



# Effects of substrate, ant and fungal species on plant fiber degradation in a fungus-gardening ant symbiosis



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## ABSTRACT

Fungus-gardening or attine ants have outsourced most of their digestive function to a symbiotic fungus. The ants feed their fungus – essentially an external digestive organ – a variety of substrates of botanical origin, including fresh and dried flowers, leaves and insect frass (processed leaves). Although plant tissues are rich in fibers (lignocelluloses, hemicelluloses, pectins and starches) and the symbiotic fungus possesses the genetic and enzymatic machinery to metabolize these compounds, the highly derived attines, the leaf-cutters (*Atta* and *Acromyrmex*), are known to produce fiber-rich waste. While leaf-cutting ants are important consumers of primary plant tissue, there have been fewer studies on physiological activity of fungi grown by closely related ant species in the genus *Trachymyrmex*, which generally grow related species of fungi, have smaller colonies and consume a wider variety of fungal substrates in addition to fresh leaves and flowers. In this study, we measured the cellulase activity of the fungus-gardening ants *Atta texana*, *Trachymyrmex arizonensis* and *T. septentrionalis*. We then quantified fiber consumption of the fungus-gardening ants *Trachymyrmex septentrionalis* and *Trachymyrmex arizonensis* by comparing the amounts and percentages present in their food and in fungus garden refuse during a controlled feeding experiment over the span of several months. Finally, we compared waste composition of *T. arizonensis* colonies growing different fungal strains, because this species is known to cultivate multiple strains of *Leucoagaricus* in its native range. The leaf-cutting ant *A. texana* was found to have lower cellulolytic activity than *T. arizonensis* or *T. septentrionalis*. Total lignocellulose and hemicellulose amounts were significantly lower in refuse piles than in the substrates fed to the *Trachymyrmex* colonies, thus these fibers were consumed by the fungal symbionts of these ant species. Although lignocellulose utilization was similar in two distinct fungal species grown by *T. arizonensis* colonies, hemicellulose utilization was higher in *T. arizonensis* colonies growing a derived leaf-cutting ant fungal symbiont than when growing a native type of symbiont. The results of this study demonstrate that fiber digestion in fungus-gardening ants is an outcome of ant-fungal interaction.

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## 1. Introduction

The success and dominance of insects in nearly all terrestrial ecosystems is largely attributed to the formation of symbioses with microorganisms (Caldera et al., 2009; Davidson et al., 2003; Douglas, 2010; Feldhaar and Gross, 2009; Janson et al., 2008; Klepzig et al., 2009). Some of the most specialized insect-microorganism symbioses are the so-called fungus-gardening

insects. The fungus-gardening or attine ants have become one of the major model systems of symbiotic evolution due to more than two decades of research devoted to elucidating co-evolutionary phylogenetic relationships (Mueller et al., 1998; Schultz et al., 2015), digestive physiology (De Fine Licht et al., 2013; de Fine Licht et al., 2010; Schiott et al., 2010), and disease biology (Currie et al., 1999, 2006; Fernandez-Marin, 2013; Hughes et al., 2008; Mueller, 2012). While many of the basal attine lineages grow species of fungi similar to free-living forms, the so-called higher attini cultivate fungi known only in association with the ants. The higher attini are composed of two major lineages: the leaf-cutting ants (*Atta* and *Acromyrmex*) and a group containing two additional genera (*Trachymyrmex* and *Sericomyrmex*). Leaf-cutting ants generally cultivate a single species of fungi called *Attamyces bromatificus*

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Kreisel in its anamorph (or asexual) form or *Leucoagaricus gongylophorus* (Möller) Singer in its teleomorph (sexual) morph (Mueller et al., 2010; Schultz et al., 2015). *Trachymyrmex* species typically cultivate several closely related species that are taxonomically unresolved but form a sister clade to *L. gongylophorus* (De Fine Licht and Boomsma, 2014; Mikheyev et al., 2008; Mueller et al., 1998; Nygaard et al., 2016; Schultz et al., 2015). While both groups of ants feed their gardens a mixture of fresh vegetation (leaves and flowers), dead plant tissue and herbivorous insect frass (de Fine Licht and Boomsma, 2010; Leal and Oliveira, 2000; Seal and Tschinkel, 2007a,b), the leaf-cutting fungus is thought to be more specialized toward the processing of leaf tissue than other higher attine fungi (De Fine Licht et al., 2013, 2014; Nygaard et al., 2016).

The metabolizing of plant fibers is a complex process that involves fungal and ant activity. While the ants possess a few glands that produce digestive enzymes, the vast majority of digestive activity in this symbiosis is conducted by the fungus, which produces exoenzymes that the ants ingest and pass through the insect gut unmodified (Aylward et al., 2015; D'Etterre et al., 2002; De Fine Licht et al., 2013; De Fine Licht and Boomsma, 2014; de Fine Licht et al., 2010; Erthal et al., 2004, 2009; Martin, 1987; Richard et al., 2005). Workers masticate the plant substrate before adding it to the top most layer of the fungal garden, which is then fertilized with fecal droplets that contain fungal digestive enzymes, before finally placing fungal mycelia that begins the fiber degradation process (de Fine Licht and Boomsma, 2010; Grell et al., 2013; Martin, 1987; Semenova et al., 2011). Ant fecal fluid is high in enzymes that have the ability to degrade starches, pectins and hemicelluloses found in plant cell walls (Martin, 1987; Rønshede et al., 2004; Schiøtt et al., 2008; Schiott et al., 2010; Silva et al., 2006). The purpose of all these enzymes, whether they are of ant or fungal origin, is to disassemble plant tissue 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 microfibrils that are entrenched within a matrix of pectin and hemicellulose (Onoda et al., 2004; Schiøtt et al., 2008). The most efficient way to break down cell walls is to first degrade pectin, which leads to biochemical changes that weaken the hemicelluloses (de Fine Licht et al., 2010; Esquerré-Tugayé et al., 2000). The attine fungus also has active laccase enzymes which are responsible for degrading lignin and other polyphenolics involved in plant defenses (De Fine Licht et al., 2013; Rønshede et al., 2004; Thurston, 1994). Therefore, it appears fungus-gardening attine ants have the proper machinery to metabolize plant cell wall compounds.

One of the surprising findings confirmed by numerous studies in recent years is that, in contrast to many other plant fiber-feeding insects (e.g. termites), the leaf-cutting fungi do not appear to be significant consumers of cellulose, the most abundant carbohydrate in leaves, even though genomic surveys have confirmed the presence of genes that code cellulase enzymes (Aylward et al., 2013, 2015; Grell et al., 2013; Nygaard et al., 2016; Suen et al., 2010) and microscopic examinations that indicate the consumption of cellulose during leaf digestion (Nagamoto et al., 2011). However, studies that have investigated the enzymatic activity of fungus garden extracts have found variable or weak evidence of cellulase activity (D'Etterre et al., 2002; De Fine Licht and Boomsma, 2014; de Fine Licht et al., 2010; Seal et al., 2014). Likewise, comparisons of lignocellulose content of harvested leaves and garden refuse did not provide evidence of significant cellulase activity (Abril and Bucher, 2002; Bucher et al., 2004). One possible explanation of little or no cellulase activity in leaf-cutting ants is that cellulase enzymes engage toward the end of the leaf harvesting process and are restricted to the lower layers of fungus gardens (Grell et al., 2013; Schiøtt et al., 2008; Schiott et al., 2010). A recent

genomic analysis illustrated that leaf-cutting fungi experienced an evolutionary reduction in lignase genes compared to lower attine or free-living fungi (Nygaard et al., 2016). Moreover, ants may interrupt fiber digestion by removing and discarding incompletely harvested leaf fragments so that overall cellulase activity is reduced and refuse depots end up being fiber-rich (de Fine Licht and Boomsma, 2010; De Fine Licht et al., 2013, 2014; de Fine Licht et al., 2010; Grell et al., 2013; Schiøtt et al., 2008; Schiott et al., 2010).

While most of our understanding of plant carbohydrate metabolism in fungus-gardening ants has focused on the leaf-cutting ants (*Atta* and *Acromyrmex*), comparatively little work has addressed the consumption of plant fibers in closely related species that may or may not illustrate similar patterns in lignocellulose digestion. For example, *Trachymyrmex* ants 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 fibers (de Fine Licht and Boomsma, 2010; de Fine Licht et al., 2010; Leal and Oliveira, 2000; Wetterer, 1994; Wilson, 1980). *Trachymyrmex* tend to have much smaller colonies than those of leaf-cutting ants, which makes them more amenable to manipulative experiments (Seal et al., 2012; Seal et al., 2014; Seal and Mueller, 2014; Seal and Tschinkel, 2007a, 2008), which can be used to understand the mechanisms that influence plant fiber digestion in these less complex symbioses. For instance, one recent experiment found significantly higher cellulase activity in *Attamyces* fungus when grown by *Trachymyrmex arizonensis* than when grown by *Atta texana*, which suggested that cellulase activity was generally influenced by ant species (Seal et al., 2014).

We report in this study significant lignocellulose digestion in *Trachymyrmex* ants using two methods. The first study examined the cellulase activity of ant fungus-garden enzyme extract from fungi grown by *Atta texana*, *Trachymyrmex arizonensis*, and *T. septentrionalis*. The second method examined the fiber content of fungus garden substrate (the food the ants collect and feed their fungus) and the fiber content of fungus garden refuse (undigested substrate) produced by *T. septentrionalis* and *T. arizonensis*. We also examine fiber consumption in *T. arizonensis* colonies that were either growing a leafcutting *Attamyces* fungus or a native 'Trachymyces'-type fungus. Both methods suggest significant cellulase activities in *Trachymyrmex* ants.

## 2. Materials and methods

### 2.1. Cellulase activity assays

#### 2.1.1. Colony collection

The *Trachymyrmex septentrionalis* colonies used in the fungus garden activity assays were collected in Texas (n = 6) and Florida (n = 2). The colonies in Texas were collected on March 21, 2011 at the University of Texas' Stengl 'Lost Pines' Biological Station (30° 5' 13.1"N 97° 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 (30° 22'46.3" N, 84° 20'6.5"W). At the time of cellulase assay, all *T. septentrionalis* colonies had been in the laboratory for one year.

*Atta texana* and *Trachymyrmex arizonensis* colonies were obtained from lab-reared newly mated queens collected after mating flights. The six *A. texana* colonies used in this study were similarly reared from newly mated queens collected after mating flights near Hornsby Bend, Texas (30° 12' 37.3"N 97° 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). 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). Maintenance and rearing of these newly mated queens to adulthood is described in detail by Seal et al. (2014). All *T. arizonensis* colonies were fed oak catkins (male flowers) *ad libitum* throughout the duration of the experiment. Colonies were fed the same type of catkins to avoid confounding the results with diet (Kooij et al., 2011).

While the *Atta texana* colonies in this study were growing native *Attamyces* fungus gardens, most of the *Trachymyrmex* colonies were growing native fungi that form a sister clade to *Attamyces* fungi (Mueller et al., 1998; Nygaard et al., 2016; Schultz et al., 2015). We generally refer to the fungi cultivated by *Trachymyrmex* ants using the provisional name 'Trachymyces' because none of these have been formally described (Seal and Mueller, 2014; Seal et al., 2014). *T. arizonensis* colonies were growing one of two types of fungus, which reflects the patterns found in nature; colonies either grow *Attamyces* or a 'Trachymyces' fungus (Seal et al., 2014). 'Trachymyces' fungus used in this study was collected from a mature *T. arizonensis* colony near Portal, AZ. *Trachymyrmex arizonensis* colonies growing *Attamyces* were supplied with fungus collected from an *Acromyrmex versicolor* colony reared from a newly mated queen collected near Tucson, AZ, USA, in 2009 (32°18.971'N, 110°53.562'W, 858 m elevation). All *T. septentrionalis* colonies in this study grew native 'Trachymyces' gardens. Sample sizes of *T. arizonensis* ant-fungal combinations are stated in subsequent sections.

### 2.1.2. Fungal enzymatic activity (cellulase) assays

Cellulase activity assays were conducted in 2012 on six *Trachymyrmex arizonensis*, eight *Trachymyrmex septentrionalis* and six *Atta texana* colonies. Cellulase activities of *T. arizonensis* and *A. texana* were published previously (Seal et al., 2014). Samples of fungus garden material of all three study species were selected from the uppermost 1.5 cm of each fungus garden because gardens are thought to exhibit spatial variation in enzymatic activity (Grell et al., 2013; Moller et al., 2011; Suen et al., 2010). Each of these three species in this analysis were growing a native fungal symbiont (*T. arizonensis* and *T. septentrionalis* colonies were growing a native 'Trachymyces' fungus and *A. texana* grew a native *Attamyces* fungus). Although *T. arizonensis* is known to grow both types of fungi, cellulase activity is not influenced by symbiont type (Seal et al., 2014). 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). Enzymes were extracted from the fungus gardens by grinding ca. 120 mg of fungus garden material in a centrifuge 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 14,000 rpm. The supernatant was then transferred to a fresh tube, which was then used for cellulase 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; Seal et al., 2014). Accordingly, 10 µl of fungus garden extract was added to 40 µl 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 incubation 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. All chemicals were obtained from Sigma Aldrich.

## 2.2. Fiber analysis

### 2.2.1. Colony collection

All colonies of *T. septentrionalis* used in the fiber analysis were collected in mid-April and early May 2014 on the University of Texas at Tyler campus in Tyler, Texas (32° 18' 46.8"N, 95° 15' 10.8"W) using colony collection methods described above. *T. septentrionalis* colonies collected at this time of year typically have reduced fungus gardens (<1 cm<sup>3</sup>) (Seal and Tschinkel, 2007a,b). All of the *T. arizonensis* colonies used in these analyses were reared from the newly mated queens collected after mating flights earlier in 2010 or 2011 (see 2.1.1) in southeastern Arizona and thus were in the lab at least 3 years at the time of the study. Four of the *T. arizonensis* colonies were growing a leaf-cutting ant fungus (*Attamyces*) whereas the remaining six *T. arizonensis* and all *T. septentrionalis* colonies were growing a native 'Trachymyces' fungus (Seal et al., 2014). Regardless of the source of the colonies, the study was completed during one season (April–August).

### 2.2.2. Colony maintenance

All experimental colonies of both *T. septentrionalis* and *T. arizonensis* were housed in Fluon® coated trays (dimensions 43 × 36 × 7 cm) to avoid escapes. The ants grew their garden in a cylindrical cavity measuring 175 cm<sup>3</sup> 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 at room temperature (Seal and Mueller, 2014; Seal and Tschinkel, 2007a,b). To equalize differences in microclimates, colonies were rotated in their shelving unit every two weeks.

### 2.2.3. Experimental groups

The two species, *Trachymyrmex septentrionalis* and *Trachymyrmex arizonensis*, were divided into three groups. Group 1 contained 11 *T. septentrionalis* colonies fed Texas Red Oak (*Quercus buckleyi*) catkins, Group 2 contained 12 *T. septentrionalis* colonies fed eastern tent caterpillar (*Malacosoma americanum* (Lepidoptera: Lasiocampidae)) frass and Group 3 contained 10 *T. arizonensis* colonies fed only oak catkins (*T. arizonensis* does not readily accept tent caterpillar frass).

### 2.2.4. Feeding

All colonies of *Trachymyrmex septentrionalis* were fed *ad libitum* after colony establishment in the laboratory. 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. Food that went uncollected by the workers was removed and weighed weekly from all *T. septentrionalis* colonies.

To account for the changes between wet and dry masses of the colonies' diets, a wet sample was collected and weighed from each new container of food. These samples were left to dry for 24 h at room temperature before a dry mass was taken. All wet masses were recorded and converted to dry masses to obtain actual

amounts of lignocellulose and hemicellulose digested by the symbiosis.

Colonies of *T. arizonensis* were included in this study to compare with fiber consumption of *T. septentrionalis*. However, because the *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 as in *T. septentrionalis* (Seal and Tschinkel, 2006), we could not be certain of the total amounts of fiber consumption without removing all fungus garden material from each colony. As a result of avoiding a potentially destructive process that would have stressed the colonies, amounts of oak catkins that were accepted by the colonies were not quantified. Therefore, we were only able to compare percentages of digested lignocellulose and hemicellulose rather than whole amounts between *T. arizonensis* and *T. septentrionalis*. Colonies of *T. arizonensis* were fed oak catkins *ad libitum* 3 times per week from May until mid-August 2014 when refuse samples were collected. *T. arizonensis* colonies had their arenas cleaned and removed of old refuse in late April 2014. While these colonies had been provided with citrus peel in the winter (December–January) of 2013–2014, colonies had been kept on a less rigorous but strict catkin diet from January to May (fed 1–3 times per week). Moreover, orange peel refuse is somewhat lighter in color than catkin or frass derived refuse (JNS, unpublished obs). As a result, we are confident that the refuse sampled in these colonies was composed of extracted catkin tissue.

#### 2.2.5. Cleaning

Refuse samples were collected from all experimental colonies beginning in June 2014. All food digested by the fungus garden and removed from the nest by workers is considered refuse. 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, identified by its color and texture, was separated from uncollected food by hand and filtered through a 2 mm sieve. Uncollected food was weighed and refuse samples were stored in labeled plastic vials.

#### 2.2.6. Fiber analysis methods

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 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. The detergent dissolves proteins as well as removes nitrogenous components and pectins at a boiling temperature, while addition of a heat-stable amylase removes starches. 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<sub>2</sub>SO<sub>4</sub>) 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.

Collected colony refuse samples were oven dried for 24 h and two 0.50 g samples from each colony were analyzed against samples of dried oak catkins or frass. To assess the amount of lignocellulose and hemicellulose accepted into the colony, the dry weights were multiplied by the average percent lignocellulose and hemicellulose present in the food source. In other words, the Van Soest method when coupled with a controlled feeding experiment provides an absolute amount of lignocellulose and hemicellulose consumed by the symbiosis. Analyses of variance and *t*-tests were

employed using Statistica version 6.1. Data were either log<sub>10</sub> or square-root transformed to meet parametric assumptions.

### 3. Results

#### 3.1. Cellulase activity

Fungus gardens grown by either *Trachymyrmex* species exhibited cellulase activities that were approximately three times higher than those from *Atta texana* fungus gardens (Fig. 1).

#### 3.2. Fiber consumption of *T. septentrionalis*

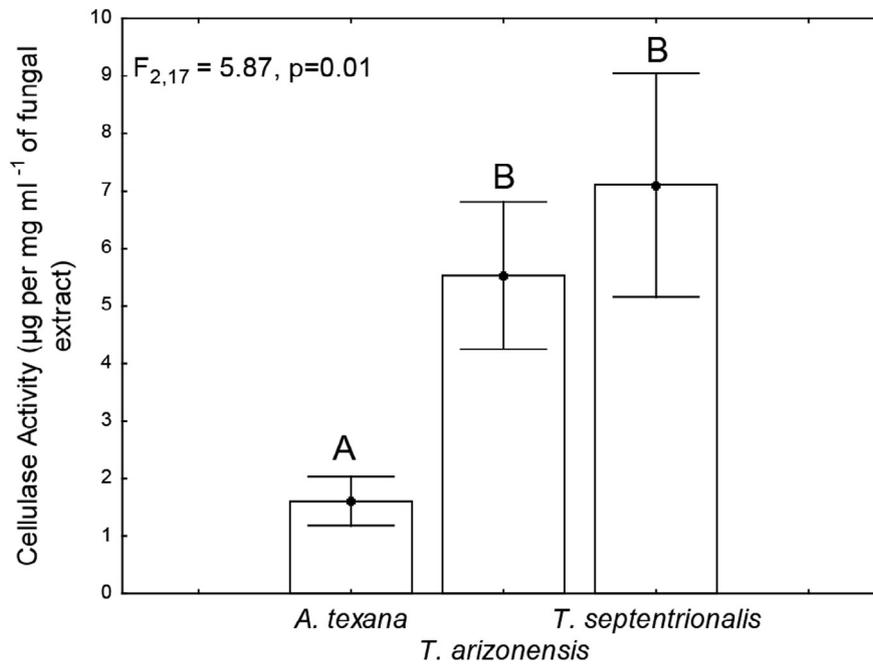
Lignocellulose and hemicellulose contents of catkins and frass were clearly different in terms of their carbohydrate composition. Frass contained significantly more lignocellulose than the oak catkins, whereas catkins were richer in terms of hemicellulose content (Table 1).

Despite the variation among the carbohydrate composition of catkins and frass, similar amounts of substrates were harvested by *Trachymyrmex septentrionalis* colonies (5.94 ± 2.73 g catkins vs. 6.81 ± 3.78 g frass harvested (mean ± 1SD) (*t*<sub>25</sub> = 0.695, *p* = 0.494). However, the amount of fibers harvested by the symbiosis or dumped in the refuse piles depended on substrate type. Colonies that were fed oak catkins accepted a mean of 1.091 ± 0.426 g of lignocellulose from all catkins that were brought into the colony, whereas an average of 0.571 ± 0.237 g of lignocellulose was found in the refuse piles (dependent *t*-test, *t*<sub>12</sub> = 7.98, *P* = 0.00004) (Fig. 2A). In other words, the ant-fungal symbiosis extracted about 48% of the lignocellulose present in the catkins. Similarly, in colonies that were fed tent caterpillar frass, 1.881 ± 0.913 g of lignocellulose was accepted into the garden whereas 1.038 ± 0.532 g of lignocellulose was found in the colonies' refuse piles (dependent *t*-test, *t*<sub>10</sub> = 5.481, *P* = 0.00027) (Fig. 2B), which amounted to an extraction of approximately 46% of the lignocellulose present in the tent caterpillar frass. Thus, the symbiosis extracted a similar percentage of lignocellulose from both diets (47.9 ± 0.103% vs 45.7 ± 0.142%, *t*<sub>22</sub> = 0.425, *p* = 0.675).

Catkin-fed colonies collected an average of 0.719 ± 0.281 g of hemicellulose, of which 0.398 ± 0.188 g ended up in the refuse pile (dependent *t*-test, *t*<sub>12</sub> = 5.82 *P* = 0.00019) (Fig. 2C). Therefore, around 46% of the hemicellulose found in catkins was extracted by the symbiosis. Interestingly, an average of 0.615 ± 0.299 g of hemicellulose from frass was collected by the ants, but only about 0.217 ± 0.113 g was found in the refuse pile (dependent *t*-test, *t*<sub>10</sub> = 5.82, *P* = 0.00017) (Fig. 2D), which equated into a removal of approximately 65% of the hemicellulose found in frass. Thus the symbiosis significantly extracted a larger percentage of hemicellulose from the hemicellulose-poorer diet (45.8 ± 0.151% vs. 64.6 ± 0.127%, *t*<sub>22</sub> = 3.259, *p* = 0.004).

#### 3.3. Interspecific comparisons of fiber consumption

The percent of lignocellulose present in catkin refuse did not differ among *T. septentrionalis* or *T. arizonensis* growing ‘Trachymyrmex’ or *Attamyces* (average percent lignocellulose content in the refuse piles of *T. septentrionalis* fed catkins was 20.2 ± 2.85% and *T. arizonensis* growing Trachymyrmex was 20.29 ± 1.69% and 21.55 ± 2.25% for *Attamyces*, *F*<sub>2,20</sub> = 0.455, *p* = 0.64) (Fig. 3). However, each ant species showed significant variation in the percent hemicellulose of fungus garden refuse material. On average, the refuse piles of *T. septentrionalis* colonies contained a significantly higher percent hemicellulose (13.43 ± 1.58%) than ‘Trachymyrmex’ 10.44 ± 0.67% or *Attamyces* (7.6 ± 0.36%) gardens grown by *T. arizonensis* (*F*<sub>2,20</sub> = 47.84, *p* < 0.0001) (Fig. 3). Thus, gardens



**Fig. 1.** Cellulase activity of *Atta texana*, *Trachymyrmex arizonensis*, and *Trachymyrmex septentrionalis*. Cellulase activity is given as µg reducing sugar per mg ml<sup>-1</sup> of fungus garden extract. Error bars correspond to ±1 SE. Data were analyzed with one-way ANOVA. Data from *A. texana* and *T. arizonensis* were previously published in Seal et al. (2014). Sample size: *A. texana* N = 6, *T. arizonensis* N = 6, *T. septentrionalis* N = 8. The two *Trachymyrmex* species were growing native 'Trachymyces' fungus whereas the *A. texana* was growing a native *Attamyces* symbiont. Cellulase activity does not differ between *T. arizonensis* colonies growing "Trachymyces" and "Attamyces" fungus (Seal et al., 2014 Fig. 3).

**Table 1**

Percent dry weights of fiber lignocellulose and hemicellulose contents of each substrate type and tests of significance. Substrate samples: N = 12 (frass), N = 11 (catkins).

	Catkins (mean ± 1SD)	Frass (mean ± 1SD)	T-test
Lignocellulose	16.88 ± 2.98	25.77 ± 3.42	t <sub>21</sub> = 4.159, p = 0.0004
Hemicellulose	11.14 ± 1.83	8.43 ± 1.27	t <sub>21</sub> = 6.613, p < 0.0001

tended by *T. arizonensis*, especially those growing *Attamyces*, appeared to have consumed more hemicellulose from the catkin diet than gardens grown by *T. septentrionalis*.

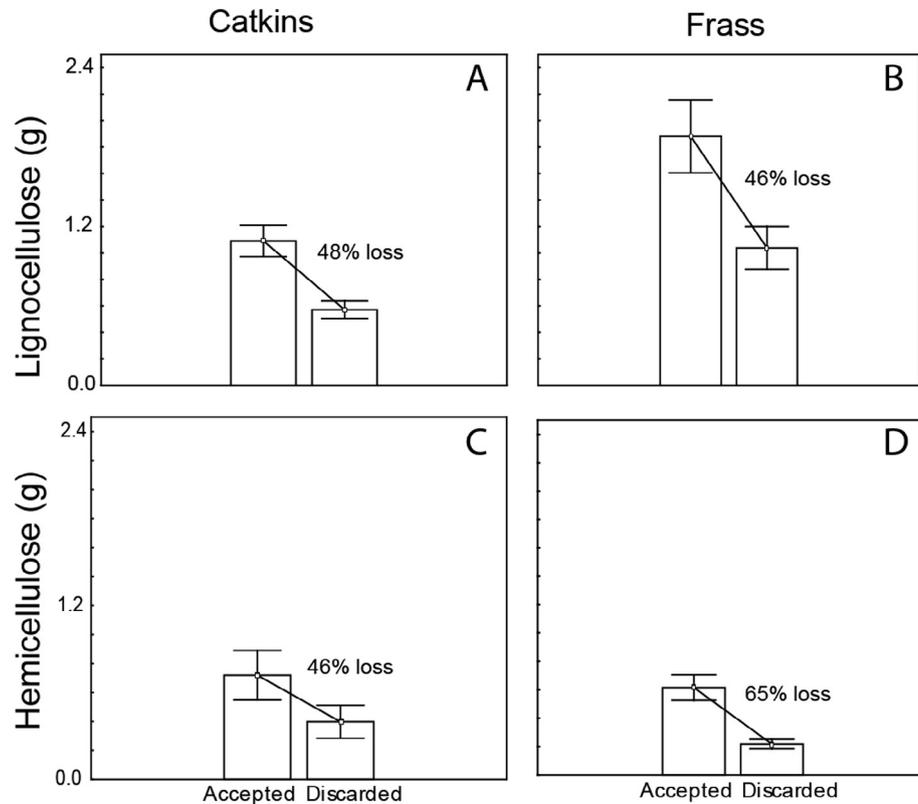
#### 4. Discussion

Our results suggest that the two *Trachymyrmex* symbioses investigated here have the ability to actively digest most plant carbohydrates, including the plant cell wall components cellulose and lignin. Our findings stand in contrast with studies that concluded little or no lignocellulose digestion after examining fiber contents of refuse dumps and leaves harvested by leaf-cutting ants (Abril and Bucher, 2002; Bucher et al., 2004; Moller et al., 2011). This study highlights that fiber digestion by fungus gardening ants is likely a complex process and probably varies among ant and fungal species. This study is to our knowledge, the first study to comprehensively examine fiber digestion in a non-leaf-cutting higher attine ant (de Fine Licht and Boomsma, 2010; De Fine Licht and Boomsma, 2014; de Fine Licht et al., 2010). While all higher attine ants grow closely related fungi (Mueller et al., 1998; Schultz et al., 2015), the substrates that the ants collect and subsequently feed their symbionts probably differ. Leaf-cutting ants (*Atta* and *Acromyrmex*) forage on leaves and other fresh plant pieces, while other higher attines forage for leaf litter and insect frass (Hölldobler and

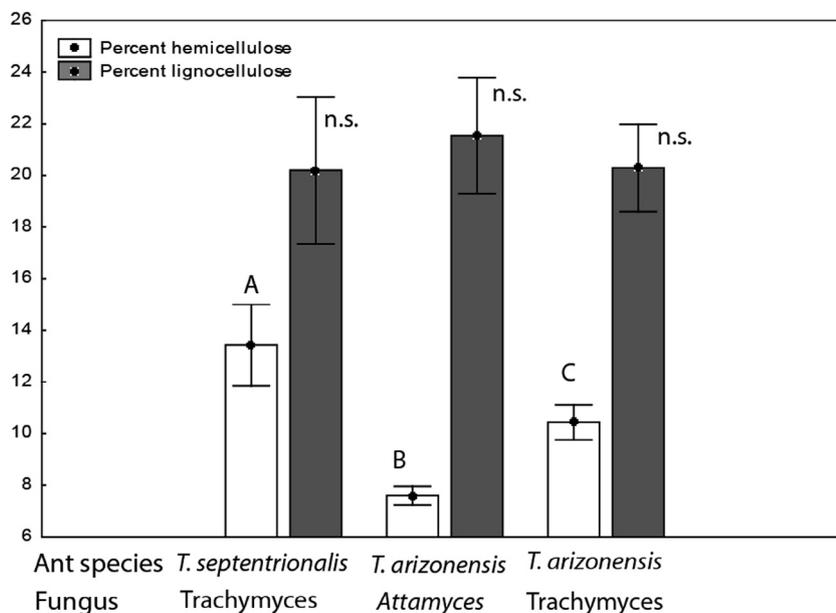
Wilson, 2011; Leal and Oliveira, 2000; Seal and Tschinkel, 2007b; Wetterer, 1994). The differences in these compounds may lead to different behavioral and digestion strategies because easily accessible and nutrient-rich compounds such as starch may be absent from dead leaves or previously consumed plant parts (frass), which in turn are likely richer in terms of fiber content (de Fine Licht and Boomsma, 2010; de Fine Licht et al., 2010; Schroeder, 1986). These results also suggest that despite a reduction in lignocellulase genes in the attini generally (Nygaard et al., 2016), the symbiosis still possesses flexibility in the digestion of plant carbohydrates.

One of the more intriguing discoveries in this study was the variability in digestion between *T. septentrionalis* colonies fed the two substrate types (Fig. 2). Both of these colony groups collected a similar amount of dry substrate biomass, yet the fiber composition varied significantly between catkins and frass. Moreover, colonies fed frass extracted more hemicellulose than those fed catkins, even though frass was relatively hemicellulose-poor compared to catkins (Table 1). This finding suggests that the ants are possibly collecting substrate independent of nutritional composition but subsequent behavioral modifications (e.g., retention time in the garden) or fungal properties (e.g., elevated hemicellulase relative to lignocellulase activity) are mechanisms that can maximize the complete extraction of hemicellulose from poor substrates. This indicates that substrate collection behavior may be a process somewhat independent and indirectly modified by fungal activity (Seal and Tschinkel, 2007a,b).

While few studies have examined in detail the role of *Trachymyrmex* ants in directing the digestion of fungus gardens (Seal et al., 2014), studies that have used *Atta* and *Acromyrmex* leaf-cutting ants as models have illustrated a strong role for ant behavior in the fiber digestion process (Moller et al., 2011; Schiøtt et al., 2008; Schiøtt et al., 2010). Ants place newly harvested material near the top of a fungus garden. Over time, deposited substrates are displaced downward as materials are deposited from above. During the downward movement, increasingly larger macronutrients are metabolized, so that oligosaccharides, starches and pectins



**Fig. 2.** Mean lignocellulose and hemicellulose amounts 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 of lignocellulose and hemicellulose. Dependent *t*-tests were used. A. ( $P < 0.00004$ ), B. ( $P < 0.00027$ ), C. ( $P < 0.00019$ ), D. ( $P < 0.00017$ ). Error bars are  $\pm 1$  standard error All y-axes are on the scale. Sample size: Colonies fed catkins  $N = 11$ , frass,  $N = 12$ .



**Fig. 3.** Mean ( $\pm$ 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. Refuse from *T. septentrionalis* colonies had the highest percent hemicellulose than *T. arizonensis* colonies growing either species of fungus, whereas the lowest percent hemicellulose was found in *T. arizonensis* colonies growing the novel *Attamyces* fungus ( $F_{2,20} = 47.8$ ,  $<0.0001$ ). Different letter correspond to significant pairwise comparisons (Tukey's post hoc test). Overall, percent lignocellulose was not significantly different among the three ant-fungal species combinations ( $F_{2,20} = 0.455$ ,  $p = 0.64$ ). n.s. = not significant (Tukey's pairwise comparisons). Sample size: *T. arizonensis*  $\times$  *Attamyces*  $N = 4$ , *T. arizonensis*  $\times$  *Trachymyces*  $N = 6$ , *T. septentrionalis*  $N = 11$ .

are among the first targets of digestion while the more recalcitrant compounds such as lignins and celluloses are targeted much later in the lower reaches (De Fine Licht et al., 2013; Grell et al., 2013;

Huang et al., 2014; Moller et al., 2011; Schiött et al., 2008; Schiött et al., 2010). This general pattern of leaf tissue degradation appears to be mirrored in the presence and activity of bacteria and

yeasts found in the garden with taxa specialized toward the digestion of smaller compounds found higher in the garden those that metabolize lignocelluloses (Aylward et al., 2012a,b; Huang et al., 2014; Mendes et al., 2012; Suen et al., 2010). The digestion and extraction of nutrients from plant tissue for ant nutrition ceases when substrates are removed and deposited in refuse piles by the ants (Scott et al., 2010; Torres et al., 1999).

The results reported in the present study indicate that ant species influence the digestive profiles of the fungus gardens, even though the causes remain unknown (De Fine Licht and Boomsma, 2014; Kooij et al., 2014; Seal et al., 2014). While enzymatic activities of fungus-gardens can be altered by the substrates ants collect and feed their gardens (Kooij et al., 2011), this does not apply in the present study because comparisons between *T. arizonensis* and *T. septentrionalis* involved colonies fed the same diets (Fig. 3). The higher hemicellulose digestion in *T. arizonensis* colonies growing *Attamyces* reported here suggests leaf-cutting ant fungi have a higher activity toward complex carbohydrates than non-leafcutting ant fungi (De Fine Licht and Boomsma, 2014; De Fine Licht et al., 2014; de Fine Licht et al., 2010; Moller et al., 2011), but interestingly, hemicellulase activities appeared higher in *T. arizonensis* generally, which suggests that ant species manage the digestion of their fungal symbionts (Seal et al., 2014). Even though the ants in this study were growing 'Trachymyces' fungi that belonged to the same phylotype ('B' of Mikheyev et al., 2008), they were growing fungi collected from their respective geographic ranges (Arizona or Texas), thus making it possible that variation in fungal physiology could explain these patterns. One possible explanation of variation in hemicellulase activity is that *T. arizonensis* leaves its substrates on the fungus garden longer than *T. septentrionalis*, which would allow for a longer extraction time. Assuming that metabolic rates are dependent on aspects of ambient conditions (e.g., temperature), the earlier the ants remove substrates, the least digested the ensuing refuse, which could account for significant quantities of undigested lignocelluloses in garden refuse (Abril and Bucher, 2002; Bucher et al., 2004; Moller et al., 2011). However, if longer extraction times were involved in hemicellulase digestion, lignocellulase activity was not affected (Fig. 3). Furthermore, it is not known at which point or why ants remove substrates from fungus gardens prior to complete digestion. Possibly, ant gardening behavior is stimulated by the release of sugars from compounds easy to digest such as starches and pectins over lignocelluloses which require more time and energetic input to be broken down into simple sugars, so that gardening workers remove substrates that may no longer be producing sugars that serve as important motivating signals (de Fine Licht et al., 2010; Roces, 1993). To account for variation in fiber digestion among ant and fungal species, additional studies will need to be performed that incorporate gene or protein/enzyme expression and activity along with behavioral observations in order to assess the mechanisms involved in the fiber digestion of *Trachymyrmex* ants and their symbiotic fungi.

Finally, another limitation of this study is that it is not known which of the microbial actors were involved in the digestion of plant fibers, because in addition to the symbiotic fungus garden, bacteria and yeasts associated with the fungus garden are known to have crucial roles in polysaccharide digestion. The methods used here do not discriminate among metabolic activity by the fungus or associated microorganisms. 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, carboxymethyl cellulose was degraded by 43% of yeasts from *A. texana* colonies and 89% of yeasts from *Acromyrmex* colonies (Mendes et al., 2012). Other yeasts of the 82 yeast taxa screened, exhibited pectinase, amylase and protease activity and (Mendes et al., 2012). Microbiome analyses of leaf-cutting ants have shown the presence

of Proteobacteria, which are capable of metabolizing cellulose and hemicellulose (Scott et al., 2010; Suen et al., 2010). Future work may wish to identify the relative contributions of the ant fungus and associated microbes toward plant fiber digestion.

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