

Ovarian activity correlates with extreme changes in cuticular hydrocarbon profile in the highly polygynous ant, *Linepithema humile*

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Abstract

In social insects, cuticular hydrocarbons of adults may vary with ovarian activity. Such variations are suggested to function in the regulation of reproduction within colonies. The modification of the CH profile with ovarian activity is usually interpreted as a signal of fertility causing workers to refrain from reproducing in the presence of the queen. We examined the effect of ovarian activity on the CH profiles in the Argentine ant *Linepithema humile*, a species where workers lack ovaries and are completely sterile. Our data show considerable differences in the CH profiles between fertile and infertile individuals. These differences are mainly qualitative. The CH profile strongly changes at the start of egg laying, both in mated and unmated queens. These results show that variation in cuticular hydrocarbons with ovarian activity in ants is not restricted to species with worker reproduction. We propose that in the Argentine ant, the cuticular hydrocarbons of laying queens correspond to a signal of fertility involved in the regulation of various aspects of reproduction, such as the rearing of new sexuals.

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1. Introduction

In social Hymenoptera, reproduction is usually monopolized by morphologically specialized queens while their worker daughters forego their own direct reproduction to participate in cooperative tasks (Wilson, 1971). Kin selection theory (Hamilton, 1964) predicts that worker sterility and helping behaviour may have been selected for, as long as workers help sufficiently to increase the offspring production of a closely related fertile queen and, by doing so, maximize their inclusive fitness. Honest fertility-signalling, which informs worker-nestmates of the presence of a healthy and productive queen is considered essential to ensure an evolutionary stable regulation of worker sterility

(Keller and Nonacs, 1993). The honest signalling hypothesis predicts an association between the pheromone output of queens and their fecundity. However, the chemical nature of the presumed fertility signal has never been identified.

Beside their role in nestmates recognition (Bonavita-Cougourdan et al., 1987; Lahav et al., 1999; Lenoir et al., 1999; Suarez et al., 2002; Vander Meer and Morel, 1998), cuticular compounds have been considered as key signals regulating reproduction in social insects (Ayasse et al., 1995; Bonavita-Cougourdan et al., 1991; Cuvillier-Hot et al., 2002; Dietemann et al., 2003; Endler et al., 2004; Hannonen et al., 2002; Heinze et al., 2002; Liebig et al., 2000; Monnin et al., 1998; Peeters et al., 1999; Sledge et al., 2001). A clear association between ovarian activity and change in CH profile has been demonstrated in several “morphologically primitive” ant species, where little or no morphological

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differences between egg-layers and infertile individuals occur, e.g. *Dinoponera quadriceps* (Monnin et al., 1998), *Harpegnathos saltator* (Liebig et al., 2000), *Diacamma ceylonense* (Cuvillier-Hot et al., 2001), *Pachycondyla inversa* (Heinze et al., 2002), *Myrmecia gulosa* (Dietemann et al., 2003). In all these species, quantitative differences in CHs between fertile and infertile individuals have been reported. Moreover, in *H. saltator* and *M. gulosa*, a few CHs were observed exclusively in fertile individuals. By contrast, the link between fertility and CH profile in the more derived subfamilies of Formicidae has been less investigated, and considerable variation occurs among species. In *Cataglyphis niger*, the CH profile of the queen appears indistinguishable from that of the workers (Lahav et al., 2001). On the contrary, queens and workers of *Cataglyphis iberica*, *Leptothorax acervorum* and *L. gredleri* show quantitative differences in the relative proportions of their cuticular hydrocarbons (Dahbi and Lenoir, 1998; Tentschert et al., 2002). In *Camponotus vagus*, the CH profiles of the queen and the workers are similar at the end of the winter, but differ in spring and summer when queens reach peak egg-laying rates (Bonavita-Cougourdan and Clément, 1994). In the slightly polygynous ant, *Formica fusca*, queens differ in their CH profiles according to their fecundity (Hannonen et al., 2002). Recently, both quantitative and qualitative differences in the CH profiles of workers and queens were reported in *Camponotus floridanus* (Endler et al., 2004).

To date, all studies regarding the influence of ovarian activity on the composition of CHs in ants involved monogynous or weakly polygynous species. Moreover, in all species studied, workers retained functional ovaries and could potentially lay (unfertilized) haploid eggs that will develop into males. The modification of the CH profile with ovarian activity has therefore been interpreted as an honest signal of fertility causing workers to refrain from reproducing in the presence of the queen(s) (Cuvillier-Hot et al., 2001, 2002; Dietemann et al., 2003; Endler et al., 2004; Hannonen et al., 2002; Liebig et al., 2000; Peeters et al., 1999).

In this study, we investigated the effect of ovarian activity on the CH profiles in the Argentine ant *Linepithema humile* (Dolichoderinae), a species forming supercolonies with thousands of reproductive queens (Markin, 1970b; Passera, 1994). Moreover, workers lack ovaries and are completely sterile (Markin, 1970a; Passera et al., 1988). Our results show that ovarian activity correlates with extreme changes in cuticular hydrocarbon profile. The differences in the CH profiles between egg-layers and infertile individuals are mainly qualitative.

2. Materials and methods

2.1. Ant maintenance and experimental units

Colonies of *L. humile* including several hundred queens, thousands of workers and large quantities of brood were collected in January and February 2001, at Port-Leucate (southern France). These source colonies were housed in artificial nests, maintained in the laboratory at 25 ± 2 °C and fed ad libitum on sugar water and maggots (Passera et al., 1988). Five experimental nests, each consisting of 20–30 queens and about 4000 workers, were constituted. After 3 weeks, they were orphaned to trigger production of male and female sexuals (Passera et al., 1988). Virgin queens (gynes) were obtained by removing female sexual pupae from the experimental nests and transferring them in small rearing units with 500 workers. Reproductives of *L. humile* reach sexual maturity very early, only 2–3 days after emergence (Passera and Keller, 1992). Within 24 h after emergence, young virgin queens were randomly assigned to one of the following treatments:

- (i) they were isolated with 10 males in small arenas. After 24 h, dealated (mated) queens were removed and placed together with 500 workers;
- (ii) they were kept with 500 workers; in this situation, virgin queens dealate within 1–14 weeks and start to lay unfertilized eggs (Passera and Aron, 1993).

2.2. Chemical analysis

Fifty workers or 10–15 queens were killed by freezing and then totally immersed for 5 min in 200–300 µl of pentane. Cuticular hydrocarbons were analysed by GC/MS using a Fisons VGAutospec mass spectrometer coupled to a Fisons GC 8065 gas chromatograph equipped with a split/splitless injector and, with a 25 m × 0.25 mm OV1 fused silica column (Rescom). Mass spectra were obtained using either electron impact (EI) (70 eV) or chemical ionization (CI) with methane as reagent gas. Oven temperature was programmed from 70 °C (isothermal for 2 min) to 320 °C at 10 °C/min, then isothermal for 10 min, using helium as carrier gas (100 kPa). Injection and detection temperatures were set at 250 °C. Hydrocarbons were identified by analysis of their mass spectra produced by both EI and CI, as well as by comparison of diagnostic ion fragments with published results on other ant species (Lenoir et al., 1997; Monnin et al., 1998). Moreover, identifications of hydrocarbons of workers, particularly trimethylalkanes, were confirmed by comparison with those previously reported for *L. humile* (Liang et al., 2001). The proportions of the different CHs were calculated from peak areas.

The following samples were analysed:

- fifty workers from the source colonies
- fifteen old mated and egg-laying queens from the source colonies
- fifteen winged virgin, non-laying queens 3 days after emergence (treatment ii)
- fifteen winged virgin, non-laying queens 3 weeks after emergence (treatment ii)
- fifteen dealated virgin, egg-laying queens (treatment ii)
- ten mated and egg-laying queens, 3 weeks after dealation (treatment i)

Each of these analyses was replicated three times.

The Nei index was calculated to estimate the degree of similarity between CH profiles:

$$I = \frac{\sum^n X_i * Y_i}{\sqrt{\sum^n X_i^2 \sum^n Y_i^2}}$$

where n , number of peaks, X_i , area (%) of peak i for sample x , and Y_i , area (%) of peak i for sample y . $I = 1$ for two strictly identical profiles and $I = 0$ for two totally different profiles. The Nei index was calculated excluding the peaks with a relative peak area of less than 1% in all CH profiles.

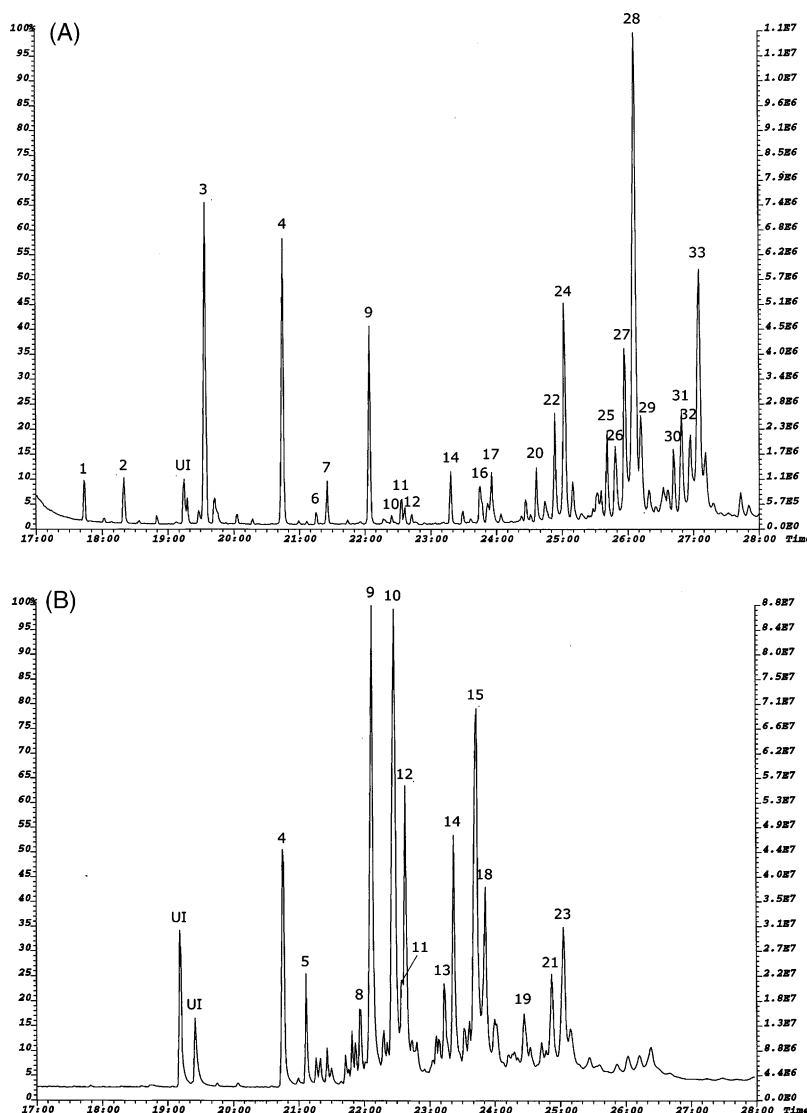


Fig. 1. GC chromatograms of cuticular hydrocarbons of *L. humile* (A) workers and (B) old mated, egg-laying queens. 1: *n*-C23; 2: 3-MeC23; 3: 13-MeC25; 4: *n*-C27; 5: 5-MeC27; 6: 3-MeC27; 7: *n*-C28; 8: xC29:1; 9: *n*-C29; 10: 5-MeC29; 11: 3-MeC29; 12: 5-MeC30; 13: xC31:1; 14: *n*-C31; 15: 5-MeC31; 16: 3-MeC31; 17: x,y-diMeC31; 18: 5-MeC32; 19: xC33:1; 20: 13- + 15- + 17-MeC33; 21: 5-MeC33; 22: 5, 15- + 5, 17-diMeC33; 23: 5-MeC34; 24: 5, 13, 17- + 5, 15, 19-triMeC33; 25: 13- + 15- + 17-MeC35; 26: 15, 19-diMeC35; 27: 5, 15- + 5, 17-diMeC35; 28: 5, 13, 17- + 5, 15, 19-triMeC35; 29: 3, 13, 17- + 3, 15, 17-triMeC35; 30: 13- + 15- + 17- + 19-MeC37; 31: 15, 19-diMeC37; 32: 5, 15- + 5, 17-diMeC37; 33: 5, 15, 19- + 5, 13, 17-triMeC37; UI: unidentified non-CH compound.

Table 1

Percentages (mean \pm SD; $n = 3$) of the peaks that mainly discriminate between workers and old mated egg-laying queens (– indicates that the compound was not detected or was less than 1% in the three replicates). Peak numbers: see Fig. 1

Peak number	Compound	Workers	Three days virgin non-laying queens	Dealated mated egg-laying queens	Old mated egg-laying queens	Three weeks virgin non-laying queens	Dealated virgin egg-laying queens
3	13-MeC25	5.91 \pm 1.59	–	–	–	–	–
5	5-MeC27	–	–	–	1.07 \pm 0.27	–	0.48 \pm 0.09
8	xC29:1	–	–	–	1.10 \pm 0.10	–	1.16 \pm 0.07
10	5-MeC29	–	–	11.42 \pm 2.60	22.27 \pm 1.03	4.62 \pm 1.12	15.78 \pm 1.20
12	5-MeC30	–	–	25.69 \pm 0.63	11.53 \pm 2.26	13.84 \pm 1.87	11.73 \pm 0.91
13	xC31:1	–	–	–	0.78 \pm 0.42	2.25 \pm 0.40	1.38 \pm 0.32
15	5-MeC31	–	–	5.49 \pm 1.78	15.99 \pm 1.89	4.31 \pm 0.84	12.91 \pm 0.57
18	5-MeC32	–	–	5.71 \pm 0.67	5.87 \pm 0.09	4.57 \pm 1.25	2.91 \pm 0.14
19	xC33:1	–	–	–	0.98 \pm 0.18	–	0.95 \pm 0.35
21	5-MeC33	–	–	3.29 \pm 1.50	2.24 \pm 0.60	–	3.14 \pm 0.29
22	5, 15- + 5, 17-diMeC33	2.57 \pm 0.94	6.28 \pm 2.72	–	–	3.67 \pm 0.35	–
23	5-MeC34	–	–	5.56 \pm 0.66	4.24 \pm 0.31	–	3.55 \pm 0.85
24	5, 13, 17- + 5, 15, 19-triMeC33	6.72 \pm 0.60	13.41 \pm 0.82	–	–	13.35 \pm 1.89	–
26	15,19-diMeC35	2.05 \pm 0.78	3.27 \pm 1.02	–	–	1.14 \pm 0.21	–
27	5, 15- + 5, 17-diMeC35	4.43 \pm 0.86	7.04 \pm 0.67	1.70 \pm 0.30	–	2.70 \pm 0.40	–
28	5, 13, 17- + 5, 15, 19-triMeC35	20.65 \pm 4.05	30.56 \pm 2.17	7.35 \pm 1.51	–	19.38 \pm 6.56	–
29	3, 13, 17- + 3, 15, 17-triMeC35	2.75 \pm 1.87	6.55 \pm 0.82	2.93 \pm 0.61	–	7.61 \pm 2.79	–
31	15,19-diMeC37	1.72 \pm 0.60	1.76 \pm 0.57	–	–	–	–
32	5, 15- + 5, 17-diMeC37	0.68 \pm 0.38	0.72 \pm 0.62	–	–	–	–
33	5, 15, 19- + 5, 13, 17-triMeC37	3.95 \pm 2.41	5.20 \pm 4.03	0.67 \pm 1.15	–	–	–

2.3. Dissection

After chemical extractions, all queens (virgin and mated) were dissected in water to check for the presence/absence of sperm in the spermatheca. Moreover, the level of ovarian activity was estimated from the number of mature oocytes and the number of yellow bodies in the ovarioles.

3. Results

CH profiles differed considerably between old mated, egg-laying queens and workers (Fig. 1, Tables 1 and 2).

GC/MS analyses of pentane extracts revealed a complex mixture of linear, methyl-branched, dimethyl-branched, and trimethyl-branched alkanes, ranging from C23 to C40, as well as a few alkenes. Queens showed considerable amounts of monomethylalkanes (5-MeC27 to 5-MeC34) that could not be detected or were less than 1% in workers. Three alkenes (C29:1, C31:1, C33:1) were also characteristic of queen CH profile. By contrast, the major compounds found in workers were dimethyl- and trimethylalkanes (diMe- and triMeC33, C35 and C37), which were absent (or less than 1%) in the queens' CH profile. An exception is the monomethylalkane, 13-MeC25, characteristic of the workers' CH profile. These differences in CH

Table 2

Similarity (mean \pm SD; Nei index) of the cuticular hydrocarbon profiles in *L. humile*. Within classes comparisons: $n = 3$; between classes comparisons: $n = 9$

	Workers	Three days virgin non-laying queens	Dealated mated egg-laying queens	Old mated egg-laying queens	Three weeks virgin non-laying queens	Dealated virgin egg-laying queens
Workers	0.905 \pm 0.039	0.856 \pm 0.076	0.231 \pm 0.046	0.118 \pm 0.033	0.685 \pm 0.104	0.169 \pm 0.042
Three days virgin non-laying queens		0.975 \pm 0.007	0.206 \pm 0.038	0.003 \pm 0.004	0.747 \pm 0.075	0.004 \pm 0.006
Dealated mated egg-laying queens			0.980 \pm 0.010	0.734 \pm 0.064	0.671 \pm 0.070	0.859 \pm 0.030
Old mated egg-laying queens				0.989 \pm 0.001	0.453 \pm 0.082	0.815 \pm 0.035
Three weeks virgin non-laying queens					0.931 \pm 0.047	0.540 \pm 0.087
Dealated virgin egg-laying queens						0.989 \pm 0.007

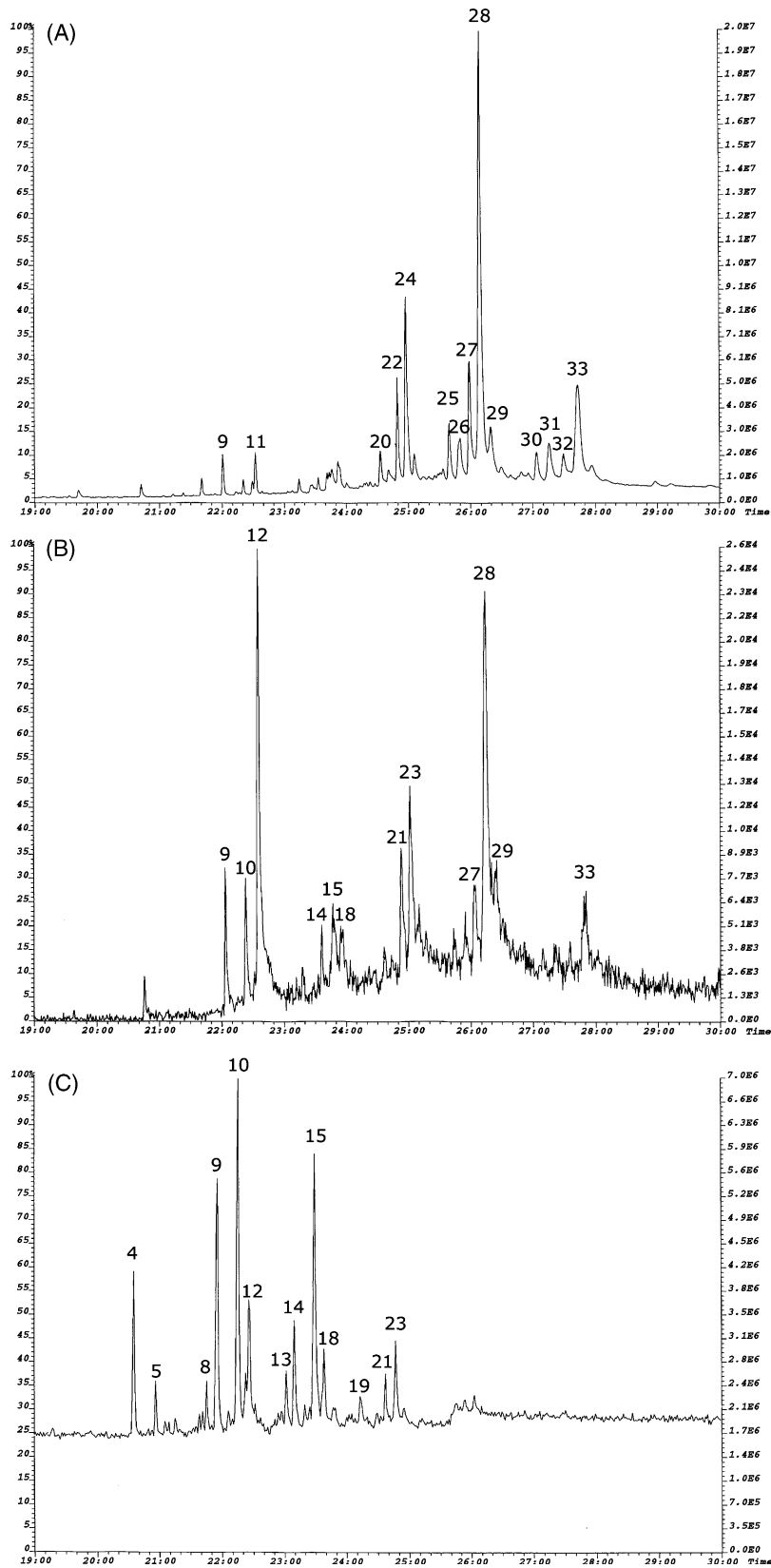


Fig. 2. GC chromatograms of cuticular hydrocarbons of *L. humile* (A) 3 days old virgin non-laying queens, (B) young mated egg-laying queens 3 weeks after dealation, and (C) old mated egg-laying queens. Peak numbers correspond to compounds identified in Fig. 1.

Table 3

Ovarian activity (mean \pm SD of the number of yellow bodies and mature oocytes) in *L. humile*

	Yellow bodies	Mature oocytes
Three days virgin non-laying queens ($n = 45$)	0 \pm 0	0 \pm 0
Three weeks virgin non-laying queens ($n = 45$)	0 \pm 0	0.1 \pm 0.4
Dealated virgin egg-laying queens ($n = 41$)	0.2 \pm 0.9	2.5 \pm 2.7
Dealated mated egg-laying queens ($n = 43$)	0 \pm 0	7.3 \pm 3.8
Old mated egg-laying queens ($n = 43$)	15.8 \pm 6.3	13.0 \pm 8.1

profiles between workers and old mated, egg-laying queens are consistent with their very low index of similarity of 0.118 (Table 2).

As shown in Fig. 2 (see also Tables 1 and 2), CH profile was closely correlated with ovarian activity. The CH profile of 3 days old virgin, non-laying queens was very similar to that of workers. Three weeks after dealation, young mated, egg-laying queens showed a cuticular profile intermediate between that of virgin non-laying queens and that of old mated, egg-laying queens. At this time, dissections revealed ovarian activity and eggs were observed in the nests (Table 3).

Similar results were obtained when considering variation in the CH profile of unmated queens with time (Tables 1 and 2). Virgin, non-laying queens 3 weeks old showed CH profiles intermediate between workers and old mated queens. By contrast, profiles of virgin, but egg-laying queens were similar to those of mated, egg-laying queens.

4. Discussion

Our results show a strong correlation of CH patterns with ovarian activity in the Argentine ant *L. humile*. Whereas the CH profile of non-laying sexual females is similar to that of sterile workers, it gradually changes both qualitatively and quantitatively once females start to lay eggs. These changes are independent of the mating status of females, since virgin egg-laying queens show a CH profile similar to that of mated egg-laying queens. During this change, the proportion of diMe- and triMeC33, C35 and C37 alkanes, which is high in workers and non-laying queens, dramatically decreases, whereas MeC27–MeC34 alkanes and three alkenes (C29:1, C31:1, C33:1), which are characteristic of old mated egg-laying queens, appear and progressively become more abundant. The occurrence of both quantitative and qualitative differences in the CH composition between laying and non-laying females has been rarely reported in social insects. In bumblebees (Ayasse

et al., 1995), social wasps (Bonavita-Cougourdan et al., 1991; Sledge et al., 2001) and most ant species studied to date (Bonavita-Cougourdan et al., 1987; Cuvillier-Hot et al., 2001; Dietemann et al., 2003; Heinze et al., 2002; Monnin et al., 1998; Peeters et al., 1999), ovarian activity is closely associated with quantitative variation in the proportions of CHs. In the ants *Harpegnathos saltator*, *Myrmecia gulosa* and *Camponotus floridanus*, however, some CHs were detected on the cuticle of fertile individuals only (Dietemann et al., 2003; Endler et al., 2004; Liebig et al., 2000). To our knowledge, the change in CH profile with ovarian development reported here in *L. humile* is among the most marked reported yet in a social insect.

In some ant species, it has been suggested that modification of the CH profile with ovarian activity could result from a selective elongation of the fatty acids, the biosynthetic precursors of CHs (Heinze et al., 2002; Liebig et al., 2000). Interestingly, in the Argentine ant, the shift proceeds in the opposite direction, since queens are characterized by shorter CHs than workers. Thus, different ant species may use different patterns of variation in cuticular hydrocarbons.

So far, a clear correlation between ovarian activity and the CH profile has been reported only in monogynous and weakly polygynous ant species, with worker reproduction. The distinctive CH profile of reproductive queens has been commonly interpreted as an honest signal of fertility, informing nestmate workers of the presence of a highly productive egg layer in the colony (see Introduction). In *L. humile*, the characteristic CH profile of fertile individuals cannot be considered as a honest signal of fecundity used by workers to repress reproduction, since workers are completely sterile in this species (Benois, 1973; Markin, 1970a; Passera et al., 1988). Nevertheless, the correlation between cuticular hydrocarbons and ovarian activity in *L. humile* may be involved in other aspects of the regulation of reproduction. (i) Previous studies have shown that reproductive queens, but not winged queens, of *L. humile* are highly attractive to nestmate workers (Aron, 1992; Keller, 1988; Keller and Passera, 1989), and that attractiveness strongly increases when queens start to lay eggs (Cariou-Etienne and Passera, 1993). Moreover, queen corpses washed in pentane became significantly less attractive than untreated ones (Cariou and Passera, 1990). These results, combined with those reported here, suggest that CHs could be used in queen recognition and attractiveness in this species. (ii) Under polygyny, differences in the CH profile between queens could be used by workers to distinguish, and possibly favour, the most fertile among the many queens of the colony (Hannonen et al., 2002). Favouring the most fecund queen(s) may enhance worker inclusive fitness if it increases colony reproductive output. If so, variation in the CH profile

between reproductive queens are expected to be honest, because both workers (receivers) and queens (emitters) benefit from acting honestly in response to the signal (Keller and Nonacs, 1993). This hypothesis predicts a correlation between chemical signals and the reproductive performance of co-breeding queens. Such a correlation has been reported in the polygynous ant *Formica fusca*, in which queens differ in the CH profiles according to their fecundity (Hannonen et al., 2002; Hannonen and Sundström, 2002). Whether such an association also occurs in the Argentine ant certainly merits further study. (iii) Cuticular hydrocarbons patterns of laying queens might also be used as a basis for regulating the production of new reproductive females. Like many other ant species, production of female sexuals in the Argentine ant is under pheromonal queen control (Vargo and Passera, 1991, 1992). Queens exert strong inhibitory effect on sexualization of female brood. The queen pheromone is transmitted by contact among colony members and its inhibitory effect is removed when queen corpses are washed in pentane. In addition, non-laying (virgin) queens are not inhibitory, whereas freshly mated, laying ones are (Vargo and Passera, 1991). These results, combined with our findings about the changes of CH profiles with ovarian activation, strongly suggest that CHs could be involved in the control of new queen production in this species. (iv) Finally, a distinctive feature of the Argentine ant reproductive strategy in its introduced range concerns queen execution. Each spring, workers execute up to 90% of the mated queens (Keller et al., 1989). Although this behaviour probably inflicts a high energetic cost on the colonies, its biological significance remains unknown. No relationship between the probability of a queen being executed and its fecundity, age or relatedness to the workers has been found (Keller et al., 1989; Reuter et al., 2001). How workers select the queens to be eliminated is also not yet established. Differences in the CH profile among queens might serve as a proximate mechanism used by workers to determine which queens will be killed. Because massive queen execution is not observed under laboratory conditions (Reuter et al., 2001), queens directly obtained from the field during the killing period could be used to test this hypothesis.

In ants, cuticular hydrocarbons are considered as the main components of the colony odour underlying nestmate recognition (Lahav et al., 1999; Suarez et al., 2002; Thomas et al., 1999; Wagner et al., 2000). Through allogrooming and/or trophallaxis, nestmates share their CHs to form the colony odour according to the “gestalt model” (Crozier and Dix, 1979; Lenoir et al., 1999). The postpharyngeal gland (PPG) plays a central role in this process by storing the exchanged CHs (Meskali et al., 1995; Soroker and Hefetz, 2000; Soroker et al., 1995, 1994). The results reported here

show that at least two subpopulations of very different CH profiles can be easily distinguished in the Argentine ant: the reproductive queens, on the one hand, and the workers and non-laying queens, on the other hand. According to the “gestalt model” (Crozier and Dix, 1979), these observations suggest that CH exchanges through allogrooming and trophallaxis between workers and queens are very limited. This seems unlikely, as contacts between both castes are frequent in this species. Alternatively, queens could synthesise high quantities of their characteristic CHs, therefore masking the main CHs of workers. The role of queens in the emergence of the colony odour has been investigated in a few species and two models have been proposed (Lahav et al., 1998; Lenoir et al., 1999). In *Camponotus floridanus*, the queen is at the origin of the colony odour (Carlin and Hölldobler, 1986). By contrast, in *Cataglyphis niger* and *C. iberica*, the contribution of the queens to the formation of colony odour is negligible (Dahbi and Lenoir, 1998; Lahav et al., 1998). The case of *L. humile* represents a third possibility in which workers and queens exhibit very different CH profiles.

The present study is the first report on the CHs of the main supercolony of Argentine ants in Europe (Giraud et al., 2002). This species was accidentally introduced both in southern Europe and in the USA, and it is expected to have independently experienced a reduction of genetic variation (Tsutsui et al., 2003, 2000). Our results show that the CH profile of the workers from the European supercolony is very similar to those reported for the introduced North American populations (Liang et al., 2001; Suarez et al., 2002). However, CHs between C14 and C22 found in significant amounts in North American colonies were not detected in our population. This difference suggests that some CHs from the native populations were lost by genetic drift during the founder event associated with introduction. A comparison between the CH profile of individuals from introduced and native populations would provide a test of this hypothesis.

In conclusion, this study shows that strong differences exist in the CH profile between fertile and non-fertile individuals of the Argentine ant *L. humile*. This is the first demonstration of a change in CH profile during ovarian activation in the subfamily Dolichoderinae and, more importantly, in a species forming very large and highly polygynous colonies with sterile workers. The biological function(s) of CHs specific to fertile individuals in *L. humile* are currently under investigation.

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