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New chemical data on the ant *Myrmecina graminicola* (Formicidae, Myrmicinae): Unusual abundance of alkene hydrocarbons and esters

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ABSTRACT

We investigated the possible origin of the specific odor of the terricolous ant *Myrmecina graminicola* by elucidating its pattern of volatiles. It appeared to have a very large hypertrophied poison gland reservoir, which contains copious amounts of acetate and propionate esters that are also present on the cuticule. The poison gland was previously reported as the source of sex pheromone, but the finding that queens and workers exhibited the same ester patterns tends to refute their role in sex attractants, and their biological significance remains elusive. The composition of cuticular hydrocarbons in this species is also highly original as it mostly comprises alkadienes and alkatrienes (70%), which is unusual and may be an adaptation to subterranean life. Neither the esters nor the cuticular hydrocarbons vary qualitatively and quantitatively between various localities in France, including Corsica.

1. Introduction

Ants in the genus *Myrmecina* (Myrmicinae) have not been well investigated. They live in humid Palearctic forests and are terricolous, generally under mosses, but can also nest deeper in the soil when it dries up (Bondroit, 1918; Wheeler, 1910; Wilson, 1971). The only two species that have been studied, *Myrmecina graminicola* in Europe (single species in France) and *M. nipponica* in Asia, exhibit similar characteristics. They are predators of various arthropods, but *M. nipponica* may also rear oribatid mites in their nest, which they consume when dead (Masuko, 1994). The colonies of the two species are small with less than 100 workers (Buschinger and Schreiber, 2002; Miyazaki et al., 2010); for example, in *M. nipponica* the average number of individuals is 30 per colony (Cronin, 2013). Both species exhibit a marked queen polymorphism. They have either a single gynomorph queen or intermorph queens, in which case they are frequently polygynous (Buschinger and Schreiber, 2002; Miyazaki et al., 2010).

Females of *M. graminicola* housed in laboratory mating cages were observed to release a pheromone from their poison gland by touching the substrate with their gaster. Males were instantly attracted, and mating typically occurred within seconds of pheromone release (Buschinger, 2003, 2005).

M. graminicola is not an aggressive species, and when a worker encounters an intruder, it rapidly adopts a thanatos posture, playing dead with legs and antennae folded like nymphaea (Forel, 1874). Due to its very hard cuticle, enemies cannot easily injure it. Forel observed colony migration along a trail, which was also recently studied by Cronin (2013).

In the present study we comparatively analyzed the content of the poison gland and of the surface washes of *M. graminicola* from various localities in France. We also compared the profiles of workers and queens. As an external control we selected *Stenamma debile* from the Alps, because it is phylogenetically close to *M. graminicola*, with comparatively small colonies and lives in the same forest habitat in the soil or beneath large rocks. This ant also present thanatos (Blatrix et al., 2013).

2. Material methods

Ants were collected at various forest sites in France: Indre et Loire (37, Chinon, 47°12′29" N, 0°17′38" E, 80m; Amboise, 47°23′23″N, 0°58′46″E, 120m and Larçay, 47°12′36″ N, 0°47′23″ E, 90m), Oise (60, Ermenonville), Pyrénées-Atlantiques (64, Viven, 43°27.50N, 0°24.44W, 150m; Ordiap 43°09′07.1" N, 0°59′16.7" W, 203m), Hautes-Alpes (05, Reallon 44°34′43.1" N, 6°22′14.0" E, 1372m) and in Corsica (20, Boticella - Ersa 42°58′32" N, 9°22′44" E, 308m). Using combined gas

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chromatography/mass spectrometry (GC/MS), we analyzed the volatiles of 38 workers and 7 queens. We analyzed also dissected poison glands of workers in pools of 5 glands (n = 3). The Dufour gland of these ants is very small, and we were not able to cleanly dissect it. *Stenamma debile* came from the Alps (Morillon, 74, 46°04′45.8" N, 6°40′57.3" E, 713m). We analyzed five extracts of *S. debile*, each containing 10 workers.

For analysis, the ants were killed by freezing and either dissected to remove the poison gland into 100 µL of hexane, or placed in a vial containing 1 ml of hexane for 1 h, after which the ants were removed, to obtain cuticular hydrocarbons and various susbstances. The solvent was evaporated, and the sample was kept dry-frozen until chromatography. Samples were run by GC/MS using either VGM250Q system (Perkin-Elmer) or GC/MS-QQQ Agilent (GC 78908, MS 7000C with a Zebron ZB-5HT) operating at 70 eV with a DB-5 fused silica capillary column. The temperature was kept at 80 °C during the initial 2 min, then raised to 300 °C at 5 °C/min and held at 300 °C for 10 min. Retention times for each hydrocarbon and equivalent chain length values (ECL) were obtained by comparison with known n-alkane standards. Individual components in the total ion scanning mode were identified from their characteristic EI-MS fragmentation patterns, compared to synthetic compounds. The synthesis of esters from long chain primary alcohols and acetic or propionic acid followed laboratory standards and were performed by Wittko Francke from the Department of Chemistry at the University of Hamburg, Germany. The relative abundance of each compound was estimated as the proportional peak area from total ion chromatograms. To obtain a simple representation of chemical distances between groups, we conducted a cluster analysis of cases (Euclidian distance, Ward method) using the mean % proportions of all peaks. We also performed Discriminant and Principal Component Analysis using the same data. All statistics were carried out with Statistica 8 for Windows. In the analyses, esters and hydrocarbons were treated separately.

3. Results

3.1. Esters identified from poison gland

The poison gland reservoir of *Myrmecina graminicola* is very large and copious, it is very fragile and frequently was fractured during dissections. The secretion contained 8 major acetates and propionates: hexadecenyl and hexadecyl acetate; hexadecenyl and hexadecyl propionate; linolenyl and oleyl acetate; linolenyl and oleyl propionate (Fig. 1 and Suppl. Table S1). Oleyl acetate (30%) and oleyl propionate (19%) were the major esters. We did not find any ester in whole body washes of *Stenamma debile*.

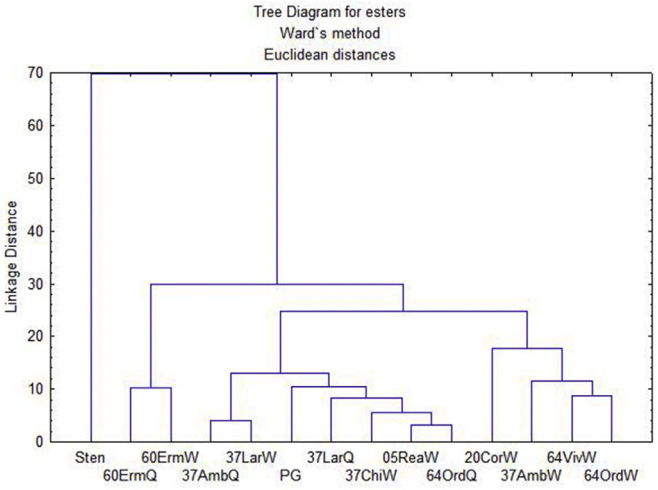

Fig. 2. Ester variation according localities for workers (W); queens (Q) and worker Poison Glands (PG). Sten = *Stenamma debile* workers; all others = *Myrmecina graminicola*; Ch = Chinon, Amb = Amboise; Rea = Reallon; Lar = Larçay; Ord = Ordiap; Viv = Viven; Erm = Ermenonville. Numbers are department codes.

Fig. 2 presents a cluster analysis of cases based on the presence of esters in *M. graminicola* from different localities and in *S. debile* as an outgroup. *Stenamma* that lacks esters is evidently very different from *M. graminicola*. It further shows that the content of poison glands of workers (PG in Fig. 2, the mean for 3 extracts) of *M. graminicola* is not chemically different from that of surface extracts of workers and queens (W and Q in Fig. 2). It also shows that the queens (Q) do not differ from workers (W), which has been confirmed by a discriminant analysis of all the data (Wilk' Lambda 0.856, F (10.62) = 1.046 with p = 0.417). Finally, there was no systematic geographical effect according to localities in France, including Corsica (indicated by department numbers) as localities cluster randomly with very low variation (Fig. 2).

3.2. Hydrocarbons

Fig. 3 shows the hydrocarbon profiles of workers of *M. graminicola* and *S. debile*, revealing that they are completely different (see Suppl. Table 2). *M. graminicola* possesses large amounts of unsaturated hydrocarbons (67.91 ± 1.69%, n = 38, with a dominance of alkadienes and alkatrienes), much more than in *S. debile* (11.85%, only alkadienes). A principal component analysis showed that the hydrocarbon

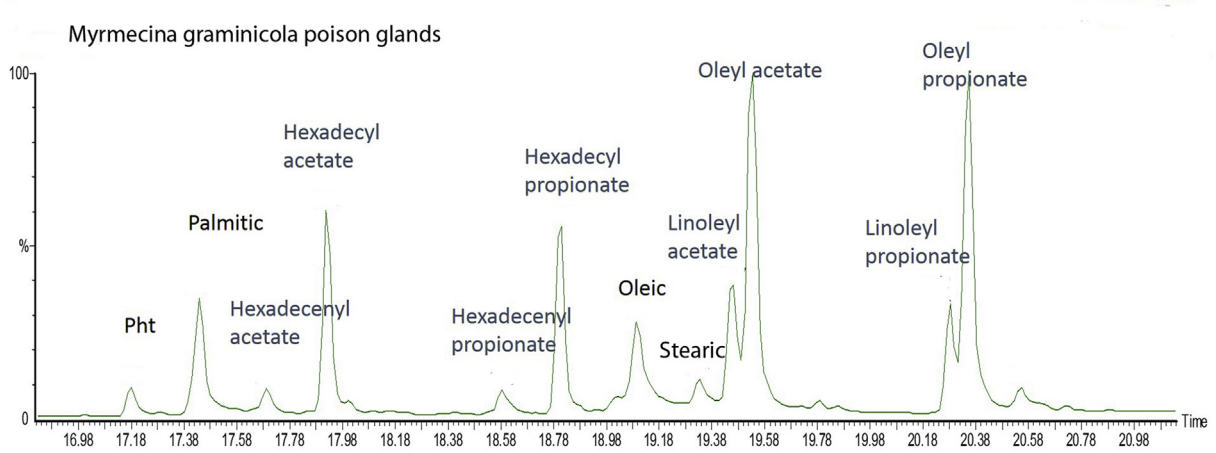

Fig. 1. Secretory composition of the poison gland of *M. graminicola*. Note the abundance of esters and the presence of the corresponding palmitic, oleic and stearic acids. Pht is a phtalate contaminant.

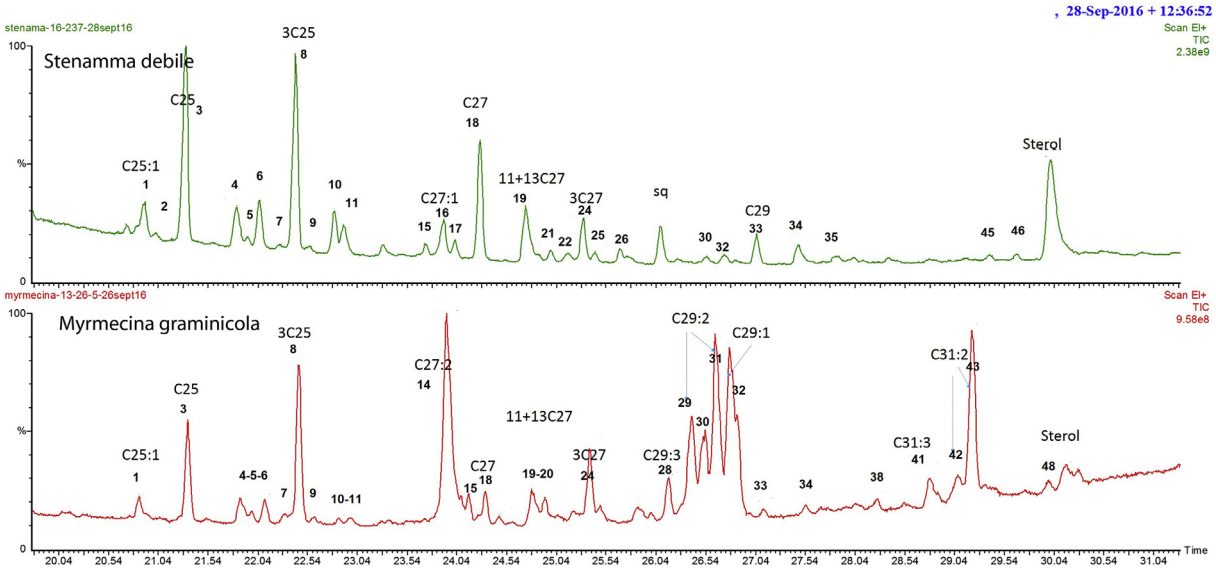

Fig. 3. Hydrocarbon profiles of *Stenamma debile* and *Myrmecina graminicola.* See Suppl. Table 2 for more information.

profiles of *M. graminicola* and *S. debile* are different on the first axis. The compounds responsible for the differences rested mainly with heptacosadiene and nonacosadiene (see Table S2 – 68% total alkenes vs 17% for *Stenamma*). Hydrocarbon profiles of *Myrmecina* originating from different localities, differed only on the axis 2 without clear geographical variations.

4. Discussion

Acetates and propionates are common esters in social insects. Dodecyl acetate has been found in parasitic bumblebees, in the genus *Psithyrus* (Martin et al., 2010). Longer chain acetates in which the alcohol moiety ranged from hexadecyl to docosyl were described from the Dufour gland of *Myrmecia* (Jackson et al., 1989) and *Bombus hypnorum* (Hefetz et al., 1993). Hexadecyl acetate has also been signaled in *Pholcus* spiders (Xiao et al., 2009). In ants the Dufour gland of *Harpegnathos saltator* contains tetradecyl propionate as well as traces of tetradecyl acetate and dodecyl acetate (Do Nascimento et al., 1993). Propionates are known from the Dufour gland of *Lasius niger* (Attygalle et al., 1987) and dodecyl acetate from the Dufour gland of some *Camponotus* and *Cataglyphis* species (Ali et al., 1988; Gökcen et al., 2002). The chemical composition of the *M. graminicola* poison gland is unique so far in having large quantities of acetates and propionates, which may give the ants the particular odor described by Forel (1874, p. 73): "*odeur très déliée, un peu framboisée*" (very untied smell, slightly raspberry). The role of the copious amounts of esters is still elusive. We exclude the possibility that they act as sex pheromone as suggested by (Buschinger, 2003, 2005), since there were no differences between queens and workers. As suggested by Cronin (2012) for *M. nipponica* we hypothesized that they are used as trail pheromone during colony migration, but our preliminary experiments were not conclusive enough to support this hypothesis. This point needs future work.

The pattern of cuticular hydrocarbons of *M. graminicola* is also special in comprising an abundance of alkadienes and alkatrienes, which is not frequent in ants (Martin and Drijfhout, 2009). We hypothesize that living under mosses and permanent subterranean life, possibly driven by competition with other ant species, prompted the production of these unsaturated hydrocarbons as protection from microorganisms (more than merely ground dwelling ants). We further postulate that since an abundance of unsaturated hydrocarbons reduces the impermeability of the cuticle (Gibbs, 1998), the species became entrapped to its rather humid habitat. This can explain why these ants are never found foraging on the ground, but stay permanently underground, and when the weather is dry, they migrate even deeper into the soil. This is in line with the ideas proposed by Menzel et al. (2017), who claimed that species from wet climates had more alkenes and fewer dimethyl alkanes than those from drier habitats, which can be explained by different waterproofing capacities of these compounds. Nevertheless, it is interesting to note that *S. debile* who have similar subterranean habitats have only 18% alkenes. It may indicate that the two species differ in their microclimates requirements, thus occupy different places.

Globally, the queens are not different from workers in the ester composition, except for one odd colony. This may hint on caste specificity and a role as fertility signal, but we cannot conclude this with only one sample. The queens did not differ from workers in hydrocarbon profiles in contrast to many other ant species (see for example d'Ettorre and Lenoir, 2010).

Analyses of *M. graminicola* ants from various localities in France including Corsica revealed a similar chemical profile for esters and hydrocarbons with low geographical variation. This is similar to the case of *Lasius niger* which exhibits the same hydrocarbon profile throughout its large geographical distribution in France (Lenoir et al., 2009) or 11 *Myrmica* species in Europe (Guillem et al., 2016).

Declarations of interest

None.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bse.2018.06.004.

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Supplementary Table S1	***Myrmecina graminicola***					
	workers		**queens**		**poison glands workers**	
	mean %	**SE**	**mean %**	**SE**	**mean %**	**SE**
Hexadecenyl acetate	3.06	0.59	3.09	0.86	1.48	0.12
Hexadecyl acetate	12.09	0.86	11.20	1.81	11.66	2.45
Hexadecenyl propionate	2.55	0.35	2.32	0.74	2.18	0.13
Hexadecyl propionate	9.01	0.55	12.26	3.91	10.19	1.19
Linolenyl acetate	13.78	1.73	11.04	1.42	10.93	3.06
Linolenyl propionate	7.95	0.66	5.93	0.60	8.98	1.91
Oleyl acetate	30.57	1.45	34.46	2.87	28.61	1.78
Oleyl propionate	19.42	1.17	18.66	1.70	25.17	2.32
Propionate	0.97	0.87	0.01	0.01	0.09	0.09
Octadecyl acetate	0.27	0.07	0.12	0.08	0.33	0.33
Octadecyl propionate	0.18	0.05	0.81	0.79	0.37	0.26
Eicosyl acetate?	0.14	0.11	0.09	0.09	0.00	0.00
total esters	100.0		100.0		100.0	
n=	38		7		3	

Supplementary Table S2		*M. graminicola* workers		*M. graminicola* queens		*Stenamma debile*	
N°	Name	mean %	SE	mean %	SE	mean %	SE
1	C25:1	0.63	0.27	0.63	0.34	8.40	2.29
2	C25:1	0.00	0.00	0.00	0.00	1.24	0.49
3	C25	10.10	1.59	9.02	1.59	10.39	2.80
4	11+13 MeC25	0.70	0.14	0.53	0.14	4.22	1.23
5	7 MeC25	0.00	0.00	0.00	0.00	0.86	0.28
6	5 MeC25	1.32	0.16	1.89	0.21	4.74	0.89
7	?	0.46	0.21	0.41	0.41	0.00	0.00
8	DiMe C25+2Me C25	0.22	0.07	0.15	0.08	0.09	0.09
9	3Me C25	6.91	0.46	8.06	0.95	19.28	3.04
10	C26	0.61	0.10	0.34	0.13	3.37	1.14
11	3,7+3,9+3,11DiMe C25	0.29	0.07	0.50	0.23	3.79	0.67
12	C27:2	0.66	0.26	0.50	0.43	0.00	0.00
13	C27:2	1.27	0.33	0.82	0.60	0.00	0.00
14	C27:2	14.15	1.62	18.88	3.37	0.00	0.00
15	4Me C26	0.00	0.00	0.00	0.00	1.47	0.34
16	C27:1	10.25	1.88	6.44	2.68	3.98	1.40
17	C27:1	1.05	0.25	0.35	0.22	1.26	0.09
18	C27	2.55	0.41	2.35	0.59	6.75	1.26
19	9+11+13MeC27	1.57	0.21	1.49	0.57	5.45	0.61
20	7Me C27	0.13	0.06	0.00	0.00	0.46	0.15
21	5Me C27	1.76	0.21	1.77	0.55	1.15	0.18
22	11,15DiMe C27	0.00	0.00	0.00	0.00	0.70	0.09
23	C28:1	0.06	0.02	0.02	0.02	0.00	0.00
24	3Me C27	3.42	0.23	2.53	0.39	6.52	1.61
25	5,15DiMe C27	0.00	0.00	0.00	0.00	0.16	0.16
26	C28	0.10	0.05	0.00	0.00	0.61	0.23
27	3,7+3,9DiMe C27	0.59	0.12	0.60	0.37	1.33	0.15
28	C29:3	2.66	0.56	3.16	0.79	0.00	0.00
29	C29:2	10.41	1.48	10.00	3.04	0.00	0.00
30	C29:2	3.80	0.92	2.02	0.57	0.38	0.11
31	C29:2	6.55	0.77	8.40	1.69	0.00	0.00
32	C29:1	9.35	1.10	9.57	2.56	0.88	0.18
33	C29	0.40	0.12	0.85	0.67	2.26	0.44
34	13+15Me C29	0.51	0.10	0.47	0.21	3.02	0.67
35	11,15DiMe C29	0.08	0.08	0.00	0.00	0.68	0.17
36	5Me C29	0.07	0.04	0.00	0.00	0.42	0.22
37	3MeC29	0.29	0.06	0.26	0.18	1.80	0.82
38	5,11DiMe C29	0.00	0.00	0.00	0.00	0.04	0.04
39	DiMe C29	0.00	0.00	0.00	0.00	0.15	0.15
40	10+12+14Me C30	0.00	0.00	0.00	0.00	2.82	2.22
41	C31:3	1.08	0.38	1.35	0.86	0.00	0.00
42	C31:2	2.15	0.39	0.87	0.37	0.00	0.00
43	C31:2	2.50	0.44	5.16	3.14	0.00	0.00
44	C31:2	0.93	0.38	0.10	0.10	0.00	0.00
45	C31:1	0.41	0.12	0.40	0.40	0.83	0.46
46	DiMe C30	0.00	0.00	0.00	0.00	0.12	0.12
47	C31	0.00	0.00	0.00	0.00	0.36	0.26
48	13+15Me C31	0.06	0.03	0.10	0.09	0.00	0.00
	TOTAL	100.0		100.0		100.0	
	n=	38		7		5	
	Total alkenes	67.91	1.69	68.67	0.96	16.97	1.70
	Total alkanes	13.76	1.94	12.56	1.84	23.76	5.01
	Total Me-alkanes	17.90	0.95	18.36	1.40	59.27	4.21
	Total	100.0		100.0		100.0	