Journal of Chemical Ecology, Vol. 26, No. 7, 2000

METAPLEURAL GLAND SECRETION OF THE LEAF-CUTTER ANT *Acromyrmex octospinosus*: NEW COMPOUNDS AND THEIR FUNCTIONAL SIGNIFICANCE

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(Received June 28, 1999; accepted March 11, 2000)

Abstract—Ants of the myrmicine tribe Attini live in symbiosis with a fungus that provides them with food. In return the ants maintain optimal growth conditions for the fungus, weed out competing microorganisms, and inhibit the growth conditions of these competitors by chemical means. We present a comprehensive analysis of metapleural gland secretions of *Acromyrmex octospinosus*, using a recently developed method for the analysis of polar compounds by gas chromatography and mass spectrometry. We show that the chemical identity and quantitative recovery of different compounds in the metapleural gland secretion depends upon the method used and the type of colony from which the samples are taken. In addition to the two compounds previously recorded in the metapleural gland secretion of *Acromyrmex* ants (indolacetic acid and myrmicacin), 20 new compounds were detected in the secretion of a random sample of workers from two laboratory colonies and two field colonies. These compounds span the whole range of carboxylic acids from acetic acid to the long-chain fatty acids but comprise also some alcohols, lactones, and keto acids. The possible function of this highly complex secretion mixture is discussed.

Key Words—Formicidae, leaf-cutter ants, *Acromyrmex octospinosus*, gas chromatography, mass spectrometry, metapleural gland, carboxylic and fatty acids, lactones, keto acids, antibiotics.

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INTRODUCTION

Leaf-cutting ants are unique among the ants because of their symbiotic interaction with a fungus where neither of the partners can survive without the other (Weber, 1966; Cherrett et al., 1989). The fungus enables the ants to use plant material indirectly as food by providing enzymes to break down plant tissue and detoxify plant secondary metabolites that are otherwise detrimental to the ants (Dowd, 1992). In addition, the fungus provides sterols, which the ants use as molting hormone precursors and membrane constituents (Maurer et al., 1992). The ants contribute to the mutualistic interaction by mechanically removing the wax layer from the harvested leaf fragments, which normally functions as a barrier to fungal infection (Quinlan and Cherrett, 1977). The ants also fertilize the younger, most active parts of the fungus garden with fecal droplets full of fungal enzymes, which they ingest from the mature parts of the garden (Boyd and Martin, 1975). Finally, they maintain, as far as possible, a competition-free environment for the mutualistic fungus by constantly weeding out alien fungi and microbial infections (Bass and Cherrett, 1994). This is essential, as the optimal conditions for the symbiotic fungus (darkness, 25°C, and pH between 4.3 and 5.0) (Powell and Stradling, 1986) are also suitable for many pathogenic bacteria and fungi, so that many of these are found in the fungus garden (Kreisel, 1972; Craven et al., 1970; Diehl-Fleig and Valim-Labres, 1993; Fisher et al., 1996; Currie et al., 1999b). The ants possess a variety of chemical defenses and secrete compounds with growth inhibiting and antibiotic properties. Specific antibiotics may come from bacterial symbionts (Currie et al., 1999a), but the metapleural gland has been implicated as a major source for general antibiotic defence substances (Maschwitz et al., 1970; Maschwitz, 1974; Beattie et al., 1985, 1986; do Nascimento et al., 1996).

The metapleural gland is a paired structure at the posterolateral end of the alitrunk that is only found in ants (Hölldobler and Engel-Siegel, 1984). Initially, it was hypothesized to produce a nestmate recognition agent (Brown, 1968) and was found to contain territorial marking pheromones in *Solenopsis geminata* (Jaffé and Puche, 1984). Later it was suggested to be involved in the antibiotic defense against microorganisms of many ant species (Maschwitz et al., 1970; Maschwitz, 1974). Several authors have since investigated the antibiotic properties of the metapleural gland secretion (Beattie et al., 1985, 1986; Veal et al., 1992; do Nascimento et al., 1996), but only a few species have been studied in sufficient detail to identify active chemical compounds and their function. Schildknecht and Koob (1971) confirmed the finding of phenylacetic acid (PAA) in the metapleural gland secretion of *Atta sexdens* (Maschwitz et al., 1970) and added β-hydroxydecanoic acid (myrmicacin), indoleacetic acid (IAA) and smaller amounts of two other β-hydroxyacids to the list of chemicals known to be present in the metapleural gland secretion of this species. Although the three

major compounds were also present in the metapleural gland secretion of *Myrmica laevinodis*, that of *Messor barbarus* contained only PAA and myrmicacin, whereas secretions of *Acromyrmex* species only seemed to contain myrmicacin and IAA. The latter result has been confirmed for *Acromyrmex octospinosus* (do Nascimento et al., 1996), but a more detailed analysis was not possible because of technical problems with their method for the analysis of polar compounds. It was also reported that proteins and peptides are likely to be present in the metapleural gland secretion of leaf-cutting ants (Maschwitz et al., 1970; do Nascimento et al., 1996). In contrast, various phenols were detected in the metapleural gland of the ant *Crematogaster deformis* and shown to have antibiotic properties (Attygalle et al., 1989). Finally, in addition to the metapleural gland, the secretion from the mandibular gland of ants has also been suggested to have antibiotic effects (Pavan, 1958; Brough, 1983; Knapp et al., 1994).

None of the previous studies have been able to uncover the entire spectrum of compounds present in the metapleural gland secretion or yielded sufficiently accurate quantitative data for intraspecific comparisons of colonies or individuals within colonies. The first aim of our study was therefore to optimize the analytical method so that a relatively complete spectrum of compounds present in the metapleural gland secretion could be identified. Our second objective was to make a detailed comparative analysis of the typical metapleural gland secretion of *Acromyrmex octospinosus* workers from both field and laboratory colonies. We have chosen *Acromyrmex octospinosus* as a model system for this study because of the relatively large size of the metapleural gland of workers (Bot and Boomsma, 1996) and because the unique symbiosis of the Attini with fungi makes special defensive functions of the metapleural gland secretion particularly likely. Two different methods were used for the extraction of the metapleural gland secretion from individual ant workers, and the influence of the extraction procedure on the quantitative recovery of different chemical compounds was tested.

METHODS AND MATERIALS

Sampling Methods. The colonies of *Acromyrmex octospinosus*, whose workers were used for these experiments, were collected in 1996 in Gamboa, Panama. After their transfer to the laboratory in Aarhus, they were housed in nestboxes in a rearing room with a constant temperature of 25°C and air humidity of ca. 70%. They were fed twice a week with the leaves and flowers of roses, *Prunus* sp. (during Danish summer), and bramble leaves, *Rubus fruticosus* (all year round) (Bot and Boomsma, 1996). The metapleural gland secretion was extracted simultaneously from a random sample of workers from all three castes (minors, media, and majors) from two laboratory-reared colonies. For compar-

ison, the metapleural gland secretion of workers from two other colonies was sampled immediately after collection in the field in Gamboa, Panama, in May 1998.

The first procedure of extracting metapleural gland secretion from individual ants (method 1) consisted of fixing the ant with a pair of forceps and piercing the wall of the bulla (the visible outer limit of the gland reservoir at the posterolateral part of the alitrunk) with a very fine insect-mounting needle. A fine glass capillary (50–70 μm diameter), connected with silicon tubes to a microliter syringe (adapted after do Nascimento et al., 1996), was then introduced into this hole and secretion was extracted into the capillary by raising the plunger of the syringe. The secretion from both reservoirs of the paired gland was extracted in one capillary, which was then sealed into a larger soft glass capillary for later gas chromatographic analysis (for details see Morgan, 1990). In the second procedure (method 2) the posterolateral part of the thorax was cut with a sterile scalpel and sealed as a whole into a soft glass capillary for further analysis. This latter method was also used on individuals from laboratory colonies, but was most suitable for the collection of gland secretion in the field.

Gas Chromatography–Mass Spectrometry (GC-MS). The analytical separation was carried out with a Hewlett-Packard 5890 gas chromatograph directly coupled to a 5970B mass selective detector (quadrupole mass spectrometer with 70 eV electron impact ionization). The system was controlled by and data accumulated on a Hewlett-Packard series 300 computer with HP 5972/5971 MSD Chemstation. Mass spectra were scanned from m/z 35 to m/z 550 with a scanning time of about 2.4 sec.

Chromatography was performed on a 15-m × 0.32-mm-ID column coated with a bonded polyethylene glycol stationary phase (0.25 μm thickness, Stabilwax, Restek) with quartz glass liners (produced in the laboratory in Keele) with Restek silanized glass wool. The samples were injected in splitless mode (injection temperature 250°C) by crushing the capillary tubes immediately inside the injector port after insertion, as described by Maile et al. (1998). The oven temperature was programmed to increase from 40°C (3 min) at 11°C/min to a maximum of 200°C. The split valve was closed before the sample was crushed and reopened 45 sec later. Helium was used as carrier gas at a flow of 1 ml/min.

The compounds were identified by comparing their mass spectra with standard MS databases. Their identity was confirmed by coinjection of synthetic standards of the acids (Sigma-Aldrich Co. Ltd., Gillingham, UK). As the chromatograms were characterized by many coeluting compounds, quantification was carried out by single-ion monitoring appropriate to each individual compound. The amounts were quantified by acquiring calibration curves of six different concentrations for each compound under the same conditions.

Organic Synthesis. All peaks, except for the two keto acids, could be identified with the above procedure. To identify and confirm the presence of 4-oxo-

octanoic and 4-oxo-decanoic acids, these compounds had to be synthesized. This was done by hydrolysis and oxidation of the corresponding δ-lactones. To this end, δ-octalactone (800 mg, Sigma-Aldrich) in acetic acid (10 ml) was prepared as solution 1 and sodium dichromate (700 mg, Sigma-Aldrich) in distilled water (0.7 ml) with concentrated sulfuric acid (0.3 ml) and acetic acid (6 ml) as solution 2. These two solutions were mixed and kept for 3 hr at room temperature. After rapid heating to 100°C, the mixture was refluxed for 1 hr. On cooling to room temperature, the mixture was poured into 75 ml of water where the keto acid precipitated. The precipitate was then washed with water and the residue was dried and recrystallized from light petroleum. The retention times and mass spectra of the two synthetic keto acids were found to be identical to the ones detected in the samples.

RESULTS

Gas chromatography of the metapleural gland secretion revealed the presence of a mixture of 21 major compounds (Figure 1; Table 1). The GC-MS analysis of a total of 138 samples of individual ants showed that all metapleural gland secretions contained acetic acid, tetradecanoic (myristic) acid, pentadecanoic acid, hexadecanoic (palmitic) acid, indoleacetic acid, δ-octalactone (in boldface type in Table 1), and an additional major compound eluting between indolacetic acid and tetradecanoic acid (peak 20 in Figure 1). The unknown compound did not show the 60 mass unit fragment that is characteristic of carboxylic acids, but it did show the fragment m/z 73, which is normally present in carboxylic acids (Figure 2). Additional fragmentation peaks were represented by the masses 85, 98, 101, and 116. The fragmentation pattern thus led us to suspect that this compound could be a derivative of a carboxylic acid. As the spectrum of this compound looks very different from that of hydroxycarboxylic acid derivatives and because there are δ-lactones present in the secretion, the assumption was made that this compound might be an oxidation product of the 4-hydroxy acids, a keto acid (4-oxocarboxylic acid) that cannot form a lactone (Table 1, peak 20). This hypothesis was confirmed by showing that the mass spectra of the synthesised keto acids were identical to 4-oxooctanoic acid and 4-oxodecanoic acid (peaks 20 and 21) from the metapleural gland secretion.

Nearly half of the mean total amount of secretion was indoleacetic acid (24–45%), followed by palmitic acid (10–25%), 4-oxooctanoic acid (9–24%), and acetic acid (3–9%, Table 1). The additional compounds (valeric acid, hexanoic acid, heptanoic acid, octanoic acid, nonanoic acid, decanoic acid, palmitoleic acid, 2-nonanone, 2-nonanol, furfuryl alcohol, and indole) could be identified in varying, but smaller amounts in most of the samples. We were also able to confirm the presence of 3-hydroxydecanoic acid, a compound detected in the

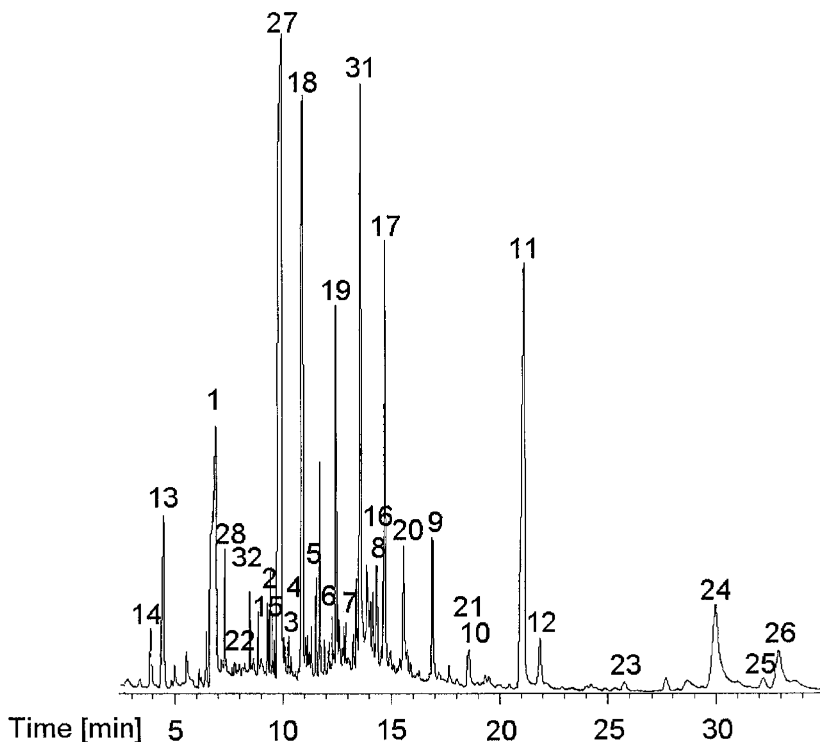

FIG. 1. Chromatogram to illustrate the chemical composition of the metapleural gland secretion of a typical *Acromyrmex octospinosus* worker. Peaks representing specific compounds are numbered in the order in which they appear in Tables 1 and 2.

metapleural gland secretion of *Acromyrmex octospinosus* by previous authors using different methods (Schildknecht and Koob, 1971; do Nascimento et al., 1996), but we could find it only in very small amounts and not in all individuals tested with the analytical method used in this study. We believe that propionic, heptadecanoic, octadecanoic, oleic, and linoleic acids (peaks 22–26, Table 2) are also genuine compounds synthesized in the metapleural gland but that these compounds may not normally reach the glandular reservoir in this form and could therefore only be detected when using method 2. The two different extraction methods produced seven further peaks (nos. 27–34; Table 2) that were inferred to be contaminants. Glycerol, acetamide, and γ-butyrolactone have been found frequently in samples of body tissue from various insect sources (E. D. Morgan, personal observation), while ethyloleate and ethyllinoleate may be artifacts produced by heating oleic and linoleic acids. Both esters and acids

TABLE 1. 21 MAJOR COMPOUNDS DETECTED IN METAPLEURAL GLAND SECRETION OF MINOR, MEDIUM, AND MAJOR WORKERS OF *Acromyrmex octospinosus* FROM TWO LABORATORY AND TWO FIELD COLONIES, EXTRACTED WITH TWO DIFFERENT METHODS[a]

		Mean secretion amount in two laboratory colonies (ng; *min–max*)				Mean secretion amount in two field colonies (ng; *min–max*)	
Compound	Peak	Method 1 (N = 72)	%	Method 2 (N = 13)	%	Method 2 (N = 53)	%
Acetic acid	1	41.64* *(0–522.6)*	***3.36***	218.2* *(0–1636.2)*	***9.43***	507.92 *(45.2–2247.9)*	***7.57***
Valeric acid	2	3.83 *(0–90.0)*	*0.30*	85.78 *(0–1080.4)*	*3.70*	44.03 *(0–839.2)*	*0.65*
Hexanoic acid	3	1.32 *(0–23.8)*	*0.10*	3.32 *(0–13.9)*	*0.14*	23.17 *(0–257.4)*	*0.34*
Heptanoic acid	4	2.83 *(0–49.7)*	*0.22*	0.36 *(0–4.8)*	*0.01*	0.13 *(0–7.3)*	*0.001*
Octanoic acid	5	4.16 *(0–120.9)*	*0.33*	2.97 *(0–23.8)*	*0.12*	24.69 *(0–292.0)*	*0.36*
Nonanoic acid	6	8.63 *(0–236.2)*	*0.69*	10.49 *(0–31.4)*	*0.45*	42.21 *(0–480.5)*	*0.62*
Decanoic acid	7	12.48 *(0–134.8)*	*1.00*	12.33 *(0–32.8)*	*0.53*	75.05 *(0–468.1)*	*1.11*
Dodecanoic acid	8	5.14 *(0–206.9)*	*0.41*	3.50 *(0–23.7)*	*0.15*	118.93 *(0-1662.9)*	*1.77*
Myristic acid	9	65.82 *(0–772.5)*	***5.31***	102.68 *(0–272.4)*	***4.43***	304.59 *(0–1305.7)*	***4.53***
Pentadecanoic acid	10	43.27 *(0–458.7)*	***3.49***	20.97 *(0–117.8)*	***0.90***	113.44 *(0–590.3)*	***1.69***
Palmitic acid	11	131.60 *(0–1298.9)*	***10.63***	217.02 *(0–605.8)*	***9.38***	1664.45 *(0–9000.0)*	***24.80***
Palmitoleic acid	12	1.40* *(0–55.3)*	*0.11*	24.21* *(0–117.9)*	*1.04*	186.56 *(0–1016.5)*	*2.78*

TABLE 1. (CONTINUED)

Compound	Peak	Mean secretion amount in two laboratory colonies (ng; *min–max*)				Mean secretion amount in two field colonies (ng; *min–max*)	
		Method 1 (*N* = 72)	%	Method 2 (*N* = 13)	%	Method 2 (*N* = 53)	%
2-Nonanone	13	5.07 *(0–31.9)*	*0.41*	8.05 *(0–27.5)*	*0.34*	23.73 *(0–433.9)*	*0.35*
2-Nonanole	14	2.37 *(0–61.2)*	*0.19*	3.67 *(0–26.1)*	*0.15*	25.29 *(0–414.1)*	*0.37*
Furfurylalcohol	15	1.06 *(0–14.4)*	*0.085*	2.53 *(0–12.4)*	*0.10*	20.50 *(0–188.0)*	*0.30*
Indole	16	1.03* *(0–37.6)*	*0.08*	40.94* *(0–160.3)*	*1.76*	104.14 *(0–900.6)*	*0.21*
Indoleacetic acid	17	558.41 *(0–5867.1)*	***45.11***	996.72 *(0–4255.9)*	***43.08***	1606.01 *(0–6968.7)*	***23.93***
γ-Octalactone	18	10.86 *(0–84.4)*	***0.87***	17.58 *(0–74.5)*	***0.75***	193.12 *(0–2061.2)*	***2.87***
γ-Decalactone	19	15.67 *(0–33.7)*	*1.26*	9.96 *(0–27.6)*	*0.43*	77.06 *(0–844.3)*	*1.14*
4-Oxooctanoic acid	20	293.52 *(0–1588.4)*	***23.71***	232.76 *(0–1692.9)*	***10.06***	643.79 *(0–5926.3)*	***9.59***
4-Oxodecanoic acid	21	26.77 *(0–296.1)*	*2.16*	84.88 *(0–929.7)*	*3.66*	271.19 *(0–5557.4)*	*4.04*
Total	***21***	***1226.00***	***100***	***2099.03****	***100***	***6070.10****	***100***

[a] See results for numbers of ants. The amount of each compound detected in an average individual [ng] and the relative proportion of each compound [%] are given. Significant differences due to the extraction method, based on *t*-test, are marked with an asterisk when they were found significant at the 95% level after Bonferroni correction. Major compounds found in almost every individual are given in bold. *N* = total amount of ants analyzed.

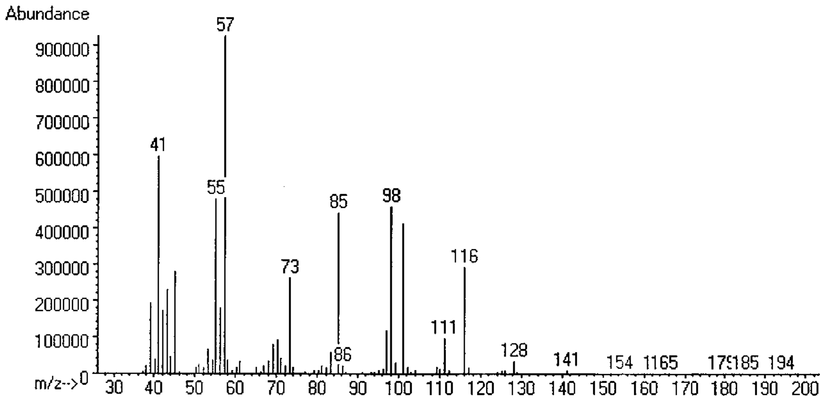

FIG. 2. Mass spectrum of 4-oxooctanoic acid with m/z 73, the typical fragmentation peak of carboxylic acids, and additional fragmentation peaks with masses 85, 98, 101, and 116.

could only be detected in the samples analyzed with extraction method 2. In contrast, 2-ethylhexanol only appeared in the samples analyzed with method 1 and was found by analysis to derive from the plastic tubes used for connecting the syringe to the glass-capillary. The two isopropyl esters, isopropyl palmitate, and isopropyl stearate also appeared in larger amounts in the samples extracted with method 1 and only in very small amounts by method 2. These compounds were found to be contaminants from the handcream used by the person handling the samples. As the glass capillaries could not be fitted into the silicon tubes with forceps, this had to be done manually, which increased this type of contamination in extraction method 1 compared to method 2 where the samples were seldom touched. Isopropyl esters are well known wetting agents used in cosmetic or medical mixtures where a better uptake of the effective agents is desired (Dettner, 1984).

To detect quantitative differences between individuals from laboratory ($N = 13$; 6 majors, 3 media, 4 minors) and field colonies ($N = 53$; 35 majors, 18 minors), both sampled with method 2, we performed individual t tests on the mean quantities of each chemical compound. After standard and sequential Bonferroni corrections (Rice, 1989) none of the quantities of each compound turned out to be significantly different, suggesting that there is no difference between laboratory and field colonies in the mean amount of each chemical component. However, there was a significant difference between laboratory and field colonies analyzed by method 2, in that we found three times as much secretion in an average worker from a field colony than in one from a laboratory colony (6070 ng vs. 2099 ng; $t = 2.2164$, $df = 64$, $P = 0.030$). To prove that this result is not due to a bias deriving from one of the three castes contributing more workers to

TABLE 2. COMPOUNDS FOUND TO BE PUTATIVE COMPONENTS (PEAKS 22–26) OR CONTAMINANTS DERIVED FROM TWO EXTRACTION METHODS (PEAKS 27–34)[a]

		Mean secretion amount in two laboratory colonies (ng; *min–max*)		Mean secretion amount in two field colonies (ng; *min–max*)
Compounds	Peak	Method 1 (N = 72)	Method 2 (N = 13)	Method 2 (N = 53)
Propionic acid	22		10.3 *(0–128.0)*	99.24 *(0–1326.7)*
Heptadecanoic acid	23			31.86 *(0–345.8)*
Octadecanoic acid	24	*	111.47* *(0–481.1)*	607.08 *(0–3422.2)*
Oleic acid	25	*	684.06* *(0–2228.9)*	4908.94 *(0–27712.6)*
Linoleic acid	26	*	224.4* *(0–919.6)*	1779.46 *(0–9327.1)*
Acetamide	27	0.99 *(0–22.3)*	214.65 *(0–495.8)*	639.39 *(0–4310.4)*
2-Ethylhexanol	28	7.71 *(0–43.7)*		
Isopropyl palmitate	29	75.42 *(0–539.6)*	2.93 *(0–11.4)*	2.77 *(0–76.5)*
Isopropyl stearate	30	74.62 *(0–648.3)*		0.23 *(0–12.3)*
Glycerol	31			516.11 *(0–4487.2)*
γ-Butyrolactone	32		0.16 *(0–2.2)*	14.42 *(0–233.7)*
Ethyl oleate	33		0.63 *(0–8.2)*	29.14 *(0–323.9)*
Ethyl linoleate	34			21.05 *(0–219.3)*

[a] The amount of each compound detected in an average individual (ng) is given. Significant differences due to the extraction method are marked with an asterisk if they were found significant at the 95% level after Bonferroni correction.

the overall analysis than the others, we performed a t test on the average total amount of major workers from the laboratory (N = 6) and the field (N = 35), both extracted with method 2. Surprisingly variances did not decrease when the analysis was restricted to a single caste, and the average major worker from the field also contained nearly three times more secretion than a major worker from the laboratory (8377 ng vs. 3175 ng; $t = 1.8661$, $df = 39$, $P = 0.069$).

We also could detect significant differences in the quantity of some of the individual chemical compounds when using the different extraction methods.

The *t* tests with Bonferroni corrections on 72 samples for method 1 (24 of each caste) and 13 samples for method 2 (6 majors, 3 media, 4 minors), from the two laboratory colonies, yielded a significant difference in the amount of acetic acid ($t = 3.2099$, $df = 83$, $P = 0.05$); palmitoleic acid ($t = 4.7962$, $df = 83$, $P = 0.05$) and indole ($t = 7.1553$, $df = 83$, $P = 0.05$) (Table 1). In addition, the amounts of three of the five compounds detected only with method 1 (most likely only found in the gland cells or the collecting sac) were found to differ significantly as well (octadecanoic acid: $t = 5.2985$, $df = 83$, $P = 0.05$; oleic acid: $t = 6.7396$, $df = 83$, $P = 0.05$; linoleic acid: $t = 6.6551$, $df = 83$, $P = 0.05$; Table 2). These differences also are reflected in the total amount of secretion sampled with either method, where method 2 yielded nearly twice the total amount of secretion from an average worker compared to method 1 (2099 ng vs. 1226 ng; $t = 1.8017$, $df = 83$, $P = 0.075$). This applies also to the total amount of secretion detected in an average major worker from a laboratory colony, which is higher, but not significantly so, when method 2 was used (3175 ng vs. 2032 ng; $t = 1.6394$, $df = 28$, $P = 0.112$).

DISCUSSION

In contrast to previous reports on only two chemical compounds in the metapleural gland secretion of two *Acromyrmex* species, β-hydroxydecanoic acid and indoleacetic acid (Maschwitz et al., 1970; do Nascimento et al., 1996), we here report on the presence of an additional 20 major and five putative minor compounds in the metapleural gland secretion of *Acromyrmex octospinosus* and confirm the occurrence of the two already known compounds. The newly discovered compounds span the whole range of carboxylic acids from C_2 to C_{18}, with two additional lactones, two 4-oxocarboxylic acids, one ketone, and two alcohols. As was pointed out by do Nascimento et al. (1996), carboxylic acids are difficult compounds to assess with gas chromatography. Because of their high polarity, the usually very small quantities available can easily get lost through adsorption onto surfaces during extraction and analysis. The successful quantitative detection of carboxylic acids in the present study was accomplished by a new analytical method (Maile et al., 1998) that uses solid injection without user-deactivated liners and glass wool in the injector, thus avoiding deactivated pre- and postcolumns. Capillaries containing glands or secretion are crushed immediately into the injector and analyzed on columns with a polar stationary phase.

In addition to method 1, which was also used by do Nascimento et al. (1996) for the extraction of metapleural gland secretion, we introduced a second, simpler method (method 2), which proved to be particularly suitable for the extraction of secretion in the field. This second method was also more effective and recovered higher amounts of secretion from individual workers: A significantly higher average amount of acetic acid, palmitoleic acid, indole (Table 1), octadecanoic

acid, oleic acid, and linoleic acid (Table 2) was detected with method 2 and the total amount of secretion found per average worker was twice as high as that recovered by method 1. This is probably due to the fact that we were only able to extract the contents of the gland reservoir with method 1, while method 2 also allowed the analysis of compounds still in the gland cells or the collecting sac. It also seems that some compounds are more abundant in the gland cells and/or the collecting sac, while others are more common in the gland reservoir (Table 1). A drawback of method 2 was the appearance of additional peaks in the chromatogram (Table 2), which were associated with body tissues of the ants. However, as only pure gland secretion was analyzed with method 1, these additional peaks could easily be identified as contaminants. We note that the amount of each component detected in the secretion was very heterogeneous in our samples (see min–max, Table 1), but that the degree of heterogeneity was the same in field and laboratory colonies and of the same magnitude in workers of only one caste (majors), independent of the extraction method used. As will be shown elsewhere (Ortius-Lechner et al., in preparation), this heterogeneity is due in part to quantitative differences between castes and to differences in age among individuals of the same caste.

Our comparison of the gland secretion of an average worker from a laboratory colony with that of a field colony worker did not reveal significant differences in the qualitative and quantitative composition of their metapleural gland secretion by the same extraction method (method 2). Although the leaf diet of field colonies is more diverse than that of laboratory colonies, this result implies that the biosynthesis of the metapleural gland compounds is independent of the type of plant food ingested. Instead, it seems likely that ant food derived from the symbiotic fungus (gongylidia), which is a more homogeneous source, is responsible for the observed, qualitatively rather constant chemical composition of the secretion. The threefold higher total amount detected in an average field colony worker compared to a laboratory colony worker analyzed by method 2 might reflect that laboratory colonies are less challenged by infections and thereby produce less of the potentially costly metapleural gland secretion. Another reason possibly accounting for the differing secretion amounts may be the different sampling times of the workers. As the ants do not seem to have muscular control over the flow of the secretion from the gland reservoir (Hölldobler and Engel-Siegel 1984; Schoeters and Billen, 1993) and the reservoir filling has been shown to be dependent on food availability (Maschwitz et al., 1970), the laboratory colony workers might not have been sampled at the exact time of highest food availability. In contrast to laboratory workers, which are fed twice a week, field colonies have continuous access to food, and it will thus be more unlikely to collect workers at times of low food availability.

Until quite recently it was common knowledge that the fungus-growing ants cultivate their symbiotic fungus in an axenic environment (Weber, 1966; Bass

and Cherrett, 1994; North et al., 1997). However, there is now growing evidence for abundant contaminants of fungus gardens by bacteria, other fungi, and yeasts, which increased our knowledge of the complexity and dynamic nature of this system (Kreisel, 1972; Craven et al., 1970; Diehl-Fleig and Valim-Labres, 1993; Fisher et al., 1996; Currie et al., 1999b). These contamination studies imply that the ants can not avoid contaminants but that they are normally able to inhibit their growth and to keep them from sporulating and overrunning the symbiotic fungus. There is some evidence that these defensive abilities may derive from the symbiotic fungus itself (Hervey and Nair, 1979), but the evidence for active defensive behavior by the ants is more substantial. Kreisel (1972) showed that symbiotic fungus gardens of *Atta insularis* are overgrown by parasitic fungi and bacteria within two days after the ants were taken away, partly because the pH of 5, which is optimal for the growth of the symbiotic fungus (Powell and Stradling, 1986; Veal et al., 1992), rises to 7 or 8 when the ants are absent. A later study by Papa and Papa (1982) confirmed this result for *Acromyrmex octospinosus* and made it clear that the ants themselves are responsible for the low pH found in the fungus garden. The evidence from Maschwitz et al. (1970) of pH 2.5 in the metapleural gland secretion of *Atta sexdens* and our finding of a broad spectrum of carboxylic acids in the secretion of *Acromyrmex octospinosus* suggests that the acidity of this secretion is used to reduce the pH of the leaf material that is brought into the colony from ca. 7–8 to 5, a value which is optimal for the symbiotic fungus but detrimental to most pathogenic strains. Recently another mutualist in this system has been discovered, a filamentous bacterium of the genus *Streptomyces* that produces specific antibiotics against a specialized fungal parasite, *Escovopsis*, which attacks the mutualistic fungus (Currie et al., 1999a). The ant workers and the queen are the carriers of this bacterium, but as yet it is unknown how the antibiotics that it produces reach their specific target. In contrast to this very specific antibiotic function of *Streptomyces*, a much broader functional significance has been attributed to the metapleural gland secretion of ants. Studies of two attine and other ant species (Maschwitz et al., 1970; Maschwitz, 1974; do Nascimento et al., 1996) and two Australian bull ant species, *Myrmecia gulosa* (Veal et al., 1992) and *M. nigriscapa* (Beattie et al., 1985, 1986), have documented the metapleural gland secretion to be effective against a wide range of common bacteria and fungi.

Until the present study, individual compounds of the metapleural gland secretion were only described for attine species and of these only indoleacetic acid and myrmicacin (hydroxydecanoic acid) from *Acromyrmex* have been tested for their functions. Myrmicacin was found to work as an herbicide and to inhibit fungal growth (Schildknecht and Koob, 1971) by specifically inhibiting mitotic progression at various stages even after metaphase (Iwanawi, 1978). However, the function of the plant hormone indole acetic acid remains controversial, with some authors claiming a negative influence on hyphal growth (Schildknecht and

Koob, 1971) and others not being able to confirm this finding, but finding a small effect only synergistically with myrmicacin (Powell and Stradling, 1986).

Concerning the wide range of acids newly identified in the metapleural gland secretion of *Acromyrmex octospinosus*, it seems clear that one of the general functions of the secretion of the metapleural gland is to reduce the pH in the fungus garden. On the other hand it has been shown that each of the acids can have antibiotic properties as well. Several studies have investigated the fungistatic and fungicidal action of fatty acids and related compounds as early as the 1940s (Cowles, 1941; Wyss et al., 1945; Spoehr et al., 1949). All studies reported an increasing antibiotic efficiency with increasing chain length of the acids, with the optimal chain length being dependent on the organism tested and the solubility of the acid. The same studies also found that the activity of fatty acids increases with decreasing pH, provided that the low pH values do not make the compound too insoluble to be effective. Koidsumi (1957) demonstrated this most convincingly by showing that the water-soluble volatile medium-chain-length acids (octanoic and decanoic acids) had the strongest inhibitory effect on spore germination and hyphal elongation, but that the activity decreased with increasing chain length. Octanoic acid was also found to increase the cuticular absorption of acetic acid present in the defensive secretion of the whip scorpion *Mastigoproctus giganteus* (Eisner et al., 1961), thereby acting as a wetting agent. As for the alcohols that we found in the metapleural gland secretion of *Acromyrmex octospinosus* (2-nonanol and furfurylalcohol, Table 1), Wyss et al. (1945) suggested that alcohols have high antibiotic activity but also are limited in their effectiveness due to their insolubility in water with increasing chain length. The picture is less clear for lactones, of which we found two in the metapleural gland secretion of *Acromyrmex octospinosus* (Table 1). They seem to be major constituents of many exocrine gland secretions in insects (Blum, 1981) and if any role could be attributed to them, it would likely be that of a deterrent in a defensive context. Only lactonic exudates of colletid and halictid bees have been thought to work as antibiotic agents when applied to the pollen stores on which the larvae develop (Blum, 1981). Bioassays will be necessary to clarify the function of the specific lactones found in this study as well as that of the two keto carboxylic acids, for which no functional record could be found.

Although many details remain to be clarified, we conclude that the metapleural gland secretion of *Acromyrmex octospinosus* has a general broad-spectrum antibiotic function, with most of its over 20 compounds being effective antimicrobial chemicals. As the secretions flow out over the ant cuticle, it is likely that they primarily protect the ants themselves, although an additional function in protecting the fungus garden cannot be ruled out, given the relatively large size of the metapleural glands in *Acromyrmex* workers (Bot and Boomsma, 1996). One such function, the general reduction in pH of the fungus garden, has now been repeatedly demonstrated. Other, more specific functions await discovery.

Acknowledgments—We thank Jeanette Bot, Dorte Bekkevold, and Jan Drachmann for help with the collection of colonies; Jeanette Bot, David Nash, Graeme Jones, and an anonymous referee for helpful comments on an earlier draft of this manuscript; the Smithonian Tropical Research Institute for providing excellent working facilities in Panama; and Panama's Instituto Nacional de Recursos Naturales Renovables for permission to sample and export the ants. Funding for this study was obtained through the research network Social Evolution of the Universities of Aarhus, Firenze, Keele, Sheffield, Uppsala, Würzburg and the ETH Zürich, financed by the European Commision via the Training and Mobility of Researchers (TMR) program.

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