ANTIMICROBIAL DEFENSE SHOWS AN ABRUPT EVOLUTIONARY TRANSITION IN THE FUNGUS-GROWING ANTS

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Understanding the relative evolutionary importance of parasites to different host taxa is problematic because the expression of disease and resistance are often confounded by factors such as host age and condition. The antibiotic-producing metapleural glands of ants are a potentially useful exception to this rule because they are a key first-line defense that are fixed in size in adults. Here we conduct a comparative analysis of the size of the gland reservoir across the fungus-growing ants (tribe Attini). Most attines have singly mated queens, but in two derived genera, the leaf-cutting ants, the queens are multiply mated, which is hypothesized to have evolved to improve colony-level disease resistance. We found that, relative to body size, the gland reservoirs of most attines are similar in size but that those of the leaf-cutting ants are significantly larger. In contrast, the size of the reservoir did not relate with the evolutionary transition from lower to higher attines and correlated at most only slightly with colony size. The results thus suggest that the relationship between leaf-cutting ants and their parasites is distinctly different from that for other attine ants, in accord with the hypothesis that multiple mating by queens evolved to improve colony-level disease resistance.

KEY WORDS: Antibiotics, Attini, comparative analysis, leaf-cutting ants, metapleural gland.

Parasites represent an important selection pressure on most organisms (Tomkins and Begon 1999; Hudson et al. 2001). However, the relative evolutionary importance in different host taxa is difficult to gauge because the expression of disease symptoms depends upon many confounding factors (e.g., parasite virulence, host condition) and will covary with host resistance. Thus, hosts exposed to greater parasite pressure may evolve better resistance mechanisms and may then in fact exhibit reduced symptoms of parasite infection. In addition, the full spectrum of parasites that an organism may be afflicted with is only very rarely known and more cryptic, avirulent parasites can often have significant effects on host life-history (e.g., Thomas et al. 2003; Hughes and Boomsma 2004a).

Given the difficulty of comparing disease symptoms across taxa, relative investment by hosts in resistance mechanisms can provide useful insight, although these too may exhibit counter-intuitive evolutionary dynamics (van Baalen 1998; Jokela et al. 2000) and may vary with host age, condition etc. (Rolff and Siva-Jothy 2003; Schmid-Hempel 2005; Baer and Schmid-Hempel 2006). Morphological, nonvariable defenses are particularly good candidates for such comparative analyses, but few animals have such structures. Ants are a notable exception. They have paired metapleural glands, which produce broad-spectrum antimicrobial compounds that accumulate in a reservoir (bulla) that is fixed in size when an adult ant ecloses (Brown 1968; Beattie et al. 1986;

Hölldobler and Wilson 1990; Veal et al. 1992; Mackintosh et al. 1995; Nascimento et al. 1996; Ortius-Lechner et al. 2000; Bot et al. 2002). Ants actively spread the secretion of this gland over their own body and, in some species, also over nestmates, brood, or mutualists (Fernandez-Marin et al. 2006). In key studies, ants with artificially closed glands were found to be significantly more susceptible to a parasite than those with open glands (Poulsen et al. 2002), and ants exposed to fungal spores actively groomed their glands more often than unexposed ants (Fernandez-Marin et al. 2006), both results confirming the importance of metapleural glands for individual defense.

The fungus-growing ants (Attini) are particularly suitable models for a comparative approach because they are a monophyletic tribe with two distinct evolutionary transitions, as well as a more gradual change in colony size. Eight of the 12 genera (lower attines) have small colonies (< 100 individuals) that use various detritus as substrate for their mutualistic fungal food (Weber 1972; Mueller 2002). The other four genera (higher attines) exhibit a more derived mutualism in which their fungal crop produces special protein-rich food bodies (Weber 1972; Mueller 2002). The two basal higher attine genera, *Trachymyrmex* and *Sericomyrmex*, have approximately monomorphic workers, make only limited use of fresh vegetation for their fungus, and have colonies either similar in size (*Trachymyrmex*) or an order of magnitude larger (*Sericomyrmex*) than lower attines (Weber 1972; Mueller 2002). The terminal higher attine clade, the *Acromyrmex* and *Atta* leaf-cutting ants, are distinctly different because they have polymorphic workers, use exclusively fresh vegetation as substrate for their fungus, and have colonies containing tens of thousands (*Acromyrmex*) or millions (*Atta*) of workers (Weber 1972; Mueller 2002).

Leaf-cutting ants also differ from all other attines in the mating frequencies of their queens. With the exception of one species of social parasite, the queens in all leaf-cutting ant species mate with multiple males (polyandry) whereas the queens of all other attines only mate with a single male (Villesen et al. 1999, 2002; Sumner et al. 2004a). The evolution of polyandry by social insect queens, and animals in general, is a fundamental puzzle in evolutionary biology because it is almost certainly costly to females whereas clear benefits are often elusive (Crozier and Fjerdingstad 2001; Simmons 2001). One of the leading explanations for polyandry by social insect queens is that, by increasing intracolonial genetic diversity, it improves colony resistance to parasites (Hamilton 1987; Sherman et al. 1988; Schmid-Hempel 2000). If this hypothesis is correct and leaf-cutting ant queens have evolved polyandry to reduce the impact of their parasites, then we might logically expect to see a similar evolutionary transition within the attines in the relative size of metapleural glands. The workers of *Acromyrmex* and *Atta* have well-developed metapleural glands that play a key role in parasite resistance (Wilson

1980; Bot and Boomsma 1996; Hughes et al. 2002; Poulsen et al. 2002; Fernandez-Marin et al. 2006), but the size of the metapleural glands of other attines is not known. Here we conduct a comparative analysis of the relative size of the metapleural gland across the Attini. We used the size of the gland bulla as a proxy for gland size. Although this is only a direct measure of the size of the reservoir, it is highly correlated with the number of gland cells in *Acromyrmex* leaf-cutting ants (Bot et al. 2001), while the size and morphology of gland cells do not appear to differ across ant species (Fanfani and Dazzini 1991; Angus et al. 1993). Furthermore, bulla size relative to body size correlates with resistance to parasites both between worker castes of the same leaf-cutting ant species (Bot and Boomsma 1996; Hughes et al. 2002; De Souza et al. 2006; Poulsen et al. 2006), and between leaf-cutting ant species (Sumner et al. 2003).

Methods

Workers were examined for four species of leaf-cutting ant (*Atta cephalotes*, *A. colombica*, *A. sexdens*, and *Acromyrmex echinatior*), five species of basal higher attines (*Trachymyrmex* cf. *zeteki*, *T. cornetzi*, *T.* sp. 3, *Sericomyrmex amabilis*, and *S.* cf. *amabilis*), and four species of lower attine (*Cyphomyrmex costatus*, *Mycocepurus smithii*, *Apterostigma dentigerum*, and *Apt.* "collare." Note that the valid names of many attine species currently await revision). Workers were sampled from two or three colonies for most species, or from single colonies of *T.* sp. 3, *Apterostigma* "collare" and *C. costatus*. All colonies came from Gamboa, Panama. Two-thirds of the leaf-cutting ants sampled were small workers (i.e., those most similar in size to the other attines). The remaining third were larger workers to provide an estimate of relative gland size over the whole worker size range.

The metapleural gland bulla of each ant was photographed and the diameter was measured (ImageJ, National Institutes of Health, Bethesda, MD) because this is highly correlated with the number of gland cells (Bot et al. 2001). As a second estimate of gland reservoir size, we also measured the distance between the bulla and the propodeal spiracle (Sumner et al. 2003), which decreases proportionally with increasing bulla diameter. To allow the measurements of metapleural gland reservoir size to be standardized for body size, a photograph and measurement was also made of the width of the ventral side of the pronotum. We first examined the relationship between bulla width and pronotum width for the different taxa using a general linear model with $\log_{10}$-transformed bulla width as the dependent variable, $\log_{10}$-transformed pronotum width as a covariate, and genus as a categorical predictor variable. As bulla width is allometric in leaf-cutting ants (Wilson 1980; Bot and Boomsma 1996), we carried out the analysis both with all leaf-cutting ant workers and with only the small workers that are similar in size to workers of the other

attines. We then examined how relative metapleural gland reservoir size (bulla width/pronotum width) related to the three predictor variables (higher versus lower attines, leaf-cutting ants vs. other attines, and colony size) using the method of phylogenetically independent contrasts, calculated using the PDAP module of the Mesquite program (Midford et al. 2003; Maddison and Maddison 2006). To do this, we replaced the categorical variables "higher vs. lower attines" and "leaf-cutting ants vs. other attines" with 0–1 dummy variables in the respective analyses. Colony sizes were the average estimated worker populations of mature colonies (Weber 1972; Murakami et al. 2000). We used the latest attine phylogeny available (Villesen et al. 2002; Sumner et al. 2004b; T. R. Schultz and S. Brady, unpubl. data), with *Trachymyrmex* sp. 3 assumed to nest within the main *Trachymyrmex* clade, and branch lengths for novel species set as equal to those of sister species for which branch length information was available. Note that these tests only had sufficient statistical power to detect large effect sizes (e.g., effect size (r) of > 0.65 with power of 0.8 at $\alpha = 0.05$).

Results

The relationship between bulla width and pronotum width differed significantly between the attine genera both overall ($F_{6,431} = 26.7$, $P < 0.0001$) and if only small leaf-cutting ant workers were included in the analysis ($F_{6,320} = 13.6$, $P < 0.0001$; Fig. 1). When analyzed using phylogentically independent contrasts and including only small leaf-cutting ant workers, the bulla/pronotum width was significantly correlated with the transition to leaf-cutting ants from the other attines ($F_{1,11} = 71.6$, $P < 0.0001$), but not with the transition from lower to higher attines ($F_{1,11} = 0.18$, $P = 0.68$; Fig. 2). Bulla/pronotum width was also not significantly correlated with colony size (albeit marginally; $F_{1,11} = 3.98$, $P = 0.071$), in spite of leaf-cutting ants having larger colonies than the other attines (Fig. 2). These relationships held both if all leaf-cutting ant workers were included in the analyses (leaf-cutting ants vs. other attines: $F_{1,11} = 54.9$, $P < 0.0001$; higher vs. lower attines: $F_{1,11} = 0.17$, $P = 0.688$; colony size: $F_{1,11} = 2.85$, $P = 0.119$),
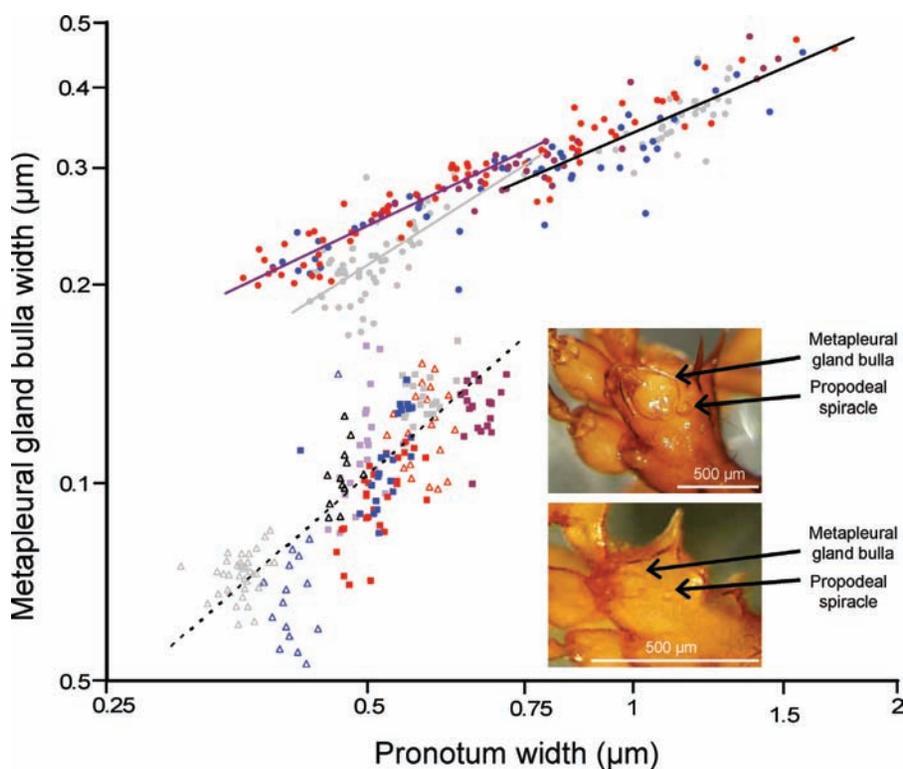

Figure 1. Relationship between metapleural gland reservoir size and body size (pronotum width) for four species of leaf-cutting ant (circles: *Acromyrmex echinatior* ●, *Atta cephalotes* ●, *A. colombica* ●, and *A. sexdens* ●), five species of basal higher attine (squares: *Trachymyrmex cf. zeteki* ■, *T. cornetzi* ■, *T.* sp. 3 ■, *Sericomyrmex amabilis* ■, and *S. cf. amabilis* ■), and four species of lower attine (triangles: *Mycocepurus smithii* △, *Apterostigma dentigerum* △, *Apterostigma* "collare" △, and *Cyphomyrmex costatus* △). Lines of best fit are $y = 1.17x - 1.14$ for nonleaf-cutting ants (dashed black line), $y = 0.572x + 0.818$ for large leaf-cutting ant workers (solid black line), $y = 0.638x + 0.671$ for small *Atta* workers (solid purple line), and $y = 0.847x + 0.047$ for small *Acromyrmex echinatior* workers (solid gray line). Inset are representative photographs of the metapleural gland bullas of a leaf-cutting ant (*Atta cephalotes*; top photo) and a lower attine (*Mycocepurus smithii*; bottom photo).
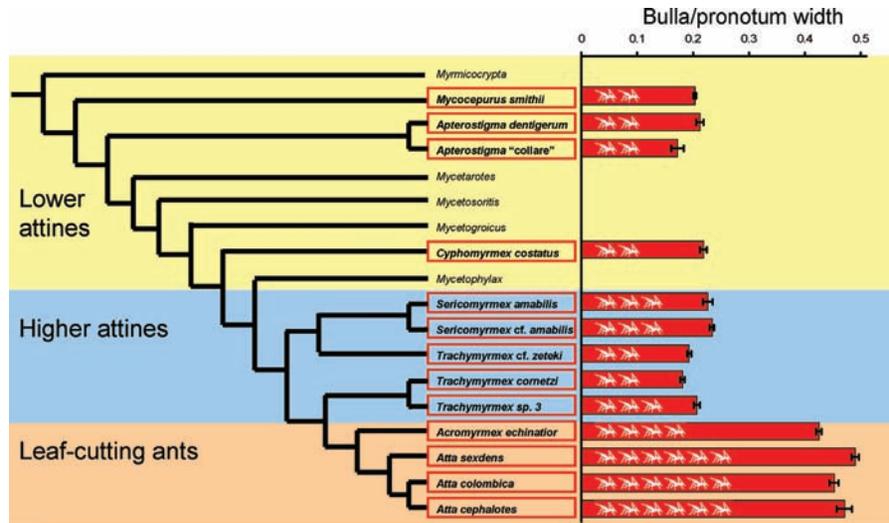
Figure 2. Phylogenetic pattern of metapleural gland reservoir size across attine ants. Of the 12 attine genera, representatives of seven were examined in this study (red boxes). The mean $\pm$ SE ratio of metapleural gland bulla width to pronotum width is given for these species. The numbers of ants within the bars indicate the typical numbers of workers per colony in powers of ten.

and if the relative distance between the bulla and the propodeal spiracle was used to estimate gland size (leaf-cutting ants vs. other attines: $F_{1,11} = 10.4$, $P = 0.008$; higher vs. lower attines: $F_{1,11} = 0.063$, $P = 0.806$; colony size: $F_{1,11} = 1.46$, $P = 0.253$).

Discussion

The fungus-growing ants show a distinct evolutionary transition in the size of the metapleural gland reservoir, with the reservoir being significantly larger, relative to body size, in leaf-cutting ants than in all other attines. In contrast, reservoir size did not correlate with the transition from lower to higher attines because the sizes in both of the basal higher attine genera, *Trachymyrmex* and *Sericomyrmex*, were no larger than those of the lower attines. Some previous comparative studies have found that investment in disease resistance increases with group size and this is taken to be because group-living animals may be exposed to greater disease transmission (Møller 2001; Møller et al. 2006; Stow et al. 2007; but see Wilson et al. 2003). There was the suggestion of a similar pattern in the Attini. Although the relationship between metapleural gland bulla width and colony size was marginally nonsignificant, *Sericomyrmex* appear to have slightly larger gland reservoirs and colonies than *Trachymyrmex*, and *Atta* larger reservoirs and colonies than *Acromyrmex*. Further work will be needed to clarify whether this trend is indeed genuine. However even if it is, the effect of colony size on disease resistance is minor compared with the dramatic transition in metapleural gland reservoir size between leaf-cutting ants and the other attines.

There are several reasons to think that the comparative pattern in metapleural gland bulla width is representative of a genuine change in host–parasite relationships within the Attini. First, bulla width is highly correlated with number of gland cells, at least in leaf-cutting ants (Bot et al. 2001), whereas the size and morphology of cells appears to differ little across ant species (Fanfani and Dazzini 1991; Angus et al. 1993). Second, bulla width relative to body size correlates strongly with disease resistance both between worker castes of the same leaf-cutting ant species (Bot and Boomsma 1996; Hughes et al. 2002; De Souza et al. 2006; Poulsen et al. 2006), and between leaf-cutting ant species (Sumner et al. 2003). Third, leaf-cutting ants are the derived members of their tribe (Chapela et al. 1994; Schultz and Meier 1995; Wetterer et al. 1998; Villesen et al. 1999, 2002; Sumner et al. 2004b). If, for example, other attines had more potent metapleural gland secretions (or other defenses) than those of leaf-cutting ants, then that would imply that leaf-cutting ants have evolved less powerful secretions at the same time as evolving larger reservoirs (and, by correlation, more gland cells) which would seem an illogical scenario.

It therefore appears most likely that the relationship with parasites is distinctly different for leaf-cutting ants than other attines. There are several possible nonmutually exclusive reasons why this may be. Leaf-cutting ants may be able to invest more in resistance, perhaps because their use of fresh vegetation as a substrate results in their fungal mutualist releasing more resources for them to invest. Alternatively, leaf-cutting ants may be exposed to more, or more damaging, parasites. Unlike other attines, leaf-cutting ant workers are polymorphic and include individuals optimized for cutting leaves that are much larger than the workers of other attines. As well as having smaller metapleural glands relative to their body size (Wilson 1980; Bot and Boomsma 1996; De Souza et al. 2006), these larger workers are also less efficient at grooming than small workers and their infrabuccal filters, which prevent microorganisms entering the crop, are less fine (Kermarrec

et al. 1986; Hughes et al. 2002). Large workers are consequently more vulnerable to parasites (Hughes et al 2002; Poulsen et al. 2006), and having large workers may therefore result in greater parasite pressure for leaf-cutting ant colonies. Finally, the risk presented by microorganisms may be greater for leaf-cutting ants because their fungal mutualists have lost competitiveness as they became specialized on a substrate of fresh vegetation and are consequently more vulnerable to competition or parasitism (Villesen et al. 2002). In this regard, it is pertinent to note that leaf-cutting ants actively groom their metapleural gland secretion on to nestmates, brood, and their mutualist fungus, whereas all other attines only groom the secretion onto their own body (Fernandez-Marin et al. 2006).

It is striking that the difference among attines in metapleural gland reservoir size mirrors the variation in queen-mating frequency: leaf-cutting ant queens mate with multiple males (polyandry) whereas those of other attines mate only once (Villesen et al. 2002; Sumner et al. 2004a). Polyandrous social insect queens have been hypothesized to evolve because the more genetically diverse colonies they produce are more resistant to parasites (Hamilton 1987; Sherman et al. 1988; Schmid-Hempel 2000). Evidence for this comes from a series of studies on bumble-bees and honey bees (Liersch and Schmid-Hempel 1998; Baer and Schmid-Hempel 1999, 2001; Tarpy 2003; Tarpy and Seeley 2006; Seeley and Tarpy 2007). The hypothesis also has good support in leaf-cutting ants because worker genotypes vary in resistance to at least one common parasite and more genetically diverse groups of workers are both more resistant and harder for the parasite to adapt to (Hughes et al. 2004; Hughes and Boomsma 2004b, 2006). However, it has also been suggested that genetic diversity may in fact make colonies more vulnerable to parasites because of the greater range of parasites that multiple genotypes will be susceptible to (Boomsma and Ratnieks 1996; van Baalen and Beekman 2006). Both polyandry and large metapleural gland reservoirs may therefore have evolved in response to greater parasite pressure, or larger gland reservoirs may have evolved because polyandry caused greater parasite pressure. Either way, the increased relative size of the metapleural gland reservoir of leaf-cutting ants demonstrates that parasites have played an important role in attine ant evolution. The patterns that we detected across genera of fungus-growing ants show that the metapleural gland of ants is an exceptionally useful structure for comparing resistance across taxa because it is fixed in size once individuals eclose as adults. Similar comparative investigations are likely to prove very useful in elucidating the evolution of host–parasite relationships for other groups.

ACKNOWLEDGMENTS

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Associate Editor: M. Van Baalen