

# Nutrition mediates the expression of cultivar–farmer conflict in a fungus-growing ant

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**Attine ants evolved farming 55–60 My before humans. Although evolutionarily derived leafcutter ants achieved industrial-scale farming, extant species from basal attine genera continue to farm loosely domesticated fungal cultivars capable of pursuing independent reproductive interests. We used feeding experiments with the basal attine *Mycocepurus smithii* to test whether reproductive allocation conflicts between farmers and cultivars constrain crop yield, possibly explaining why their mutualism has remained limited in scale and productivity. Stoichiometric and geometric framework approaches showed that carbohydrate-rich substrates maximize growth of both edible hyphae and inedible mushrooms, but that modest protein provisioning can suppress mushroom formation. Worker foraging was consistent with maximizing long-term cultivar performance: ant farmers could neither increase carbohydrate provisioning without cultivars allocating the excess toward mushroom production, nor increase protein provisioning without compromising somatic cultivar growth. Our results confirm that phylogenetically basal attine farming has been very successful over evolutionary time, but that unresolved host–symbiont conflict may have precluded these wild-type symbioses from rising to ecological dominance. That status was achieved by the evolutionarily derived leafcutter ants following full domestication of a coevolving cultivar 30–35 Mya after the first attine ants committed to farming.**

social evolution | crop domestication | symbiosis | geometric framework | *Mycocepurus smithii*

Resources derived from mutualists often enhance the ecological dominance of partnered species (1, 2), but can also lead to conflicts between them (3, 4). Such conflicts typically stem from diverging reproductive interests between hosts and symbionts, which may arise for two reasons. First, there is a fundamental conflict over symbiont mixing because any host compartment that is accessible for multiple symbiont strains incurs a risk of symbionts competing for host resources (5) or of one of them free-riding on the services of the other (6, 7), to the detriment of the host. The most harmonious mutualisms are therefore expected to always involve a single strain of clonal symbiont per individual host. Typical examples are the mitochondria and plastids that were domesticated by early eukaryote protists (8, 9), and nutritional symbionts of insects with specialized diets that are clonal within host individuals but genetically variable across hosts (10–12). Second, when mutualisms evolve vertical symbiont transmission, hosts are selected to suppress tendencies of their symbiont to continue investing in traits favoring horizontal transmission, because to quote Axelrod and Hamilton, “. . . [a]ny symbiont that still has a transmission ‘horizontally’ . . . would be expected to shift from mutualism to parasitism. . .” (13). Such selfish symbiont traits that use host resources to enhance independent reproductive success without offering the host any returns (14) have been likened to parasitic virulence (5), highlighting that mutualisms are forms of reciprocal exploitation unless reproductive interests are completely aligned (9, 15).

Host–symbiont conflicts over horizontal symbiont transmission are particularly acute when symbionts are multicellular eukaryotes

whose sexual fruiting bodies represent considerable investments. An illustrative example is offered by the fungus-growing attine ants, whose dispersing queens transmit symbionts vertically and whose farming workers therefore actively suppress wasteful formation of inedible mushrooms (16, 17). Another example is provided by two independently derived lineages of fungus-growing termites that both evolved vertical transmission of *Termitomyces* cultivars while terminating mushroom production (18). However, although such correlated adaptive states are consistent with theoretical predictions, and have been tested and confirmed in some plant–microbe symbioses (6, 7), no experimental work has tested whether allocation conflicts affect the ecological dynamics of animal ectosymbioses in similar ways.

Here, we present such a test, using the common and widely distributed (Argentina to northern Mexico) basal fungus-growing ant *Mycocepurus smithii* as a model (19). This ant species is notable for being parthenogenetic (19), allowing controlled experimentation with genetically uniform worker ants. It also rears an unusual variety of fungal symbiont clones that are not irreversibly domesticated and apparently continue to exchange genes with free-living relatives (16, 20). Cultivars have thus retained their full sexual potential so that ant farmers have explicit potential conflicts with their crops over mushroom production that may damage symbiotic performance when expressed.

The phylogenetically basal “lower attine” lineages to which *M. smithii* belongs evolved more than 50 Mya when the ants

## Significance

**Early subsistence farming implied significant physiological challenges for Neolithic farmers until they genetically isolated their crops through artificial selection and polyploidization. The attine ants faced analogous challenges when they adopted fungus farming 55–60 Mya. Whereas evolutionarily derived attine lineages irreversibly domesticated cultivars approximately 25 Mya and ultimately realized industrial-scale farming, basal lineages retained small-scale farming, diversified, and now coexist with advanced fungus-farmers in most New World tropical ecosystems. We show that management of independent sexual reproduction in cultivars constrained farming productivity, echoing early human farming of unspecialized low-productivity crops. Loss of cultivar gene exchange with nondomesticated relatives likely reduced host–symbiont conflict over reproduction, fostering the rise of ecologically dominant ant-agriculture.**

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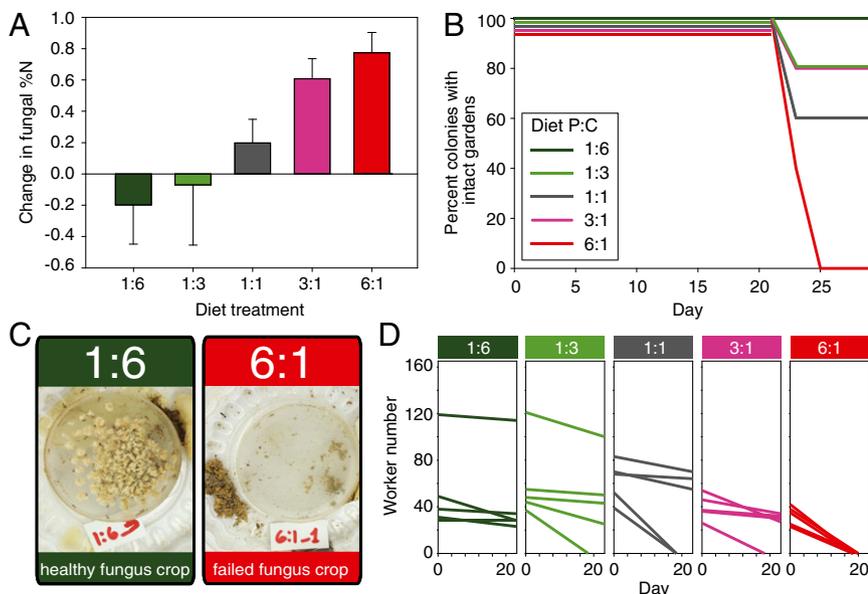
Data deposition: The sequences reported in this paper have been deposited in the GenBank database. For a list of accession numbers, see Table S9.

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**Fig. 4.** Garden nutrition, performance of cultivars, and worker mortality in the diet experiments of Fig. 2. (A) The %N of fungal crops (and thus their protein content) increased when workers harvested protein-rich substrate, and (B and C) this was associated with a higher likelihood of crop failure, and (D) with higher rates of worker mortality (see text for statistics). The pictures of experimental colonies (viewed from above) in C contrast a 1:6 P:C diet [thriving fungus garden hung by workers from the lids of the Petri dish, as is typical for healthy field gardens hanging from the ceiling of nest cavities (green image; see also Fig. 2A)], and a 6:1 P:C diet [dead fungus garden discarded from the nest in an external trash pile and with all brood having been discarded as well (red image)].

symbiosis protein-averse. However, despite this general aversion, workers were inclined to harvest more protein and less carbohydrate than what could have maximized somatic hyphal growth. These patterns of restrained nutrient mixing appear consistent with the necessity for farming ants to curtail potential host-symbiont conflict over independent symbiont reproduction and with a productivity cost for the entire farming symbiosis to maintain stable mutualistic cooperation.

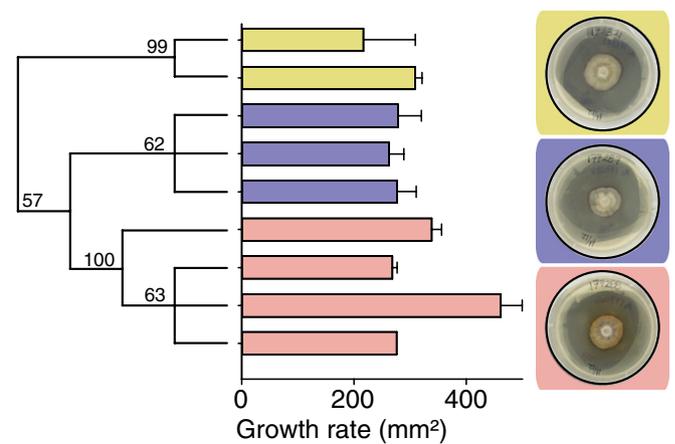
## Discussion

**The Questions Asked and the Answers Obtained.** We used a state-of-the-art nutritional geometry approach to understand how foragers of fungus-growing ants adjust garden provisioning to suppress independent reproduction by their cultivars in ways that also compromise symbiotic productivity. Our results expand the existing set of geometric framework approaches (31–33) by: (i) providing a map of the nutrient requirements of fungal cultivars reared without farming ants (Fig. 1); (ii) using this map to track collective foraging and provisioning decisions of ant farmers in laboratory experiments offering specific nutritional blends (Fig. 2), and in the field (Fig. 3); and (iii) evaluating the negative consequences of improperly biased nutrition for the combined performance of the *Mycocrepus* farming symbiosis (Fig. 4).

The results that we obtained yielded relatively clear answers to the four questions we posed. First, the cultivars of *M. smithii* thrived when provisioned with carbohydrate-biased substrate and suffered with increasing protein concentrations. Second, ant foraging preferences largely matched fungal preferences with workers harvesting substantial amounts of carbohydrates when they could do so without simultaneously overharvesting protein from the same source, a strategy that avoided wasteful mushroom production without excessive costs. Third, this behavior appeared to match general adaptations to low-protein availability of saprophytic leaf-litter fungi on the forest floor, as expected because the cultivars of *M. smithii* remain closely related to free living fungi (16, 20, 34). At the same time, naturally available foraging targets, such as insect frass and dead plant fragments, are sufficiently variable in P:C ratios to allow differential garden provisioning, as we simulated in our garden-provisioning experiments. Fourth, differential provisioning and nutrition of ants and cultivars does appear to allow fine-tuned garden provisioning so that the expression of host-symbiont conflict over cultivar reproduction can be avoided, because hyphal growth remained sustainable under higher protein levels than mushroom formation. However, it also appeared that *Mycocrepus* ant farmers face fundamental

productivity constraints because the carbohydrate-rich, protein-poor substrates that maximize growth rates of edible hyphae also enabled the production of costly mushrooms when intake rates were high.

During the first 30–35 My of attine evolution, the ants produced a monophyletic radiation of specialized fungus farmers. However, their fungal cultivars were not genetically isolated from free-living fungi and could therefore not coevolve to any significant degree with their farmers as happened during the last approximately 25 My, after the ancestor of the higher attine ants came to initiate a new radiation based on a truly domesticated cultivar lineage (16, 23–25, 35–38). Our study illustrates how this early interaction asymmetry between mutualistic partners has likely constrained ecological performance, which may help explain both the low rates of fungus garden metabolism (39) and the typically low rates of crop provisioning (four workers per nest per hour) (Table S7) in lower attine ants relative to evolutionarily derived *Atta* leafcutter ant colonies, where teeming foraging



**Fig. 5.** Somatic growth across *M. smithii* cultivar lineages. A sample of cultivars from nine sympatric colonies of *M. smithii* exhibited substantial genetic diversity without differences in growth rate (hyphal area in square millimeters after 30 d + SE) in a phylogenetically controlled analysis (Blomberg's  $K = 0.419$ ;  $P = 0.845$ ) (for details, see *SI Methods* and Table S9). The tree is a majority rule consensus maximum-likelihood chronogram with nonparametric bootstrap support values.

highways mobilize thousands of workers to carry leaf fragments to their nests. Although these productivity constraints may well have prevented the lower attines from obtaining an ecological footprint in Neotropical ecosystems comparable to the leafcutter ants, the substantial radiation of farming strategies and species diversity represented by the extant lower attines also suggests that these early farmer lineages gained control over the expression of selfish symbiont traits soon after becoming farmers.

The stoichiometric and geometric framework approaches developed for this study highlight a mix of evolutionary adaptations and constraints associated with ant subsistence farming. Our present study clarifies some of the nutritional mechanisms by which lower attine ants may have tamed their cultivars as long as gene flow with free-living fungal relatives precluded proper domestication. However, the precise mechanisms by which *M. smithii* ant workers regulate garden nutrition and suppress mushroom production need further study, both in the laboratory where we have far from exhausted the opportunities offered by the geometric framework approach, and in the field, where further studies could manipulate foraging on frass and dead plant material to explore health consequences for natural *M. smithii* colonies.

**Remarkable Parallels with the Challenges of Human Subsistence Farming.** The earliest human subsistence farmers faced severe physiological challenges (40, 41), in part because plant-based agricultural diets provided more carbohydrates and less protein than the lean game that dominated diets of ancestral hunter-gatherers (31). Modern humans appear to retain these “outdated” nutritional adaptations, regulating the intake of protein more strongly than carbohydrates, and thus overeating sugars, starch, and lipids to reach set targets for protein consumption when navigating today’s nutritional landscape that has become even more biased toward carbohydrates (42). The extant relatives of the earliest small-scale fungus-farming ants in the present study echo this “leveraging” of protein intake (Fig. 2), although the mechanisms likely differ because attine ants appear to have an upper protein tolerance set by fungal symbionts adapted to low protein conditions (Figs. 2 and 4). *M. smithii* workers also appeared to regulate protein harvest more tightly, and at lower levels, than nonfarming ant species (33, 43, 44), which may present additional physiological challenges because the ants need substantial amounts of protein to fuel colony growth.

We have shown that *Mycocrepinus* ant farmers could neither increase carbohydrate provisioning without cultivars allocating the excess toward mushroom production, nor increase protein provisioning without compromising somatic cultivar growth. The ants appear to navigate nutritional intake trade-offs in a prudent and possibly fitness-maximizing manner, just as human subsistence farmers have always tried to do. The phylogenetically basal forms of attine farming have remained very successful over evolutionary time, despite not reaching ecological dominance, quite possibly because they evolved in an empty niche and could gradually respond to selection for the highest possible productivity. When higher attine ants eventually emerged, they gradually moved into another, functionally herbivorous niche, so did not compete for the same resource base, which may have contributed to the coexistence of ancestral subsistence farming and industrial-scale farming of attine lineages in many neotropical ecosystems. In contrast, the cultural evolution of human agriculture proceeded rapidly and modern industrial-scale farming almost inevitably replaces subsistence farming because the two agricultural practices compete for the same land, water, and fertilizers.

## Methods

We used a geometric framework approach to assess the nutritional requirements for maximizing hyphal growth and mushroom production in pure clonal cultivar inoculates isolated from a large colony of *M. smithii* (177198) collected from the rainforest at Soberania National Park, Panama (N 9.11489, W 79.69784), and grown on Petri dishes filled with a standard potato dextrose agar (PDA) medium. We offered nine different P:C ratios (1:9, 1:6, 1:3, 1:2, 1:1, 2:1, 3:1, 6:1, 9:1) and four concentrations (8, 20, 40, and 60 g/L), yielding 36 replicated culture conditions in which we estimated hyphal growth rates and probabilities of mushroom production (modified from ref. 32) (*SI Methods* and

*Table S1*). We placed 5-mm-diameter plugs of pure fungal culture in 60 × 15-mm Petri dishes containing 12 mL of 36 sterile synthetic agar-based diets. We sealed the dishes with parafilm (diet × dilution:  $n = 12$ ), checked them every few days for contamination, and removed infected agar from some plates while discarding plates if overrun with contaminants.

After 32 d we photographed noncontaminated plates ( $n = 360$ ) and estimated fungal expansion rate (fungal area after 32 d, mm<sup>2</sup>) with ImageJ (NIH Image; v1.49g) (as per ref. 32). After 80 d we photographed all instances of mushroom growth on the same plates ( $n = 47$ ), and used percent surviving plates with mushrooms for each diet treatment as the dependent variable in subsequent analyses. We used least-square regressions with both linear and quadratic terms to evaluate how fungal expansion rates and percentages of surviving fungus plates with mushrooms varied across the 36 diet protein and carbohydrate combinations (32). Nonparametric thin-plate splines that do not constrain the shape of the response surface were determined with the “fields” package v.2.14.0 in R and used to map fungus growth and mushroom production across the 36 diets (32, 45, 46).

We measured growth rate over 30 d on PDA of pure fungal cultures isolated from nine *M. smithii* colonies ( $n = 5$  dishes per colony) using the protocols described above. To test for fungal strain growth differences, we isolated DNA from these fungal samples with 10% (wt/vol) Chelex extractions (47) followed by PCR amplification of the conserved nuclear large subunit (LSU) rRNA. The resulting PCR products were sequenced at Beijing Genomics Institute (BGI) Europe, producing sequences that were deposited in GenBank (see *Table S9* for corresponding accession numbers). We used these sequences to construct a chronogram using the function *phyloSig* from the *ape* package in R v3.0.8 to test for a phylogenetic signal in fungal growth rates across colonies using Blomberg’s *K* (48).

We extended the geometric framework logic described for fungal cultivars to the entire farming symbiosis. We first cataloged the substrate harvested by *M. smithii* workers during approximately 20 h of observations at 22 colonies in Soberania Park from November 2013 to December 2013 (*Table S7*). We then collected additional substrate for analyses of elemental N (%N) from October 2014 to December 2014. This process allowed us to estimate the percent crude protein in harvested substrate (*Table S8*), assuming that proteins contain, on average, 16% N (49). We also harvested entire *M. smithii* colonies from May 2014 to June 2014 and established them in plastic containers with ad libitum water and ground polenta as forage for garden maintenance.

We performed a P:C diet laboratory feeding experiment within the geometric framework (32, 34, 50) to analyze the foraging strategies by which workers prioritize protein and carbohydrates when harvesting nutritionally defined agar-based substrates. The diets contained 20 g/L macronutrients and known P:C ratios modified from the literature (50) (*Table S5*). We used a no-choice experiment confining colonies to a single P:C diet (1:6, 1:3, 1:1, 3:1, or 6:1), simulating field conditions with a constant P:C ratio of substrate. We replaced diets every day and estimated cumulative protein and carbohydrate harvest of colonies from dietary P:C ratios and dry:wet mass ratios of control diet pieces (*SI Methods*).

We used general linear mixed models (GLM) to test for diet treatment effects on: cumulative diet harvest (total diet, protein, and carbohydrates), final worker number, final colony mass (adult ants + brood + fungus gardens), and final brood mass (*SI Methods*, Fig. S1, and *Table S6*). We included initial colony mass as a covariate and colony ID as a random factor because the largest of the 20 collected colonies was initially divided into five sub-colonies and distributed across diet treatments, yielding 25 experimental colonies. We estimated initial worker number, retrospectively, from dead workers collected during the experiment and final survivors. Changes in colony demography were observed over 29 d, whereas diet harvest was measured over 22 d because crop failure on high-protein diets precluded measurements toward the end of the experiment. We analyzed the proportion of colonies with failed crops at the end of the experiment using a GLM with a binomial distribution with a logit link function, and diet protein availability as ordinal variable. Fungal samples were collected from each colony for %N analysis on the first and last days of the experiment, and a Kruskal–Wallis test was used to examine changes in fungal crop %N for each dietary P:C treatment.

Nutritional substrate selected by workers could potentially offer a biased estimate of fungal resource provisioning if workers used substantial amounts of carbohydrates (but not proteins) to fuel their own metabolic demands. We tested this conjecture by comparing the carbon mass in harvested sucrose with estimates of carbon mass respired as CO<sub>2</sub> by workers in the 1:6 and 6:1 P:C diet treatments over 22 d. Mass-balance calculations (detailed in *SI Methods*), based on metabolic data from a previous study (39), indicated that worker maintenance metabolism required a small fraction of the harvested sucrose carbon mass, from  $2.17 \pm 1.00\%$  to  $5.96 \pm 4.25\%$  in the

high-carbohydrate 1:6 P:C and low-carbohydrate 6:1 P:C diets, respectively, suggesting that the observed intake targets were not greatly influenced by worker energy demands. This inference is consistent with farming ants representing a very small part of a colony-farm's total biomass and energy consumption (39).

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