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Active use of the metapleural glands by ants in controlling fungal infection

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Insect societies face constant challenges from disease agents. Ants deploy diverse antimicrobial compounds against pathogens and the key sources are metapleural glands (MGs). Are MG products passively secreted and used indiscriminately or are they selectively used when ants are challenged by pathogens? In 26 species from five subfamilies, ants use foreleg movements to precisely groom the MG opening. In the absence of experimental infection, MG grooming rates are low and workers groom themselves after contacting the MGs. The derived leaf-cutter ants (*Atta* and *Acromyrmex*) also groom their fungal gardens, substrata (leaves), queens and nest-mates after MG grooming. *Atta* respond to a challenge by fungal conidia by increasing the rate of MG grooming, but do not do so when an inert powder is applied. This increase occurs in the first hour after a potential infection, after which it returns to baseline levels. Ants with open MGs produce more infrabuccal pellets (IP) than ants with sealed MGs and conidia within pellets from the former are less likely to germinate. Thus, ants selectively groom their MGs when disease agents are present, suggesting that they also selectively use their MG secretions, which has important implications for understanding the evolution of hygienic behaviour in social groups.

Keywords: metapleural gland; pathogens; antibiotics; hygiene; social evolution; ants

1. INTRODUCTION

Insect societies have confronted and solved problems that are analogous to the major public-health challenges that humans face in controlling infectious diseases and their resistance to antimicrobial compounds (Cohen 1992; Neu 1992). Fungus-growing ants (Attini) are members of a complex mutualism, which includes a symbiotic fungus that is cultivated as a food source (Belt 1874) and an actinomycete bacterium which is cultured for its metabolites that have antibiotic properties (Currie *et al.* 1999*a*). The partners in this mutualism have been stable for 50–65 million years, with a few signs of disease resistance among an array of generalist fungi and bacteria that threaten the ants' agricultural system (Currie *et al.* 1999*b*; Schultz *et al.* 2005). In addition to the actinomycetes, the ants utilize an array of behavioural and chemical defences against pathogens (e.g. Schildknecht & Koob 1971; Currie & Stuart 2001; Hart & Ratnieks 2001; Hughes *et al.* 2002), as do other social insects (e.g. Rosengaus *et al.* 2000). One of the main chemical defences involves secretions from the metapleural glands (MGs), which are paired structures that are diagnostic for identifying both fossil and extant ants (Formicidae), apart from some taxa with secondary losses (Wilson *et al.* 1967; Hölldobler & Wilson 1990; Grimaldi & Agosti 2000). The morphology and physiology of the glands vary among and sometimes within species (Brown 1968; Hölldobler & Engel-Siegel 1984; Sumner *et al.* 2003). Empirical studies have shown that control of infection is a primary function of the MGs, which synthesize an array of antimicrobial compounds (e.g. Schildknecht & Koob 1971; Maschwitz 1974; Mackintosh *et al.* 1995; do Nascimento *et al.* 1996). In some species it may have other roles, such as producing alarm pheromones for colony defence or compounds to maintain group identity via kin recognition (Brown 1968; Jaffé & Puche 1984).

The manner in which the ants use their MG antibiotic secretions is unclear. It is generally assumed that ants do not control the release and application of MG compounds, but that the compounds passively flow non-stop from the orifice and spread over the cuticle, where nest-mates distribute them further during social grooming (Attygalle *et al.* 1989; Schoeters & Billen 1993; North *et al.* 1997). In contrast, Brown (1968) and others (e.g. Fernández-Marín *et al.* 2003) observed that some ants precisely groom the MG opening and they hypothesized that ants actively regulate the use of MG secretions by controlling when they groom the gland opening. To explore whether ants in general actively groom their MGs, we surveyed 26 species representing five subfamilies of ants. Next, we experimentally tested the hypothesis that ants increase the rate of MG grooming during a microbial infection, assuming that secretions are transferred by grooming to combat and prevent the establishment and spread of disease within the colony.

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2. MATERIAL AND METHODS

(a) *Comparative survey of metapleural gland use in ants*

Colonies of 26 ant species representing five subfamilies of Formicidae were collected in Puerto Rico and Panamá, and then transplanted and maintained in artificial nests using standard methods (Weber 1972). We used a stereomicroscope to observe ants on an *ad libitum* basis. We defined MG grooming as follows: an ant rubbed its foreleg over the opening (meatus) of the MGs and passed the foreleg to the glossa. For each species, we recorded which individuals or objects were contacted following MG grooming (i.e. self, brood (eggs, larvae, pupae), conspecific workers, queens, nest materials and food).

(b) *Metapleural gland grooming following fungal infection in* Atta

To determine if MG grooming changes in frequency following an inoculation of conidia, we used 33 young queen-right colonies (*ca* four months old, with 25–46 workers (mean = 37.8) of *Atta columbica*), infected with conidia from the following fungi: *Aspergillus tamarii*, *Eupenicillium javanicum*, *Escovopsis* sp. (collected from an *A. columbica* garden with cultivar), *Escovopsis* sp. (from an *Acromyrmex octospinosus* garden with cultivar), *Penicillium citrinum*, *P. paxilli*, *P. simplicissimus*, *Paecilomyces lilacinus* and *Metarhizium anisopliae*. Unless indicated otherwise above, fungi were isolated from dead queens of *A. columbica*, and cultured using standard methods to obtain pure cultures (following Chapela *et al.* 1994; Currie *et al.* 1999*b*). All fungi were subsequently identified by morphological features and, in some cases, by confirmatory internal transcribed spacer sequence (White *et al.* 1990). Dry conidia from a *ca* 1 mm^2 area of pure culture were placed on a piece of parafilm (*ca* 5×5 mm^2). The parafilm was held with sterile forceps and the surface with conidia was gently brushed over the entire fungus garden and brood to disperse the conidia. An area of *ca* 1 mm^2 of a 5×5 mm^2 piece of parafilm was covered with inert talcum powder (Johnson & Johnson Co.), and applied as above. To control for the application procedure, we used new pieces of parafilm without conidia or powder and contacted the garden and brood as above. Five minutes after each treatment, we recorded the frequency of MG grooming for 15 min. Three *A. columbica* colonies were tested for each treatment and controls.

(c) *Temporal patterns of metapleural gland grooming following fungal infection in* Atta

To assess the temporal response of workers to inoculations of conidia, we used colonies of *A. columbica*, *A. sexdens* and *A. cephalotes* that were *ca* 5–7 months old to create sub-colonies, each of which contained a young queen and 40 workers from three size classes (20 workers with head-width less than 1.0 mm; 12 workers 1.1–1.4 mm and 8 workers 1.5–1.8 mm). These sub-colonies were housed in individual plastic boxes with 1.0 g of fungus garden and 10 pupae or larvae. We applied two clean parafilm pieces (each *ca* 5×5 mm^2) as control blanks using methods described above, and then recorded the frequency of MG grooming behaviour for 1 h. We then applied two new parafilm pieces, containing *ca* 20×10^6 *Penicillium* sp. conidia, using the same methods. After 5 min, we recorded the frequency of MG grooming continuously during each of the next 3 h by ants in an area of *ca* 3.5 cm diameter of the garden.

(d) *Metapleural gland grooming and infrabuccal pellet production by* Atta *in response to different concentrations of conidia*

To test whether MG grooming increases with increasing concentration of conidia, we recorded the responses of *A. columbica* workers to five different concentrations of *Penicillium* sp. conidia (*ca* 3, 6, 12, 24 and 36×10^6 conidia). We used 13 different sub-colonies for each fungal concentration, with conidia applied as described above; each sub-colony contained 40 workers as above, but without a queen. After inoculation, we recorded the frequency of MG grooming by all workers for 1 h in the sub-colony and the production of infrabuccal pellets (IP).

(e) *Effect of metapleural gland grooming by* Atta *on viability of conidia*

To determine the effect of MG grooming during a microbial infection, we established two sub-colonies with 20 workers each (range of head widths, 1.3–1.8 mm) from 33 *A. columbica* colonies. In one sub-colony, the ants' MGs were open, while in the other, the openings were sealed following the methods of Poulsen *et al.* (2002). Eleven pairs of sub-colonies were challenged with conidia of *Penicillium* sp., 11 pairs with those of *A. tamarii* and 11 pairs were used as controls without conidia to determine if sealing the MGs increased MG grooming in the absence of fungi. We used *ca* 4 mm^2 area of dry conidia, which contained *ca* 1.2×10^7 conidia of *A. tamarii* and 2.5×10^8 conidia of *Penicillium* sp. because the conidia of the two species have different sizes (4.5 and 1.1 μm diameter, respectively). As described above, we recorded MG grooming for 1 h post-inoculation. Ants remove detritus and other materials from their nests and bodies via grooming behaviour, and this detritus is packed into infrabuccal pellets (IP) in the infrabuccal pocket (Janet 1895; Bailey 1920; Little *et al.* 2006). To test whether the production of IPs and germination rates of conidia in the IP are associated with MG grooming for 3 h post-inoculation, we isolated the workers from the sub-colonies used above, and then removed the IP with a flame-sterilized needle and counted them. To determine the germination rate, each IP was placed singly on a Petri dish with sterile media (potato dextrose agar, 19.5 $g\,l^{-1}$, DIFCO; without antibiotics), and germination rates were recorded after 24 h.

(f) *Statistical analyses and voucher specimens*

The statistical analyses were made using STATISTICA (Statsoft Inc.), indicated in the text. The data met all the assumptions for parametric statistical tests, except pellet numbers, which were log-transformed prior to analysis. Data are given as means with standard errors. Vouchers of the ants are deposited in the Dry Reference Collection, Smithsonian Tropical Research Institute.

3. RESULTS

(a) *Comparative survey of metapleural gland use in ants*

Twenty-six ant species from five sub-families rubbed their forelegs (metatarsi) over the slit (meatus) of the MG orifice (figure 1), which involved a complex suite of leg movements. A worker ant partially extended its legs to raise the body from the substrate, and then flexed the foreleg at the femora–tibial joint to bring the posterior surface of the metatarsus in contact with the opening of

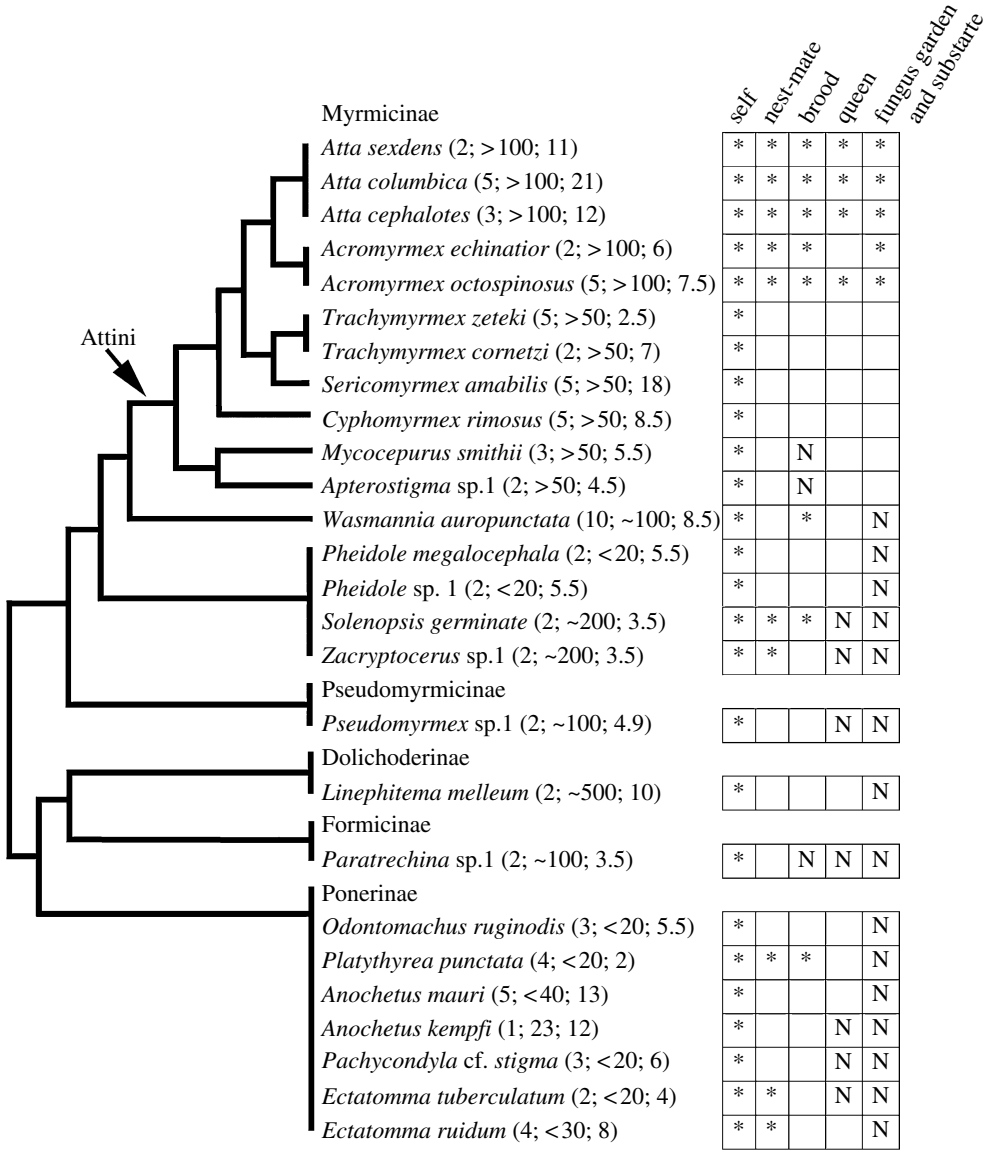

Figure 1. The phyletic distribution of metapleural gland grooming for 26 ant species, prior to contact with the following potential targets: self, nest-mate workers and queens and brood. For attine ants, two additional targets were the fungus garden and substrate. N, not applicable—no objects or individuals belonging to the target group were present during the observations. All colonies contained workers and reproductives, except *Anochetus kempfi*, *Pachycondyla*, *Ectatomma tuberculatum*, *Solenopsis*, *Zacryptocerus*, *Paratrechina* and *Pseudomyrmex*, which contained only workers. Following each species in parentheses is the number of colonies observed per species, the estimated number of workers per colony and the total time of observation (in hours), respectively. Phylogenetic relationships follow Schultz & Meier (1995), Wetterer *et al.* (1998) and Grimaldi & Agosti (2000).

the ipsilateral MG (in Ponerinae both the metatarsus and tibia contact the slit). Subsequently, the ant rubbed the metatarsus over this opening, and then extended the leg to bring it in contact with the lateral surface of the glossa. All of the species showed this behaviour, albeit at low frequency. In most taxa subsequent grooming was restricted to their own bodies. However, for five species of the derived leaf-cutters *Atta* and *Acromyrmex*, additional targets included the fungal gardens, garden substrata, queens, brood and nest-mates (figure 1).

(b) *Metapleural gland grooming following fungal infection in* Atta

A. columbica ants increased the frequency of MG grooming when exposed to conidia from nine fungal species relative to talcum powder and blank controls (ANOVA with least significant difference (LSD) *post hoc* comparisons, $F_{2,10}=6.34$; $p=0.0001$; figure 2).

(c) *Temporal patterns of metapleural gland grooming following fungal infection in* Atta

Workers of all the three *Atta* species increased MG grooming during the first hour after a *Penicillium* sp. inoculation, after which the frequency of MG grooming was not different from baseline levels for all the three species (figure 3; ANOVA with repeated measures: *A. columbica*, $F_{3,18}=36.45$, $p<0.0001$; *A. sexdens*, $F_{3,12}=15.68$, $p=0.00018$; *A. cephalotes*, $F_{3,9}=20.49$, $p=0.0002$).

(d) *Metapleural gland grooming and infrabuccal pellet production by* Atta *in response to different concentrations of conidia*

Workers from sub-colonies infected with different concentrations of *Penicillium* sp. responded differently with respect to frequency of MG grooming (ANOVA, $F_{4,60}=2.595$, $p=0.045$), and the production of IP (ANOVA, $F_{4,60}=23.04$, $p<0.0001$; figure 4).

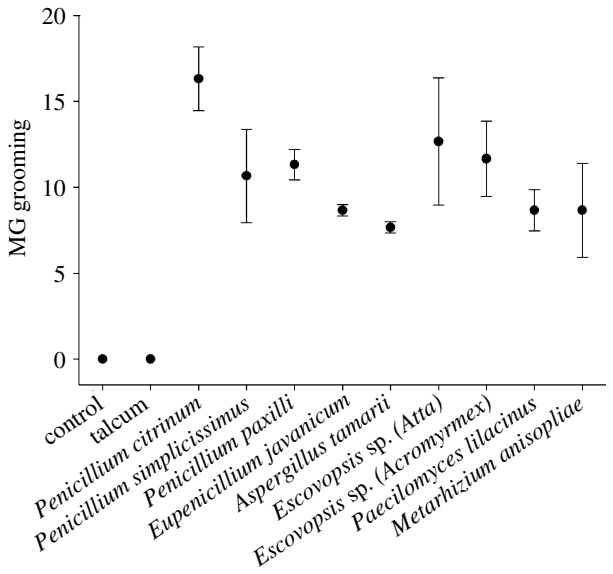

Figure 2. Metapleural gland grooming events for 15 min following inoculation with dry conidia from nine fungi, relative to baseline rates (control) or when challenged by an inert powder (talcum). Ants significantly ($p=0.0001$) increased the grooming rate when challenged with conidia, but not inert powder.

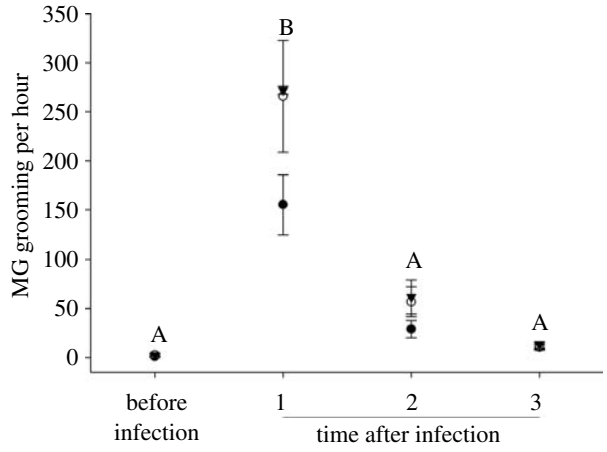

Figure 3. Metapleural gland grooming by workers of three *Atta* species following inoculation of *Penicillium* sp. conidia. Closed circles indicate *Atta cephalotes*; open circles indicate *A. sexdens*; and closed triangles indicate *A. columbica*. Different letters indicate significant differences ($p<0.05$) within species using Tukey's Honestly Significant Difference test.

(e) *Effect of metapleural gland grooming by* Atta *on viability of conidia*

The frequency of MG grooming differed between workers with sealed versus open MGs across treatments (ANOVA with repeated measures, $F_{1,30}=7.46$, $p=0.004$), and a planned comparison showed that grooming frequency did not differ between open and sealed MGs in the absence of conidia ($F_{1,30}=0.01$, $p=0.91$; figure 5). The overall frequency of MG grooming also differed with respect to the fungi used in the treatments (ANOVA with repeated measures, $F_{2,30}=78.5$, $p<0.0001$; figure 5). Ants produced more IP when the MGs were open versus sealed (ANOVA with repeated measures, $F_{1,20}=52.38$; $p<0.0001$; figure 6), and planned comparisons showed that for each species (*Penicillium* sp. and *A. tamarii*) the production of IP was different when MGs were open or sealed ($F_{1,20}=37.7$, $p<0.0001$, and $F_{1,20}=16.76$, $p=0.0005$, respectively). The germination of conidia from IPs produced by ants with open or sealed MGs differed significantly 24 h after pellet production for *Penicillium* sp. (ANOVA with planned comparisons, $F_{1,20}=9.14$, $p=0.006$) and *A. tamarii* (ANOVA with planned comparisons, $F_{1,20}=13.9$, $p=0.001$).

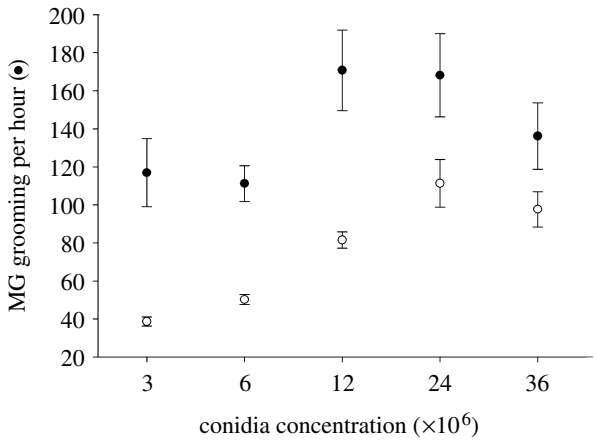

Figure 4. Metapleural gland grooming (closed circles) and infrabuccal pellet production (open circles) by *Atta columbica* workers in response to inoculation with different concentrations of conidia from *Penicillium* sp.

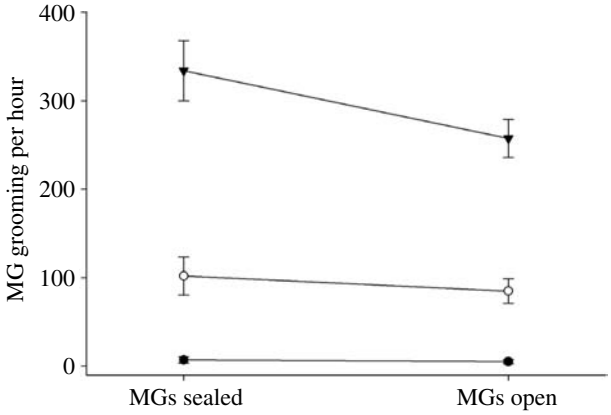

Figure 5. Metapleural gland grooming by *Atta columbica* workers after infection with dried conidia, or in the absence of experimental infections (closed circles). Open circles indicate *Penicillium* sp., and closed triangles indicate *Aspergillus tamarii*. Ants with sealed glands groomed the opening significantly more often than those with unsealed glands ($p=0.004$).

4. DISCUSSION

MG grooming behaviour entails a series of coordinated foreleg and body movements that are strikingly different from the leg movements involved in self-grooming, which have been described for diverse Hymenoptera, including other ants (see Farish 1972; Jander 1976; Basibuyuk & Quicke 1999). This complex behaviour occurs in ants representing most major lineages, and we hypothesize that it is a part of the formicid groundplan. We also hypothesize that it functions to transfer secretions from the MGs to the forelegs, and from the latter to the mouthparts and then to subsequent targets, although this remains to be documented. Our comparative survey shows that ants of different lineages groom themselves after MG grooming.

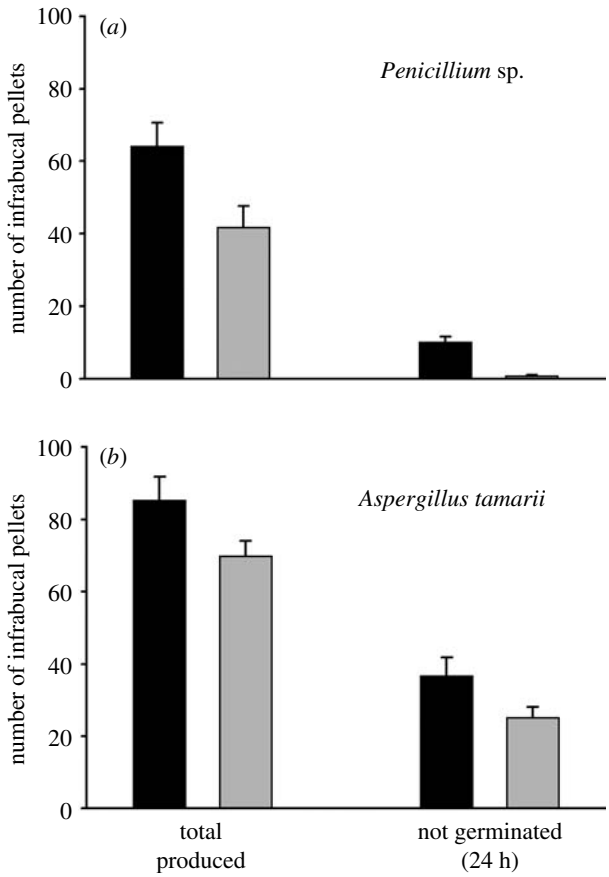

Figure 6. The overall production of infrabuccal pellets by ants with open MGs (black bars) was significantly greater 3 h after infection, relative to ants with experimentally sealed MGs (grey bars; $p < 0.0001$). After 24 h, infection pellets from ants with sealed MGs were more likely to germinate than those from ants with open MGs, regardless of whether conidia were from (*a*) *Penicillium* sp. ($p = 0.006$) or (*b*) *Aspergillus tamarii* ($p = 0.001$).

The derived leaf-cutter ants (*Acromymex* and *Atta*) also groom their fungal gardens, substrata, queens and nestmates following MG grooming, but more basal genera do not do so, suggesting that the use of MG secretions has expanded during attine evolution. This expanded role complements weeding and grooming behaviours that ants use to physically disinfect the nest and garden (Bailey 1920; Bass & Cherrett 1994; Currie & Stuart 2001) as they accumulate conidia and hyphae in IP, which are later discarded.

Our comparative observations imply that ants actively control MG use, and experimental manipulations with *Atta* confirmed this active-use hypothesis. MG rates increase significantly in the first hour after ants are challenged with dry conidia, relative to baseline rates, but they do not increase in response to a challenge with an inert powder (talcum). After 2–3 h of infection, grooming rates do not differ from baseline levels, showing that they regulate MG use. MG grooming is also associated with increased formation of IP and decreased germination of conidia from the IP, as inferred from results that compare germination rates from pellets produced by ants with their MGs open versus those with the openings experimentally sealed. Thus, our results contradict the assumption that the MG compounds are passively secreted and thus indiscriminately distributed during social grooming (e.g. Beattie *et al.* 1986; Schoeters & Billen 1993; Poulsen *et al.* 2002). This passive-use hypothesis is surprising for two reasons. First, these secretions are energetically expensive to produce, as inferred from studies of metabolic rates (Poulsen *et al.* 2002), and from comparative studies which show that the MGs are evolutionarily lost when no longer used (e.g. in parasitic species; Brown 1968; Hölldobler & Engel-Siegel 1984). Second, given that indiscriminate use of antimicrobials by humans is invoked as a causal factor underlying the recurrent emergence of resistant pathogens (Cohen 1992; Neu 1992; Bergstrom *et al.* 2004), it follows that indiscriminate use of antimicrobial secretions by ants could lead to the evolution of resistant diseases, and a drastic reduction in the efficacy of their biochemical arsenal, with a consequent decrease in relative fitness.

Just as ants evolved the ability to culture bacteria to produce antibiotics long before humans (Currie *et al.* 1999*a*; Santos *et al.* 2004), our results suggest that they may have solved problems associated with the potential misuse of antibiotics as well, since selective MG grooming minimizes their use of antimicrobial compounds. Although the ants' arsenal (e.g. antimicrobials secreted by exocrine glands) is apparently fixed over ecological time, it nevertheless is extremely diverse. In theory, ants can apply a 'cocktail' mixture of antimicrobials, including antibiotic metabolites by bacteria (Currie *et al.* 1999*a*) and fungi (Hervey & Nair 1979), and an array of compounds from different exocrine glands, each of which has multiple components (Hölldobler & Wilson 1990; Orthius-Lechner *et al.* 2000). Recent theoretical models have shown that mixing of two or more different antibiotics is more likely to prevent the evolution of resistant disease agents as compared with the sequential cycling of the same antibiotics (Bergstrom *et al.* 2004; Levin & Bonten 2004). We speculate that these models help to explain why attines selectively use a mixture of different compounds to combat disease and thus impede the evolution of resistant pathogens in the attine–fungus mutualism.

Thorne & Traniello (2003) hypothesized that the origin of the MG was a key innovation in ant evolution, since its role in disease management permitted the evolution of increased colony size (Hölldobler & Wilson 1990), with the attendant problems of disease transmission in large social groups (Schmid-Hempel 1998). The importance of this innovation was questioned by Baroni Urbani (1989), who suggested that the relative importance of MG function decreased throughout ant evolution concurrent with the origin of other antibiotic-producing exocrine glands (Hölldobler & Engel-Siegel 1984). Our results contradict his hypothesis, because even within attine ants, the more derived species have expanded their use of MG secretions. As hypothesized above, the evolution of new exocrine glands, while retaining the use of the MGs, is advantageous since it would diversify the compounds used against different pathogens. Comparative (phylogenetic) studies are needed to assess the relationships among relative disease risk, the evolution of resistance by pathogens, and hygienic responses by ants with different social organizations (colony size), to better understand how, when and where ants use MG secretions, and in what dosage, when challenged with disease agents.

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