

The metapleural gland of ants

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ABSTRACT

The metapleural gland (MG) is a complex glandular structure unique to ants, suggesting a critical role in their origin and ecological success. We synthesize the current understanding of the adaptive function, morphology, evolutionary history, and chemical properties of the MG. Two functions of the MG, sanitation and chemical defence, have received the strongest empirical support; two additional possible functions, recognition odour and territorial marking, are less well supported. The design of the MG is unusual for insects; glandular secretions are stored in a rigid, non-compressible invagination of the integument and the secretion is thought to ooze out passively through the non-closable opening of the MG or is groomed off by the legs and applied to target surfaces. MG loss has occurred repeatedly among the ants, particularly in the subfamilies Formicinae and Myrmicinae, and the MG is more commonly absent in males than in workers. MG chemistry has been characterized mostly in derived ant lineages with unique biologies (e.g. leafcutter ants, fire ants), currently precluding any inferences about MG chemistry at the origin of the ants. A synthetic approach integrating functional morphology, phylogenetic transitions and chemical ecology of the MGs of both the derived and the unstudied early-branching (basal) ant lineages is needed to elucidate the evolutionary origin and diversification of the MG of ants.

Key words: antibiotic secretion, ants, chemical defence, evolution, metapleural gland, recognition odour, territoriality.

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I. INTRODUCTION

Social insects have evolved an astounding diversity of exocrine glands to mediate social organisation and competitive interactions (Hölldobler & Wilson, 1990; Jackson & Morgan, 1993; Billen & Morgan, 1998; Billen, 2009). One of these glands - the metapleural gland (MG) - is found only in ants (Formicidae) (Fig. 1). Because homologues of the MG are unknown from other insect lineages, its unique presence in ants suggests a critical role of this gland in their origin and ecological success (Wilson, 1987; Hölldobler & Wilson, 1990; Ward, 2007).

The opening of the MG is generally a rather conspicuous external feature and therefore serves as an unambiguous character for ant identification (Fig. 2). Other characteristics used for ant identification [elbowed antennae, petiole (nodular waist), and eusociality] are each also shared with other insect lineages. Only the MG is diagnostic as a key synapomorphy for the ant family Formicidae, including fossilized lineages (Grimaldi, Agosti & Carpenter, 1997; Bolton, 2003; Engel & Grimaldi, 2005; Ward, 2007). For example, the presence of a MG in the Sphecomyrminae places this extinct subfamily near the ancestor of extant ant lineages, but the apparent absence of the MG in the ant-like, extinct lineage Armaniinae suggests a somewhat more distant relationship (Ward, 2007).

The MG likely originated once in the ancestor of the Formicidae at least 120–140 million years ago (Brady *et al.*, 2006). The gland is thought to be present ancestrally in all castes (males, queens, and workers) (Wheeler, 1928; Tulloch, 1935; Taylor, 2007), but was secondarily lost independently in diverse lineages (Brown, 1968; Hölldobler & Engel-Siegel, 1985), most notably within the subfamilies Formicinae and Myrmicinae. Absence of the MG occurs most frequently in males; when present in males, the MG is smaller than in queens and workers (Hölldobler & Engel-Siegel, 1985). To our knowledge, there exists no ant species where the gland is present only in the males. Likewise, the MG is generally more developed in queens than in workers. Ant lineages differ with respect to the hypothesized primary function of the MG (Table 1), gland architecture, and relative size, ranging from reduced or absent MGs in many social-parasitic ants to hypertrophied MGs used in defence in the *Crematogaster* subgenus *Physocrema*. A full understanding of MG evolution, therefore, requires integrated functional-morphological, chemical, behavioural, and phylogenetic analyses. We review here the accumulated evidence that may contribute to such a synthetic analysis of the MG, then conclude with the most promising areas for future research on this ant-specific gland.

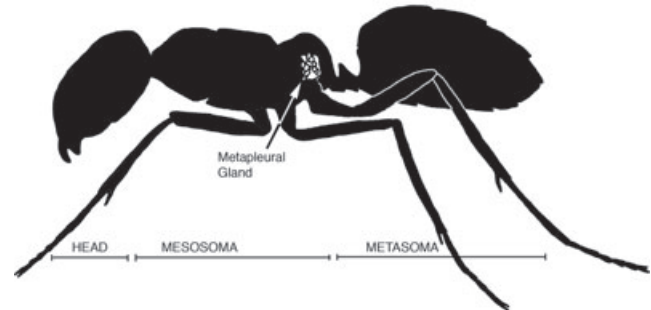


Fig. 1. Position of the metapleural gland (MG) in the posterior mesosoma of a typical ant (*Linepithema humile*). Modified from Pavan & Ronchetti (1955).

Myrmecologists were aware of the presence of the MG over 100 years ago (Meinert, 1861; Janet, 1898*a, b*; Wheeler, 1910). Early work focused on the morphology and histology of the gland (see also Tulloch, 1936; Pavan & Ronchetti, 1955; Whelden, 1957*a, b*, 1960, 1963*a, b*; Tulloch, Shapiro & Hershenov, 1962), referring to the gland also as the metathoracic and sometimes erroneously, as the metasternal gland [Wheeler (1910) attributes this error to Carlo Emery, and indeed Emery (1900) refers to the MG as the “ghiardola metasternale”; the gland actually resides in the lower plate of the metapleuron (Tulloch, 1935)]. For nearly a century, the function of the MG remained enigmatic despite a growing list of hypotheses derived mainly from anatomical studies (Janet, 1898*a, b*; Tulloch, 1936; Gösswald, 1953; Tulloch *et al.*, 1962; Brown, 1968) (Table 1). Ulrich Maschwitz’s seminal work nearly 40 years ago provided the first experimental tests of these hypotheses (Maschwitz, Koob & Schildknecht, 1970; Maschwitz, 1974), rejecting some widely accepted hypotheses (e.g. colony and species recognition) and concluding that the main function of the gland is antibiotic secretion. Although antisepsis and hygiene are currently believed to be the primary functions of the MG in ants (e.g. Hölldobler & Wilson, 1990; Schlüns & Crozier, 2009), the accumulated evidence suggests diverse functions of the MG among ant lineages and among ant castes (Table 1).

II. FUNCTIONS OF THE METAPLEURAL GLAND

Four hypotheses on MG function have been considered seriously (Table 1), and these hypotheses are not mutually exclusive: colony or species recognition; territory or nest-entrance marking; antisepsis; and chemical defence. Only the last two hypotheses have received uncontroversial empirical

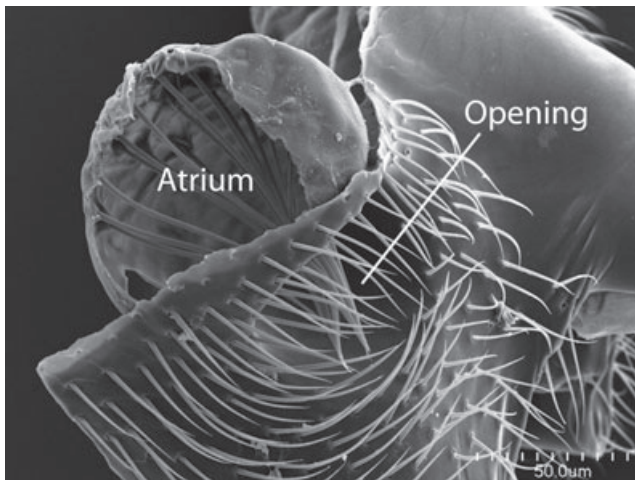


Fig. 2. Scanning electron micrograph of the metapleural gland (MG) of *Tapinoma erraticum*. A brush composed of bristle-like hairs passes through the atrium of the MG ending at the opening of the atrium. Image courtesy of Roberto A. Keller, American Museum of Natural History.

support. Additional hypotheses, such as trail-pheromone production or nutrient secretion for larvae, are now widely dismissed because of insufficient evidence, but some plausible hypotheses remain untested (Table 1). In many ant species, the MG secretions may well serve more than one function.

(1) Recognition odour

Early researchers hypothesized that the MG produces pheromones mediating nest-mate or species recognition (Janet, 1898*a,b*; Gösswald, 1953; Tulloch *et al.*, 1962; Brown, 1968). Although nest-mate and species recognition are fundamentally different processes, Brown (1968) reasoned that an individual lacking the MG may emit fewer recognition odours, increasing its chances of acceptance by another ant colony. The recognition odour hypothesis could therefore explain the absence of the MG in ants whose life histories require infiltration of other ant nests. For example, parasite queens must gain acceptance into a heterospecific host colony (Brandt *et al.*, 2005; Nash & Boomsma, 2008), army-ant males (e.g. *Dorylus* or *Eciton* spp.) must enter conspecific colonies to reach wingless queens that are surrounded by large numbers of workers (Rettenmeyer, 1963; Kronauer, 2009), and slave-raider workers (e.g. *Polyergus* spp.) must enter heterospecific *Formica* spp. nests to steal pupae as slaves (d’Ettorre & Errard, 1998).

The recognition odour hypothesis was weakened when Hölldobler & Engel-Siegel (1985) showed that the MG was absent in some ant lineages (e.g. the genus *Oecophylla*, most *Camponotus* spp.) that are known to be aggressive and capable of discrimination between nest-mates and non-nest-mate competitors. Absence of the MG in these ants therefore implies that either the MG is not involved in recognition, or that recognition is mediated by some other odour source in these MG-less species. Moreover, Hölldobler & Engel-Siegel

(1985) showed that the MG is absent in males of diverse ant lineages, not only in army ant species where males enter conspecific nests for mating. The majority of male ants that lack the MG or possess it in a reduced state do not enter heterospecific nests and in fact mate in the open (males lacked the MG in 15 out of 20 species examined by Hölldobler & Engel-Siegel, 1985). The accumulated evidence suggests that absence of the MG evolved multiple times in male ants, apparently irrespective of their need to enter foreign colonies for mating (see Section IV), thus weakening the hypothesis that the MG mediates nest-mate or species recognition in ants.

The recognition odour hypothesis was tested by Maschwitz *et al.* (1970) who conducted a series of behavioural experiments to assess the role of MG secretions in both nest-mate and species recognition. When workers of *Myrmica laevinodis* or *Myrmica rubra* were exposed to filter paper containing MG secretions from either species, workers explored the conspecific secretions to the same extent as the heterospecific secretions. [The interpretation of these experiments is complicated by taxonomic ambiguity: *Myrmica laevinodis* was later considered a subspecies of *M. rubra*, and the old *M. rubra* is now named *M. ruginodis* (Seifert, 1988, 2007).] Maschwitz *et al.* (1970) further showed that anaesthetized workers onto which MG secretions were applied from heterospecific workers were not attacked and did not elicit noticeable alarm behaviours (e.g. open mandible threat) when they were returned awake to their natal nest. In addition, *M. rubra* workers that were treated with MG secretions of heterospecific *M. laevinodis* were attacked when inserted into a *M. laevinodis* nest. Lastly, workers of *Formica rufa* and *Formica polyctena* from which the MGs were surgically removed were attended non-aggressively by conspecifics, but were attacked by heterospecifics. The results indicated that MG secretions were insufficient to elicit acceptance by a colony, thus contradicting the recognition odour hypothesis.

Subsequent to this work, the recognition odour hypothesis has been largely discarded and there have been no further attempts to test for contributions of MG odours to colony recognition. This may be unfortunate, as the gland’s secretions may contribute to the odour bouquet of a colony and thus modulate recognition in some ant species.

(2) Territory and nest-entrance marking

Tulloch *et al.* (1962) suggested that the MG secretes colony-specific pheromones used in territory marking. Territorial marker pheromones in ants are typically deposited directly onto the substratum through controlled use of an applicator such as the sting or the legs (Morgan, 2009; Billen, 2009). Such pheromones are secreted by diverse glands, for example the poison, Dufour’s, pygidial, sternal, hindgut, rectal, tibial, and tarsal glands (Hölldobler & Wilson, 1990). A common feature of all these secretions is that their application can be controlled by the ants. The best-studied example is the African weaver ant *Oecophylla longinoda* that marks

Table 1. Hypotheses on the function of the metapleural gland.

Hypotheses with empirical support for some ant lineages	
Recognition odour	Janet (1898 <i>a, b</i>), Gösswald (1953), and Brown (1968) hypothesized that the MG produces pheromones that mediate nestmate or species recognition. Several ant lineages without MGs have life histories that require infiltration of other ant nests (e.g. social parasites, army-ant males), which suggested to Gösswald (1953) and Brown (1968) that an individual lacking the MG may emit fewer recognition odours, increasing its chances of acceptance during nest infiltration. Maschwitz <i>et al.</i> (1970) failed to find behavioural-experimental support for the recognition odour hypothesis. Indirect support for this hypothesis therefore derives entirely from the absence in some (but not all) nest-infiltrating ant lineages, which can also be explained by other hypotheses (e.g. antiseptis).
Territory and nest entrance marking	Tulloch <i>et al.</i> (1962) proposed that the MG secretes colony-specific pheromones used in territorial marking. The MG secretions of several myrmicine species are thought to function as territorial or nest-entrance markers that regulate aggressive interactions between conspecific, neighbouring colonies (Jaffe & Puche, 1984; Jaffe <i>et al.</i> , 1986; Cammaerts & Cammaerts, 1998, 2001), but the experimental evidence remains controversial. Territorial or nest-entrance marking by MG secretions has not been tested for ants outside the subfamily Myrmicinae.
Antiseptis and hygiene	Maschwitz <i>et al.</i> (1970) and Maschwitz (1974) hypothesized that the MG serves a general antibiotic function by suppressing ant diseases and other detrimental microbes in the nest. The MG secretions of many ant species inhibit microbes, due to a combination of bacteriostatic acidity and possible non-specific antibiotics of MG secretions. Although only derived ant lineages have been tested for antibiotic properties of MG secretions, it is widely believed that the primary and original function of the MG is antiseptis.
Chemical defence	Ant species in the <i>Physocrema</i> subgenus of <i>Crematogaster</i> have hypertrophied MGs that secrete deterrent chemicals. When threatened, these ants extrude a droplet of whitish and sticky liquid from the MG opening, which can be retracted back into the MG by the ants (Maschwitz, 1974). These MG secretions contain diverse phenolic compounds that are toxic to invertebrate predators (Attygalle <i>et al.</i> , 1989). Hypertrophy and phenolic secretions of the MG are best studied in the Asian <i>Crematogaster</i> (<i>Physocrema</i>) ants, but some Neotropical <i>Crematogaster</i> (<i>C. acuata</i> , <i>montezumia</i>) also appears to have enlarged MGs (Hosoishi & Ogata, 2009).
Hypotheses that remain inadequately tested	
Mating pheromone	Hölldobler & Engel-Siegel (1985) speculated that the MG may produce mating pheromones in males as a secondary function (in addition to a primary antiseptic function) and that these pheromones could evolve under sexual selection. Because the MG chemistry of male ants is completely unstudied, this hypothesis remains untested. While mating-pheromone production applies to males and females, this explanation can be ruled out for workers, which do not mate and are often sterile.
Trail pheromone	Tulloch <i>et al.</i> (1962) suggested that the MG may secrete trail pheromones. However, the laterally oriented and elevated position of the MG on the ant body does not permit controlled application of the MG secretions to a trail, unlike the diverse trail-pheromone glands in ants (Morgan, 2008). Maschwitz <i>et al.</i> (1970) showed that much of the MG effluent is deposited inadvertently by ants on the ground, but that such depositions did not elicit trail-following behaviour in <i>Myrmica</i> spp.
Support of antibiotic-producing bacteria	Poulsen <i>et al.</i> (2003) hypothesized that, in some specialized ant lineages such as leafcutter ants, a derived, secondary function of the MG may be the support and sheltering of disease-suppressing mutualistic microbes. No microbes have been found to date in any MG using either microscopic techniques (Stow & Beattie, 2008; various Australian ants) or molecular screens (Mueller <i>et al.</i> , 2008; <i>Atta</i> spp.).
Hypotheses disregarded because of lack of supporting evidence	
Food production for larvae	Smith (1857) believed that the MG secretes “saccharine fluids” to feed larvae. This hypothesis has subsequently been disregarded because the elevated position on the metathorax and the known chemical composition of MG secretions seem incompatible with larval feeding.
Food storage chamber	Donisthorpe (1941) suggests that, analogous to the crop of honeypot ants, the hypertrophied MG of <i>Crematogaster difformis</i> could function as a pocket for the reception and storage of sugar liquid. This suggestion was based on the observation that workers vigorously lick each other’s metathoraces (where the MGs are located). However, chemical analysis of the MG secretion of <i>C. difformis</i> identified a mixture of defensive phenolic compounds, but no sugary substances (Maschwitz, 1974; Attygalle <i>et al.</i> , 1989).
Sound resonator	Because the atrium of the MG is a rigid integumental invagination that is filled largely with air rather than secretion, Nachtwey (1961, 1963 <i>a, b</i>) speculated that the air-filled atrium could function as a resonator in sound perception or sound production (e.g. stridulation). However, the volume of the air-filled atrium is too small to function as an effective resonator for substrate-borne vibratory communication in ants (Flavio Roces, personal communication).

arboreal territories through direct application of colony-specific pheromones in the rectal fluid and increases the rate of rectal fluid deposition in new territory (Hölldobler & Wilson, 1978). The elevated position of the MG on ants precludes direct application of MG secretions onto targets, but indirect application may occur through controlled spreading of MG secretions by leg movements, paralleling

the hypothesized application of antibiotic MG secretions to the brood and garden in fungus-growing ants (Fernández-Marín, Zimmermann & Wcislo, 2003; Fernández-Marín *et al.*, 2006, 2009). The use of territorial pheromones appears to be a derived trait (Jaffe & Puche, 1984), because early-branching (basal) genera such as poneroid ants are thought to recognize their territory using visual cues and environmental

odour, but not *via* glandular territorial pheromones (Jaffe & Marcuse, 1983).

MG secretions appear to be used to mark nest entrances or territories in some ant species, including *Tetramorium caespitum*, *T. impurum* (Cammaerts & Cammaerts, 2001), *Pheidole pallidula* (Cammaerts & Cammaerts, 1998), *Solenopsis geminata* (Jaffe & Puche, 1984), and *Pseudomyrmex triplarinus* (Jaffe, Lopez & Aragort, 1986). These studies tested the ability of workers to differentiate between non-native and native territories (Jaffe & Puche, 1984) and showed that workers are more aggressive when their own nest entrance had been marked by non-native ants (Cammaerts & Cammaerts, 1998, 2001). Different extracts (head, thorax, metathorax, legs, and metasoma) were used to investigate the origin of these marker pheromones, but only extracts from the metathorax and the hind legs increased aggressive tendencies (Cammaerts & Cammaerts, 2001). Although these observations suggest that the MG could contribute to territorial or nest-entrance marking, many details remain unclear. For example, the mechanism of application of the putative MG-derived, territorial marker onto the substratum is unknown for *Solenopsis geminata* (Jaffe & Puche, 1984), and the source of the marker can be narrowed down to the thoracic region but not specifically to the MG (*Pseudomyrmex triplarinus*, Jaffe *et al.*, 1986; *Tetramorium caespitum*, *T. impurum*, Cammaerts & Cammaerts, 2001). MG extracts elicited territorial responses only in *S. geminata*, but not in *S. invicta* (Jaffe & Puche, 1984). Studies that manipulate the flow of MG secretions (e.g. by experimentally sealing the MG opening) seem most promising to test the role of MG secretions in colony-specific marking of territories and nest entrances. Chemical analyses should also verify the hypothesized flow or application of MG secretions *via* the legs onto the marked substratum.

(3) Antisepsis and hygiene

Maschwitz *et al.* (1970) first hypothesized that the MG secretions have a general antiseptic function. Numerous studies (see Appendix S1) have documented antibiotic properties of MG secretions against diverse microbes (yeast, bacteria, fungi). Although some studies failed to find evidence of antibiotic activity (e.g. Diehl & Junqueira, 2001), it is widely believed that the primary function of MG secretions is sanitation by suppression of ant diseases or other microbes in the nest environment.

MG secretions are highly acidic (Maschwitz, 1974), and antimicrobial effects of the secretions may be largely due to this acidity. For example, *Atta sexdens* have MG secretions with a pH of 2.5, *Myrmica laevinodis* and *M. rubra* workers with a pH of 3.0–3.5 (Maschwitz *et al.*, 1970), *Crematogaster scutellaris*, *C. inflata*, *C. difformis* and diverse ponerine workers with a pH of 3–4 (Maschwitz, 1974), and *Myrmecia gulosa* with a pH of 3.5 (Mackintosh *et al.*, 1995). *Aenictus fergusonii* is the only known ant with MG secretions that are not acidic (Maschwitz, 1974). Several studies on fungus-growing ants have shown that MG secretions lower the pH in the fungus garden (Bot *et al.*, 2002; Ortius-Lechner *et al.*, 2000; Powell & Stradling, 1986; Papa & Papa, 1982; Maschwitz *et al.*,

1970), which would help suppress bacterial growth because many bacteria are inhibited at low pH levels. MG secretions of leafcutter ants contain a diversity of carboxylic acids (Schildknecht & Koob, 1971; do Nascimento *et al.*, 1996; Ortius-Lechner *et al.*, 2000, 2003), and these acids should lower the pH on the ant integument and possibly in the nest environment if the ants distribute sufficient amounts of MG secretions.

Acidity is a property of many other ant secretions. Nearly half of the known glandular secretions of ants contain carboxylic acids (Hermann & Blum, 1981). Consequently, many other ant glands secrete compounds that are weakly bacteriostatic or, in the case of formic acid in the poison gland of formicine ants, significantly bactericidal. A bacteria-suppressing effect of a particular acidic glandular secretion therefore may not be its primary function. For example, the primary function of formic acid in the poison gland of formicine ants is presumably defence, not antisepsis. Documentation of an *in vitro* antibiotic effect of an acidic secretion is therefore insufficient to conclude antibiosis as the primary function of the MG; this would require testing of the contribution of acidity to antibiosis separate from the contributions of particular antibiotics, which is experimentally challenging.

Powell & Stradling (1986) showed that removal of fungus-growing ant workers resulted in a pH increase in the ants' garden and eventual garden destruction by parasitic fungi. This could suggest that pH-lowering MG secretions are important in garden health, but other ant factors (e.g. grooming, secretion from other glands) that may also prevent garden deterioration cannot be ruled out in this particular experiment. Although Powell & Stradling's (1986) results are consistent with a contribution of MG acidity to hygiene, further work is needed to establish the effect of acidity independent of the effects of specific antibiotics in MG secretions.

The mechanisms underlying the antibiotic action of MG secretions are largely unknown, except for a membrane-destabilizing effect documented in an *in vitro* assay (Veal, Trimble & Beattie, 1992). Absorption of the active MG compounds through the phospholipid membrane disrupts membrane structure and function (Mackintosh *et al.*, 1995), causing the cells to burst in both prokaryotes and eukaryotes. Mackintosh *et al.* (1995) discuss several possible mechanisms for how the ants may protect themselves against the generalized antibiotic effects of their own MG secretions, such as a modified integument forming an effective barrier or secretions that become active and harmful only after exposure to the external environment.

Brown (1968) reported that some ants groom the MG opening with their legs, and such grooming was confirmed in subsequent studies of dozens of species from six ant subfamilies (Farish, 1972; Basibuyuk & Quicke, 1999; Fernández-Marín *et al.*, 2003, 2006). MG grooming permits active dispersion of MG effluent, rather than passive and uncontrolled flow (Fernández-Marín *et al.*, 2006, 2009). By increasing the rate of MG grooming during microbial infection, ants seem to be able to upregulate dispersion

of MG secretions (Fernández-Marín *et al.*, 2006, 2009). Increased rates of MG grooming occur in defence against both virulent and more benign diseases, supporting the view that MG secretions have broad-spectrum antimicrobial properties (Fernández-Marín *et al.*, 2009).

Preventive measures that serve as a first line of defence should be typical for eusocial lineages with perennial colonies such as ants (Beattie *et al.*, 1986; Boomsma, Schmid-Hempel & Hughes, 2005; Cremer, Armitage & Schmid-Hempel, 2007). MG secretions therefore may be designed to be more effective against early developmental stages of pathogens (e.g. spores that have yet to germinate) than later developmental stages (e.g. growing hyphae). Empirical tests have confirmed this prediction for MG secretions (Veal *et al.*, 1992; Beattie *et al.*, 1986; Bot *et al.*, 2002), and MG secretions therefore appear to target fungal spores before hyphal growth of a spreading infection becomes a serious problem.

If MG secretions serve primarily antiseptic functions, one might expect that the caste that is most involved in pathogen defence (e.g. small nest workers in many species; gardeners in leafcutter ants) have larger MGs. This has been documented for some leafcutter ants where minima workers have disproportionately large MGs compared to major workers (Wilson, 1980; Bot & Boomsma, 1996; Hughes *et al.*, 2008). Moreover, small workers of the leafcutter *A. octospinosus* are more effective at grooming than large workers. In infection bioassays, small *A. octospinosus* workers are more resistant to pathogens than are larger workers (Hughes, Eilenberg & Boomsma, 2002; Poulsen, Hughes & Boomsma, 2006). In contrast to the above studies, Diehl & Junqueira (2001) found no survival differences among *Atta sexdens piriventris* sub-castes when challenged with the entomopathogen *Beauveria bassiana*, and the disproportionately larger MGs of these small workers apparently do not provide greater disease protection.

Apart from caste-dependent factors, environmental factors may also modulate MG function. In *Atta sexdens piriventris*, MG secretions vary throughout the year, with the highest levels of MG secretion during warmer months (Diehl & Junqueira, 2001). In *Acromyrmex octospinosus*, workers in field colonies produce greater quantities of MG secretions compared to workers in laboratory colonies, but the MGs of these two types of workers do not show qualitative chemical differences (Ortius-Lechner *et al.*, 2000). The observed quantitative differences could be due to differences in food availability, temperature, disease pressure, or any other spatially or seasonally varying factor. It is possible that both the better nutrition and low disease prevalence in the laboratory colonies strengthen colony immunity, lowering secretory flow in the MGs of laboratory workers.

Among the closest relatives to ants, bees and some vespoid wasps are important plant pollinators, whereas very few ants pollinate plants (see Rico-Gray & Oliveira, 2007, and references therein; de Vega *et al.*, 2009). To explain this difference, Beattie *et al.* (1984) proposed that the antibiotic MG secretions kill pollen or inhibit pollen germination,

rendering ants ineffective as plant pollinators unless they have reduced or absent MGs. Consistent with this hypothesis, reduction and absence of MGs is found in some pollinating ants, such as males of *Myrmecia urens* that pollinate the orchid *Leporella fimbriata* through pseudocopulation (Peakall, Beattie & James, 1987) and *Camponotus compressus* workers that pollinate coconuts (Patel, 1937). However, reduction or absence of the MG among ant pollinators is not the norm, and effective ant pollinators with well-developed MGs are not uncommon (e.g. *Formica lasioides*, *F. oregonensis*, Kincaid, 1963; *Formica argentea*, Hickman, 1974; *Proformica longiseta*, Gómez & Zamora, 1992). Moreover, contact of *Crematogaster auberti* and *Pheidole pallidula* ants with functional MGs does not reduce pollen viability in the ant-pollinated plant *Cytinus hypocistis*, whereas contact with the MG-less *Camponotus pilicomis* greatly reduces pollen viability (de Vega *et al.*, 2009). Based on this small survey of pollinating ants, it appears that possession of a functional MG does not preclude an evolutionary transition to ant pollination. The observed rarity of pollinating ant species appears better explained by the fact that ant workers are flightless and cannot readily vector pollen among different plants, thus limiting the distance that ants can disperse pollen compared to flying pollinators such as bees and wasps.

(4) Chemical defence

Crematogaster ants in the subgenus *Physocrema* have hypertrophied MGs (Janet, 1898b; Donisthorpe, 1941; Hosoishi & Ogata, 2008, 2009), and MG secretions of *Crematogaster difformis* and *Crematogaster inflata* contain a mixture of phenolic compounds that are noxious to predators (Attygalle *et al.*, 1989; Jones *et al.*, 2005). When *C. (Physocrema) inflata* workers are attacked, they expel from the MG opening a droplet of a whitish, viscous, sticky secretion which they can retract back into the MG (Maschwitz, 1974; Buschinger & Maschwitz, 1984). These MG droplets deter hostile ants and vertebrate predators (Maschwitz, 1974; Ito *et al.*, 2004), and volatile emissions from the droplets alarm nestmates (Maschwitz, 1974). The secretions repel ant predators by acting both as a chemical deterrent and as a glue (Maschwitz, 1974). Despite extensive grooming, ants smeared with the MG secretions of *C. inflata* are unable to remove the sticky secretion (Maschwitz, 1974; Ito *et al.*, 2004). The repellent nature of MG secretions is best documented for *Crematogaster (Physocrema)* ants (Maschwitz, 1974; Attygalle *et al.*, 1989).

Although both *C. difformis* and *C. inflata* use MG secretions mainly in defence, the secretions serve additional functions in these species. Volatile odours from the MG induce alarm behaviour in nest-mates of *C. inflata*, but not in *C. difformis* (Maschwitz, 1974) where the alarm pheromone is secreted by the mandibular gland. *C. difformis* is thought to use a two-gland system for defence. The head, neck, appendages, and metasoma are protected by secretions from the Dufour's gland, whereas the thorax and petiolar-postpetiolar region are protected by secretions from the MG (Maschwitz, 1974; Jones *et al.*, 2005).

(5) A pluralistic view of metapleural-gland function

To date, a disproportionate number of studies have investigated antibiotic functions of MG secretions. Most of these studies focused on ant lineages with uniquely derived biologies (e.g. fungus-growers such as *Atta* and *Acromyrmex* leafcutter ants, and solitarily foraging predators such as the bulldog ant *Myrmecia gulosa*). Because of their derived biologies, the function(s) of the MG in these groups may be uniquely modified and thus may not be representative for ants at large. Further studies of a more representative sample across the ant phylogeny are needed, particularly of the early-branching (basal) ant lineages for which there is currently no information. Without such studies, it will be difficult to elucidate the original function of the MG.

While the recent literature has favoured antibiosis as the single, primary function of the MG, insect exocrine glands often serve several concurrent functions (Hermann & Blum, 1981; Hölldobler & Wilson, 1990). Multiple functions of the MG should therefore not be ruled out *a priori* for ants. Sanitation and territorial marking are two functions of single glands hypothesized for a number of ant species (Jaffe & Puche, 1984; Cammaerts & Cammaerts, 1998), and pheromones used in communication can have strong antibiotic properties (Cole, Blum & Roncadori, 1975). MG secretions could have primary and secondary functions that differ among castes or among life stages (nurse ant *versus* forager), such as sex pheromone production in addition to antibiotic production (Hölldobler & Engel-Siegel, 1985). Alternatively, MG function could be concentration-dependent [e.g., a low concentration of MG secretion could promote the growth of the symbiotic fungus in attine ants, whereas a high concentration may inhibit growth (Powell & Stradling, 1986)]. Lastly, of course, the different compounds in the secretory mix could serve different functions. Even a single compound can simultaneously serve several roles. For example, indoleacetic acid in the MGs of leafcutter ants may contribute to bacteriostasis by lowering the pH of the secretion, but may also facilitate fungal growth in leafcutter gardens because of its auxin properties (Schildknecht *et al.*, 1973). Because many ant species may possess such functional complexity in MG secretion (e.g. between castes), the recent research emphasis on antibiotic effects may have precluded fruitful investigations into additional functions.

III. MORPHOLOGY OF THE METAPLEURAL GLAND

Hölldobler & Engel-Siegel's (1985) comparative-morphological study of 59 ant species (24 genera, six subfamilies) still represents the landmark morphological work on the MG. Several studies expanded on this work by examining additional species and by providing detailed histological and ultrastructural analyses (e.g. Billen & van Boven, 1987; Fanfani & Valcurone Dazzini, 1991*a,b*; Schoeters & Billen, 1992, 1993; Bot *et al.*, 2001; Gusmão, Caetano & Nakano,

2001; Vieira, Bueno & Camargo-Mathias, 2010*a, b*). These studies show that the MG consists of four parts: (a) a cluster of unicellular glandular cells, (b) a membranous collecting sac or collecting recess (not present in all species), which receives the secretion from the secretory cells; (c) a larger, sclerotized atrium into which the collecting sac empties; and (d) an externally visible orifice. In those ant species that do not possess a membranous collecting sac (one-chamber design), the glandular cells empty directly into the sclerotized atrium.

(1) One-chamber and two-chamber metapleural glands

Many ants exhibit a two-chamber MG design with a clear constriction between a membranous collecting sac and a sclerotized atrium (Fig. 3). Such compartmentalization into separate collecting and storage sub-compartments is a general design of other exocrine glands in ants (e.g. poison gland; Schoeters & Billen, 1993). By contrast, some ants exhibit a simpler, one-chamber design with no clear separation between collecting sac and atrium (Fig. 2). In this simpler design, the secretory cells empty directly into the sclerotized atrium (Billen & van Boven, 1987; Schoeters & Billen, 1992).

Schoeters & Billen (1992) pointed out that the two-chamber MG design may permit controlled release of secretion through application of pressure from thoracic muscles onto the pliable collecting sac (see also Schoeters & Billen, 1993; Bot *et al.*, 2001). Such controlled release is not possible in the one-chamber design, because the atrium is a

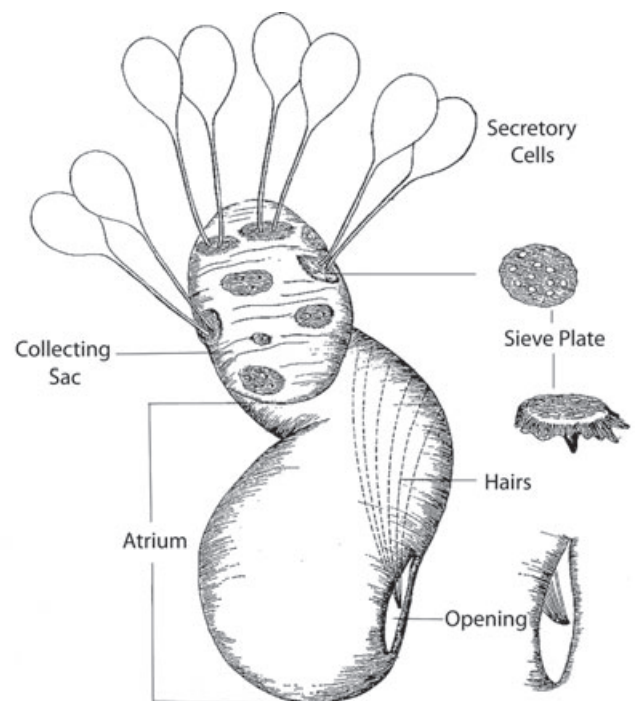


Fig. 3. Reconstruction of the two-chambered metapleural gland (MG) of *Myrmecia nigrocincta*. Modified from Tulloch *et al.* (1962).

highly sclerotized, rigid chamber, formed as an invagination of the metapleural integument. The uncompartimentalized, simple MG design therefore could represent a more ancestral state, perhaps evolutionarily derived from an integumental patch of cuticular glands that gradually became recessed and eventually invaginated to form a rigid chamber.

The one-chamber design has been described in poneroid ants (*Amblyopone pallipes*, Whelden, 1957b; *Diacamma* spp., Schoeters & Billen, 1992), doryline ants (nine species of *Dorylus* spp.; Billen & van Boven, 1987), and dolichoderine ants (*Dolichoderus quadripunctatus*, *Linepithema humile*, *Tapinoma erraticum*; Fanfani & Valcurone Dazzini, 1991b; Roberto Keller personal communication). By contrast, two-chamber designs have been described in the subfamilies Myrmeciinae (*Myrmecia nigrocincta*, *M. pilosula*, Tulloch *et al.*, 1962; Hölldobler & Engel-Siegel, 1985; *Nothomyrmecia macrops*, Hölldobler & Engel-Siegel, 1985), Myrmicinae (*Acromyrmex octospinosus*, *Aphaenogaster rudis*, *Atta bisphaerica*, *A. laevigata*, *A. sexdens*, *Crematogaster striatula*; Hölldobler & Engel-Siegel, 1985; Fanfani & Valcurone Dazzini, 1991a; Schoeters & Billen, 1993; Bot *et al.*, 2001), and Formicinae (*Myrmecocystus mendax*, Hölldobler & Engel-Siegel, 1985). These phylogenetic patterns seem to support the view that the one-chamber design is ancestral among ants. However, only a comprehensive survey across the entire diversity of ant genera, including the early-branching (basal) ant lineages, can hope to reconstruct evolutionary transitions in morphological complexity of the MG. In fact, additional designs that may not fit into a division between one-chamber and two-chamber designs appear to exist, as for example the MG designs with irregular membranous, fingerlike expansions in doryline and ecitonine army ants (Whelden, 1963a,b; Roberto Keller, personal communication), in some ectatommine ants (Whelden, 1960), and in some ponerine ants (Roberto Keller, personal communication).

(2) External morphology

The external morphology of the MG varies greatly among ant lineages (Fig. 4). In many ants, the gland empties *via* an oval or slit-shaped opening, located either just above the articulation of the hind coxa, or somewhat more dorsal-posteriorly below the propodeum in the recess between the articulation of the hind coxa and the petiole. Slit-shaped or oval openings give the impression of a gland that is designed for broad, brush-like release of the secretion, rather than point application.

The MG opening in many ant lineages deviates from this general slit-like design of the opening, suggesting modified functions. For example, the MG opening can be large and round (*Diacamma* spp., Schoeters & Billen, 1992; some dolichoderines, Fanfani & Valcurone Dazzini, 1991b), such that the MG atrium appears as a relatively exposed and unprotected invagination of the metapleural integument, rather than as a sequestered receptacle designed for storage and controlled release of secretion. In lineages exhibiting army-ant-like morphology and behaviour (e.g. *Leptanilla*, dorylomorphs) and in *Myrmecia* spp., a carina-like lip flanks

the slit-like MG opening from above (Brady & Ward, 2005); this flange may protect the glandular atrium from accidental contamination, facilitate collection of the secretion by legs for subsequent application, reduce evaporation, or serve other functions. In ecitonine army ants, the flange extends anteriorly as a ridge onto the mesosoma, perhaps to facilitate flow of the secretion in an anterior direction (Phil Ward, personal communication). Keller (2008) describes variations of this flange in other ant lineages, noting phylogenetically informative modifications of the structure and its carina-like extensions. Keller (2008) also describes a flap emanating from the ventral margin of the MG orifice. This flap extends upward, sometimes covering the opening almost completely except for a narrow slit opening towards the posterior or above, rather than towards below. In addition to such morphological variations in shape of the MG opening, the opening can be oriented in different directions (Fig. 4). For example, the MG opens towards the side and onto the coxa in some ants, but towards the posterior and onto the propodeum in other ants (Keller, 2008; Phil Ward, personal communication). In sum, the metapleural gland opening and its associated structures offer a rich diversity of unexplored characters for phylogenetic analyses, perhaps even allowing differentiation among closely related species. For example, queens of closely related *Atta* species can have MG openings that differ markedly in size, whereas the workers of the corresponding species do not show this difference (Gusmão, Caetano & Nakano, 2001).

In leafcutter ants, *Crematogaster* (*Physocrema*) ants, and many others, the glandular atrium is enlarged and forms a conspicuous bulge (bulla) above the MG opening. Such hypertrophied glands occupy a significant volume of the posterior mesosoma. At the other extreme, the MG opening can be small, indicated externally merely by a small pore that is easily overlooked. For example, the inquiline parasite *Teutomyrmex schneideri* was thought to have no metapleural gland (Gösswald, 1953; Brown, 1968), but a small pore is actually present just above the hind coxa where a MG would be found (S.H. Yek, C. Rabeling, & U.G. Mueller, personal observations). Without dissection, it is unclear whether this pore is a vestigial feature or is associated with a functional gland, as discussed by Brown (1968).

Whether large and round, narrowed to a slit, or partially covered by a lip, the MG atrium appears less protected from contamination with particles and chemicals than the typical exocrine glands of ants (Billen & van Boven, 1987; Billen, 2009). The reasons for the generally large opening of the MG are unclear. Rapid release of large quantities of secretion is one explanation for a large opening (e.g. in the *Crematogaster* subgenus *Physocrema*). An alternative hypothesis may be that the large opening may allow simultaneous entry of air into the atrium while releasing the secretion (Schoeters & Billen, 1992). Indeed, the atrium always contains some air (Janet, 1898a,b; Gösswald, 1953; Maschwitz *et al.*, 1970; Poulsen *et al.*, 2002b), depending presumably on a balance between secretory inflow and outflow. In a study by Poulsen *et al.* (2002b) on *Acromyrmex octospinosus*, for example, most garden workers of a laboratory colony had approximately one-third

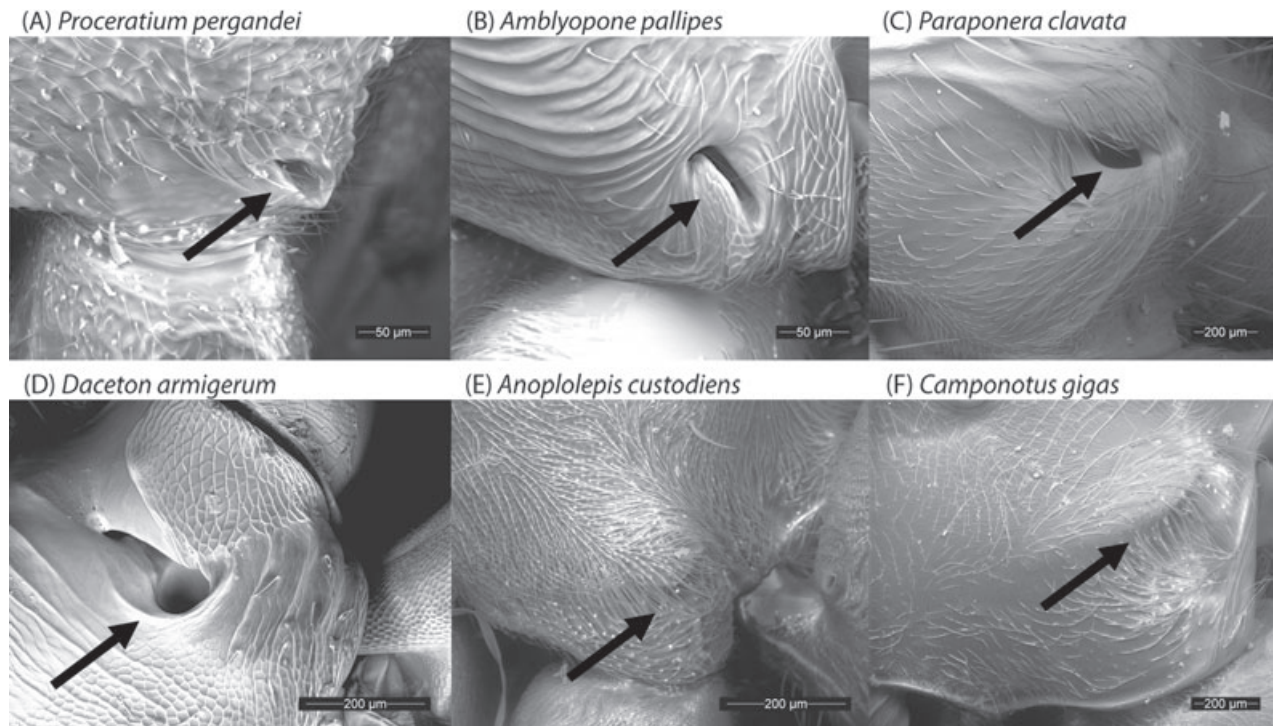


Fig. 4. Morphological diversity of metapleural gland openings (arrows) of workers in a sample of early-branching (basal) and derived ant lineages. (A) *Proceratium pergandei*, (B) *Amblyopone pallipes*, (C) *Paraponera clavata*, (D) *Daceton armigerum*, (E) *Anoplolepis custodiens*, (F) *Camponotus gigas*. Images courtesy of Jeffrey Sossa-Calvo, Smithsonian Institution.

of the atrium volume filled with secretion, 5% of workers had a completely empty atrium, and only about 4% had a completely filled atrium. Interestingly, atria are completely filled with air in starved workers, but re-accumulate secretion when the workers resume feeding (Maschwitz *et al.*, 1970). In *Acromyrmex octospinosus* workers, food deprivation leads rapidly to reduced secretory rates (Bot & Boomsma, 1996), and secretory rates seem to decline with age (Bot *et al.*, 2001). The relative proportion of air and secretion in the atrium therefore is variable during an ant's life, and an atrium filled partially with air is a normal condition. Perhaps the interface between the secretion and the air in the metapleural atrium serves some unknown purpose.

Another variable feature among ant lineages is the area just below the glandular opening, which is frequently smooth (Schoeters & Billen, 1993; Gusmão *et al.*, 2001), even in species with a rugulose, pitted, or otherwise roughened integument. In *Protanilla* spp., a smooth trench extends anteriorly from the MG opening (the metapleural trench; Bolton, 1990), perhaps to direct the flow of the MG secretion towards the meso- and meta-coxae. In *Atta* spp. leafcutter ants, a cuticular ridge just below the MG opening directs the secretory flow directly onto the hind coxa; this design is thought to aid the spread of the secretion by coxal movements (see Schoeters & Billen, 1993). Lastly, rows of hairs are frequently arranged just outside the opening, but hairs can also originate inside the atrium (Meinert, 1861; Pavan & Ronchetti, 1955; Hölldobler & Engel-Siegel, 1985; Billen & van Boven, 1987; Fanfani & Valcurone Dazzini,

1991a,b) (Fig. 2). By contrast, *Atta bisphaerica*, *A. capiguara*, and *A. sexdens rubropilosa* leafcutter ants are devoid of hairs at the orifice (Schoeters & Billen, 1993; Gusmão *et al.*, 2001). Hairs at the MG opening may capture and hold the outflowing secretion, prevent accidental entry of contaminant particles into the large opening of the gland, aid in the channeling and distribution of the secretion, or serve sensory functions.

In *Tapinoma erraticum*, *Iridomyrmex purpureus*, *Linepithema humile*, *Myrmecia pilosula*, and *M. nigrocincta*, bristle-like hairs originate at the back of the atrium and project through the atrium towards the MG opening (Tulloch *et al.*, 1962; Hölldobler & Engel-Siegel, 1985; Fanfani & Valcurone Dazzini, 1991b; Keller, 2008; Figs 2 & 3). Such hairs are assumed to guide the secretion through the atrium to the MG opening, and Hölldobler & Engel-Siegel (1985) therefore call these hairs “dispenser bristles”. A single row of hairs (*Myrmecia nigrocincta*; Tulloch *et al.*, 1962) or two parallel rows of hairs (*Myrmica rubra*; Tulloch, 1936) originate from the lateral, internal surface of the atrium, whereas a brush of hairs originates at the back of the atrium in *Tapinoma erraticum* (Fig. 2). The tips of these hairs converge at a common point at the centre of the external opening, and in *Lasius flavus* the converging hairs appear like a pointed, hollow brush (Wheeler, 1910). In such designs, the secretion may not flow along the walls of the atrium, but could be suspended along hairs in an otherwise air-filled atrium.

It is surprising that, despite the importance of the MG in ant identification, we are still lacking a comparative functional-morphological analysis of the structures associated

with the MG opening. It is also surprising that the presence or absence of the gland in males is rarely reported in taxonomic descriptions, whereas its presence/absence in females and workers is generally noted. We are also lacking a comprehensive comparative analysis of MG chemistry to allow us to infer ancestral states and identify subsequent transitions to derived states. Keller's (2008) survey suggests rich variation in MG morphology among ant lineages that could provide important clues for diverse MG functions when combined with behavioural and ecological information (see also Fig. 4). A comprehensive analysis should focus not only on the external and internal morphology of the MG, but also on the structures that interact with the MG, such as the legs, which may pick up and spread the secretion (Brown, 1968; Fernández-Marin *et al.*, 2006, 2009). We are surprised to find that studies on the MG have so far largely ignored the design of the body surfaces that are likely to facilitate removal of the MG secretion.

(3) Internal morphology

The MG is divided into three parts, clusters of secretory (exocrine) cells, a collecting sac/recess into which the cells secrete, and an atrium (Fig. 3). The glandular cells may be numerous enough to engulf the collecting sac, which then takes the appearance of an internal lumen. If few secretory cells are present, they appear as a fan-shaped cluster attached to a portion of the collecting sac. The average cluster is comprised of 10–30 secretory cells, and several such tightly packed clusters can be grouped into larger secretory structures producing a cauliflower-like appearance (Schoeters & Billen, 1992, 1993; Gusmão *et al.*, 2001; Bot *et al.*, 2001). Each cell cluster empties into the collecting sac through its own sieve-like plate (Billen & van Boven, 1987; Gusmão *et al.*, 2001; Fig. 3). The cell clusters and the collecting sac are generally located higher and more anteriorly than the atrium [but see Whelden's (1963*a, b*) description of the MG of *Eciton burchellii* queens], perhaps to facilitate a passive flow downwards to the glandular opening located at the inferior-posterior end of the atrium.

The intracellular structure of the secretory cells has been studied in very few ant species (*Myrmecia nigrocincta*, Tulloch *et al.*, 1962; *Dorylus* spp., Billen & van Boven, 1987; *Atta bisphaerica*, *A. laevigata*, and *A. sexdens*, Schoeters & Billen, 1993). The secretory cells show an end apparatus typical for insect secretory cells, consisting of an intracellular collecting ductule and a surrounding sheath of microvilli (Tulloch *et al.*, 1962; Schoeters & Billen, 1992). Ultrastructural differences between contiguous cells suggest asynchronous secretory activity (Schoeters & Billen, 1992). As in most ant glands, the secretory cell cytoplasm is rich in smooth endoplasmic reticulum and mitochondria (Billen & van Boven, 1987).

Each secretory cell of the MG possesses an intracellular ductule that collects the secretion (see Billen, 1991 for a diagram of this kind of exocrine cell). The intracellular ductule extends *via* a duct cell into the collecting sac, a pliable membranous receptacle that can expand or contract when receiving or releasing the glandular secretion. Ductules

do not fuse (Billen & van Boven, 1987), and each secretory cell therefore empties individually into the collecting sac. As far as is known, no muscles attach to the collecting sac that could directly control the release of the glandular secretion, nor are there nerve endings terminating at the secretory tissue that could regulate glandular activity (Schoeters & Billen, 1993). However, Wheeler (1910, p. 38) includes a ganglion located next to the secretory cells in a drawing of the MG of *Lasius flavus*. This may be a misidentification as no such ganglion has been reported in any subsequent histological study.

From the collecting sac, the secretion flows into the sclerotized atrium. The sclerotization of the atrium can be extensive, perhaps to protect the ant from the acidic or toxic secretion it contains. In *Diacamma* spp., for example, the atrium wall is actually thicker than the exoskeleton (Schoeters & Billen, 1992). The atrium is positioned immediately below the integument and can appear externally as a bulge (bulla) of the integument. An atrium is supplied by only one collecting sac (or one secretory area with sieve plates for one-chamber species). Many researchers have commented on the similarity of the atrium wall to the integument (Janet, 1898*a, b*; Whelden, 1957*a, b*; Billen & van Boven, 1987; Schoeters & Billen, 1992; Keller, 2008), suggesting that the atrium is a relatively unmodified integumental invagination. Because this structure usually is mostly filled with air, we prefer to use the term 'atrium', rather than 'reservoir' used more commonly in the literature.

Janet (1898*a, b*) and Schoeters & Billen (1993) describe a narrow groove in the wall of the atrium of *Myrmica rubra* and *Atta sexdens*. This groove appears to function as a gutter to guide the flow from the collecting sac to the MG opening. As described above, other ants such as *Lasius flavus*, *Myrmecia pilosula*, *M. nigrocincta*, and *Linepithema humile* have bristle-like hairs passing through the atrium that could function to guide the secretory flow through the atrium (Wheeler, 1910; Tulloch *et al.*, 1962; Hölldobler & Engel-Siegel, 1985; Fanfani & Valcurone Dazzini, 1991*b*). The hairs are sometimes arranged as a hollow brush (Wheeler, 1910) (Fig. 2), perhaps drawing the secretion towards the MG opening by means of capillary forces, aiding in the evaporation of chemicals, or facilitating chemical interactions between the secretion and air inside the atrium. We are not aware of a species which has both bristle-guides and a gutter in the MG atrium: these two features therefore may represent alternative designs to channel the secretory flow through the atrium towards the opening.

The efflux from the atrium onto the body surface is thought to be passive (but see below), as the atrium is a rigid structure that resists compression (Hölldobler & Engel-Siegel, 1985; Billen & van Boven, 1987). However, thoracic muscles adjacent to the pliable collecting sac may compress the sac and hence indirectly modulate the efflux from the atrium (Bot *et al.*, 2001). Schoeters & Billen (1992, 1993) discuss how the action of specific metathoracic muscles could apply pressure to the glandular tissue and the collecting sac, causing the secretion to be released into the atrium and towards the MG opening. In *Crematogaster (Physocrema) difformis* and *C. inflata*,

workers can expel a defensive secretory droplet, retain it at the rounded, glandular orifice, then retract it back into the atrium (Buschinger & Maschwitz, 1984; Maschwitz, 1974). The neuromuscular mechanisms underlying this controlled expulsion and retraction have not been studied.

The size of the secretory tissue differs among castes. Queens have about twice the number of glandular cells than the average worker (Hölldobler & Engel-Siegel, 1985; Angus, Jones & Beattie, 1993; Appendix S2). For the handful of species for which male glands have been examined, males have only about 20–50% of the number of glandular cells as the corresponding workers (Appendix S2). We are not aware of any species where the gland is absent in workers but present in sexual females, and we know only of *Lasius fuliginosus* where the gland is pronounced in workers but absent (or greatly reduced) in sexual females (Appendix S2).

Among ant species, larger ants tend to have larger glands with more secretory cells, whereas cell number appears to be independent of ant colony size (number of workers) (Angus *et al.*, 1993). Among species in the fungus-growing ant tribe Attini, relative MG bulla size (bulla size standardized by pronotum width) is not correlated with colony size ($P = 0.07$; Hughes *et al.*, 2008). However, relative to body size, leafcutter ant species have significantly larger MGs compared to non-leafcutter species, indicating that MG size increase was linked to the origin of leafcutter fungiculture (Hughes *et al.*, 2008). These patterns suggest that MG size evolution is not driven by factors associated with colony size, but rather by factors associated with body size and microbial interactions (see Section II.3).

The relationship between MG size and worker caste has been studied in only a few species. Secretory-cell number appears independent of worker size in *Orectognathus versicolor* (minors, medias, and majors each have about 80 cells per MG; Hölldobler & Engel-Siegel, 1985; Appendix S2), but cell number increases with worker size in *Acromyrmex octospinosus* leafcutter ants, where the largest workers have about 2–3 times the number of cells than the smallest workers (Bot *et al.*, 2001) (Appendix S2). The increase in cell number with leafcutter worker size is allometric, however, such that relative to body size, the smallest workers have proportionately larger MGs compared to larger workers (Bot & Boomsma, 1996; Bot *et al.*, 2001; Gusmão *et al.*, 2001; Hughes *et al.*, 2008). This greater investment into MG function by the smallest *Acromyrmex octospinosus* workers may be linked to the garden- and nest-sanitation activities of this caste (see Section II.3). In *Acromyrmex octospinosus*, bulla size and number of secretory cells are highly correlated ($r = 0.84$), justifying measurement of the externally visible bulla as a proxy for the size of the MG (Bot *et al.*, 2001; Hughes *et al.*, 2008).

(4) Functional morphology

The location of the MG opening low at the latero-posterior end of the mesosoma, often below the level of the petiole insertion, could be a starting point for a functional-morphological analysis. No conspicuous homologous glands

are currently known from this area in the putative vespid sister lineages of ants (Ward, 2007; Pilgrim, van Dohlen & Pitts, 2008), precluding comparative inferences about MG function at the origin of the ants.

The location of the MG opening in the centre of the ant is unusual. Only one other gland is known to empty in this area, the mesopleural gland, which is present only in dacetine and phalacromyrmecine ants (Bolton, 2003). By contrast, most ant glands empty at the mouth, near the tip of the metasoma, or on the legs; these locations permit easy contact with the substratum for point-application of a secretion. The central and elevated position of the MG is less compatible with certain hypothesized MG functions, such as the production of a trail pheromone - known trail pheromones in ants are secreted from structures that permit controlled application and that are close to the substratum, such as the tip of the metasoma or the legs (Hölldobler & Wilson, 1990). However, the central, elevated position of the MG is compatible with other functions, such as the production of recognition pheromones, antibiotics, or defensive secretions to protect the vulnerable petiolar region. Other unusual features of the MG compared to other exocrine glands may also inform hypotheses of MG function: (1) a large, non-closable opening; (2) a frequently slit-like opening, suggesting brushlike application rather than point application; (3) rigidity of the atrium, precluding contraction and controlled glandular discharge; and (4) opening of the gland low near the coxae or the petiole.

For protection of body surfaces against diseases, a logical design would be to have small glands distributed across the entire integument (like the numerous wax-secreting glands that cover the insect body), rather than two central gland openings from which secretions must be spread across the body by laborious and time-consuming grooming. The presence of only two MG openings therefore suggests other functions. The single, large opening of each MG could be better explained if the gland's primary function is to supply secretions that can be targeted by grooming to specific problem sites, such as infected body parts, brood, or specific areas in the nest (Fernández-Marín *et al.*, 2006, 2009). However, the presumed uncontrolled oozing of secretion from the MG opening then remains unexplained (Maschwitz *et al.*, 1970). To validate this widely accepted assumption, it will now be important to measure the uncontrolled efflux relative to the active spreading of MG secretions by grooming. We note that the original study by Maschwitz *et al.* (1970) merely documented that MG secretions applied to the surface of the mesosoma seem to flow passively onto the substratum *via* the ants' legs, but passive efflux of the viscous MG secretion through the MG opening has never been quantified directly.

Expanding on reports of active spreading of MG secretions by leg grooming motions (Brown, 1968; Maschwitz *et al.*, 1970), Fernández-Marín *et al.* (2006) recently confirmed the so-called MG grooming in 26 ant species from five subfamilies. MG grooming involves a series of coordinated fore leg and body movements. A worker ant partially extends its legs to raise the body from the substratum, flexes the

fore leg at the femoral-tibial joint to bring the posterior surface of the metatarsus in contact with the opening of the ipsilateral MG, rubs the metatarsus over the opening, then brings the leg into contact with the lateral surface of the glossa (Fernández-Marín *et al.*, 2006). The occurrence of this behaviour across major ant lineages suggests that it arose early during ant evolution (see also Farish, 1972).

IV. EVOLUTIONARY LOSSES AND REGAINS OF THE METAPLEURAL GLAND

A survey of the early-branching (basal) ant lineages reveals that the MG was ancestrally present in ant workers, females, and probably also males (Bolton, 2003), but that the gland was lost in many lineages during ant evolution. We estimate that about 20% of ant species do not have a MG. Losses in workers have occurred sporadically (Appendix S2), for example during evolutionary transitions to social parasitism. These parasitic lineages are not particularly speciose. Prominent ant lineages without a MG are found in the Camponotini (Maschwitz, 1974; Hölldobler & Engel-Siegel, 1985), including the genera *Camponotus* (about 1000 described species) and *Polyrhachis* (about 500 species). This group comprises about 15% of described ant species diversity and is thought to have derived from a common ancestor with MGs (Ellen Schläuns, unpublished data). Because the MG is absent in the vast majority of species in the hyperdiverse genus *Camponotus*, the MG-less condition apparently did not prevent the radiation of this ecologically successful lineage.

(1) Evolutionary losses in social parasites

Gösswald (1953) first suggested that the MG may contribute to colony odour (“Nestgeruch”) in non-parasitic ants; gland loss would reduce odour levels for socially parasitic ants and thus improve the parasite’s chances of successfully entering a host nest. Gösswald (1953) and Brown (1968) further noted that MG loss appears to have occurred most frequently in workerless inquiline ants (permanent social parasites), and less often in temporary social parasites which differ in their treatment of the host queen. Whereas an inquiline ant does not kill the host queen but coexists intimately with the host (the host workers rear inquiline reproductives), a temporary social parasite eventually kills the host queen, the parasitic stage is transitory, and the parasite workers ultimately take over the colony as the host workers die out (Buschinger, 2009). In a brief survey of parasitic ants, we confirmed MG loss in 67% of inquiline species, but only in 9% of temporary social parasites (Appendix S3). Because most inquiline social parasites in our survey were myrmecine ants whereas most temporary social parasites were formicine ants (Appendix S3), an analysis of phylogenetically independent contrasts of a larger sample is needed to substantiate this intriguing association between inquilinism and MG loss.

Interestingly, two inquiline *Acromyrmex* species of comparatively recent origin have retained small workers. These

socially parasitic workers have smaller MGs compared with their respective non-parasitic sister species, yet the respective parasitic queens have MGs of similar size to host queens (Sumner, Hughes & Boomsma, 2003; de Souza *et al.*, 2006). Parasite queens are exposed to similar risks as non-parasite queens during their mating flight and nest-searching phase, and undergo the additional risk of invasion of the host colony, whereas parasite workers never or rarely leave the host colony. The convergent reduction in worker MGs in these two recently evolved *Acromyrmex* inquilines supports the view that a transition to inquiline life results in selection for a reduction in MG size in workers, presumably because maintenance of such secretory structures is costly (Sumner *et al.*, 2003; Poulsen *et al.*, 2002b).

In sum, the many independently evolved socially parasitic ants provide a rich testing ground for elucidating MG function. The presence of a MG in some parasitic ants indicates that MG-derived odours do not preclude infiltration of host colonies. More importantly, evolutionary maintenance of the MG in workers of temporary social parasites but frequent reduction of the MG in inquilines (see Appendix S3) appears to contradict the recognition odour hypothesis, but is consistent with some other hypothesis (e.g. antibiosis).

(2) Evolutionary losses and regains in formicine ants

Hölldobler & Engel-Siegel (1985) noted two additional attributes of MG-less ant lineages: nest-weaving and arboreal life. In nest-weavers, workers use larval silk to construct nests, for example, by weaving live leaves together into a protective envelope. Nest-weaving occurs in formicine genera such as *Oecophylla*, *Polyrhachis*, and several lineages in the genus *Camponotus* (Johnson, Agapow & Crozier, 2003; Robson & Kohout, 2005). Under the most likely evolutionary scenario, nest-weaving has been gained four times and lost once in the subfamily Formicinae, and loss of the MG is associated significantly with these four transitions to nest-weaving (Johnson *et al.*, 2003).

Hölldobler & Engel-Siegel (1985) suggested that arboreal nesting could permit reduction or loss of the MG because pathogen pressures may be less severe for arboreal ants than ground-nesting ants. However, many successful arboreal ant lineages (e.g. pseudomyrmecines) have well-developed MGs, whereas some successful ground-dwelling lineages do not (e.g. *Camponotus* spp.) (see also discussion on MGs and arboreality in Orivel & Dejean, 1999). These two observations weaken the arboreality hypothesis and indicate that arboreal nesting *per se* is not a major factor influencing MG loss. The analysis of species-specific factors that drive MG loss is complicated by the fact that nest-weaving and arboreality are correlated (most nest weavers are also arboreal).

A particularly interesting case is the large formicine genus *Camponotus* which comprises nearly 1000 described species most of which do not possess MGs. A few *Camponotus* species have unreduced MGs such as the South-East Asian giant rainforest ant *Camponotus gigas* (Hölldobler & Engel-Siegel, 1985). *C. gigas* nests in the soil at tree bases, under fallen

logs, or in tree cavities, but forages arboreally (Pfeiffer & Linsenmair, 1998, 2000, 2001). Other exceptions include *Camponotus sericeus* (Dey & Coumar, 2008), which nests in the ground, is strictly diurnal, and is widely distributed across Africa, the Middle East, and India (Mody & Linsenmair, 2003); *Camponotus thadeus* (Shattuck, 2005), a recently discovered species that inhabits high-elevation rainforest in Australia and appears to nest arboreally (Shattuck, 2005); and 10 species in the *Camponotus* subgenus *Myrmonesites* (Brian Fisher, personal communication), all from Madagascar. No nesting information is known from these Malagasy ants (Brian Fisher, personal communication). These four exceptional camponotine lineages with MGs offer interesting test cases for elucidating ecological factors in MG evolution.

(3) Metapleural gland of males

Hölldobler & Engel-Siegel (1985) reported that the MG is absent in most males. By contrast, our survey indicates that the MG may be present in males of more lineages than currently realized (Appendix S2), including early-branching (basal) ant lineages such as the proceratiines, amblyoponines, and ponerines. This supports the ancestral presence of the MG in ant males (Wheeler, 1928; Taylor, 2007), with multiple losses during their subsequent evolution. A comprehensive survey of males of the early-branching (basal) ant subfamilies (Martialinae, Leptanillinae, Proceratiinae, poneroids) is needed to confirm this pattern. Unfortunately, males are currently unknown for the most early-branching (basal) ant subfamily Martialinae (Rabeling, Brown & Verhaagh, 2008), males of the Leptanillinae and Proceratiinae are difficult to collect, and the presence of the MG in leptanilline males can only be determined by careful dissection (Baroni Urbani, 1977). Space constraints to accommodate the large flight muscles in the mesosoma of males could drive MG loss. An alternative explanation for MG loss in males is that males are short-lived and therefore have a reduced requirement for hygienic defences compared to long-lived workers and queens (Maschwitz *et al.*, 1970; Hölldobler & Engel-Siegel, 1985).

V. CHEMISTRY OF THE METAPLEURAL GLAND

The chemical constituents of MG secretions have been analysed in only a handful of species, with most analyses focused on ants with derived biologies such as leafcutter ants (43 compounds), fire ants (19 compounds), and *Crematogaster* (*Physocrema*) spp. (16 compounds). MG chemistry differs significantly among these three groups (Appendix S4). Carboxylic acids and fatty acids of various chain lengths dominate in leafcutter and fire ants, whereas phenolic compounds dominate in the *Crematogaster* (*Physocrema*) species. Proteinaceous compounds comprise a significant fraction of MG secretions in *Atta* spp. leafcutter ants (Maschwitz *et al.*, 1970; do Nascimento *et al.*, 1996), and it is presently unclear whether proteinaceous additions occur in the MG secretions of other ants.

(1) Metapleural gland chemistry of leafcutter ants

The most surprising insight emerging from a comparison of leafcutter MG chemistry is how few chemicals are shared among attine species (Appendix S4). Only five chemicals were consistently found from the MG secretions of several leafcutter species (3-hydroxydecanoic acid, indoleacetic acid, phenylacetic acid, 3-hydroxydodecanoic acid, and heptadecanoic acid). The first three of these acids are commonly reported as major constituents of MG secretions in leafcutter ants, whereas 3-hydroxydodecanoic acid and heptadecanoic acid represent minor constituents.

3-hydroxydecanoic acid, also called myrmicacin, was the first compound identified from the MG of attine ants (*Atta sexdens*, Schildknecht & Koob, 1971). Myrmicacin was later also discovered in three other attine species (*Atta cephalotes*, *Acromyrmex octospinosus*, and *Acromyrmex subterraneus*) (do Nascimento *et al.*, 1996; Ortius-Lechner *et al.*, 2000). Due to its anti-microbial activity, myrmicacin was initially hypothesized to function as an “herbicide” in attine gardens (Schildknecht & Koob, 1971; Iizuka, Iwadare & Orito, 1979). However, a specific herbicidal role of myrmicacin in attine gardens now appears unlikely for three reasons. First, myrmicacin is also found in non-attine ants that have no need of “herbicides” in their nest (e.g. *Messor barbarus* and *Labidus coecus*, Beattie *et al.*, 1984). Second, myrmicacin disrupts molecular flow through cell walls, the function of Golgi vesicles, and mitosis (see Nakamura, Miki-Hirosige & Iwanami, 1982 and references therein), suggesting a non-specialized, antibiotic effect typical for most organic acids. Third, even though myrmicacin seems to be a major compound in the MGs of *Atta* species, only small quantities of myrmicacin are found in *Acromyrmex* spp. leafcutter ants (Ortius-Lechner *et al.*, 2000), suggesting that it has a minor role in these close relatives of *Atta* leafcutter ants.

Indoleacetic acid (IAA) has been found in four species of attine ants: *Atta sexdens*, *Atta cephalotes*, *Acromyrmex octospinosus*, and *Acromyrmex subterraneus*. The detection of IAA was surprising because it is a well-known plant growth hormone (heteroauxin) (Schildknecht *et al.*, 1973). IAA is the major constituent in *Acromyrmex octospinosus* (24–25% of total MG secretion) but a minor one in *Atta* species (do Nascimento *et al.*, 1996). Despite its known auxin properties in plants, the functional role of IAA in leafcutter ants remains unclear. Initial studies claimed a negative influence on hyphal growth of the cultivated fungus (Schildknecht & Koob, 1971), while later studies found inhibition only at higher concentrations but growth enhancement at lower levels (Schildknecht *et al.*, 1973). Other studies detected a small inhibitory effect of IAA only when interacting synergistically with myrmicacin (Powell & Stradling, 1986).

Phenylacetic acid (PAA) is a major constituent of MG secretions in *Atta sexdens* and *Atta cephalotes* where it comprises 72% and 80% of the total secretion, respectively, but PAA is absent in *Acromyrmex octospinosus*. PAA also exhibits heteroauxin activities (Wightman & Lighty, 1982), but is weaker than IAA. Interestingly, PAA occurs also in other ant glands, such as the mandibular gland of the harvester ant

Pogonomyrmex rugosus (Fales *et al.*, 1992). The role of PAA as an anti-microbial agent is unclear. However, PAA undoubtedly contributes to the acidity of the MG secretion and therefore also to its general anti-microbial activity.

Although some closely related leafcutter species show qualitative similarities in MG chemistry (e.g. similar acidic composition in *Atta sexdens* and *Atta cephalotes*; Appendix S4), quantities of specific compounds vary substantially among leafcutter species. This suggests conservation of acidic constituents among closely related species, but possible modulation of the quantity of these constituents depending on behavioural roles (nurse ant *versus* forager) or environmental condition (healthy *versus* diseased colony). Consistent with this view of behavioural and environmental modulation, differences in MG chemistry did not correlate with genetic differences among workers from the same *Acromyrmex* colony (Ortius-Lechner *et al.*, 2003).

MG secretions between laboratory and field workers have only been compared in the leafcutter ant *Acromyrmex octospinosus* (Ortius-Lechner *et al.*, 2000). MG secretions were qualitatively similar between these workers, but field workers carried on average three times more MG secretion than laboratory workers (6070 ng *versus* 2099 ng). This quantitative difference could be due to several factors, such as higher pathogen pressures in the field or the greater diversity of leaves harvested by field workers, which in turn could influence microbial influx into a nest (van Bael *et al.*, 2009). By contrast, laboratory colonies exist in a more hygienic environment, may be less challenged by pathogens, and may therefore produce a reduced quantity of the potentially metabolically costly MG secretion (Poulsen *et al.*, 2002a, b).

(2) Metapleural gland chemistry of fire ants

The MG secretions of fire ants (*Solenopsis invicta* and *Solenopsis geminata*) contain a high percentage of fatty acids, along with methyl-branched and unbranched alkanes (Appendix S4). Several of these chemicals are shared between these two species. Interestingly, Cabrera *et al.* (2004) detected low levels of cuticular hydrocarbons in MG secretions, but they did not believe that the MG synthesizes cuticular hydrocarbons, and instead attributed hydrocarbon presence to accidental contamination entering through the large opening of the MG. Under this view, the MG opening allows for a two-way flow, permitting efflux of MG secretions but also entry of extrinsic chemicals derived from the ants (e.g. cuticular hydrocarbons) or derived from the nest environment. The likelihood of entry into the MG atrium presumably depends on a variety of interacting factors, such as the size and position of the MG opening, as well as the retention properties of the matrix in the MG atrium.

One hypothesized function of the MG of *S. geminata* is to secrete territorial markers (see Section II.2; Jaffe & Puche, 1984). A role in territorial marking was also suggested for *S. invicta* because of similarities in MG fatty acid composition to *S. geminata* (palmitic acid, linoleic acid, oleic acid, and stearic acid) and alkane composition (tricosane, tetracosane, and pentacosane). To our knowledge, no behavioural

experiments on the role of MG secretion in territoriality have been carried out on *S. invicta*. Likewise, no anti-microbial assays are available for the MG secretions of any *Solenopsis* ant, although the strong acidity of the MG secretions suggests a possible anti-bacterial effect.

(3) Metapleural gland chemistry of *Crematogaster* (*Physocrema*) spp.

Species in the *Physocrema* subgenus of *Crematogaster* have a swollen propodeum accommodating hypertrophied MGs (Donisthorpe, 1941; Hosoishi & Ogata, 2008, 2009). Two species in this group (*Crematogaster difformis* and *C. inflata*) are known to exude repellent droplets of whitish liquid from their hypertrophied MGs (see Section II.4). The major chemical components of the MGs are mainly alkylphenols (*C. difformis*) and alkylresorcinols (*C. inflata*), chemicals with known anti-microbial and repellent properties that are found also in other biological sources (Attygalle *et al.*, 1989; Jones *et al.*, 2005 and references therein). Likewise, mellein (3,4-dihydro-8-hydroxy-3-methylisocoumarin), found in the MG secretions of *C. difformis*, is known from diverse insects, including other species of ants, termites, and moths (Blum *et al.*, 1982; Attygalle *et al.*, 1989). Mellein occurs in other ant glands such as the mandibular gland of *Camponotus femoratus*, an ant that builds ant-gardens (Seidel, Epstein & Davidson, 1990). It is generally believed that the hypertrophied glands of *Crematogaster* (*Physocrema*) spp. arose from glands with a primary antimicrobial function (Maschwitz, 1974; Attygalle *et al.*, 1989; Jones *et al.*, 2005).

(4) Proteinaceous components of metapleural gland secretions

The presence of proteinaceous compounds in MG secretions was first noted by Maschwitz *et al.* (1970), consistent with later ultrastructural analyses documenting a rich endoplasmic reticulum in the secretory cells of the MG that implicated significant peptide or protein synthesis (Schoeters & Billen, 1993). Do Nascimento *et al.* (1996) found that MG secretions of leafcutter ants consisted mainly of peptide components. Peptide identities remain unknown. Veal *et al.* (1992) heated the MG secretion of *Myrmecia gulosa* to 100°C and treated it with proteolytic enzymes, before testing for anti-microbial activity. Anti-microbial activity did not differ before and after heat and enzyme treatment, suggesting that the peptides in the MG secretions of *M. gulosa* do not contribute to its antibiotic effects.

VI. CONCLUSIONS

- (1) Relative to other ant glands such as the well-researched poison gland or the diverse trail-pheromone glands (Morgan, 2009), current understanding of the evolution, comparative physiology, and chemical ecology of the MG is meagre. At present, the accumulated information on the MG does not permit

a test of the hypothesis that the MG functioned as an adaptive innovation in the origin, diversification, and evolutionary success of ants (Wilson, 1987; Hölldobler & Wilson, 1990; Ward, 2007).

- (2) A disproportionate number of studies have focused on testing antibiotic functions of MG secretions, largely disregarding other plausible functions (Table 1). Most empirical work to date focused on ant lineages with specialized biologies, such as fungus-growing ants or bulldog ants. Other studies simply assumed a primary function of antibiosis. Analyses of a representative phylogenetic sampling are needed to establish the generality of antibiotic properties of MG secretions, particularly of the early-branching (basal) ant lineages, where MGs remain completely unstudied.
- (3) Clues for possible functions of the MG could be gleaned from tracking the fate of MG secretions on ants, in the nest, and in their territory. Manipulation of the flow of MG secretions in carefully designed behavioural and microbial experiments could elucidate the role of the MG in antibiosis and territorial marking; such manipulation of secretory flow is possible by experimentally closing the MG opening, as pioneered by Poulsen *et al.* (2002b). Such studies could be coupled with chemical analyses to verify the hypothesized passive flow of MG secretions *via* the hind legs onto the substratum, or its active spread *via* MG grooming by the fore legs and application to specific sites (Fernández-Marín *et al.*, 2006, 2009).
- (4) Current phylogenetic patterns suggest that early-branching (basal) ant lineages had a simple one-chamber MG design compared to more derived lineages (two-chamber MG design), but only a comprehensive comparative analysis capturing the true diversity in MG morphology will be able to confirm this. Such comparative analyses should also include structures that interact with the MG secretions, such as the areas near the MG opening and the fore legs involved in MG grooming.
- (5) MG secretions are strongly acidic and thus inherently antibacterial. This does not prove a primary antibiotic function of the MG, just as the antimicrobial properties of the highly acidic and defensive poison gland in formicine ants does not indicate a primary antibiotic function. Elucidation of the functions of MG secretion will require documentation of antibiotic effects *in situ*, ideally separating general acidity effects from specific antibiotic effects of particular chemical compounds. In addition, the roles of the proteinaceous constituents in MG secretions remain to be characterized for any ant. Such comparative chemical-microbial ecology will require the development of standardized analytical methods.
- (6) All chemical studies to date investigated the MG secretions of workers but not of male and female reproductives. Male MG morphology remains completely unstudied for any ant. Comparative analyses of male

MG chemistry and functional morphology will inform hypotheses of MG evolution, particularly because loss of the MG in males appears to have occurred more frequently than in workers and queens. Comparative studies of males with unique biologies (e.g. dimorphic males with distinct life histories) may play a key role in the understanding of MG function in males.

- (7) An integrated approach drawing on functional morphology, phylogenetic history, comparative behaviour, and chemical ecology of a representative sampling of ant lineages, including the early-branching (basal) ant lineages, is needed to elucidate the evolutionary origin, diversification, transitions, and diverse adaptive functions of the MG of ants.

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IX. SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article.

Appendix S1. Antibiotic properties of metapleural gland secretions.

Appendix S2. Presence or absence of the metapleural gland in different castes (worker, soldier, queen, male) and, for species with metapleural glands, the number of secretory cells in one of the paired glands.

Appendix S3. Presence and absence of the metapleural gland in reproductive females of inquiline social parasites and temporary social parasites.

Appendix S4. Chemicals identified in metapleural gland secretions of different ant species.

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