Functional Ecology 2007
21, 988–997

Co-evolution and the superorganism: switching cultivars does not alter the performance of fungus-gardening ant colonies

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Summary

1. The fungus-gardening ants and their fungi represent a highly co-evolved, vertically transmitted mutualism. Mutualisms such as these are thought to be reciprocal antagonisms, so that the ants and the fungus can be expected to have some conflicting interests.
2. This paper reports the results from a cultivar switch experiment that documented the effects of switching the native cultivar from the basal 'higher-attine' *Trachymyrmex septentrionalis* with a derived cultivar from the leaf-cutting ant, *Atta texana*. If the cultivars have been modified significantly during the adaptive radiation of this clade, then they should differ in their ability to produce ant and fungal biomass. If the ants can perceive differences in cultivar performance, then their food preference may also change as a result of the switch. Lastly, if conflict is present in this mutualism, then the sex ratio in the switched colonies should be male biased.
3. Our results showed that food preference was not altered by the new cultivar. The *A. texana* cultivar did not change the performance of *T. septentrionalis* colonies relative to colonies that were cultivating conspecific fungal cultivars. Foragers preferred insect faeces and oak staminate flowers over fresh leaves or flowers. Most variation in colony performance was attributable to substrate type. Sex ratio of offspring was not affected by fungal cultivar. With respect to fungal growth, the *A. texana* cultivar appeared to be more of a generalist, while the *T. septentrionalis* cultivar performed better on substrates preferred by the ants than those unpreferred.
4. The results of this study indicate that cooperation and not conflict has been more important in shaping the evolutionary ecology of this mutualism. Although the cultivars were certainly genetically and physiologically distinct, these differences did not account for variation in the production of ant biomass or ant behaviour. The emerging picture thus indicates that this mutualism should be viewed as a highly integrated superorganism that is more than the sum of its parts.

Key-words: Attini, colony performance, frass, food preference, mutualism

Functional Ecology (2007) **21**, 988–997
doi: 10.1111/j.1365-2435.2007.01294.x

Introduction

Mutualisms are currently viewed as reciprocal antagonisms in which the species comprising the mutualism are exploiting the others, but all actors receive some benefit (Bronstein 1994, 2001). Some of the best-studied obligate mutualisms support this view. For example, figs and yuccas must sacrifice some of their seeds to the specialist pollinators (fig wasps and yucca moths, respectively) in order for successful pollination to occur (Bronstein 2001). This view has recently been applied toward the agricultural insects that have co-evolved mutualisms with specific strains of fungi (Herre *et al.* 1999; Mueller *et al.* 2001; Mueller 2002). These insects are obligately dependent on specific fungi that they cultivate inside their nests on dead vegetation, fresh leaves, flowers, fruit pulp or insect faeces (Martin 1987; Mueller & Gerardo 2002; Mueller *et al.* 2005). The fungus digests these plant materials into compounds that the insects can easily metabolize (Martin 1987).

In the case of fungus-gardening ants (tribe Attini), Mueller (2002) proposed that the fungi are not passive and have domesticated the ant as much as the ants have domesticated the fungus. He outlined potential bases of conflict between the ants and their fungal cultivar.

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Accordingly this mutualism evolved because the fungus used the ants as a dispersal agent while the ants used the fungus as food. Founding queens take a piece of the parental garden tucked into their infrabuccal pockets (Quinlan & Cherrett 1978; Little *et al.* 2003) prior to mating flights. The ant fungi appear to be closely related to non-cultivated soil and litter fungi, and many Hymenoptera typically contain fungal spores in their infrabuccal pockets (Mueller *et al.* 2001).

Cultivar switch experiments have been proposed as tools to test conflict and cooperation (Mueller 2002; Mehdiabadi, Hughes & Mueller 2006). Since the fungus is propagated only by female offspring, it would be in the fungus' interest to produce female biased brood as early as possible during colony life because males and workers are not directly involved in the propagation of new fungus gardens. Specifically there should be a shift toward male-biased brood, because the ants will act as if they were 'still engaged in a tug-of-war with [their] native cultivar'. However, this time their cultivar will essentially be 'impotent' to some degree because it has not had time to evolve mechanisms to deal with ants' divergent interests (Mueller 2002).

Alternatively, conflicts present during the evolution of this mutualism may have largely disappeared during its subsequent adaptive radiation. Vertically transmitted mutualisms are thought to lack conflict because neither member has an incentive to sabotage the other – both benefit from successful reproduction (Herre *et al.* 1999). The mutualism between ants and fungus might be best described as a macroscopic endosymbiosis or superorganism, much in the way eukaryotic cells evolved from merging of prokaryotic cells (Margulis & Sagan 2002; Dyall, Brown & Johnson 2004).

Evidence for either metaphor is largely lacking, though some evidence suggests little role for conflict. A recent cultivar switch study among related species of *Cyphomyrmex* did not produce expected alteration of sex ratio or timing of reproduction (Mehdiabadi *et al.* 2006). Furthermore, the conflict model implicitly assumes that the co-evolution of ants and cultivars has been reciprocal (i.e. one-to-one); however horizontal transfer of cultivars is possibly widespread throughout the major clades of the attini (Green, Mueller & Adams 2002; Mikheyev, Mueller & Abbott 2006). Many cultivar lineages have been reported to be interchangeable among the ant genera, especially among the higher attines (Weber 1972; Stradling & Powell 1986; Mehdiabadi *et al.* 2006), yet few experiments have documented the effects of such switches, other than those by Mehdiabadi *et al.* (2006).

This paper investigated colony performance when the cultivar of the fungus-gardening ant, *Trachymyrmex septentrionalis* McCook, was switched to a cultivar from the leaf-cutting ant, *Atta texana* Buckley. This study tested for evidence of ant–fungus conflict as envisioned by Mueller (2002) by testing for a male biased sex ratio in colonies cultivating a 'new' cultivar. Additionally this study examined the possibility that the cultivar can modify ant behaviour. This project built on an earlier finding (Seal 2006; Seal & Tschinkel 2007a) that *T. septentrionalis* foragers did not prefer fresh vegetation. One possibility was that non-leaf cutting fungi are not adapted for an exclusive diet of leaves, a diet that is more suitable for the leaf cutting fungi (Powell & Stradling 1986; Martin 1987). Therefore, *T. septentrionalis* foragers from colonies cultivating the *A. texana* fungus may prefer leaves.

Materials and methods

STUDY SPECIES

Trachymyrmex septentrionalis is a common ant in eastern North America, occupying a region that extends from south Florida and Texas to 40° N (Weber 1972). This species is among the most abundant ants in longleaf pine sandhills of the Apalachicola National Forest (ANF) in northern Florida – a hectare may contain over 1000 nests (Seal & Tschinkel 2006). This ant has a seasonal phenology with a dormant period in the winter (November to March) and with sexual production occurring during the spring (May to June) (Seal & Tschinkel 2007b). Therefore experiments performed in the spring should have consequences for the production of sexual brood, a direct correlate of fitness.

All *T. septentrionalis* colonies were collected in the Wakulla District of the Apalachicola National Forest *c.* 15 km south of Tallahassee, FL (30°22′ N, 84°22′ W) in early March 2003, just after the ants had ended their winter dormancy. The *A. texana* cultivars came from two incipient colonies collected in August 2003 from the Kisatchie National Forest (Evangeline District), Rapides Parish, LA (31°14′ N, 92°37′ W). *Atta texana* is a leaf-cutting ant found in upland pine forests in western Louisiana and southeastern Texas (Moser 2006). Although there are notable differences between longleaf pine forests west and east of the Mississippi River (Harcombe *et al.* 1993; Peet & Allard 1993), the habitat of *A. texana* is comparable to *T. septentrionalis* habitat in north Florida because both are characterized by deep sandy soils and plant species adapted to xeric conditions. Therefore *A. texana* ants are likely to feed their fungus substrates similar to those found in Florida.

COLLECTION AND MAINTENANCE OF COLONIES

All *T. septentrionalis* colonies were collected by excavating a 1-m^3 pit *c.* 30 cm from the nest entrance. Tunnels and fungus garden chambers were found by carefully removing soil from the face of the pit toward the entrance with a trowel and kitchen spoons. All tunnels were followed until all ants had been collected. This entire process took about 45–90 min per colony and resulted in a > 95% chance of collecting the queen of this monogyne (single-queened) species.

Colonies were housed in the laboratory under standard conditions. Each colony was housed in a tray coated with Fluon © (Northern Products, Woonsocket, RI) along the sides to prevent escapes. The ants grew their garden in a cylindrically-shaped, 175 cm^3 depression in a polystyrene box lined with dental plaster. The top of this chamber was completely covered with a piece of Plexiglas. Two 9 mm diameter holes were drilled in the side of each plaster nest for the ants to enter and exit the fungus garden chamber. As colonies grew larger, additional plaster nests were added and interconnected with 5 cm segments of clean, rubber hoses. A 10 mm test tube half filled with water and plugged with cotton was placed in each tray. The plaster nest was watered weekly by filling each of four 9 mm diameter holes located in each of the four corners.

SUBSTRATES

Upon collection, fungal substrates were stored in the freezer (–20 °C). This experiment used four substrates that were collected about the same time as the ant colonies. Frass (faecal pellets) from eastern tent caterpillars (*Malacosoma americanum* F. (Lasiocampidae)) was obtained by rearing several colonies on cherry leaves (*Prunus serotina* Ehrh.). Catkins (staminate flowers) were obtained from bluejack oak (*Quercus incana* Bartr.). Flowers came from eastern redbud (*Cercis canadensis* L.) trees and leaves from early spring growth of bluejack oak trees. All of these plant types were collected from sandhill sites in the ANF.

PREFERENCE DETERMINATION

The substrate preference of foraging ants was conducted after the colonies had been acclimated to laboratory conditions (*c.* 5–7 days). Preferences were conducted a second time to monitor the effects of cultivating a new cultivar – about 1 month after the first preference tests (or 3 weeks after the cultivar switch). In all instances, preference thus refers to the substrates that the ants collected preferentially over others. The preference of the fungus was not measured in this study.

Preferences were determined by exposing 40 colonies to equal substrate amounts of substrate pairs. Pairs were composed of one substrate thought to be preferred and one unpreferred substrate. Preferences were inferred by counting the number of pieces (pieces of frass, catkin bud, or ant-sized snippets of leaf or flower) removed by foragers from waxed weighing papers placed near the nest entrance. Choices were inferred when an ant carried a piece off the paper. At this point the ant and the substrate item were removed temporarily to a box outside the tray, so that neither the substrate nor the ant could influence the behaviour of other ants. The order in which each colony was tested was random.

Replicated goodness-of-fit tests (*G*-Test, (Sokal & Rohlf 1995)) determined whether the preferences of foragers were consistent with patterns indicated by prior studies (Seal 2006; Seal & Tschinkel 2007a) and to determine whether preferences were statistically consistent across all colonies. Replicated goodness-of-fit tests are analogous to analyses of variance because they test for significant variation within (G_H (heterogeneity)) and among experimental units (G_P (pooled)). Specifically they test whether G_H adds significant variation to the total (G_T).

EFFECTS OF SUBSTRATES ON GARDEN AND ANT COLONY PERFORMANCE

Colonies were fed daily *ad libitum* one type of substrate by placing the substrates on wax paper near the nest entrance. Colonies received either substrate that was preferred or unpreferred. Wet weights of substrates were converted to dry weights using constants obtained by drying small amounts of substrates for 48 h under ambient conditions. Amounts not collected by the ants after 2 days, as well as pieces deposited in the refuse piles were collected and weighed. In this way it was possible to measure the amount of substrate collected by the ants and therefore consumed by the fungus garden.

Feedings were conducted until new offspring (sexuals and new workers) emerged and could be seen walking about the fungus garden. At this point colonies were killed by freezing, their contents sorted by hand under a microscope and subsequently dried in an oven, weighed and counted.

Response variables

MEASURES OF ANT PERFORMANCE

The main response variables were the total weights, energetic contents and average percent fat of ant offspring. Energetic content of brood was obtained by extracting the body fat from adult ants in a Soxhlet extractor using diethyl ether for 48 h (Soxhlet 1879). Energetic contents of ant biomass were obtained by multiplying lean weights by 18·87 J/mg and fat weights by 39·33 J/mg (Peakin 1972) and summing. A maximum of 10 female, male and new worker offspring were chosen from each colony for extraction. The actual number of sexuals extracted in each colony depended on how many developed offspring were present. Only the darkest females, males and new workers in each nest were selected. This was deliberately non-random since female alates are known to dramatically increase their weight and fat content from eclosion to dispersal (Tschinkel 1993). Female fat contents were specifically compared to Seal & Tschinkel (2007b) who reported a mean value of 25% body fat for *T. septentrionalis* newly mated queens. This provided an estimate of the maturity of female offspring in each colony and the probable readiness of the colony to reproduce.

MEASURES OF FUNGAL PERFORMANCE

Fungus garden traits included fungus garden weight and an estimate of the amount of chitin (percent and total amount). Chitin is the main constituent of fungal cell walls and its quantity in a substrate is frequently used as an indicator of fungal biomass in soil or wood, among other substrates (Plassard, Mousain & Salsac 1982). Moreover the ant fungi do not appear capable of digesting chitin readily (Martin 1987), making this structural compound essentially a metabolic dead-end. Therefore chitin was used to estimate the total amount of fungal biomass that resulted from the experimental manipulations in this study.

The chitin assay we used tested free aldehydes resulting from the acid (6N HCl) hydrolysis of chitin and subsequent deamination of the glucosamine residues by nitrous acid (HNO_2) (Plassard *et al.* 1982; Vignon *et al.* 1986). Free aldehydes form a stable complex with MBTH (3-Methyl-2-benzothiazolone hydrazone hydrochloride), which turns blue in the presence of ferric chloride ($FeCl_3$). The assay samples were then read in a Beckman–Coulter DU 640 Spectrophotometer at 650 nm. The amount of chitin in each sample was estimated by interpolating the absorbance of each sample onto a standard curve constructed by subjecting five dilutions (range: 0·0625–1·0 mg mL^{-1}) of purified chitin (Sigma-Aldrich, St Louis, MO) to the procedure outlined above.

EFFECTS OF SUBSTRATES ON GARDEN AND ANT COLONY PERFORMANCE

Forty *T. septentrionalis* colonies were used in this experiment. Twenty received one of two *A. texana* cultivars and the other 20 received one of two *T. septentrionalis* cultivars, the latter half representing control switches to account for the effect of removing a colony's cultivar and replacing it with another. The *T. septentrionalis* cultivars came from laboratory stock colonies collected from the same general vicinity as the experimental colonies.

Cultivar switches were completed as follows. Fungus gardens were removed with sterile forceps and replaced with a standardized piece (*c.* 1 cm^3) of either *A. texana* or *T. septentrionalis* cultivar. Since fungus gardens in this seasonal species are extremely small in early spring; the new garden was several times larger (5–10 times, Seal & Tschinkel 2007b) than their original garden. Consequently it was easy to observe the fate of this garden in their plaster nests. As a precaution, small nuclei of fungus garden material tended by ants away from this garden were periodically removed with sterile forceps.

In addition to conducting preference tests after the colonies had been acclimated to laboratory conditions, preferences were evaluated after the colonies had been switched onto the new cultivar, or *c.* 3 weeks into the experiment.

Molecular verification of switches

DNA EXTRACTION, PCR AND SEQUENCING

Approximately 1–2 weeks before the end of the experiment, small portions of fungus garden material were removed from each colony in the experiment and placed in RNAlater (Ambion, Inc.) and stored at –20 °C. For DNA extraction, *c.* 50–75 mg of garden material (~100 µL volume) was removed from the DNALater, freeze-dried and pulverized. Total DNA was extracted by the method of Rehner & Buckley (2003) and adjusted to a final concentration of –5 ng/µL.

The nuclear ribosomal internal transcribed spacer (ITS) sequence was determined for each cultivar. The ITS was amplified by the polymerase chain reaction and sequenced with primers ITS5 (5′-GGAAGTAAAAGTCGTAACAAGG) and ITS4 (5′-TCCTCCGCTTATTGATATGC) (White *et al.* 1990) using the amplification protocol of Rehner & Buckley (2005). The PCR products were fractionated on a 1·5% NuSieve agarose gel (BioWhittaker, Rockland, ME) in a low EDTA Tris–acetate buffer (40 mM Tris–acetate, 0·1 mM EDTA) and extruded from the gel by centrifugation after two freeze-thaw cycles.

Sequencing reactions were performed with ABI BigDye 2·0 (Applied Biosystems, Foster City, CA) according to the manufacture's instruction Cycle sequencing products were separated from residual reaction components by ethanol precipitation suspended in deionized formamide, heat denatured, and run on an ABI 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA). DNA sequences were assembled and edited using Sequencher 4·1 (Gene Codes Corp., Ann Arbor, MI) and multiple sequence alignments were constructed with the MegAlign module of dnastar 5 (LaserGene, Madison, Wisconsin).

STATISTICAL ANALYSIS

All analyses were conducted with Statistica version 6·1 (Statsoft 2003). Measures of ant and fungal performance were analysed with full model anova that consisted of cultivar, preference, cultivar × preference interaction and substrate nested within preference. This design is a nested factorial and as such, the nested factor is a random factor (Sokal & Rohlf 1995). Effects were removed from the model (pooled) if they produced *P*-values > 0·25 in the procedure outlines by Underwood (1997). Generally, the nested terms and preference × cultivar interactions were pooled, but in some cases the main effect of preference was not significant but significant variation nevertheless occurred among substrates. In this instance, the model consisted of cultivar, substrate and the interaction between the two. Power analyses were conducted using *G*-Power (Faul & Erdfelder 1992). Data were $\log_{10}$ or square root transformed to meet parametric assumptions, except for percentages (sex ratio), which were arc–sin square root transformed.

Results

SWITCH SUCCESS

DNA yield and quality from samples from two colonies had degraded and were therefore unsuitable for PCR. BLAST analysis of the ITS sequence obtained from colonies cultivating the *A. texana* cultivar was an identical or near-identical match to sequences from other *Atta* cultivars (e.g. *Leucoagaricus gongylophorus* AY 642802). The two *T. septentrionalis* ITS types shared 46·5% and 43·4% identity to the *A. texana* cultivar ITS, and 75·7% identity to one another. No closely matching sequences for either *T. septentrionalis* cultivar is currently deposited in GenBank. However, the top 20 BLAST hits to either *T. septentrionalis* ITS sequence types had *E* values ranging from $7e^{-80}$ to $2e^{-77}$ and were to species of Agaricales, including several other attine fungal cultivars and free-living species of *Leucoagaricus*. Also, the ITS of both *T. septentrionalis* cultivars closely matched sequences from other North American *T. septentrionalis* cultivars isolated into pure culture (S.A. Rehner, unpublished data). Therefore 18 out of 20 *T. septentrionalis* colonies were without a doubt cultivating the *A. texana* cultivar and the remaining 20 were cultivating the *T. septentrionalis* cultivar. These two colonies were excluded from further analyses (see below). The switch method used in this study therefore had a success rate ≥ 90%.

PREFERENCES

Preferences were clearly distinct. Foragers prefer oak catkins (415 pieces collected (47%)) and caterpillar frass (291 pieces, 33%) over oak leaves (81 pieces, 9%) and redbud flowers (98 pieces, 11%) ($G_P = 342{\cdot}3$, df = 1, $P < 0{\cdot}0001$; $G_T = 417$, df = 1, $P < 0{\cdot}0001$). Exceptions occurred when two colonies gathered more frass than leaf pieces but were otherwise not statistically significant (18 frass *vs* 10 leaf pieces, $G = 2{\cdot}2$, df = 1, $P > 0{\cdot}12$; 11 frass *vs* 4 leaf bits, $G = 3{\cdot}4$, df = 1, $P > 0{\cdot}06$) or when one colony failed to exhibit a preference (five pieces each of catkins and flowers). Nevertheless no colonies preferred leaves or flowers; therefore the test of heterogeneity among colonies was not significant ($G_H = 30{\cdot}4$, df = 39, $P > 0{\cdot}73$). A single exception occurred when one colony chose eight pieces of catkin and two pieces of flower ($G = 3{\cdot}85$, df = 1, $P = 0{\cdot}05$).

The cultivar switch did not influence the preference behaviour of the ants. Approximately 3 weeks after the cultivar switch, colonies still preferred catkins (270 pieces collected, 44%) and frass (252 pieces, 41%) over leaves (50 pieces, 8%) and flowers (46 pieces, 7%). Therefore, the switch did not result in the expected change of preferences ($G_P = 337$, df = 1, $P < 0{\cdot}0001$; $G_T = 436$, df = 1, $P < 0{\cdot}0001$). Although the test of heterogeneity among colonies was significant ($G_H = 99{\cdot}7$, df = 37, $P < 0{\cdot}0001$), no colonies changed their preferences to leaves or flowers. Only one colony cultivating the *A. texana* fungus failed to exhibit a significant preference by collecting equal amounts of frass and oak leaves. The seven remaining colonies that failed to exhibit a preference were cultivating the *T. septentrionalis* cultivar. Two of these collected equal amounts and the five other collected more preferred substrates, but not significantly.

ADJUSTING FOR AN IMBALANCED DESIGN

The imbalanced design produced by excluding two colonies was dealt with using a method recommended by Underwood (1997). 'Dummy' variables were created by taking the mean values for each group to which the missing data belonged. This altered neither the mean nor the variance in each of these groups (Underwood 1997); however, the error degrees of freedom for each test involving these colonies were reduced by two.

PERFORMANCE MEASURES: ANT BIOMASS WEIGHTS AND ENERGETICS

The effect of the *A. texana* cultivar on the production of *T. septentrionalis* ant biomass (sexual and new worker brood) was minimal; most significant variation was attributable to preference (Table 1). Colonies produced *c.* 40% more ant biomass on preferred substrates than on unpreferred (246 ± 95 *vs* 173 ± 98 mg (mean ± 1 SD), respectively). Similarly, the energetic content of brood was not significantly affected by cultivar, rather preferred substrates yielded more Joules of sexual and new worker brood than unpreferred substrates (4131 ± 2051 *vs* 2609 ± 2448 J, respectively; Table 1). However, the *A. texana* cultivar was just as productive on preferred (202 ± 87 mg) and unpreferred substrates (187 ± 91 mg), whereas the *T. septentrionalis* cultivar produced more ant brood on preferred substrates (290 ± 85 mg) than unpreferred substrates (159 ± 108 mg) (significant interaction term, Table 1). This suggests that leaf-cutting ant cultivar is better able to convert fresh plant tissue into ant biomass than *T. septentrionalis* cultivars.

Seventeen of the eighteen colonies growing an *A. texana* cultivar produced sexual offspring, all of which produced female sexuals. Eight of these colonies produced only female brood. Eighteen of the colonies growing *T. septentrionalis* cultivar produced sexuals but only fourteen of these produced any females, with four producing only female brood. Male production in other words appeared to be more abundant in colonies growing a *T. septentrionalis* cultivar, contrary to the suggestion of Mueller (2002).

Sexual biomass was not significantly affected either by cultivar type or preference (Table 1). Most variation in sexual production was attributable to substrate type within preference and an interaction between preference and cultivar. These effects appear to be due to lower amounts of sexual biomass produced on oak leaves, than on flowers, catkins or frass. More sexual

Table 1. *F*-statistics and *P*-values from analyses of variance for most colony performance measures. Interactions or nested terms with *P*-values > 0·25 were pooled. Significant tests ($\alpha = 0{\cdot}05$) are highlighted in bold

Variable	Cultivar	Preference	Substrate (preference)	Cultivar × Preference
Ant biomass (g)	$F_{1,34} = 0{\cdot}57$, $P > 0{\cdot}45$	**$F_{1,34} = 4{\cdot}87$, $P < 0{\cdot}05$**	$F_{2,34} = 1{\cdot}48$, $P > 0{\cdot}24$	$F_{1,34} = 3{\cdot}74$, $P = 0{\cdot}06$
Ant energetic content (J)	$F_{1,34} = 0{\cdot}09$, $P > 0{\cdot}75$	**$F_{1,34} = 5{\cdot}77$, $P < 0{\cdot}05$**	$F_{2,32} = 0{\cdot}88$, $P > 0{\cdot}42$	**$F_{1,34} = 4{\cdot}48$, $P < 0{\cdot}05$**
Sexual biomass (g)	$F_{1,32} = 0{\cdot}11$, $P > 0{\cdot}74$	$F_{1,2} = 2{\cdot}02$, $P > 0{\cdot}29$	**$F_{2,32} = 4{\cdot}02$, $P < 0{\cdot}05$**	**$F_{1,32} = 4{\cdot}91$, $P < 0{\cdot}05$**
Female biomass	$F_{1,32} = 0{\cdot}40$, $P > 0{\cdot}53$	$F_{1,2} = 2{\cdot}38$, $P > 0{\cdot}26$	$F_{2,32} = 3{\cdot}17$, $P > 0{\cdot}06$	**$F_{1,32} = 4{\cdot}2$, $P < 0{\cdot}05$**
Male biomass	$F_{1,30} = 0{\cdot}86$, $P > 0{\cdot}35$	$F_{1,2} = 1{\cdot}09$, $P > 0{\cdot}41$	$F_{2,32} = 1{\cdot}3$, $P > 0{\cdot}29$	$F_{1,32} = 0{\cdot}55$, $P > 0{\cdot}47$
% male biomass	$F_{1,34} = 2{\cdot}47$, $P > 0{\cdot}12$	$F_{1,34} = 1{\cdot}66$, $P > 0{\cdot}20$	$F_{2,32} = 0{\cdot}89$, $P > 0{\cdot}42$	$F_{1,34} = 1{\cdot}51$, $P > 0{\cdot}22$
Fungus garden weight	**$F_{1,28} = 5{\cdot}71$, $P < 0{\cdot}05$**	**$F_{1,28} = 44{\cdot}2$, $P < 0{\cdot}001$**	$F_{2,26} = 0{\cdot}60$, $P > 0{\cdot}56$	**$F_{1,28} = 4{\cdot}96$, $P < 0{\cdot}05$**
Percent chitin	**$F_{1,26} = 5{\cdot}52$, $P < 0{\cdot}05$**	$F_{1,2} = 0{\cdot}05$, $P > 0{\cdot}85$	**$F_{2,26} = 12{\cdot}73$, $P < 0{\cdot}01$**	–
Total chitin	$F_{1,32} = .004$, $P > 0{\cdot}95$	$F_{1,2} = 4{\cdot}87$, $P > 0{\cdot}15$	**$F_{2,26} = 5{\cdot}89$, $P < 0{\cdot}01$**	**$F_{1,32} = 8{\cdot}79$, $P < 0{\cdot}001$**
Ant biomass × substrate^{-1}	$F_{1,30} = 0{\cdot}03$, $P > 0{\cdot}87$	$F_{1,2} = 1{\cdot}36$, $P > 0{\cdot}25$	$F_{3,30} = 0{\cdot}682$, $P > 0{\cdot}40$	$F_{3,30} = 0{\cdot}512$, $P > 0{\cdot}48$
Ant (J) × substrate^{-1}	$F_{1,30} = 0{\cdot}39$, $P > 0{\cdot}54$	$F_{1,2} = 0{\cdot}29$, $P > 0{\cdot}59$	$F_{3,30} = 0{\cdot}17$, $P > 0{\cdot}91$	$F_{3,30} = 1{\cdot}13$, $P > 0{\cdot}35$
Chitin × substrate^{-1}	$F_{1,30} = 0{\cdot}802$, $P > 0{\cdot}37$	$F_{1,2} = 3{\cdot}55$, $P > 0{\cdot}20$	**$F_{2,32} = 5{\cdot}89$, $P < 0{\cdot}01$**	–

output was produced in *T. septentrionalis* cultivars receiving preferred substrates and all *A. texana* cultivars than *T. septentrionalis* cultivars receiving unpreferred substrates (Fig. 1). This pattern was mirrored in the production of female biomass, which did not vary due to cultivar or preference; rather most variation appears attributable to low production in the *T. septentrionalis* cultivars fed leaves or flowers (unpreferred substrates, Table 1). Male biomass on the other hand did not vary between cultivars, substrates or the interaction between cultivars and substrates (Table 1).

Neither cultivars nor substrates appear to have affected the sex ratio (percent total male biomass/(female + male biomass)) of sexual offspring. Although more male biomass was produced in colonies growing the *T. septentrionalis* cultivar (25 ± 9%) (mean ± SE), range: 0%–100%) than in those cultivating the *A. texana* cultivar (9 ± 5%, range: 0%–96%), these results were not statistically significant (Table 1). The power of the cultivar test was rather low; however, with a value of *c.* 0·25. Therefore the probability of making a type II error was high (concluding that there is no trend when one in fact exists) ($B = 1 - 0{\cdot}25 = 0{\cdot}75$). Even if there were a significant trend in the data here, it would be in the opposite direction than indicated by Mueller (2002), since the sex ratio is male biased in the colonies on the conspecific cultivar.

Female alate offspring were on average fatter in colonies growing the *T. septentrionalis* cultivar (20·3 ± 6% (*A. texana*) *vs* 25·5 ± 5% (*T. septentrionalis*) ($F_{1,29} = 5{\cdot}57$, $P < 0{\cdot}05$, $r^2 = 0{\cdot}15$)). This pattern was also observed in the average percent fat of male offspring (5·4 ± 4·2% (*A. texana*) *vs* 9·4 ± 4·1% (*T. septentrionalis*) ($F_{1,17} = 4{\cdot}66$, $P < 0{\cdot}05$, $r^2 = 0{\cdot}22$)). Preference type was not a significant effect (22·3 ± 7% (preferred) *vs* 22·7 ± 6% (unpreferred) ($F_{1,31} = 0{\cdot}03$, $P > 0{\cdot}86$)). Although missing cells prevented adequate analysis of substrates and interactions, it appears that variation in fat content is mostly attributable to cultivar.

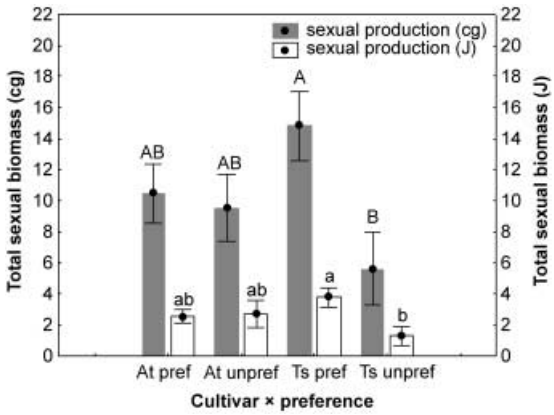

Fig. 1. Mean total sexual biomass (± SE) measured as dry weight (cg) and Joules. Categories indicate each cultivar and preference combination. Shaded bars indicate centigrams of sexual biomass and unshaded bars indicate energetic values (J). Of the 18 colonies cultivating the *Atta texana* cultivar, eight were receiving preferred substrates and 10 the unpreferred substrates. Of the remaining 20 colonies cultivating the *Trachymyrmex septentrionalis* cultivar, 10 received preferred substrates and the other 10, unpreferred substrates. Significant differences are denoted by different letters ($P < 0{\cdot}05$, Tukey's HSD tests).

FUNGUS GARDEN TRAITS

Fungus gardens were heaviest in colonies receiving preferred substrates relative to those receiving unpreferred substrates (9·23 ± 4 *vs* 3·63 ± 1·96 g, respectively, Table 1). Fungus gardens growing the *T. septentrionalis* cultivar were also heavier than gardens growing the *A. texana* cultivar (7·65 ± 5·26 *vs* 5·21 ± 2·37 g, respectively; Table 1). Substrates were pooled because they exhibited little variation other than that due to preference (Table 1). *Trachymyrmex septentrionalis* and *A. texana* fungus gardens receiving unpreferred substrates were the lightest, whereas the heaviest gardens were *T. septentrionalis* gardens receiving preferred

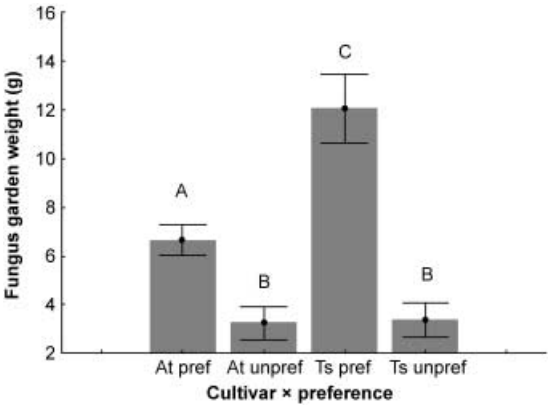

Fig. 2. Mean (± SE) fungus garden weight (g) by each cultivar and preference combination. Of the 18 colonies cultivating the *Atta texana* cultivar, eight were receiving preferred substrates and 10 the unpreferred substrates. Of the remaining 20 colonies cultivating the *Trachymyrmex septentrionalis* cultivar, 10 received preferred substrates and the other 10, unpreferred substrates. Significant differences are denoted by different letters ($P < 0{\cdot}05$, Tukey's HSD tests).

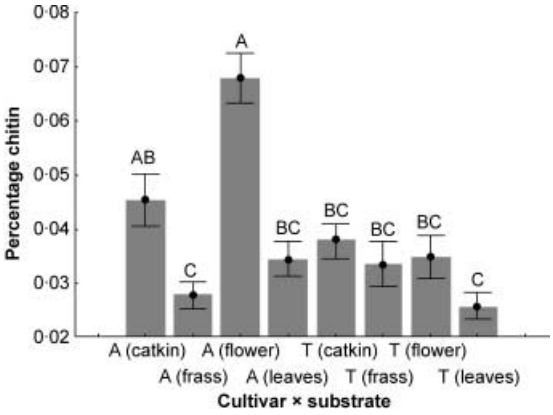

Fig. 3. Mean (± SE) percent chitin in each fungus garden by cultivar substrate combination. Significant differences are denoted by different letters ($P < 0{\cdot}05$, Tukey's HSD tests). A, *A. texana* cultivar; T, *T. septentrionalis* cultivar; cat, catkins, and so on. In all groups depicted here, $n = 5$, except $n = 4$ for colonies cultivating the *Atta texana* cultivar receiving catkins and frass.

substrates (significant interaction term, Table 1). *Atta texana* cultivars receiving preferred substrates were slightly lower but above the unpreferred substrates (Fig. 2).

Colonies growing the *A. texana* cultivar contained a higher percent chitin than colonies growing the *T. septentrionalis* cultivar (4 ± 2% *vs* 3 ± 1%, respectively; $F_{1,26} = 5{\cdot}52$, $P < 0{\cdot}05$). Variation in percent chitin was influenced mainly by substrate nested within preference ($F_{2,26} = 12{\cdot}73$, $P < 0{\cdot}001$) that was independent of preference ($F_{1,26} = 0{\cdot}05$, $P > 0{\cdot}85$), making it apparent that the substrates were not uniform within preference. For this reason, the effect of preference was dropped. Subsequent analysis for this variable was conducted on a model that contained the effects of cultivar, substrate and their interaction. Cultivar type interacted in a complex fashion with substrate so that the best substrate and cultivar combination for the production of chitin was the *A. texana* cultivar growing on catkins or redbud (two-way ANOVA, $F_{3,30} = 6{\cdot}37$, $P > 0{\cdot}01$, adjusted $r^2 = 0{\cdot}66$; Fig. 3). The lowest percent chitin was found in the *A. texana* cultivar growing on frass and *T. septentrionalis* cultivar growing on oak leaves. Notably differences were found between the two cultivars when grown on the same substrate. The *A. texana* cultivar contained about double the percent chitin found in *T. septentrionalis* cultivars when growing on redbud flowers.

Fungus gardens of both cultivar types did not differ significantly in terms of total chitin content (0·218 ± 0·12 (*A. texana*) *vs* 0·264 ± 0·207 (*T. septentrionalis*)) (Table 1). Although preferred substrates did not produce significantly more chitin, there was significant variation among the substrates (Table 1) so that more chitin was produced in the *T. septentrionalis* gardens fed preferred substrates than those fed unpreferred

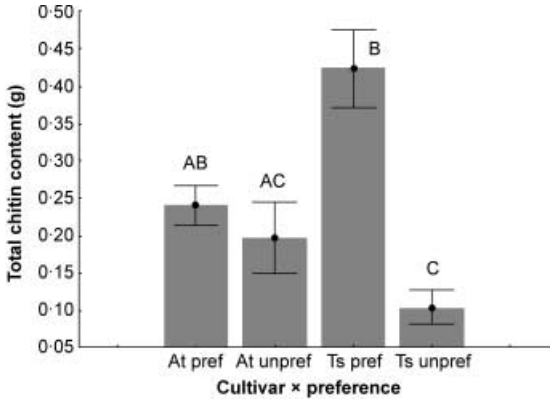

Fig. 4. Mean (± SE) total chitin amount in each fungus garden by cultivar preference combination. Significant differences are denoted by different letters ($P < 0{\cdot}05$, Tukey's HSD tests). A, *Atta texana* cultivar; T, *T. septentrionalis* cultivar; cat, catkins, and so on. In all groups depicted here, $n = 10$, except $n = 8$ for colonies cultivating the *Atta texana* cultivar on preferred substrates.

substrates of either cultivar (Fig. 4). The *A. texana* cultivars produced similar amounts of fungal biomass on preferred and unpreferred substrates. Unpreferred substrates on both cultivars were not significantly different.

EFFICIENCIES OF CULTIVARS AND SUBSTRATES IN PRODUCING FUNGAL AND ANT BIOMASS

It appears that each substrate and cultivar is just as efficient at producing ant biomass, on a weight or energetic basis. The efficiency of the production of ant biomass or energetic content per gram of substrate did not vary significantly with regard to cultivar, substrate

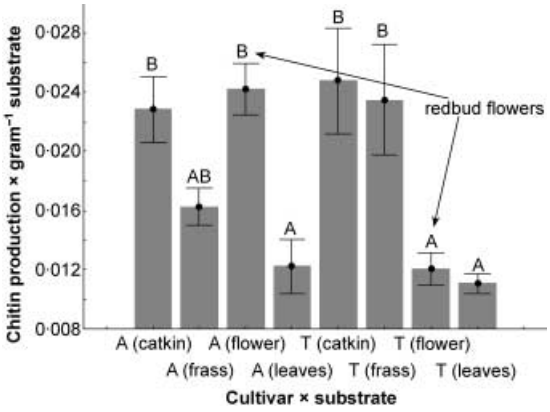

Fig. 5. Mean (± SE) efficiency of the conversion of substrate biomass toward the production of the total amount of chitin in each fungus garden by cultivar substrate combination. In all groups depicted here, $n = 5$, except $n = 4$ for colonies cultivating the *Atta texana* cultivar receiving catkins and frass. Significant differences are denoted by different letters ($P < 0{\cdot}05$, Tukey's HSD tests). A, *Atta texana* cultivar; T, *T. septentrionalis.*

or the interaction (Table 1). Neither cultivar type nor preference differed in their efficiency to convert substrate biomass into chitin (Table 1). However there was significant variation among substrates within preference (Table 1). Consequently, the main effect of preference was dropped from the model and a model containing substrates, cultivar and their interactions subsequently analysed. Cultivar was not an important factor in the efficiency of chitin production ($F_{1,30} = 1{\cdot}19$, $P > 0{\cdot}28$) but significant variation occurred among substrates ($F_{3,30} = 12{\cdot}35$, $P < 0{\cdot}001$) and the interaction with cultivar ($F_{3,30} = 6{\cdot}63$, $P < 0{\cdot}001$). Oak leaves were the least efficient toward the production of chitin and this was true for both cultivars. A similar lack of efficiency was recorded for flowers on the *T. septentrionalis* cultivar (Fig. 5). This interaction appeared to be the consequence of the redbud flowers, which were nearly twice as efficient toward the production of chitin in the *A. texana* cultivar as in the *T. septentrionalis* cultivar (Fig. 5).

Discussion

Mueller (2002) and Mueller *et al.* (2001) argue that the fungus should not be viewed as a passive member of the mutualism. Rather the fungus may manipulate the ants to its own ends. The data presented in this study instead shows that the ability of the fungus to influence ant behaviour is actually quite limited. There is no evidence of conflict – in fact the evidence points toward complete cooperation between ants and fungi. The cultivars appear to be virtually identical from the perspective of the ant. This is surprising since the data presented here indicated that the cultivars differed physiologically. The *A. texana* cultivars were clearly more efficient at converting redbud (*C. canadensis*) flowers into fungal biomass.

There was no evidence for a 'tug-of-war' between ants and fungi over sex ratio or sexual reproduction. On the contrary, it appeared that the *A. texana* cultivar might be better suited toward the production of sexuals, especially females. Although differences were not statistically significant and lacked sufficient power, the direction of differences was in the opposite direction to that predicted by Mueller (2002), since more male biomass was produced on the *T. septentrionalis* cultivar.

The results from this study are similar to an experiment designed to test for conflicts between ants and fungi in the closely related *Cyphomyrmex* species (Mehdiabadi *et al.* 2006). These authors did not find expected differences with regard to sex ratio or sexual production. One explanation was that *Cyphomyrmex* ants appear to have switched cultivar lineages repeatedly over evolutionary time (Mueller, Rehner & Schulz 1998), so that ant–cultivar conflict has not had enough time to evolve. However, the current study used higher attine species that have been cultivating the same lineage of fungi for 40–50 million years (Chapela *et al.* 1994; Mueller *et al.* 1998), which would presumably be long enough for conflict to evolve (Mehdiabadi *et al.* 2006). It would appear then that manipulation of the sex ratio might reduce the performance of the symbiosis.

The results in this study indicate that ants have probably adapted to their cultivar to a certain degree especially with regard to substrate choice, even though this does not appear to influence their overall performance. *Trachymyrmex septentrionalis* colonies were more productive on preferred substrates (frass and catkins) than on unpreferred substrates (leaves and flowers) when they grew their cultivar. However these differences disappear when they cultivate the *A. texana* strain. Additionally, females were fatter in gardens cultivating the *T. septentrionalis* cultivar, which indicates that these females had probably matured and were ready to fly (Seal & Tschinkel 2007b). The pattern for average male fat also support this claim, in several field and lab experiments, average male fat rarely exceeds 11% (J. N. Seal, unpublished data). These colonies therefore would have had an earlier bout of reproduction than colonies on the *A. texana* cultivar, perhaps conferring a selection advantage (Bourke & Franks 1995).

The phylogenetic differences are a surprising result, since leaf-cutting fungi should be specialized to metabolizing leaf tissue. Leaf-cutting ants feed their fungus primarily leaves. This claim has been supported by physiological studies (Martin 1987) and anecdotal accounts of cultivar switches (Stradling & Powell 1986). The non-leaf-cutting cultivars should be adapted to a wider-variety of substrates that includes leaves and flowers. It seems that the evolution of leaf-cutting was not accompanied by a loss of the ability to process frass, a substrate that less derived ants feed their fungus, rather it was accompanied by the ability to metabolize a wider spectrum of substrates. One explanation is that very young colonies of leaf-cutting ants contain small monomorphic workers that are not able to cut tough

leaves (Wetterer 1994). Some leaf-cutting ants may even collect frass when leaves are unavailable (Wetterer, Gruner & Lopez 1998). In this regard it may not be adaptive to have a cultivar specialized solely for a leaf diet.

Focusing on conflict potentially causes us to overlook some very important features of this mutualism that sets this ant apart from other ants, if not organisms generally. Within their geographic range, attines tend to be highly abundant and extremely important members in their ecological community in terms of the amount of biomass they harvest, soil turnover, influences on plant communities, nutrient recycling or even their status as agricultural pests (LaPointe, Serrano & Jones 1998; Wirth *et al.* 2003; Seal & Tschinkel 2006). Indeed, the data in this study indicate that cooperation is a far more important element than conflict in their evolutionary ecology. We agree with Boucher (1988) who suggested that a general emphasis on predation and parasitism has perhaps been an impediment, if not a detriment, to understanding mutualisms and their apparent cooperative nature (Boucher, James & Keeler 1982; Bronstein 2001). Rather, focus must emphasize the mutualism or superorganism as a discrete entity. Focusing on conflict or one member of the mutualism is just as non-sensical as studying mitochondria to understand the behaviour of eukaryotic organisms, because highly integrated systems are more than the sum of their parts (Camazine *et al.* 2001). Our understanding of these mutualisms would thus be more complete if our interpretations viewed the members as a group or a superorganism.

Acknowledgements

Steve Rehner of the USDA Biocontrol Laboratory in Beltsville, Maryland generously took the time in conducting the molecular assays that were critical in determining the success of the cultivar switches. Without his help, this study in current form would have been difficult. Adrian A. Smith devoted many hours in sorting ants from fungus under a microscope and will likely need new eyeglasses in the near future. We also thank Alice A. Winn, Don Levitan, Fran James and Frank Johnson for their comments throughout the many phases of this project. Ulrich Mueller, Sasha Mikheyev and Sergio Sanchez-Pena each provided their insight in cultivar switching. Margaret Seavy and Steve Miller of the FSU Department of Biological Science Analytical Laboratory assisted with the use of the spectrophotometer. Tom Miller provided a vortexer. William H. Outlaw and Danielle Sherdan provided additional support with the chitin assay. Kier Klepzig and Lary Roton were extremely helpful in collecting the incipient *A. texana* colonies. The USDA Forest Service allowed us to collect colonies in the Apalachicola National Forest. This project was partly supported by a FSU Dissertation Research Grant to JNS and carried out during the tenure of a Florida State University PEG (Program Enhancement Fund) Grant and NSF (DEB-0212085) Grant to WRT.

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Received 13 December 2006; revised 5 April 2007; accepted 25 April 2007
Editor: Hefin Jones