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Lignocellulose Metabolism in a Fungus-Gardening Ant Symbiosis

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LIGNOCELLULOSE METABOLISM IN A FUNGUS-GARDENING ANT SYMBIOSIS

by

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Abstract

LIGNOCELLULOSE METABOLISM IN A FUNGUS-GARDENING ANT SYMBIOSIS

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Fungus-gardening ants exhibit a unique interaction with specific fungi in an obligatory symbiosis; ants feed and prune their fungal symbiont in exchange for nourishment. Cellulose is the most abundant plant compound found on earth and may serve as a rich carbon source to ants. Recent studies show that leaf-cutting (*Atta* and *Acromyrmex*) ants' fungus does not typically digest cellulose, but there have been few comparisons to the other higher attine fungus gardening ants. In this study, I measured the lignocellulose consumption of the higher fungus-gardening ants *Trachymyrmex septentrionalis* and *Trachymyrmex arizonensis*. Colonies were fed either a diet of Texas Red Oak (*Quercus buckleyi*) catkins (staminate flowers) or tent caterpillar (*Malacosoma americanum*) frass to assess the ability of the symbiosis to digest plant-based carbohydrates. The Van Soest fiber analysis method was used to evaluate the amount of lignocellulose and hemicellulose content of substrates and fungus garden refuse, *i.e.*, organic matter before and after assimilation in the fungus garden, respectively. The

evidence supports the hypothesis that these two species of *Trachymyrmex* colonies are significant metabolizers of cellulose since levels of lignocellulose and hemicellulose were significantly lower in refuse piles than in the substrates the colonies were fed. Fungus gardens of *T*. *septentrionalis* extracted about 46% and 47% of the lignocellulose present in the catkins and frass, respectively, as well as 43% of the hemicellulose found in catkins and 67% of the hemicellulose that was found in frass.

Chapter One

Introduction

Many examples of mutualisms can be found throughout nature, but not many as well-studied and intriguing as the symbiotic mutualism between fungus-gardening ants (tribe Attini) and basidiomycete fungi. Mutualisms are a type of symbiosis where interspecific interactions benefit both parties (Bronstein, 1994; Bronstein et al., 2006). In this case, attine ants protect their fungal symbiont from harmful bacteria and microbes by continuously cleaning and pruning the garden (Hölldobler and Wilson, 2010). The ants also cultivate the fungus through vertically transmitting the garden from parent to daughter colony, as the fungus is not always able to reproduce sexually (Mikheyev et al., 2006; Hölldobler and Wilson, 2010). In return, the fungus is used as a source of food and provides the colony with enzymes that are able to digest complex macromolecules that the ants are unable to digest on their own (Martin, 1987a; de Fine Licht and Boomsma, 2010; de Fine Licht et al., 2010).

Attine ant species are characterized by their interaction with specific basidiomycete fungi, where both species are entwined in a beneficial mutualism. Higher attine ants are composed of the most derived leaf-cutting ants of the genera *Atta* and *Acromyrmex* as well as fungus-gardeners *Trachymyrmex* and *Sericomyrmex*. Leaf-cutting ants cultivate a single species of fungi called *Attamyces bromatificus* in its asexual form or *Leucoagaricus gongylophorus* in its sexual morph (Fisher et al., 1994; Silva et al., 2006). *Trachymyrmex* species cultivate several closely related *Leucocoprinus* species that

are not taxonomically resolved and colloquially referred to as 'Trachymyces' (Mikheyev et al., 2008; 2010; Schultz and Brady, 2008). While non-attine ants forage for themselves, fungus-gardening ants forage to feed their fungal symbiont. Leaf-cutting ant workers cut fresh plant material to fertilize their garden, while fungus-gardeners find leaf litter and insect frass for their fungal partner (Hölldobler and Wilson, 2010). Workers masticate the plant substrate before adding it to the top most layer of the fungal garden (Kooij et al., 2011). Ants also fertilize the garden with a fecal droplet, which contains fungal digestive enzymes and place fungal mycelia on newly collected substrate to begin degradation (Boyd and Martin, 1975; Quinlan and Cherrett, 1977; Bass and Cherrett, 1994; Erthal et al., 2004). The fungus produces swollen hyphal tips, called gongylidia, that are filled with nutrients from the broken down plant material that the ants provided to the fungus (Bass and Cherrett, 1995; Schiott et al., 2008; Stradling and Powell, 1986). The gongylidia are thought to be consumed by all ant castes and have been shown to account for most of the leaf cutting ant's nutritional needs (Silva et al., 2003). This is especially true for the larvae, which are fed on a diet composed exclusively of gongylidia (Quinlan and Cherrett, 1979; Bass and Cherrett, 1995). Worker ants have been found to supplement their diet with both fungus and plant sap that is ingested while chewing up fresh substrate before inoculation into the garden (Littledyke and Cherrett, 1976; Quinlan and Cherrett, 1979; Bass and Cherrett, 1995).

Fertilizing the fungal garden has a dual purpose; the myriad of fungally-derived enzymes residing within the ant's rectum both break down plant phenolic defenses and digest plant nutritional compounds. Laccases are known to break down secondary, toxic plant compounds and have been found to be expressed in the hyphal tips of the

Acromyrmex echinator fungus (de Fine Licht et al., 2013). Workers defecate these laccase enzymes onto recently cut plant material to detoxify any phenolic resistances (de Fine Licht et al., 2013). As another demonstration of the symbiotic relationship, the ants and fungus have enzyme repertoires that degrade different compounds within the substrate (Richard et al., 2005). The ants target substances with lower molecular weights, such as oligosaccharides, whereas fungally-derived enzymes work on polysaccharides (Richard et al., 2005, D'Ettorre et al., 2002). For example, some of the first enzymes that plant material comes into contact with are alpha-amylases secreted by the ant's labial glands. To degrade starch found in plants, workers chew up newly foraged substrate with the help of alpha-amylase before inoculation into the garden (D'Ettorre et al., 2002; Erthal et al., 2004; Kooij et al., 2011). Specifically, alpha-amylase hydrolyses alpha-linked polysaccharides to produce maltose which is hydrolyzed by a maltase to produce glucose, one of the most important carbon sources for ants (Silva et al., 2003). Interestingly, this alpha-amylase of ant origin is not found in the fecal fluid, but an alpha-amylase produced by the fungus can be found when analyzing fecal droplets, which suggests that while the fungal alpha-amylase is ingested it is not digested by the ants (Bacci et al., 2013). Bacci et al. (2013) found the opposite while investigating the origins of maltase, as the fungal maltase was digested by the ant, while the ant maltase was upregulated to be deposited onto substrate through a fecal droplet. Overall, their study provided additional evidence for the complexity of the numerous enzymes used by the ant-fungal mutualism through the assimilation of nutrients from plant matter.

Once worker ants have added their own unique enzymes to a masticated piece of plant material, they add the result to the garden with a fecal droplet (Boyd and Martin,

1975; Quinlan and Cherrett, 1977; Bass and Cherrett, 1994; Erthal et al., 2004). Masticating plant material allows the fungal enzymes to begin digestion, as the fungi does not form any specific structures to remove plant waxes or break down cell walls without the aid of the ants (Erthal et al., 2009). Fecal droplets contain a mixture of enzymes, most of which are of fungal origin that pass through the ant's digestive system unaltered (Boyd and Martin, 1975b; Quinlan and Cherrett, 1977; Bass and Cherrett, 1995; Erthal et al., 2004). For example, Rønhede et al. (2004) demonstrated that fecal droplets of *Acromyrmex echinatior* and *Atta colombica* ants contained carboxymethylcellulases, laccases, proteases and pectinases that are of fungal and not ant origin. Likewise, pectinolytic enzymes of fungal origin were found in the gongylidia, so that there were still a significant amount of functional enzymes after digestion by the ants (Schiott et al., 2010). Xylanolytic and cellulolytic enzymes have been found in the fecal fluid in numerous other studies that may also be of fungal origin (Martin et al., 1975; D'Ettorre et al., 2002; Richard et al., 2005). These physiological behaviors are made possible by the rectal epithelium not having any secretory functions, but rather have the rectum acting as a repository for the amassing of enzymes (Erthal et al., 2004).

The purpose of all these enzymes, whether they are of ant or fungal origin, is to disassemble plant forage for usable carbon sources by the symbiosis. Plant cell walls account for 30-50% of dry leaf mass and are composed of polysaccharides in the form of cellulose microfibers that are entrenched within a matrix of pectin and hemicellulose (Onoda et al., 2004; Schiott et al., 2008). Hemicellulose differs from cellulose by being composed of heterogeneous polymers as opposed to the homogenous glucose monomers that comprise cellulose chains (Saha, 2003). Xylan is a hemicellulose composed of units

of xylose, a pentose sugar, making it a major structural polysaccharide (Collins et al., 2005). Pectins are a group of polysaccharides which are rich in galacturonic acid (Willats et al., 2001). Pectins occur in primary cell walls as well as the middle lamellae between primary cell walls and act as an adhesive between cell walls, controlling cell porosity and expansion of cellulose microfibers (Willats et al., 2001). Other cell wall components are comprised of lignin, an aromatic alcohol polymer embedded within the cellulose to add structural support, and starch, which stores energy in the form of glucose for plants (Nelson et al., 2008).

The most efficient way to break down this highly resistant structure is to first degrade pectin, which leads to biochemical changes that weaken the hemicellulolitic structure (Esquerre-Tugaye et al., 2000; de Fine Licht et al., 2010). Ant fecal fluid is high in pectinolytic enzymes that have the ability to degrade pectin found in plant cell walls (Martin et al., 1975; Rønhede et al., 2004; Schiott et al., 2010). Hemicellulose is then broken down by the symbiotic fungus through xylanase (Martin et al., 1975; D'Ettorre et al., 2002; Richard et al., 2005; Schiott et al., 2008). There is also evidence that starch, another plant cell wall component, is broken down by alpha-amylases (D'Ettorre et al., 2002; Silva et al., 2003; Erthal et al., 2004; Kooij et al., 2011; Bacci et al., 2013). The attine fungus also has active laccase enzymes which are responsible for degrading lignin, a polyphenolic compound (Thurston, 1994; Rønhede et al., 2004; Henrik et al., 2013). These laccase digesting enzymes have a dual purpose, as they have the ability to digest possible toxic plant compounds as well as breakdown lignin, an important structural component of plant cell walls (Rønhede et al., 2004; de Fine Licht et al., 2013).

Therefore, it appears fungus-gardening attine ants have the proper tools to metabolize plant cell wall compounds.

Part of the success of these ants was due to a group of common ancestors which were generally hunter and gatherers to become functional herbivores by forming a symbiosis with a saprophytic fungus specialized toward a diet of plant tissue (Mueller et al., 2001; Shik et al., 2014). In contrast to the many other plant fiber-feeding insects that form a symbiosis with bacteria and fungi, e.g. termites, it is far from clear that the fungus-gardening ants are utilizing the most abundant carbohydrate in leaves and on earth as a whole: cellulose (Martin, 1987b). Various studies have described the ability of leaf-cutting ants and their fungi to digest cellulose on both synthetic and natural diets (Bacci et al., 1995; D'Ettorre et al., 2002; Silva et al., 2003; Schiott et al., 2008). In contrast, evidence for the digestion of cellulose by the symbiosis of leaf-cutting ants is lacking (Abril and Bucher, 2002; Rønhede et al., 2004; Bucher et al., 2004; Erthal et al., 2004 & 2009; Richard et al., 2005; de Fine Licht et al., 2009 & 2013; Schiott et al., 2010; Kooij et al., 2011; Semenova et al., 2011; Mendes et al., 2012; Bacci et al., 2013). Therefore, it is generally accepted that leaf-cutting ants do not metabolize cellulose. Interestingly, cultivar switching studies have shown significantly higher cellulase activity by fungus-gardening ants in the genus *Trachymyrmex* when growing either their own fungus (Trachymyces) or *Atta texana* fungus (*Attamyces*) than *Atta texana* growing their own fungus (Appendix A; Seal et al., 2014). As a result, cellulose metabolism in the non-leaf-cutting fungus-gardening ants may occur (de Fine Licht et al., 2010). One explanation of this is that the leaf-cutting ants feed their fungal symbiont fresh cut plant material which may contain higher amounts of readily metabolized carbohydrates, such as starches and

pectins (Leal and Oliveira, 2000; de Fine Licht and Boomsma, 2010). Whereas the non-leaf-cutting ants, such as *Trachymyrmex* and *Sericomyrmex* lack the large mandibles to harvest leaves and instead must gather fallen leaves, flowers and caterpillar frass, which are probably relatively poor in starches, but rich in celluloses (Leal and Oliveira, 2000; de Fine Licht and Boomsma, 2010).

This study investigates the ability of the fungus gardening ants *Trachymyrmex septentrionalis* to digest plant polysaccharides, as very little is currently known concerning their ability to digest cellulolytic compounds, although they are known to provide their gardens with fresh leaves, flowers and caterpillar frass (Seal and Tschinkel, 2007b). In this project, plant fiber metabolism was estimated by comparing the original amounts of lignocellulose and hemicellulose in collected food materials (oak flowers and caterpillar frass) to the amount recovered in refuse piles.

Chapter Two

Methods

Trachymyrmex septentrionalis is the most widely distributed attine ant species in the eastern United States, ranging from Illinois to New Jersey (Hölldobler and Wilson, 2010) and all the way south to Texas and Florida (McCook, 1880; Rabeling et al., 2007; Seal and Tschinkel, 2006). *T. septentrionalis* is a conspicuous species in pine forests throughout the southeastern United States (Seal and Tschinkel, 2006 & 2010; Seal et al., 2015). Colonies are characterized by several hundred relatively small (size) workers and one reproductive queen. *Trachymyrmex arizonensis* represents a lesser studied but nevertheless common higher attine fungus-gardening species. *T. arizonensis* occurs in mountainous areas of the Sonoran and Chihuahua deserts located in Arizona, New Mexico and Mexico (Wheeler, 1907 and 1911; Rabeling et al., 2007).

All colonies of *T. septentrionalis* were collected in mid-April and early May 2014 on the University of Texas at Tyler campus in Tyler, Texas. Nests were located by scanning along the edge of wooded pathways looking for a characteristic half-moon shaped tumulus of excavated sand around a small nest entrance (Tschinkel and Bhatkar, 1974). Colonies were collected by digging a small pit roughly 30cm from the nest entrance and digging horizontally towards the tunnels and fungus chambers. Once the initial chamber and connecting tunnel were located, the small pit was widened with a shovel, taking extra care to dig parallel to the chambers. This was performed for ease of

locating the deeper chambers. The soil was carefully removed using a spade and small spoons as not to collapse any tunnels. All ants were collected using an aspirator, paying special attention to locating the queen, and transferred into a moist plaster-lined box. Fungus was collected with soft forceps and placed into the box with its colony. All boxes were placed in a cooler before transport to the laboratory. This collection process averaged 75-90 minutes and typically resulted in the collection of all the workers and the queen. Chamber dimensions (length, width and height) and depths were recorded. All of the *T. arizonensis* colonies were reared from newly mated queens collected after mating flights earlier in 2010 or 2011 in southeastern Arizona and thus were in the lab at least 3 years at the time of the study.

All experimental colonies of both *T. septentrionalis* and *T. arizonensis* were housed in Fluon© coated trays to avoid escapes. The ants grew their garden in a cylindrical cavity measuring 175 cm^3 in a polystyrene box lined with dental plaster and topped with a Plexiglas lid. To allow for ant movement to and from the fungus garden chamber, two 9 mm diameter holes were drilled in the side of each plaster nest. Additional plaster nests were added and interconnected with 5 cm segments of clean, rubber hoses as colony sizes expand. Four 9 mm diameter holes were drilled into each corner of the plaster nest to allow for watering. All colonies were kept in the lab under standard conditions (Seal and Mueller, 2014; Seal and Tschinkel, 2007a; Seal and Tschinkel, 2007b).

The two species, *Trachymyrmex septentrionalis* and *Trachymyrmex arizonensis*, were divided into three experimental groups. Group 1 contained 15 *T. septentrionalis* colonies being fed Texas Red Oak (*Quercus buckleyi*) catkins, Group 2 contained 13 *T.*

septentrionalis colonies being fed tent caterpillar (*Malacosoma americanum*) frass and Group 3 contained 18 *T*. *arizonensis* colonies being fed oak catkins. *T*. *arizonensis* does not readily accept tent caterpillar frass to feed its fungus as it is not an available food source in its natural habitat (personal observation).

As *T*. *septentrionalis* colonies were growing and establishing their gardens from early May to mid-June, they were fed 3 times per week. When the colonies shifted to brood care in mid-June, they were fed twice per week through the end of August. To account for the changes between wet and dry masses of the colonies' diets, a wet sample was collected and weighed for each new container of food. These samples were left to dry for 24 hours before a dry mass was taken. Colonies were fed 1-2 grams of their experimental diets by weighing out a wet sample on an electronic scale. All diets were then transferred to a small weigh boat located next to their nest entrance. All wet masses were recorded and converted to dry masses to obtain actual amounts of lignocellulose and hemicellulose digested by the symbiosis. Because the digestion method only gives a percentage of lignocellulose or hemicellulose found in the food or refuse pile, dry masses are needed to calculate the exact amount of each carbohydrate. Food that went uncollected by the workers was removed and weighed weekly from all *T*. *septentrionalis* colonies. The dimensions of the fungus garden were also measured weekly to assess the growth and health of the garden and colony. To equalize differences in microclimates, colonies were rotated in their shelving unit every two weeks. Because all of the *T*. *arizonensis* were established laboratory colonies, they were fed oak catkins *ad libitum* 3 times per week from May until mid-August 2014. Amounts of catkins fed were not weighed because accurate results to compare against *T*. *septentrionalis* colony

metabolism could not be obtained without completely destroying the *T. arizonensis'* fungus garden. All experimental colonies were watered weekly by filling up the four holes in the corners of their nests with distilled water.

Refuse samples were collected from all experimental colonies beginning in June 2014. When a colony was selected for cleaning, it was removed from its tray and placed in a temporary Fluon© lined tray. All dead ants were removed from the trays, and the refuse was separated from uncollected food by hand and filtered through a 2mm sieve. Uncollected food was weighed and refuse samples were stored in plastic vials, labeled and frozen for future analysis.

To assess the effectiveness of the symbiosis to digest specific carbohydrate components, the ANKOM Neutral Detergent Fiber (NDF) technique and Acid Detergent Fiber (ADF) technique were used (Goering and Van Soest, 1970; Van Soest et al., 1991). This was performed in collaboration with the Forage Physiology Laboratory at Texas A&M's Agrilife Research and Extension Center at Overton. This technique separates digestible plant cell contents such as starch, protein, sugar, pectin and lipids from less digestible cell wall components such as hemicellulose, cellulose and lignin (Goering and Van Soest, 1970; Van Soest et al., 1991). The detergent dissolves proteins as well as remove nitrogenous components and pectins at a boiling temperature, while addition of a heat-stable amylase removes starches (Goering and Van Soest, 1970; Van Soest et al., 1991). The final fiber residues consist of cellulose, hemicellulose and lignin fractions of all samples. The ADF residues correspond to the cellulose and lignin ("lignocellulose") content after digestion with sulfuric acid (H_2SO_4) and CTAB (cetyltrimethylammonium bromide) to dissolve hemicellulose and soluble minerals. Therefore, ADF values

correspond to lignocellulose content of a sample while values for hemicellulose content are obtained by subtracting ADF values from NDF values.

Chapter Three

Results

Trachymyrmex septentrionalis colonies were found to be significant metabolizers of lignocellulose and hemicellulose. Colonies that were fed oak catkins accepted a mean of 1.1g of lignocellulose from all catkins that were brought into the colony. On average, 0.6g of lignocellulose was found in the refuse piles (P value= 0.000004) (Fig. 1A). In other words, the ant-fungal symbiosis extracted about 46% of the lignocellulose present in the catkins. There were similar findings for lignocellulose levels in colonies that were fed tent caterpillar frass; 1.9g of lignocellulose was accepted into the garden and 1g of lignocellulose were found in the colonies' refuse piles (P value= 0.00027) (Fig. 1B). This amounts to an extraction of approximately 47% of the lignocellulose present in the tent caterpillar frass. Likewise, hemicellulose was also metabolized by all colonies on both diets. The amount of hemicellulose foraged by the catkin-fed colonies was 0.7g on average, with a mean of 0.4g in the refuse (P value= 0.000019) (Fig. 1C). Therefore, around 43% of the hemicellulose found in catkins were extracted by the symbiosis. Lastly, hemicellulose was significantly metabolized by colonies whose diet consisted of frass pellets. An average of 0.6g of hemicellulose was placed on the symbiosis for digestion and only about 0.2g were found after assimilation (P value= 0.00017) (Fig. 1D). This equates to a removal of approximately 67% of the hemicellulose that is found in frass by the symbiosis.

Colonies of *T*. *arizonensis* were included in this experiment to compare results of the colonies' ability to digest carbohydrates against *T*. *septentrionalis* colonies. Colonies of *T*. *arizonensis* colonies were reared in the laboratory for over three years (from 2011) and do not exhibit clear seasonal cycles of garden build-up in the summer and take-down in the fall like *T*. *septentrionalis* colonies (Seal and Tschinkel, 2006). Acceptance rates of oak catkins were not quantified because gardens would have to have been destroyed, likely stressing the colonies. Therefore, data presenting comparisons between *T*. *arizonensis* and *T*. *septentrionalis* are given as percentages. The percent of lignocellulose present in the refuse of *T*. *arizonensis* was not significantly different from catkin refuse in *T*. *septentrionalis* colonies (average percent lignocellulose content in the refuse piles of *T*. *septentrionalis* fed catkins was 20.2% and *T*. *arizonensis* was 20.8%, $p= 0.58$) (Fig. 2A). There was however a significant difference between the amount of hemicellulose digested by each species. On average, colonies of *T*. *septentrionalis* had 13.4% hemicellulose in their refuse material, compared to only 9.3% hemicellulytic material found in *T*. *arizonensis* refuse piles ($p < 0.0001$) (Fig. 2B). Thus, gardens tended by *T*. *arizonensis* possibly consumed more hemicellulose than those by *T*. *septentrionalis*.

Several refuse samples were also taken from wild colonies that foraged naturally. Two samples were obtained from colonies excavated and transported into the lab (summer colonies), where the worker ants created their own refuse piles. These colonies were not fed for two weeks, thus all refuse they produced was from the garden substrates the ants collected in the field. Another two samples were found outside known *T*. *septentrionalis* colonies' nest entrances (field colonies). The summer and field colonies show a much higher percentage of lignocellulose in their refuse piles, 53% and 60% on

average, respectively, whereas refuse from the laboratory colonies averaged only 25% lignocellulose content (averages from colonies fed catkins and frass) (Table 1). Hemicellulose percentages in the refuse from the summer and field colonies contained an average of 6% total hemicellulose. These values were most similar to the laboratory colonies fed frass but quite unlike those fed catkins (Table 1).

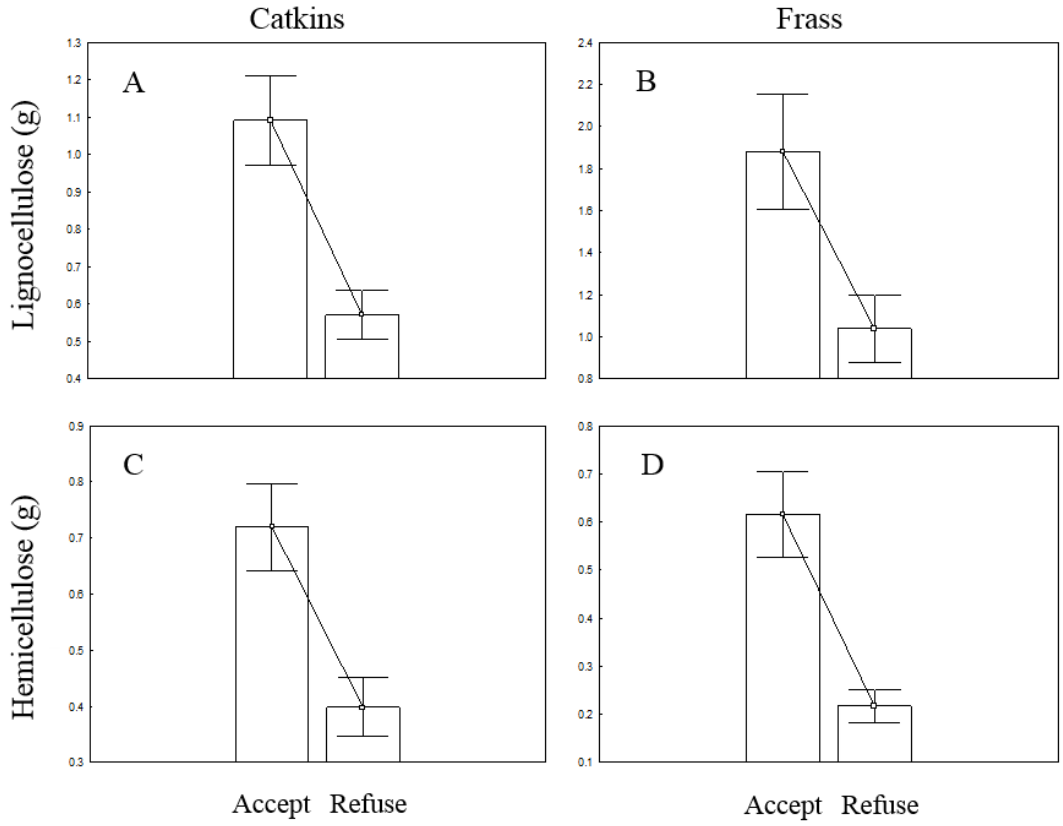

Figure 1.

Figure 1. Mean lignocellulose and hemicellulose activity, accepted into the colony ('Accept') and found in refuse piles ('Refuse'), for *Trachymyrmex septentrionalis* colonies fed on diets of oak catkins and tent caterpillar frass. All colonies digested a significant amount lignocellulose and hemicellulose. Dependent t-tests were used. A. ($p<0.000005$), B. ($p<0.0005$), C. ($p<0.00005$), D. ($p<0.0005$). Error bars are ±1 standard error.

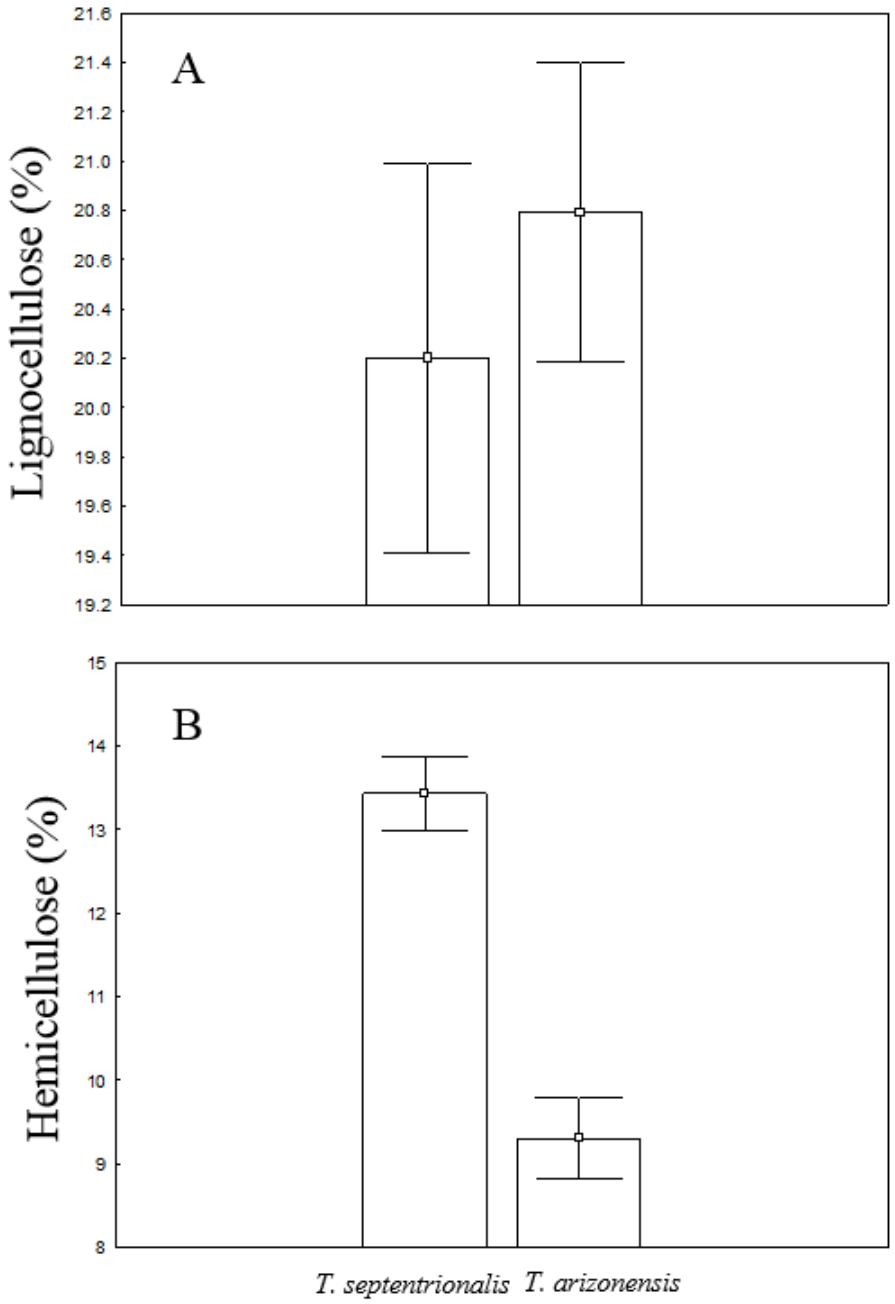

Figure 2.

Figure 2. Mean (± SE) percent lignocellulose and hemicellulose present in the garden refuse (i.e. undigested by fungus gardens) of laboratory colonies of *T. septentrionalis* and *T. arizonensis* fed on a diet of oak catkins. A. There was no significant difference of lignocellulose digestion by either species ($p = 0.58$, independent t-test). B. *T. arizonensis* colonies metabolized a significantly higher percentage of hemicellulose than colonies of *T. septentrionalis* ($p < 0.000005$, independent t-test).

ID	Lignocellulose (%)	Mean ±SD	Hemicellulose (%)	Mean ±SD
Summer 6	58.6	53.5 ±7.5	4.9	6.3 ±2
Summer 7	48.1		7.8	
Field 1	59.5	60 ±0.7	5.8	6.2 ±0.6
Field 2	60.5		6.7	
T. sept Catkin (average)	20.2	25.2 ±7.1	13.4	9.9 ±5
T. sept Frass (average)	30.2		6.4	

Table 1.

Table 1. Percent lignocellulose and hemicellulose found in wild colonies that foraged naturally compared to average values of laboratory colonies of *T. septentrionalis*. Summer colonies were dug up and brought into the lab. Field samples were found outside *T. septentrionalis* nest entrances.

Chapter Four

Discussion

Our results suggest that *Trachymyrmex septentrionalis* colonies have the ability to digest most plant carbohydrates, especially the plant cell wall components cellulose and lignin. This is interesting as there is an ongoing debate among myrmecologists about the ability of attine ants to digest cellulose. There are several studies that have shown cellulose digestion. Two of these studies show digestion of cellulose on artificial media (Bacci et al., 1995; D'Ettorre et al., 2002). Other studies show polysaccharidase activity of the fungus garden, including pectinase, amylase, xylanase and cellulase activity (Silva et al., 2003; Schiott et al., 2008). In contrast, a study performed by Abril and Bucher (2002) found no fungal growth on pure cellulose media as well as a much lower ratio of lignin to cellulose in ant refuse piles, compared to animals that do digest cellulose, such as termites. As an extension of this study, Bucher et al. (2004) used cellulose as an indicator for nutrient balances found in refuse piles as cellulose is not digested by the fungus garden. They found that only soluble carbon sources and phenols were

metabolized by the symbiosis, while there were still high amounts of cellulose found in refuse piles (Bucher et al., 2004).

It is important to note that there have been few comparisons of carbohydrate metabolism in other higher attine species such as *Trachymyrmex* and *Sericomyrmex*. While all higher attine species grow basidiomycete fungi of closely related *Leucoagaricus* species, what the ants forage for and feed to their symbiont differs (Fisher et al., 1994; Silva et al., 2006). Leaf-cutting ants forage on leaves and other fresh plant pieces, while other higher attines forage for leaf litter and insect frass (Hölldobler and Wilson, 2010). The differences in these compounds may lead to novel digestion techniques because easily accessible and nutrient-rich compounds such as starch may be absent from fallen leaves or previously consumed plant parts (frass) (Schroeder, 1986).

A limitation of studying enzymatic digestion comes from transplanting colonies from the field to the lab, as enzyme profiles may quickly be altered due to dietary shifts observed by Kooij et al (2011). Moreover, colonies in the wild are more likely to forage on diverse substrate types as opposed to a single type (De Fine Licht et al., 2010). Additional studies will need to be performed in order to assess the cellulose digestion capability of *T. septentrionalis* colonies in the field to rule out potential laboratory artifacts. Because it will be impossible to weigh all substrate accepted into a colony and know its carbohydrate composition, gene activation studies performed on the fungus at different times throughout the colonies' reproductive season may give some insight. Nevertheless, that cellulase activity occurs in the lab suggests that the laboratory environment causes genes to be activated whereas in the field they are not.

Outside of the ants and fungus, bacteria and yeasts may play a part in polysaccharide digestion. Yeasts found in leaf-cutting ant nests have enzymes capable of digesting plant polysaccharides that can be used by the ants as food (Mendes et al., 2012). For example, CMCellulose (carboxymethyl cellulose) was degraded by 43% of yeasts from *A*. *texana* colonies and 89% of yeasts from *Acromyrmex* colonies, which may produce more readily available sugars like glucose (Mendes et al., 2012). Other yeasts of the 82 yeast taxa screened, exhibited enzymatic activity by pectinases, proteases and amylases (Mendes et al., 2012). Metagenomic characterization of the microbiome of the leaf cutting-ant *Atta colombica* has shown presence of Proteobacteria capable of metabolizing cellulose and hemicellulose (Suen et al., 2010). However, the relative contribution of the ant fungus and associated microbes is currently unknown.

As interest in biofuels has piqued, the possibility of understanding how the microbial community residing within the attine symbiosis functions, could lead to novel methods in attaining the goal of renewable energy from cellulosic biofuels (Suen et al., 2010). Bioethanols are produced by fermentation of released sugars from lignocelluloses and can be used as a source of fuel (Sethi and Scharf, 2013). However, it is currently too expensive to replace a substantial amount of petroleum with biofuels due to the cost of cellulases and pretreatment processes (Sticklen, 2008). Therefore, there is a need for a naturally occurring source of lignocellulytic conversion techniques. The higher attine ant symbiosis may provide this service, as *T*. *septentrionalis* and leaf-cutting ants have been shown to rapidly digest large quantities of plant biomass for a carbon fuel source.

As one of the most widely distributed attine ants in North America, *T*. *septentrionalis* provide many ecosystem services. Nutrients in refuse piles were found to

be high in Ca, Mg, Na, P, Fe, Mn, K, C and N, which could provide plants with previously unavailable, but essential compounds (Torres et al., 1999). *T*. *septentrionalis* ants may also contribute to high soil turnover rates, as colonies have been shown to excavate over 1 metric ton of soil per year in a single hectare of pine forest in Northern Florida (Seal and Tschinkel, 2006). These ants are also potentially responsible for breaking down considerable amounts of fiber; with roughly 1,100 nests in an average hectare of forests of the Apalachicola National Forest, and about 1.5g of lignocellulose accepted per colony, over 1.6kg of crude fiber is being broken down per hectare (Seal and Tschinkel, 2006). An additional 700g of hemicellulose are being degraded per hectare. After assimilation, 1.2kg of lignocellulose and hemicellulose can be found in refuse piles of a single hectare of forest. Future work may want to quantify relative contribution of fiber degradation of *T*. *septentrionalis* with regard to other decomposers in the community.

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Appendix A: Cellulase Activity of *Atta texana*, *Trachymyrmex arizonensis* and *T. septentrionalis*

Materials and Methods

Colony Collections

Atta texana and *Trachymyrmex arizonensis* colonies were obtained from lab-rearing newly mated queens collected after mating flights. Queens were provided with approximately 50 g of garden (*T*. *arizonensis* queens), or 200 g of garden (*A*. *texana* queens; owing to the much larger size of *Atta* queens; (Seal, 2009).

Two *T*. *arizonensis* colonies were reared from queens collected in 27-28 July 2011 and four from queens collected 25-27 July 2010. All *T*. *arizonensis* colonies were collected at the Southwest Research Station, near Portal, Arizona (31° 53.025' N, 109° 12.374' W, 1646 m elevation) one day after the first heavy rain of the summer monsoon season. Queens were supplied with fungus from a *T*. *arizonensis* colony collected in July 2010 at the Southwest Research Station. Upon collection, all *T*. *arizonensis* queens were supplied with one melanized pupa obtained from a mature *T*. *arizonensis* colony in the laboratory, placed with ca. 8 cm^3 of fungus into a 4 cm Petri dish (garden chamber) that was inserted inside a 9 cm Petri dish. All of the space between these two Petri dishes was filled with cotton saturated with sterile water. *Trachymyrmex arizonensis* queens were fed and cleaned daily until the first workers appeared (Seal and Tschinkel, 2007c). Colonies were kept in these nesting containers until the first callow workers appeared (ca. 6 weeks later), then colonies were transferred to 7x7 cm plastic boxes with a 5mm-thick bottom of moistened dental plaster (Marjoy Enterprises, San Antonio, Texas). After approximately

one year of age, colonies were moved to larger nesting containers used in previous studies on *Trachymyrmex* ants (plaster-lined, cylindrically-shaped, 196 cm^3depressions in a square plastic box (11 x 11 x 3 cm) (Seal and Mueller, 2014 (Seal and Tschinkel, 2007a, b) All *T. arizonensis* colonies were fed *ad libitum* oak catkins (*Quercus shumardii*) throughout the duration of the entire study.

The six *Atta texana* colonies used in this study were similarly reared from newly mated queens collected after mating flights near Hornsby Bend, Texas ((30º 12' 37.3"N 97º 38' 28.07"W). These six colonies were reared from queens collected on 18 May 2010 (n=1), 13 May 2011 (n=4) and April 2007 (n=1). Freshly collected queens were placed in square plastic boxes (7 x 7 x 3 cm) lined with 5mm of dental plaster, which was moistened twice weekly. Because *Atta* queens do not forage during the founding phase (Fernández-Marín et al., 2004; Seal, 2009), they were given substrate only after the first workers emerged. After worker emergence, colonies were connected to a foraging arena via a plastic tube and fed and cleaned at least twice a week. *Atta texana* colonies were fed substrates similar to those fed the *T. arizonensis* colonies, except they were periodically supplied also with pear leaves (Bradford pear, *Pyrus calleryana*).

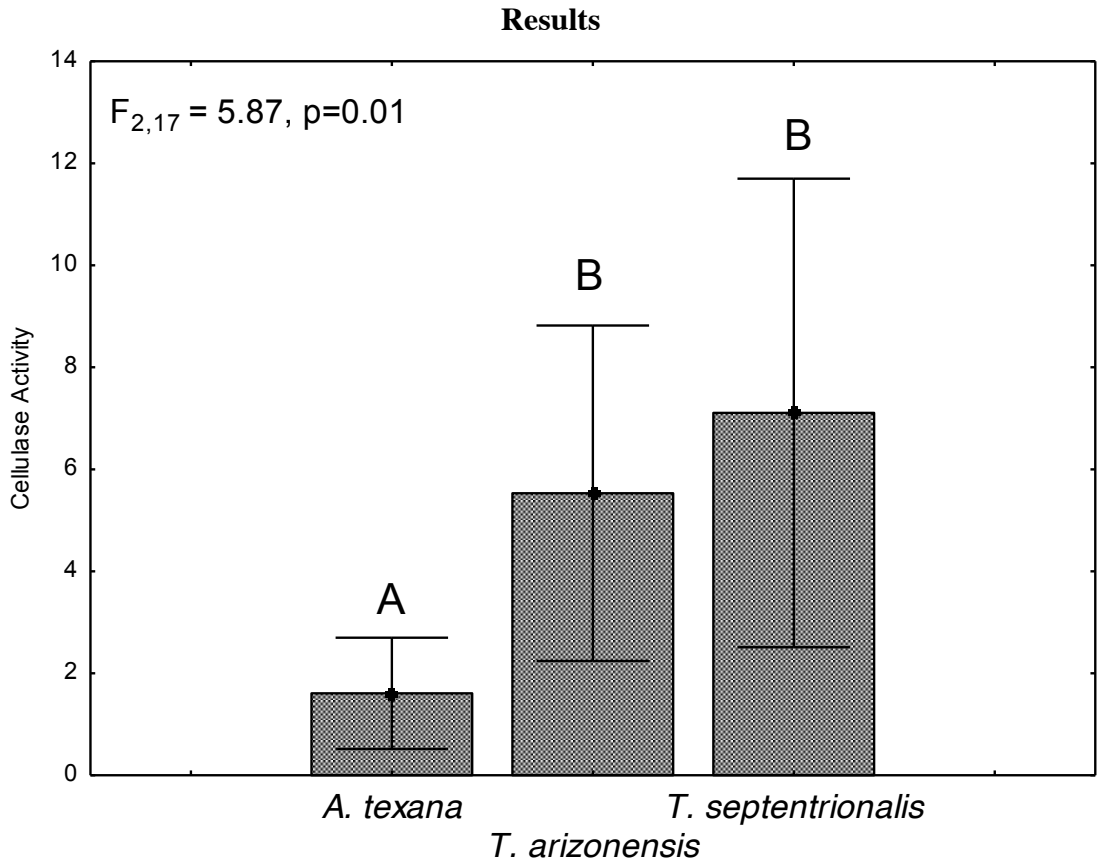
Two of the *T. septentrionalis* colonies were collected in Florida and six in central Texas. In both of these populations, colonies were collected just after the ants ended their winter dormancy. The colonies in Texas were collected on March 21, 2011 at the University of Texas' Stengl 'Lost Pines' Biological Station (30º 5' 13.1"N 97º 10' 25.5"W) and the colonies in Florida were collected March 7-9 2011 in the Wakulla District of the Apalachicola National Forest near Tallahassee, Florida (30º22'46.3" N,

84º20’6.5”W). At the time of assay, all *T*. *septentrionalis* colonies had been in the laboratory for one year.

Fungal Enzymatic Activity (Cellulase) Assays

Cellulase activity assays were conducted in 2012 when all colonies were at least one year old (Seal et al. 2014). Because fungus gardens are thought to exhibit spatial variation in enzymatic activity (Moller et al., 2011; Suen et al., 2010), fungus garden material was selected from the uppermost 1.5 cm of each fungus garden, which approximated a sample from the top one-third of a garden. Most fungally-derived digestion occurs in the upper-most part of the garden, whereas relative activity of enzymes derived from non-cultivar microbes (e.g., bacterial biofilms) seems to be greater in the lower portions (Moller et al., 2011; Suen et al., 2010). Furthermore, because enzymatic activities in gardens depend on the substrates usedby the ants (Kooij et al., 2011), all ant colonies of both species were fed a strict diet of oak catkins for 4 weeks prior to enzymatic assays. Finally, gardens were also sampled at least 48 hours after the last feeding, so that there were no freshly deposited substrates in the gardens. Enzymes were extracted from the fungus gardens by grinding ca. 120mg of fungus garden material in an Eppendorf tube containing 500 ml of 20 mM phosphate buffer (pH=6.9) after the removal of visible eggs, larvae and pupae. Extracts were centrifuged at 4°C for 15 min at 14000 rpm. The supernatant was then transferred to a fresh tube, which was then used for both the carbohydrase and proteinase activity assays. Each colony was sampled four times over the course of two weeks, and the average enzymatic activity was taken from these four values to provide an estimate for each colony.

Cellulase activity was measured using the dinitrosalicylic acid method, which assays reducing sugar concentration (Miller, 1959; Silva et al., 2003). Accordingly, 10 µl of fungus garden extract was added to 40µl of water, and 50 µl of 1% (w/v) (= 500 µg of substrate) CM cellulose solution and incubated at room temperature for 60 min. The hydrolysis of cellulose solution were terminated by adding 50 µl of 96 mM DNS (dinitrosalicylic acid) solution and incubating at 99° C for 15 min. At high temperature, the DNS dye changes color depending on the concentration of reducing sugars (the darker the color, the higher concentration of reducing sugars). Control samples were treated by adding the DNS and the enzyme extract before immediate incubation at 99° C. After incubation, 50 µl of each sample was added to 150 µl of water and then read in a spectrophotometer at 540 nm. Amounts hydrolyzed were interpolated using a standard curve for glucose.



Cellulase activity was significantly higher in both *Trachymyrmex* species than in *Atta texana*. Error bars correspond to ±1 SD.

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