Ant–fungal species combinations engineer physiological activity of fungus gardens

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ABSTRACT
Fungus-gardening insects are among the most complex organisms because of their extensive co-evolutionary histories with obligate fungal symbionts and other microbes. Some fungus-gardening insect lineages share fungal symbionts with other members of their lineage and thus exhibit diffuse co-evolutionary relationships, while others exhibit little or no symbiont sharing, resulting in host–fungus fidelity. The mechanisms that maintain this symbiont fidelity are currently unknown. Prior work suggested that derived leaf-cutting ants in the genus Atta interact synergistically with leaf-cutter fungi (Attamyces) by exhibiting higher fungal growth rates and enzymatic activities than when growing a fungus from the sister-clade to Attamyces (so-called Trachymyces), grown primarily by the non-leaf cutting Trachymyrmex ants that form, correspondingly, the sister-clade to leaf-cutting ants. To elucidate the enzymatic bases of host–fungus specialization in leaf-cutting ants, we conducted a reciprocal fungus-switch experiment between the ant Atta texana and the ant Trachymyrmex arizonensis and report measured enzymatic activities of switched and sham-switched fungus gardens to digest starch, pectin, xylan, cellulose and casein. Gardens exhibited higher amylase and pectinase activities when A. texana ants cultivated Attamyces compared with Trachymyces fungi, consistent with enzymatic specialization. In contrast, gardens showed comparable amylase and pectinase activities when T. arizonensis cultivated either fungal species. Although gardens of leaf-cutting ants are not known to be significant metabolizers of cellulose, T. arizonensis were able to maintain gardens with significant cellulase activity when growing either fungal species. In contrast to carbohydrate metabolism, protease activity was significantly higher in Attamyces than in Trachymyces, regardless of the ant host. Activity of some enzymes employed by this symbiosis therefore arises from complex interactions between the ant host and the fungal symbiont.

KEY WORDS: Attini, Cellulose, Co-evolution, Enzyme activity, Pectin, Starch, Symbiosis, Xylan

INTRODUCTION
Co-operation among unrelated organisms (mutualistic symbioses) has led to many key evolutionary innovations with far-reaching ecological consequences. One of the larger mysteries is understanding how such complex organisms maintain homeostasis and are able to function as distinct units (Douglas, 2010; Kültz et al., 2013; McFall-Ngai et al., 2013). Of the numerous examples of such complexity, the fungus-gardening insects have evolved obligate macro-symbioses with specific clades of fungi, and use fungal symbionts essentially as an external digestive organ that allows the insect to thrive on otherwise non-digestible substrates, such as structural carbohydrates of plants (e.g. cellulose) (Aanen et al., 2002; Aylward et al., 2012a; Aylward et al., 2012b; Bacci et al., 1995; Farrell et al., 2001; De Fine Licht and Biedermann, 2012; Martin, 1987a; Mueller et al., 2005). One of the most striking attributes of these symbioses is the degree of physiological integration: the insect host functions as a distributor of fungal enzymes, which digest plant fibers external to the insect’s body (Aanen and Eggleton, 2005; Aylward et al., 2012b; De Fine Licht and Biedermann, 2012; De Fine Licht et al., 2013; Martin, 1987b; Schiøtt et al., 2010).

The fungus-gardening (attine) ants exhibit several macroevolutionary trends with regard to their farmed symbionts. Although vertical transmission of symbionts and generalized fidelity between clades of ants and clades of fungi is the norm, horizontal transmission is quite extensive, especially among the phylogenetically early branching lineages (Green et al., 2002; Kellner et al., 2013; Mehdibadi et al., 2012; Mikheyev et al., 2010; Mueller et al., 2010; Mueller et al., 1998; Schultz and Brady, 2008). One of the more profound evolutionary and ecological transitions occurred in the derived lineages (the ‘higher Attin’), the most complex of which are found in the clade containing leaf-cutting ants (genera Atta and Acromyrmex). These ants exhibit large queens, pronounced variation in worker size and colony sizes of immense proportions (>1 million workers); as a result, fungus-growing ants may exert enormous ecological impacts (Hölldobler and Wilson, 2011; Meyer et al., 2011; Seal, 2009; Wilson, 1980; Wirth et al., 2003). Leaf-cutter ants generally cultivate a single species of fungus, called Attamyces bromatificus (hereafter, Attamyces) in its anamorph (vegetative, clonal) growth form and Leucocoprinus gongylophorus in its teleomorph (sexual) form (Mueller et al., 2010; Mueller et al., 2011b; Mueller et al., 1998). All leaf-cutting ants are thought to have descended from a common ancestor shared with several Trachymyrmex species in the Trachymyrmex ‘septentrioralis’ group, of which many inhabit North America (Rabeling et al., 2007; Schultz and Brady, 2008). Ants in the genus Trachymyrmex are characterized by relatively small colonies (hundreds of workers), modest or no worker polymorphism, and occasional population sizes that may make them ecologically relevant (Besher and Traniello, 1994; Leal and Oliveira, 1998; Seal and Tschinkel, 2006; Seal and Tschinkel, 2010; Torres et al., 1999). Trachymyrmex ants typically cultivate a more diverse assemblage of closely related Leucocoprinus lineages (Mikheyev et al., 2010; Mikheyev et al., 2008; Mueller et al., 1998; Schultz and Brady, 2008). None of these have been taxonomically resolved; hence we refer to them provisionally as ‘Trachymyces’.

The leaf-cutting ant fungi are thought to be specialized toward the rapid metabolism of starches, hemicelluloses and proteins found in...
leaves, whereas the non-leaf-cutting fungi have lower metabolic activities toward these substrates and may have a greater ability to digest cellulose (Bacci et al., 2013; D’Ettorre et al., 2002; Erthal et al., 2004; Erthal et al., 2009; De Fine Licht et al., 2013; De Fine Licht et al., 2010; Richard et al., 2005; Schiøtt et al., 2008; Schiøtt et al., 2010). These physiological differences seem to be linked with dietary preferences associated with the ants; leaf-cutter ants provide their garden with fresh leaves, whereas the non-leaf-cutters provide their fungus with various dried plant debris, flower parts and caterpillar excrement (Hölldobler and Wilson, 2011; Leal and Oliveira, 2000; Seal and Tschinkel, 2007b).

Although the two-higher-attine ant lineages generally exhibit fidelity to their fungal lineages (i.e. leaf-cutter ants tend to cultivate *Attamyces*; *Trachymyrmex* ants tend to cultivate *Trachymyces*), several observations are inconsistent with a strict 1:1 co-evolution. Colonies can be experimentally switched to non-native fungi, and surveys indicate that switches can occur naturally on ecological and evolutionary scales (Mehdiabadi et al., 2012; Mikheyev et al., 2010; Mueller et al., 2011b; Mueller et al., 1998; Seal and Tschinkel, 2007a; Stradling and Powell, 1986; Weber, 1956) (supplementary material Fig. S1). The only replicated experiments that addressed whether higher-attine fungal lineages were adapted to their ant hosts suggested that diseases constrain symbiont switching in two species of *Trachymyrmex* ants (Seal and Mueller, 2014; Seal and Tschinkel, 2007a). One preliminary study suggested that some Trachymyces cultivars can lower the performance of leaf-cutting ant colonies, but this study suffered from a low sample size (Sánchez-Peña, 2005). These findings prompted our study of the enzymatic properties of gardens from fungal-switched colonies of *Atta* and *Trachymyrmex* ants. We investigated the potential for constraints in symbiont switching by testing three hypotheses, described below.

The ‘host-independent hypothesis’ postulates that ant species’ identity is of lesser importance or not important in influencing fungal enzyme activity in gardens, and consequently predicts that *Attamyces* gardens will exhibit higher enzymatic activities regardless of the ant species growing them (and conversely, *Trachymyces* should always show lower enzyme activities regardless of the ant host growing them). Evidence for this hypothesis derives from an *in vitro* study (fungus grown on artificial media) (Stradling and Powell, 1986) and *in vivo* quantification of enzymatic activity of native ant–fungus associations (De Fine Licht et al., 2010) showing that *Attamyces* has indeed higher growth rates and enzymatic activities compared to non-leaf-cutting fungi. Additional support derives from non-replicated experiments where the addition of fresh *Attamyces* garden to a garden-deprived *Trachymyrmex urichi* colony resulted in increased ant colony growth (Stradling and Powell, 1986), as well as reduced colony and garden growth when *Atta* colonies were forced to cultivate *Trachymyces* gardens (Sánchez-Peña, 2005). A problem with this hypothesis is that by itself it does not explain why *Trachymyrmex* ants would persist in growing a physiologically inferior fungal symbiont (rather than switch to *Attamyces*) strains cultivated by sympatric leaf-cutter ants; it only explains why *Atta* ants may avoid the apparently ‘inferior’ *Trachymyces*. Because *Attamyces* appears to have swept by lateral transfer through leaf-cutter ant populations (Mikheyev et al., 2010), a generally superior *Attamyces* could have spread likewise through *Trachymyrmex* populations.

The ‘adaptive combination hypothesis’ postulates that only the specific ant–fungus combinations observed in nature confer high fitness (i.e. *Atta* ants cultivating *Attamyces* fungus; *Trachymyrmex* ants cultivating *Trachymyces* fungi). Under this hypothesis, these natural combinations exhibit higher fungal enzymatic activities than novel combinations (*Trachymyces × Atta*, or *Attamyces × Trachymyrmex*). While enzymatic activity may be typically higher in *Attamyces* than in *Trachymyces*, artificial experimentally induced combinations would result in lower activities. Evidence supporting this hypothesis derives from fungus-switch experiments where *T. septentrionalis* colonies growing *Attamyces* did not increase their reproductive output compared with colonies growing *Trachymyces* (Seal and Tschinkel, 2007a), which was possibly due to infections by pathogens (Seal and Mueller, 2014). These experiments did not examine the reverse switch (*Atta* colonies growing *Trachymyces*) (but see Sánchez-Peña, 2005) or investigate the effects of switches in other *Trachymyrmex* species.

A third possibility, termed the ‘complex synergism hypothesis’, is that enzymatic responses to ant–fungus combinations may depend on complex interactions between ants and symbiotic fungi. According to this hypothesis, the combinations that exhibit higher enzymatic activities do not reflect phylogeny, so higher activities may result when *Atta* ants are growing *Trachymyces* or *Trachymyrmex* ants are growing *Attamyces*.

We tested these three hypotheses by performing a 2×2 reciprocal symbiont-switch experiment with colonies of the leaf-cutting ant *Atta texana* Buckley 1860 and the non-leaf-cutting ant *Trachymyrmex arizonensis* (Wheeler 1907) that were growing either *Attamyces* or *Trachymyces*. The weight of the evidence supports the complex synergism hypothesis because enzymatic activity is influenced by ant–fungus interactions.

## RESULTS

### Enzymatic activity

The two species of fungi exhibited different catabolic properties toward carbohydrates and proteins when cultivated by the two species of ants. Amylase (starch hydrolysis) activity was significantly dependent on fungal species – *Attamyces* had much higher amylase activity than *Trachymyces* – but was not dependent on the species of ant host cultivating either fungus. There appeared to be a synergistic effect of fungal and ant species (significant interaction term), which was caused by the lower amylase activity of *Trachymyces* gardens when cultivated by *A. texana* compared with the amylase activity of the three other types of garden (*Attamyces* cultivated by *A. texana*, or either *Trachymyces* or *Attamyces* cultivated by *T. arizonensis* (Fig. 1, Table 1). Thus, amylase activity was significantly reduced when *Trachymyces* was grown by *A. texana*. *Trachymyrmex arizonensis* had similar amylase activity regardless of the type of fungus this ant species was growing.

Pectinase activity was significantly influenced by ant and fungal species and also exhibited significant synergistic effects (Table 1). Pectinase activity was highest in the *A. texana × Attamyces* combination where it was ~30% higher than in all other groups (Fig. 2, Table 1). *Trachymyrmex arizonensis* colonies had comparable pectinase activity regardless of fungal species grown, unlike the pattern of amylase activity (Fig. 1). In contrast to the patterns for amylase and pectinase, cellulase activity of the fungus garden appeared to be influenced by the ant species growing it. Cellulase activity was significantly higher in *T. arizonensis* colonies regardless of fungal species than in *A. texana* growing *Attamyces* (Fig. 3, Table 1). Xylanase activity did not appear to be influenced by ant or fungal species (Table 1; supplementary material Fig. S2). Furthermore, all xylanase effects except fungal symbiont species were significantly heteroscedastic. Thus, the xylanase *P*-values reported here may be inflated.
**Attamyces** gardens exhibited protease activity that was approximately twice that of Trachymyces gardens and this appeared to be independent of ant species [**Attamyces** 1545±536×10^3 U, Trachymyces 834±384×10^3 U; Table 1; supplementary material Fig. S3]. There might have been a weak interaction between ant and fungal species in protease activity (protease activity being higher in **T. arizonensis** colonies growing **Attamyces** than in conspecific colonies growing Trachymyces, F_{1,9}^*=5.95, P<0.03); however, because variances were heteroscedastic, attempts to make firm conclusions here introduce the risk of a Type I Error. Consequently, the interaction was removed from the model.

**Fungus garden growth rates**
Despite differences among enzymatic activities, fungus garden growth rates did not differ among the colonies of either ant species.

**Trachymyrmex arizonensis**
Over the course of the year, the growth rate of gardens of colonies collected in 2011 was not dependent on fungal species when cultivated by **T. arizonensis** (fungus: F_{1,6}=0.964, P=0.361; Fig. 4A). Because gardens grew in size steadily during the 1 year experiment, time was a significant variable (F_{10,60}=72.25, P<0.0001, Greenhouse–Geisser E=0.145,145,871, P<0.0001, Huynh–Feldt E=0.211,13,12,68, P=0.08).

**Atta texana**
Colonies growing Trachymyces grew slightly larger gardens over the first 12 months of their lives than those growing **Attamyces**, but this was statistically insignificant (F_{1,10}=4.02, P=0.07; Fig. 4B). Years were pooled because of low sample size. Time was significant (F_{8,80}=43.96, P=0.0001, Greenhouse–Geisser E=0.385,3,13,0, P<0.0001, Huynh–Feldt E=0.639,11,5,1,0, P<0.0001) and there was a significant interaction between garden volume and time (F_{8,80}=3.22, P<0.01, Greenhouse–Geisser E=0.385,13,0, P<0.05, Huynh–Feldt E=0.639,11,5,1,0, P<0.05). However, closer examination indicated that the significant interaction was largely due to differences between the first 2 months and all successive measurements. Thus, time was the most significant variable explaining the variation in the increase of garden volume.

**DISCUSSION**
The activity of particular enzymes in gardens of **A. texana** and **T. arizonensis** appears to be an emergent feature of ant–fungal interaction. Amylase and pectinase activities were higher when **A. texana** was done only in cases where the factorial test was significant to avoid inflating the significance of the interaction.

**Table 1. Summary of factorial ANOVA tests of enzyme activities of gardens of **Atta texana** and Trachymyrmex arizonensis** ants cultivating either **Attamyces** or Trachymyces fungus

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Ant species</th>
<th>Fungal species</th>
<th>Ant × fungus interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Starch</td>
<td>F_{1,9}=2.32, P=0.09</td>
<td>F_{1,9}=11.71, P=0.003</td>
<td>F_{1,9}=6.91, P=0.02</td>
</tr>
<tr>
<td>Pectin</td>
<td>F_{1,9}=9.42, P=0.006</td>
<td>F_{1,9}=14.95, P&lt;0.001</td>
<td>F_{1,9}=8.09, P=0.01</td>
</tr>
<tr>
<td>Cellulose</td>
<td>F_{1,9}=23.64, P=0.0001</td>
<td>F_{1,9}=0.0003, P=0.99</td>
<td>F_{1,9}=5.50, P=0.03</td>
</tr>
<tr>
<td>Xylan</td>
<td>F_{1,9}=1.36, P=0.27</td>
<td>F_{1,9}=0.006, P=0.94</td>
<td>F_{1,9}=1.23, P=0.31</td>
</tr>
<tr>
<td>Protein</td>
<td>F_{1,9}=0.245, P=0.63</td>
<td>F_{1,9}=7.89, P=0.01</td>
<td>–</td>
</tr>
</tbody>
</table>

Interactions with P>0.25 were pooled and only main effects are listed here. Significant tests (α=0.05) are highlighted in bold. To account for the unbalanced design (only five colonies of **T. arizonensis** were growing **Attamyces**, whereas all other sample sizes were N=6) and to make up for the ‘missing’ colony, a dummy variable was instead used (see Materials and methods); as a result, one degree of freedom had to be removed from the corresponding F-tests. This was done only in cases where the factorial test was significant to avoid inflating the significance of the interaction.
were analyzed on square-root transformed data. Error bars are ±1 s.d. Differences are denoted by different letters (P<0.05, Scheffé’s test). Data were analyzed on square-root transformed data. Error bars are ±1 s.d.

texana grew Attamyces than when growing Trachymyces, consistent with both the host-independent hypothesis (Attamyces has higher enzymatic activity regardless of the ant species growing it) and the adaptive combination hypothesis (activities are highest in natural combinations), but the corresponding pattern was not observed for T. arizonensis. Trachymyrmex arizonensis gardens exhibited similar amylase and pectinase activities regardless of the fungal species being cultivated. The weight of the observations therefore supports the complex synergism hypothesis, because the activity of each enzyme appears to follow idiosyncratic patterns as a result of ant-fungus combinations. In other words, neither fungal species by itself nor ant species by itself determines enzymatic activities of the symbiosis; rather, activities result from an interaction between ant and fungal species.

The weight of the accumulated evidence therefore does not support Stradling and Powell’s (Stradling and Powell, 1986) hypothesis (i.e. the host-independent hypothesis) that Attamyces have intrinsically higher enzymatic activities and growth rates than Trachymyces, and that this superiority of Attamyces played a key role in the evolution of leaf cutting (Figs 1–4). Trachymyrmex arizonensis exhibited similar fungus garden growth rates regardless of fungal species cultivated. The equivalence of growth of the two fungal species when grown by T. arizonensis may explain why, while Attamyces may be more productive in vitro (Stradling and Powell, 1986), it has not swept through the T. arizonensis population. This may also explain why some T. arizonensis colonies in the field grow Attamyces (U.G.M., unpublished data; supplementary material Fig. S1). However, the host-independent hypothesis was supported by observations in the A. texana colonies. The reduced enzymatic performance of A. texana growing Trachymyces might explain why A. texana is known to grow only Attamyces, even though our observations show that A. texana does not seem to have difficulty in growing Trachymyces in the laboratory [unlike an earlier report on Attamyces mexicana colonies switched in the lab to a Trachymyces fungus (Sánchez-Peña, 2005)].

What then constrains cultivar switching? One possibility is that interactions among the microbiota associated with the ants and/or fungi constrain ant and fungal combinations. This possibility was raised when T. septentrionalis and Trachymyrmex turritifex experienced fitness reductions after adopting Attamyces fungus (Seal and Mueller, 2014), which apparently was the result of invasion by weedy fungi destroying gardens, though the exact mechanism of garden decline was not known with certainty in that study. Trachymyrmex arizonensis likely associate with different microbial communities that are distinct from other species of Trachymyrmex, as do other attine species (Ishak et al., 2011; Rodrigues et al., 2011; Sen et al., 2009), which may or may not be able to withstand invasions by competitor fungi (Haeder et al., 2009; Mueller, 2012; Rodrigues et al., 2009). Another non-exclusive possibility is that ants may not apply the appropriate triggers to novel fungal
symbionts that would stimulate the synthesis of the diverse enzymatic machinery required for the metabolism of carbohydrates, lignocelluloses and proteins that Attamyces is known to possess (Aylward et al., 2013). For example, pectinase activity was lower in *T. arizonensis* colonies growing *Attamyces* than in *A. texana* colonies growing *Attamyces* (Fig. 2). Thus ant–fungus switches may result in mismatches at the physiological and genomic level (i.e. negative inter-genomic epistasis (Heath, 2009; Linksvayer, 2007; Wade, 2007).

One of the more surprising findings of this study sustains an ongoing debate on whether *Attamyces* can metabolize cellulose. A number of studies report significant cellulase activity for *Attamyces*-cultivated fungi grown on a variety of natural and artificial diets (Bacci et al., 1995; D’Ettorre et al., 2002; Martin and Weber, 1969; Schiott et al., 2008; Silva et al., 2003), whereas other studies failed to find significant cellulase activity (Abri1 and Bucher, 2002; Bucher et al., 2004). The results presented here are intriguing because they suggest that *T. arizonensis* colonies growing *Attamyces* on natural substrates (catkins) can in fact metabolize cellulose because *Attamyces* grown by *T. arizonensis* exhibited higher cellulase activity than *Attamyces* grown by *A. texana*. *Attamyces* is known to possess at least three genes for cellulose degradation (Aylward et al., 2013). Therefore, one possible explanation of our observations is that when *Attamyces* is grown by *Trachymyrmex* ants, cellulase genes are activated or are more actively expressed than when grown by *Atta* ants. Some authors have raised a second possibility that leaf-cutting ants never allow their *Attamyces* gardens to digest cellulose because the ants remove older sections of gardens and place them in refuse depots once simpler compounds (starches, pectins, etc.) are exhausted (De Fine Licht et al., 2010; Moller et al., 2011). Evaluation of this hypothesis requires knowledge of whether *Atta* and *Trachymyrmex* ants differ in how much time they give their fungus to get to a point in the garden cycle when cellulose is extracted from substrates. A third possibility is that switching gardens between host species may cause a reshuffling of auxiliary microbes in garden biofilms such that cellulose-metabolizing microbes [e.g. yeasts (Mendes et al., 2012)] are either more abundant or more active in gardens grown by *T. arizonensis*. Considering recent interest in the role of fungus gardens in cellulose metabolism (Aylward et al., 2012a; Suen et al., 2010), the fungal symbioses of *T. arizonensis* ants might be important sources of cellulases, meriting further study.

**MATERIALS AND METHODS**

This study employed reciprocal symbiont switch experiments conducted on the leaf-cutter ant *A. texana* and the higher-attine ant *T. arizonensis*. Colonies of both species were obtained by rearing newly mated queens collected immediately after mating flights (Seal, 2009; Seal and Tschinkel, 2007c) in 2010 and 2011. Colonies were maintained in plaster-lined boxes and fed *ad libitum* in a manner similar to previously published methods (Seal and Tschinkel, 2007a; Seal and Tschinkel, 2007b). Queens were fed and nests cleaned daily until the first workers appeared, after which colonies were fed at least twice weekly with oak catkins (staminate flowers of Quercus shumardii, a naturally occurring fungal substrate that many fungus-gardening ants will accept and feed their fungus; J.N.S., unpublished observation).

*Trachymyrmex arizonensis* is a common species along mid-elevations (1000–2000 m) in mixed oak–juniper–pine forests in the Sonoran Desert and western portions of the Chihuahuan Desert (Rabeling et al., 2007). *Atta texana* is found throughout central, east and south Texas, western Louisiana and northeastern Mexico (Mueller et al., 2011a; Mueller et al., 2011b; Sanchez-Peña, 2010). *Atta texana* cultivates a single fungal species typical of the vast majority of leaf-cutting ants (*Leucocephrinus gongylorrhous*) (Fisher et al., 1994; Mikheyev et al., 2006; Mueller et al., 2011a; Mueller, 2002; Pagnocca et al., 2001); however, this species rarely reproduces sexually – we refer to it by its anamorph (asexual) form, *Attamyces bromaticus* Kreisel (Mueller et al., 2010; Mueller et al., 2011b; Seal et al., 2012; Seal and Mueller, 2014). In contrast, *T. arizonensis* cultivates a Trachymyrmex fungus typical for most (but not all) *Trachymyrmex* species that is placed in a taxonomically unresolved *Leucocephrinus* clade that is the sister clade to the *Attamyces* clade. Ongoing surveys, however, indicate that some populations of *T. arizonensis* cultivate *Attamyces* instead of Trachymyrmex (supplementary material Fig. S1; U.G.M., in preparation). *Trachymyrmex arizonensis* likely exchanges symbionts with the sympatric leaf-cutter ant *Acromyrmex versicolor* (supplementary material Fig. S1; U.G.M., in preparation). Although the two ant species used in this study are not sympatric, these ants and their cultivated fungi are representative of the two major clades of the higher Attni (Schultz and Brady, 2008). Despite population differentiation between *Attamyces* from Texas and Arizona (Mueller et al., 2011b), this genetic differentiation is substantially less than the differentiation between *Attamyces* and *Trachymyrmex* (Mikheyev et al., 2006; Mikheyev et al., 2008; Mueller et al., 2011; Mueller et al., 2011b) (supplementary material Fig. S1).

Queens were randomly assigned to either the *Attamyces* or *Trachymyrmex* condition and provided with ~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). *Atta texana* queens were supplied with either *Attamyces* collected from an *A. texana* nest near Austin, TX, USA, in May 2007 (30°12.622′N, 97°38.469′W, 125 m elevation) or Trachymyrmex fungus collected from a *T. septentrionalis* nest collected in April 2010 at the University of Texas Stengl ‘Lost Pines’ Biological Station (30°05.218′N, 97°10.425′W, 141 m elevation).

The experimental switches on *T. arizonensis* used in this study were conducted on eight colonies started with queens collected on 27–28 July 2011. Five of these were reared on *Attamyces* and three on *Trachymyrmex*. Because of unequal mortality among these queens, we also include data on 10 colonies reared similarly from queens collected on 25–27 July 2010 (all of which were cultivating *Trachymyrmex*); three of these colonies were randomly selected for enzymatic activity analysis. All *T. arizonensis* colonies were collected at the Southwest Research Station, near Portal, AZ, USA (31°53.025′N, 109°12.374′W, 1646 m elevation), 1 day after the first heavy rain of the summer monsoon season. 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). Colonies growing *Trachymyrmex* 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 cm³ of fungus into a 4 cm Petri dish (garden chamber) that was insulated 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 7×7 cm plastic boxes with a 5 mm-thick bottom of moistened dental plaster (Marjoy Enterprises, San Antonio, TX, USA). After approximately 1 year of age, colonies were moved to larger nesting containers used in previous studies on *Trachymyrmex* ants (plaster-lined, cylindrically shaped, 196 cm³ depressions in a square plastic box, 11×11×3 cm) (Seal and Mueller, 2014; Seal and Tschinkel, 2007a; Seal and Tschinkel, 2007b). All *T. arizonensis* colonies were fed oak catkins (*Quercus shumardii*) *ad libitum* throughout the duration of the entire experiment. Colonies of the two species were fed the same type of catkins to avoid confounding the results with diet (Kooij et al., 2011).

The 12 *Atta texana* colonies used in this study were similarly reared from newly mated queens collected after mating flights near Hornsby Bend, TX, USA (30°12′37.3′′N, 97°38′28.0′′W). Of the colonies growing *Trachymyrmex*, three were collected on 13 May 2011 and three were collected on 16–18 May 2010, whereas the six colonies growing *Attamyces* came from cohorts 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 (7×7×3 cm) lined with 5 mm 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 to the *T. arizonensis* colonies, except they were also periodically supplied with pear leaves (Bradford pear, *Pyrus calleryana*).

**Fungus garden growth measurements**

Colonies were monitored monthly over the course of the first year of their lives. Volumes of gardens were measured monthly in both species. Fungus garden volumes were estimated by measuring the maximum dimensions (length, width and height) in each nest box at monthly intervals. The top of the plaster nest chamber was completely covered with a piece of Plexiglas. The width and length of each fungus garden were measured with a ruler placed on top of each nest cover (a piece of Plexiglas that contained five pre-drilled 1 mm holes, 2 cm apart, from the center of the cover) (Seal and Mueller, 2014; Seal and Tschinkel, 2007a; Seal and Tschinkel, 2007b).

**Fungal enzymatic activity assays**

Enzymatic activity assays were conducted in 2012 when all colonies were at least 1 year old. 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 uppermost part of the garden, whereas relative activity of enzymes derived from non-cultivars 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 used by the ants (Kooij et al., 2003). Accordingly, 10ml of fungus garden extract was added to 40ml of water and 50μl of 1% (w/v) (=500μmol) of 1% (w/v) (=500μmol) 1M of NaOH. Absorbance was measured at 440 nm at 25°C. Control samples were prepared identically except enzyme extract was added immediately before the application of TCA. Relative protease activity was calculated from the difference between treatment and control absorbances.

The methods used to estimate enzyme activity do not differentiate between enzymes secreted by the cultivated fungus or associated microbial biofilms. The metabolic activities of fungus gardens are likely quite diverse (Aylward et al., 2012a; Aylward et al., 2012b). Yeasts inhabiting the garden may be important contributors of cellulase activity and perform a variety of functions in extracting energy frompectins (Mendes et al., 2012). Although it is unclear whether the distinction between compounds produced by *Leucocoprinus* spp. versus other microbes inhabiting the fungus gardens is important to the ants, because the ants are potentially consuming whatever they find in the fungus garden, the data presented here correspond to an ‘extended phenotype’ of enzymatic activity in the symbiosis *senso lato*. As a result, when the statistical tests report a significant ‘fungus’ effect (or ‘ant’ effect), it should be interpreted as an effect influenced by the fungus garden and possibly its associated biofilms.

**Statistical analyses**

The problem of unequal sample sizes resulting from only five switched *T. arizonensis* colonies (whereas all other ant × fungal combinations had N=6) was solved using Underwood’s technique of creating a dummy variable calculated from a mean value of each group to which the missing data belonged (Seal and Tschinkel, 2007a; Underwood, 1997). This method alters neither the mean nor the variance (Underwood, 1997), but to avoid rejecting the null hypothesis falsely (Type I Error), the test is made more conservative by subtracting one degree of freedom from the corresponding F-test for each of the missing values generated.

Garden volumes were analyzed with a repeated measures ANOVA, with time as the repeated measure and with fungal symbiont species as main effect. Volumes were repeatedly measured on all colonies in this experiment at approximately 1 month intervals. In addition to tests for standard parametric assumptions, we tested for sphericity, which tests for equality of variances among the repeated measures (Keselman et al., 2001; Sokal and Rohlf, 1998). In instances where sphericity tests were rejected, we report Greenhouse–Geisser- and Huynh–Feldt-corrected F-statistics and P-values. Data were either log10 or square-root transformed to meet parametric assumptions.

**Fungus samples**

Small samples (hyphae or spores) were collected with sterile forceps from the emerging cultures and placed in Chelex resin (Sigma-Aldrich) and heated to near boiling for 90 min. Pure fungal cultures were identified by PCR amplification of the ITS gene using primers ITS4 and ITS5 (Mueller et al., 1998; Sen et al., 2009; White et al., 1990), then sequencing at the Microbial Facility supplied the plate reader.

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**Competing interests**

The authors declare no competing financial interests.

**Author contributions**

J.N.S. and U.G.M. conceived and designed the experiments. M.S. developed and modified the enzymatic assays. J.N.S. executed the experiments and enzymatic assays. J.N.S. and U.G.M. wrote and revised the manuscript, with significant input by M.S.


