name with extension

Contents

### Images

Blueberry staining images..... Ericoid mycorrhizal structures stained with ink and vinegar