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Reduced biological control and enhanced chemical pest management in the evolution of fungus farming in ants

Hermógenes Fernández-Marín[1,2,3,4,*], Jess K. Zimmerman[5], David R. Nash[4], Jacobus J. Boomsma[4] and William T. Wcislo[1,2,*]

[1]*Smithsonian Tropical Research Institute, PO Box 0843-03092, Balboa, Ancón, Panama*
[2]*STRI, MRC 0580-12, Unit 9100, PO Box 0948, DPO AA 34002-9998, USA*
[3]*Department of Biology, and* [5]*Institute for Tropical Ecosystem Studies, University of Puerto Rico, PR 00936, USA*
[4]*Department of Biology, Centre for Social Evolution, University of Copenhagen, Universitetsparken 15, 2100 Copenhagen, Denmark*

To combat disease, most fungus-growing ants (Attini) use antibiotics from mutualistic bacteria (*Pseudonocardia*) that are cultured on the ants' exoskeletons and chemical cocktails from exocrine glands, especially the metapleural glands (MG). Previous work has hypothesized that (i) *Pseudonocardia* antibiotics are narrow-spectrum and control a fungus (*Escovopsis*) that parasitizes the ants' fungal symbiont, and (ii) MG secretions have broad-spectrum activity and protect ants and brood. We assessed the relative importance of these lines of defence, and their activity spectra, by scoring abundance of visible *Pseudonocardia* for nine species from five genera and measuring rates of MG grooming after challenging ants with disease agents of differing virulence. *Atta* and *Sericomyrmex* have lost or greatly reduced the abundance of visible bacteria. When challenged with diverse disease agents, including *Escovopsis*, they significantly increased MG grooming rates and expanded the range of targets. By contrast, species of *Acromyrmex* and *Trachymyrmex* maintain abundant *Pseudonocardia.* When challenged, these species had lower MG grooming rates, targeted primarily to brood. More elaborate MG defences and reduced reliance on mutualistic *Pseudonocardia* are correlated with larger colony size among attine genera, raising questions about the efficacy of managing disease in large societies with chemical cocktails versus bacterial antimicrobial metabolites.

Keywords: Attini; mutualism; *Pseudonocardia*; metapleural gland; public health; social complexity

1. INTRODUCTION

Disease agents are important selective forces on social evolution (Hamilton 1982; Schmid-Hempel 1998; Fefferman *et al.* 2007; Stow *et al.* 2007). Pathogens can determine upper limits of host group size, because increasing density of individuals results in elevated contact rates that facilitate high rates of pathogen transmission (Hamilton 1982; Schmid-Hempel 1998; Boomsma *et al.* 2005), potentially leading to epidemics within genetically homogeneous groups (Schmid-Hempel 1998; Hughes *et al.* 2002). Social insects use diverse methods to prevent and combat disease agents, such as grooming or other hygienic behaviours and the use of antimicrobial compounds from glandular secretions or external sources (e.g. bacteria; Oi & Pereira 1993; Currie *et al.* 1999*a*; Mueller *et al.* 2005). The evolution of larger colony sizes places greater demands on 'public health' adaptations (e.g. Fefferman *et al.* 2007; Stow *et al.* 2007), but trade-offs associated with different disease management strategies are not well understood.

The fungus-growing ants (Attini) provide an opportunity to explore the evolutionary relationships between changes in hygienic strategies and social complexity (Mueller *et al.* 2005). This tribe includes 12 genera and over 210 species (Schultz & Brady 2008), among which colony size varies by six orders of magnitude (Weber 1972). Colony size is partly associated with queen mating frequency, such that gynes from basal and transitional genera mate once, while those from derived leafcutter genera mate multiply, so that genetic heterogeneity is greater in colonies with larger numbers of workers (Villesen *et al.* 2002), which is advantageous in disease management (Hughes & Boomsma 2006; Mattila & Seeley 2007).

The attine ants have an obligate and ancient (approx. 50 million years BP; Mueller 2002; Schultz & Brady 2008) mutualism with basidiomycete fungi, which are cultivated as a food source and defended from competitors and pathogens. They also have an ancient association with actinomycete *Pseudonocardia* bacteria (Currie *et al.* 1999*b*, 2006), which ants of most genera culture in specialized structures on their cuticle. Available evidence indicates that *Pseudonocardia* metabolites are narrow-spectrum antimicrobials active against *Escovopsis*, a potentially virulent specialized fungus that attacks the ants' mutualistic fungus (Currie *et al.* 1999*b*, 2003*a*, 2006). In addition to *Escovopsis*, an array of generalist pathogens may attack both the fungal symbiont and the ants (Currie *et al.* 1999*b*; Jaccoud *et al.* 1999; Currie & Stuart 2001;

*Authors and address for correspondence: Smithsonian Tropical Research Institute, PO Box 0843-03092, Balboa, Ancón, Panama (hermogenes_f@hotmail.com, wcislow@si.edu).

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Hughes & Boomsma 2004). Exocrine gland secretions, especially from the metapleural glands (MG), are broad-spectrum antimicrobials (do Nascimento *et al.* 1996; Bot *et al.* 2002; Fernández-Marín *et al.* 2006). In short, ants reduce the pathogen load within nests by (i) monitoring contaminant levels (Fernández-Marín *et al.* 2006), (ii) grooming their bodies and those of adult and immature nest-mates (Bailey 1920; Weber 1972; Quinlan & Cherrett 1977), (iii) actively regulating antimicrobials from exocrine glands (Fernández-Marín *et al.* 2006), (iv) weeding the garden (Bass & Cherrett 1994; Currie *et al.* 2006), and (v) deploying antibiotics from *Pseudonocardia* (Currie *et al.* 1999*a*, 2003*b*; Little *et al.* 2006).

In the present paper, we examine the relative use of *Pseudonocardia* metabolites and MG secretions in disease management strategies, and associated trade-offs among derived and basal attine genera. We explore the evolutionary relationships between large-scale farming and the relative investment in hygienic strategies involving direct chemical and behavioural control of pathogens versus bacteria-derived antibiotics.

2. MATERIAL AND METHODS

(a) *Relative abundance of* Pseudonocardia

Nests from nine species representing five genera were collected in Soberanía National Park, Panama: *Atta colombica* ($n=10$ colonies); *Atta sexdens* ($n=5$); *Atta cephalotes* ($n=7$); *Acromyrmex octospinosus* ($n=10$); *Trachymyrmex zeteki* ($n=9$); *Trachymyrmex* cf. *cornetzi* ($n=10$); *Sericomyrmex* sp. 1 ($n=4$); *Sericomyrmex amabilis* ($n=10$); and *Cyphomyrmex longiscapus* ($n=6$). Ant colonies were transferred to plastic containers where they were maintained using standard laboratory methods (Weber 1972). Using a stereomicroscope, we scored 25–150 worker ants per colony for the presence of a 'conspicuous white bloom' on the propleural plates or other thoracic areas, which is the standard criterion for the presence of *Pseudonocardia* (Currie *et al.* 1999*b*, 2006; Poulsen *et al.* 2003*b*). To measure relative abundance at the colony level, we used the proportion of workers with visible actinomycetes.

(b) *MG grooming following fungal inoculation with* Penicillium

From each colony, we established sub-colonies in transparent plastic boxes (7.4×7.4×3.1 cm) that each contained 40 workers, 1 g of fungus garden (except for *C. longiscapus*, for which we used 0.5 g), six larvae and six pupae (Fernández-Marín *et al.* 2006). We used two pieces of parafilm (each approx. 5×5 mm^2) to transfer conidia to each garden. On each piece, we placed an approximately 3 mm^2 piece of pure culture (potato dextrose agar, PDA media; see below) of *Penicillium* sp. 1 with conidia, and each sub-colony thus received a total of 1.5–2.3×10^7 dry conidia. To transfer the dry conidia, each piece of parafilm was held with sterile forceps and gently brushed over the fungus garden and brood. During the next hour, we recorded MG grooming by all workers observed in a 3.5 cm diameter field of view of a stereomicroscope (7×). We recorded MG grooming for 1 hour as a baseline measure prior to inoculations, but first we manipulated the garden with two parafilm pieces that lacked conidia to control for physical changes to the garden. MG grooming is defined as a worker ant partially extending its legs to raise the body from the substrate, and then flexing the foreleg at the femorotibial joint to bring the posterior surface of the metatarsus into contact with the opening of the paired MG.

(c) *MG grooming after infection with* Metarhizium *and* Escovopsis

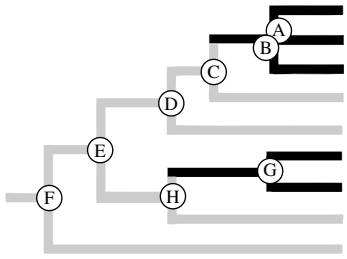
To ascertain MG grooming rates when challenged by a fungal pathogen (*Escovopsis*) and an entomopathogen (*Metarhizium anisopliae*), we used *A. colombica*, *Ac. octospinosus*, *S. amabilis* and *T. zeteki*. From each of 10 nests per species, we established two sub-colonies containing 20 workers and 1 g of fungus garden. *Atta* and *Acromyrmex* have polymorphic workers, so we used workers with head widths between 1.3 and 1.5 mm and the same body colour (and therefore approximately the same age; Poulsen *et al.* 2002, 2003*b*). We used a two-factorial treatment, where we added three larvae and three pupae to one replicate sub-colony, and left the other without brood. Five sub-colonies per species were inoculated with approximately 1.5×10^6 dry conidia of *M. anisopliae*, and five were inoculated with approximately 1.5×10^6 dry conidia of an appropriate *Escovopsis* strain (see below). Inoculations for both pathogens were done with the same procedure as for *Penicillium*. For each sub-colony, we recorded MG grooming during 90 min after inoculation. We also noted the targets that workers contacted with their legs immediately after each bout of MG grooming (i.e. garden, brood, nest-mate workers, a worker's own body).

We used the following pure cultures: a contaminant weed, *Penicillium* sp. 1, isolated from the cuticle of a queen of *A. colombica*; *M. anisopliae* strain Ma275 obtained from the University of Copenhagen, isolated from beetles; and *Escovopsis* strains isolated from the corresponding ant host species. Dry conidia were applied because inoculation by suspension with water plus detergent, as usually used (Currie *et al.* 2003*b*; Hughes & Boomsma 2004), induces lower MG grooming rates. All fungal cultures were grown and maintained in Petri dishes with PDA (19.5 g per 0.5 l of distilled water) without antibiotics.

Mature colony sizes were taken from the following sources: *C. longiscapus* (Mueller & Wcislo 1998); *T. zeteki* and *T.* cf. *cornetzi* (H. Fernández-Marín, E. B. Gomez, J. J. Boomsma, D. Nash & W. T. Wcislo 2008, unpublished data); *Sericomyrmex* sp. 1 and *S. amabilis* (Murakami *et al.* 2000; H. Fernández-Marín 2004–2006, unpublished data); *A. colombica* (Lewis 1975; Fowler *et al.* 1986); *A. sexdens* and *A. cephalotes* (Weber 1972); and *Ac. octospinosus* (Lewis 1975).

(d) *Statistical analyses and voucher specimens*

Rates of MG grooming before and after *Penicillium* infection were analysed using general linear models (GLMs) with Poisson errors (JMP 7.02, SAS Inc., Cary, NC, USA), using the number of grooming events per hour as the dependent variable. Comparisons of actinomycete cover among species were made using GLM with binomial errors, with the number of actinomycete-coated workers from each colony as the dependent variable and the total number of workers examined in each colony as the binomial denominator. The relationship between MG grooming rate after infection and relative abundance of actinomycetes was assessed using Spearman's rank correlation. Where colony size was used as a predictor variable, it was normalized by logarithmic transformation. The phylogenetically independent relationship between MG grooming rate after infection and relative abundance of actinomycetes was assessed using linear



ant clade	MG grooming		actinomycetes	colony size
	before infection	after infection		
Atta cephalotes	0.9 ± 0.5	180.5 ± 133.7	0.0 ± 0.0	3 000 000
Atta sexdens	2.6 ± 1.2	265.8 ± 127.1	0.0 ± 0.0	5 000 000
Atta colombica	1.8 ± 0.8	235.0 ± 112.0	0.0 ± 0.0	2 500 000
Acromyrmex octospinosus	0.3 ± 0.2	12.0 ± 8.1	0.75 ± 0.1	40 000
Trachymyrmex cf. *cornetzi*	0.0 ± 0.0	2.3 ± 1.2	0.95 ± 0.4	105
Sericomyrmex amabilis	0.8 ± 0.4	176.9 ± 27.7	0.028 ± 0.03	2000
Sericomyrmex sp. 1	0.5 ± 0.5	25.0 ± 8.9	0.008 ± 0.016	2000
Trachymyrmex zeteki	1.2 ± 0.8	8.4 ± 3.1	0.96 ± 0.08	78
Cyphomyrmex longiscapus	0.0 ± 0.0	0.0 ± 0.0	0.95 ± 0.5	30

Figure 1. Mapping traits onto the attine phylogenetic tree (from Schultz & Brady 2008) shows the independent recurrence of a reduction in visibly cultured *Pseudonocardia* bacteria, which is associated with increased MG grooming rates (black lines). Mapped traits are mean rates of MG grooming following exposure to conidia of *Penicillium* sp. 1, proportion of workers with visible bacteria and estimated colony sizes. Data are presented as mean ± s.d., except estimated colony size. Letters of nodes in the phylogeny correspond to those in the inset of figure 2.

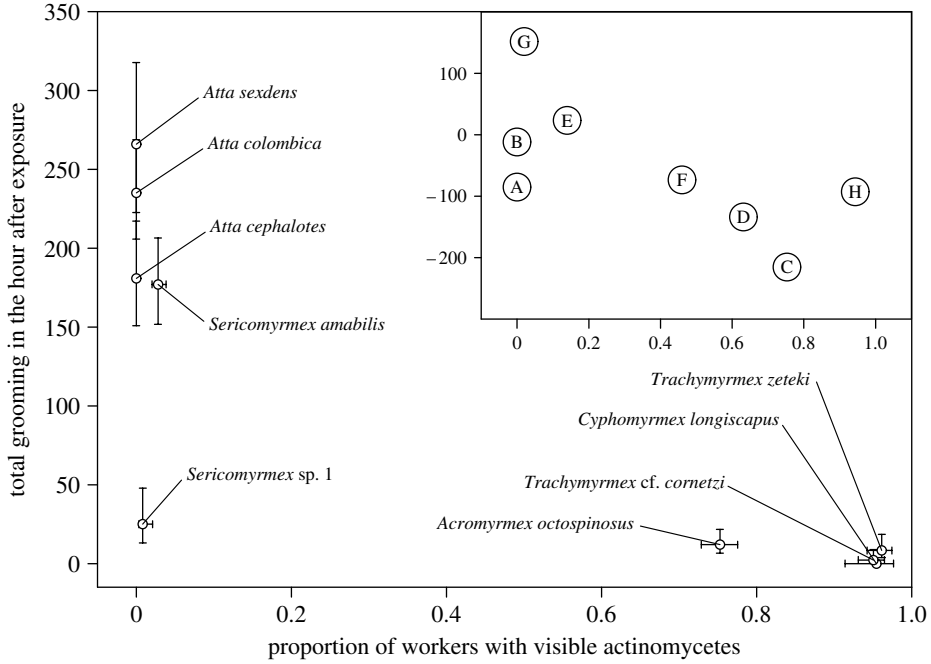


Figure 2. MG grooming rates by attine workers (mean ± s.e., based on log-transformed data) following exposure to *Penicillium*, compared with the proportion of workers with visible *Pseudonocardia* bacteria (mean ± s.e., based on logit-transformed data). The inset graph shows the relationship between the positivized phylogenetically independent contrasts for the two variables, based on the phylogenetic tree presented in figure 1, with each data point labelled with the node letter from the phylogeny.

correlation through the origin (Garland *et al.* 1992) between phylogenetically independent contrasts for both variables, generated using the PDAP v. 1.14 module (Midford *et al.* 2008) of the program Mesquite v. 2.5 (Maddison & Maddison 2008), based on the phylogenetic tree presented in figure 1; branch lengths were estimated from Schultz & Brady (2008).

Comparisons among species of baseline and post-exposure rates of MG grooming for ant species with (*Acromyrmex* and *Trachymyrmex*) and without visible actinomycetes (*Atta* and *Sericomyrmex*) following exposure to *Escovopsis* and *Metarhizium* were examined using logistic regression. Since worker ants either groomed at a high rate or hardly groomed at all, each colony was classified as grooming (more than 10 grooming events in 90 min; mean = 123.6) or not (less than five grooming events; mean = 0.529), based on a *k*-means mixture analysis (JMP 7.02). Ant colony was nested within species and pathogen treatment to take into account differences in grooming behaviour between colonies. To compare targets following MG grooming, we used only treatments with brood, and employed a GLM and multinomial logistic regression analyses. Where appropriate, all GLMs were corrected for overdispersion in the data. Voucher specimens are deposited in the dry reference collection, Smithsonian Tropical Research Institute, Panama.

3. RESULTS

A high percentage of *Acromyrmex*, *Trachymyrmex* and *Cyphomyrmex* workers had a conspicuous white bloom of *Pseudonocardia* on their exoskeletons, while workers of *Atta* lacked it (figures 1 and 2). *Sericomyrmex* had a few (less than 2.5%) workers with fine white lines of bacteria on the head or thorax, but no bloom. The proportion of workers with *Pseudonocardia* differed significantly between species (GLM with binomial errors: likelihood ratio, LR, $\chi^2_8 = 1812.56$, $p < 0.0001$), with 27 per cent of the variation between species explained by differences in average colony size, with a smaller proportion of workers from species with large average colony size having visible *Pseudonocardia* (LR $\chi^2_1 = 60.83$, $p < 0.0001$).

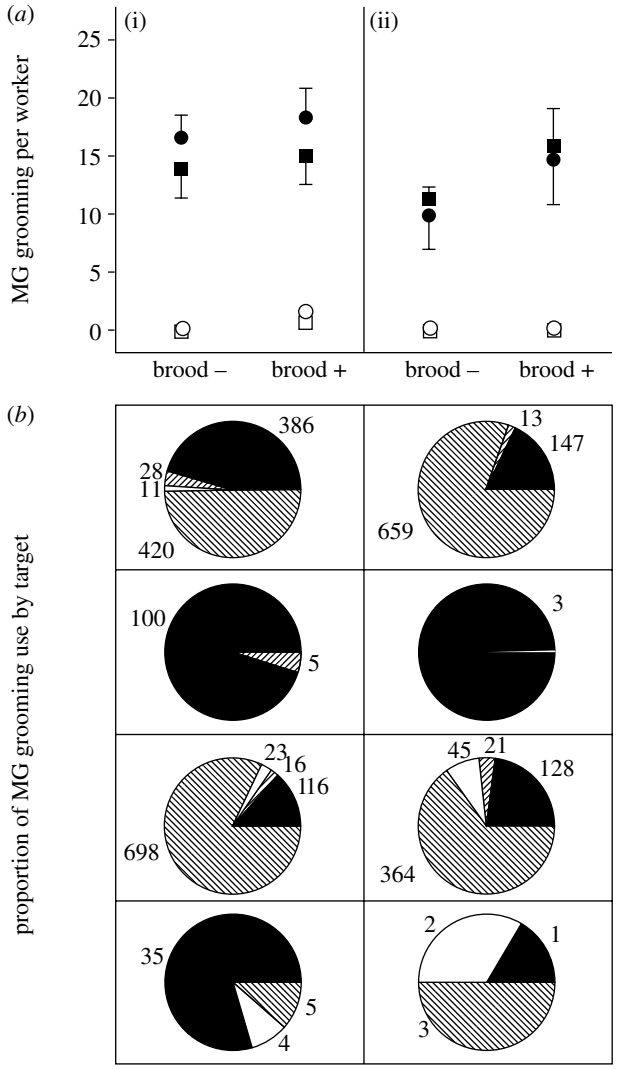


Figure 3. (*a*) MG grooming rates (mean ± s.e.) in attine ants after infection with (i) *Metarhizium anisopliae* and (ii) *Escovopsis* in sub-colonies with (brood +) and without (brood −) brood: filled circles, *Atta colombica*; open circles, *Acromyrmex octospinosus*; filled squares, *Sericomyrmex amabilis*; open squares, *Trachymyrmex zeteki*. Prior to infection, all MG grooming rates for all species were less than 0.5. (*b*) Proportion of different targets (brood: filled; fungus garden: left-hatched; fellow workers: right-hatched; self: open) that are contacted after MG grooming, with the summed total of MG events.

Prior to inoculation with *Penicillium* sp. 1, the ant species differed somewhat in baseline rates of MG grooming (GLM with Poisson errors: LR $\chi^2_8 = 23.92$, $p = 0.0024$), but grooming rates were universally low; the maximum recorded number of grooming movements was six in 1 h. After the inoculation, there was a significant increase in MG grooming rates, the magnitude of which differed significantly between species (LR $\chi^2_8 = 218.7$, $p < 0.0001$; figure 2), with the greatest increase in species without visible *Pseudonocardia*. This led to a negative correlation between species-specific MG grooming rates after infection and relative abundance of *Pseudonocardia* (Spearman's $\rho = -0.763$, $p = 0.017$; figures 1 and 2), which was also present when the effects of phylogenetic relatedness were removed ($R = -0.687$, $p = 0.047$; figure 2 inset). Workers in *Atta* and *S. amabilis* sharply increased MG grooming frequency; the increase for *Sericomyrmex* sp. 1 was less pronounced, while *Ac. octospinosus*, *T. zeteki*, *T.* cf. *cornetzi* and *C. longiscapus* workers hardly increased grooming rates (figures 1 and 2).

Prior to challenging with the ant pathogen *M. anisopliae* and the garden pathogen *Escovopsis* spp., MG grooming rates of all species were universally low (less than five events in 60 min), so all colonies fell into our 'did not groom' category. However, after challenging we found significant differences among species in MG grooming rates, comparing taxa with visible *Pseudonocardia* (*Ac. octospinosus* and *T. zeteki*) versus those without them (*A. colombica* and *S. amabilis*; logistic regression, LR $\chi^2_3 = 96.5$, $p = < 0.0001$; figure 3*a*). Planned comparisons showed that ants from sister genera differed in MG grooming frequency when infected with pathogens (*A. colombica* versus *Ac. octospinosus*: LR $\chi^2_1 = 44.68$, $p < 0.0001$; *S. amabilis* versus *T. zeteki*: LR $\chi^2_1 = 61.08$, $p < 0.0001$). Likewise, there were significant differences in MG grooming rates depending on the substrate (the presence or absence of brood; LR $\chi^2_1 = 15.36$, $p < 0.0001$; figure 3*a*). There were no significant differences in MG grooming rates, comparing the fungal pathogen *Escovopsis* and the entomopathogen *Metarhizium* (LR $\chi^2_1 = 0.0018$, $p = 0.966$; figure 3*a*). A decrease in *Pseudonocardia* abundance was associated with an expanded range of targets following MG grooming in *Atta* and *Sericomyrmex* (figure 3*b*). *Trachymyrmex* also have multiple targets, but the frequencies are very low in comparison with genera that lack abundant *Pseudonocardia* (figure 3*b*). Brood are primary targets of MG grooming in *Ac. octospinosus*. Comparisons among targets showed significant differences among species (LR $\chi^2_3 = 89.91$, $p < 0.0001$). There were no significant differences between pathogens (LR $\chi^2_1 = 0.007$, $p = 0.9$), but there was a significant interaction between species and pathogens (LR $\chi^2_3 = 125.79$, $p < 0.0001$; figure 3*b*).

4. DISCUSSION

(a) *Trade-offs in social control of pathogens*

Sanitary behaviour in ants includes grooming to collect debris that are gathered in the form of waste pellets in the infrabuccal cavity and then removed (Bailey 1920; Fernández-Marín *et al.* 2006; Little *et al.* 2006). Most ants complement these behaviours by deploying antimicrobial exocrine products, particularly those from the MG (Hölldobler & Wilson 1990; Fernández-Marín *et al.* 2006). Increased MG grooming is associated with increased pellet production when ants are challenged with entomopathogenic fungi (Fernández-Marín *et al.* 2006). This novel behaviour may represent an evolutionary innovation that was necessary because MG secretions, at least in *Acromyrmex*, inhibit both the ants' fungal symbiont (Bot *et al.* 2002) and *Pseudonocardia* bacteria (Poulsen *et al.* 2003*a*), which otherwise provide fungus-growing ants with an additional source of antimicrobial compounds (Currie *et al.* 1999*b*, 2006). Much attention has focused on how *Pseudonocardia* antibiotics control *Escovopsis*, but our results suggest that *Escovopsis* is also targeted by MG products, consistent with the demonstration that MG compounds from *Acromyrmex* reduce germination of *Escovopsis* (Bot *et al.* 2002). Preliminary results also indicate that germination of *Escovopsis* conidia within infrabuccal pellets is inhibited

by *Atta* and *Sericomyrmex* workers to the same degree (more than 80%; H. Fernández-Marín 2008, unpublished data) as in taxa with abundant *Pseudonocardia* (Little *et al.* 2006). In taxa with abundant actinomycetes, MG products are targeted primarily to brood, whereas *Atta* and *Sericomyrmex* expand the range of targets to include the fungal gardens and fellow workers, in addition to the brood.

The negative association between frequency of induced MG grooming and relative abundance of *Pseudonocardia* implies that *Pseudonocardia* may be effective against generalist pathogens, or that the ants use another exocrine source against them. The former possibility is unlikely, as isolates of bacteria from *Ac. octospinosus* have no detectable inhibitory effects against an array of generalist fungal pathogens (Currie *et al.* 1999*b*), although the generality of this result for all attine-associated *Pseudonocardia* has recently been questioned (Mueller *et al.* 2008). The latter possibility has not been thoroughly investigated, but mandibular gland secretions may have antiseptic activity (North *et al.* 1997).

(b) *Is there decreasing reliance on an ancient bacterial mutualism in favour of MG chemical control?*

Currie *et al.* (2006, p. 81) reported that *Pseudonocardia* bacteria are 'associated with all attine-ant species examined' and 'occur on specific locations on the cuticle of a given ant species'. This contradicts the electronic supplementary materials of Currie *et al.* (2006), which stated that *Atta* and *Sericomyrmex* have 'no filamentous bacteria or morphological structures' on the exoskeleton. Our results are consistent with the latter observation. These taxa are derived from ancestors that possessed these structures, which indicates that they were evolutionarily lost. Recent molecular studies have failed to detect *Pseudonocardia* in *Atta* spp., including those species in our study (Mueller *et al.* 2008), which is inconsistent with *in vitro* evidence that isolates of actinomycetes from *Atta* are active against *Escovopsis* (Currie *et al.* 1999*b*). *In vivo* evidence is lacking, and nothing is known of the dosage-dependent efficacy of bacteria cultured from different ant taxa. Trace quantities of *Pseudonocardia* in some taxa may have retained specific disease control functions, and a recent study has shown that both *Atta* and *Acromyrmex* can transfer actinomycetes to the fungus garden during treatment of leaf fragments by workers (Mangone & Currie 2007). Such transfers occurred in only 10–25 per cent of the trials for both genera, however, so they are unlikely to play a primary role in defence against pathogens.

A loss or drastic reduction in visible bacteria, and an increased reliance on chemical control of pathogens by fungus-growing ants, may be related to three factors.

Health care costs. Using both bacterial and MG antimicrobials may account for up to 40 per cent of the basal metabolism of *Acromyrmex* leafcutting ants, with approximately equal costs for each defence (Poulsen *et al.* 2002, 2003*a*,*b*). The evolution of novel chemical compounds from MG secretions with broad-spectrum activity would be selected if they improve sanitation, reduce hygienic costs or both. We lack detailed comparative information on the diversity and function of MG secretions and other exocrine glands (see Schildknecht & Koob 1971; do Nascimento *et al.* 1996; Ortius-Lechner *et al.* 2000) that could test such hypotheses (but see North *et al.* 1997).

Minimizing resistance. The best public health strategy to deploy narrow-spectrum antibiotics is to apply a strong dose during an infection to kill a specific pathogen, but otherwise not use the medication (Levy 1998). Broad-spectrum antibiotics, by contrast, create unfavourable habitat for pathogen growth and most applications are general hygiene sanitation measures, which normally do not create resistance problems (Hoffken 2000; Bergstrom & Feldgarden 2007). Intriguingly, these rules of thumb of proper antibiotic use seem to be reversed in attine ants. The active use of MG secretions appears to be regulated for minimal dosage and maximal efficiency (Fernández-Marín *et al.* 2006), which may indicate that either some unknown MG compounds have narrow-spectrum antibiotic functions, or MG secretions are too costly to be used in a more profligate preventive way. A putative increased reliance on MG secretions in *Atta* and *Sericomyrmex* implies that these genera should have larger MGs relative to respective sister genera, but gland size is only slightly larger in *Atta* and *Sericomyrmex* (Hughes *et al.* 2008), suggesting that quality of secretions rather than quantity has been decisive. The use of actinomycete-derived antibiotics appears to be less precisely regulated, although it is possible that production of metabolites could be upregulated when needed. Although the abundance of bacteria may change in response to increasing disease challenge (Currie *et al.* 2003*b*), this continual presence hardly appears to be a fine-tuned mechanism and may help to explain why resistant strains of pathogens may occasionally appear (Little *et al.* 2006).

Antagonistic interactions in the fungus-growing ant mutualism. A black yeast is also associated with at least some attine ants (Little & Currie 2008). This yeast may antagonize the ants' mutualistic bacteria, which would inhibit their ability to control *Escovopsis* (Little & Currie 2008). This antagonism may have necessitated new hygienic strategies, in particular when black yeast infections are chronic, as would be expected in colonies with large numbers of workers.

(c) *The evolution of public health strategies and social organization*

Our results suggest that there has been evolutionary divergence in the use of antibiotic sources, which parallels changes in the social structure of attines. Large-scale farming of a genetically homogeneous crop by group members who are genetically related poses special problems for disease management and public health strategies. We speculate that the domestication of *Pseudonocardia* was a major evolutionary advance at the origin of attine fungiculture *ca* 50 Myr ago, but that later developments towards complex agricultural societies required additional mechanisms for disease control, including an increased reliance on chemical and behavioural defences. This was accompanied by a dramatic transition in the mating system of queens, from exclusively single mating to obligate multiple mating in the highly derived *Acromyrmex* and *Atta* leafcutters, leading to a substantial increase in genetic diversity among workers (Villesen *et al.* 2002; Mattila & Seeley 2007) with at least some documented advantages for resistance to disease

(Hughes & Boomsma 2006). There are substantial differences in sizes of mature colonies when comparing those taxa with abundant actinomycetes versus elevated rates of MG use. Colony size is an important factor in ant life history, including defensive strategies against pathogens (Hughes *et al.* 2002). Limited data suggest that MG grooming rates and relative abundance of actinomycetes do not change as colonies mature. Within species, variation in MG grooming rates and relative abundance of actinomycetes are not associated with variation in colony size in four species of *Trachymyrmex* (H. Fernández-Marín, G. Bruner, E. Gomez, D. R. Nash, J. J. Boomsma & W. T. Wcislo 2008, unpublished data). In *A. colombica*, workers from three- to six-month old colonies have similar patterns of MG use to those of workers from colonies older than 24 months (H. Fernández-Marín, unpublished data), and overall tasks do not change with the age of colonies (Augustin & Lopes-Santos 2008). The decay and collapse of mutualistic relationships are not uncommon (see discussion in Mueller *et al.* 2005; Sachs & Simms 2006; Kost *et al.* 2007) and raise questions about compensatory changes that may be associated with the evolution of large colonies and social complexity across the fungus-growing ants.

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