Multiple Mutualist Effects in an Ant-Plant-Bacteria System

by

Mitchel Trychta

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Department of Ecology and Evolutionary Biology
University of Toronto

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Abstract

Plants simultaneously partake in multiple mutualisms, and partner species that share a focal host are capable of indirectly affecting one another, potentially affecting the outcome of mutualisms. Context-dependence is thought to largely determine the nature of these multiple mutualist effects as well as focal host resource allocation in each partner. We factorially manipulated the ant “bodyguards” and nitrogen-fixing rhizobia that associate with the legume Chamaecrista fasciculata in a field experiment to determine how each mutualistic partner affected the other. Rhizobia treatment impacted the identity and abundance of ants. Both rhizobia treatment and ant treatment impacted plant survival. Additionally, ant and rhizobia treatment affected the weight of nodules housing rhizobia, but in a way that depended on plant size. Our results show that belowground mutualists can influence plant interactions with aboveground mutualists, and vice versa. This study highlights the importance of incorporating multiple mutualist effects into our understanding of mutualisms.
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In an effort to create a more equitable environment I will conclude by acknowledging my privilege as a White upper-class male. While conducting this work I unequivocally benefited from privilege associated with my social-location and socioeconomic status. These advantages come at a steep cost to all minority groups. Failing to openly address that I benefited from such inequality would be a grave injustice. That being said, this paragraph does not to reconcile discrimination in STEM fields and higher education, but it does recognize my responsibility to contribute to a more equitable future.
# Table of Contents

Acknowledgments ........................................................................................................ iii  
Table of Contents ......................................................................................................... v  
List of Tables ................................................................................................................ vi  
List of Figures ................................................................................................................ vii  
List of Appendices ........................................................................................................ ix  
Introduction ................................................................................................................. 1  
Methods ......................................................................................................................... 4
  Study System .............................................................................................................. 4
  Experimental Design .................................................................................................. 5
  Experimental Plants .................................................................................................. 6
  Data Collection ......................................................................................................... 8
  Data Analysis ............................................................................................................ 10
Results ............................................................................................................................ 12  
Discussion .................................................................................................................... 13  
Tables ............................................................................................................................ 21  
Figures .......................................................................................................................... 25  
Literature Cited ............................................................................................................ 33  
Appendix 1: Supplementary Tables ............................................................................ 38  
Appendix 2: Supplementary Figures ............................................................................ 40
List of Tables

Table 1. Ant abundance model results ................................................................. 21

Table 2. Monomorium viridium abundance model results ...................................... 21

Table 3. Forelius pruinosus abundance model results ........................................... 21

Table 4. Herbivore abundance model results .......................................................... 22

Table 5. Aphid abundance model results ............................................................... 22

Table 6. Leaf number model results ................................................................. 22

Table 7. EFN sugar production model results ...................................................... 23

Table 8. Plant survival model results ................................................................. 23

Table 9. Plant damage model results ................................................................. 23

Table 10. Nodule weight model results .............................................................. 24

Table 11. Aboveground plant biomass model results .......................................... 24

Table 12. Plant height model results ................................................................. 24

Table A1. Plant mortality .................................................................................... 38

Table A2. Ant species abundance ...................................................................... 38

Table A3. EFN production ................................................................................ 39
List of Figures

Figure 1. *Chamaecrista fasciculata* at the field site ......................................................... 25

Figure 2. Distinct EFN shape and coloration in *C. fasciculata* ........................................ 25

Figure 3. Effects of treatment on ant abundance ..................................................................... 26

Figure 4. Ant abundance plotted against plant size ................................................................. 26

Figure 5. *Monomorium viridium* and *Forelius pruinosus* abundance by treatment .......... 27

Figure 6. *M. viridium* abundance plotted against plant size ................................................. 27

Figure 7. *F. pruinosus* abundance plotted against plant size .................................................. 28

Figure 8. Effects of treatment on herbivore abundance .......................................................... 28

Figure 9. Effects of treatment on aphid abundance ................................................................. 29

Figure 10. Effects of treatment on leaf number over time ....................................................... 29

Figure 11. Effects of treatment on EFN sugar production ......................................................... 30

Figure 12. Interaction plot of plant survival model ................................................................. 30

Figure 13. Effects of treatment on plant damage ................................................................. 31

Figure 14. Effects of treatment on nodule weight ................................................................. 31

Figure 15. Interaction plot of nodule weight model ................................................................. 32

Figure 16. Abundance of *M. viridium* and *F. pruinosus* across treatments over time .......... 32

Figure A1. Correlation matrix of data..................................................................................... 40

Figure A2. Ant abundance plotted against nodule weight ..................................................... 40

Figure A3. *M. viridium* abundance plotted against *F. pruinosus* abundance ...................... 41
Figure A4. Effects of treatment on plant height over time ......................................................... 41

Figure A5. Effects of treatment on aboveground plant biomass................................................. 42

Figure A6. *Chamaecrista fasciculata* illustration by S.E.R. for M.R.T ...................................... 43
List of Appendices

Appendix 1. Supplementary tables ........................................................................................................ 38

Appendix 2. Supplementary figures ....................................................................................................... 40
Multiple Mutualist Effects in an Ant-Plant-Bacteria System

Introduction:
Research on the ecology and evolution of species interactions has traditionally focused on pairwise interactions, but the emphasis is gradually shifting to studying multispecies interactions. The complex ecological and evolutionary dynamics inherent in multi-species mutualisms has led to the study of multiple mutualist effects (MME). The MME framework incorporates the explicit mechanisms by which mutualists directly and indirectly interact, as well as the associated costs and benefits of these interactions (Afkhami et al. 2014). MMEs are mediated by community composition, with changes in the relative abundance and identity of mutualist partners potentially shaping how a focal host allocates resources to its associated partners (Strauss & Irwin 2004; Ossler et al. 2016, Keller & Lau 2017).

Multiple mutualists can have additive or non-additive effects on focal partners (Afkhami et al. 2014). Gustafson and Casper (2006) found a synergistic effect of two species of mycorrhizal fungi on the growth of big bluestem grass, *Andropogon gerardii*; plant growth exceeded the additive expectation from separately measuring the effects of each mycorrhizal fungus. Similarly, a study examining coral defense by mutualistic crustaceans found that multiple crustacean species prevented more coral tissue loss than the additive effect of each crustacean species on its own (McKeon et al. 2012). In these examples, having several partners provided synergistic benefits to the focal host, but conflict among mutualistic partners can also result in redundant or reduced host performance when interacting with multiple mutualists (Stachowicz & Whitlatch 2005; Morris et al. 2007). For example, in plants that associate with both ant “bodyguards” and pollinators, partnering with the highest quality ant defender significantly reduced pollinator visitation rates, lowering plant fitness (Ness 2006).

Conflicting selective pressures as well as underlying constraints may limit the focal host from allocating resources optimally to either partner. The presence of multiple mutualist partners may place a single host trait under conflicting or synergistic selection pressures (Strauss & Irwin 2004). If the cost to the focal mutualist of interacting with multiple partners exceeds the benefit of multiple partners compared to a single partner, then the focal mutualist should interact with
either partner in isolation (Dutton et al. 2016). Under these circumstances, focal mutualists may allocate limited resources to one partner over the other (Laird & Addicott 2007). However, the local biotic and abiotic context determines the degree to which trade-offs in partner resource allocation scale with the costs and benefits incurred by the focal host as well as additive and non-additive effects (Dutton et al. 2016; Keller & Lau 2017).

To date, limited empirical work has used a MME framework to assess how above and belowground partners collectively shape host fitness in natural systems (Orrell & Bennett 2013). The ant-legume-rhizobia system has been the focus of a few MME studies examining focal host fitness as well as direct and indirect interactions between partners. Legumes engage in mutualisms both above ground with insect pollinators and defenders, and below ground, with rhizobia and mycorrhizal fungi. More than half of all 740 legume genera associate with rhizobia (Afkhami et al. 2018), nitrogen-fixing bacteria housed by plants in specialized root nodules. Interacting with rhizobia generally increases plant fitness and can both increase the direct chemical defenses of host plants (Ballhorn et al. 2008) and accelerate chemical defense induction (Van der Ent et al. 2009). Additionally, rhizobia can mediate resource allocation trade-offs in host plants, altering plant physiology to increase shoot biomass and soluble leaf proteins (Thamer et al. 2011; Godschalx et al. 2015). Studies suggest that plants with increased levels of nodulation likely produce more jasmonic acid, a signaling molecule that increases herbivore resistance (Kessler & Baldwin 2002; Hause & Schaarschmidt 2009). Not all rhizobia strains are of equal quality; nonetheless, legume and rhizobia fitness interests appear to be well aligned, with mostly positive correlations between rhizobia and plant fitness (Friesen 2012).

Many plants that associate with rhizobia also partake in a facultative relationship with ants above ground. These plants produce extrafloral nectar (EFN) from specialized structures aptly referred to as extrafloral nectaries (EFNs). A study by Yamawo et al. (2015) suggests that in the absence of ant mutualists, plants bearing EFNs invest more heavily in chemical defensive traits, but in the presence of ants, these plants invest predominantly in indirect defenses. Ants often prefer to forage on plants with EFNs, and some ant species are more likely to visit EFNs that secrete more soluble sugars or proteins (Heil 2011). Extrafloral nectar production should carry some inherent cost to the plant, it requires plant resources to produce EFNs and secrete a sugar rich solution containing amino acids and proteins; however, empirical evidence is somewhat equivocal, with perhaps more support for ecological than direct costs (Rutter &
Rausher 2004; Assunção et al. 2014; Dutton et al. 2016). For example, Dutton et al. (2016) found that extrafloral nectar production trades off with floral nectar production in two species of *Turnera*, but had no direct effects on seed set.

Thus, in areas where ants and or herbivores are scarce, allocation of resources to EFN is expected to be minimized. Legumes can be locally adapted to ant communities, with differential allocation to EFN based on ant identity and abundance (Bentley 1977; Rios et al. 2008). Rudgers and Strauss (2004) found that across multiple populations of wild cotton, the net outcome of facultative ant-plant mutualisms depended on the abundance of ants and the level of herbivore damage, with ant presence acting as a selective agent on EFN traits in the host plant. Depending on the ant species, EFN composition, and various environmental factors, the response of foraging ants to invertebrate herbivores on the focal host plant can range from rapid and aggressive to entirely indifferent; thus, the benefits and costs of investing in ant mutualists are fairly variable (Kelly 1968; Rios et al. 2008; Shenoy et al. 2012; Godschalx et al. 2015).

Focal hosts may have a limited resource pool to compensate both ants and rhizobia. This may result in competition between above and below-ground mutualist partners. If partners compete for limited focal host resources, each partner may evolve direct or indirect mechanisms to reduce focal host provisioning in other partners or may even adapt by interacting preferentially with focal hosts with minimal exposure to other partners (Mack & Rudgers 2008). A recent study showed that ants became more abundant on host plants as nodule number decreased (Keller et al. 2018). This suggests that provisioning one mutualist partner, such as rhizobia, with resources can incur indirect costs to the host, by limiting the resources available to direct towards rewarding ant mutualists and possibly deterring ant partners (Mack & Rudgers 2008).

Several studies have shown that rhizobia can decrease EFN production in plants, resulting in reduced attendance of ant defenders, illustrating that belowground mutualists can affect aboveground mutualists (Gehring & Bennett 2009; Summers & Mondor 2011). In prior studies, plants hosting rhizobia produced fewer EFNs or less EFN in response to herbivory than plants lacking rhizobia; consequently, ants preferred to forage on rhizobia-free plants (Summers & Mondor 2011; Godschalx et al. 2015). There is less evidence suggesting that ants can impact how plants allocate resources to belowground mutualists. However, insect community
composition can affect the costs and benefits accrued by the focal host when associating with rhizobia (Heath & Lau 2011; Simonsen & Stinchcombe 2014).

Further research is needed to clarify how the direct and indirect effects of multiple mutualists impact focal host fitness (Afkhami et al. 2014). Few studies to date have taken to the field to understand how biotic components, such as partner abundance, mediate outcomes and selective forces in multiple mutualist systems. Furthermore, studies need to consider both herbivory and plant performance when assessing ant-plant interactions (Chamberlain & Holland 2009). The two measures are not universally associated, and it is essential to consider how the direct and indirect interactions plants have with other mutualists, as well as herbivores, shape the outcomes of these interactions (Morris et al. 2007).

Here, we experimentally manipulated the abundance of ants and rhizobia interacting with the legume *Chamaecrista fasciculata* in a two-by-two factorial experiment, and measured plant performance, species-specific ant abundance, plant damage, and the allocation of plant resources into both ant and rhizobia partners. To our knowledge this is one of the few field studies to investigate multiple mutualist effects in a legume-microbe-ant system and only the second detailing *C. fasciculata*'s simultaneous relationship with bacteria and ant mutualists (Keller et al. 2018). This work aimed to answer the following questions: (1) Does partner abundance impact plant resource allocation into either partner? (2) Does greater resource allocation into aboveground or belowground mutualists have direct fitness consequences for the focal host? (3) Do belowground mutualists impact the identity and abundance of foraging insects on aboveground plant parts? The results of this study will help clarify the direct and indirect effects linking above and belowground mutualists to focal host fitness.

**Methods:**

**Study System.** *Chamaecrista fasciculata* (Fabaceae), commonly known as the partridge pea, is an annual herbaceous legume that thrives in open disturbed areas including prairies and sandy savannahs (Foote & Jackobs 1966; Galloway & Fenster 2000; Figure 1). *Chamaecrista fasciculata* is found throughout the southeastern United States and parts of northern Central America.

Varying with the local climate, *C. fasciculata* germinates between late March and early June, and flowers from July to September, with fruits maturing from August into October.
(Barton 1986; Kelley 1986; Rutter & Rausher 2004). With the formation of the third or fourth true leaf, most leaves bear a single circular extrafloral nectary at the base of the petiole. The color and precise shape of the extrafloral nectaries varies across populations, ranging from light green to dark reddish-brown, as well as circular to noticeably oblong (Trychta, personal observation; Figure 2). The production of EFNs and extrafloral nectar occurs throughout the growing season in conjunction with the production of new leaves (Barton 1986; Kelley 1986; Rutter & Rausher 2004).

Racemes originating from the leaf axils produce up to eight yellow hermaphroditic flowers, which bloom for one day, lack nectar, and are exclusively buzz pollinated (Hobson 1972). Although the morphological structure of *C. fasciculata* flowers is invariant, there are noticeable differences in pigmentation of the stamen, carpel, and the base of the petals across populations (Trychta, personal observation). High-quality sites can support *C. fasciculata* densities of up to 100 plants per square meter (Keller 2014), and mature plants can exceed 50 cm tall and bear over 500 leaves (Barton 1986; Rutter & Rausher 2004).

Throughout its range, *C. fasciculata* interacts with various species of mutualistic ants (Barton 1986; Kelly 1986; Rios et al. 2008). These ants are attracted by the production of sugar-rich extrafloral nectar. Work done throughout the southern-central US has shown that the effects of ant presence on *C. fasciculata* fitness varies geographically, (Barton 1986; Kelly 1986; Rios et al. 2008). *Chamaecrista fasciculata* also often simultaneously associates with nitrogen-fixing rhizobia (*Bradyrhizobium* spp.) in root nodules (Harris et al. 1985).

**Experimental Design.** We conducted a field experiment in the spring and summer of 2017, transplanting experimentally manipulated *C. fasciculata* into an arid scrubland community at Archbold Biological Station, along the Lake Wales Ridge in Central Florida (27.18°N, 81.35°W). The climate of the field site is warm and humid, with average maximum summer temperatures ranging from 33 – 33.6°C, and more than sixty percent of the 1365.25 mm of annual precipitation falling between June and September (Archbold Biological Station Climate Data Records 2017). Surface temperatures in scrub often exceed 40°C during midday in the summer and can exceed 48°C even in the winter (Wiescher 2010). The field site was a white-sand scrubland that had been subject to a controlled burn the year prior. The site itself was a sandy clearing spanning a 2 x 200 meter transect which bisected early successional patches of
palmetto scrub. The local community included native *C. fasciculata* plants, with and without root nodules, and a variety of ant visitors.

This field experiment had a two-by-two factorial design: each plant was treated with either high or low-density rhizobia inoculate, and ants were either allowed on or excluded from plants. Originally each of the four treatment groups was to have 30 plants each. However, due to an unexpectedly low germination rate and unforeseen mammalian herbivory, at the end of the experiment the number of replicates per treatment group was reduced by roughly half, with the high rhizobia and ant exclusion treatment having 15 replicates, the high rhizobia with ants treatment having 12 replicates, the low rhizobia and ant exclusion treatment having 13 replicates, and the low rhizobia with ants treatment having 21 replicates (Table A1).

Each experimental plant was randomly assigned to a location along the transect. Plants were planted approximately 3 meters apart. To prevent larger mammalian herbivores from disturbing the experiment each plant was initially enclosed in a cage (0.35 m high x 0.35 m diameter) made of 2.5 x 2.5 cm hardware cloth (Lowes, Mooresville, NC, USA), and after a few weeks these enclosures were replaced with larger cages (1 m high x 1 m diameter) constructed out of 2.5 x 2.5 cm chicken-wire (Lowes, Mooresville, NC, USA) and secured with landscape staples.

Transplanting took place in one afternoon, June 2\textsuperscript{nd} 2017, to coincide with the start of the rainy season. Just prior to planting the bottom quarter of each plant’s shuttle pot was carefully removed so as to not damage the roots or nodules and allow for root growth into the local substrate. Each plant pot was then buried leaving 3 cm of the pot above the sand and the sides of the pot belowground encircling the root mass.

*Experimental Plants.* *Chamaecrista fasciculata* plants were grown from a mix of seed obtained from the National Plant Germplasm System. At the time, The National Plant Germplasm System did not have any seeds of *C. fasciculata* from Archbold or Florida. To minimize plant mortality in the field we opted to use lines that naturally occurred under abiotic conditions similar to the Florida scrublands (i.e. temperature, precipitation, soil nutrient availability). Additionally, we used The National Plant Germplasm System trait data records of height, width, branching, foliage, and hardiness to select plant lines with a range of values in each category. Altogether we used four plant lines derived from populations in Northeast Texas, Northern Louisiana,
Southwest Arkansas, and central Mississippi. Twice the number of seeds needed for the field experiment underwent the following process: surface sterilization by briefly dipping seeds in 95% ethanol followed by soaking in a 10% bleach solution for ten minutes before rinsing seeds for 2 minutes. Seeds were then scarified, dampered, and cold stratified in petri dishes lined with damp filter paper (Galloway & Fenster 2000). Seeds were left in a 4º C fridge for 12 days.

Seeds were then planted in Jiffy webflat trays (Growers Solution, Cookeville, TN, USA) and placed outside, underneath a porch to germinate. Every other day until being moved to the field seedlings were bottom-watered and trays were checked for germination. After 21 days, 101 seeds had germinated and were then transplanted into trade-gallon Thermoform Shuttle Pots (Growers Solution, Cookeville, TN, USA) and moved to full sun conditions. Plug trays as well as the gallon pots were filled with a 1:1:1 mix of potting soil (Harvest, Waltham, MA, USA), white torpedo sand (Lowes, Mooresville, NC, USA) and calcined clay (Turface-Pro, Buffalo Grove, IL, USA). Potting soil was autoclaved prior to use in an effort to reduce the abundance of microbes in the soil.

Dr. Katy Heath germinated C. fasciculata seeds in soil from the Archbold scrublands, and after four weeks of initial growth, the plants were harvested, and all root nodules were plated on yeast-mannitol media. She isolated five strains of rhizobia from the harvested nodules using the protocol described in Heath et al. (2010). Prior to inoculation each isolated strain was cultured in liquid yeast-mannitol media for 72 hours at room temperature and then moved to 4º C fridge to slow growth. The five strains of rhizobia were mixed together in equal volumes just prior to use. Experimental plants were inoculated with these local strains of rhizobia 28 days after planting by pipetting 1 ml of treatment-specific inoculate at the base of the seedling and then lightly watering until the soil was damp to the touch. Plants assigned to the high-rhizobia treatment were inoculated with 1-ml rhizobial of culture diluted by a factor of $10^{-2}$, and those assigned to the low-rhizobia treatment were inoculated with 1-ml of culture diluted to $10^{-6}$.

The tops of the pots were wrapped in Parafilm (Pechiney Plastic Packaging, Chicago, IL, USA), and a coating of Tanglefoot (Scotts Miracle-Gro, Marysville, OH, USA) were applied to the Parafilm wrapping on the pots of plants in the ant exclusion treatment. The Parafilm wrappings or Tanglefoot barriers were reapplied on all pots as necessary.
Data Collection. The experiment ran for nine weeks, from early June to early August 2017. We measured plant height, leaf number, and leaf damage every seven days, and at the end of the experiment to assess how they changed in response to ant and rhizobia treatments. Plant height (cm) was measured by starting at the base of the plant in the pot and recording the distance to the tallest point of growth. Only leaves that had the size and coloration of mature foliage were considered when assessing leaf number. Herbivore damage was measured by counting the total number of leaves on each plant that had more than a quarter of their original surface area damaged or removed (the most common forms of damage being browning and tissue removal). Throughout the experiment we collected survival data for the 101 plants originally moved to the field.

Measures of extrafloral nectar production were taken repeatedly during the field experiment, with sampling constrained by plants needing to be fairly mature to produce the minimum volume of nectar necessary for dilution and use on a refractometer. Therefore, EFN production was measured a total of seven times between late July and early August. Twenty-four hours prior to measuring extrafloral nectar production, the ten most recently developed extrafloral nectaries were wiped clean with filter paper, and a coat of Tanglefoot was applied to the Parafilm base on each pot to prevent ants from collecting EFN during the 24-hour window.

Due to the extreme daytime heat and its impact on nectar volume, samples were collected prior to sunrise from 03:30 - 05:30. Using 30 microliter capillary tubes (Drummond Scientific Co., Broomall, PA, USA) the 10 nectaries in the highest vertical position on the plant were sampled. The internal volume of the micro-capillary tube was determined in accordance with the manufacturers provided dimensions and was corroborated by trials in the field and lab. EFN volume was recorded using digital calipers to measure the length of the capillary tube filled with EFN to the hundredth of a mm (Mitutoyo Co., Kawasaki, Japan). The EFN was deposited into a pre-chilled 1.5-ml micro-centrifuge tube filled with 40 microliters of sterilized deionized water, and then placed in a cooler until returning from the field. After sampling each plant, the Parafilm and Tanglefoot were stripped away, and reapplied to the ant-excluded plants.

A handheld digital field refractometer with a range from 0 – 85 degrees Brix was used to determine the Brix value for each diluted sample to the tenth of a percent while adjusting for the ambient air temperature (Bellingham and Stanley Limited, Tunbridge Wells, Kent, UK).
Samples were vortexed and then placed onto the refractometer for analysis, with the refractometer being cleaned and blanked with sterilized deionized water prior to each reading. Measures of EFN volume and EFN Brix values were analyzed in accordance with the methods outlined by Dutton et al. (2016), providing the mass in micrograms of sugar in each EFN sample.

Measures of insect abundance and identity were taken at least twice a week for the duration of the field experiment. Data were recorded between 09:00 – 11:00, when insect activity was highest. Ant attendance was measured via scan sampling. Plants were observed from top to bottom, after which the ant species and the number of ants for that species was recorded. Ants were not removed after observations, and the plants were disturbed as little as possible. We performed several tests of this process to ensure that ants could accurately be identified to species level in the field; we sampled ants foraging nearby or on native C. fasciculata at the field site, ants were identified in the field, the same ants were then identified again in the lab using a dissecting scope, with the final identification confirmed by resident myrmecologist Dr. Mark Deyrup. Any ant that could not be immediately identified in the field was brought back to the lab for further examination as well as Dr. Deyrup’s final confirmation.

The abundance and identity of insect herbivores was measured using the same method as above, and each herbivore was identified to order (Hemiptera, Lepidoptera, Coleoptera, or Orthoptera). The criterion for noting non-ant insects as herbivores was only their presence on the plant. Small Hemipterans such as aphids were measured apart from other large bodied Hemipterans. Thus, Hemiptera refers to any non-aphid-sized members of the family. Measures of abundance for aphid-sized hemipterans were binned into five classes (Class 1 = none, Class 2 = < 10, Class 3 = < 20, Class 4 = < 50, Class 5 = > 50).

Plants were extracted from the field on August 6th, 2017. Aboveground and belowground biomasses were bagged and stored separately. The aboveground biomass was inspected for incidentally collected insects, cleaned of all debris, dried in a drying oven at 40 ºC for 48 hours, and weighed to the nearest one-hundredth of a gram.

The belowground biomass was subjected to nodule sampling. Since their initial transplanting into the field the root systems had expanded rapidly to consist of many filamentous root sections adhered to the taproot. The fine structure of roots retained a substantial amount of local soil, therefore, obtaining accurate counts of nodule number required extensive cleaning of
the roots. A robust effort was made to clean the roots, including soaking, various levels of
sifting, and removing debris by hand. However, each method proved highly destructive, resulting
in a considerable loss of fine and hardy root mass and associated nodules. Ultimately, the root
system of each plant was briefly cleaned by hand, removing larger debris, after which a total of
thirty nodules from each plant were harvested at random. The thirty nodules were collectively
weighed to a one-hundredth of a gram and then stored in amber vials filled with cotton and silica
beads, after which they were placed in a refrigerator. We did not determine the abundance of
each strain in the thirty nodules collected from each plant.

Data Analysis. All statistical analyses were performed in R (version 1.0.153). The package used
to run each model depended on the distribution of the data and is indicated below for each
model. When necessary, data were log-transformed to meet normality assumptions. Here we
present the significant main or interaction effects from each model. For each analysis, we started
with the most complex model, then used the stepAIC function to simplify the model (MASS
package), removed non-significant interactions, and re-tested the simplified model. The final
dataset includes only the plants that survived the entire field season, with the exception of the
plant survival data. A plant was considered dead when more than two-thirds of its leaves had
senesced since the last measurement; dead plants were given a value of zero for survival while
the 61 plants that survived were coded with survival values of one. Plant survival data was
analyzed using a generalized linear model with a binomial distribution, the model included
rhizobia treatment, ant treatment, and the spatial location of each plant as fixed effects.

We analyzed the effects of rhizobia and ants on plant performance using a two-by-two
factorial approach and focused on three measures of plant size: height, leaf number, and
aboveground biomass. Compared to other measurements of plant performance (height and
aboveground biomass), leaf number seemed to be the most robust measure of plant performance.
leaf number was closely associated with the number of extrafloral nectaries on each plant, and
we predicted that it may be indicative of increased resource allocation in ant partners.
Consequently, leaf number at harvesting is our selected measure of plant performance for this
experiment.

Height and leaf number were measured repeatedly throughout the experiment, and
therefore necessitated the use of a linear mixed effect model in the lme4 package that accounted
for repeated measures. Both models included rhizobia treatment, ant treatment, the spatial location of each plant, and plant genotype as fixed effects, time was also a fixed covariate and a part of the correlation structure modelled using the function corCAR1. Both models had plant ID as a random factor nested within time to account for multiple measurements on each plant. Analysis of aboveground biomass required the use of a linear model, which used both treatments and plant genotype as fixed effects. Likewise, treatment effects on nodule weight were analyzed using the same linear model structure with the addition of the number of leaves at the last harvesting as a covariate, and the removal of genotype from the model.

The effect of treatments on sugar mass in extrafloral nectar was tested using a generalized linear model with negative binomial distribution (MASS package). The final model used the summed mass of sugar in micrograms across every time point rounded to the nearest whole number as the response variable, with genotype and the number of leaves at harvest as covariates. Similarly, testing for a treatment effect on leaf damage also used a GLM with a negative binomial distribution, with the summed number of damaged leaves recorded over the duration of the field experiment as the response variable, treatments as fixed effects, as well as the number of leaves at the last measurement, the spatial location of each plant, and plant genotype as fixed effects.

We analyzed treatments effects on total ant abundance as well as species-specific ant abundance using generalized linear models with negative binomial distributions. We calculated ant abundance by taking the summed number of ants recorded on each plant across all observation periods; likewise, species-specific ant abundance was the summed number of either Monomorium viridium or Forelius pruinosus ants recorded on each plant throughout the study. A zero-inflated negative binomial error structure (ZINB) was used to account for zero inflation as well as overdispersion in models of overall ant abundance and Monomorium viridium abundance (PSCL package). No ants were recorded on plants in the ant-exclusion treatment; therefore, we excluded data from ant-treatment plants, and used rhizobia treatment in the original fixed effect structure to construct all three models of ant abundance. Additionally, the model of ant abundance also included the number of leaves at the last measurement as well as the spatial location of each plant as covariates. In the model of Monomorium viridium abundance we included Forelius pruinosus abundance, the number of leaves at the last measurement, the spatial location of each plant, and plant genotype as covariates. The model of Forelius pruinosus
abundance included the number of leaves at the last measurement, the spatial location of each plant, and the number of leaves at the last measurement as covariates.

We used a generalized linear model with negative binomial distribution to analyze treatment effects on herbivore abundance, with the sum number of herbivores recorded across all four orders for each plant as the response variable, ant treatments as a fixed effect, and the number of leaves at the last measurement as a covariate. Finally, a cumulative link model used to analyze aphid abundance (ordinal package), which included only the total weight of all EFN sugar sampled per plant as a predictor.

Results:

Across all plants, *Monomorium viridium* accounted for 42 percent of all ants, while *Forelius pruinosus* made up 51 percent, and the remaining 7 percent was split among several species of ants in the genera *Dorymyrmex* and *Nylanderia* (Supplementary Table A2). There was little overlap between the two most common species of ants across plants, with only 12 percent of plants attracting both *M. viridium* and *F. pruinosus*. Plant size alone was not a significant predictor of ant abundance; however, there was a significant interaction between rhizobia treatment and plant size, with the number of ants increasing more rapidly with plant size in the low-rhizobia than the high-rhizobia treatment (Table 1, Figure 3, Figure 4).

Plants in the high-rhizobia treatment hosted *M. viridium* in greater numbers than low-rhizobia plants. The abundance of *F. pruinosus* significantly impacted *M. viridium* abundance, with increases in *F. pruinosus* reducing *M. viridium* abundance. Additionally, *M. viridium* abundance varied significantly with plant genotype (Table 2, Figure 5, Figure 6). *Forelius pruinosus* abundance was significantly impacted by rhizobia treatment, with low rhizobia plants attracting more *F. pruinosus*. *Forelius pruinosus* abundance was greater on the largest plants and was significantly impacted by plant location (Table 3, Figure 5, Figure 7).

Despite treatment effects on ant abundance, this did not translate into treatment effects on herbivore abundance. Herbivore abundance was not significantly different across treatments, but it did vary in response to plant size, with larger plants having more herbivores (Table 4, Figure 8). Aphid abundance did not significantly differ across treatments (Table 5, Figure 9).
Plants grew similarly in all treatments. Repeated measures of plant size showed significant temporal autocorrelation, but ant and rhizobia treatments did not have significant effects on leaf number over time (Table 6, Figure 10) or leaf number at harvest. On average, over a 24-hour period, C. fasciculata plants produced 4.76 μl of EFN, containing 782 μg of sugar (Table A3). However, neither the ant or rhizobia treatment had significant effects on extrafloral nectar production, which was only significantly impacted by plant genotype (Table 7, Figure 11, Table A3).

Plant mortality differed between treatments and with the spatial location of the plant in the field. The significant interaction between rhizobia treatment and ant treatment suggests that the negative effects of high-rhizobia treatment and ant exclusion have on survival are reduced when plants are subjected to both treatments in combination (Table 8, Figure 12). There was a significant interaction between plant size and ant treatment on plant damage, with ants increasing plant damage more on larger plants. Additionally, plant genotype significantly impacted the amount of plant damage (Table 9, Figure 13). Unsurprisingly, herbivore abundance was positively correlated with plant damage, suggesting that more herbivores resulted in more damage (Figure A1).

There was a significant three-way interaction among rhizobia treatment, ant treatment, and plant size on nodule weight, meaning that the interactive effect of rhizobia and ants on nodule weight depended on plant size (Table 10, Figure 14, Figure 15). Specifically, when plants were large, the low-rhizobia treatment resulted in heavier nodules than the high-rhizobia treatment when ants were present, but the reverse was true when ants were excluded. When plants were small, the converse was true: the high-rhizobia treatment resulted in heavier nodules when ants were present, but the low-rhizobia treatment resulted in heavier nodules when ants were absent. The significant main effect of rhizobia treatment is therefore only true when ants were absent, and plants were small.

**Discussion:**

Although the rhizobia and ant treatments did not affect plant performance as measured by leaf number at harvest, there was an interactive effect between treatments and plant survival. Plants treated with a higher density of rhizobia attracted more ants, especially more M. viridium, showing that belowground mutualists can affect the abundance and species identity of
aboveground partners. Altering the abundance of either above or belowground mutualists did not impact plant production of EFN, and thereby did not alter resources allocated to ant bodyguards. However, the three-way interaction among ant treatment, rhizobia treatment, and plant size on nodule weight suggests there may be a trade-off between growing leaves and growing nodules that is most pronounced when both rhizobia and ants are most abundant; the (negative) slope of the relationship between nodule weight and leaf numbers is steepest in the high-rhizobia treatment when ants are present. Additionally, ants showed a strong effect on nodulation in plants in the low-rhizobia treatment, increasing nodule mass when ants are present, but demonstrating a root-to-shoot resource trade-off when ants are absent. Finally, focal host survival was significantly different across treatments, indicating that associating with rhizobia and ants may influence the feeding preferences of mammalian herbivores. Examining legume-ant and legume-rhizobia mutualisms in combination yielded evidence supporting that belowground symbionts can impact the abundance and identity of aboveground partners, and that the presence of aboveground mutualists can affect focal host resource allocation in belowground symbionts.

We found evidence that ant identity and abundance changed in response to rhizobia treatment, plant size, and the occurrence of other ant species; consequently, these results emphasize, that within naturally occurring ant-legume-rhizobia systems, the identity and abundance of each partner is a product of indirect interactions among aboveground and belowground partners (Laird & Addicott 2007). Overall, ants were more abundant on high-rhizobia plants. This could be a result of the rhizobia treatment influencing EFN chemistry. Godschalx et al. (2015) showed that plants hosting rhizobia were larger and made more nutrient-rich extrafloral nectar than rhizobia-free plants. Optimal foraging theory predicts that ants should prefer high-rhizobia plants that produce EFN with the highest energetic value, however for ants the preferred ratio of sugars to proteins can vary by species (Kay 2004). Similarly, augmenting the presence of belowground nutrient-fixing partners, as attempted in the rhizobia treatment groups, has been shown to alter visitation rates of insect pollinators as well as shift pollinator identity, likely in response to changes in floral nectar chemistry (Cahill et al. 2008). EFN and floral nectar share several chemical components such as sugars, proteins, and secondary metabolites, and variations in nectar chemistry are known to impact interactions between plants and associated insect mutualists (Gonzalez & Heil 2009; Heil 2015; Stevenson et al. 2017).
Hence, differing ant abundance and identity among rhizobia treatments suggests that rhizobia may be indirectly impacting EFN chemistry.

Our study found that ant identity and species-specific abundance changed in response to rhizobia treatment. *Monomorium viridium* attendance was highest on high-rhizobia plants but decreased on plants that hosted *Forelius pruinosus*. Conversely, *Forelius pruinosus* attendance was highest on larger plants and low-rhizobia plants, but also differed in response to plant location. If *M. viridium* is the competitively dominant of the two common ant mutualists, increased abundance of *M. viridium* on high-rhizobia plants could result from rhizobia altering focal host traits in a manner that increased ant attraction. *Monomorium viridium* and *F. pruinosus* differ morphologically, behaviorally, and in their primary food source, which may explain why *M. viridium* and not *F. pruinosus* were found foraging on most plants in the high-rhizobia treatment.

Being slender and roughly 2 mm in length, *M. viridium*, the metallic training ant is a black ant of smaller stature that is easily distinguished by the metallic green sheen of its gaster (Deyrup 2016). Being the smallest of all the ants observed, the realized niche of *M. viridium* may be constrained in ant communities where body size determines competitive ability. This species nests in well drained sandy areas (Dubois 1986), where intense daytime heat and reduced thermal tolerance limits *M. viridium* to foraging in the morning and early evening (King 2007). *Monomorium viridium* are predaceous ants, consuming insects as well as collecting honeydew. They are often observed working in large coordinated teams forming narrow files capable of traversing long distances (Deyrup 2016).

*Forelius pruinosus* prefers semiarid habitats, such as the Florida scrub. Individuals are gray in color, fast moving, and larger than *M. viridium* (Deyrup 2016). *Forelius pruinosus* is adapted to forage during periods of extreme daytime heat, and colony activity is highest at times when heat has forced most ant species to retreat belowground. Compared to *M. viridium*, *Forelius pruinosus* are predominantly scavengers that prefer dead insects to live ones. Furthermore, ant activity is not as organized, with workers moving between the nest and resources individually irrespective of recruitment levels at the resource. Nonetheless, *F. pruinosus* workers move quickly, and defend resources vigorously, capable of displacing other ants from resources such as nectar or an insect carcass (Deyrup 2016).
Being dietary generalists, both *F. pruinosus* and *M. viridium* likely forage best in recently disturbed areas. Izhaki et al. (2003) found that *Forelius pruinosus* thrives in post-fire conditions and excluded other species of generalist ants, including *M. viridium*. The dominance of *Forelius pruinosus* post-fire had long-term impacts on ant species richness, reducing ant diversity while maintaining high *F. pruinosus* abundance for up to six months after a low-intensity fire (Izhaki et al. 2003). The field site for our study was exposed to high-intensity fires, the most recent being less than a year prior. There is a limited understanding of how fire frequency and intensity impact ant communities, so it is possible that there were elevated numbers of *F. pruinosus* in the field that suppressed *M. viridium*.

*Monomorium viridium* and *F. pruinosus* did not co-occur on the same plants and there was minimal turnover between the two species on each plant through time. In ant communities of upland Florida, behavioral dominance is likely mediated by body size, trophic level, abiotic limitations, and habitat preferences (Kaspari 2001; King 2007). In a study assessing the ant communities in the Florida scrub, Wiescher et al. (2011) found that at surface temperatures over 40°C the dominant ant species cease foraging, thereby allowing subordinate ants to forage more extensively. Furthermore, the study points to *M. viridium* being competitively dominant to *F. pruinosus*. This dominance thermal tolerance trade-off is drawn from subordinate ants exhibiting higher thermal tolerances, taking greater thermal risks, and reaching peak foraging densities at higher overall temperatures.

If *M. viridium* is a competitively dominant ant species, the increased abundance of *M. viridium* on the high-rhizobia plants suggest that high-rhizobia plants are higher quality foraging sites than low-rhizobia plants. Preferential foraging by *M. viridium* may have permitted subordinate ants, such as *F. pruinosus*, to forage on the remaining unoccupied plants. When *M. viridium* not present to competitively exclude other ants, *F. pruinosus* may prefer to forage on the largest of plants, which likely contained the highest density of available resources. As low-rhizobia plants matured, *M. viridium* abundance began to increase while *F. pruinosus* abundance gradually declined, suggesting that low-rhizobia plants increase in quality as they reach sufficient size (Figure 16).

*Monomorium viridium* and *F. pruinosus* abundance was impacted by rhizobia treatment, and *Forelius pruinosus* may have partnered with plants that *M. viridium* discerns to be lower
quality. In a similar study, *C. fasciculata* plants supplemented with rhizobia inoculate attracted more ants than plants that acquired rhizobia from the natural soil environment (Keller et al. 2018). Larger plants often have more EFNs and thus regularly produce more EFN, therefore ants should prefer to forage on the largest of plants with the highest EFN production. However, this is not reflected in the data; plant size did not impact *M. viridium* abundance. Nonetheless, the response of *F. pruinosus* abundance to plant size as well as rhizobia treatment and the clear response of *M. viridium* abundance to treatment may be the result of *M. viridium* excluding all other ants from foraging on the highest quality plant partners. *F. pruinosus* may have been limited to foraging on low-rhizobia plants, opportunistically foraging on larger plants.

Our observed differences in plant survival between treatments suggests that allowing ants on plants as well as inoculating plants with the low-rhizobia treatment increased plant survival. At least 90% of the plant mortality in this experiment was associated with damage by mammalian herbivores. Various studies posit that mutualistic ant defenders deter mammalian herbivores and ultimately increase plant survival (Janzen 1966; Madden & Young 1992; Palmer et al. 2008), therefore one possible explanation for our findings is that ants did effectively defend plants from generalist mammalian herbivores. Our results also suggest the high-rhizobia treatment reduced plant survival, possibly because rhizobia are capable of influencing aboveground herbivore communities (Heath & Lau 2011). Associating with rhizobia at different levels can impact plant nutritional quality which may influence feeding preferences across herbivore classes differently (Dean et al. 2014). The high-rhizobia treatment may have made plants more attractive to generalist mammalian herbivores, which were capable of trimming *C. fasciculata* to no more than stubs overnight, dramatically reducing plant survival.

The spatial location of the plant in the field site also impacted survival, as plants neared the center of the scrubland habitat survival decreased. Plants with spatial locations over 60 were farther from the field station compound and roadways and were located in a more thickly vegetated area. This area likely held a higher density of mammalian herbivores, which contributed to the effect of space on plant survival. It is also important to mention that plants had only been inoculated several days prior to transplanting into the field, and that most of the mortality associated with the mammalian herbivores occurred within the first 72 hours of transplanting the plants into the field and applying ant exclusion treatments. Furthermore, these
results contrast to our other findings which suggest that there was no difference in herbivore abundance or plant damage in response to either treatment.

Several factors could explain why we did not observe differences in plant performance, EFN production, or herbivory in response to ant treatments. EFN production and herbivore damage in *C. fasciculata* can vary in response to a plant being locally adapted to interacting with ant partners and insect herbivores (Rutter & Rausher 2004; Abdala-Roberts & Marquis 2007). Among the four lines of *C. fasciculata* used for this work, EFN production as well as herbivore damage have been found to vary in respect to the plant genotype; consequently, this is likely the result of each line being locally adapted to the ecological conditions of its original geographic location. Furthermore, the outcome of mutualistic interactions, especially plant defense by ants, is often highly context-dependent. Not all ant partners are of equal benefit; particular species are more effective against specific herbivores (Miller 2007). Attracting only one species of ant may mitigate benefits provided by ant defense if that ant species is not suited to deterring a particular herbivore (Rico-Gray & Oliveira 2007; Chamberlain & Holland 2009; Rosumek et al. 2009).

Context-dependency is pervasive throughout ant-plant mutualisms, especially in facultative systems. According to a meta-analysis by Chamberlain and Holland (2009), ant-plant associations were found to impact plant performance in a predominantly positive manner. However, the degree to which plants experienced reductions in herbivory was affected by variation in plant rewards, latitude, and ant species richness. Contrary to these findings, our study indicates that ants had no effect on herbivory, irrespective of the scrublands’ high ant species richness and sub-tropical climate. Nonetheless, our inability to detect an effect may be an artifact of the low sample size and the short duration of the study.

Baker-Meio and Marquis (2012) examined EFN production across three naturally co-occurring varieties of *Chamaecrista desvauxii*. In this congeneric species, only plants with the largest extrafloral nectaries produce enough nectar to attract a sufficient number of mutualistic ants to significantly improve plant fitness; removing EFNs from these particular plants resulted in a significant decrease in plant fitness and an increase in herbivory. Ants significantly improved plant fitness only when focal host resource allocation to ant partners was high as compared to other plants of the same species in the local population, demonstrating that within a single species of focal host plants the benefits of associating with ants fluctuate. Perhaps the
experimental plants were not producing enough EFN as compared to the local population, and therefore they experienced no benefit from attracting ants.

The effects of ant presence on plant fitness and EFN production in *C. fasciculata* varies with herbivore pressure and the abundance and identity of ant mutualists. Prior studies suggest that when *C. fasciculata* is grown in the presence of abundant herbivores and a diverse array of ant partners, there is a significant increase in EFN production that decreases herbivory and increases growth (Barton 1986; Kelly 1986; Rios et al. 2008). A study using the legume *Vicia faba* found that plants need to produce enough extrafloral nectar to attract four or more mutualistic ants every ten minutes to experience a significant reduction in herbivore abundance (Katayama & Suzuki 2004). Chamberlain et al. (2014) found that the context-dependency in mutualisms, such as between rhizobia and *C. fasciculata*, varied mostly in respect to study location and not in relation to the presence of third-party species, such as mutualist ants. Furthermore, a multiyear study assessing how mutualistic ants affect herbivory and plant performance in the congener *Chamaecrista nictitans* found that ants did not reduce insect herbivory or increase plant fitness (Ruhren 2003).

Similarly, rhizobia treatment did not affect plant performance, EFN production, or herbivory. Other studies have found that under various conditions rhizobia can improve host plant fitness, increase plant defenses, and alter EFN composition (Thamer et al. 2011; Godschalx et al. 2015; Keller et al. 2018). However, the benefits of rhizobia can fluctuate, and the capacity for rhizobia to improve plant fitness is known to be affected by composition of the local herbivore community (Heath & Lau 2011). Host plants are liable to experience increased herbivory in response to hosting rhizobia (Kempel et al. 2009), reducing the net benefit of hosting rhizobia (Thamer et al. 2011; Simonsen & Stinchcombe 2014). These aspects and the relatively short duration of this experiment may have attributed to the rhizobia treatment having no effect on plant performance, EFN production, or herbivory.

Resource allocation to belowground mutualists was mediated by a three-way interaction between treatments and plant size; the data suggested that there is a trade-off in focal host resource allocation, with plants reducing resource allocation in belowground partners as they mature. Plants in the high-rhizobia treatment showed reduced nodule weight as they grew more leaves, with the presence of ants increasing the strength of this trade-off. In the high-rhizobia
treatment, plants had more potential rhizobial partners. Thus, they may have been more selective in forming associations with rhizobia, dedicating more resources to strains that were most beneficial. Prior work indicates that as nitrogen becomes sufficiently abundant legumes are capable of autoregulating nodule formation, optimizing plant growth and limiting excessive investment in rhizobia (Mortier et al. 2012). Consequently, the high-rhizobia plants may have obtained sufficient nitrogen early on and limited further resource allocation in rhizobia while directing more resources in aboveground growth.

Many mature plants in the local Chamaecrista fasciculata population had few or no nodules (Trychta, personal observation). The root-to-shoot (or at least, leaf-to-nodule) trade-off occurring in high-rhizobia treatment plants as well as limited nodulation in natural plants may have been caused by elevated nitrogen levels in the local environment. Having been subject to a high-intensity controlled burn in the year prior, the field site could have contained an abundance of available resources, especially nitrogen, thus prompting both local and experimental plants to scale back resource allocation to rhizobia as they grew sufficiently large. For plants in the low-rhizobia treatment, leaves and nodule weight were also negatively correlated in the absence of ants. However, when ants were present, nodule weight scaled positively with leaf number, suggesting that aboveground mutualists are capable of moderating focal host resource allocation to belowground mutualists.

This work contributes to the small, but growing body of research exploring multiple mutualist effects in natural systems. Our data are unique as they show that species identity and abundance of ant mutualists responded indirectly to rhizobia treatment, corroborating that belowground mutualists can influence focal host interactions with aboveground mutualists. Furthermore, the presence of ants affected nodule weight differently across rhizobia treatments, demonstrating that aboveground mutualists can influence focal host interactions with belowground mutualists. Unexpectedly, plant performance, EFN production, and herbivory did not respond to ant or rhizobia treatments, but did impact plant survival, suggesting that the net outcomes of naturally occurring multiple mutualist systems may not be as context-dependent as previously thought. Consequently, we should strive to incorporate multiple mutualist effects into future studies in an effort to expand our understanding of mutualisms beyond the pairwise context and provide additional insights into how mutualisms shape natural communities.
### Tables

**Table 1: Ant Abundance Generalized Linear Model Results**

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<td>Coefficient Estimate</td>
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<td>Ant Abundance</td>
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**Table 2: Monomorium viridium Abundance Generalized Linear Model Results**

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**Table 3: Forelius pruinosis Abundance Generalized Linear Model Results**

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Table 4: Herbivore Abundance Generalized Linear Model Results

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Table 5: Aphid Abundance Cumulative Link Model Results

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Table 6: Leaf Number Linear Mixed Model Results

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### Table 7: EFN Sugar Production Generalized Linear Model Results

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### Table 8: Plant Survival Generalized Linear Model Results

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<td>Rhizobia * Ant</td>
<td>2.29</td>
<td>2.43</td>
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### Table 9: Plant Damage Generalized Linear Model Results

<table>
<thead>
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<th></th>
<th>Model Summary</th>
<th>Type III Anova</th>
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<tr>
<td></td>
<td>Coefficient Estimate</td>
<td>Z-Value</td>
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<tr>
<td>Plant Damage</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rhizobia Treatment</td>
<td>0.52</td>
<td>1.62</td>
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<tr>
<td>Ant Treatment</td>
<td>0.42</td>
<td>1.37</td>
</tr>
<tr>
<td>Final Leaf Number</td>
<td>0.004</td>
<td>2.14</td>
</tr>
<tr>
<td>Distance</td>
<td>0.003</td>
<td>1.64</td>
</tr>
<tr>
<td>Genotype</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rhizobia * Final Leaf Number</td>
<td>-0.0037</td>
<td>-1.42</td>
</tr>
<tr>
<td>Ant * Final Leaf Number</td>
<td>-0.0052</td>
<td>-2.12</td>
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**Table 10: Nodule Weight Linear Model Results**

<table>
<thead>
<tr>
<th></th>
<th>Coefficient Estimate</th>
<th>T-Value</th>
<th>Chi-Sq</th>
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<tr>
<td><strong>Nodule Weight</strong></td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>Rhizobia Treatment</td>
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<td>-2.16</td>
<td>1.46</td>
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<td>0.036</td>
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<tr>
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<td>-1.09</td>
<td>0.37</td>
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<td>0.28</td>
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<tr>
<td>Final Leaf Number</td>
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<td>Rhizobia * Ant</td>
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<td>Rhizobia * Final Leaf Number</td>
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<td>Ant * Final Leaf Number</td>
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<td>1.42</td>
<td>0.64</td>
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<td>0.16</td>
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<td>Rhizobia * Ant * Final Leaf Number</td>
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<td>-3.09</td>
<td>3.009</td>
<td>1</td>
<td>0.003</td>
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**Table 11: Aboveground Plant Biomass Linear Model Results**

<table>
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<tr>
<th></th>
<th>Coefficient Estimate</th>
<th>T-Value</th>
<th>Chi-Sq</th>
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<td><strong>Aboveground Plant Biomass</strong></td>
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<tr>
<td>Rhizobia Treatment</td>
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<td>Ant Treatment</td>
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<td>1.28</td>
<td>14.36</td>
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<td>0.21</td>
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<td>Genotype</td>
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<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Rhizobia * Ant</td>
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<td>-1.68</td>
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**Table 12: Plant Height Linear Mixed Model Results**

<table>
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<th>T-Value</th>
<th>Chi-Sq</th>
<th>df</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Plant Height</strong></td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>Time</td>
<td>0.04</td>
<td>14.36</td>
<td>206.37</td>
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<td>Rhizobia Treatment</td>
<td>-0.27</td>
<td>-0.084</td>
<td>0.71</td>
<td>1</td>
<td>0.4</td>
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<td>Ant Treatment</td>
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<td>-0.66</td>
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<td>1</td>
<td>0.51</td>
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<tr>
<td>Genotype</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Distance</td>
<td>0.0014</td>
<td>0.61</td>
<td>0.38</td>
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<td>0.54</td>
</tr>
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<td>Time * Rhizobia</td>
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<td>1.009</td>
<td>1.02</td>
<td>1</td>
<td>0.31</td>
</tr>
<tr>
<td>Time * Ant</td>
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<td>0.5</td>
<td>0.09</td>
<td>1</td>
<td>0.77</td>
</tr>
<tr>
<td>Rhizobia * Ant</td>
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<td>1.49</td>
<td>2.23</td>
<td>1</td>
<td>0.14</td>
</tr>
<tr>
<td>Time * Rhizobia * Ant</td>
<td>-0.006</td>
<td>-1.06</td>
<td>1.13</td>
<td>1</td>
<td>0.29</td>
</tr>
</tbody>
</table>
Figures

Figure 1: *Chamaecrista fasciculata*, photo taken from the field site at Archbold Biological Station. A *Monomorium viridium* worker is barely visible on EFN of third leaf on the right-hand side. Photographed by Dustin Angell.

Figure 2: *Chamaecrista fasciculata* plants from field experiment. Note the difference in EFN color and shape between the two plants. Photographed by Mitchel Trychta.
Figure 3: Violin plots showing the effect of rhizobia treatment on ant abundance. X-axis, rhizobia treatment. Y-axis, total number of ants recorded during experiment ($n_{\text{High}} = 12$, $n_{\text{Low}} = 21$), line is the group mean.

Figure 4: Ant abundance plotted against plant size for each rhizobia treatment. The lines show the smoothed conditional mean for each rhizobia treatment. X-axis, number of leaves at harvest. Y-axis, total number of ants recorded during experiment.
Figure 5: Boxplot of the effect of rhizobia treatment on *M. viridium* and *F. pruinosus* abundance. X-axis, rhizobia treatment. Y-axis, total number of ants recorded during experiment.

Figure 6: *Monomorium viridium* abundance plotted against plant size for each rhizobia treatment. The lines show the smoothed conditional mean for each rhizobia treatment. X-axis, number of leaves at harvest. Y-axis, total number of *M. viridium* ants recorded during experiment.
Figure 7: *Forelius pruinosus* abundance plotted against plant size for each rhizobia treatment. The lines show the smoothed conditional mean for each rhizobia treatment. X-axis, number of leaves at harvest. Y-axis, total number of *F. pruinosus* ants recorded during experiment.

Figure 8: Violin plots showing the effects of ant (control/exclusion) and rhizobia (high/low) treatment on herbivore abundance (*n* High x E = 15, *n* High x C = 12, *n* Low x E = 13, *n* Low x C = 21), line is the group mean.
Figure 9: plot showing the effects of ant (control/exclusion) and rhizobia (high/low) treatment on aphid abundance.

Figure 10: Effects of treatment on leaf number log$_2$ (no.), over time.
**Figure 11:** Violin plots showing the effects of ant (control/exclusion) and rhizobia (high/low) treatment on EFN sugar production, line is the group mean.

**Figure 12:** Plant survival depended on ant (control/treatment) and rhizobia (high/low) treatment. X-axis: location at field site, zero being nearest the start of the scrublands and one-hundred being at the very center, Y-axis: Plant survival (1: survived to harvest, 0: died before harvest).
Figure 13: Violin plots showing the effects of ant (control/exclusion) and rhizobia (high/low) treatment on plant damage, line is the group mean.

Figure 14: Violin plots showing the effects of ant (control/exclusion) and rhizobia (high/low) treatment on nodule weight $\log_2 (g)$, line is the group mean.
Figure 15: Nodule weight depended on ant (control/exclusion) and rhizobia (high/low) treatment. X-axis: Number of leaves, Y-axis: nodule weight $\log_2 (g)$.

Figure 16: Abundance of *M. viridium* and *F. pruinosus* throughout the experiment. X-axis, days since planting. Y-axis, mean number of ants recorded ($n_{high} = 27$, $n_{low} = 34$).
Literature Cited


DuBois, M.B., 1986. A revision of the native New World species of the ant genus Monomorium (minimum group) (Hymenoptera: Formicidae). *University of Kansas science bulletin (USA)*.


Orrell, P. and Bennett, A.E., 2013. How can we exploit above–belowground interactions to assist in addressing the challenges of food security?. *Frontiers in plant science, 4*, p.432.


Appendix 1 – Supplementary Tables

Table A2: Plant Mortality

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Initial Number of Plants</th>
<th>Number of Plants at Harvest</th>
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<tbody>
<tr>
<td>Total</td>
<td>101</td>
<td>61</td>
</tr>
<tr>
<td>Ant Control * High Rhizobia</td>
<td>26</td>
<td>12</td>
</tr>
<tr>
<td>Ant Control * Low Rhizobia</td>
<td>25</td>
<td>21</td>
</tr>
<tr>
<td>Ant Treatment * High Rhizobia</td>
<td>25</td>
<td>15</td>
</tr>
<tr>
<td>Ant Treatment * Low Rhizobia</td>
<td>25</td>
<td>13</td>
</tr>
</tbody>
</table>

Table A2: Ant Species Abundance

<table>
<thead>
<tr>
<th>Ant Species</th>
<th>Count</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>All</td>
<td>746</td>
<td></td>
</tr>
<tr>
<td><em>Monomorium viridium</em></td>
<td>317</td>
<td>42.50%</td>
</tr>
<tr>
<td><em>Forelius pruinosus</em></td>
<td>380</td>
<td>50.90%</td>
</tr>
<tr>
<td><em>Dorymyrmex bureni</em></td>
<td>3</td>
<td>0.40%</td>
</tr>
<tr>
<td><em>Dorymyrmex flavopunctus</em></td>
<td>18</td>
<td>2.40%</td>
</tr>
<tr>
<td><em>Nylanderia phantasma</em></td>
<td>8</td>
<td>1.10%</td>
</tr>
<tr>
<td><em>Nylanderia arenivaga</em></td>
<td>20</td>
<td>2.70%</td>
</tr>
</tbody>
</table>
Table A3: EFN Production

<table>
<thead>
<tr>
<th>EFN Measures</th>
<th>Average Volume (µl)</th>
<th>Average Sugar (µg)</th>
</tr>
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<tbody>
<tr>
<td>All Plants</td>
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<td>782</td>
</tr>
<tr>
<td>Texas</td>
<td>2.8</td>
<td>584</td>
</tr>
<tr>
<td>Louisiana</td>
<td>3.81</td>
<td>601</td>
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<td>Arkansas</td>
<td>6.78</td>
<td>1108</td>
</tr>
<tr>
<td>Mississippi</td>
<td>6.26</td>
<td>956</td>
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</table>
Appendix 2 – Supplementary Figures

**Figure A1:** Correlation rates across various components in the dataset pertaining to only the plants for which ants were not excluded. Positive correlations are displayed in blue and negative correlations in red. Color intensity and the size of the circle are proportional to the correlation coefficients.

**Figure A2:** The effect of rhizobia treatment on nodule weight (g) plotted against ant abundance (correlation coefficient = -.28).
Figure A3: The effect of rhizobia treatment on the abundance of *M. viridium* plotted against the abundance of *F. pruinosus* (correlation coefficient = -.27).

Figure A4: Effects of treatment on plant height \( \log_2 \) (cm), over time (\( n_{\text{High} \times N} = 15, n_{\text{High} \times Y} = 12, n_{\text{Low} \times N} = 13, n_{\text{Low} \times Y} = 21 \)).
Figure A5: Violin plots showing the effects of ant (control/exclusion) and rhizobia (high/low) treatment on aboveground plant biomass, line is the group mean.
Figure A6: Chamaecrista fasciculata, illustrated by S.E.R. for use in MSc thesis of M.R.T.