Evol Ecol
DOI 10.1007/s10682-013-9665-8

ORIGINAL PAPER

Instability of novel ant-fungal associations constrains horizontal exchange of fungal symbionts

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Received: 7 January 2013 / Accepted: 20 July 2013

Abstract One of the more fascinating features of fungus-gardening ants (Attini: Formicidae) is their fidelity to their lineage-specific fungal symbionts. Among the derived higher-attine ants (leafcutter ants and close relatives), it is thought that most leaf-cutting ants grow *Attamyces* fungus whereas most *Trachymyrmex* ants grow 'Trachymyces' fungus, but there exist exceptions to this clade-to-clade correspondence between ants and fungi. The exceptions are inconsistent with strict one-to-one coevolution, which suggests that ants sometimes are able to switch to novel fungi. Such switches appear to be largely constrained and ants are generally faithful to their species-specific fungi. Prior experiments demonstrated no clear fitness consequences of growing novel fungi over the short-term when the ant *Trachymyrmex septentrionalis* was symbiont-switched by forcing it to grow *Attamyces* leaf-cutter fungus. We hypothesized that long-term ant-fungal fidelity is constrained either by physiological differences among fungal species or by garden diseases that symbiont-switched ants cannot control. Repeat experiments in a different location show that *T. septentrionalis* colonies switched to grow *Attamyces* exhibit sudden declines in garden biomass and consequent fitness reductions due to garden destruction by pathogens, whereas control colonies (*Trachymyrmex* ants cultivating Trachymyces fungus) do not show parallel garden declines. These patterns are mirrored in symbiont-switch experiments conducted on colonies in *Trachymyrmex turrifex*. Disease microbes selecting on ant-cultivar combinations therefore can constrain switches to novel cultivars and maintain combinations that are more resistant to disease.

Electronic supplementary material The online version of this article (doi:10.1007/s10682-013-9665-8) contains supplementary material, which is available to authorized users.

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Published online: 01 August 2013

Keywords Attini · Coevolution · Colony performance · Disease · Fungi · Immunity · Symbiosis

Introduction

Fungus-gardening insects are among the most highly integrated symbioses. Some beetles, termites, and ants evolved fungiculture with specific mutualistic fungi (Farrell et al. 2001; Aanen et al. 2002; Mueller et al. 2005), which the insects tend and grow as their major food source. The integration between the insects and their farmed fungi is thought to be so extensive that they can best be described as highly integrated super organisms so that the insects and fungi can no longer lead separate lives (Seal and Tschinkel 2007b; Hölldobler and Wilson 2008; Hölldobler and Wilson 2011; Aylward et al. 2012b) and are in fact nutritionally interdependent (Martin 1987; Aanen and Eggleton 2005; de Fine Licht et al. 2010; Schiøtt et al. 2010; Aylward et al. 2012b; de Fine Licht and Biedermann 2012).

Of the fungus-faming insects, attine fungiculture is best understood at the macroevolutionary level due to a number of co-phylogenetic analyses of ant and fungal partners (Mueller et al. 1998; Schultz and Brady 2008; Mikheyev et al. 2010; Mehdiabadi et al. 2012). Attine ants propagate their fungal cultivars as clonal monocultures in gardens, which are transmitted vertically between generations by dispersing queens that carry fungal inocula in their mouths (Quinlan and Cherrett 1978; Mueller et al. 1998, 2010; Green et al. 2002; Little et al. 2003). This leads to tight ant-fungus associations over short times, but these tight associations are disrupted regularly through horizontal cultivar exchange; in such ant-fungus combinations of recent origin, the ant and fungal partners have co-propagated only for short time, limiting the potential for co-evolution (Mueller et al. 1998; Mikheyev et al. 2010).

Much of the discourse about attine ant-fungus symbiosis has been based on the assumption of clade-to-clade specificity between ants and fungi (Mueller and Gerardo 2002; Currie et al. 2003b; Schultz and Brady 2008). Most basal ant lineages show relatively frequent horizontal transfer of cultivars among attine lineages and even recent domestication of novel fungi from free-living populations (Mueller et al. 1998). On the other hand, the derived higher-attine lineages, which is composed of two major clades, the monophyletic leaf-cutting ants *Atta* and *Acromyrmex* and the non-leafcutter, paraphyletic genera *Trachymyrmex* and *Sericomyrmex*, show some clade-to-clade correspondence between ants and fungi (Mueller and Gerardo 2002; Schultz and Brady 2008; Mueller et al. 2010). Nevertheless, horizontal transfer of symbionts between higher attine taxa has been documented (Mikheyev et al. 2007, 2010; Mueller et al. 2011b; UGM, unpublished data). The vast majority of leaf-cutting ants (*Atta* and *Acromyrmex*) that have been analyzed so far cultivate a single species of fungus, which in teleomorph (sexual) form is called *Leucocoprinus gongylophorus* A. Møller (Mueller et al. 2010, 2011b). Leaf-cutting ant fungus rarely reproduces sexually (Fisher et al. 1994; Pagnocca et al. 2001; Mueller 2002; Mikheyev et al. 2006) and as a result, we use here the name of the anamorph (asexual) form, *Attamyces bromatificus* Kreisel. In contrast, the non-leaf-cutting, higher-attine genera typically cultivate a more diverse assemblage of taxonomically unresolved, but closely related *Leucocoprinus* lineages (Chapela et al. 1994; Mueller et al. 1998; Mikheyev et al. 2008; Schultz and Brady 2008). We refer here to the fungi cultivated by *Trachymyrmex* ants using the provisional name 'Trachymyces'.

The accumulated evidence for ant:fungal specialization has been ambiguous so far. The earliest observations indicated mutual symbiont compatibility between *Trachymyrmex* and *Atta* ants despite higher growth rates in *Attamyces* cultivated by either leafcutter or *Trachymyrmex* ants (Weber 1956; Stradling and Powell 1986). The only experiment that specifically addressed the issue whether higher attine fungi were indeed adapted to their ant hosts produced ambiguous results for the test species *Trachymyrmex septentrionalis* McCook (Seal and Tschinkel 2007a). The main finding was that *T. septentrionalis* colonies growing either native Trachymyces or switched *Attamyces* symbiont produced comparable amounts of ant biomass, yet the colonies growing *Attamyces* collected less forage and grew smaller gardens (Seal and Tschinkel 2007a). Thus colonies cultivating an *Attamyces* may have higher fitness because it reduced the amount of substrate needed and the time spent outside the nest for foraging, which could lower risk to predation (Rao 2000) and desiccation (Hood and Tschinkel 1990). These observations beg the question why colonies in the wild persist growing Trachymyces fungus because it seems biologically possible for switches to occur naturally (e.g., Mikheyev et al. 2010) and *Attamyces* may be a superior clone than Trachymyces. Thus the fact that switches are rare would indicate that there are constraints preventing this from happening.

A limitation of Seal and Tschinkel (2007a) was that it was conducted only over a 3-month period and comprised only one reproductive bout, which might be an insufficient fitness measure for perennial species such as ants (Bourke and Franks 1995). Moreover, long-term fitness consequences of cultivar switching were not measured in this experiment. The single line of evidence suggesting that ants were adapted to their lineage-specific fungus was the finding that the female sexual offspring (gynes) of *T. septentrionalis* colonies growing *Attamyces* had lower fat stores than gynes produced by control colonies cultivating a native *Trachymyces* cultivar (Seal and Tschinkel 2007a), which in turn had similar fat stores of gynes in natural nests (Seal and Tschinkel 2007c). Logically, *T. septentrionalis* colonies growing Trachymyces would have a demographic advantage (the gynes would reach maturity earlier) over those growing *Attamyces*.

Ant-fungal fidelity may be constrained by several non-mutually exclusive mechanisms. First, many of the digestive enzymes in attine ants appear to be fungally derived (fungal enzymes pass unharmed through the ant gut and are defecated onto the garden; (Martin 1987; Erthal et al. 2004, 2009; Rønhede et al. 2004; Schiøtt et al. 2008, 2010; de Fine Licht and Boomsma 2010; de Fine Licht et al. 2010). Thus, when a colony adopts a new fungus, the ants adopt the enzymes of the new fungus, though it is not clear whether the enzymes of the newly adopted fungus will adequately digest substrates the ants habitually feed to the new symbiont. For example, leaf-cutting ants typically feed their gardens leaves, which could be compositionally different than the substrates *Trachymyrmex* ants would feed their gardens (e.g., insect frass, fruit pulp, flowers or dead vegetation, and occasionally fresh-leaf fragments) (Leal and Oliveira 2000; Seal and Tschinkel 2007b). Thus, there may be long-term detrimental effects that result from symbiont-substrate mismatches.

The second hypothesis recognizes that the attine symbiosis is in fact a consortium of thousands of microbe species rather than a simple association consisting of only ant and fungal species (Rodrigues et al. 2008; Sen et al. 2010; Ishak et al. 2011; Aylward et al. 2012b). The long-term clonal monocultures exhibited by attine ants (Poulsen and Boomsma 2005; Mueller et al. 2010; Seal et al. 2012) present problems for disease control (Mueller et al. 2005), especially because attine gardens often contain specialized garden parasites (Currie et al. 1999a, 2003b) or generalist 'weedy' fungal invaders (Rodrigues et al. 2008, 2011). As a result, ants are thought to have evolved strategies to manage disease outbreaks that employ specialized behaviors, glandular secretions that may have

antimicrobial properties, or antibiotic producing microbes, or a combination of all three (Currie et al. 1999a, 2003b; Currie and Stuart 2001; Santos et al. 2004; Little et al. 2006; Hughes et al. 2008; Mueller et al. 2008; Fernández-Marín et al. 2009; Barke et al. 2010; Yek et al. 2012). Some types of microbes are associated with the ants and grow on specialized areas on the ants' body (Currie et al. 2006; Mueller et al. 2008) whereas others seem to grow in the fungus garden matrix (Santos et al. 2004; Haeder et al. 2009; Rodrigues et al. 2011). For example, a number of microbes associated with either ants or gardens secrete antifungal antibiotics (Haeder et al. 2009; Rodrigues et al. 2009; Sen et al. 2009; Barke et al. 2010). Consequently, there may be detrimental consequences that result from mismatches among the ants, fungi as well as ant- and garden-associated microbiota.

We here present observations from a repeat of the symbiont-switch experiment on *T. septentrionalis* conducted originally (Seal and Tschinkel 2007a). We also expanded the experiments to include the species *Trachymyrmex turrifex* Wheeler. Unlike the findings in Seal and Tschinkel (2007a), all our experimentally fungus-switched colonies suffered reductions in fitness, both in standing worker biomass and in sexual production. Gardens of switched colonies became generally infected, especially by non-specialized garden weeds, whereas native ant-fungal combinations appear to be more resistant to the invasion of 'weedy' species. Diseases therefore appear to constrain fungus-switches and help maintain long-term fidelity of natural ant-fungal combinations.

Materials and methods

Symbiont-switch experiments were conducted with two *Trachymyrmex* species (*T. septentrionalis* and *T. turrifex*). *T. septentrionalis* is a common ant in sandy soils of eastern North America, occupying a region that extends from Texas and Florida to New York and at least west to southern Illinois (Weber 1972; Rabeling et al. 2007). It is conspicuously the only higher-attine species in the US whose population occurs entirely in the US (Rabeling et al. 2007). *T. septentrionalis* is among the most abundant and conspicuous ants in pine forests throughout the southeastern US (Seal and Tschinkel 2006, 2010). Its western limits overlap with those of *T. turrifex*, which is found in clay and sandy soils only west of the Mississippi River, ranging from western Louisiana, throughout most of Texas, to northeastern Mexico (Rabeling et al. 2007; Sanchez-Peña 2010). Unlike *T. septentrionalis, T. turrifex* does not completely take down its garden in winter (JNS unpublished data). In Texas and western Louisiana, both species are sympatric with the leaf-cutting ant, *Atta texana* Buckley. *A. texana* is the northern-most representative of its genus and occupies a distribution somewhat congruent with that of *T. turrifex* (Mueller et al. 2010, 2011a, b; Sanchez-Peña 2010).

Experimental methods

Fungus-switching of experimental colonies used the same method described by Seal and Tschinkel (2007a). Colonies were collected at the end of dormancy (mid to late March in Florida and Texas). Unlike Seal and Tschinkel (2007a), colonies in this study were not sacrificed. Colonies had their native fungus garden removed with sterile forceps and replaced with ca. 300 mg (1 cm^3) of *Attamyces* garden from an *A. texana* colony. The switches reported here used an *A. texana* source colony collected near Austin, Texas in May 2007. The effect of removing a colony's cultivar and replacing it with a different cultivar (*i.e.*, the colony disturbance associated with the switch) was evaluated in control switches, in which the

control colonies had their cultivar removed and replaced with a *T. septentrionalis* cultivar from a different conspecific colony. All control colonies received a fungus from their respective populations and clade; for example, all colonies from Texas used in this study cultivated Trachymyces from phylotype 'A' (Mikheyev et al. 2008) and were accordingly supplied fungus from a source colony from Texas growing the same phylotype, whereas colonies from Florida were growing fungus from either phylotype 'A' or 'B' and thus were similarly treated. Fungal genotypes were identified by PCR amplification and sequencing of the ITS gene using primers ITS4 and ITS5 (White et al. 1990; Sen et al. 2009).

Colony collections

Trachymyrmex septentrionalis colonies were collected in Florida and in central Texas. Eleven *T. septentrionalis* colonies from Florida and 12 from Texas were used as experimental colonies.

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 (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) studied previously in detail (Seal and Tschinkel 2006, 2007a, b, 2008).

Twenty *T. turrifex* colonies were collected May–April 2010 at the University of Texas' Brackenridge Field Laboratory (30°17′7.15″N, 97°46′52.3″W). Following collection, all colonies were briefly chilled (4 °C) and weighed. Eleven colonies had their native fungus replaced with *Attamyces* fungus and the remaining nine colonies received Trachymyces from a single *T. turrifex* colony. The *T. turrifex* colonies were switched in October 2010. To control for different initial garden sizes, all control *T. turrifex* colonies had their fungus gardens removed and replaced with ca. 0.5 cm^3 fungus from a single source *T. turrifex* colony growing Trachymyces. Control *T. turrifex* colonies received approximately half the amount of Trachymyces than *T. septentrionalis* because the former tend to have smaller colonies than the latter. This was not a source of bias because quantitative interspecific differences were not of interest.

Each colony was housed in a tray coated with Fluon © (Northern Products, Woonsocket RI) along the sides to prevent escapes. Colonies grew their garden in a cylindrically-shaped, 196 cm^3 depression in a square plastic box (7.5 × 7.5 × 3 cm) lined with dental plaster (Fig. S1). The top of this chamber was completely covered with a piece of plexiglass. The width and length of each fungus garden was measured with a ruler placed on top of the plexiglass cover. The height of the garden was obtained by extending an ethanol and flame sterilized straight wire marked in 5 mm increments through five 1 mm diameter holes predrilled into each plexiglass cover (2 cm apart, from the center of the cover). The average height was then obtained from these five measurements. Water was supplied weekly in pre-drilled holes located at each of the four corners. Colonies were kept at ambient at approximately 24–25 °C in a room that was otherwise unheated or unhumidified. These conditions are suitable for laboratory experiments with *Trachymyrmex* ants (Seal 2006; Seal and Tschinkel 2007a, b). Colonies were fed a mixture of substrates highly preferred by *T. septentrionalis*, oak catkins (staminate flowers) and feces from the eastern tent caterpillar reared on cherry and pear leaves (*Prunus* and *Pyrus* spp., respectively) (Seal and Tschinkel 2007a, b, 2008). Catkins were obtained every spring from the Texas live oak (*Quercus fusiformis*.) and Texas red oak (*Quercus texana*). Upon collection, fungal substrates were stored in ziplock bags at 20 °C. Colonies were fed pre-weighed amounts of

substrate, which was converted to dry weights using constants obtained by drying small amounts of substrates for 48 h under ambient conditions.

As a positive control to test for possible contaminated *Attamyces* fungus, we report data on *A. texana* colonies cultivating the same clone *Attamyces* fungus from the same source colony employed in the *Trachymyrmex* switches reported here. Because of the rarity of small *A. texana* colonies in central Texas and the extreme large size of mature colonies, which limits sample size, we report data on *A. texana* colonies growing *Attamyces* fungus reared from queens collected after mating flights in May 2010 and 2011 [mating flights in *A. texana* generally occur 2–3 months after *T. septentrionalis* ends dormancy (Mintzer 1987; Seal and Tschinkel 2008)]. In 2010 these incipient queens were kept in the same room as the eventual experiments on the *Trachymyrmex* spp. reported here, whereas those collected in 2011 were kept in a neighboring room since these were part of another experiment. Thus, we report the growth of *A. texana* from the year before and after the experiments reported here. These queens were collected after a mating flight near Hornsby Bend, Texas (30°12′37.3″N, 97°38′28.07″W). Upon collection, the *A. texana* queens were placed in clean, clear plastic boxes (4.5 × 4.5 × 2.5 cm) lined with dental plaster and received ca. 200 g of fungus garden material. As *Atta* queens do not forage during the founding phase (Huber 1905; Fernández-Marín et al. 2004; Seal 2009), they were supplied with fungal substrate only after the first workers emerged. Otherwise they were fed and cleaned at least twice a week in a manner identical to that described above in the *Trachymyrmex* colonies.

Performance measures

The main performance measures in this study were the volume of fungus garden measured over time, ant-colony weights (workers and queen minus fungus garden) before and after the experiment, and, if any reproductive's were produced, the numbers and weights of male and female sexual offspring. Colony weights before and after the experiment were conducted during the dormant period (November–January) when gardens of laboratory colonies of both species are at their nadir (J.N. Seal, unpublished data). The total amount of fungal substrate (food that the ants feed the fungus) collected by the ants was calculated by subtracting the amount not collected and the amount of garden refuse produced from the total amount of substrate provided to each colony. Sexuals are typically produced under laboratory conditions by *T. septentrionalis* (Seal and Tschinkel 2007a, b), but the production of sexuals by *T. turrifex* colonies in the laboratory seems less predictable (JN Seal, unpub. data) since only two colonies produced sexual brood (two individuals). Laboratory colonies also exhibit mating flight behavior, which usually occurs 1–3 days after weekly watering. During these events, the ants aggregate outside of the nest entrances and the alates (winged females and males) become negatively geotropic. Alates were collected as they appeared for these flights, then frozen, dried, weighed, and counted. Although the vast majority of alates flew during June–August, it became evident during the colony weighing during the dormant season (October–January) that not all alate females flew since some colonies contained a number of de-alate females that were not present when colonies were collected. These were counted but not removed due to possible confusion with the queen.

Mycological techniques

Small fragments (0.5 cm^3) were removed with sterile forceps from each experimental garden because the fungus-switched colonies exhibited behaviors unlike those observed in

a previous switch-study by Seal and Tschinkel (2007a). Colonies either were lackluster and exhibited very little growth, or underwent sudden crashes after building up large gardens. Gardens were hypothesized to be unhealthy and possibly infected by pathogens or parasites. The small garden fragments were placed on PDA plates and observed for contaminates within the first 48 h. Small samples (hyphae or spores) were collected with sterile forceps from the emerging cultures and placed in Chelex resin (Sigma–Aldrich, St. Louis, Missouri) and heated near boiling for 90 min. Pure fungal cultures were identified by PCR amplification of the ITS gene using primers ITS4 and ITS5 (White et al. 1990; Mueller et al. 1998; Sen et al. 2009), sequencing at the University of Texas at Austin University DNA Sequencing Facilities. Sequences were identified using BLAST at NCBI Genbank.

Statistical analysis

Garden volumes were analyzed with a repeated measures ANOVA with time as the repeated measure and cultivar and population as additional effects. Volumes were repeatedly measured on all colonies in this experiment at approximately 1-week intervals. In addition to testing whether volumes differed between weeks as gardens grew or declined, the ANOVA also tested for interactions between week and cultivar and population. In addition to tests for standard parametric assumptions, we tested for sphericity, which tests for equality of variances among the repeated measures (Sokal and Rohlf 1995; Keselman et al. 2001). In instances where sphericity tests were rejected, we report Greenhouse-Geisser and Huynh–Feldt-corrected F-statistics and p values. All data were $\log_{10}$ transformed to meet parametric assumptions.

Problems of unbalanced sample-sizes in the factorial analysis were solved by the following method. Missing cells were filled by using a dummy variable calculated from the mean value for each group to which the missing data belonged. 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. For example, this method was required in the analysis of male number and weight because only five colonies from Florida were switched onto *Attamyces* fungus whereas the other fungus and population combinations consisted of six colonies. Thus, to conduct a balanced factorial analysis, a dummy variable had to be created for male production from a 'sixth Florida colony growing *Attamyces*.

Results

The most surprising result of this study was that each species responded differently when growing *Attamyces*. Overall, performance of *T. septentrionalis* and *T. turrifex* colonies was lower when growing *Attamyces* (Figs. 1, 2).

Trachymyrmex septentrionalis

The experiment on *T. septentrionalis* produced results during the first few weeks that were consistent with those of Seal and Tschinkel (2007a, b). Colonies readily adopted the *Attamyces* fragments; however, colonies growing *Attamyces* either did not thrive or built up large gardens that then suffered rapid and sudden declines in garden-volume (Fig. 1, S3). Over the course of the experiment, colonies growing *Attamyces* had smaller volumes

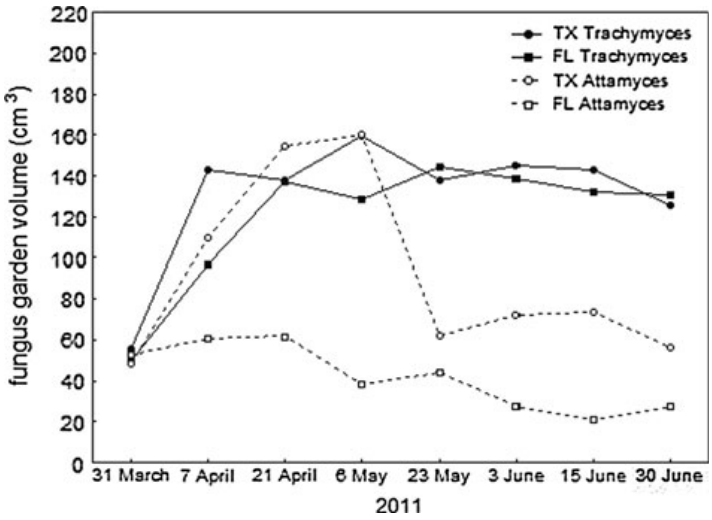

Fig. 1 Volume (cm^3) of experimental fungus gardens of *T. septentrionalis* during the switch experiment conducted in 2011. *Filled symbols* and *solid lines* indicate colonies growing Trachymyces whereas *open symbols* and *dashed lines* correspond to colonies growing *Attamyces*. *Circles* indicate colonies from Texas (TX) and *squares* indicate colonies from Florida (FL). *Error bars* have been omitted here for visual clarity, but are shown in Figure S3. Data were analyzed after $\log_{10}$ transformation. Colonies growing *Attamyces* fungus had smaller volumes than those growing Trachymyces ($F_{1,22} = 11.79$, $p = 0.002$). The volume of fungus garden material declined after 6 weeks in colonies growing *Attamyces* fungus but remained more constant in colonies growing Trachymyces

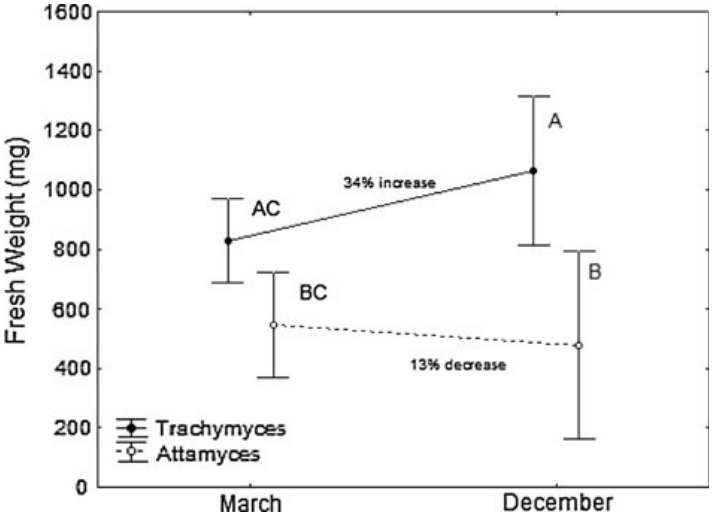

Fig. 2 Change in worker number of *T. septentrionalis* colonies during the experiment conducted in 2011. Significant differences are denoted by *different letters* ($p < 0.05$, Scheffé's test). Fresh weights of colonies did not differ in at the beginning of the experiment in March 2011, whereas by December 2011 (the end of the experiment colonies) colonies growing Trachymyces had increased their standing worker-biomass, and colonies growing *Attamyces* experienced no growth if not slight losses in standing biomass

than those growing Trachymyces ($F_{1,22} = 11.79$, $p = 0.002$, Fig. 1, S3). Since gardens increased in size as workers added substrate, time was a significant predictor of garden volume ($F_{7,\ 154} = 4.45$, $p < 0.0001$; Greenhouse-Geisser $E_{2.16,\ 47.4} = 0.308$, $p = 0.02$, Huynh–Feldt $E_{2.51,\ 55.1} = 0.358$, $p = 0.01$). On the other hand, the interaction between time and type of fungus did not meet the threshold for rejection, though barely

($F_{7,\ 154} = 2.79$, $p = 0.009$; Greenhouse-Geisser $E_{2.16,\ 47.4} = 0.308$, $p = 0.07$, Huynh–Feldt $E_{2.51,\ 55.1} = 0.358$, $p = 0.06$). Thus, it is unclear whether the two types of fungus have different growth rates when grown by *T. septentrionalis*. Origin of the ant colonies (Florida or Texas populations) was not a significant predictor of fungus garden volume and could therefore be removed from the statistical model (Table S1). Even though the effect of population was not statistically significant, a perhaps important observation was that Florida colonies did not exhibit the previously observed rapid increase in fungus garden growth (Seal and Tschinkel 2007a, b), whereas Texas colonies did when cultivating either *Attamyces* or Trachymyces (Fig. 1a). Rather, colonies from Florida remained small and somewhat stunted, lacking the more three-dimensional structure of typical fungus gardens (Fig. S1). The largest of these gardens grown by Florida colonies looked less stunted than the others by retaining its volume for over 6 weeks, but no brood could be observed inside the garden. On the other hand, colonies from Texas growing *Attamyces* cultivated vibrant three-dimensional gardens, which were for the first 5 weeks very similar in size to gardens of control colonies growing Trachymyces (Fig. 1). After these 5 weeks, switched gardens from Texas exhibited sudden and complete garden declines; over the course of 48 h, the ants in the colonies deconstructed the gardens and deposited the fungal fragments into refuse piles outside the nest chambers. Workers in these colonies piled the brood (sexual pupae and late-instar larvae) in the center of the nest box. Colonies that experienced garden-crashes were repeatedly given fresh *Attamyces* garden (ca. 1 cm^3) during the following 2 months (at about biweekly intervals), but gardens did not regain their pre-crash size. Rather, following a garden crash, most workers entered an inactive state, did not forage and did not tend the replacement *Attamyces* garden. Three of these inactive colonies growing *Attamyces* eventually died over the dormant period (all workers and queen) whereas the remaining 20 inactive colonies survived until the following spring (8 *Attamyces*, 12 Trachymyces). Two colonies from Florida were removed from the experiment and allowed to grow Trachymyces after suffering mortality and were reduced to <50 workers. Interestingly, the six remaining colonies growing *Attamyces* reactivated themselves in February and March 2012, grew gardens only to suffer catastrophic declines weeks later (Fig. S4). However, only two of the six surviving colonies growing *Attamyces* produced sexual brood compared to eight of the 10 growing Trachymyces (two of the twelve colonies growing Trachymyces failed to initiate garden growth and were excluded from further observation) (Figure S5).

Generally, there was strong evidence that the reduced garden growth in switched colonies led to reductions in colony performance—fungus-switched colonies collected less fungal substrate, had smaller gardens and produced fewer offspring. More substrate was accepted in colonies growing Trachymyces and colonies from Florida growing *Attamyces* collected the least (Tables 1, 2). *T. septentrionalis* colonies growing *Attamyces* appeared to lose standing colony biomass (weight of all workers and queen) at the end of the experiment, since colonies growing Trachymyces were heavier than colonies growing *Attamyces* (Fungal species: $F_{1,21} = 21.3$, $p < 0.0001$, Fungal species × time interaction $F_{1,\ 21} = 10.8$, $p = 0.003$; Fig. 2). Colonies growing either fungal species did not differ in weight at the start of the experiment but colonies growing Trachymyces were nearly twice the size of colonies growing *Attamyces* by the end. Population (Florida or Texas) did not have a significant effect on colony weights and was accordingly removed from the statistical model (Table S2).

Trachymyrmex septentrionalis colonies growing *Attamyces* produced significantly fewer sexuals than colonies growing Trachymyces (Tables 1, 2). Colonies from Florida growing *Attamyces* produced no sexual offspring of either sex, whereas some sexuals were produced in switched colonies from Texas. Nevertheless, the numbers and weights of

Table 1 Number (mean ± SD) of sexuals produced by experimental *T. septentrionalis* colonies from Florida and Texas, as well as corresponding weights (in mg) of garden substrate collected by workers

Variable	Florida ants on *Attamyces*	Florida ants on Trachymyces	Texas ants on *Attamyces*	Texas ants on Trachymyces
Female number	0	9.5 ± 13.2	22.3 ± 21.2	93 ± 95.14
Male number	0	35.2 ± 31	21 ± 36.9	3.33 ± 2.94
Female production (mg)	0	17.5 ± 25.1	31.9 ± 32.1	149.2 ± 158.9
Male production (mg)	0	18.4 ± 16.6	12.9 ± 21.9	2.55 ± 2.5
Substrate collected (mg)	5,783.2 ± 2,754.2	12,730.9 ± 1,902.4	10,428 ± 6,030.1	9,759.6 ± 3,688.51

Colonies were cultivating either *Attamyces* or Trachymyces fungus. Note the lower sexual production of Florida colonies growing *Attamyces* relative to colonies with other ant-fungus combinations

Table 2 F-statistics and p values for each factor in the analyses of variance of *T. septentrionalis* performance measures

	Ant population (Texas vs Florida)	Fungal species (Trachymyces vs *Attamyces*)	Ant × fungus interaction
Female number	$\mathbf{F_{1,20} = 28.82, p < 0.0001}$	$\mathbf{F_{1,20} = 9.87, p = 0.005}$	–
Male number	$F_{1,19} = 0.123, p = 0.728$	$\mathbf{F_{1,19} = 7.49, p = 0.01}$	$\mathbf{F_{1,19} = 14.62, p < 0.0001}$
Total sexual number	$\mathbf{F_{1,20} = 9.075, p = 0.006}$	$\mathbf{F_{1,20} = 13.69, p = 0.001}$	–
Female weight	$\mathbf{F_{1,20} = 17.96, p = 0.0004}$	$\mathbf{F_{1,20} = 8.81, p = 0.008}$	–
Male weight	$F_{1,20} = 0.21, p = 0.89$	$F_{1,20} = 3.51, p = 0.08$	$F_{1,20} = 11.92, p = 0.002$
Total sexual weight	$\mathbf{F_{1,20} = 14.5, p = 0.001}$	$\mathbf{F_{1,20} = 13.99, p = 0.001}$	–
Substrate accepted	$F_{1,20} = 0.256, p = 0.62$	$\mathbf{F_{1,20} = 4.99, p = 0.04}$	$\mathbf{F_{1,20} = 5.24, p = 0.03}$

Significant p values in bold ($\alpha < 0.05$). Dashed entries correspond to pooled effects ($p > 0.20$, see text for details)

female and male sexuals exhibit similar patterns. Variation in sexual biomass and number of individuals indicated significant effects of ant population and cultivated fungal species (Tables 1, 2). The production of female sexual offspring was higher in colonies from Texas and on native Trachymyces than in colonies from Florida or on *Attamyces* (Tables 1, 2). More males were produced in colonies growing Trachymyces than colonies growing *Attamyces,* and there was also an interaction between fungal species and population, which was largely driven by significantly greater male-production in Florida on Trachymyces than on *Attamyces*. Otherwise the production of males in colonies in Texas did not seem to differ (Tables 1, 2). Similar to the findings of Seal and Tschinkel (2007c), the average weight of a female alate reared on *Attamyces* was lower than field-collected queens (Seal and Tschinkel 2007a) but not lower than females reared on Trachymyces ($F_{2,\ 26} = 8.23$, $p = 0.002$, Fig. 3; Table 1).

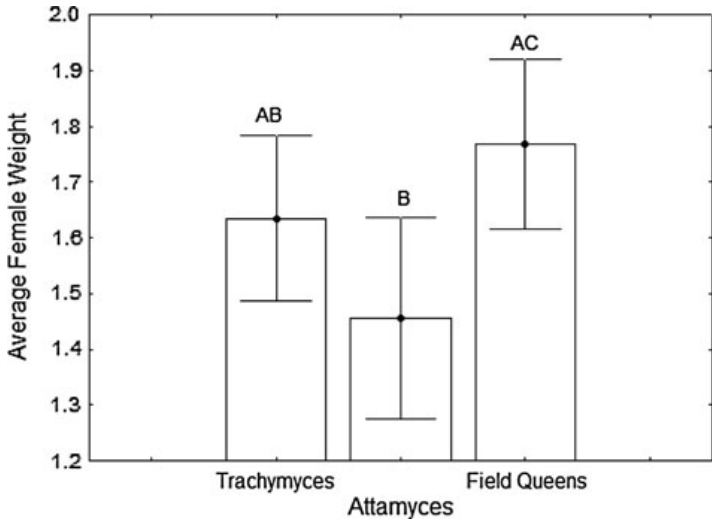

Fig. 3 Average weight of gynes (female sexual offspring) produced in the 2011 experiment compared to average weights of field-collected individuals from a previous study in Florida (data from Seal and Tschinkel 2007c). Significant differences are denoted by *different letters* ($p < 0.05$, Scheffé's test). *Error bars* indicate standard errors

Trachymyrmex turrifex

It was more obvious that *T. turrifex* colonies formed a clear synergism with their typical type of Trachymyces fungus. Similar to *T. septentrionalis*, time was a significant predictor of *T. turrifex* fungus garden volume ($F_{8,\ 144} = 33.42$, $p < 0.0001$, Greenhouse-Geisser $E_{4.62,\ 83.22} = 0.578$, $p < 0.0001$, Huynh–Feldt $E_{6.76,\ 121.74} = 0.845$, $p < 0.0001$; Fig. 4) and Trachymyces gardens were larger than *Attamyces* gardens ($F_{1,\ 18} = 148.8$, $p < 0.0001$; Fig. 4). The significant interaction between time and species of fungus largely seemed driven by an increase in volume by both fungal species over the starting volume but subsequent decreases on *Attamyces* volumes and according increases in Trachymyces gardens ($F_{8,\ 144} = 25.67$, $p < 0.0001$, Greenhouse-Geisser $E_{4.62,\ 83.22} = 0.578$, $p < 0.0001$, Huynh–Feldt $E_{6.76,\ 121.74} = 0.845$, $p < 0.0001$; Fig. 4).

Additionally, worker mortality was higher in colonies cultivating *Attamyces.* While fungal species was not significant ($F_{1,18} = 3.83$, $p = 0.07$), there was a significant interaction with time ($F_{1,18} = 102.5$, $p < 0.0001$) and time was also a significant factor ($F_{1,18} = 7.56$, $p = 0.013$).

Average colony size fell by 48 % (initial worker number—final worker number/final worker number) compared to a 275 % increase in colonies growing Trachymyces (Fig. 5). Only two *T. turrifex* colonies produced any sexual brood (2 gynes each in one colony growing Trachymyces). All colonies produced worker brood, even those growing *Attamyces*; pupae and larvae were regularly observed nestled on the *Attamyces* gardens, typical brood-rearing behavior for *Trachymyrmex*.

Atta texana positive control

The *A. texana* incipient colonies exhibited positive growth during the subsequent 7 month period. Growth was similar in both years ($F_{1,6} = 0.87$, $p = 0.38$), increased over time ($F_{6,36} = 16.09$, $p < 0.0001$) and did not interact significantly with time ($F_{6,36} = 1.55$,

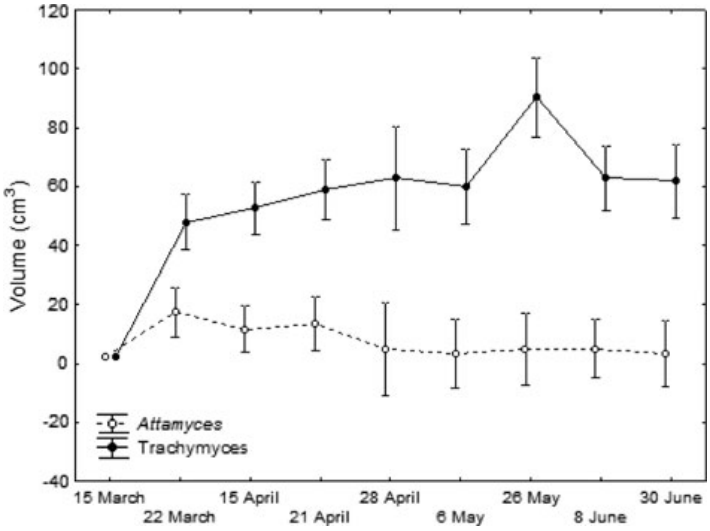

Fig. 4 Volume (cm^3) of fungus gardens in *T. turrifex* colonies growing either *Attamyces* or Trachymyces fungus during the course of the experiment. Unlike the situation in *T. septentrionalis*, *Attamyces* gardens grown by *T. turrifex* increased briefly in size but remained small during the remainder of the experiment whereas those growing Trachmyces grew and retained large gardens ($F_{1,\ 18} = 148.8$, $p < 0.0001$). The significant interaction between fungal species and time appeared to have been driven by above average garden volume of Trachymyces colonies on May 26 relative to Attamyces gardens and initial volume (15 March) ($F_{8,\ 144} = 25.67$, $p < 0.0001$, see text for corrected p values). For simplicity, not all significant pairwise comparisons are illustrated. *Filled* and *open symbols* indicate Trachymyces or *Attamyces* respectively. Data were analyzed after $\log_{10}$ transformation. *Error bar*s correspond to 95 % confidence intervals

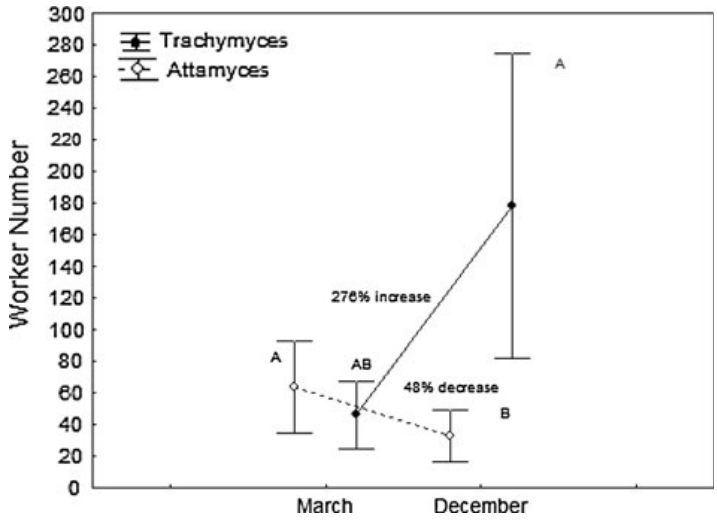

Fig. 5 Change in worker number during the course of the symbiont switch experiment on *T. turrifex* colonies. Colonies growing *Attamyces* lost biomass relative to those growing Trachymyces ($F_{1,18} = 102.5$, $p < 0.0001$). Significant differences are denoted by *different letters* ($p < 0.05$, Scheffé's test). Fresh weights of colonies did not differ in at the beginning of the experiment in March 2011, whereas by December 2011 (the end of the experiment colonies) colonies growing Trachymyces appeared to have increased their standing worker-biomass whereas colonies growing *Attamyces* experienced no growth if not slight losses in worker numbers

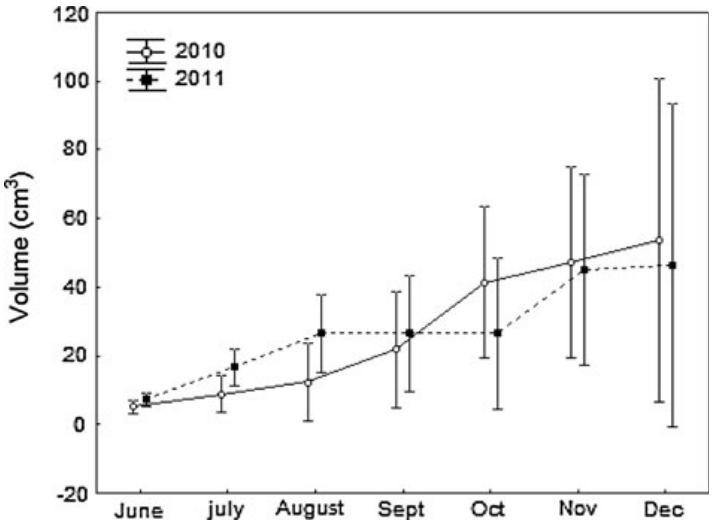

Fig. 6 Volume (cm^3) of *Atta texana* growing *Attamyces* during 7 month periods in 2010 and 2011 (n = 4 both years). Open and closes symbols correspond to newly mated queens collected in 2010 and 2011 from Hornsby Bend, Texas, respectively. *Error bars* correspond to 95 % confidence intervals. Data were analyzed after $\log_{10}$ transformation. The only significant effect is time; in both years incipient colonies exhibited positive growth over time ($F_{6,36} = 16.09$, $p < 0.0001$) and growth neither varied between years ($F_{1,6} = 0.87$, $p = 0.38$) nor interacted significantly with time ($F_{6,36} = 1.55$, $p = 0.19$)

$p = 0.19$). In other words, garden declines were not observed in *A. texana* × *Attamyces* combinations (Fig. 6).

Mycological examination of fungus gardens

Fungus garden fragments taken from declining *Trachymyrmex* gardens and inoculated on agar plates indicated that the declining gardens were primarily infected with *Syncephalastrum racemosum* (Zygomycota: Syncephalastraceae), a species known to be an important weed of higher-attine gardens (Rodrigues et al. 2008, 2009). This species was found to be present in all gardens of *T. septentrionalis* and *T. turrifex* regardless of fungal species being cultivated. *Syncephalastrum* was found in 3 colonies of *A. texana* growing *Attamyces*, but none of these showed overt signs of infection. The first hyphae of *S. racemosum* were observed growing from the fungal fragments within the first 24 h of inoculation on PDA agar. Interestingly, *S. racemosum* emerged from inocula on agar only after 48 h in the unswitched colonies, whereas it appeared within the first 24 h in switched colonies (Fig. S2). The *S. racemosum* strains were similar in appearance across all cultures of fungal fragments growing on the PDA plates. As a result, five of these were randomly chosen for DNA sequencing. ITS sequences from these five *S. racemosum* cultures were 100 % identical (*E* values ranged from $4e^{-89}$ to $4e^{-168}$) to those reported by Sen et al. (2009) (Genbank accession number FJ948130.1 and also to the ITS sequence of isolates obtained from non-ant sources at GENBANK (e.g., JQ724503.1, HM999985.1 and JN942874.1) Because the disease investigations were originally unplanned, the presence of other weedy species in gardens was not systematically evaluated, though in some colonies other fungal species such as *Fusarium oxysporum*, *Aspergillus flavus*, *Acremonium polychromum* were isolated from the same garden fragments along with *S. racemosum*. These sequences were 100 % identical to those reported by Rodrigues et al. (2011).

Discussion

One of the more striking observations in this study was the initial success of *T. septentrionalis* and *T. turrifex* colonies when experimentally switched to *Attamyces* cultivar, but these colonies performed poorly or experienced catastrophic losses of fungus gardens after a period of garden buildup typical for spring-collected *T. septentrionalis* colonies (Seal and Tschinkel 2007a, b). It would seem that *Trachymyrmex* colonies are able to grow *Attamyces* gardens and produce brood, but they seem unable to do so indefinitely under our laboratory conditions. Consequently, *Trachymyrmex* ants would appear to encounter constraints to growing novel fungal symbionts.

The observations appear at odds with those reported by Seal and Tschinkel (2007a) who did not observe any obvious consequences of such symbiont switching. *Attamyces* gardens grown by *T. septentrionalis* in that study neither crashed nor exhibited signs of infection (Seal 2006; Seal and Tschinkel 2007a). In the current study, however, the overall lower performance and sudden garden declines of switched colonies growing *Attamyces* seemed to implicate disease, because these colonies took on characteristics of unhealthy or sick colonies (Fig. S1B). One possible explanation for the different results is that a decade of keeping attine species in the same lab (in Texas) led to a buildup of pathogens that then also infected our source and experimental colonies, whereas the colonies of Seal and Tschinkel (2007a) were kept under comparatively disease-free conditions (in Florida). Another possibility is that naturally occurring (e.g., airborne) microbiota in Texas and Florida are different and generate different disease pressures. On the other hand, infections and garden declines might be an inevitable result of fungus-switching, regardless of population. If pathogen buildup has a role in the Texas laboratory, these diseases do not seem to affect *A. texana* colonies growing *Attamyces* or *Trachymyrmex* colonies growing *Trachymyces*, since neither of these combinations resulted in garden declines. Although the *A. texana* queens were collected toward the end of the experiments with *Trachymyrmex* colonies and some *A. texana* colonies were infected with *Syncephalastrum*, this did not seem to influence the growth of the *Atta* colonies. Thus ant-fungal combinations had different responses to the same laboratory environment.

Although Seal and Tschinkel (2007a) did not conclude that *T. septentrionalis* and native symbionts represented reciprocal adapations, some findings were nevertheless not at variance with the results reported here. For example, Seal and Tschinkel (2007a) reported that colonies growing *Attamyces* produced gynes leaner than gynes produced from Trachymyces, which could have set switched colonies up for a fitness disadvantage. Similarly, colonies growing *Attamyces* in the present study also were leaner, which seemed to be directly due to the catastrophic garden collapses and it was unlikely that they would have fatted up since they lacked a garden upon which to feed. Hypothetically, these two similar outcomes (leaner gynes from *Attamyces* colonies) could indicate that the colonies in Seal and Tschinkel (2007a) were also dealing with infections and had that experiment had been allowed to run longer, gardens may have crashed as well. However, these conclusions could only be supported by repeating the experiment in Florida as well as investigating the microbial communities of both populations to rule out the effects of local conditions or laboratories.

It is unclear whether the garden-invading fungus *S. racemosum* was the primary cause of garden decline or a secondary infection. Although a comprehensive survey in central Texas did not find *S. racemosum* in gardens of three attine species, including *T. septentrionalis* (Rodrigues et al. 2011), *S. racemosum* is known to occur in central Texas (Kuehn and Koehn 1988) and to infect attine colonies in southern Brazil (Rodrigues et al. 2008).

S. racemosum infected laboratory colonies of *T. zeteki* in a nearby room as our experiments (Sen et al. 2009). Moreover *S. racemosum* apparently is a cosmopolitan generalist with an affinity with soil (Miller et al. 1957; Fell and Hunter 1979; Kuehn and Koehn 1988; Migahed 2003; Saravanakuma and Kaviyarasan 2010). Thus, it is unlikely that *S. racemosum* is a specialized disease of ant fungi and is most likely a generalist competitor (Rodrigues et al. 2008). *S. racemosum* was isolated from all colonies in our experiment regardless of the type of fungus they were growing, but may have been more abundant in colonies growing *Attamyces*, because it took nearly twice as long for *S. racemosum* hyphae to appear on the agar plates. It would seem that native ant x symbiont combinations are more resistant to invasions by weeds.

Attine ants are thought to minimize infections and prevent colony failures by a combination of specialized behaviors (e.g., weeding) and chemical suppression [antibiotics and metapleural gland secretions (Fernández-Marín et al. 2006, 2009; Little et al. 2006; Hughes et al. 2008; Yek et al. 2012)]. Interestingly, the catastrophic garden failures in *T. septentrionalis* occurred when most of the brood had pupated and thus had stopped feeding. Because worker foraging tendencies decrease after colonies produce sexual pupae (presumably because the larvae have stopped feeding; (Seal and Tschinkel 2007b, 2008), it would seem also possible that general colony-level behavioral changes affected general sanitation and thus facilitated the catastrophic losses in garden volume. Interestingly, colonies seemed to recover for a short time after reactivation during the following spring (March–June 2012) and that gardening behaviors (weeding, garden buildup, etc.) can override whatever deficiencies or pathogens that lead to garden decline the previous year. Future work may wish to address whether behaviors solely are responsible for these patterns or perhaps other factors such as anti-microbial secretions are also involved.

One additional explanation of garden failure is that the microbiota of *T. septentrionalis* are mismatched with *Attamyces* garden, which may have facilitated the invasion by fungal garden weeds such as *S. racemosum*. For example, the microfungal communities associated with Trachymyces (from *T. septentrionalis*) and *Attamyces* (from *A. texana*) are rather distinct (Rodrigues et al. 2011). Similarly, *T. septentrionalis* ants and their Trachymyces gardens are known to harbor distinct bacterial communities (Ishak et al. 2011). Although the role of associated microbes is unknown in the context of the functioning of the overall symbiosis, many are known to produce antibiotics or are thought to serve nutritional roles (Currie et al. 1999b; Santos et al. 2004; Haeder et al. 2009; Oh et al. 2009; Rodrigues et al. 2009; Sen et al. 2009; Barke et al. 2010; Aylward et al. 2012a). Because antibiotics from integument-inhabiting microbes can severely suppress the growth of the cultivated fungus (Sen et al. 2009), Trachymyces fungus may be somewhat resistant to *Trachymyces* ant-microbiomes, whereas *Attamyces* fungus may be more sensitive to the same microbes when cultivated by *Trachymyrmex* ants. Furthermore, the microbial community of *Attamyces* cultivated by *A. texana* might be more conditioned to the chemical forms of weed and pathogen suppression than the biological control thought to be performed by *Trachymyrmex* ants (Fernández-Marín et al. 2009). Mismatch between cultivar and antibiotics derived from ant-associated microbiomes therefore may have stressed *Attamyces* garden in our experiments.

Regardless of the cause (inability of ants to prevent invasion of garden diseases, mismatch between ant-microbiomes and cultivar), our observations implicate a degree of specialization between *Trachymyrmex* ants and Trachymyces and that there appear to be constraints in growing novel fungal symbionts. Instability seems to be associated with interactions among the microbes associated with the ants and their fungus. Although microbial interactions have long been though to be very important in the stability of the

symbiosis especially those that depict interactions between specialized pathogens (*Escovopsis*) and antibiotic producing bacteria in the genus *Pseudonocardia* (Currie et al. 2003a, b, 2006; Cafaro et al. 2011), it is unclear whether these models are appropriate for North American *Trachymyrmex* species. Although *Pseudonocardia* is present among several other abundant microbes on the integument of *T. septentrionalis* ants (Ishak et al. 2011), *Escovopsis* is not known to occur in the range of *T. septentrionalis* (Mikheyev et al. 2008; Ishak et al. 2011; Rodrigues et al. 2011). Rather, antibiotics produced by integumental bacteria might be part of a generalized immune system employed by insects (Kaltenpoth 2009; Mueller 2012) and co-evolutionary stability between the ants and fungus may be conferred by interactions between ant-associated and fungus-associated microbiota.

The different responses to symbiont switching between *T. septentrionalis* and *T. turrifex* could be explained by differences in colony-level behaviors and social organization. *T. septentrionalis* colonies growing *Attamyces* had gardens for at least 4 weeks that were initially similar in size to colonies growing Trachymyces, whereas *T. turrifex* colonies growing *Attamyces* quickly declined and retained gardens much smaller than those growing native Trachymyces. *T septentrionalis* and *T. turrifex* are not closely related (Rabeling et al. 2007). The former belongs to the *septentrionalis*-group of *Trachymyrmex*, of which most North American *Trachymyrmex* species belong (Rabeling et al. 2007), whereas *T. turrifex* belongs to the primarily tropical *T. urichi* group (Brandão and Mayhé-Nunes 2007). Notably, *T. septentrionalis* has a colony cycle that is most likely an adaptation to a temperate environment. Gardens are miniscule during the winter and over the course of 4 weeks in early spring, the ants rapidly increase the size of their gardens and would seem to undergo pronounced changes in colony-level behavior (Seal and Tschinkel 2006, 2007b, 2008). Consequently, *T. septentrionalis* is probably under intense selection to rapidly grow a sizeable fungus garden in a relatively short time period when resources are plentiful and might have adaptations to do so, such as more intense weeding behaviors as well as different sets of microbes or types of secretions. Although not as well understood as the biology of *T. septentrionalis*, *T. turrifex* is not known to exhibit much seasonality. For example, *T. turrifex* does not exhibit pronounced dormancy during the winter months (JNS and UGM, unpublished observation) nor does it appear to exhibit complex foraging behaviors (Waller 1989). Generally, colony-level behaviors and social organization of these two species therefore may influence their different responses to growing an alien fungus.

Acknowledgments Constructive comments by the reviewers and editors greatly improved the manuscript. We also thank Andre Rodrigues for sharing his knowledge about attine-garden weeds; Walter Tschinkel for use of his laboratory; and John Crutchfield, Larry Gilbert, and Steven Gibson for facilitating the colony collections at Brackenridge Field Laboratory and at Stengl Lost Pines Field Station. The USDA Forest Service allowed us to collect colonies in the Apalachicola National Forest. Funding was provided by NSF award IOS-0920138 to JNS and UGM.

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