

Complexity in an obligate mutualism: do fungus-gardening ants know what makes their garden grow?

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Abstract Fungus gardening ants make clear choices among fungal substrates (food for their fungus). It has been proposed, but never demonstrated, that these ants are collecting the best for their symbiotic fungus and the production of ant biomass (fitness). The goal of this study was to determine whether preferred substrates lead to higher fitness in the attine, *Trachymyrmex septentrionalis*. Preferences exhibited by foragers were established. Colonies were fed a single substrate or a mixture of substrates during the entire course of the experiment, which ended when sexual offspring appeared in the nest. The response variables were numbers and weights of ant offspring and the chitin content of fungus gardens. Preference was not strongly related to fitness. The preferred oak catkins produced the highest amounts of ant and fungal biomass, but the ants collected much more material than needed, which indicates that forager activity is decoupled from fitness. The preferred caterpillar feces were rejected shortly after the feedings began. The unpreferred oak leaves were just as effective at producing ant and fungal biomass as catkins. Leaves are possibly unpreferred because they are expensive to cut. The unpreferred huckleberry flowers were inferior but did not cause rejection behavior. The mixed diet was just as productive as catkins or leaves. This study indicates that foragers possess a default mechanism to prefer catkins and frass, which can be quickly changed if substrates are bad. In contrast, there does not appear to be a similar mechanism causing substrates to become preferred quickly.

Keywords Attini · Colony productivity · Food preference · Foraging · Self-organization · *Trachymyrmex septentrionalis*

Introduction

Social insects are classic central-place foragers because they make repeated foraging trips to and from a central location—the nest. As such, they are expected to be under selection to optimize behaviors that result in the highest net profit in some currency, such as energy or the highest output of winged, dispersing sexuals by the colony (Stephens and Krebs 1986; Kramer 2001). A general criticism of optimality approaches is that they often assume that individual social insects are capable of complex behaviors and that optimization occurs at the level of the individual (Bonabeau et al. 1997; Bonabeau 1998; Kramer 2001). Although there are certainly constraints in the cognitive ability of individual social insects, which may restrict them to simple behaviors, complex behaviors may arise from self-organizing processes among the interacting individuals in a colony (Bonabeau et al. 1997; Camazine et al. 2001). Accordingly, behaviors of individuals in response to their environment may be simple stimulus-response (“if-then”) behaviors (Deneubourg et al. 1999), and complex behaviors may be essentially emergent phenomena (Bonabeau et al. 1997; Camazine et al. 2001). This implies that *individual* worker performance may be poorly related to fitness, but a foraging strategy may still be adaptive if their behavior maximizes aspects of *colony* performance. In other words, optimal foraging and self-organization are not necessarily mutually exclusive; rather, they may complement each other by shifting emphasis to colonies rather than individuals.

Leaf-cutting ants are dominant herbivores in warm latitudes of the western hemisphere (Hölldobler and Wilson

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1990; Wirth et al. 2003). Naturalists have long observed that these ants make clear choices among available leaves and often employ complex recruiting strategies in the process (Hubbell et al. 1980; Rockwood and Hubbell 1987; Hölldobler and Wilson 1990). The question for several decades has been why these ants prefer certain leaves over other seemingly equivalent leaves (Cherrett 1968; Rockwood 1976; Roces 2002). A complicating factor is that these ants are collecting leaves not for their own direct consumption, but for their symbiotic fungus (Martin 1987; Silva et al. 2003; Richard et al. 2005). Foragers of these ants must somehow “know” which substrates meet the needs of their fungus garden and their own nutritional demands.

One possibility is that these choices reflect the optimal gain of resources (Rockwood and Hubbell 1987). The implication is that individual leaf-cutting ants can be expected to “know”, and therefore, choose the best leaves for the production of ant and fungal biomass. In support of this notion were positive correlations with the leaf preference of leaf-cutting ant foragers and leaf nutrients (Berish 1986), water content (Bowers and Porter 1981), relatively nontoxic plant secondary metabolites (Hubbell et al. 1984; Howard 1987, 1988; Folgarait et al. 1996), and leaf age (Nichols-Orians and Schultz 1990; Nichols-Orians 1992). Negative correlations were found between preference and tough leaves (Waller 1982) and toxic plant metabolites (Hubbell et al. 1983; Howard 1987, 1988; LaPointe et al. 1996). However, these findings describe broad patterns. Outside of extreme cases where ants avoid toxic leaves, (Hubbell et al. 1983; LaPointe et al. 1996), nutrient content and secondary chemistry are not strongly related to preference (Howard 1987, 1988).

In violation of optimal foraging predictions (Kramer 2001) is the discovery that leaf-cutting ant foragers do not maximize the amount of leaf tissue harvested during their foraging trips (Kacelnik 1993; Burd 2000; Roces 2002). Foragers cut smaller leaves and ran faster when recruiting to more nutritious leaves (Roces and Nuñez 1993) and when starved or exposed to unfamiliar leaves (Roces and Hölldobler 1994). While leaf-cutting ants frequently use “bucket-brigades” to transfer leaf bits along foraging trails (Hubbell et al. 1980; Anderson et al. 2002), transport time was actually greater than when an individual ant carried pieces back to the nest (Röschard and Roces 2003). These findings are only problematic to optimal foraging theory if one hypothesizes that performance of individual foragers has been optimized.

The goal of this study is to determine the consequences of forager preference on colony fitness. If individual performance has been optimized, then we should expect congruence between forager behavior (food preference) and colony fitness. On the other hand, if colony performance is

the optimized variable, then worker performance may be indirectly related to fitness. To address this issue, we compared the effects of substrates that foragers either preferred or did not prefer on the colony and fungal performance of *Trachymyrmex septentrionalis*.

Ants in the genera *Trachymyrmex* and *Sericomyrmex* along with the leaf cutters (*Acromyrmex* and *Atta*) comprise the “higher attine” group (Fig. 1) (Chapela et al. 1994; Mueller et al. 1998; Wetterer et al. 1998). In contrast to the leaf cutters, these ants have much smaller colony sizes; typically a few hundred workers compared to the thousands if not millions in *Atta* and *Acromyrmex* colonies (Hölldobler and Wilson 1990). All higher attines appear to share the same fungus cultivar lineage, which possesses swollen hyphal tips that serve as food for the ants (gongylidia). They also share many of the specialized garden parasites (Mueller et al. 1998; Adams et al. 2000; Currie et al. 2003). In addition, higher attines tend to cultivate their garden on fresh vegetation (Leal and Oliveira 2000), to which their fungus appears physiologically adapted, in contrast to other attines (Martin 1987). In summary, there are many similarities that these genera have with the leaf cutters that make them ideal for studies of the functional relationships among foragers, the colony and the fungus garden.

Materials and methods

Study species

T. septentrionalis is a common ant in sandy soils of eastern North America, occupying a region that extends to 40° N (Weber 1972). This species is among the most abundant ants in longleaf pine-turkey oak sandhills of the Apalachicola National Forest (ANF) located in northern Florida—a hectare may contain more than 1,000 nests (Seal and Tschinkel 2006b). Like most temperate ants, this ant has a seasonal phenology with a dormant period in the winter (November–March) and sexual production in the spring (May–June). Sexual production is a highly synchronized event in early summer with few colonies producing sexuals later in the season (Seal and Tschinkel 2006a). Fungus gardens during the dormant period are also greatly reduced (<1.0 cm³) (J. N. Seal, unpublished data). Therefore, these ants should be under selection to seek out the best substrates for fungal growth, and experiments performed in the spring should have consequences for the production of sexual brood, a direct correlate of fitness. All colonies were collected in the Wakulla District of the Apalachicola National Forest located approximately 15 km south of Tallahassee, Florida (30°22' N, 84°22' W) in early March 2003, just after the ants ended their winter dormancy.

Collection and maintenance of colonies

Colonies were collected by excavating a 1-m³ pit approximately 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 monogynous (colonies contain a single queen) species. Between 2001 and 2004, more than 250 colonies were collected in this manner.

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 cm³ depression in a box lined with dental plaster. The top of this chamber was completely covered with a piece of plexiglass. A 9-mm diameter hole was drilled in the side of each plaster nest for the ants to enter and exit the fungus garden chamber. Additional plaster nests were added and interconnected with 5-cm segments of clean, rubber hoses as colonies grew larger gardens. 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

All substrates were collected in the period from late February until mid-March 2003. Four types of substrate were used in this study: oak catkins (staminate flowers), caterpillar frass (insect feces), flowers, and leaves. Upon collection, fungal substrates were stored in the freezer (−20°C). Catkins and early spring leaves were obtained from the bluejack oak (*Quercus incana* Bartr.). Frass was obtained by lab-rearing larvae of the orange-striped tussock moth caterpillar [*Orgyia detrita* Guérin-Méneville (Lymantriidae)] on turkey oak leaves. *O. detrita* egg masses were collected from buildings in Gainesville, Florida (ca 200 km away) but were fed leaves collected near Tallahassee. Flowers were obtained from the dwarf huckleberry [*Gaylussacia dumosa* Andr. (Ericaceae)]. All of these plant types are highly typical of Florida sandhills (Myers 1990), and in most cases, *T. septentrionalis* ants have been observed to collect these substrates.

Preference determination

Preference tests were conducted after the colonies had been acclimated to laboratory conditions (approximately 5–7 days). Preferences were determined by exposing colonies to equal substrate amounts of two substrate

types, measured by the number of pieces (pieces of frass, catkin bud, or pre-cut ant-sized snippets of leaf or flower) by placing these items on a piece of waxed weighing paper 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. In this way, *neither the substrate nor the ant could have influenced the behavior of other ants.*

Preliminary studies conducted in 2002 indicated that certain substrate types were preferred over others. Specifically, *T. septentrionalis* foragers preferred caterpillar frass and oak catkins over fresh leaves and flowers (Seal 2006). This pattern was confirmed statistically in this study by using pairwise trials between a preferred substrate and an unpreferred substrate, with the condition that one of the substrates had been assigned for the feeding experiment (see below).

The pairwise method proceeded by placing equal amounts of a preferred and an unpreferred substrate on waxed paper near the entrance of the ant nest. Preferences were detected with goodness-of-fit tests [G -Test, (Sokal and Rohlf 1995)]. Replicated goodness-of-fit tests (Sokal and Rohlf 1995) were used to determine whether preferences were statistically consistent across all colonies, and we used each trial from each colony as a replicate. 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, it tests whether G_H adds significant variation to the total (G_T). A significant G_H statistic would imply significant variation in preference among colonies, whereas an insignificant value would indicate that preferences are uniform among colonies.

Effects of substrates on garden and ant colony performance

Colonies were fed daily ad libitum by placing the substrates on wax paper near the nest entrance. Wet weights of substrates were converted to dry weights using constants obtained by drying small amounts of substrates for 48 h under room temperature. Amounts not collected by the ants after 2 days and 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. Four groups of six colonies each received exclusively one of four substrates, while the remaining 13 colonies received a mixture of the four substrates (total $N=37$). This mixture group was established to determine any possible side effects of feeding colonies one substrate, which does not occur under natural conditions.

Feedings were conducted until new offspring (sexuals and new workers) eclosed 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. Workers in the refuse piles were counted as being dead while the colony was alive. These dead ants were generally different in appearance than those killed by the freezing as they were often missing appendages or body parts.

Response variables

Measures of ant performance

The main response variables were the total weights and numbers of ant offspring. Adult ants had their body fat extracted for 48 h in a Soxhlet extractor using diethyl ether. Ten dark workers (old workers) and a maximum of ten of virgin female, male and new worker offspring were chosen from each colony for extraction. Energetic contents of ants were obtained by multiplying lean weights by 18.87 J/mg and fat weights by 39.33 J/mg (Peakin 1972) and summing.

Measures of fungal performance

Fungus garden traits were fungus garden weight and an estimate of the amount of chitin (percent and total amount). Chitin is the main constituent of fungal cell walls (Raven et al. 1999), and its quantity in a substrate is frequently used as an indicator of fungal biomass in soil or wood, among other substrates (Plassard et al. 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, it estimates the total amount of fungal biomass that was an outcome of the experimental manipulations in this study.

We employed a test specific to the free aldehydes that result from the acid (6-*N* HCl) hydrolysis of chitin and subsequent deamination of the glucosamine residues by nitrous acid (HNO₂) (Plassard et al. 1982; Vignon et al. 1986). Free aldehydes form a stable complex with 3-methyl-2-benzothiazolone hydrazone hydrochloride (MBTH), which turns blue in the presence of ferric chloride (FeCl₃). The 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 g ml⁻¹) of purified chitin (Sigma-Aldrich, St. Louis MO).

Statistical analysis

All analyses were conducted with Statistica version 6.1 (Statsoft 2003). Data were log₁₀ transformed to meet para-

metric assumptions; otherwise nonparametric tests (i.e., Kruskal–Wallis) were employed.

Results

Preferences

Workers within each colony generally exhibited clear preferences with nearly all preferring catkins and frass over leaves and flowers ($G_P=154.2$, $df=1$, $p<0.0001$; $G_T=164.4$, $df=1$, $p<0.0001$, Fig. 1). No colonies contained workers that consistently preferred leaves or flowers; therefore, the test of heterogeneity among colonies was nonsignificant ($G_H=10.2$, $df=23$, $p>0.99$).

Changes during colony development

Foragers appear to have the capacity to change their preference. During the first day of feedings, ants in colonies receiving the *O. detrita* frass completely covered their fungus garden with pieces of frass, a typical behavior observed in other treatments; however, within 2 days, this substrate was rejected. Not only did the ants remove the frass from the fungus garden, they removed the frass from the waxed paper and deposited it in their refuse piles along the tray's corner. To avoid losing an entire group due to starvation, which was likely, as they were not building up their garden, the diet of this group was augmented with a 50:50 blend of oak catkins and frass. The initial intention was to determine whether the ants would collect frass along with the catkins; however, by the end of the experiment, it became clear that little, if any, of the frass had been

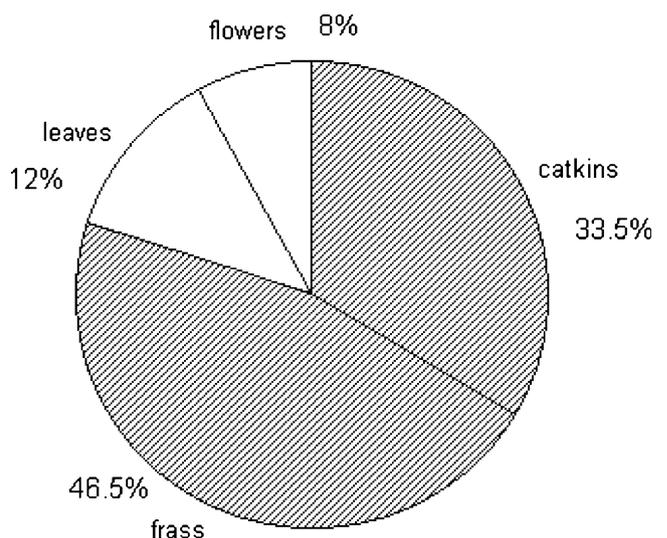


Fig. 1 Percentages of total amount of substrate collected. Shaded areas indicate preferred substrates and unshaded areas indicate unpreferred substrates

incorporated into the fungus garden. Nearly 90% of these colonies' diet consisted of catkins: three collected no frass, while the other three had diets composed of 5–10% frass. Therefore, colonies in the *O. detrita* frass treatment will be henceforth referred as the “low-catkin” diet and the catkin diet proper, the “high-catkin” diet. Neither the bluejack oak leaves nor the huckleberry flowers, unpreferred substrates, were rejected.

Substrate preferences, more or less, were reflected in the colonies receiving a mixed diet. It became immediately obvious during the beginning of the experiment that the colonies would feed exclusively on catkins if left to their own devices. It was surprisingly difficult to force the colonies onto a fixed diet composed of an even mixture of all four substrates because the ants clearly preferred catkins and appeared to ignore other substrates for several days after receiving catkins. This was one reason why the amount of leaves made available was less than 25% (Table 1)—we needed to conserve our leaf supply for the leaves-only diet. This qualification notwithstanding, the colonies' diet was composed of approximately 60% catkins and 9–18% for the other three substrates (Table 1). Approximately 80% of the catkins offered were accepted by the colony, compared to 74% of the leaves, 44% of the flowers, and 25% of the frass. Catkins, leaves, and flowers comprised more than 90% of their diet (Table 1).

Performance measures

The weight of the fungus garden was positively related to the weight of substrate collected by the colonies (log fungus garden weight = $0.07 + 0.79 \times \log$ substrate accepted; $R^2 = 0.89$, $p < 0.0001$). Not surprisingly then, colonies on the high-catkin diet had grown the largest gardens because they had collected the most substrate, and those receiving flowers, the least substrate and the smallest fungus gardens (Fig. 2). Intermediate in size were gardens in colonies fed oak leaves, those on the low-catkin diet (unpreferred), and those receiving the mixture of all four substrates ($F_{4,32} = 12.06$, $p < 0.0001$, Fig. 2). It is unlikely that colony size (the number of workers) had a role in the patterns of fungus garden weight, as only those colonies on the mixed diet were significantly smaller than those in the remaining four

groups (old worker weight: $F_{4,32} = 2.48$, $p = 0.06$ or old worker number: $F_{4,32} = 2.75$, $p = 0.045$). Colonies receiving the leaves had the most workers. Mortality was also not different among all groups ($F_{4,32} = 0.454$, $p = 0.76$).

Total ant biomass production (energetic content and total weight of all offspring) was similar in all groups, except for the significantly lower amounts in the flower group ($F_{4,32} = 7.7$, $p < 0.001$, Fig. 3a). Energetic content of offspring largely mirrored this pattern except that offspring from the flowers and the mixture had lower energy content than those from other substrates ($F_{4,32} = 5.54$, $p = 0.002$, Fig. 3b). All colonies on flowers produced only worker brood (except for a single colony that produced four males). Neither female number nor biomass varied among the four remaining treatments ($F_{3,24} = 1.45$, $p = 0.25$; $F_{3,24} = 1.55$, $p = 0.22$, respectively). Male number ($F_{4,16} = 1.01$, $p = 0.43$), male biomass ($F_{4,15} = 0.68$, $p = 0.62$), and total new worker biomass ($F_{4,30} = 2.15$, $p = 0.10$) and new worker number ($F_{4,30} = 2.27$, $p = 0.09$) did not differ among the treatments.

Fungus gardens in the flower treatment contained the lowest percent chitin (1.2%) or fungal biomass per gram of fungus garden, whereas the four remaining groups were higher but still similar to each other ($>2\%$) ($F_{4,32} = 11.2$, $p < 0.001$; Fig. 4a). Total chitin (= percent chitin \times fungus garden weight) was greatest in the high-catkin diet, lowest in the flower diet, and intermediate in the remaining three groups ($F_{4,32} = 27.3$, $p < 0.001$; Fig. 4b).

One measure of the consequence of choices is the efficiency of the conversion of substrate into ant and fungal biomass, as measured by the amounts of biomass produced per gram of substrate collected. A low-catkin diet or an oak-leaf diet is clearly more efficient for the production of brood than the high-catkin diet ($F_{4,32} = 6.26$, $p < 0.001$; Fig. 5). Whereas leaves appear to be most efficient for the production of fungal biomass (chitin), only flowers are significantly less efficient ($F_{4,32} = 3.85$, $p = 0.01$, Fig. 5).

Discussion

The emerging picture indicates a complex relationship between substrate preference and productivity of colonies and fungus gardens. Individual worker performance is not a

Table 1 Average amounts and percent of substrates accepted by colonies receiving a mixed diet

Substrate	Amount provided (g) (± 1 SD)	Percent (%) of total provided (± 1 SD)	Amount accepted (g) (± 1 SD)	Percent (%) of total accepted (± 1 SD)
Flowers	3.25 \pm 1.18	25 \pm 3	1.55 \pm 1.34	18 \pm 5
Leaves	1.47 \pm 1.03	10 \pm 3	1.1 \pm 0.9	13 \pm 4
Catkins	5.73 \pm 1.84	45 \pm 4	4.6 \pm 2	60 \pm 7
Frass	2.69 \pm 1.37	20 \pm 3	0.66 \pm 0.4	9 \pm 5
Total	13.2 \pm 5.3		7.88 \pm 4.5	

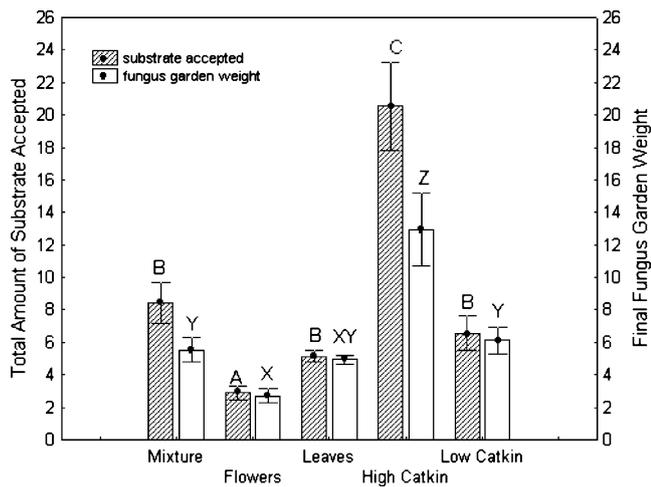


Fig. 2 Mean total amounts of substrate (\pm SE) accepted and final fungus garden weight. *Shaded bars* indicate the weight of substrate accepted and *open bars* indicate final fungus garden weight. Significant differences are denoted by *letters* (A–C for the amount of substrate accepted and X–Z for fungus garden weight) ($P < 0.05$, Tukey's HSD tests). There were six colonies in all single substrate groups and 13 in the mixed diet

variable that appears to be maximized because foragers are making decisions somewhat independent of substrate quality. Two of the four substrates (catkins and leaves) were clearly good substrates for the production of ant and fungal biomass. However, only catkins were preferred, and the ants collected an amount far greater than they subsequently could use. The other preferred substrate (caterpillar frass) was rejected shortly after their first feeding. It does not seem that foragers by themselves are capable of making correct decisions.

This study illustrates that these ant colonies are indeed composed of several parts that operate somewhat independently of the other. It is only at the colony level that information about the suitability of various substrates becomes processed and subsequent decisions made. In other words, decisions that workers make are not necessarily the same ones that colonies eventually reach. This study is consistent with a conclusion made by Wirth et al. 2003, (p. 130): preferences reflect a colony learning details about its environment, which change temporally and spatially. Ants appear to try various substrates in the vicinity of their nests to determine those that are best for the production of ant and fungal biomass. For colony performance to be optimized, it appears critical that workers interact with their nestmates and/or their fungus garden.

The observations in the high-catkin diet colonies strongly suggest that substrate preference exhibited by foragers is decoupled from colony performance. These gardens do not appear to have led to significant increases in ant production, relative to colonies in the low-catkin diet. It is tempting to suggest that catkins are a nutritionally

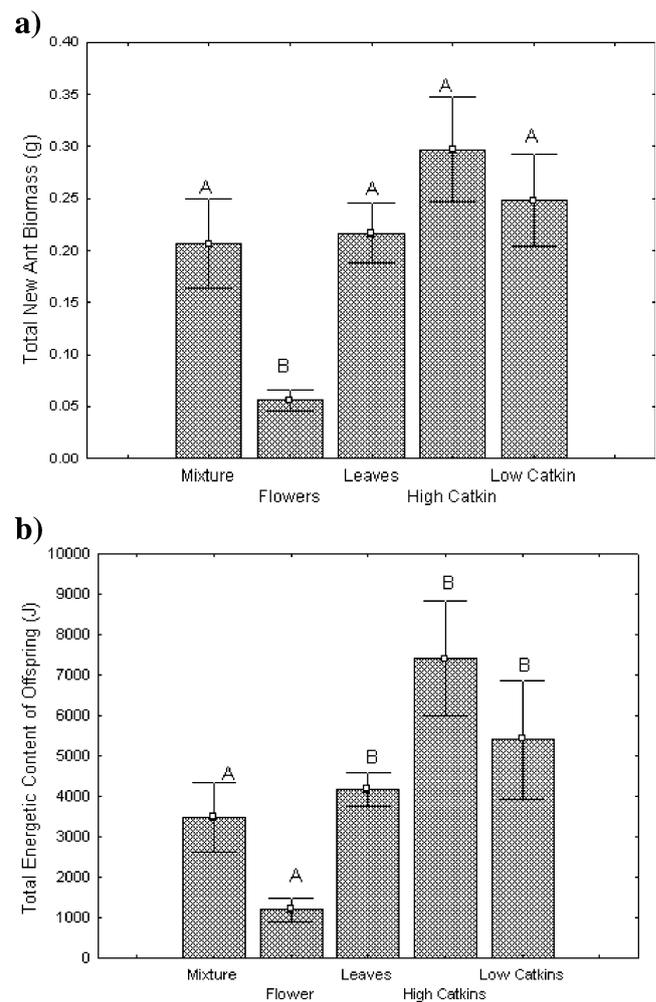


Fig. 3 a Mean total ant biomass (\pm SE) for each treatment. **b** Mean total energetic content (\pm SE) of brood. Significant differences are denoted by *letters* ($P < 0.05$, Tukey's HSD tests). There were six colonies in all single substrate groups and 13 in the mixed diet

deficient diet, and therefore, must have been collected in large numbers to produce an adequate amount of offspring. However, the data do not support this, as the low-catkin diet colonies produced similar amounts of biomass and more chitin than those on the high diet. This appears inefficient in the long run because these large gardens were not used for the production of brood, and these colonies were the least efficient producers of ant brood on a per gram basis. It seems additionally that fungal production is decoupled from ant performance. One colony on the high-catkin diet had, in fact, begun to dismantle these older gardens on the day it was killed. It is possible that foragers were stimulated to build these large gardens by enhanced hyphal growth, as the high-catkin diet colonies contained the highest percent chitin, approaching 3% in some colonies.

The adaptiveness of the high collection rates may lie in the suitability of this substrate and its relative rarity.

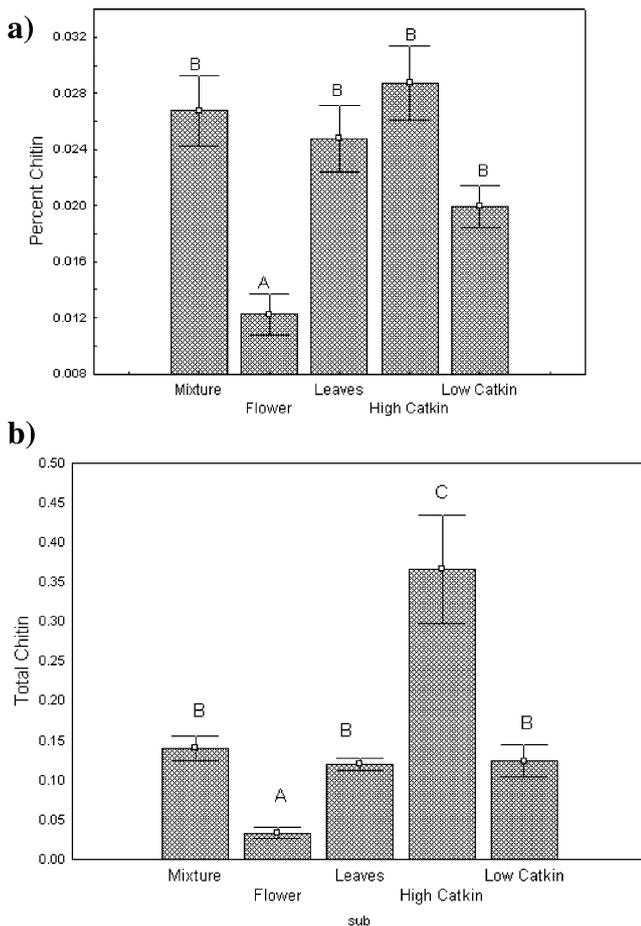


Fig. 4 **a** Mean percent chitin (\pm SE) in each fungus garden by treatment. **b** Mean total chitin (\pm SE) in each fungus garden by treatment. Significant differences are denoted by letters ($P < 0.05$, Tukey's HSD tests). There were six colonies in all single substrate groups and 13 in the mixed diet

Although oaks are the most abundant woody species after pines in sandhills (Myers 1990), catkins do not fall to the ground until after they have dehisced. These ants do not collect dehisced catkins or climb up trees to collect catkins. They, therefore, rely on clumps that have been blown off the tree or those on clonal ground-hugging oaks (*Quercus minima* and *Quercus pumila*). Catkins and frass may be easily harvestable once the ants discover a cache by simply snipping off catkin buds or carrying off frass morsels through the use of recruiting trails. In other words, ants rarely will have an unlimited supply of catkins at their disposal.

What stimulates a worker to select a substrate? Possibly, odors and motivation can guide an ant in making choices so that *T. septentrionalis* workers choose substrates that give off characteristic odors, as reported in leaf-cutting ants (Roces 1990, 1994; Roces and Nuñez 1993). This may certainly explain their predilection toward catkins. Such a mechanism may also explain why foragers initially preferred the *O. detrita* frass, but subsequently, rejected it.

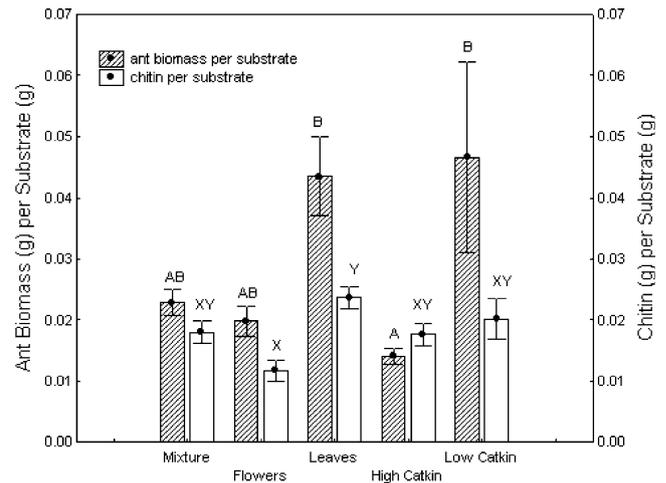


Fig. 5 Mean efficiencies of each substrate (\pm SE) for the production of ant and fungal biomass. Shaded bars correspond to the amount of ant biomass produced per gram of substrate and open bars indicate the efficiency for the production of chitin. Significant differences are denoted by different letters ($A-B$ for ant biomass and $X-Y$ for chitin) ($P < 0.05$, Tukey's HSD tests). There were six colonies in all single substrate groups and 13 in the mixed diet

Possibly, *O. detrita* frass looked and smelled like good frass, as some frass types are certainly excellent substrates, such as tent caterpillar frass (*Malacosoma americanum*) (Seal 2006). The behavior observed in this study appears rather similar to the “delayed rejection” behavior reported when a preferred substrate (citrus pulp) was laced with a fungicide (Ridley et al. 1996; North et al. 1999). After several days, the ants learned to avoid citrus pulp, even when citrus pulp not impregnated with fungicide was provided (Ridley et al. 1996; North et al. 1999). One possibility in the present study is that the ants were not familiar with the *O. detrita* frass as the caterpillar eggs were collected approximately 200 km from the ant colony collection site. However, these caterpillars were reared on (and therefore, produced the frass from) leaves collected in the vicinity of the ant colonies used in this study.

These data suggest that the default strategy is to collect items that give off specific chemical cues, the evaluation of which (i.e., their suitability) occurs at a later time. This phase is essentially exploratory. In the absence of positive or negative feedback, some substrates may never leave this phase. The ability of ants to remember traits of substrates and their interaction with the fungus garden may be quite limited. Foragers are at least 1-year old in this species, as most workers are produced a month or more after sexuals (J. N. Seal, unpublished data)—those in this study were produced no more recently than the previous summer. It is possible that foragers must learn or relearn the characteristics of their environment each spring.

Huckleberry flowers were inferior by any measure of ant or fungal output. Because these flowers are very common

(the adult plant is a low, spreading, clonal shrub), they would appear to be quite plentiful from the ants' perspective. These flowers have been observed inside fungus gardens of excavated field colonies, and foragers can be routinely observed cutting out corollas each spring (J. Seal, unpublished data). Probably important is the fact that the ants have never been observed to recruit to these flowers as they will typically do to fresh growth on small oaks [bluejack and runner oak (*Q. pumila*)], bracken fern (*Pteridium aquilinum*), and catbrier (*Smilax* spp.) (J. Seal, unpublished data). It is unlikely that these flowers are providing necessary but rare micronutrients, as the nutrient balance hypothesis (Powell and Stradling 1991; Stradling and Powell 1992) predicts, as the performance on mixed diet was similar to groups fed catkins (high and low diets) or leaves. In short, it is not immediately obvious why the ants collect huckleberry flowers.

Why are leaves not preferred substrates even though they appear to be suitable? It does not appear that preference is strictly guided by substrate nutrient content. These differences may reflect different collecting and handling costs. Leaves might not be preferred because by midsummer, leaves of oaks and other deciduous trees typically toughen and become unharvestable by many herbivorous insects (Feeny 1970; Schroeder 1986). The evolution of a folivorous diet in the Attini is correlated with the evolution of large, polymorphic workers that are quite suitable for the harvesting of leaf tissue (Wilson 1980; Wetterer 1994). Even when the leaves are soft, it takes an individual *T. septentrionalis* ant as much as 15 min to cut a piece of leaf, compared to as little as a few seconds to cut a catkin bud (J.N. Seal, unpublished data). Learning that leaves are difficult to harvest must happen early in development, if it is not a hard-wired trait, as ants did not prefer leaves that were pre-cut approximately to easily transportable sizes. This assumes that the ants can distinguish the chemical signatures (e.g., odors) of leaves and catkins, even though the leaves and catkins came from the same individual plants and were also stored in the same containers. Worker experience could have a role in shaping their preference so that if this experiment encompassed more than one reproductive bout, foragers might have learned to prefer leaves.

To some extent, this study has raised many questions about how this mutualism is organized. Foragers of *T. septentrionalis* exhibit preferences toward substrates that are not necessarily the best. Although there is a positive correlation between the collection rate of one preferred substrate and the ensuing amount of fungal biomass, these large gardens do not necessarily result in the highest amount of ant biomass. The other preferred substrate was evidently unsuitable and was rejected for unknown reasons. Although it seems possible that workers can quickly learn

to reject substrates, there does not appear to be a similar mechanism for the learning and subsequent adoption of good substrates, i.e., it is not a straightforward process. At the very least, other factors are involved in making suitable substrates, such as leaves, unpreferred. It is possible that the negative effects of the frass and huckleberry flowers were only evident because colonies had no other food items that could mitigate their negative effects on colony performance. In more natural conditions, foragers could still collect these substrates with little effect on colony performance, if other foragers collected larger amounts of highly productive substrates such as catkins. It is possible that catkins contain significant quantities of compounds easily digested by the fungus, such as starches (Silva et al. 2003); if so, the fungus may give off cues that stimulate foragers to collect more. It is possible that under natural conditions foragers make initially random choices but eventually fixate on those that interact positively with the fungus garden. If the ants can learn to discriminate among substrates and can retain memory, then older workers (those that are at least 1 year old) should prefer catkins, whereas younger, inexperienced workers may collect huckleberry flowers or other poor substrates before they learn the good substrates that they or nestmates subsequently collect. Future work may also want to conduct studies under field conditions that may account for these factors, which are just some of the hypotheses that could be tested on these fascinating complex agricultural societies.

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