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Natural History Note

Dynamic Disease Management in *Trachymyrmex* Fungus-Growing Ants (Attini: Formicidae)

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abstract: Multipartner mutualisms have potentially complex dynamics, with compensatory responses when one partner is lost or relegated to a minor role. Fungus-growing ants (Attini) are mutualistic associates of basidiomycete fungi and antibiotic-producing actinomycete bacteria; the former are attacked by specialized fungi (*Escovopsis*) and diverse generalist microbes. Ants deploy biochemical defenses from bacteria and metapleural glands (MGs) and express different behaviors to control contaminants. We studied four *Trachymyrmex* species that differed in relative abundance of actinomycetes to understand interactions among antimicrobial tactics that are contingent on the nature of infection. MG grooming rate and actinomycete abundance were negatively correlated. The two species with high MG grooming rates or abundant actinomycetes made relatively little use of behavioral defenses. Conversely, the two species with relatively modest biochemical defenses relied heavily on behavior. Trade-offs suggest that related species can evolutionarily diverge to rely on different defense mechanisms against the same threat. Neither bacterial symbionts nor MG secretions thus appear to be essential for mounting defenses against the specialized pathogen *Escovopsis*, but reduced investment in one of these defense modes tends to increase investment in the other.

Keywords: metapleural glands, fungal symbiont, hygiene, grooming, weeding, Actinomycetales, Attini, *Escovopsis*.

Introduction

The hygienic and therapeutic strategies that a host can use to prevent or combat disease depend on the nature of infections and on the availability and costs of alternative defense mechanisms. Sessile organisms (e.g., plants) or temporarily sessile organisms (e.g., nesting animals) provide interesting models to explore the evolutionary trade-offs that may lead to one defense being prioritized over another, because escape from enemies by fleeing is generally not possible. Plants, for example, deploy a diverse array of biochemical, morphological, and biotic defenses derived from symbionts, even among closely related species (reviewed in Coley and Barone 1996; Stamp 2003; Fine et al. 2006; Agrawal 2007). Each of these alternatives carries a cost (Heil 2002), and thus many species confront energetic trade-offs associated with investment in one defensive mechanism or another or with differential investment in somatic defenses versus reproductive effort (e.g., Itino and Itioka 2001; Fincher et al. 2008). Similar trade-offs are known in sponges (e.g., Leong and Pawlik 2011) and other animals. A comparison of tree and house sparrows (*Passer*), for example, showed that tree sparrows exhibit a relatively weak nonspecific inflammatory response to malarial blood parasites and a relatively strong inducible immune response, relative to house sparrows, and vice versa (Lee et al. 2006).

Studies of eusocial insects are especially relevant to understanding trade-offs in antimicrobial strategies. Colony sizes range from a few related individuals to tens of thousands or even millions, living in crowded conditions favorable for pathogen transmission (Schmid-Hempel 1998; Boomsma et al. 2005; Cremer and Sixt 2009). Considerable evidence now suggests that polyandry may have secondarily evolved in response to disease pressure in eusocial insects with large, long-lived colonies (Hughes and Boomsma 2004; Tarpy and Seeley 2006). In a colony with a single multiply-mated queen, the genetic diversity in the long-lived chimeras of patrilines remains approximately constant throughout the colony's life span, which may facilitate the intracolony transmission of pathogens (Hughes and Boomsma 2006; Mattila and Seeley 2007; Reber et al. 2008). It is therefore no surprise that eusocial

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animals have evolved a diverse assembly of behavioral and biochemical mechanisms to control pathogens (Oi and Pereira 1993; Cremer et al. 2007; Reber et al. 2008). Many prior studies have focused on one defense strategy over another, and hence the coordination and integration of these multiple defense strategies is not yet well understood (e.g., Boomsma et al. 2005).

The obligate mutualism between attine ants and their fungal cultivars is ideally suited to explore how ants coordinate and integrate different elements of disease control (Weber 1972; Currie et al. 1999*b*; Bot et al. 2002; Hart et al. 2002; Mueller 2002; Little and Currie 2008) and how their societies adjust and optimize integrated pest management strategies under different or changing social and environmental conditions (Fernández-Marín et al. 2009). Attine ants are members of a mutualistic consortium (sensu Kozo-Polyansky [1924] 2010), along with leucocoprinous or pterulaceous (Basidiomycotina) fungi that are cultivated as food, and most species harbor filamentous Actinomycetales bacteria, which are cultured on their exoskeletons, to obtain antibiotics active against pathogens (Currie et al. 1999*a*, 1999*b*, 2006; Mueller et al. 2008; Mattoso et al. 2012). The ant fungal gardens are attacked by a parasitic microfungus (*Escovopsis*, Ascomycotina; Currie et al. 2003). Strains of *Escovopsis* differ in their virulence, and here we assess whether ants adjust their antimicrobial tactics according to the virulence of the pathogen. Both the cultivar and the ants are also attacked by an array of generalist pathogens (see Fernández-Marín et al. 2006, 2009 and references therein). A black yeast occurs in gardens of some attines that may antagonize the mutualistic actinomycete bacteria and thus reduce the ants' abilities to suppress *Escovopsis* infections (Little and Currie 2007, 2008).

Attine ants actively monitor their fungal cultivar, brood, and nestmates for signs of disease, and they weed the former and groom the latter to remove contaminants (e.g., Currie and Stuart 2001). They also plant pieces of healthy fungal cultivar over contaminated areas of the garden (see "Definitions of Behaviors" below), which may modify local competitive dynamics. Finally, when they encounter noncultivar fungal conidia, they apply antimicrobial secretions from exocrine glands, especially the paired metapleural glands (MGs; Fernández-Marín et al. 2006, 2009). MG secretions and bacterial metabolites have been viewed as dual complementary antimicrobial strategies of attine ants (Mueller et al. 2005, 2008; Currie et al. 2006; Fernández-Marín et al. 2006). Recent work has suggested that some members of the antibiotic-producing bacterial community are specific against *Escovopsis* while others have broad-spectrum activity (Haeder et al. 2009; Oh et al. 2009; Barke et al. 2010; Schoenian et al. 2011; Mattoso et al. 2012). Comparative analysis has further revealed a negative interspecific correlation between the proportion of workers per colony with visible actinomycetes and the frequency of MG grooming (Fernández-Marín et al. 2009), suggesting that the simultaneous use of multiple antimicrobial defenses may be too expensive to be a prudent strategy. That study contrasted representatives of the higher attine genera *Trachymyrmex* and *Acromyrmex* (with visibly abundant actinomycete bacterial cover in workers) with those of *Atta* and *Sericomyrmex* (which lack or have few visible bacteria; Mueller et al. 2008; Fernández-Marín et al. 2009). These comparisons showed that visible bacterial cover was greatly reduced or lost in genera with relatively larger colony sizes (for a discussion of breakdown of mutualisms, see Sachs and Simms 2006; Fernández-Marín et al. 2009). These genera, however, also differ greatly in numerous other aspects of their natural history, including differences in mature-colony size, queen mating frequency, diet, and worker caste differentiation, which made it difficult to generalize those prior results.

Here we aim to help resolve this ambiguity with a comparative study of four species of *Trachymyrmex* from central Panama that are relatively similar in social structure and life history but differ in abundance of actinomycetes, ranging from not visible to highly abundant on the exoskeleton. Specifically, we tested whether (1) the visible abundance of cuticular bacteria across species was inversely associated with the frequency of MG grooming, (2) weeding and fungus-planting behaviors might compensate for a reduction in visible abundance of actinomycetes, (3) ant hygienic behavior changes when colonies are infected with different *Escovopsis* strains, (4) the size of the MG reservoir (bulla) increases with a decrease in visibly abundant actinomycetes, and (5) reduced abundance of cuticular actinomycetes is more likely to occur in species with larger colonies.

Material and Methods

Ant Colony Collection and a Précis of Trachymyrmex *Natural History*

The four species of *Trachymyrmex* in this study are abundant in central Panama, although studies of their natural history have been fragmentary (Dijkstra and Boomsma 2003; Fernández-Marín et al. 2004, 2006; Hughes et al. 2008; Pérez-Ortéga et al. 2010). Colonies of *Trachymyrmex cornetzi*, *Trachymyrmex* sp. 3, and *Trachymyrmex zeteki* were collected in open and disturbed areas or forested regions, near Gamboa and Parque Nacional Soberania in central Panama, that receive about 1,800 mm rain per year. Colonies of *Trachymyrmex* sp. 10 were collected near Cocoli, in a drier forest and adjacent open areas that receive about 1,500 mm of rain per year (for details of the site, see Condit et al. 2004). Cocoli is near the Pacific coast of Panama City, about 20

km from the Gamboa/Soberania site. At the Cocoli site, there are other attines, including species representing *Mycocepurus*, *Apterostigma*, *Myrmicocrypta*, *Cyphomyrmex*, *Sericomyrmex*, *Trachymyrmex*, *Atta*, and *Acromyrmex*. At this site, we have no data on cultivar sharing.

In all species, the fungus gardens were usually suspended from rootlets that entered the chamber, or were attached to the ceiling (see Fernández-Marín et al. 2007), but parts of larger gardens contacted the chamber floor. Nests had a single entrance, which in mature colonies generally connected to a single fungus-garden chamber in *T. cornetzi* and *Trachymyrmex* sp. 3, one to four chambers in *T. zeteki*, and three to nine chambers in *Trachymyrmex* sp. 10. To obtain colony size estimates (numbers of worker), we collected 18 nests of *Trachymyrmex* sp. 10, 17 nests of *Trachymyrmex* sp. 3, 20 nests of *T. cornetzi*, and 128 nests of *T. zeteki*.

Nests were excavated with a geology pick and forceps, and when necessary, an electric aspirator was used to collect workers. The fungus garden was carefully collected to minimize any structural disturbance or introduction of contaminants from the soil. Ants were housed in plastic boxes using standard methods (Weber 1972). Three times per week, ants were fed with rice, corn, and pieces of fresh leaves and supplied with water. All nests were used within 3 weeks after collection.

For three colonies of *T. zeteki* and two colonies each of *T. cornetzi*, *Trachymyrmex* sp. 3, and *Trachymyrmex* sp. 10, we recorded the abundance of visible actinomycetes and measured head width, antennal scape length, pronotum width, and MG bulla length and width for 50–127 workers per nest. Morphological measurements were made to the nearest 0.01 mm with a Leica Wild M10 stereomicroscope.

Abundance of Actinomycetes and Escovopsis

We scored the abundance of visible actinomycetes on 16–50 workers from each of 8–15 colonies per species in 2007, and in 2008 workers were sampled from 10–14 colonies per species (see appendix for details). To score actinomycete cover, we used a scale developed by Poulsen et al. (2002, 2003*a*, 2003*b*; see also Currie et al. 2003; Fernández-Marín et al. 2009), which ranged from 0 (no visible actinomycetes) to 12 (exoskeleton completely covered). We also isolated actinomycetes and quantified their abundance in fungus gardens and on ant workers by counting colony-forming units (CFUs), following standard methods (Biani et al. 2009), from five randomly selected workers and 0.05 g of fungus garden from each of 10 nests. Under a laminar-flow fume hood, the method involved placing a sample in 10 mL of a solution of sterile water with 0.85% sodium chloride, 0.2% peptone, and 0.05% Tween 80 (a surfactant that facilitates the suspension of spores and cells in water; Sigma, St. Louis). Dilutions were plated on chitin agar (10 g chitin from crab shells, 0.5 g peptone, 0.1 g yeast extract, 0.01 g $FePO_4 \cdot H_2O$, 250 mL filtered seawater, 15 g agar), with 0.2 g/L of Nystatin (Bristol-Myers Squibb) as an antimycotic. Actinomycete colonies were grown at 25°C, and the number of CFUs was counted 4 weeks later.

We recorded the prevalence and intensity of *Escovopsis* infections in nests of each ant species by isolating 50 pieces of fungus garden per nest (3–5 mm diameter) and culturing 10 inoculations per petri dish; each petri dish contained potato dextrose agar (PDA, 19.5 g/500 mL of sterilized water) without antibiotics. We counted the number of healthy and *Escovopsis*-infected pieces after 10 days. *Escovopsis* infection was identified by mycelium growth and confirmed by conidia production. The number of nest samples per species was 12 for *T. cornetzi*, 13 for *Trachymyrmex* sp. 10, 3 for *Trachymyrmex* sp. 3, and 12 for *T. zeteki*.

Definitions of Behaviors

Self- and allo-grooming behaviors are common hygienic behaviors of insects but were not included in this study. "Self-grooming behavior" is not very specific, and it occurs when an ant rubs one of several legs across various body regions (e.g., Jander 1976); "allo-grooming behavior" involves similar limb movements, but the targets are adult or immature nestmates.

"Fungal grooming behavior" involves ants "licking" the fungus garden surface to remove any particles that are presumably contaminated (Currie and Stuart 2001). "Metapleural gland (MG) grooming" serves to transfer MG secretions to a source of infection and is characterized by a highly stereotypical behavior: worker ants extend their legs to raise the body from the substrate and then flex a foreleg along the femorotibial joint to bring the posterior surface of the metatarsus into contact with the opening of one of the MGs. "Fungus-planting behavior" involves a worker cutting a piece of healthy fungus garden and transplanting it to an infected area of a garden, which is distinct from behavior associated with the initiation of new garden growth on freshly collected substrate. "Weeding behavior" occurs when a piece of the garden is removed and placed in a garbage dump away from the garden.

Pathogen-Switching Experiments

A total of 12 colonies for each species were used in a pathogen-switching experiment. From each colony, we created a single subcolony of 20 workers, 0.5 g of fungus garden, and six brood items (three larvae and three pupae). Three of the 12 subcolonies were then infected with each of the *Escovopsis* strains isolated from nests of the four

Trachymyrmex species (i.e., there were three replicates per ant species–*Escovopsis* strain combination).

A semicircular piece of fungus garden (0.5 g, ~2.5 cm in diameter) with brood was placed on a sterile petri dish (100 mm × 15 mm) with sterile forceps. A piece of parafilm containing ~2×10^6 dry *Escovopsis* conidia was then gently rubbed over this garden fragment, after which sterile forceps were used to add 20 randomly chosen workers. After an acclimation period of around 10 min, we recorded the number of ants grooming the garden and the rates of MG grooming, fungus-planting behavior, and weeding behavior per worker for six consecutive periods of 10 min, with the aid of a 70× stereomicroscope.

We examined *Escovopsis* prevalence in infrabuccal pellets after the switching experiments described above. Infrabuccal pellets consist of trash collected by the ants, including abnormal fungal fragments, bacterially infected items, and other materials collected during grooming. These items are compacted and stored in the infrabuccal cavity just behind the mouthparts and subsequently regurgitated (Bailey 1920). When a colony is infected with fungal conidia, the infrabuccal pellet is primarily composed of infectious conidia (Little et al. 2003, 2006; Fernández-Marín et al. 2006). Using a stereomicroscope and a sterile loop, we gently removed each fresh infrabuccal pellet (4–7 h after infection) from ants from all subcolonies and inoculated them onto sterile petri dishes with PDA medium. Pellets are fragile, so we discarded those that broke during manipulations (never more than four pellets per subcolony). Each plate was monitored daily for up to 10 days, and the presence of *Escovopsis* (defined by sporulation) in the pellets was recorded.

Inhibition of Escovopsis *sp. Sporulation by the Fungal Cultivar*

To determine whether the fungal symbiont reduced *Escovopsis* sporulation, 1 g of fungus garden from 32 colonies of each of the four *Trachymyrmex* species was placed onto separate sterile petri dishes. Each petri dish also had a ring of cotton wool moistened with water placed around its rim to maintain humidity. The petri dishes were then divided randomly into four groups of eight per ant species, and each group was inoculated with 6.0×10^4 dry conidia of *Escovopsis* from gardens of each of the *Trachymyrmex* species in a complete factorial design. Ten days after inoculation, the fungus gardens including *Escovopsis* were suspended in 80 mL of a solution of Tween (0.2%); then, 1 mL of this solution was sampled and transferred to a hemocytometer to count the number of sporulated conidia. Counts were made only of replicates in which *Escovopsis* was the dominant fungus, and we excluded replicates in which *Trichoderma*, *Rhizopus*, or other fungi had overgrown the petri dish (range of excluded replicates per species, 0–6; for details, see appendix). While we cannot be certain that the *Escovopsis* sporulating on the plates was not already present in the fungus garden, the morphology and color of the *Escovopsis* germinated corresponded to that inoculated in all cases.

Statistical Analyses and Voucher Specimens

Many of the measured variables were nonnormally distributed or showed strong heteroskedasticity, in which case they were analyzed with nonparametric Kruskal-Wallis tests. Pairs of species were then compared by Mann-Whitney *U*-tests with Bonferroni-corrected significance levels. Because of the lack of a robust species-level phylogeny, we treated each species as an independent data point and did not implement comparative methods to take phylogenetic data into account.

The number of CFUs cultured from fungus fragments and workers and infrabuccal-pellet germination rates were compared by means of generalized linear models with Poisson and binomial error structure, respectively, corrected for overdispersion. To examine whether the size of the MG is related to actinomycete abundance both between and within species, we examined the allometric relationship between a composite measure of body size (the first principal component from an unrotated principal-component analysis of $\log_e$[head width] and $\log_e$[pronotum width]) and MG size ($\log_e$[bulla diameter × bulla width]). Residuals from this analysis were used to test whether there was a relationship between MG size relative to body size and visible actinomycete abundance, using a nested ANCOVA. The number of *Escovopsis* conidia sporulating was log transformed to normalize their distribution and compared between fungal and *Escovopsis* strains with two-way ANOVA.

Ant vouchers were deposited in the Museo de Invertebrados, Universidad de Panamá.

Results

The intensity of *Escovopsis* infection differed significantly between the different *Trachymyrmex* species (table 1; Kruskal-Wallis test: $H = 9.35$, df $= 3$, $P = .025$). The species also differed significantly in colony size ($H = 20.34$, df $= 3$, $P < .001$; fig. 1*A*) and the number of colony-forming units cultured from fungus gardens and workers after 4 weeks (fig. 1*B*; generalized linear model: between species: likelihood ratio (LR) $\chi^2 = 13.1$, df $= 3$, $P = .004$; fungus vs. workers: LR $\chi^2 = 1.77$, df $= 1$, $P = .184$; interaction: LR $\chi^2 = 2.67$, df $= 3$, $P = .445$). Visible actinomycete cover differed significantly both between species ($H = 38.0$, df $= 3$, $P < .001$; fig. 1*C*) and among nests within

Table 1: Summary of *Escovopsis* infection and disease management strategies for the four *Trachymyrmex* species studied (means ± SD)

	Trachymyrmex species					
	T. cornetzi	*T.* sp. 10	*T.* sp. 3	*T. zeteki*	H_3	P
Escovopsis infection (%)	43.1 ± 7.8	17.1 ± 3.4	10.0 ± 4.2	17.8 ± 5.9	9.35	.025
Visible actinomycete abundance	2.43 ± .22	.0 ± .0	2.95 ± .23	5.94 ± .45	38.0	<.001
Number of workers on garden	12.5 ± 1.0	10.2 ± .9	14.7 ± .6	13.3 ± .7	12.9	<.001
MG grooming events worker^{-1} h^{-1}	.09 ± .08	5.51 ± .79	.32 ± .17	.26 ± .26	33.4	<.001
Weeding events worker^{-1} h^{-1}	3.62 ± .5	1.1 ± .4	1.79 ± .3	1.17 ± .2	21.4	<.001
Fungal planting events worker^{-1} h^{-1}	3.34 ± .73	.32 ± .11	1.55 ± .58	.36 ± .12	16.9	<.001

Note: Differences between species were assessed with Kruskal-Wallace tests with 3 df (H_3). MG = metapleural gland.

species for *Trachymyrmex cornetzi* ($H = 265.3$, df = 22, $P < .001$), *Trachymyrmex* sp. 3 ($H = 93.8$, df = 18, $P < .001$), and *Trachymyrmex zeteki* ($H = 407.4$, df = 22, $P < .001$), but not for *Trachymyrmex* sp. 10, where only a few individuals in one nest had any visible actinomycetes ($H = 0.516$, df = 27, $P > .5$). There was no significant difference in visible actinomycete abundance between workers examined in 2007 and 2008 for any of the species (Kruskal-Wallis test: all $P > .28$).

Trachymyrmex species differed in their strategies to combat pathogen exposure. After infection, the four *Trachymyrmex* showed significant differences in numbers of workers tending the infected gardens per 10-min period ($H = 12.85$, df = 3, $P = .005$), so behavior rates per worker were used in the other analyses to remove this effect. The four species differed significantly in their per-worker rates of MG grooming ($H = 33.4$, df = 3, $P < .001$), weeding ($H = 21.4$, df = 3, $P < .001$), and fungus planting ($H = 16.9$, df = 3, $P < .001$), but these did not differ between different strains of *Escovopsis* (MG grooming: $H = 0.56$, df = 3, $P > .5$; weeding: $H = 2.59$, df = 3, $P = .459$; fungus planting: $H = 0.98$, df = 3, $P > .5$).

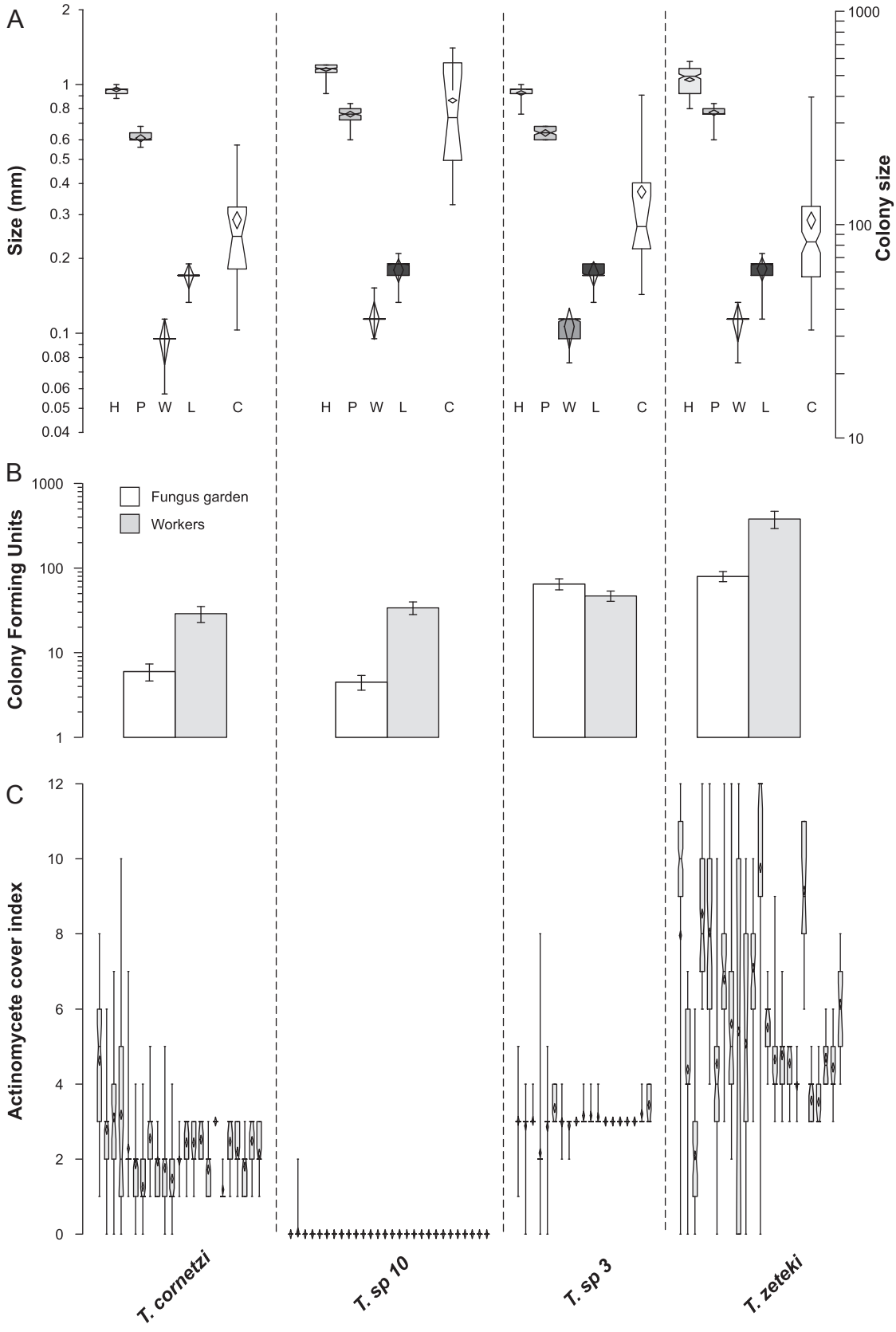
There was no overall relationship between use of MG grooming and median visible actinomycete abundance within three of the species, because one of the two measures was always invariant (fig. 2). For the fourth species, *T. cornetzi*, there was a negative relationship that was marginally insignificant (Spearman's $\rho = -0.657$, $n = 8$, $.05 < P < .10$). For the four species combined, however, there was a clear negative association between MG grooming rates and visible actinomycete abundance (fig. 2); *T. zeteki* had the highest level of actinomycete coverage and showed no MG grooming, while *Trachymyrmex* sp. 10 had the highest rate of MG grooming and no visible actinomycetes. Across all species, the relationship between MG grooming rates and visible actinomycete abundance is well described by an indifference curve based on a Cobb-Douglas utility function (orthogonal regression: $U = 0.607$; lack of fit: $\chi^2 = 4.46$, df = 30, $P = .999$).

Difference in the inhibition of *Escovopsis* spore germination from infrabuccal pellets by the different *Trachymyrmex* species was marginally insignificant (fig. 3; LR $\chi^2 = 7.38$, df = 3, $P = .061$), but there was a significant difference between different *Escovopsis* strains (LR $\chi^2 = 8.07$, df = 3, $P = .045$) and a significant interaction between *Escovopsis* strain and *Trachymyrmex* species (LR $\chi^2 = 20.04$, df = 3, $P = .018$). There was a trend for greater germination of *Escovopsis* strains when confronted with the species from which they were harvested (the combinations along the leading diagonal in fig. 3), compared with other species, that was marginally insignificant (LR $\chi^2 = 3.68$, df = 1, $P = .055$), although the proportion of explained variance was small.

The size of the MG increased with body size for all four *Trachymyrmex* species (fig. 4; nested ANCOVA: $F_{3,21.2} = 106.71$, $P < .001$), but the slope of this relationship differed between species ($F_{3,21.2} = 3.11$, $P = .026$); at the same body size, species differed significantly in MG size ($F_{3,21.2} = 17.88$, $P < .001$). The relationship between MG size and body size within a species was not significantly different between different nests ($F_{5,21.2} = 1.72$, $P = .127$).

The relationship between relative MG size and visible actinomycete cover varied across the three species with visible actinomycete cover (nested ordinal logistic ANCOVA: LR $\chi^2 = 6.58$, df = 2, $P = .037$). There was no correlation between residual MG size and residual actinomycete cover (calculated by subtracting the median actinomycete cover for each colony from the actinomycete cover of each member of that colony) for *T. cornetzi* (Spearman's $\rho = -0.115$, $n = 61$, $P = .377$) and *Trachymyrmex* sp. 3 ($\rho = +0.084$, $n = 112$, $P = .375$), but there was a significant positive correlation for *T. zeteki* ($\rho = +0.170$, $n = 247$, $P = .008$; fig. 5).

The number of *Escovopsis* conidia sporulating was significantly different in fungus gardens derived from the nests of the different *Trachymyrmex* species ($F_{3,73} = 23.9$, $P = .0005$) but did not depend on the species of *Trachymyrmex* from which the *Escovopsis* was derived ($F_{3,73} = 1.17$, $P = .325$), and there was no interaction between



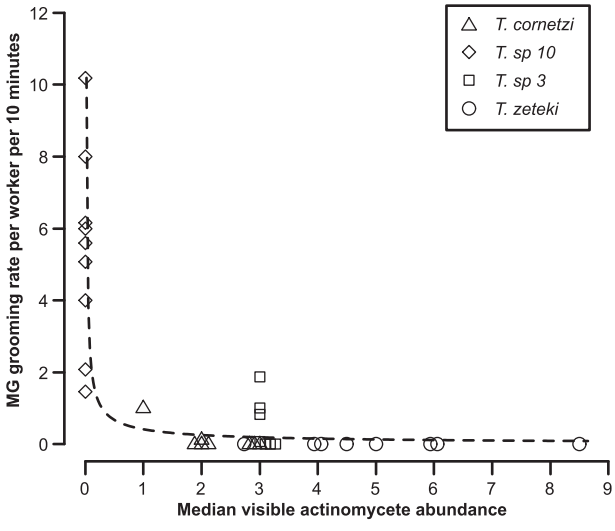


Figure 2: Negative correlation between median metapleural gland (MG) grooming rates by attine workers following exposure to *Escovopsis* spores, compared with the median relative abundance of visible actinomycete bacteria on the workers' exoskeleton (Spearman's $\rho = -0.618$, $P < .001$). Each symbol represents the data from a single colony. Overlapping symbols are slightly offset along the *X*-axis. The dashed line is a fitted indifference curve based on the Cobb-Douglas utility function (see text for details).

Escovopsis strain and fungus garden strain ($F_{9,73} = 0.298$, $P = .973$; fig. 3*B*).

Discussion

Our results show that variation in the relative abundance of visible actinomycetes is associated with differential deployment of MG secretions, weeding, and fungus-planting behaviors to control garden disease in *Trachymyrmex* ants. Such observations support previous work on disease management by attines, which demonstrated a trade-off between MG grooming rates and actinomycete abundance among genera, in that *Atta* and *Sericomyrmex* have coopted MG secretions to target a specialist pathogen (*Escovopsis*) that is controlled by actinomycete antibiotics in other attines (Fernández-Marín et al. 2009).

Here we document a species-level example of this trade-off between MG secretions and actinobacterial antibiotics. We do not know to what extent our comparisons are phylogenetically independent, because *Trachymyrmex* is paraphyletic (Schultz and Brady 2008), with some species being more closely related to *Sericomyrmex* than to other *Trachymyrmex*. A current phylogenetic hypothesis places *Trachymyrmex zeteki* in the same clade as *Sericomyrmex*, with *Trachymyrmex cornetzi* in another clade (Schultz and Brady 2008). While *Sericomyrmex* virtually lacks visible actinomycetes, *T. zeteki* has twice as many as *T. cornetzi* (table 1). It therefore seems unlikely that phylogenetic constraints are important for disease management strategies; rather, our results suggest that gains and losses of actinomycete cover can evolve quickly.

Our observations are potentially complicated by two additional considerations. First, one of the main trade-offs involved *Trachymyrmex* sp. 10 (with low levels of actinomycete cover) versus *T. cornetzi*, *T. zeteki*, and *Trachymyrmex* sp. 3 (all with higher levels of cover). This comparison is potentially confounded because the former species was collected in a habitat drier than that from which the latter three species were collected. We have no evidence to date of habitat-specific pathogens attacking *Trachymyrmex*. After our studies, we encountered a site about halfway between Gamboa and Cocoli where all four species co-occur sympatrically (H.F.-M., personal observation), which will enable us to test directly for habitat-specific pathogens. For other fungus-growing ants (*Atta*), phylogenetic studies have shown that the evolutionary history of *Escovopsis* does not track the evolutionary history of the ants (Taerum et al. 2007). Some strains of *Escovopsis* that attack *Atta colombica*, *Atta cephalotes*, and *Atta sexdens* group together in phylogenetic analyses. All of these host species have lowland tropical distributions, but *A. sexdens* prefers drier habitats (Weber 1972). Thus, at least for one of the specialized parasites, there is no evidence for habitat-specific pathogens that could drive observed differences.

Our study used two methods to estimate actinomycete abundance that yielded apparently contrasting results: *T. cornetzi* and *Trachymyrmex* sp. 3 had higher values for actinomycete cover than did *Trachymyrmex* sp. 10, which showed virtually no actinomycete cover, yet workers of all three species showed similar numbers of colony-forming units (CFUs). Counting CFUs is a culture-dependent method that is well known to underestimate bacterial abundance, because the culture medium is likely to sustain growth of some bacteria but not others. Instead, a culture-

Figure 1: Measures of worker size, colony size, and actinomycete abundance in Panamanian *Trachymyrmex*. *A*, Box plots showing the distribution in the size of *Trachymyrmex* workers (H = head width, P = pronotum width) and their metapleural glands (MGs; W = MG bulla width, L = MG bulla length), along with colony size (C). *B*, Colony-forming units (mean ± SE) of actinomycete bacteria developing after 3 weeks of culture of fragments of fungus garden (open bars) or worker ants (filled bars). *C*, Variation of visible actinomycete cover within and between *Trachymyrmex* species. Each box plot shows the distribution of cover classes within a single colony of the appropriate species. For box plots, the narrowest section of each box represents the median value, with the extremes of the box giving the 25% and 75% quartiles and the whiskers giving the extremes of the distribution. Means are marked with diamonds.

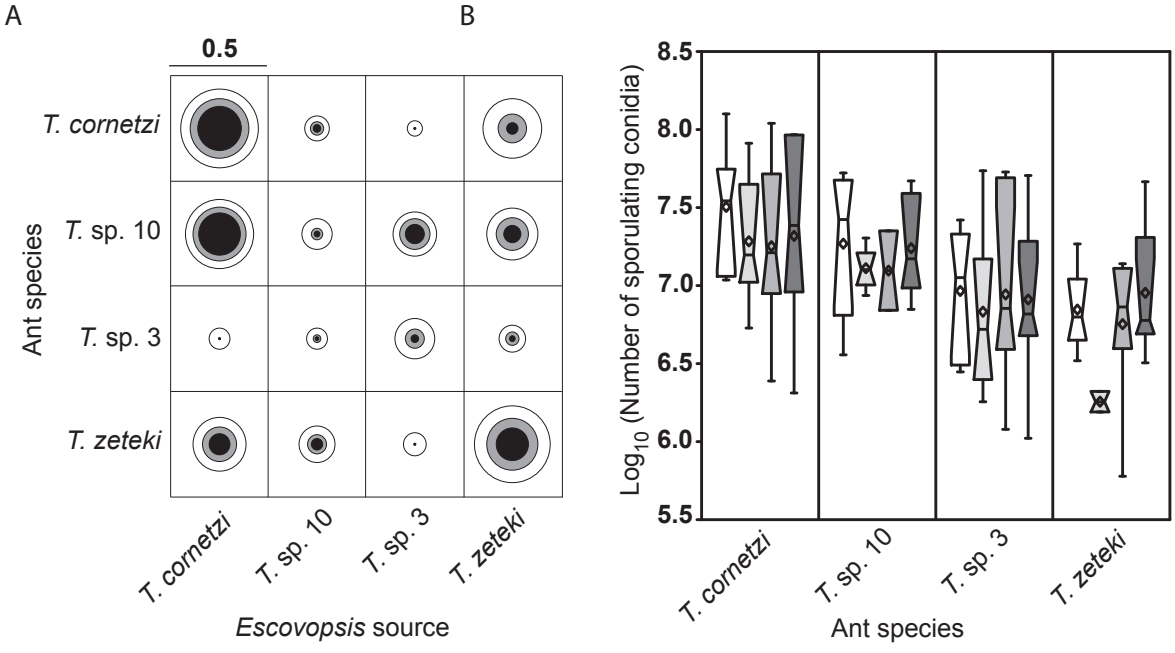


Figure 3: *A*, Pellet germination rates. *Escovopsis* strains derived from each *Trachymyrmex* species were used to infect every other *Trachymyrmex* species. The diameter of the gray circle represents the mean rate of germination, with the black and white circles representing ±1 SE. *B*, Box plots showing the number of sporulating conidia produced by *Escovopsis* derived from the fungus gardens of four *Trachymyrmex* species ("*Escovopsis*") when used to infect garden fragments of their own and the other three *Trachymyrmex* species ("Ant species"). The narrowest section of each box represents the median value, with the extremes of the box giving the 25% and 75% quartiles and the whiskers giving the extremes of the distribution. Means are marked with diamonds.

free method, such as visually estimating the white bacterial cover on workers' exoskeletons or next-generation sequencing, will possibly yield a more reliable result (e.g., Cafaro and Currie 2005; Currie et al. 2003; Kost et al. 2007; Mueller et al. 2008; Haeder et al. 2009; Sen et al. 2009; Barke et al. 2010).

When we removed *Trachymyrmex* sp. 10 from our analyses, the negative correlation between MG grooming rate and actinomycete abundance was not significant, suggesting that MG secretions may play a minimal role in controlling *Escovopsis* in some *Trachymyrmex*. Overall, the differential abundance of actinomycetes among *Trachymyrmex* species might thus be explained by two hypotheses that are not mutually exclusive. First, different species may have actinomycetes that produce antibiotics with differing degrees of efficacy in controlling *Escovopsis*, and they incur different costs to maintain the bacteria, so that species could have relatively few actinomycetes that produce more potent antibiotics, but this has not been tested. Second, *Trachymyrmex* species could complement their actinomycete antibiotics with other hygienic behaviors or sources of antimicrobial compounds. We found that *T. cornetzi* and *Trachymyrmex* sp. 3, both species with intermediate actinomycete abundance, had higher frequencies of weeding and fungal-planting behaviors, compared with *T. zeteki* (with abundant actinomycetes and relatively low rates of MG grooming) and *Trachymyrmex* sp. 10 (nearly no visible actinomycetes but high rates of MG grooming), consistent with the second hypothesis. While nothing is known of the dose-dependent effects of actinomycete antibiotics or MG compounds, it is interesting to note that even closely related *Trachymyrmex* species differ in their glandular chemistry (Adams et al. 2012), although whether this is the case for MG secretions remains to be tested.

Complementarity of multiple defenses in attine ant public health management raises new questions about the relative metabolic costs of alternative defenses, as suggested by Fernández-Marín et al. (2009). This complementarity hypothesis was developed for the interaction between MG secretions and actinomycete maintenance, but our results imply that similar arguments may apply to trade-offs with weeding and grooming behaviors, although we lack data on the metabolic costs associated with behavior. The functional role of weeding and grooming as an alternative to employing antimicrobial compounds is clear, because this behavior removes pathogens and infected fungal-garden pieces (Bailey 1920; Bass and Cherrett 1994; Currie and Stuart 2001). In contrast, the functional role of fungus-

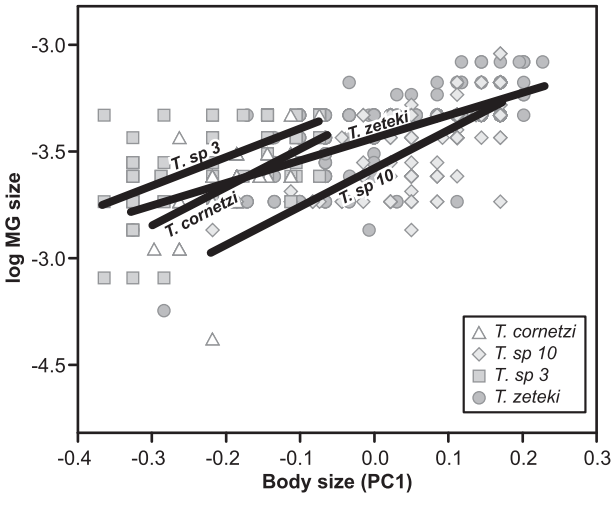


Figure 4: Relationship between body size of *Trachymyrmex* worker ants (first principal component; see text for details) and the logarithm of metapleural gland (MG) size. Least squares regression lines are shown for each species.

planting behavior is not clear, although covering behavior could physically minimize the dispersion of pathogenic conidia. In addition, such behavior may inhibit germination/growth of the pathogen by modifying microenvironmental conditions to render them unfavorable to development of the pathogen, analogous to the growth of tumors under some microenvironmental conditions but not others (Polyak et al. 2008). The latter hypothesis is consistent with the fact that some fungal cultivar strains inhibit the growth of some *Escovopsis* strains (fig. 3*B*; Gerardo et al. 2004, 2006), although the mechanism by which they do so is not known.

An understanding of the trade-offs among different defensive strategies by attines is impeded by limited data on (1) the costs of the diverse behavioral and biochemical strategies using a common currency such as metabolism, (2) dose-dependent efficacy of different antimicrobial compounds, (3) differing degrees of virulence among strains of pathogens, and (4) evolutionary lability of behavioral and biochemical defenses. Synthesis of MG secretions accounts for more than 15% of basal metabolic energy in *Acromyrmex* (Poulsen et al. 2002), and maintaining actinomycete bacteria carries approximately the same cost (Poulsen et al. 2003*a*, 2003*b*), but nothing is known of the energetic costs of prophylactic and hygienic behaviors (Fernández-Marín et al. 2007). Trade-offs among defensive strategies are most thoroughly studied for plants' antiherbivore defenses, which are shaped by the relative diversity and abundance of specialist and generalist enemies (see references in "Introduction").

Coevolution among fungus-growing ants, their fungal cultivars, and actinomycete bacteria has been likened to an arms race with respect to *Escovopsis* infections (Cafaro et al. 2011), with considerable emphasis on biochemistry. Our results highlight the fact that sanitary behaviors, such as weeding, grooming, and fungus planting, are underappreciated additional weapons in the ants' arsenal that can modify the dynamics of the race (e.g., Little et al. 2006). Further complications for understanding the dynamics of this symbiosis include factors associated with the acquisition of new fungal cultivars by farming ant populations. Relatively basal species such as *Mycocepurus smithii* grow at least five different cultivars (Mueller et al. 1998), while *Cyphomyrmex* (Green et al. 2002) and *Acromyrmex* species (Poulsen et al. 2009) may exchange fungal cultivars, although each mature attine colony appears to host only a single cultivar (Poulsen and Boomsma 2005; Mueller et al. 2010). Switching fungal cultivars increases genetic heterogeneity across colonies, which may make the symbiosis as a whole less susceptible to population-wide sweeps of specific fungal pathogens (Hamilton 1982; Mueller et al. 2005). Such population structures of garden symbionts across colonies will tend to maintain genetic diversity for mutually adaptive responses of ants, fungus, and pathogens (Thompson 2005).

At present, we lack organized data on the genetic diversity of symbiont strains cultivated by *Trachymyrmex*

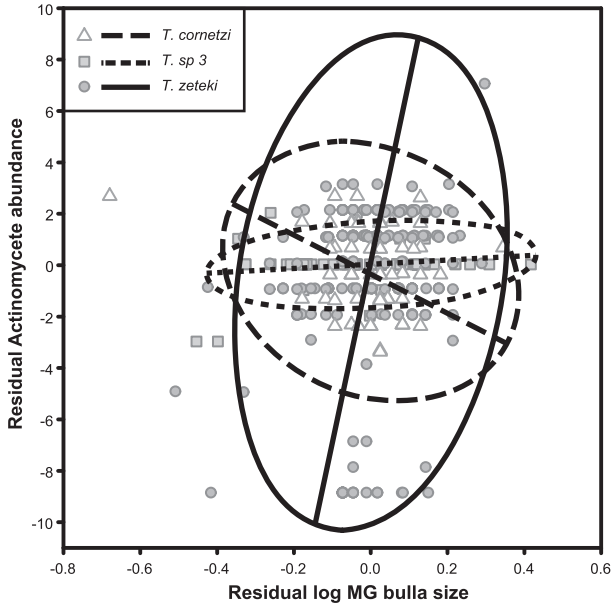


Figure 5: Relationship between metapleural gland (MG) size (removing the effects of worker body size by using the residuals from the regressions in fig. 4) and actinomycete cover (after removing the effect of differences between colonies; see text for details) for *Trachymyrmex* workers. The 95% confidence ellipses and reduced major axes for the data are shown for each species.

species in central Panama. If diversity of fungal symbionts itself has a role in disease management, then species that make minimal use of actinomycetes and MG grooming (e.g., *T. cornetzi* and *Trachymyrmex* sp. 3) should have a higher diversity of cultivar strains, while species that make extensive use of actinomycytes or MG grooming (e.g., *T. zeteki* and *Trachymyrmex* sp. 10) should have a limited diversity of fungal symbiont strains. The genetic diversity of *Escovopsis* strains attacking Panamanian *Trachymyrmex* symbionts is also unknown, but provisional data suggest that there is probably no tight long-term association between *Escovopsis* strains and the fungal cultivar, because the same strains of *Escovopsis* can be found in both closely and distantly related attines (e.g., *Apterostigma* [Gerardo et al. 2006] and *Atta* [Taerum et al. 2007]). Although the large-scale co-occurrence patterns of attine ants, cultivars, *Escovopsis*, and *Pseudonocardia* are increasingly well understood (Currie et al. 2006; Cafaro et al. 2011), there is much left to be done before we understand the local and regional dynamics of the host-mutualist-parasite interactions that govern the attine ant farming symbiosis.

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APPENDIX

Methods to Assess Inhibition of Escovopsis *sp. Sporulation by the Fungal Cultivar*

In vitro assays were used to assess whether fungal cultivars are able to inhibit sporulation of *Escovopsis*, from con- and heterospecific nests. In some cases, the petri dishes were overgrown by contaminants and were discarded, as follows. With respect to infections using *Escovopsis* from *Trachymyrmex zeteki* nests, we eliminated two replicates from *Trachymyrmex* sp. 10, two replicates from *T. zeteki*, no replicates from *Trachymyrmex* sp. 3, and three replicates from *Trachymyrmex cornetzi*. For infections using *Escovopsis* from *T. cornetzi* nests, we eliminated two replicates from *Trachymyrmex* sp. 10, four replicates from *T. zeteki*, one replicate from *Trachymyrmex* sp. 3, and two replicates from *T. cornetzi*. For infections using *Escovopsis* from *Trachymyrmex* sp. 3, we eliminated six replicates from *Trachymyrmex* sp. 10, one replicate from *T. zeteki*, one replicate from *Trachymyrmex* sp. 3, and two replicates from *T. cornetzi*. Finally, for infections using *Escovopsis* from *Trachymyrmex* sp. 10, we eliminated three replicates from *Trachymyrmex* sp. 10, six replicates from *T. zeteki*, two replicates from *Trachymyrmex* sp. 3, and two replicates from *T. cornetzi*.

Methods to Score Abundance of Actinomycetes

Over two years, we collected a sample of ants from different nests for each of the four study species, to make comparisons among species and between years. The number of colonies from which workers were sampled in 2007 and 2008, respectively, are 13 and 11 colonies for *Trachymyrmex* sp. 10, 12 and 11 colonies for *T. cornetzi*, 8 and 10 colonies *Trachymyrmex* sp. 3, and 15 and 14 colonies for *Trachymyrmex zeteki*. For 2007 collections, we examined 50 workers per colony, except for *T. cornetzi* ($n = 25$ workers). In 2008, we examined 25, 16–50, 25, and 20–25 workers per colony for *Trachymyrmex* sp. 10, *T. cornetzi*, *Trachymyrmex* sp. 3, and *T. zeteki*, respectively.

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